ACTA CHEMICA SCANDINAVICA 17 (1963) S1–S8

Über den Einfluss des Kohlensäuredrucks auf den Quantenbedarf der Photosynthese

OTTO WARBURG und GÜNTER KRIPPAHL

Max-Planck-Institut für Zellphysiologie, Berlin-Dahlem, Deutschland

Lässt man den Kohlensäuredruck von 0.25 % einer Atmosphäre, bei dem Emerson im Jahr 1941 die Energieausbeute bei der Photosynthese gemessen hat, auf 5 % einer Atmosphäre steigen — das ist der Kohlensäuredruck, bei dem in Dahlem die Energieausbeute gemessen worden war — so steigt die Energieausbeute auf das 4-fache. Die schlechten Energieausbeuten, die Emerson bei der Nachprüfung der Dahlemer Arbeiten im Jahr 1941 erhielt und die James Franck im Jahr 1941 als die thermodynamisch maximal möglichen erklärte, waren also die Folge davon, dass Emerson bei der Nachprüfung der Dahlemer Arbeiten einen Kohlensäuredruck anwandte, der 20-mal niedriger war, als bei den Dahlemer Arbeiten und der auch 20-mal niedriger war, als der Kohlensäuredruck, bei dem sowohl wir in Dahlem als auch Emerson in Urbana die zu den Versuchen verwendete *Chlorella* gezüchtet hatten.

Eine neue Methode zur Messung der Energieausbeute bei der Photosynthese ist für die Zwecke dieser Arbeit entwickelt worden.

Da der Quantenbedarf der Photosynthese das Fundament ist, auf dem heute in Dahlem die Chemie der Photosynthese aufgebaut wird, haben wir eine kürzlich zur Messung des Quantenbedarfs eingeführte[1] Ein-Gefässmethode weiterentwickelt, die darauf beruht, dass in gesonderte Abteile der Manometriegefässe konzentrierte Bicarbonat-Carbonatgemische eingefüllt werden, die definierte Kohlensäuredrucke erzeugen und sie konstant halten.

Ein geeignetes Manometriegefäss ist in Fig. 1 abgebildet. Der Hauptraum enthält wenig *Chlorella* (3 mm^3), die in viel Flüssigkeit (7 cm^3 Kulturlösung, pH 4,3) suspendiert ist; der Anhänger enthält 3-molare Bicarbonat-Carbonatgemische; der Gasraum enthält anfangs Luft und nach kurzem Schütteln denjenigen Kohlensäuredruck, der der Gleichgewichtsdruck des Bicarbonat-Carbonatgemischs im Anhänger ist. Damit sich dieses Gleichgewicht schnell einstellt, gibt man vor Schluss der Hähne in den Anhänger einige mg des Ferments Cartase, das die Reaction der Kohlensäure mit Wasser katalysiert. So wird der Kohlensäuredruck in den Manometriegefässen durch die Bicarbonat-Carbonatgemische konstant gehalten, und es können Druckänderungen, die bei Belicht-

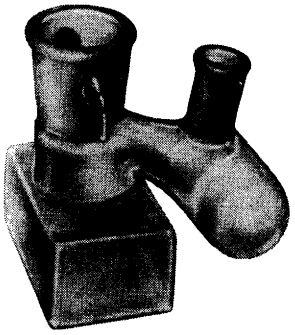


Fig. 1. Manometriegefäss zur Messung des Quantenbedarfs der Photosynthese mit der Ein-Gefässmethode. (Fecit Peter Ostendorf). Gesamtvolumen 25,9 cm³. Im Hauptraum 7,0 cm³ Zellsuspension, im Anhänger 3,2 cm³ 3-molares Bicarbonat-Carbonatgemisch und zwar:
85 Bicarb. + 15 Carbonat zur Erzeugung des CO_2-Drucks 4,7 % einer Atmosphäre
40 Bicarb. + 60 Carbonat „ „ „ „ „ 0,24 % einer Atmosphäre

$$k_{O_2}{}^{20} = 1{,}5 \text{ mm}^2; \; k_{CO_2}{}^{20} = 2{,}354 \text{ mm}^2.$$

ung auftreten, nur Änderungen des Sauerstoffdrucks sein. Dann kann die Sauerstoffentwicklung im Licht nach der Formel

$$\dot{x}_{O_2} = h_{O_2} \cdot k_{O_2}$$

berechnet werden.

Bei der Bestimmung des Quantenbedarfs brennt über dem Thermostaten ununterbrochen eine "Tageslichtlampe", deren Licht das ganze Manometriegefäss diffus ausleuchtet und so schwach ist, dass es die Atmung der 3 mm³ Zellen gerade kompensiert. Zur Messung des Quantenbedarfs wird dem diffusen sehr schwachen blaugrünen Licht ein Licht*inkrement* hinzugegefügt, ein roter Lichtstrahl, dessen Quantenintensität bolometrisch gemessen ist und der durch den Boden des Manometriegefässes vertikal in die Zellsuspension eindringt. Der von den Zellen absorbierte Bruchteil dieses Strahls wird in der Ulbricht'schen Kugel gemessen, in deren Mittelpunkt[2] die Manometriegefässe bestrahlt und bewegt werden, wie bei der Messung des Quantenbedarfs. So erhält man, mit Hilfe *eines* Manometriegefässes, auf sehr einfache Weise und sehr genau den Quantenbedarf der Sauerstoffentwicklung bei der Photosynthese

$$\frac{1}{\Phi} = \frac{\text{Mole Quanten absorbiert}}{\text{Mole Sauerstoff entwickelt}}$$

Zur Bestimmung des Quantenbedarfs werden nur junge *Chlorella*-Kulturen benutzt, deren Züchtungsbedingungen im experimentellen Teil angegeben sind und beachtet werden müssen. Zur Schonung werden die geernteten Zellen nicht centrifugiert, sondern lediglich vor dem Einbringen in die Manometriegefässe auf die Konzentration 3:7000 mm³ verdünnt. Dann wachsen die Zellen in den bewegten Manometriegefässen während der Bestimmung der Quantenausbeuten. Durch mehrfache Messungen der Lichtabsorption im Fortschritt der Versuche, die beliebig lange dauern können, haben wir dem Wachstum der Zellen Rechnung getragen, wie es in Protokoll 2 beschrieben ist.

Es ist bemerkenswert, dass bei dieser Versuchsanordnung trotz relativ hoher Lichtintensitäten gute Ausbeuten erhalten werden. Zum Beispiel war bei 9-facher Überkompensation der Atmung durch das rote Lichtinkrement der Quantenbedarf $1/\Phi = 3{,}8$, bei 14-facher Überkompensation $1/\Phi = 4{,}6$ und bei 20-facher Überkompensation $1/\Phi = 6{,}8$, was wahrscheinlich damit zusammenhängt, dass bei der geringen Zelldichte alle Zellen hinreichend blaugrünes Licht[3] erhalten. Jedenfalls verschwinden bei unserer Versuchsanordnung die Unsicherheiten, die mit der Ruheatmung der Zellen zusammenhängen; und die Sauerstoffentwicklung im Licht, zu der wegen der Kompensation der Atmung keine Dunkelatmung zu addieren ist, liegt ganz im positiven und ist ein sicheres Mass des Energiegewinns.

Die neue Versuchsanordnung ist besonders geeignet zur Untersuchung des Einflusses des Kohlensäuredrucks auf den Quantenbedarf. Denn um den Kohlensäuredruck zu variieren, hat man nur nötig, die Zusammensetzung der Bicarbonat-Carbonatgemische in den Anhängern zu variieren, während man alles andere konstant lässt. Es ist ein weiterer methodischer Fortschritt, dass man nunmehr, trotz eines grossen chemischen Verbrauchs an Kohlensäure in den belichteten Manometriegefässen, den Einfluss sehr kleiner Kohlensäuredrucke auf den Quantenbedarf untersuchen kann, da die im Licht verbrauchte Kohlensäure aus dem grossen Kohlensäurevorrat der Puffergemische der Anhänger schnell ersetzt wird.

Zwei Kohlensäuredrucke waren es, die uns besonders interessierten: der Kohlensäuredruck von etwa 5 % einer Atmosphäre (500 mm Wasser), das ist der Kohlensäuredruck, bei dem *Chlorella* am besten wächst und bei dem sie nach unsern Vorschriften überall gezüchtet wird. 5 % einer Atmosphäre ist auch der Kohlensäuredruck, bei dem wir im Jahr 1923 in Dahlem die ersten Messungen des Quantenbedarfs der Photosynthese durchgeführt haben[4]. Der zweite Kohlensäuredruck, der uns interessierte, war der Kohlensäuredruck von 0,25 % einer Atmosphäre (25 mm Wasser), bei dem Emerson[5] im Jahr 1941 in den U. S. A. den Quantenbedarf der Photosynthese gemessen hat.

In der Tabelle 1 und den Figuren sind einige Versuche beschrieben, bei denen der Dahlemer Kohlensäuredruck und Emersons Kohlensäuredruck gegeneinander

Tabelle 1. Quantenbedarf der Photosynthese bei variierten Kohlensäuredrucken.

CO_2 – Druck mm Brodie	mm^3 Quanten pro Minute eingestrahlt	$\frac{1}{\Phi} = \frac{\text{Quantenbedarf}}{\frac{mm^3 \text{ Quanten absorbiert}}{mm^3 O_2}}$
470	7	3,78
470	15	3,92
470	24	4,60
470	31	5,0
470	50	6,8
24	15	16
24	24	14
24	31	16

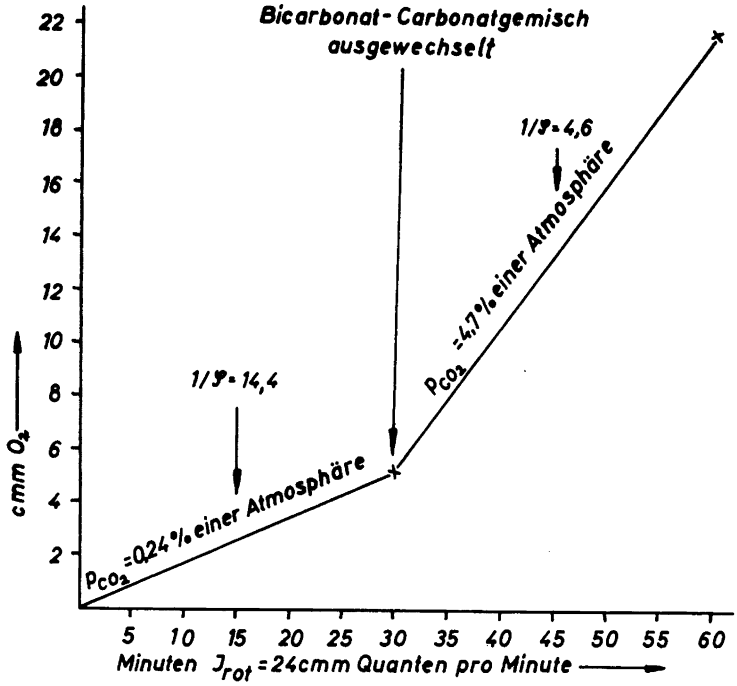


Fig. 2. Messung des Quantenbedarfs bei variierten Kohlensäuredrucken. Zuerst 0,24 %, dann 4,7 % einer Atmosphäre, erzeugt mit Bicarbonat-Carbonatgemischen im Anhängergefäss nach Figur 1. Berechnung des Quantenbedarfs Protokoll 1. Auch ohne Rechnung sieht man aus der Fig., dass die Sauerstoffentwicklung auf das 4-fache steigt, wenn man Emersons Kohlensäuredruck durch den Dahlemer Kohlensäuredruck ersetzt. Der niedrige Kohlensäuredruck ist Emersons Kohlensäuredruck, der höhere Kohlensäuredruck ist der Dahlemer Kohlensäuredruck.

ausgetauscht wurden, während alles andere konstant blieb. Wie man sieht, stieg die Sauerstoffentwicklung auf das 4-fache (Fig. 2), wenn man von Emersons Kohlensäuredruck auf den Dahlemer Kohlensäuredruck überging; und die Sauerstoffentwicklung sank auf 1/4 (Fig. 3), wenn man von dem Dahlemer Kohlensäuredruck auf Emersons Kohlensäuredruck überging.

Bekanntlich hat J. Franck[6] die niedrigen Ausbeuten Emersons bei dem Kohlensäuredruck von 0,25 % einer Atmosphäre für die maximal möglichen gehalten und deshalb die Dahlemer Ausbeuten, die bei einem 20-mal höheren Kohlensäuredruck gemessen worden waren, für falsch erklärt.

1948 bestätigten wir die Dahlemer Ergebnisse bei dem Kohlensäuredruck von 5 % einer Atmosphäre in gemeinsamen Versuchen mit amerikanischen Gelehrten[7] in Urbana, Bethesda und Woods Hole. Später wiederholten wir unsere Versuche in Dahlem bei jedem Fortschritt der Methoden und bestätigten sie von neuem[8]. Aber alle bisherigen Methoden übertrifft die hier beschriebene Ein-Gefässmethode an Einfachheit und Überzeugungskraft. Nunmehr kann jeder, der fähig

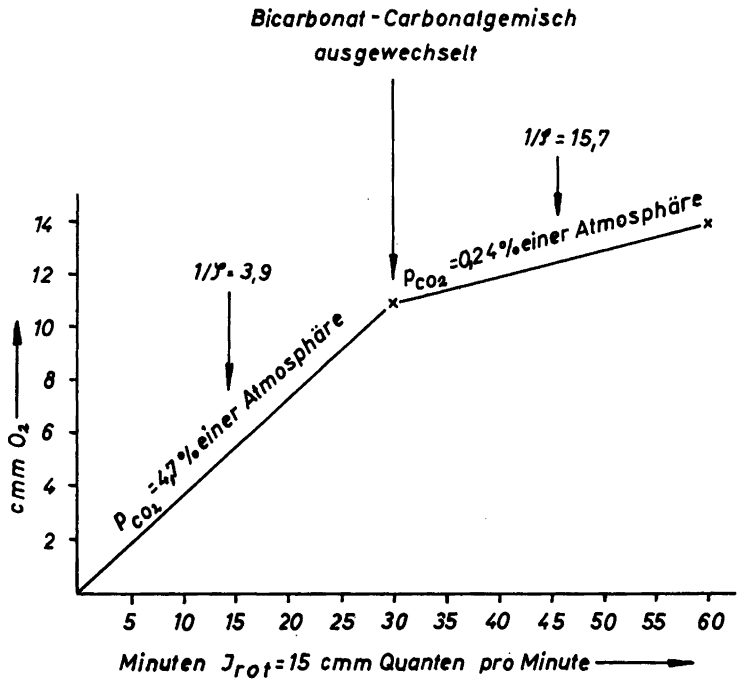


Fig. 3. Messung des Quantenbedarfs bei variierten Kohlensäuredrucken. Zuerst bei 4,7 % und dann bei 0,24 % einer Atmosphäre. Berechnung des Quantenbedarfs in Protokoll 2. Auch ohne Rechnung sieht man aus der Figur, dass die Sauerstoffentwicklung auf ¼ sinkt, wenn man den Dahlemer Kohlensäuredruck durch Emersons Kohlensäuredruck ersetzt. Der niedrige Kohlensäuredruck ist Emersons Kohlensäuredruck, der höhere Kohlensäuredruck ist der Dahlemer Kohlensäuredruck.

ist, ein Manometer abzulesen, am Manometer *sehen* — und wäre er unfähig, eine Messung oder eine Rechnung auszuführen — wie die Sauerstoffentwicklung einer gegebenen *Chlorella*suspension auf das 4-fache steigt, wenn man Emersons Kohlensäuredruck durch den Dahlemer Kohlensäuredruck austauscht, während alles übrige konstant bleibt. "In den Naturwissenschaften", sagt Pasteur, "ist die Zeit der oberste Richter."

Experimentelle Einzelheiten

Chlorella pyrenoidosa wurde in Kulturlösung K + Microelementen suspendiert[9] und in 250 cm³ Kulturkolben unter Durchleitung von 5 Vol % CO_2-Luft bei 25 °C im Licht einer 160 Watt Xenon-Hochdrucklampe bei 20 cm Abstand gezüchtet. Die Einsaat war 60 mm³ Zellen, die Ernte nach 24 Stunden war 200 mm³. Zum Versuch wurden die Zellen nicht centrifugiert, sondern mit Kulturlösung K (ohne Microelemente) auf 3 mm³ : 7000 mm³ verdünnt. Je 7,0 cm³ dieser Suspension wurde in die Gefässe Fig. 1 eingefüllt. Die Temperatur des Mess-Thermostaten war 20 °C.

Eine 100-Watt Tageslichtlampe brannte ununterbrochen während der Versuche, die beliebig lange dauern konnten, über dem Thermostaten in einem Abstand von den Manometriegefässen,

der so reguliert wurde, dass die Atmung der Zellen gerade kompensiert war. Der hierzu erforderliche Lampenabstand nahm in der ersten Zeit dauernd zu, das heisst, die Lichtwirkung nahm dauernd zu; erst wenn sie konstant geworden war, begann die Messung des Quantenbedarfs — der Zusats des Lichtinkrements und die dadurch bewirkte Messung der Sauerstoffentwicklung. Das Lichtinkrement war ein roter Lichtstrahl des Bezirks 645 mμ, der bolometrisch gemessen war und aus dem Licht einer 500 Watt Xenonhochdrucklampe isoliert war durch 5 cm fliessendes Wasser, 2 cm 2 % $CuSO_4 \cdot 5H_2O$ und eine Interferenzscheibe. Die Konstanz der am Konstanthalter brennenden Lampe wurde während der Versuche durch einen abgezweigten Lichtstrahl kontrolliert, dessen Photostrom mit einem Silica-Element von Bruno Lange gemessen wurde.

Protokoll 1. Versuch vom 17.4.62. 3 mm³ *Chlorella* in 7,0 cm³ Kulturlösung pH 4,3 im Hauptraum, 3,0 cm³ 3-molares Bicarbonat-Carbonat 40 + 60 und 2 mg Cartase in 0,2 cm³ H_2O im Anhänger, sodass der CO_2-Druck = 24 mm Brodie. Gasraum dann 0.24 % CO_2-Luft. Wellenlänge 645 mμ, von der die Zellen 10,5 % absorbieren.

Licht-Intensität		$k_{O_2}^{20} = 1{,}5$ mm²	
T(ageslicht)	5′	0	mm
T	5′	0	”
T + J_{645} = 24	60′	+10	60′ : +10–3 = +7 mm = 10,5 mm³
T – –	10′	+ 1	$\frac{1}{\Phi} = \frac{60\times24\times0{,}105}{10{,}5} = 14{,}4$
T – –	10′	+ 1	
T + J_{645} = 24	15′	+ 3,0	30′ : +6,5 – 3 = +3,5 mm = 5,25 mm³
” ” ”	15′	+ 3,5	$\frac{1}{\Phi} = \frac{30\times24\times0{,}105}{5{,}25} = 14{,}4$
T – –	10′	+ 1	
T – –	10′	+ 1	

Nunmehr Gemisch 40 + 60 im Anhänger ausgetauscht gegen Gemisch 85 + 15, sodass CO_2-Druck 470 mm Brodie und Gasraum dann 4,7 % CO_2-Luft.

		$k_{O_2}^{20} = 1{,}5 \times 1{,}04 = 1{,}56$ mm²	
T	5′	+ 1	mm
T	10′	0	30′ : +10,5 – 0 = 10,5 mm = 16,4 mm³.
T + J_{645} = 24	15′	+ 5,0	$\frac{1}{\Phi} = \frac{30\times24\times0{,}105}{16{,}4} = 4{,}6$
” ” ”	15′	+ 5,5	
T – –	10′	0	10′ : + 5,0 – 0 = 5,0 mm = 7,8 mm³.
T + J_{645} = 50	5′	+ 2,5	$\frac{1}{\Phi} = \frac{10\times50\times0{,}105}{7{,}8} = 6{,}75$
” ” ”	5′	+ 2,5	

Die Atmung der 3 mm³ Zellen war in 30 Min – 1,2 mm³ O_2, die O_2-Entwicklung im Licht bei J = 24 war + 16,4 mm³, die Atmung war also durch den roten Lichtstrahl 13,6-fach überkompensiert. Bei J = 50 war die Atmung nach der entsprechenden Rechnung 19,5-fach überkompensiert.

Protokoll 2. Versuch vom 28.6.62. Anordnung wie in dem Versuch Protokoll 1, nur wurden andere Intensitäten des roten Lichtstrahls angewandt und die Reihenfolge der Kohlensäuredrucke war umgekehrt, zuerst der hohe und dann der niedrige Kohlensäuredruck.

Gasraum 4,7 % CO_2-Luft. $k_{O_2}^{20} = 1{,}56$ mm² Absorption 645mμ=9,5 %

T(ageslichtlampe)	15′	0	mm
$T + J_{645} = 15$	5′	vor	
T „ „	15′	+ 3,5	30′ + 8 − 1 = 7 mm = 10,9 mm³.
T „ „	15′	+ 4,5	$\frac{1}{\Phi} = \frac{30\times15\times0{,}095}{10{,}9} = 3{,}92$
T − −	5′	vor	
T − −	15′	+ 1	
$T + J_{645} = 31{,}2$	5′	vor	
$T + J_{645} = 31{,}2$	15′	+ 6,5	30′ +14 − 2 = 12 mm = 18,7 mm³.
T „ „	15′	+ 7,5	$\frac{1}{\Phi} = \frac{30\times31{,}2\times0{,}10}{18{,}7} = 5{,}0$
T − −	5′	vor	
T − −	15′	+ 1,0	
$T + J_{645} = 7{,}0$	5′	vor	
T „ „	60′	+11,5	60′ + 11,5 − 4 = 7,5 mm = 11,7 mm³.
T − −	5′	vor	$\frac{1}{\Phi} = \frac{60\times7\times0{,}105}{11{,}7} = 3{,}78$
T − −	15′	+ 1	

Nunmehr Gasraum 0.24 % CO_2-Luft. $k_{O_2}^{20} = 1{,}50$ mm². Absorption 645 mμ = 10,5 %

T − −	15′	+ 1	
$T + J_{645} = 15$	15′	vor	30′ + 3 − 1 = 2 mm = 3 mm³.
T „ „	30′	+ 3	$\frac{1}{\Phi} = \frac{30\times15\times0{,}105}{3} = 15{,}7$
T − −	5′	vor	
T − −	15′	0	
$T + J_{645} = 31{,}2$	5′	vor	30′ + 5,0 − 0,5 = 4,5 mm = 6,75 mm³.
T „ „	30′	+ 5	$\frac{1}{\Phi} = \frac{30\times31{,}2\times0{,}115}{6{,}75} = 15{,}9$
T − −	5′	vor	
T − −	15′	+ 0,5	Endabsorption 11,5 %

Die Lichtabsorption richtig gezüchteter Zellen in dem Manometriegefäss soll für 3 mm³ Zellen auf einer Fläche von 8 cm² nicht mehr als etwa 10 % betragen; bei wesentlich höheren Chlorophyllgehalten sind die Ausbeuten schlechter. Die Lichtabsorption wurde mit der Ulbricht'schen Kugel, wie in der Einleitung erwähnt, gemessen[2].

Die Kohlensäuredrucke in den Gefässen wurden erzeugt durch 3 cm³ 3-molare Bicarbonat-

Carbonatgemische in dem Anhänger. Die mathematische Theorie[19] der Ein-Gefässmethode kann in die Gleichung zusammengefasst werden

$$K_{O_2} = k_{O_2} \times \left[\frac{R}{k_{CO_2}} \right]$$

$$\left[k_{CO_2} + \gamma \times k_{O_2} \right]$$

wo K_{O_2} die Gefässkonstante für Sauerstoff für *beliebige* Werte der Retention r der Kohlensäure bedeutet, während k_{O_2} die einfache Gefässkonstante für Sauerstoff und der eingeklammerte Ausdruck die Korrection ist, mit der k_{O_2} zu multiplicieren ist, um K_{O_2} zu erhalten. Praktischen Wert hat die Eingefässmethode nur dann, wenn der eingeklammerte Ausdruck sich dem Wert 1 nähert. Bei den beiden von uns angewandten Kohlensäuredrucken von 24 und 470 mm Brodie ist die Retention $R = 3 \times r$ so gross, dass $K_{O_2} = k_{O_2} \times 1$ für den CO_2-Druck 24 mm und $K_{O2} = k_{O2} \times 1.04$ für den CO_2-Druck 470 mm Brodie ist.

In den Protokollen 1 und 2 bedeutet "T" Tageslichtlampe, die während der ganzen Dauer der Versuche ununterbrochen brannte, J bedeutet die Quantenintensität in mm³ Quanten pro Minute des eingestrahlten roten Lichtstrahls der Wellenlänge 645 mμ, a ist der von den Zellen absorbierte Bruchteil dieses Strahls. Waren durch den Strahl in t Minuten x_{O_2} mm³ Sauerstoff entwickelt worden, so war der Quantenbedarf der Sauerstoffentwicklung

$$\frac{1}{\Phi} = \frac{t \times J \times a}{x_{O_2}}$$

Nach Zugabe oder Fortnahme des roten Lichtstrahls wurde meistens 5 Minuten bis zur Einstellung des neuen stationären Zustands gewartet. Erst dann begann die Messung des Quantenbedarfs.

LITERATUR

1. Warburg, O. *Weiterentwicklung der zellphysiologischen Methoden*, Georg Thieme, Stuttgart und Interscience, New York. 1962. Im folgenden als *"Weiterentwicklung"* citiert.
2. *"Weiterentwicklung"*, S. 237.
3. *"Weiterentwicklung"*, S. 248 und 261.
4. Warburg, O. und Negelein, E. *Z. Physik. Chem. (Leipzig)* **102**, 236, 1922 und **106**, 191, 1923.
5. Emerson, R. und Lewis, C. M. *Am. J. Botany*, **28** (1941) 789.
6. Franck, J. und Gaffron, H. *Advan. Enzymol.*, **1**, 199, 1941.
7. *"Weiterentwicklung"*, S. 75 bis 128 (gemeinsam mit Dean Burk).
8. *"Weiterentwicklung"*, S. 248, 261, 414, 447, 484, 578, 582.
9. *"Weiterentwicklung"*, S. 452.
10. *"Weiterentwicklung"*, S. 578.

Eingegangen am 5. Februar 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S9–S16

Chemical Paleogenetics

Molecular "Restoration Studies" of Extinct Forms of Life

LINUS PAULING and EMILE ZUCKERKANDL*

*Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California, USA***

Attention is attracted to the possibility of reconstructing the amino-acid sequence of ancestral polypeptide chains by virtue of a comparison between the amino-acid sequences of related polypeptide chains found in contemporary organisms. A tentative partial structure is proposed for two ancestral hemoglobin polypeptide chains. Some perspectives of paleobiochemistry are outlined.

In different hemoglobin polypeptide chains, derived either from one individual organism (man) or from different vertebrate species, identical amino-acid residues are often found in corresponding positions along the chains (*cf.* Braunitzer[1]). This occurs too frequently to be due to chance, and it appears to be too constant a feature over long periods of evolutionary time to be attributable to convergence by natural selection from primitively heterologous polypeptide starting materials. Consequently, the homology is plausibly interpreted by the assumption of the past existence of common polypeptide-chain ancestors, controlled by common ancestral genes. At least a few times during evolution an evolutionarily effective duplication, *i. e.*, a duplication that has been spread at least temporarily by natural selection, of either a hemoglobin gene or a chromosome carrying such a gene is thought to have occurred. The resulting daughter genes are considered to have differentiated by independent mutation (Itano[2]; Ingram[3]; Zuckerkandl and Pauling[4]).

On the basis of this hypothesis, the degree of difference between two homologous polypeptide chains is a measure of the relative time at which the common ancestor of the structural genes controlling these chains existed, and it is also, within large limits of error, a measure of this time in absolute units (Zuckerkandl and Pauling[4]). We now direct attention to two further types of information that can be derived from the comparison of different homologous polypeptide chains. First, it is possible to determine, with some probability, the amino-acid sequence of their presumed common polypeptide-chain ancestor. Second, when two polypeptide chains do not possess the same amino-acid residue at a certain

* On leave from Centre National de la Recherche Scientifique, Paris.
** Contribution No. 2957.

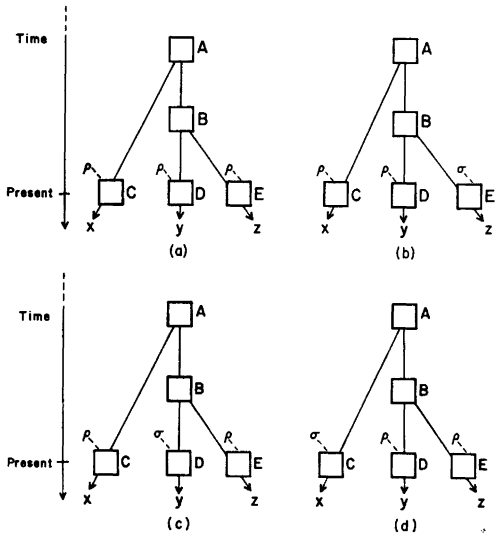


Fig. 1. Different evolutionary relationships of the amino-acid residues ϱ and σ found at corresponding sites in the homologous polypeptide chains C, D, and E. See text.

molecular site, it is possible to determine in which line of descent the mutation responsible for this difference has occurred since the epoch of the common ancestor.

If the amino-acid residue is the same in two chains at a given molecular site, there is a certain likelihood that this residue was also present in the common ancestor of the chains*. If three or more chains are compared and the same residue is found at corresponding sites in two or more chains whereas it differs in one chain, there is a certain likelihood that the mutation responsible for the difference occurred in the line of descent leading to the chain showing this difference, and that it occurred since the time of the most recent molecular ancestor that can be assumed to relate the variant chain to one of the others. The further apart the organisms from which homologous polypeptide chains are derived, the more significant are the identities found, not only because they then suggest that the residue at the molecular site considered has remained the same for a long evolutionary time and therefore must have an important function within the molecule, but also because in widely different forms the occurrence of convergence at the molecular level appears to us to be a negligible possibility.

In Figure 1 three lines of molecular descent, x, y, and z, are represented, and the respective common molecular ancestors at two branching points are A and B. Squares stand for related polypeptide chains. Two different amino-acid residues, ϱ and σ, are considered for one given molecular site in the homologous polypeptide chains, C, D, and E. These chains are located on the time scale at a

* Convergence effects may possibly intervene under some circumstances even at the molecular level, but are not considered to be likely to affect an important proportion of amino-acid residues in polypeptides.

level representing present time. The following probable statements can be made about the four situations pictured: Situation (a): the common chain-ancestors A and B had residue ϱ at the molecular site under consideration. Situation (b): the chain ancestors A and B had residue ϱ; a mutation to residue σ has occurred in evolutionary lineage z after it became distinct from evolutionary lineage y. Situation (c): the chain ancestors A and B had residue ϱ; a mutation to residue σ occurred in evolutionary lineage y after lineage z branched off from lineage y. Situation (d): the chain-ancestor B had residue ϱ, but on the basis of the evidence the nature of the residue in chain-ancestor A is undetermined; further related polypeptide chains have to be drawn into the comparison to permit a conclusion to be reached. The probability of correctness of the other deductions can be increased by use of information about other chains.

Three out of the four types of chains that make up human hemoglobin molecules (there are four chains of two types per molecule), namely the α-, β-, and γ-chains, have been defined by their N-terminal sequence (Schroeder[5]). This sequence may change partially or totally during evolution, and yet the structural genes that control it may still be homologous. We need to be able to refer to homologous structural genes irrespective of the actual amino-acid sequence of their polypeptide products. Let us propose, therefore, to speak of the I^{α}, II^{β}, III^{γ}, and IV^{δ}-hemoglobin-chain genes and the corresponding polypeptide chains, the superscript representing in each case the reference for homology considerations. The common ancestor of two or more of these genes is then designated by juxtaposition of the symbols referring to the genes derived by duplication. Thus, the II^{β}–IV^{δ}-gene is the common ancestor of the II^{β}-gene and the IV^{δ}-gene; the II^{β}–III^{γ}–IV^{δ}-gene is the common ancestor of the III^{δ}-gene and the II^{β}–IV^{δ}-gene; and the I^{α}–II^{β}–III^{δ}–IV^{δ}-gene is the common ancestor of the I^{α}-gene and the II^{β}–III^{γ}–IV^{δ}-gene (Fig. 2).

Tables 1a, 1b, and 1c illustrate the procedure by presenting two partially and tentatively reconstructed ancestral hemoglobin polypeptide chains. The evidence on which these reconstructions are based are the human α-, β-, γ-, and δ-chains, the sperm-whale myoglobin chain (see Schroeder[7] for references and latest information on the structure of these chains), the horse α-chain (Braunitzer and Matsuda, in: Cullis *et al.*[8]), and the human myoglobin chain (Hill[9]). One of us (L. P.[10]) has listed earlier probable residues for 70 loci in the common precursor

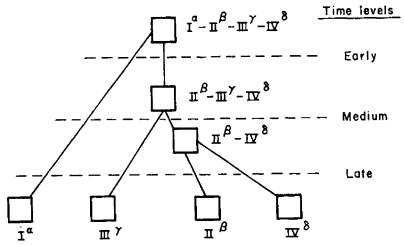


Fig 2. Schematic representation of the successive gene duplications that are presumed to have lead to the hemoglobin genes found in man. *Cf.* Ingram[3]. See text.

Table 1 a. Tentative partial structure of two chain-ancestors of the human hemoglobin polypeptide chains. The numbering of the residues is the one usually applied to the human α-chain. The abbreviations for the amino acids are those commonly employed, except for asparagin (asg) and glutamine (glm). Other abbreviations: E = early epoch; M = medium epoch; L = late epoch; abs = absent. "None", in column (e), means: probably no evolutionrily effective mutation occurred at the site under consideration in the line of descent leading from the ancestral genes to the human genes. The residues and comments are placed in parentheses when the conclusion reached is partly based on the consideration of human and sperm whale myoglobins.

(a) Residue number
(b) Partial sequence of the II^{β}–III^{γ}–IV^{δ}-chain (late form)
(c) Partial sequence of the I^{α}–II^{β}–III^{γ}–IV^{δ}-chain (late form)
(d) Chain(s) in whose direct ancestry the mutation(s) seem to have occurred
(e) Nature of the substitution
(f) Qualitative evaluation of the time of mutation
(g) From evidence relating to a number of different animals (cf. Gratzer and Allison[6])
(h) Not after the time of ancestor common to horse and man
(i) After the time of ancestor common to horse and man
(j) Amino-acid residue x present at a homologous site in the two myoglobins
(k) Polypeptide chain I^{α} or II^{β}–III^{γ}–IV^{δ}

(a)	1	1a	2			5					10		12
(b)	val	his	leu	thr	pro	glu	asp	lys	?	?	val	thr	ala
(c)	val	?	leu	?	pro	?	asp	lys	?	?	val	?	ala
(d)	III^{γ}	(k)	III^{γ}	(k)	III^{γ} and horse I^{α}	(k)	II^{β}—IV^{δ}				III^{γ}	(k)	III^{γ}
(e)	val→gly		leu→phe		III^{γ}: pro→glu; horse I^{α}: pro→ala		asp→glu	none			val→ileu		ala→ser
(f)	M or L	E(g)	M or L	E or M(h)	Man: M or L Horse: (i)	E or M(h)	M					E or M(h)	M or L

(a)	13		15					20					25			28
(b)	leu	try	gly	lys	val	asg	val	abs	abs	?	glu	?	gly	gly	glu	ala
(c)	?	try	gly	lys	val	?	?	?	?	?	glu	?	gly	?	glu	ala
(d)	(k)		I^{α} of horse			(k)	(k)				III^{γ}			(k)		III^{γ}
(e)		none	gly→ser	none	none						glu→asp		none		none	ala→thr
(f)	E or M(h)		(i)			E or M(h)	E or M(h)				M or L			E or M(h)		M or L

Table 1 b. Continuation of Table 1 a. See Table 1 a for legend.

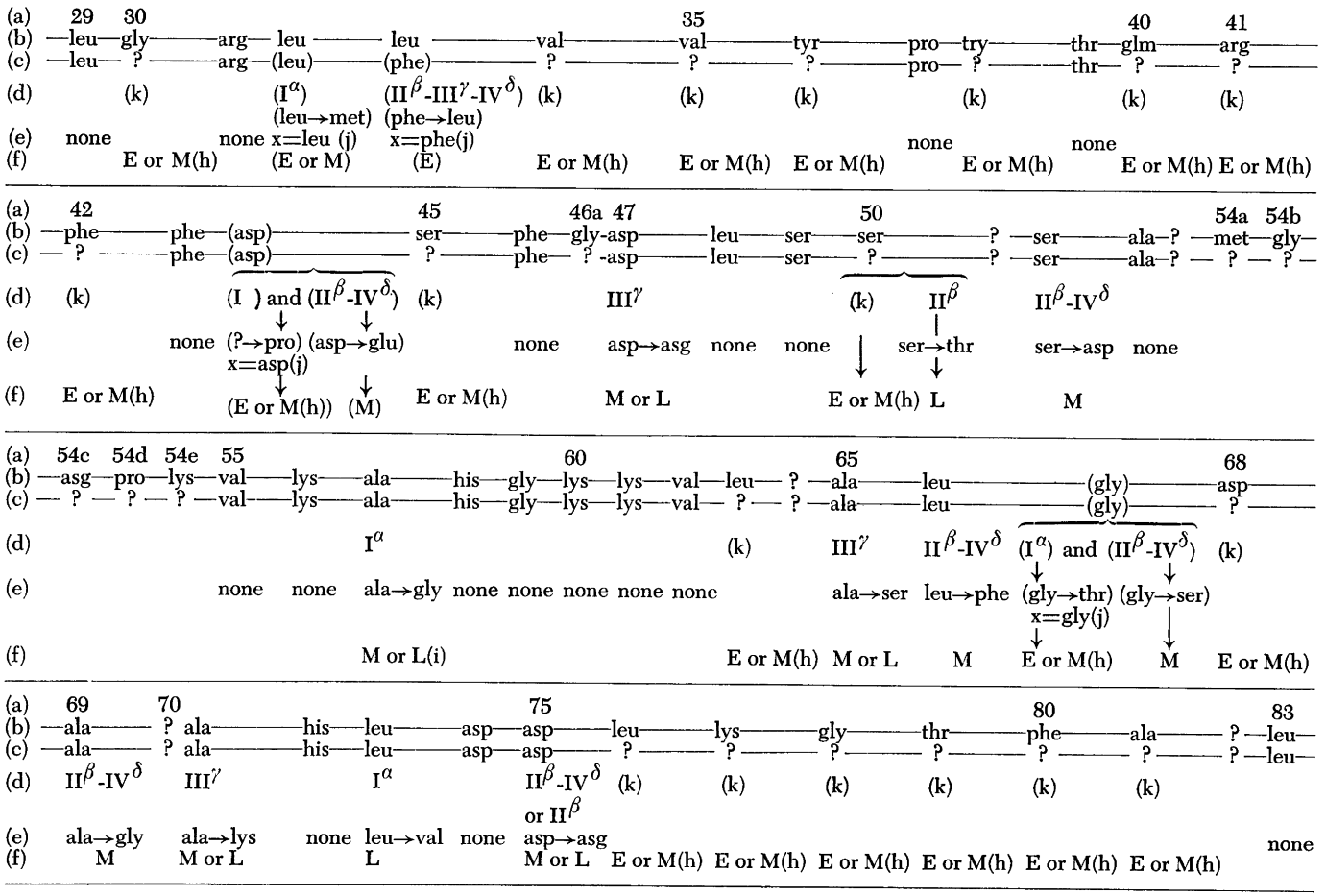


Table 1 C. Continuation of Table 1 a. See Table 1 a for legend.

(a)	84	85					90					95				99
(b)	—ser—	glu—	leu—	his—	cys—	asp—	lys—	leu—	his—	val—	asp—	pro—	glu—	asg—	phe—	lys—
(c)	—ser—	? —	leu—	his—	(ala)—	? —	lys—	leu—	? —	val—	asp—	pro—	? —	asg—	phe—	lys—
(d)		(k)			(II^{β}-III^{γ}-IV^{δ})	(k)			(k)				(k)			II^{β}-IV^{δ} or II^{β}
(e)	none		none	none	(ala→cys) (x=ala (j))		none	none		none	none	none		none	none	lys→arg
(f)		E or M(h)			(E)	E or M(h)			E or M(h)				E or M(h)			M or L

(a)	100					105					110					115
(b)	—leu—	leu—	gly—	asg—	val—	leu—	val—	? —	val—	leu—	ala—	? —	his—	phe—	gly—	lys—
(c)	—leu—	leu—	(ser)—	? —	? —	leu—	? —	? —	? —	leu—	ala—	? —	his—	? —	? —	? —
(d)			(II^{β}-III^{γ}-IV^{δ})	(k)	(k)		(k)		(k)					(k)	(k)	(k)
(e)	none	none	(ser→gly) (x = gly (j))			none				none	none					
(f)			(E)	E or M(h)	E or M(h)		E or M(h)		E or M(h)					E or M(h)	E or M(h)	E or M(h)

(a)	116				120					125					130	
(b)	—glu—	phe—	thr—	pro—	? —	val—	glm—	ala—	ser—	? —	glm—	lys—	? —	val—	ala—	gly—
(c)	— ? —	phe—	thr—	pro—	? —	val—	? —	ala—	ser—	? —	(asp)—	lys—	? —	? —	ala—	? —
(d)	(k)						(k)		II^{β}-IV^{δ} or II^{β}		(II^{β}-III^{γ}-IV^{δ})			(k)	III^{γ}	(k)
(e)		none	none	none				none	ser→ala		asp→glm (x = asp (j))	none			ala→thr	
(f)	E or M(h)						E or M(h)		M or L		(E)			E or M(h)	M or L	E or M(h)

(a)	132	133		135					140	
(b)	—val—	ala—	? —	ala—	leu—	? —	ser—	lys—	tyr—	his
(c)	—val—	? —	? —	? —	leu—	? —	ser—	lys—	tyr—	?
(d)		(k)		(k)			II^{β}-IV^{δ}	III^{γ}		(k)
(e)	none				none		ser→his	lys→arg	none	
(f)		E or M(h)		E or M(h)			M	M or L		E or M(h)

of α-chains and non-α-chains. A qualitative estimate is made of the time at which a given mutation supposedly occurred. (In a majority of cases the changes probably involve more than one substitution, according to the genetic codes proposed by Yukes[11] and by Smith[12]). Three periods are distinguished: early, medium, and late, according to the time off the mutation or mutations in relation to the successive gene duplications (Fig. 2).

Fossil remains no doubt express the activity of only a fraction of the genes of a given organism (although perhaps a significant fraction) and this fraction cannot be analyzed into its components. Paleobiochemistry, through molecular restoration studies on the basis of existing related polypeptide chains, provides the means of investigating the structure of such components for any part of the genome of extinct organisms. This holds, however, only in relation to structural genes, as long as the object of such studies is confined to the polypeptide products rather than extended to the genic material itself. Yet, once the structures of ancestral polypeptide chains are known, it will in the future be possible to synthesize these presumed components of extinct organisms. Thus one will be able to study the physico-chemical properties of these molecules and to make inferences about their functions. For instance, the oxygen affinity and its dependence on pH of ancestral hemoglobins might be studied as well as the affinity of ancestral enzymes for various substrates and the probable nature of these substrates in past evolutionary history. As information about various paleogenes belonging to a given group of extinct organisms will accumulate, some deductions concerning these organisms will be possible in relation to levels of biological integration higher than the level of individual macromolecules. When a fossil record is available, knowledge about the organisms concerned will go far beyond what has so far been believed possible. Important information will also be provided about forms that have left no fossil record whatsoever, such as many soft-bodied animals.

When a gene-duplication has occurred and one of the duplicate genes is not needed for carrying out the function for which its partner suffices, the polypeptide chain synthesized under its control may undergo a change in structure, including a change in spatial conformation, and the latter change especially may sometimes lead to the appearance of a new function. If the new function is retained by natural selection, it appears likely that during the subsequent evolutionary period many more mutational changes will be preserved by the corresponding gene than by genes whose functions have long been established. The latter genes are submitted to forces of selection that are conservative most of the time. Two reasons may be advanced for a more rapid alteration of the amino-acid sequence in a conformationally and functionally altered polypeptide: First, a newly evolved function is likely to be perfectible through further structural changes; second, while over very long periods of evolution the amino-acid sequence of homologous polypeptide chains can be deeply transformed even when the spatial conformation is kept nearly constant, as the comparison of myoglobin and hemoglobin suggests, a faster transformation is to be expected when the spatial conformations differ, since in that case the residues required to remain unchanged in order to preserve a given spatial conformation are not any more the same in the precursor polypeptide and in the polypeptide derived

from it. Can paleobiochemical studies be anticipated to trace the filiation between genes that control polypeptides endowed with different functions? In a polypeptide whose function has been replaced by another one the amino-acid sequence may eventually be modified to such an extent that it becomes unrecognizable in terms of the molecular ancestor. Thus the comparison of the amino-acid sequences in contemporary polypeptide chains endowed with different functions probably will lead only in a minority of cases to the detection of the evolutionary relationships that actually obtain, *viz.*, mainly when the acquisition of a different molecular function by a polypeptide has been relatively recent. In such cases convincing homologies in amino-acid sequence will still be found, although the spatial conformations of the proteins may differ. Even then the discovery of polypeptides related by evolution but functionally different might appear to involve either an unlikely coincidence or a formidable task. This task could, however, be substantially reduced, if a systematic investigation were made of the amino-acid sequences in polypeptides that have been shown by genetic studies to be controlled by closely linked genes, notably by genes controlled by a common operator gene (Jacob and Monod[13]). The functions carried out by the genes included in a given operon (the unit controlled by an operator gene) can be quite diversified, and it is to be expected that the spatial conformations of the corresponding polypeptides are diverse also. Yet, since at least some genes derived through the duplication of a mother gene will probably remain closely linked during a considerable evolutionary time and since some of them may change in their function, the chances of discovering homologies between apparently unrelated proteins are likely to be greatest in a survey of the amino-acid sequences of polypeptides controlled by closely linked genes. An effort in that direction may result in a worthy contribution to the theory of evolution, in that it might show that even apparently unrelated proteins can indeed have a common molecular ancestor. Thus suggestive evidence would be furnished in support of the view that most or all apparently heterologous genes derive ultimately from a common gene-ancestor.

REFERENCES

1. Braunitzer, G., Hilschmann, N., Rudloff, V., Hilse, K., Liebold, B. and Müller, R. *Nature* **190** (1961) 480.
2. Itano, H. A. *Adv. Protein Chem.* **12** (1957) 216.
3. Ingram, V. M. *Nature* **189** (1961) 704.
4. Zuckerkandl, E. and Pauling, L. In Kasha, M. and Pullman, B. *Horizons in Biochemistry*, Academic Press, New York and London, 1962, p. 189.
5. Schroeder, W. A. *Fortschr. der Chem. Org. Naturstoffe* **17** (1959) 322.
6. Gratzer, W. B. and Allison, A. C. *Biol. Rev. Cambridge Phil. Soc.* **35** (1960) 459.
7. Schroeder, W. A. *Ann. Rev. Biochem.* **32** (1963). *In press.*
8. Braunitzer, G. and Matsuda, G. *Cf.* Cullis, A. F., Muirhead, H., Perutz, M. F. and Rossmann, M. G. *Proc. Roy. Soc. London* **A 265** (1962) 161.
9. Hill, R. *Personal communication* (1962).
10. Pauling, L., communicated at Hemoglobin Workshop, Arden House, Columbia University, Nov. 1962. *Cf.* Ingram, V. M., Richards, D. W. and Fishman, A. P. *Science* **138** (1962) 996.
11. Jukes, T. H. *Proc. Natl. Acad. Sci. U. S.* **48** (1962) 1809.
12. Smith, E. *Proc. Natl. Acad. Sci. U. S.* **48** (1962) 859.
13. Jacob, F. and Monod, J. *J. Mol. Biol.* **3** (1961) 318.

Received March 29, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S17–S22

Life Span of Tissue Cells

G. HEVESY

Institute of Organic Chemistry and Biochemistry, University, Stockholm, Sweden

The life cycle of tissue cells, for example those of the liver, is determined by measuring the rate of incorporation of ^{32}P, ^{14}C, of ^{3}H-thymidine or other suitable markers into DNA of the cells. An alternative method is the measurement of the rate of loss of the labelled DNA and thus of labelled cells from a uniformly labelled organ. Both methods and the results obtained by them are discussed.

Besides expressing his cordial wishes on this memorable day for the happiness and further success of Hugo Theorell, the author wishes to contribute to this volume by a discussion of the problem of the life span of tissue cells.

At the end of the thirties J. Ottesen and the present writer were interested in the determination of the life span of erythrocytes by labelling these with a stably adhering radioactive marker. The disappearance of the radioactivity should in such a system indicate the end of the life span of the erythrocyte. The time however which ^{32}P, ^{24}Na, ^{42}K and some other radioactive tracers, which were at that time at our disposal, spend in non-nucleated red corpuscles is much shorter than is the life span of the latter, correspondingly we had to abandon our plan to determine the life span of the mammalian red corpuscle by the above mentioned method. Earlier investigations carried out by us suggested that the radioactive label attached to the DNA of nucleated red corpuscles is stable and we therefore concentrated our interest on the determination of the life span of the nucleated erythrocytes of the hen[1]. The plasma orthophosphate of the hen was kept on a constant specific activity level and at intervals the latter was compared with the specific activity of the DNA phosphorus of the erythrocytes. After the lapse of about 1 month the two specific activities were found to be equal indicating that the full renewal of the erythrocyte DNA and thus of the erythrocytes took place in the course of about 1 month. One year later Rittenberg and Shemin[2] found in the stable ^{15}N a very suitable tracer of the hemin of hemoglobin and determined in a classical investigation the life span of the red corpuscle of the man to be about 120 days. When I shortly afterwards mentioned our results with Ottesen at the Solvay Congress in Brussels I met an incredulous audience.

The objection to our results (an entirely unjustified one) was that if the red corpuscle of the man prevails for 120 days the nucleated red corpuscle of the hen should live longer than 120 days, thus very much longer than 30 days. Soon after Rittenberg repeated our experiments using ^{15}N as a tracer and corroborated our results. After the availability of ^{14}C, ^{15}N was in most cases replaced by the first mentioned isotope in life span determinations of erythrocytes. Several other methods of determination of life span became available as well. In a pioneer work Paul Hahn and collaborators[3] observed that ^{59}Fe released from the dying red corpuscle is reutilized in the formation of new erythrocytes, thus ^{59}Fe could not be used as a tracer in the determination of the life span of red corpuscles. When much later ^{59}Fe of very high specific activity became available it was shown that about 2 % of the ^{59}Fe released by the dying erythrocyte is not re-utilized, or not reutilized within a reasonable time, in the formation of new red corpuscles. It became now possible to use even ^{59}Fe as a tracer in the life span determinations of red corpuscles. Nowadays mostly ^{51}Cr and DF ^{32}P are used for that purpose. All these methods found a far-reaching application and we have today quite an extended knowledge of the life span of the red corpuscle both under physiological and pathological conditions. We have very much less knowledge on the life span of tissue cells. In determining the life span of erythrocytes we introduce in several of the methods applied a labelled component into the heme during its formation which is renewed only when the erythrocyte is destroyed. In the determination of the life span of nucleated erythrocytes an alternative method is the introduction of a labelled component into the DNA molecule of the nucleus in the course of its formation. The latter procedure is the only one which can be applied in the determination of the life span of tissue cells. The DNA molecules of the latter, apart from a few very special cases, are not renewed during the life of the cell. The disappearance of the label of the DNA molecule indicates thus the termination of the life span of the tissue cell. The human red corpuscles have the same life span apart from a small fraction which is decaying at random. In the liver, for example, we find cells of very different life time. The parenchymal cells live longer than the reticulo-endothelial cells and indications are not lacking that even within these two groups we find cells of different life time. It therefore encounters difficulties to state the life time of liver cells. We can however state after the lapse of a day what fraction of those originally present are still present or of those present after 100 days how many still prevail after the lapse of 200 days and so on.

The first investigation in which the incorporation of ^{32}P into DNA was studied by the present writer and J. Ottesen[4] brought out the great difference in the rate of incorporation of ^{32}P into DNA and into the acid-soluble components of the liver of the fully grown rat. The latter takes place at a fairly rapid or even very rapid rate, the former at a very slow rate only. In the above mentioned experiments in the course of 4 days about 4 % only of the liver cells of the fully grown rat were found newly formed. These results were interpreted as indicating that while ^{32}P incorporation into acid-soluble components of the liver is due to a molecular renewal that into DNA is to be ascribed to a new formation of tissue cells. The rate of cell formation can thus be easily measured. Possible errors of the method will be discussed later.

Shortly afterwards Lucie Ahlström, Hans von Euler and the present writer[5] carried out experiments taking 2 hours only and found a 0.13 % renewal. Hammarsten and the present writer[6] found a renewal rate of 0.105 % of the DNA molecule of the liver in experiments taking 2 hours. These values are somewhat larger than those found in experiments taking 4 days, where the corresponding (2-hour) value was 0.08 only. The incorporation of ^{32}P into DNA takes place 5–7 hours during the late interphase. In the experiments taking 2 hours we measure not only labelled finally formed cells but also such in the late interphase, while in the experiments taking 4 days happenings during 5–7 hours can almost be disregarded. Differences as mentioned above are to be expected as we have different types of liver cells and these are renewed at different rates. As the most labile ones are already replaced by labelled cells in experiments of short duration, a prolonged experiment cannot be expected to lead to as high per hour renewal figures as found in experiments of short duration. The renewal rate was in all the above investigations assumed to be equal to the ratio of specific activity of the DNA phosphorus at the end of the experiment and the mean value of cellular orthophosphate phosphorus during the experiment. It was assumed that the extracellular labelled orthophosphate first mixes with the intracellular ortophosphate and participates then in the synthesis of organic phosphorus compounds. This assumption can however not be maintained. Ahlström and associates[5] when determining the rate of formation of liver cells in newly born (4 day old) rapidly growing rats found a very high formation rate, 2 % in the course of 2 hours. In the strongly growing rat we can determine the weight increase of the liver in the course of the experiment and calculate, under the assumption that the DNA content increases proportionally with the weight of the organ, the DNA increase in the course of 2 hours. This was found to make out 0.7 times only the figure calculated from radioactive data. As a possible explanation of this difference the view was put forward that beside a formation of labelled, thus new, cells some turnover of DNA molecule takes place as well, which is partly responsible for the high ^{32}P incorporation into DNA. Soon, however, it was found that this assumption cannot be maintained as a renewal of the DNA molecule only takes place in some very special cases. The difference between the measured increase in the DNA content and that calculated from radioactive data is due to another reason than to a turnover of the DNA molecule. It is due to an erroneous assumption when carrying out the calculation from radioactive data. The extracellular ortophosphate does not first mix with the cellular orthophosphate and is then incorporated into organic phosphorus compounds as assumed in the above consideration, but the synthesis of these starts at once after intrusion of the labelled orthophosphate into the cell. The mean specific activity of the reacting orthophosphate is thus larger than assumed when carrying out the calculation leading to too high formation figures and the ratio calculated correspondingly smaller. Thus the percentage of DNA formed in the liver of 4 days old rats in the course of 2 hours is not 2 but $0.7 \times 2 = 1.4$. Possible errors involved in the method described when applied to the measurement of the rate of formation of the liver cells are: a) That even in the fully grown animal some growth takes place, thus the formation of some of the labelled DNA molecules is not due to replacement but to additional formation of cells. b) Polyploid nuclei

may be formed in the course of the experiment. In this case the formation of some of the labelled DNA molecules is not due to the synthesis of new cells but to an increase in the DNA content of cells already present. When determining the rate of renewal of tissue cells it is therefore preferable not to measure the rate of labelling of DNA but the rate at which the DNA label and thus the labelled cells are lost.

Measurement of the rate of loss of labelled tissue cells

Tissue cells are renewed and thus labelled at a very different rate, which for many liver cells is so slow that the life time of the mouse does not suffice to achieve a labelling of a part of the cells. We can, however, label pregnant mice with the result of a uniform distribution of the label in the organs of the offspring taking place. We take, for example, a group of mice the DNA of which is labelled with ^{14}C in utero when 50 days old, another when it reached an age of 100 days and determine the ^{14}C content of the DNA of the liver in both groups. The difference in the ^{14}C content of the DNA of the liver of the two groups indicates the DNA loss and thus the cell loss in the course of 50 days. This method was applied by Forssberg, Dreyfus and the present writer[7]. 20 pregnant rats were labelled by injecting 10 μC of adenine-2-^{14}C. The results of experiments in which the first group is taken 30 days, the second 116 days post partum is seen in Table 1.

Table 1. Daily percentage loss of DNA purine ^{14}C by the liver of mice between 30 days post partum and 116 days post partum.

Liver	Spleen	Brain
0.44	0.42	0.21

A possible error of experiment which would result in a reduction of the measured daily DNA purine ^{14}C loss is the reutilization of the released purine ^{14}C. As the labelled purines will have to compete with the non-labelled ones present, furthermore RNA is a much more powerful competitor for adenine than is DNA, the formation rate of labelled RNA molecules making out more than 30 times that of labelled DNA molecules[6], the probability of reutilization of released purine ^{14}C is a restricted one. Cronkite *et al.*[8] found released labelled thymidine not to be reincorporated into DNA.

The half life of the liver cells of the mouse works out from Table 1 to be 114 days. However this is a resultant of different life times. What we can conclude is that out of 100 cells present in the liver at 30 days post partum 86 days later only 62 are present. In the course of the first 30 days of our experiments with growing mice the percentage daily liver cells increase made out 3 %. Later the daily increase more and more slowed down. Hand in hand with this increase due to growth a daily loss of about 0.5 % of the labelled liver cells took place. The latter get replaced by new-formation. We know from much evidence obtained from studies of partially hepatectomized mice and other animals that loss of liver cells is followed by a replacement. In the rat 32 hours after partial hepa-

tectomy an intense new formation of cells can be observed, the liver regaining ultimately its original size. It is probable that the liver cells removed in the course of phagocytosis or by physiological death are replaced by a similar mechanism as the cells removed by surgical interference. As to the mechanism of the replacement of the through hepatectomy removed cells different views were put forward. The following explanation is that of Paul Weiss[9].

Compensation of the removed liver cell

Weiss assumes that the growth rate is proportional to the concentration of an intracellular specific catalyst, a "template". Under normal conditions the compounds remain confined within the cell. Each cell also produces "antitemplates" to this compound which can inhibit the former species by combining with them into inactive complexes. The "antitemplates" in contrast to the templates are released from the cell and get into the extracellular space and into circulation. They carry the specific tag of their producer cell type which endows them with selective affinity for any cell of the same type. They are in constant production so as to make up for their extracellular decomposition and final excretion. If a part of the liver is removed, less "antitemplate" will be formed and reach the circulation. Correspondingly the liver cells are now exposed to less circulating "antitemplates" than was the case prior to hepatectomy and they shall start to divide. This will result in an accumulation of additional amounts of "antitemplate" which in turn shall more and more inactivate "templates". Growth rate will decline in all cells belonging to that particular strain bathed by the common humoral pool. When stationary equilibrium between intracellular and extracellular concentration is reached, growth will cease. While the hepatectomized liver grows until the original size is reached but not further, an increase in the size of the undamaged liver of one partner of a pair of parabiosed rats following removal of the liver of the other partner takes place. Furthermore an increase in the mitotic figure of the livers of normal rats injected intraperitoneally with serum from partially hepatectomized animals is observed. The "growth-stimulating" effect of embryo extract on the proliferation of tissue cultures and also of organs demonstrated by an increased incorporation of ^{32}P into DNA of various organs[10], is accounted for by the fact that, since embryo extract contains cell debris of all organs the growth of any tissue explanted in it would be favoured.

Formation and loss of liver cells

A possible explanation of the observation that in growing liver a not negligible daily cell loss takes place, is indicated by results obtained in the investigation of the ^{32}P incorporation into the DNA of the liver cells under toxic conditions. Kelly *et al.*[11] studied the effect of endotoxins on the rate of formation of labelled DNA, so did Meissner[12] that of tubercle bacteria. The rate of formation of labelled cells was found appreciably accelerated in these cases, the increased cell formation being partly due to the compensation of cell loss.

It is quite possible that even in the normal mice investigated toxins coming from the intestine or from other sites get partly phagocytised by liver cells which

get in the course of the phagocytic process destroyed and then rebuilt. A cell loss observed would thus be a result of elimination of toxins. The possibility can however not be excluded that the loss and following renewal of liver cells is a physiological process showing some resemblance to molecular renewal. The results of experiments with bacteria free mice may decide between these two alternatives.

REFERENCES

1. Hevesy, G. and Ottesen, J. *Nature* **156** (1945) 689.
2. Shemin, D. and Rittenberg, D. *J. Biol. Chem.* **166** (1946) 627.
3. Hahn, P., Bale, W. F. and Balfour, W. M. *Amer. J. Physiol.* **135** (1942) 600.
4. Hevesy, G. and Ottesen, J. *Acta Physiol. Scand.* **5** (1943) 237.
5. Ahlström, L., v. Euler, H. and Hevesy, G. *Arkiv Kemi* **19 A** (1944) No. 9.
6. Hammarsten, E. and Hevesy, G. *Acta Physiol. Scand.* **11** (1946) 335.
7. Forssberg, A., Dreyfus, G. and Hevesy, G. *Arkiv Kemi* **18** (1961) No. 11.
8. Cronkite, E. P., Bond, V. P., Fliedner, P. M. and Rubini, J. R. *Scand. J. Clin. Lab. Invest.* **8** (1959) 263.
9. Weiss, P. *Biological Specificity and Growth,* Princetone University Press, Princetone, 1955, p. 195.
10. Payne, A. H., Kelly, L. S. and Jones, H. B. *Cancer Res.* **122** (1952) 661.
11. Kelly, L. S., Dobson, E. L., Finney, C. R. and Hirsch, J. D. *Amer. J. Physiol.* **198** (1960) 1134.
12. Meissner, J. *Arch. Exptl. Pathol. Pharmakol.* **237** (1959) 104.

Received February 9, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S23–S26

The Metal Ion Status of Rat Liver Lactic Dehydrogenase

C. S. VESTLING, W. T. HSIEH*, H. TERAYAMA**
and J. N. BAPTIST***

Biochemistry Division, University of Illinois, Urbana, Illinois, USA

Earlier experiments indicated that pure rat liver lactic dehydrogenase (LDH) was not strongly inhibited by various metal chelators. The present report supplies analytical data obtained by emission spectrographic analyses and demonstrates that this particular LDH contains only small and variable amounts of several metal ions, depending upon previous treatment. The effects of several metal chelating agents are described.

Experiments which provide information about the catalytic reaction sites of enzymes are of the greatest interest. In the case of highly purified dehydrogenases from various sources, the suggestion has been made by Vallee and coworkers[1,2] and by others[3–6] that a divalent cation at the reaction site may play an important role in the catalysis. This role is usually considered to involve chelation of coenzyme or substrate on the reaction site. If this suggestion is valid, one would expect that a pure dehydrogenase would show at least one mole of metal ion per mole of reaction sites. Examples of this kind of relationship have been reported for yeast alcohol dehydrogenase[7,8], horse liver alcohol dehydrogenase[9,10], rabbit muscle lactic dehydrogenase[11], and beef liver glutamic dehydrogenase. A contrary point of view with respect to a role for zinc in pig heart, rat and rabbit skeletal muscle lactic dehydrogenases was presented by Pfleiderer, Jeckel and Wieland[13]. These investigators found no appreciable inhibition of these particular dehydrogenases by metal chelating agents and no significant amounts of zinc after ashing.

It is, therefore, a matter of interest to examine another pure dehydrogenase and determine its metal ion status.

EXPERIMENTAL AND RESULTS

Emission spectrographic analyses

Rat liver LDH was prepared by either the procedure of Gibson *et al.*[14] or more recently by the modified method of Hsieh and Vestling[15]. Preparations of maximum specific activity were

* In part from the Ph. D. thesis of W. T. Hsieh (1963).
** Present address: Department of Biochemistry, University of Tokyo, Tokyo, Japan.
*** In part from the Ph. D. thesis of J. N. Baptist (1957). Present address: Research Laboratories, W. R. Grace and Company, Clarksville, Maryland.

used. Davisson *et al.*[16] described the physico-chemical characterization of maximally pure LDH. The more convenient recent isolation method[15] involves (1) extraction of fresh or frozen rat liver with 0.5 M NaCl – 20 % ethanol; (2) ammonium sulfate fractionation; (3) ethanol fractionation; (4) carboxymethyl cellulose column fractionation with ionic strength gradient elution in the presence of 0.001 M 2-mercaptoethanol, pH 6.0; and (5) DEAE-cellulose column fractionation in 0.02 M phosphate – 0.001 M 2-mercaptoethanol, pH 7. From these steps recoveries of approximately 35 % of extractable LDH are realized, and the crystalline enzyme shows catalytic and physico-chemical properties identical to those of the pure LDH obtained by the Gibson[14] procedure.

In preparation for the metal content studies reagent grade ammonium sulfate and potassium chloride were recrystallized twice from 0.001 M Versene-Na_2 (disodium ethylenediamine tetraacetate). Glass-distilled, deionized water was used throughout, and glassware was soaked overnight in acid dichromate solution and thoroughly rinsed.

LDH of maximum purity prepared according to Gibson *et al.*[14] was treated as follows[17]: First it was salted out with purified ammonium sulfate and then dissolved in 0.1 M KCl and dialyzed on a rocking dialyzer at 4°C against 20 to 50 volumes of 0.1 M KCl with four changes during a 24-hour period. The first two dialyzing solutions were 0.1 M KCl – 10^{-4} M Versene-Na_2. After dialysis the LDH retained its maximum specific activity. This preparation was sent to Dr. B. L. Vallee, Biophysics Research Laboratory, Peter Bent Brigham Hospital, Boston, for emission spectrographic analysis by the porous cup spark excitation procedure. The authors are deeply indebted to Dr. Vallee for this assistance. The results, shown in Table 1, Column 1, indicate the presence of fractional molar quantities of Zn, Fe and Al and larger quantities of Mg and Ca (See Reference 1, p. 254.). Rat liver LDH has a molecular weight of 126 000[16] and shows two binding sites for NAD^+ or NADH per mole[18–21]. Accordingly, it would appear in the case of this particular LDH that Zn, Fe and Al are not involved in stoichiometric quantities at the catalytic site. The status of Mg and Ca will be clarified with the presentation of the data in Columns 2 and 3 in Table 1.

Recently pure LDH has been prepared by the Hsieh-Vestling procedure and subjected to the following steps. First, it was precipitated by the addition of purified ammonium sulfate and then dissolved in 0.05 M potassium phosphate-

Table 1. Rat liver lactic dehydrogenase. Emission spectrographic analyses.

	Moles of metal per mole of LDH (See text)		
Metal	1	2	3
Zn	0.37	0.35	0.37
Mg	4.03	7.05	1.35
Ca	10.0	10.0	1.32
Fe	0.38	Trace	Not detected
Al	0.41	Not detected	,, ,,
Sr	0.06	,, ,,	,, ,,
Ba	0.03	,, ,,	,, ,,
	Present but not determined: Na, K Not detected: Cd, Co, Cr, Mn, Mo, Ni, Pb, Sn, P Not determined: Cu	Trace: Cu Not detected: Na	Trace: Cu

0.001 M Versene-Na_2 – 0.001 M 2-mercaptoethanol, pH 7.6 and dialyzed for 12 hours at 4°C against three changes of 20–50 volumes of the same solution (rocking dialyzer used in all cases). Then it was dialyzed for 12 hours at 4°C against three changes of 0.1 M KCl. The dialyzed solution was assayed and then dried to constant weight in a platinum crucible (desiccator and 100°C oven). The weight of protein was obtained by subtracting the weight of KCl. The dried sample was submitted to Dr. S. W. Melsted and H. L. Motto, Department of Agronomy, University of Illinois, for emission spectrographic analysis. The authors are grateful for this assistance. The dried sample was ignited at 400°C overnight, and a rotating disk electrode with spark excitation was used. The results are presented in Table 1, Column 2. It is apparent that this LDH sample contained about the same quantities of Zn and Ca and more Mg than the earlier sample.

In order to see if further attempts to remove metal ions without loss of enzyme activity would be successful the following experiment was carried out: Pure LDH prepared by the Hsieh-Vestling method was precipitated by the addition of ammonium sulfate and dissolved in 0.05 M potassium phosphate – 0.001 M 2-mercaptoethanol, pH 7.6. The solution was dialyzed for 12 hours at 4°C against three changes of 20–50 volumes of 0.05 M potassium phosphate–10^{-4} M 1,10-phenanthroline, pH 7.6. The dialysis was continued for 12 hours against three changes of 0.05 M potassium phosphate – 0.001 M 2-mercaptoethanol–10^{-4} M Versene-Na_2, pH 7.6. A third 12-hour dialysis was carried out in the same way against 0.1 M KCl before submitting the sample to the Agronomy Department for analysis. Enzyme assays were varried out at each dialysis step. The specific activity of the pure LDH prior to dialysis was 9.15×10^4 μmoles NADH/min/mg protein; this value fell to 8.4×10^4 at the end of the dialysis. The total recovery of LDH during the dialysis steps was 92 %. The results shown in Table 1, Column 3, indicate a rather tenacious retention of a fractional molar quantity of Zn and sharply lowered levels of Mg and Ca. Again the conclusion must be drawn that Zn is probably not involved at the reaction site and that all three metals can be reduced to low levels of concentration without any effect on enzyme activity.

Terayama and Vestling[20] reported that several chelating agents were without marked effect on rat liver LDH activity. These agents included: Versene, 1,10-phenanthroline, 8-hydroxyquinoline, and α,α'-dipyridyl. Diethyldithiocarbamate, pyrophosphate, citrate, and salicylate were equally ineffective. Even after pre-incubation for one to three hours relatively slight inhibition – if any – was observed. In Table 2, data of this kind are presented. It is of interest to note that phenanthridine (5-aza-phenanthrene) is a very weak inhibitor; it is also not a chelator. This result is in contrast to that reported by Yielding and Tomkins[22] for glutamic dehydrogenase.

Effect of metal chelators

Terayama and Vestling[20] also noted that hydrosulfide ion inhibition of LDH could not be interpreted as evidence for metal ion participation in the catalysis, but rather that hydrosulfide ion inhibition could be reversed by any process which involved the removal or displacement of the SH^- ion in its apparent interaction with NAD^+ bound to the LDH reaction site.

Table 2. Effect of various agents on rat liver LDH Activity measured under standard assay conditions from the lactate side. Each cuvette contains: Sodium D,L-lactate, 0.11 M; sodium diethyl barbiturate, 0.03 M; NAD^+, 2×10^{-4}; LDH, approximately 10^{-9} M; pH 8.6; 25°C.

Reagent	Concentration M	Inhibition %
Versene-Na_2	10^{-2}	0
1,10-Phenanthroline	10^{-3}	5 to 10
,,	$10^{-2.5}$	10 to 20
Phenanthridine	10^{-4}	5 to 10
Diethyldithiocarbamate	10^{-2}	20
$ZnCl_2$	10^{-6}	0
,,	10^{-4}	10
,,	10^{-2}	90

Preliminary studies of the ultraviolet absorption and fluorescence spectra of pure LDH in combination with 1,10-phenanthroline and/or Zn^{++} support the general conclusion that the residual Zn^{++} which remains in the LDH molecule is available for interaction with 1,10-phenanthroline.

The authors are privileged to contribute this report to a collection of papers in honor of Professor H. Theorell, in whose laboratory one of them (C. S. V.) spent a very interesting period of investigation. These studies have been supported by a grant (C-1856) from the *National Cancer Institute* of the *U. S. Public Health Service.*

REFERENCES

1. Vallee, B. L. In Boyer, P. D., Lardy, H. and Myrbäck, K. *The Enzymes,* Academic Press, New York, 1960, Vol. 3, p. 241.
2. Vallee, B. L. *Federation Proc.* **20** (1961) 71.
3. Theorell, H. *Federation Proc.* **20** (1961) 967.
4. Plane, R. A. and Theorell, H. *Acta Chem. Scand.* **15** (1961) 1866.
5. Wallenfels, K. and Sund, H. *Biochem. Z.* **329** (1957) 48.
6. Wallenfels, K. and Sund, H. *Biochem. Z.* **329** (1957) 59.
7. Vallee, B. L. and Hoch, F. L. *J. Am. Chem. Soc.* **77** (1955) 821.
8. Vallee, B. L. and Hoch, F. L. *Proc. Natl. Acad. Sci. U. S.* **41** (1955) 327.
9. Theorell, H., Nygaard, A. P., and Bonnichsen, R. *Acta Chem. Scand.* **9** (1955) 1148.
10. Vallee, B. L. and Hoch, F. L. *J. Biol. Chem.* **225** (1957) 185.
11. Vallee, B. L. and Wacker, W. E. C. *J. Am. Chem. Soc.* **78** (1956) 1771.
12. Vallee, B. L., Adelstein, S. J. and Olson, J. A. *J. Am. Chem. Soc.* **77** (1955) 5196.
13. Pfleiderer, G., Jeckel, D. and Wieland, Th. *Biochem. Z.* **330** (1958) 296.
14. Gibson, D. M., Davisson, E. O., Bachawat, B. K., Ray, B. R. and Vestling, C. S. *J. Biol. Chem.* **203** (1953) 397.
15. Hsieh, W. T. and Vestling, C. S. *Biochem. Prep.* **11** (1964) *In press.*
16. Davisson, E. O., Gibson, D. M., Ray, B. R. and Vestling, C. S. *J. Phys. Chem.* **57** (1953) 609.
17. Baptist, J. N. *Ph. D. Thesis,* University of Illinois, 1957.
18. Ihnen, E. D. *Ph. D. Thesis,* University of Illinois, 1960.
19. Karnes, C. Y. *Ph. D. Thesis,* University of Illinois, 1960.
20. Terayama, H. and Vestling, C. S. *Biochim Biophys. Acta* **20** (1956) 586.
21. Vestling, C. S. *Methods Biochem. Analy.* **10** (1962) 137.
22. Yielding, K. L. and Tomkins, G. M. *Biochim. Biophys. Acta* **62** (1962) 327.

Received March 28, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 27–S 33

Thermodynamics and Molecular Kinetics of Liver Alcohol Dehydrogenase

KEITH DALZIEL*

Department of Biochemistry, University of Sheffield, Sheffield, 10, Great Britain

The effect of temperature on the initial rate of the liver alcohol dehydrogenase reaction in the range 8–36°C has been studied at pH 7.1. Heats, entropies and free energies of activation have been calculated from the variation with temperature of the parameters in the initial rate equation for the reaction in both directions. Thermodynamic data have been interpreted in terms of the Theorell-Chance mechanism. A satisfactory thermodynamic balance is demonstrated by comparison with independent data for the overall equilibrium, and the reactions of the enzyme with the coenzymes are discussed in terms of the absolute reaction rate theory.

The foundations of modern kinetic studies of coenzyme-substrate reactions were largely laid in 1951 in the papers by Theorell and Bonnichsen[1] and Theorell and Chance[2] on liver alcohol dehydrogenase. After much uncertainty[3,4], due mainly to the unsuspected effects of impurities in coenzyme preparations[5,6], and on the basis of further theoretical work[7,8], there is now fairly convincing evidence that, provided pure coenzymes are used, initial rate data for liver alcohol dehydrogenase at pH 6.0–9.0 are consistent with the mechanism proposed by Theorell and Chance. This is the limiting case of a compulsory order mechanism in which ternary enzyme-coenzyme-substrate complexes do not limit the maximum rate[2,8]. The chief evidence in its support is as follows: the rate of combination of reduced coenzyme and enzyme measured directly by rapid reaction technique agrees reasonably well with the value calculated from initial rate measurements on the overall reaction[2]; the maximum rate in each direction is equal to the rate of dissociation of the product coenzyme complex calculated from initial rate parameters for the reverse reaction[9]; the same values for maximum rates and for Michaelis constants for coenzymes are obtained with different substrates, which give quite different overall rates in rate-limiting concentrations[4]; values for the dissociation constants of the enzyme-coenzyme complexes calcu-

*Sorby Research Fellow of the Royal Society.

lated from initial rate data are in satisfactory agreement with those obtained by direct equilibrium measurements in absence of substrate[9,10].

In this paper, the variations of initial rate parameters for liver alcohol dehydrogenase with temperature at pH 7.1 are reported, and interpreted on the basis of the Theorell-Chance mechanism.

EXPERIMENTAL PROCEDURE

Crystalline alcohol dehydrogenase was prepared from horse liver and assayed as described previously[11]. Nicotinamide adenine dinucleotide (NAD) was purchased from Sigma Chemical Company ("β-DPN, 98 %"), and purified by ion-exchange chromatography[12]. The reduced coenzyme ($NADH_2$) was freshly prepared as described elsewhere[5].

Initial rate measurements were made with a recording fluorometer, and detailed descriptions have been given[4] of the apparatus, technique and method of estimation of the kinetic coefficients in the initial rate equation[8]

$$E/v_0 = \Phi_0 + \Phi_1/S_1 + \Phi_2/S_2 + \Phi_{12}/S_1S_2 \quad (1)$$

for the reduction of acetaldehyde (S_2) by $NADH_2$ (S_1), and the coefficients Φ_0' etc. in the analogous initial rate equation for the oxidation of ethanol by NAD.

The fluorescence intensities of both the Perspex fluorescent standard used in the fluorometer and $NADH_2$ in solution vary considerably with temperature, and calibration over the temperature range studied was necessary. The method was described previously[11], and the empirical relation

$$F_T/F_S = F_R = Ac - 0.0055Ac^2$$

was established between the concentration, c, and the fluorescence F_T of an $NADH_2$ solution relative to that of the standard, F_S. The calibration factor dF_R/dc, required to convert the measured initial rate of change of fluorescence dF_R/dt, into rate of change of $NADH_2$ concentration dc/dt, is

$$dF_R/dc = A - 0.011Ac$$

Calibration at temperatures from 7 to 37°C showed that A varies linearly with temperature, to within 2 % (Fig. 1). The calibration factor was calculated for each temperature and initial $NADH_2$ concentration from the appropriate value of A.

The highest and lowest reactant concentrations used were: $NADH_2$, 21 and 1 μM; acetaldehyde, 2600 and 13 μM; NAD, 330 and 2 μM; ethanol, 8000 and 200 μM. Each experiment consisted of triplicate series of measurements with from 3 to 5 different concentrations of both coenzyme and substrate, making from 9 to 25 different measurements in each series.

The sensitive fluorometric method, first developed by Theorell and Nygaard[13], is essential for reasonably accurate estimates of initial rate parameters for liver alcohol dehydrogenase, other than maximum rates. Since, for example, the Michaelis constants ($\Phi_1\Phi_0$) for $NADH_2$ and NAD are only 13 μM and 3 μM, respectively, at pH 7.1 spectrophotometry is hardly a

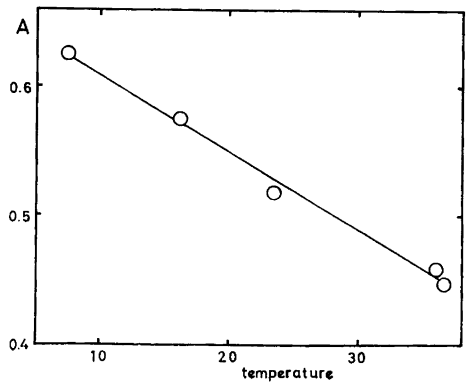


Fig. 1. Calibration of the fluorometer for $NADH_2$ measurements over a range of temperature. Variation with temperature of the constant A in the empirical equation $dF_R/dc = A - 0.011Ac$. (see text and reference 10).

feasible method. On the other hand, the highest concentration of $NADH_2$ that can be used in the fluorometer is limited by quenching, and the true maximum rate is significantly greater than the highest measured rate. For this reason, and as a check on the large temperature corrections for the fluorometer calibration, maximum rates of the $NADH_2$-acetaldehyde reaction at three temperatures were also determined by spectrophotometric initial rate measurements with 40–120 μM $NADH_2$.

The ionic strength of the reaction mixtures was 0.1 with respect to sodium phosphate buffer of pH 7.1 at 25°C. The change of pH with temperature[14] is −0.025 from 20 to 38°C.

Two distinct sets of experiments at temperatures from 8 to 36°C were carried out, separated by almost a year. Enzyme and $NADH_2$ were freshly prepared by the same methods for each set, but different NAD preparations were used. For the first set a commercial preparation was employed; for the second, following the discovery of a competitive inhibitor in commercial preparations[6], the NAD was purified by ion-exchange chromatography[12].

RESULTS

The kinetic coefficients in eqn. 1 have the dimensions of reciprocal velocity constants: $1/\Phi_0$ may be formally regarded as a first order rate constant (the maximum specific rate), $1/\Phi_1$ and $1/\Phi_2$ as second order rate constants, and $1/\Phi_{12}$ as a third order rate constant. The variation of these quantities with temperature is shown in the Arrhenius plots of Figs. 2 and 3 and the activation energies are given in Table 1.

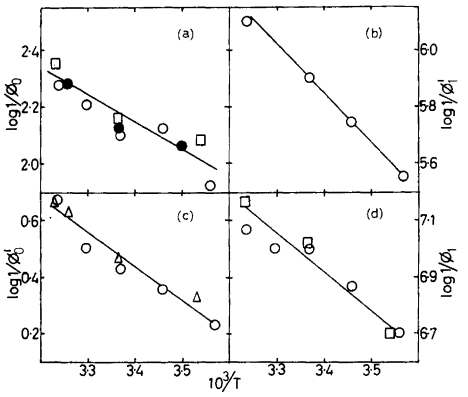


Fig. 2. Arrhenius plots showing the variation with temperature of the reciprocals of kinetic coefficients in Equation 1. (a) $1/\Phi_0$ (sec^{-1}), the maximum specific rate of the $NADH_2$-acetaldehyde reaction, measured in two series of experiments in the fluorometer (O, □) and by spectrophotometric initial rate measurements (●). (b) $1/\Phi_1'$ ($M^{-1}sec^{-1}$) from fluorometric initial rate measurements on the NAD-ethanol reaction with commercial NAD. (c) $1/\Phi_0'$ (sec^{-1}), the maximum specific rate of the NAD-ethanol reaction, from two series of initial rate measurements in the fluorometer with commercial (O) and chromatographically purified (△) NAD. (d) $1/\Phi_1$ ($M^{-1}sec^{-1}$) from two series of fluorometric initial rate measurements on the NADH-acetaldehyde reaction.

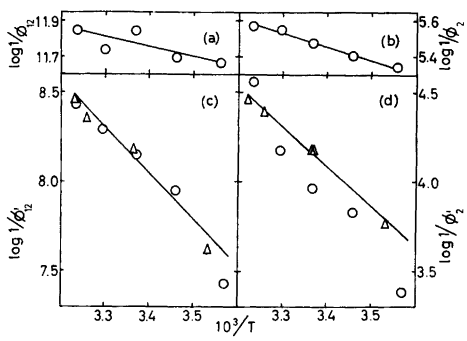


Fig. 3. Arrhenius plots showing the variation with temperature of initial rate parameters. In (a) and (b), values for $1/\Phi_{12}$ ($M^{-2}sec^{-1}$) and $1/\Phi_2$ ($M^{-1}sec^{-1}$) were obtained in a single set of experiments on the $NADH_2$-acetaldehyde reaction. In (c) and (d), measurements of the corresponding parameters for the NAD-ethanol reaction were made with commercial (O) and chromatographically purified (△) NAD.

Table 1. Apparent activation energies from the temperature variation of initial rate parameters at pH 7.1.

Parameter	Φ_0	Φ_1	Φ_2	Φ_{12}	Φ_0'	Φ_1'	Φ_2'	Φ_{12}'
E, kcal × mole^{-1}	4.4	6.3	3.5	~2.5	5.4	7.9	10.3	12.0

In Fig. 2(a), values for the maximum specific rate of the $NADH_2$ – acetaldehyde reaction, $1/\Phi_0$, obtained by spectrophotometric and fluorometric measurements are in satisfactory agreement. Figs. 2(c), 3(c) and 3(d) show parameters for the NAD-ethanol reaction obtained with commercial and chromatographically purified NAD. In accordance with earlier theory and experiment[9,12] only for the parameter Φ_2', (Fig. 3(d)) were significantly different values obtained with purified and commercial NAD. In this case, the activation energy was calculated from the measurements with purified NAD only, since the slope could be affected by the nucleotide impurity in commercial NAD if the heat of dissociation of its enzyme compound is different from that of the enzyme-NAD compound[12].

The precision of the activation energies in Table 2 is probably no better than ±1 kcal in general. The estimates of Φ_{12} are very approximate[4], but the difference between the apparent activation energies for $1/\Phi_{12}$ and $1/\Phi_{12}'$ is clearly significant, and there is an analogous difference between the temperature effects on $1/\Phi_2$ and $1/\Phi_2'$ (Fig. 3).

DISCUSSION

From evidence summarised in the introduction, the reciprocals of the kinetic coefficients Φ_0 and Φ_1 in equation (1) may be identified with the specific rate of dissociation of the product coenzyme-enzyme complex and the specific rate of formation of the reactant coenzyme-enzyme complex respectively. That is, $1/\Phi_1 = k_1$, $1/\Phi_0 = k_{-1}'$, $1/\Phi_1' = k_1'$, and $1/\Phi_0' = k_{-1}$, where the rate constants are defined by the following reaction sequence:

$$\mathrm{E} + \mathrm{NADH_2} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{E \cdot NADH_2}$$

$$\mathrm{E \cdot NADH_2} + \mathrm{CH_3CHO} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E \cdot NADH_2 \cdot CH_3CHO} \underset{k'}{\overset{k}{\rightleftharpoons}}$$

$$\rightleftharpoons \mathrm{E \cdot NAD \cdot C_2H_5OH} \underset{k_2'}{\overset{k_{-2}'}{\rightleftharpoons}} \mathrm{E \cdot NAD} + \mathrm{C_2H_5OH}$$

$$\mathrm{E \cdot NAD} \underset{k_1'}{\overset{k_{-1}'}{\rightleftharpoons}} \mathrm{E} + \mathrm{NAD}$$

If, as seems certain, the reaction of substrate with bound coenzyme involves ternary complexes, even though these are not rate limiting under maximum rate conditions, the coef-

Table 2. Heats, entropies and free energies of activation at pH 7.1 and 23.5°C.

Reaction	Specific rate	ΔH^* kcal × mole^{-1}	ΔS^* e. u.	ΔF^* kcal × mole^{-1}
$E + NAD \rightarrow E \cdot NAD$	$1/\Phi_1' = 9 \times 10^5 M^{-1}sec^{-1}$	7.3	− 9	10.0
$E \cdot NAD + C_2H_5OH \rightarrow E \cdot NADH_2 + CH_3CHO$	$1/\Phi_2' = 1.5 \times 10^4 M^{-1}sec^{-1}$	9.7	− 9	12.3
$E \cdot NADH_2 \rightarrow E + NADH_2$	$1/\Phi_0' = 2.7$ sec^{-1}	4.8	−42	17.2
$NAD + C_2H_5OH \rightarrow NADH_2 + CH_3CHO$	Sum	21.8	−60	39.5
$E + NADH_2 \rightarrow E \cdot NADH_2$	$1\Phi_1 = 1 \times 10^7 M^{-1}sec^{-1}$	5.7	− 9	8.4
$E \cdot NADH_2 + CH_3CHO \rightarrow E \cdot NAD + C_2H_5OH$	$1/\Phi_2 = 3 \times 10^5 M^{-1}sec^{-1}$	2.9	−26	10.6
$E \cdot NAD \rightarrow E + NAD$	$1/\Phi_0 = 125$ sec^{-1}	3.8	−38	15.0
$NADH_2 + CH_3CHO \rightarrow NAD + C_2H_5OH$	Sum	12.4	−73	34.0

ficients Φ_2 and Φ_2' will be functions of several rate constants[8], *e. g.* $\Phi_2 = (k_{-2}k_{-2}' + k_{-2}k' + k_{-2}'k)/k_2k_{-2}'k$. No information is available about the relative values of these rate constants. The activation energy derived from $1/\Phi_2$ will be a function of those for the several forward and reverse steps involved and will correspond to the height of the highest energy barrier above the energy level of the reacting bound coenzyme and substrate.

Heats, entropies and free-energies of activation in Table 2 were calculated from the activation energies of Table 1 and the best estimates[9] of the rate constants at 23.5°C by the relations of the absolute reaction rate theory[15,16]:

$$\Delta H^* = E - RT = E - 0.6 \text{ (kcal} \times \text{mole}^{-1}) \quad (2)$$

$$\Delta S^* = 2.3R \text{ (log } k - \log k_B T/h) + \Delta H^*/T \quad (3)$$
$$= \Delta H^*/T - 4.57 \text{ (13.22} - \log k)$$

$$\Delta F^* = \Delta H^* - T\Delta S^* \quad (4)$$

Thermodynamic data for the overall oxidation of ethanol by NAD may be calculated as the differences between the sums of these pseudo-thermodynamic quantites for the forward and reverse steps in Table 2, and are $\Delta H = 21.8–12.4 = 9.4$ kcal, $\Delta S' = -60 + 73 = 13$ e. u. and $\Delta F' = 39.5 - 34.0 = 5.5$ kcal. These quantities relate to an arbitrary standard state of the hydrogen ion of pH 7.1 and to the apparent equilibrium constant at pH 7.1, $K_0/(H^+) = (NADH)(CH_3CHO)/(NAD^+)(C_2H_5OH)$. The standard data for pH 0, or 1 molal activity of the hydrogen ion, are

$$\Delta H^\circ = \Delta H = 9.4 \text{ kcal}$$
$$\Delta S^\circ = \Delta S' - 2.3R(\text{pH}) = 13–33 = -20 \text{ e. u.}$$
$$\Delta F^\circ = \Delta F' + 2.3RT(\text{pH}) = 5.5 + 9.6 = 15.1 \text{ kcal} \times \text{mole}^{-1} \text{ at } 23.5°C$$

and relate to the pH-independent equilibrium constant $K_0 = (NADH)(H^+)(CH_3CHO)/(NAD^+)(C_2H_5OH)$. From direct studies of the variation of K_0 with temperature, Bäcklin[17] found $\Delta H^\circ = 7.13$, $\Delta S^\circ = -26.5$ and $\Delta F^\circ = 15.0$. The values obtained from the six kinetic parameters agree as well as could be

Table 3. Thermodynamic data for the oxidation of ethanol by NAD at pH 7.1 and. 23.5°C, calculated from activation data of Table 2.

Reaction	ΔH kcal×mole^{-1}	$\Delta S'$ e. u.	$\Delta F'$ kcal×mole^{-1}
$E + NAD \rightleftharpoons E \cdot NAD$	3.5	29	−5.0 (−5.2[a])
$E \cdot NAD + C_2H_5OH \rightleftharpoons E \cdot NADH_2 + CH_3CHO$	6.8	17	1.7
$E \cdot NADH_2 \rightleftharpoons E + NADH_2$	−0.9	−33	8.8 (8.8[a])
$NAD + C_2H_5OH \rightleftharpoons NADH_2 + CH_3CHO$	9.4 (7.1[b])	13 (6.5[b])	5.5 (5.4[b])

[a] Calculated from the data of Theorell and McKee[10].
[b] Calculated from the data of Bäcklin[17].

expected from the combined experimental errors. This is a further test of the Haldane relation $K_0/(H^+) = \Phi_0\Phi_1\Phi_2/\Phi_0'\Phi_1'\Phi_2'$, which is characteristic of the Theorell-Chance mechanism, and depends upon the identification of $1/\Phi_0$ and $1/\Phi_0'$ with the rates of dissociation of the enzyme-coenzyme compounds[8].

Thermodynamic data in Table 3 for the three successive reactions in the oxidation of ethanol by NAD, according to the Theorell-Chance mechanism, were similarly calculated as the differences between the activation data (Table 2) for the forward and reverse steps, and again refer to the arbitary standard state of pH 7.1. The parameters Φ_2 and Φ_2', although functions of several rate constants, are related[9] to the apparant equilibrium constant for the overall reaction of bound coenzymes and substrates at fixed pH by $K = \Phi_2/\Phi_2' = (E \cdot NADH_2)(CH_3CHO)/(E \cdot NAD)(C_2H_5OH)$, and thus thermodynamic data for this reaction can be calculated as the differences between activation data derived from these two parameters (Table 2). The largest free energy change accompanies the dissociation of the stable compound $E \cdot NADH_2$. Oxidation of ethanol by $E \cdot NAD$ involves little free energy change. This important fact and its possible physiological significance was pointed out long ago by Theorell and Bonnichsen[1] in other terms: the binding of the coenzymes by the enzyme favours substrate oxidation thermodynamically because the dissociation constant of $E \cdot NADH_2$ is smaller than that of $E \cdot NAD$. Judged by initial rate data[12], this is true for a number of dehydrogenases, and may be a general phenomenon of importance in metabolism. From the high yields of alcohol dehydrogenase and other dehydrogenases from liver, it seems likely that in the tissue the coenzymes will be largely present in the bound forms[1].

Although the free energy change accompanying ethanol oxidation by $E \cdot NAD$ is small, there is a large increase of heat content compensated by an increase of entropy. This step is mainly responsible for the heat content change in the overall reaction. The oxidation of ethanol by $E \cdot NAD$ is favoured by increase of temperature, K at pH 7.1 increasing from 0.05 at 23.5°C to 0.08 at 38°C. No significant enthalpy change accompanies the combination of enzyme and $NADH_2$, but $E \cdot NAD$ appears to be slightly endothermic. The value $\Delta H = 3.5$ kcal in Table 3 was obtained from activation energies derived from $1/\Phi_0$ and

$1/\Phi_1'$. The dissociation constant for E · NAD is also related[8] to the kinetic parameters by $K_1' = \Phi_{12}'/\Phi_2'$, and the activation energies from these two parameters (Table 1) give $\Delta H = 1.7$ kcal. However, no account has been taken of pH effects on the rate constants and dissociation constants, which for E · NAD, in contrast to E · $NADH_2$, are considerable[9] near pH 7.1. There is evidence that combination of NAD (but not $NADH_2$) requires ionisation of an acidic group in the enzyme with p$K \sim 6.4$, and that the pK values of this and another weaker group are shifted to lower levels in the compound[9,10]. The small ΔH value at pH 7.1 may represent the heats of ionisation of these groups, and activation data from true, pH-independent rate constants would be expected to give a smaller value of ΔH.

ΔS for the formation of both enzyme-coenzyme compounds are large positive values (Table 3). Also, whilst the entropy of activation for the combination of the enzyme with both coenzymes is the expected value for a bimolecular reaction, the reverse, dissociation reactions have large negative ΔS^* values (Table 2). These findings are consistent with neutralisation of the two negative charges on the phosphate groups of the coenzymes by positively charged binding sites in the enzyme, and with considerable charge separation on formation of the activated complexes in the dissociation reactions. The latter is largely responsible for the stability of the compounds. The possibility of a structural contribution to the large entropy changes cannot, of course, be dismissed[18].

Acknowledgement. I am greatly indebted to Mrs. B. Sutton for technical assistance.

REFERENCES

1. Theorell, H. and Bonnichsen, R. *Acta Chem. Scand.* **5** (1951) 1105.
2. Theorell, H. and Chance, B. *Acta Chem. Scand.* **5** (1951) 1127.
3. Dalziel, K. and Theorell, H. *Biochem. J.* **66** (1957) 34 P.
4. Dalziel, K. *Biochem. J.* **84** (1962) 244.
5. Dalziel, K. *Biochem. J.* **84** (1962) 240.
6. Dalziel, K. *Biochem. J.* **84** (1962) 69 P.
7. Alberty, R. A. *J. Amer. Chem. Soc* **75** (1953) 1928.
8. Dalziel, K. *Acta Chem. Scand.* **11** (1957) 1706.
9. Dalziel, K. *J. Biol. Chem. In press.*
10. Theorell, H. and McKee, J. S. M. *Acta Chem. Scand.* **15** (1961) 1811.
11. Dalziel, K. *Biochem. J.* **80** (1961) 440.
12. Dalziel, K. J. *Biol. Chem.* **284** (1963) 1538.
13. Theorell, H. and Nygaard, A. P. *Acta Chem. Scand.* **8** (1954) 877.
14. Hastings, A. B. and Sendroy, J. *J. Biol. Chem.* **61** (1924) 695.
15. Eyring, H. *J. Chem. Phys.* **3** (1935) 107.
16. Glasstone, S. Laidler, K. J. and Eyring, H. *The Theory of Rate Processes,* McGraw-Hill Book Co., New York, 1941.
17. Bäcklin, K.-I. *Acta Chem. Scand.* **12** (1958) 1279.
18. Laidler, K. J. *The Chemical Kinetics of Enzyme Action,* Clarendon Press, Oxford, 1958.

Received March 6, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 34–S 40

Interpretation of Relaxation Times

ROBERT A. ALBERTY, GAD YAGIL, WARREN F. DIVEN and MARK TAKAHASHI

Department of Chemistry, University of Wisconsin, Madison 6, Wisc., USA

When a reaction system is displaced from equilibrium it returns to equilibrium in the quickest possible way. If the displacements from equilibrium are small the kinetics are characterized by a number of relaxation times which is equal to the number of independent concentration variables. These relaxation times depend upon the various rate constants and equilibrium concentrations and are to be identified with single steps only in special cases. The expressions for the two relaxation times for a simple system of general interest in connection with enzyme kinetics are given and simplified for various special cases.

For a complicated reaction system the approach to equilibrium is described by a very complicated equation, if indeed the simultaneous rate equations can be integrated. However, in connection with the development of relaxation methods for studying very fast reactions Eigen[1–4] has pointed out that if the initial displacement of the various concentration variables from their equilibrium values is small, their subsequent change with time may be expressed as a sum of exponential terms. Each term is characterized by relaxation time τ (the time for that term to fall to 1/e of its initial value), and the number of relaxation times is equal to the number of independent concentration variables. The general theory for the calculation of relaxation times has been given[5,6], and these methods have been applied to enzymatic reactions[7,8]. However, the subject is sufficiently complicated so that it appears desirable to discuss the application of these ideas to a relatively simple system of general interest.

$$\begin{array}{ccc} & \overset{k_{12}}{\rightleftharpoons} & \\ 1.\ A + B + C & \underset{k_{21}}{} & 2.\ AB + C \\ \Big\| k_{41} \quad \Big\| k_{14} & & k_{32} \quad \Big\| k_{23} \\ & \underset{k_{43}}{} & \\ 4.\ AC + B & \underset{k_{34}}{\rightleftharpoons} & 3.\ ABC \end{array} \qquad (1)$$

The system can exist in four states and the rate constants indicate the initial and final states for each reaction. In this mechanism, A could represent enzyme, B coenzyme and C hydrogen ion; or A could represent antibody, B hapten with C not representing anything, that is A and AC would be isomers. The time variation of any concentration variable after an initial small displacement from equilibrium (as by a sudden temperature change[9]) would be given by

$$c_i - \bar{c}_i = \Delta c_i = K_{1i}e^{-t/\tau_1} + K_{2i}e^{-t/\tau_2} + K_{3i}e^{-t/\tau_3} \quad (2)$$

where $\bar{c}_i$ is the equilibrium concentration.

The expressions for the relaxation times for system 1 would be obtained as follows: There are 6 concentration variables and 3 conservation relations so that there are 3 independent concentration variables, 3 independent rate equations and 3 relaxation times. Any three independent rate equations for the system are written down. These rate equations are linearized by replacing each concentration (X) by (X) + Δ(X), where (X) is the equilibrium concentration and Δ(X) is the displacement from equilibrium. When products of concentrations are multiplied out, terms involving Δ^2 are ignored because the displacements from equilibrium are small. In this way the three differential equations may be reduced to the form

$$\frac{d\Delta(A)}{dt} = a_{11}\Delta(A) + a_{12}\Delta(AB) + a_{13}\Delta(ABC) \quad (3)$$

$$\frac{d\Delta(AB)}{dt} = a_{21}\Delta(A) + a_{22}\Delta(AB) + a_{23}\Delta(ABC)$$

$$\frac{d\Delta(ABC)}{dt} = a_{31}\Delta(A) + a_{32}\Delta(AB) + a_{33}\Delta(ABC)$$

According to the theory of linear differential equations the three relaxation times are obtained by solving the determinant

$$\begin{vmatrix} a_{11} + \frac{1}{\tau} & a_{12} & a_{13} \\ a_{21} & a_{22} + \frac{1}{\tau} & a_{23} \\ a_{31} & a_{32} & a_{33} + \frac{1}{\tau} \end{vmatrix} = 0 \quad (4)$$

Since the a's are functions of rate constants and equilibrium concentrations this would lead to very complicated expressions for the general case. However, considerable simplification results if the number of steps is smaller or some of the steps are so fast that certain substances can be considered to remain in equilibrium during a relaxation process. Also fast steps can be treated on the assumption that slow steps do not occur. Certain special cases derived from the general system can be treated separately.

One step. The simplest special case is one in which there is a single reaction[1-3].

$$A + B \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} AB$$

$$\frac{1}{\tau} = k_{21} + k_{12}\,[(\bar{A}) + (\bar{B})] \tag{5}$$

where $(\bar{A})$ and $(\bar{B})$ represent equilibrium concentrations.

For the isomerization reaction $A \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} A'$

$$\frac{1}{\tau} = k_{21} + k_{12} \tag{6}$$

so that changing a bimolecular step to a unimolecular step has the effect of replacing the sum of concentrations by 1.

Two steps. There are three qualitatively different sequences of two reactions in scheme 1.

$$A + B + C \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} AB + C \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} ABC \tag{7}$$

$$AC + B \underset{k_{14}}{\overset{k_{41}}{\rightleftharpoons}} A + B + C \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} AB + C \tag{8}$$

$$AC + B \underset{k_{34}}{\overset{k_{43}}{\rightleftharpoons}} ABC \underset{k_{23}}{\overset{k_{32}}{\rightleftharpoons}} AB + C \tag{9}$$

Each of these systems is characterized by two relaxation times which are obtained by solution of a quadratic equation. The complete expressions are quite complicated functions of the rate constants and equilibrium concentrations and allow for a possibility that would not allow the separate experimental determination of τ_1 and τ_2, that is, the possibility that these parameters are of the same magnitude. In general $1/\tau_1 + 1/\tau_2 = a_{11} + a_{22}$ so that if $\tau_2 \rangle\rangle \tau_1$ there will be a relatively simple expression for τ_1. Such expressions are given below. Another special case which simplifies the mathematics is that in which the square root term $(1-x)^{1/2}$ arising in the solution of the quadratic may be approximated with $1 - x/2$. In this way the following expressions are obtained for mechanism 7.

$$\frac{1}{\tau_1} = k_{21} + k_{12}\,[(\bar{A}) + (\bar{B})] + k_{32} + k_{23}\,[(\overline{AB}) + (\bar{C})] \tag{10}$$

$$\frac{1}{\tau_1\,\tau_2} = k_{21}\left\{k_{32} + k_{23}\,(\overline{AB})\right\} + k_{12}\,[(\bar{A}) + (\bar{B})]\left\{k_{32} + k_{23}\,[(\overline{AB}) + (\bar{C})]\right\} \tag{11}$$

These expressions indicate that studies of the dependencies of τ_1 and τ_2 on various concentration variables could yield the four independent rate constants in this mechanism. If τ_1 and τ_2 are not very different the complete expressions should be used to evaluate the rate constants. In any case the values of the various rate constants must be substituted into the quadratic expressions to make sure that the approximation used was satisfactory.

If one of the steps is equilibrated much faster than the other, these expressions take on simpler forms. If $k_{21} + k_{12}\,[(\bar{A}) + (\bar{B})] \rangle\rangle k_{32} + k_{23}\,[(\overline{AB}) + (\bar{C})]$, τ_1 is given by equation 5, and τ_2 by:

$$\frac{1}{\tau_2} = k_{32} + k_{23}\left\{(\overline{AB}) + \frac{(\bar{C})\,[(\bar{A}) + (\bar{B})]}{K_{21} + (\bar{A}) + (\bar{B})}\right\} \tag{12}$$

where $K_{21} = k_{21}/k_{12}$. Equation 12 may be derived directly, as discussed below, without solving a quadratic equation.

A further special case of interest occurs when B is buffered. For example, B might be hydrogen ion in a buffered solution and the rates of proton gain by A and loss by AB may be fast compared with other steps in the mechanism. In this case

$$\frac{1}{\tau_1} = k_{21} + k_{12}\,(\mathrm{B}) \tag{13}$$

$$\frac{1}{\tau_2} = k_{32} + k_{23}\left[(\overline{AB}) + \frac{(\bar{B})\,(\bar{C})}{K_{21} + (\bar{B})}\right] \tag{14}$$

Terms in $[(\bar{A}) + (\bar{B})]$ are replaced with $(\bar{B})$ because $(\bar{B})$ does not change during the reaction, and the bimolecular reaction A + B becomes a pseudo-unimolecular reaction with a rate constant proportional to $(\bar{B})$.

If the second step is fast $k_{21} + k_{12}\,[(\bar{A}) + (\bar{B})] \langle\langle k_{32} + k_{23}\,[(\overline{AB}) + (\bar{C})]$

$$\frac{1}{\tau_1} = k_{32} + k_{23}\,[(\overline{AB}) + (\bar{C})] \tag{15}$$

$$\frac{1}{\tau_2} = k_{12}\,[(\bar{A}) + (\bar{B})] + k_{21}\frac{[K_{32} + (\overline{AB})]}{K_{32} + (\overline{AB}) + (\bar{C})} \tag{16}$$

where $K_{32} = k_{32}/k_{23}$.

If in addition C is buffered

$$\frac{1}{\tau_1} = k_{32} + k_{23}\,(\bar{C}) \tag{17}$$

$$\frac{1}{\tau_2} = k_{12}\,[(\bar{A}) + (\bar{B})] + \frac{k_{21}K_{32}}{K_{32} + (\bar{C})} \tag{18}$$

If AB in mechanism 7 is in a steady state one of the rate equations is eliminated ($d(AB)/dt = 0$), and the expression for the steady state relaxation time becomes

$$\frac{1}{\tau} = \frac{k_{21}\,k_{32} + k_{12}\,k_{23}\{(\bar{C})\,[(\bar{A}) + (\bar{B})] + (\bar{A})\,(\bar{B})\}}{k_{21} + k_{23}\,(C)} \tag{19}$$

If in addition C is buffered, the term in (A)(B) in the numerator is eliminated.

The mechanism

$$A + B \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} AB \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} AB' \tag{20}$$

may be considered to be a special case of mechanism 7. In the expressions for τ_1 and τ_2 terms in $[(\bar{AB}) + (\bar{C})]$ are replaced by 1 since the bimolecular reaction of AB with C has been replaced by unimolecular reaction. The term in $k_{23}(\bar{AB})$ vanishes. The relaxation times (*cf.* Ref. 4) given by expanding the square root term are

$$\frac{1}{\tau_1} = k_{21} + k_{23} + k_{32} + k_{12}\,[(\bar{A}) + (\bar{B})] \tag{21}$$

$$\frac{1}{\tau_1\,\tau_2} = k_{21}\,k_{32} + k_{12}\,(k_{23} + k_{32})\,[(\bar{A}) + (\bar{B})] \tag{22}$$

In another special case one of the reactants in the bimolecular reaction undergoes isomerization rather than the product, as in reaction 20.

$$AB' + C \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} AB + C \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} ABC \tag{23}$$

In this case the relaxation times given by expanding the square root term are

$$\frac{1}{\tau_1} = k_{21} + k_{12} + k_{32} + k_{23}\,[(\bar{AB}) + (\bar{C})] \tag{24}$$

$$\frac{1}{\tau_1\,\tau_2} = k_{32}\,(k_{12} + k_{21}) + k_{21}\,k_{23}(\bar{AB}) + k_{12}\,k_{23}\,[(\bar{AB}) + (\bar{C})] \tag{25}$$

Three steps. If one of the reactions in system 1 is eliminated there will still be three relaxation times, and the solution of a cubic equation will be required unless there is a big difference in the rates of equilibration of different steps. If two of the steps are fast it is just as easy to consider all four steps.

Four steps. We will consider the case where the reactions involving C are considerably faster than the reactions involving B, and that C is buffered. In considering the long relaxation time A and AC may be assumed to be in equilibrium, and the same will be true for AB and ABC. The relaxation process con-

sidered is simply a slow equilibration between the two equilibrium pairs A + AC and AB + ABC. This may be considered as an example of a simple bimolecular association with

$$\frac{1}{\tau} = k_{\text{dissoc}} + k_{\text{assoc}} [(\bar{A}) + (\overline{AC}) + (\bar{B})] \tag{26}$$

The sum $(\bar{A}) + (\overline{AC})$ is included in the concentration term since these two forms are in equilibrium and must be treated together. In the absence of C this expression must reduce to that for the upper line of scheme 1, namely equation 5, and at very high C concentrations the relaxation must be achieved by the lower path so that

$$\frac{1}{\tau} = k_{34} + k_{43} [(\overline{AC}) + (\bar{B})] \tag{27}$$

This is satisfied if the following forms are used for k_{dissoc} and k_{assoc}

$$k_{\text{dissoc}} = \frac{k_{21} + k_{34}(\bar{C})/K_{32}}{1 + (\bar{C})/K_{32}} \tag{28}$$

$$k_{\text{assoc}} = \frac{k_{12} + k_{43}(\bar{C})/K_{43}}{1 + (\bar{C})/K_{43}} \tag{29}$$

where $K_{32} = k_{32}/k_{23}$ and $K_{43} = k_{43}/k_{34}$. If C is not buffered more complicated expressions are obtained.

When certain reactants may be assumed to be in equilibrium the expression for the relaxation times may be derived directly without deriving the more general expression and simplifying. For instance in reaction 7, if the first step is in rapid equilibrium, one can write for the instantaneous equilibrium at any time during the slow relaxation

$$K_{21} = \frac{k_{21}}{k_{12}} = \frac{[(\bar{A}) + \Delta(A)]\,[(\bar{B}) + \Delta(B)]}{(\overline{AB}) + \Delta(AB)} = \frac{[(\bar{A}) + \Delta(A)]\,[(\bar{B}) + \Delta(A)]}{(\overline{AB}) + \Delta(AB)} \tag{30}$$

where the concentrations are expressed as final equilibrium values plus a displacement. This yields the following relation between $\Delta(A)$ and $\Delta(AB)$

$$\Delta(A) = \Delta(AB)\,\frac{K_{21}}{[(\bar{A}) + (\bar{B})]} \tag{31}$$

Using this relation, one can describe the slow relaxation by a single differential equation in $\Delta(ABC) = -[\Delta(A) + \Delta(AB)]$:

$$\frac{\mathrm{d}\Delta(ABC)}{\mathrm{d}t} = k_{23}\left[(\overline{AB}) - \Delta(ABC)\,\frac{(\bar{A}) + (\bar{B})}{K_{21} + (\bar{A}) + (\bar{B})}\right][(\bar{C}) - \Delta(ABC)] - k_{32}[(\overline{ABC}) - \Delta(ABC)] \tag{32}$$

This equation leads directly to equation 12 for τ_2. Expressions for the slow relaxation time in other cases where a fast step can be assumed to be in equilibrium can be derived in a similar way.

It has been impossible to give all the forms for the expressions for the relaxation times of these and related cases in this short paper, but the authors will be happy to supply the additional expressions in mimeographed form to those who are interested.

Acknowledgements. The authors are indebted to the *Public Health Service* and the *National Science Foundation* for financial support of this work.

REFERENCES

1. Eigen, M., Kurtze, G., and Tamm, K. Z. *Elektrochem.* **57** (1953) 103.
2. Eigen, M. Z. *Physik. Chem. (Frankfurt)* **1** (1954) 176.
3. Eigen, M. *Discussions Faraday Soc.* **17** (1954) 194.
4. Eigen, M. *Discussions Faraday Soc.* **24** (1957) 25.
5. Eigen, M. Z. *Elektrochem.* **64** (1960) 115.
6. Eigen, M. and De Mæyer, L. In Weissberger, A. *Technique of Organic Chemistry,* 2nd Ed., Interscience Publ., New York, 1963, Vol. VIII.
7. Hammes, G. G. and Alberty, R. A. *J. Am. Chem. Soc.* **82** (1960) 1564.
8. Hammes, G. G. and Fasella, P. *J. Am. Chem. Soc.* **84** (1962) 4644.
9. Czerlinski, G., and Eigen, M. Z. *Elektrochem.* **63** (1959) 652.

Received March 25, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S41–S46

On Bacterial "Cytochromoids"*

MARTIN D. KAMEN

University of California at San Diego, La Jolla, California, USA

A new type of haem protein, extractable as a soluble di-haem protein conjugate, is found in purple photosynthetic bacteria. The physical and chemical properties of two representative samples, obtained from the facultative photoheterotrophe, *Rhodospirillum rubrum*, and the obligate photoanaerobe, *Chromatium*, are reviewed briefly and discussed in relation to the recommendation for establishment of a new class of haem proteins to be called "cytochromoids".

There has never been a dearth of phenomena for the biochemist to ponder when he has had occasion to consider problems of structure and function presented by the haem proteins. Now there come more in the wake of studies on variant haem proteins which occur distributed widely throughout the group of purple photosynthetic bacteria, and which have been known since 1953 when the first specimen was observed in cell-free extracts prepared from the facultative photoheterotrophe, *Rhodospirillum rubrum*[1,2]. Subsequent researches have established a complex of properties[3] for these proteins which have prompted the Commission on Enzymes of the International Union of Biochemistry to propose[4] a new class of haem proteins to be termed "cytochromoids", defined as "Haemoproteins with a haemoglobin-like spectrum and a reactivity with ligands which do not react with cytochrome *c*".

In this article, this nomenclature will be adopted, in place of the previous "RHP-type". The properties of cytochromoids will be surveyed and present knowledge of structure and function discussed briefly, as based on studies of two representative proteins – the first, labeled "I", from *R. rubrum* and the second, labeled "II", from the strict photoanaerobe, *Chromatium* (strain D). Space available prohibits detailed presentations of data; for these the reader is referred to the bibliography.

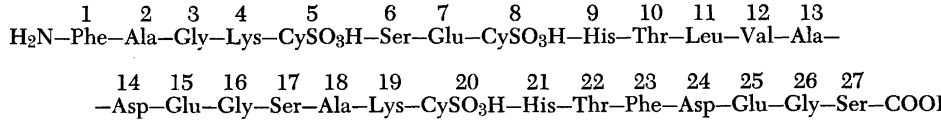
* Researches on which this article is based have been supported by grants-in-aid from the *National Institutes of Health* (C-3649 and C-5992), the *National Science Foundation* (G-6441 and G-19642), and the *Charles F. Kettering Foundation*. Some of the observations reported were obtained by the writer while in residence at various times from 1956–1962 in the laboratories of the Biochemical Section of the Nobel Institute in Stockholm. The writer is indebted to Professor H. Theorell for making these facilities available.

The prosthetic groups

Most studies have been limited by the minute quantities of pure protein available. At present, the nature of the prosthetic haems must be inferred more or less indirectly from spectrochemical studies[3]. It is certain that cytochromoids I and II are di-haem proteins. This conclusion follows from measurements[5] of molar absorbancy indices at the characteristic maxima of absorption exhibited by the reduced (ferro-) forms both in the native state, and as derivative haemochromogens formed by partial denaturation in alkali alone or by ligand interactions with pyridine and cyanide after denaturation in alkali. It is also certain that the prosthetic haems are bound covalently, because fission does not occur by the usual treatment with acid-acetone. Attempts to obtain the free haems by fission in the presence of dilute acid and heavy-metal salts (Ag^+ or Hg^{++}), a treatment effective to the extent of approximately 80 % or better for cytochrome *c* and myoglobin types of haem protein, have been disappointing[6]. Yields no greater than approximately 20 % have been obtained with a mixture of products which, after conversion to porphyrins, include a major fraction as a porphyrin very similar to haematoporphyrin and two others as yet unidentified. However, recent experiments, which employ a modification of the original conditions of the Paul procedure[7], have resulted in much improved yields (up to 70 % of total haem) with only a single product, haematohemin, as judged by chromatography and spectroscopic criteria.

From the position of the alpha maxima (550 mμ) of the pyridine haemochromogens, it appears certain that the majority of the substituent groups on the peripheries of the pyrrole moieties are not electron-withdrawing (unsaturated). However, it cannot be stated unequivocally that there is not a single such group (vinyl, or partially shielded unsaturated chain residue) present.

A promising approach to the eventual characterization of the haem groups is based on the partial enzymic hydrolysis of the cytochromoids after preparatory extensive denaturation by boiling or treatment with organic solvents. The cytochromoids are resistant to enzymic attack in the native state. The only procedure which is effective without prior denaturation is that with pepsin, which of course involves incubation in acid solvent (pH $\approx$ 1.9). Even such treatment leaves a large core peptide of some 27 residues which still contains both haem groups[8]. Further directed degradation to obtain the amino acid sequence requires first that the haem groups be split off (by performic acid oxidation). The resultant peptide reveals the following sequence:



Small but significant quantities of a monohaem peptide which is not resistant to enzymic attack, and which is made up of the sequence including residues 2–10, occur. This, together with the remarkable resemblance of this portion of the long peptide to sequences encountered invariably in *c*-type cytochromes[9,10] renders very plausible the possibility that one haem is attached, as in cytochrome *c*,

through thioether linkages to the cysteinyl residues 5 and 8. The placement of the other haem is still unknown. A possibility that it is linked by a single saturated bond either to serine 17 or cysteine 20, or to both by two saturated bonds, is obvious. No positive clue can be obtained from the absorption spectrum of the di-haem peptide which is that of the usual *c*-type haemochromogen, except for an anomalously high ratio of absorbancy of the oxidized form (haemichrome) at the 530 mμ maximum to that of the reduced alpha peak at 550 mμ[8].

Determinations of the paramagnetic susceptibility and electron spin resonance measurements on the native forms of I and II show[11] that both haems contain iron in the high spin form when the cytochromoids are reduced, but that there is an equilibrium mixture of high and low spin forms in the oxidized cytochromoids. These results are reminiscent of the behavior of alkali haematin[12]. No measurements have been made as yet on the peptic di-haem peptide.

When sufficient quantities of peptide are available, efforts will be made to obtain the two monohaem peptides expected from the above sequence studies. Such experiments, coupled with the improved cleavage procedures and application of the recently-developed proton spin resonance analysis[13], should clarify the nature of the two haem groups.

Attempts to resolve the prosthetic haems as attached in the native proteins have been only partially successful. Thus, low temperature absorption spectra[14] of reduced cytochromoid I show clearly resolution of the Soret absorption band into two components with maxima at 422 mμ and 433 mμ. However, no comparable resolution of two weakly differentiated maxima at 549 mμ and 565 mμ in the visible alpha region is affected. Differential reduction[15], in the presence or absence of CO, also fails to give evidence that two haem groups differ appreciably either in reactivity with CO, or in midpoint potentials at neutral pH ($E_{m,7}$ = = −8 to −5 mV).

Ligands interactions

As observed repeatedly[3], the responses of cytochromoids to all of the usual ligand reagents are negative, except for certain limited interactions with CO and NO, both small, uncharged molecules. The only other molecules, or solvent species, which may have access to the reactive sites, are molecular oxygen — as evidenced by the ready autoxidizability of I and II, hydronium ion — as seen[5] in the complex spectroscopic changes which occur when the oxidized cytochromoids are subjected to gradual changes in pH from 6 to 10, and possibly molecular hydrogen — because catalytic reduction by hydrogen is observed in the presence of palladium.

The changes noted with change in pH do not require that protons from the solvent medium actually penetrate to the active center, however. It is also possible that these effects are the results of configurational changes transmitted through the protein structure by alterations at the surface exposed to the solvent.

The oxidized cytochromoids act as though they are completely inaccessible to all charged ligand reagents. Thus, unlike other haem proteins with typical "open" spectra (myoglobins, peroxidases, *etc.*), no spectroscopic changes are noted in the presence of a long list of such reagents, *e. g.*, N_3^-, CN^-, HS^-, F^-, *etc.*[3]

The reduced cytochromoids appear even more inaccessible inasmuch as they

fail to respond spectrally to deprotonation[5], as do the oxidized forms. Large uncharged ligands, *e. g.*, nitrosobenzene[16], 4-methylimidazole[3], *etc.*, are also unreactive.

Even in the reactions which do take place, as with CO and NO, many anomalies occur[16]. Thus, while CO forms a typical haemochromogen with ferrocytochromoids, its affinity is much lower than for other haem compounds; thus, pressures for half saturation of I are $\approx$ 0.015 atmospheres, whereas they are only approximately $^1/_{10}$ this much for typical haemoglobin interactions. Even at atmospheric pressure, association is only approximately 90 % complete. Moreover, the photodissociation of the CO-complex is pH-sensitive, a phenomenon not noted with any other haem compound, as far as the writer is aware. At pH 4.8, the degree of association is twice that at pH 7.0.

The reactions with NO are complex and in many respects unlike any hitherto observed with other haem proteins[16]. The affinity of ferri- or ferro-cytochromoid I for NO is less than a tenth that displayed by the reduced form for CO – a relation just the reverse of that usually found in other haem compounds. The major interaction is one displayed by the ferri-form which appears to react with production of a pH-sensitive charge transfer adduct, *e. g.*, $Fe^{+++} + NO = Fe^{++} \cdot NO^+$, as in the case of ferricytochrome *c*[17]. The typical spectrum of a haemochromogen is produced. However, the residual ferro-form cannot be recovered by alkalination, as is found for the ferricytochrome *c*-NO adduct. Instead, the ferri-cytochromoid-NO adduct is already markedly unstable at pH 7, unlike the ferricytochrome *c* compound, and dissociates to yield the ferri-cytochromoid. As the pH is lowered progressively, the NO adduct is increasingly stabilized.

The ferrocytochromoid reacts weakly with NO to form a haemochromogen-like compound(s) of unknown structure which is not affected by changes in pH. At pH $>$ 11, irreversible changes which involve degradation of the haem moieties occur for both ferri- and ferro-forms.

In view of these limited ligand interactions, the wisdom of accentuating the *c*-type cytochrome character of these proteins by use of the term "cytochromoid" is evident. Taken with the results of the fission experiments, and sequence studies, the "cytochrome-like" character of the cytochromoids can be considered as outweighing the "peroxidase-like" character deduced from spectroscopic and magnetic properties.

Function

Every major function, other than oxygen transport or storage, has been suggested for the cytochromoids. These include roles as peroxidases, oxidases, and components of electron transport, with or without coupling to phosphorylation.

The peroxidase function has been eliminated as a reasonable possibility by the observations that peroxidase activity does not fractionate with cytochromoids I and II[1,3] and that peroxidation of ascorbate in the presence of cytochromoids I and II proceeds at rates much less than those observed for such pseudo-peroxidases as myoglobin and denatured cytochrome *c*[18].

Arguments for and against an oxidase function have appeared in the literature and need not be reviewed here[1, 3, 16, 19–21]. The most recent findings strongly

contraindicate an oxidase function. These are: (1) cytochromoid I is not present in significant amounts in non-photochemical aerobic cells[22,23]; (2) spheroplasts of *R. rubrum* with strong oxidase activity contain no cytochromoid I; (3) a cytochromoid found in *Rhodopseudomonas palustris,* is not auto-oxidizable; (4) immune sera specific for cytochromoid I react with extracts from light-grown cells but not with those from dark-grown cells[26,26].

The weight of evidence in favor of a strictly catalytic role in electron transport coupled to phosphorylation is considerable. Kinetic studies[14,27] show that cytochromoid I is a substrate for a flavin-mediated haem protein reductase which is a member of the electron-transport chain linked to photophosphorylation. Depleted chromatophores, with a low rate of photophosphorylation, can be activated by addition of either cytochrome c_2 or cytochromoid I, both endogenous haem proteins of *R. rubrum,* but the activity-concentration function shows the cytochromoid as more likely situated somewhere in the middle of the chain (as consistent with its midpoint potential, $E_{m,7} \approx 0$), rather than at the terminal end, as is the case with cytochrome c_2. (Many data implicate cytochrome c_2 as the terminal substrate for the photo-oxidase system but a discussion of these lies outside the scope of the present article). No data exist which run counter to a role for cytochromoids as intracellular electron carriers.

A definitive determination of the specific role of cytochromoid I in *R. rubrum* metabolism is difficult because of the very close coupling between oxidative metabolism and photoactivation processes[27]. Further progress will depend on development of techniques for resolution of the electron transport systems which are associated with dark and light metabolism.

General conclusions

On the basis of the evidence at hand, the creation of a new class of haem proteins to include cytochromoids appears well justified. Although the properties of the class must remain defined largely on the basis of those displayed by just two members, cytochromoids I and II, the data available are comparable with those which have been considered adequate for characterization of the more conventional classes of haem proteins. Thus, *c*-type cytochromes until recently were described almost entirely on the basis of the properties exhibited by horse-heart or yeast cytochrome *c*. A similar situation holds for the haemoglobins, chlorocruorins, myoglobins, peroxidases, catalases, *etc.,* in each of which relatively few specimens have been examined with a degree of thoroughness.

It is premature to attempt a more specific description than is implied in the present definition of cytochromoids. Already it is evident that there is a considerable spread in physicochemical properties of cytochromoids as a class. Thus, the amino acid compositions[28] and sedimentation and diffusion constants[3] are markedly different in cytochromoids I and II. More important, the *R. palustris* cytochromoid has a relatively high mid-point oxidation potential ($E_{m,7} \approx 250$ mV) and is not auto-oxidizable. However, such variations within a class of haem proteins are no more unusual than those which exist in the class of *c*-type cytochromes, for which similar greatly disparate degrees of autoxidizability and spreads in range of oxidation potential are found.

The apparent limitation in distribution of cytochromoids is also likely to vanish

as research continues. On comparative biochemical grounds, it can be anticipated that cytochromoids may exist in the groups of micro-organisms related to purple photosynthetic bacteria, such as the chemosynthetic facultative anaerobes which reduce nitrates, and the chemosynthetic anaerobes which reduce sulfates, as well as in the green photosynthetic bacteria. While attempts to reveal the presence of cytochromoids in such systems have been negative to the present, such results have been derived from studies on limited quantities of micro-organisms and prove only that the amounts of cytochromoids which may be present are much less than in the photosynthetic apparatus of the purple bacteria, in which the amounts of cytochromoids are very large. Recently, an apparently authentic example of a cytochromoid in a nonphotosynthetic facultative anaerobe, *Pseudomonas denitrificans*, has been described[29]. The possibility that cytochromoids may occur in conventional aerobic systems is not remote and has even been mentioned in one case, that of a new haem protein from liver microsomes[30].

REFERENCES

1. Vernon, L. P. and Kamen, M. D. *J. Biol. Chem.* **211** (1954) 643, 663.
2. Kamen, M. D. and Vernon, L. P. *Biochim. Biophys. Acta* **17** (1955) 10.
3. Kamen, M. D. and Bartsch, R. G., Falk, J., Lemberg, R. and Morton, R. In *Haematin Enzymes*, Pergamon Press, New York, 1961, p. 419.
4. *Report of the Commission on Enzymes, I. U. B. Symposium Series*, Pergamon Press, Oxford 1961, Vol. 20, Chapt. 5.
5. Horio, T. and Kamen, M. D. *Biochim. Biophys. Acta* **48** (1961) 226.
6. Barrett, J. and Kamen, M. D. *Biochim. Biophys. Acta* **50** (1961) 563.
7. Morrison, M. *J. Biol. Chem.* (1963). *In press.*
8. Dus, K., Bartsch, R. G. and Kamen, M. D. *J. Biol. Chem.* **237** (1962) 3083.
9. Tuppy, H. and Paléus, S. *Acta Chem. Scand.* **9** (1955) 353.
10. Tuppy, H. Z. *Naturforsch.* **126** (1954) 784.
11. Ehrenberg, A. and Kamen, M. D. *In preparation.*
12. George, P., Beetlestone, J. and Griffith, J. S., ref. (3), p. 105.
13. Caughey, W. and Koski, W. S. *Biochemistry* **1** (1962) 923.
14. Horio, T. and Kamen, M. D. *Biochemistry* **1** (1962) 1141.
15. Horio, T. and Kamen, M. D. *J. Biol. Chem.* **236** (1961) 2783.
16. Taniguchi, S. and Kamen, M. D. *Arch. Biochim. Biophys. In press* (1963).
17. Ehrenberg, A. and Szcepkowski, T. W. *Acta Chem. Scand.* **14** (1962) 1684.
18. R. Henderson. *Unpublished observations.*
19. R. G. Bartsch and M. D. Kamen. *J. Biol. Chem.* **230** (1958) 41.
20. Horio, T. and Taylor, C. P. S. *J. Biol. Chem.* (1963). *In press.*
21. Chance, B., see ref. (3), p. 433.
22. Geller, D. M. *J. Biol. Chem.* **237** (1962) 2947.
23. Taniguchi, S. *Unpublished observations.*
24. DeKlerk, H., Bartsch, R. G. and Kamen, M. D. *Unpublished observations.*
25. Newton, J. W. *Biochim. Biophys. Acta* **42** (1960) 34.
26. Orlando, J. A., Levine, L. and Kamen, M. D. *Ibid.* **46** (1961) 126, and *unpublished observations.*
27. Horio, T. and Kamen, M. D. *Biochemistry* **1** (1962) 144.
28. Bartsch, R. G., Coval, M. C. and Kamen, M. D. *Biochim. Biophys. Acta* **51** (1961) 241.
29. Suzuki, H. and Iwaski, H. *J. Biochem. (Tokyo)* **52** (1962) 193.
30. Omura, T. and Sato, R. *J. Biol. Chem.* **237** (1962) 1375 and *private communication.*

Received January 25, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S47–S50

Formation of Porphyrin *c* and Porphyrin-Protein from Reduced Protoporphyrin

T. L. POPPER and H. TUPPY

Institut für Biochemie der Universität Wien, Vienna, Austria

Cysteine and protoporphyrin reduced with sodium amalgam or titanous chloride have been found to react under mild conditions to give porphyrin *c*.

It has also been shown that reduced protoporphyrin combines covalently with bovine serum albumin, which was used as a model sulfhydryl-containing protein. Proteolytic digestion of the resulting porphyrin-protein yielded peptides containing bound porphyrin.

As shown by Theorell[1] in 1938, the covalent linkages between protein and prosthetic group in cytochrome *c* are due to the addition of sulfhydryl groups of cysteine residues in the protein moiety across the vinyl groups of a protoheme molecule. He succeeded in isolating porphyrin *c*, the compound formally derived from one protoporphyrin and two cysteine molecules, from cytochrome *c* and in synthetizing it by heating protoporphyrin with cysteine in the presence of mineral acid[2]. Other methods [3,4] of preparing this compound have also involved rather harsh conditions.

Sano and Granick[5] observed that when protoporphyrinogen and cysteine in phosphoric acid were illuminated with white light, a porphyrin *c*-type compound was formed, which, however, was not further investigated.

In this laboratory in the course of an investigation of the formation of thioethers from reduced protoporphyrin, the reaction of the latter with cysteine and with bovine serum albumin was studied in detail.

MATERIALS AND METHODS

For the *preparation of protoporphyrin* the method of Ramsey[6] was used. Pure protoporphyrin was prepared by hydrolysis of recrystallized protoporphyrin dimethylester. Commercial preparations of crystalline bovine serum albumin (Sigma lots A 70B–088, 090) were used without further purification. Pepsin was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, and titanous chloride from Riedel-de Haën AG, Seelze, Hannover, as a 15 % solution in hydrochloric acid. Sephadex G-50 was purchased from Pharmacia, Uppsala, Sweden. The hydrochloric acid used for hydrolysis was distilled over $SnCl_2$.

All spectrophotometric measurements were made in a Beckman Model DU spectrophotometer.

Reduction of protoporphyrin with sodium amalgam. 5.5 mg (10 μmoles) of protoporphyrin in 10 ml of 0.1 N NH_4OH was heated at 80°C for 10 min with 25 g of freshly prepared 3 % sodium amalgam. The brownish solution was filtered and adjusted to pH 6.5–7.5. All these operations were carried out in an atmosphere of nitrogen. The neutralized solution was immediately used for further reactions.

Reaction with cysteine. 157.5 mg (1 mmole) of L-cysteine hydrochloride in 10 ml of water was adjusted to pH 7, and quickly added to the above prepared solution of reduced protoporphyrin. The solution was thoroughly mixed and allowed to stand at 20°C for 2 h under an atmosphere of nitrogen. The solution was concentrated *in vacuo* to 6 ml and adjusted to pH 1.6, when most of the protoporphyrin, contaminated by lesser amounts of porphyrin *c*, precipitated and was removed by centrifugation. The red supernatant was brought to pH 3.35 and the precipitate, which contained the bulk of porphyrin *c*, was separated by centrifugation and dried (1.7 mg).

Purification of the crude porphyrin c by partition chromatography. To 5 g of carefully washed[7] and dried Hyflo Supercel 1.8 g of the lower layer of a freshly prepared mixture of butanol, glacial acetic acid and water (4 : 1 : 5) was added. After thorough mixing, the Hyflo Supercel was suspended in a suitable volume of the upper phase of the solvent system so as to form a slurry, and was poured into a chromatography column 1.2 cm in diameter. After settling, the Hyflo Supercel formed a column 13.2 cm high. On the top of this column was placed 1.7 mg of the above-described precipitate dissolved in the smallest possible amount of the upper phase. The main component formed a narrow, reddish-brown band which moved down the column with an R_F value of approx. 0.8, while a small amount of colored material stayed on the top. The effluent containing the main band was collected and evaporated to dryness under reduced pressure.

Identification of porphyrin c. An aliquot of the main fraction dissolved in 1 N HCl showed absorption maxima at 406, 552.5 and 594 mμ (*Ref.* 4, 406, 553 mμ). Paper chromatography was carried out by the method of Eriksen[8]. Descending chromatograms were run with Schleicher and Schüll paper 2043b in a 2,6-lutidine-water system at 21°C under an atmosphere of ammonia. The following R_F values were observed: protoporphyrin 0.66; porphyrin *c*[4] 0.21; and porphyrin *c* obtained above 0.22.

Reduction of protoporphyrin with titanous chloride and reaction with cysteine. 2.8 mg (5 μmoles) of protoporphyrin were stirred at 20°C, under an atmosphere of nitrogen, in 30 ml of a 1 : 1 : 1 mixture of 1 N HCl, ethanol and titanous chloride solution. After 30 min 79 mg (0.5 mmole) of L-cysteine hydrochloride in 5 ml water were added and the mixture was stirred for 1.5 h under nitrogen. The dark solution was brought to pH 1.5 and extracted successively with small portions of ethyl acetate and chloroform. The organic solutions were combined, washed with water, and evaporated to dryness *in vacuo*. The reddish-brown residue was subjected to partition chromatography as described above. A broad reddish-brown band with an R_F value of approx. 0.85 was eluted. After evaporation of the solvent the residue was identified as porphyrin *c* by means of paper chromatography and absorption spectroscopy.

Reaction with serum albumin. 11.2 mg (20 μmoles) of protoporphyrin were reduced with sodium amalgam and neutralized as described above. To the resulting solution were added 650 mg (10 μmoles) of bovine serum albumin in 10 ml of water. The mixture was allowed to stand for 2 h at 20°C under nitrogen. The solution was then exposed to air, adjusted to pH 9.1, and 4 mg of sodium dodecyl sulfate were added. A gel-filtration column (30 × 4.2 cm) was prepared by suspending Sephadex G-50 in a 0.02 M sodium borate buffer (pH 9.1) containing 1.25 μmoles/ml of sodium dodecyl sulfate. The red solution was placed on the top of this column and the same buffered detergent solution was used for development. Whereas protoporphyrin stayed on the upper fourth of the column as a strong reddish-brown zone, another red band moved down rapidly with an R_F value of approx. 0.95. This was collected in the smallest possible volume of eluent and had λ_{max} 538 mμ, while protoporphyrin in the same solvent showed λ_{max} 541 mμ. The eluate was concentrated *in vacuo* to 15 ml, mixed with 150 ml of ethanol and allowed to stand for a few h. The pink precipitate was removed by centrifugation, washed with ethanol and ether and dried.

Enzymatic hydrolysis of the porphyrin-serum albumin adduct and isolation of porphyrin-containing peptides. The protoporphyrin-serum albumin adduct (625 mg) obtained above was dissolved in 35 ml of 0.01 N HCl. A solution of 24 mg pepsin in 15 ml of 0.01 N HCl was added, the mixture was adjusted to pH 2.0 and incubated at 37°C for 16 h. Then the deep red solution was brought to pH 3.9, centrifuged, and the dark precipitate suspended in water

and dissolved by adjusting to pH 8.0. This procedure was repeated twice, the precipitate being finally taken up in a volume of 0.4 ml.

Talc was purified by the method of Witter and Tuppy[9], suspended in water and poured into a column 14 × 2.4 cm. The dark solution (0.4 ml, pH 8) obtained by dissolving the porphyrin-containing peptides was transferred to the column. The red peptides were adsorbed on the upper part of the column, which was then thoroughly washed with water. The red material was subsequently eluted from the talc with 50 % aqueous ethanol containing 2 % ammonia. This eluate was evaporated to dryness *in vacuo*. The dark residue was subjected to partition chromatography on 15 g of Hyflo Supercel as described above for the crude porphyrin *c*. When the chromatogram was developed with the upper phase of a freshly prepared mixture of butanol, glacial acetic acid and water (4 : 1 : 5) a broad band was observed to move downward with an R_F value of approx. 0.7, while a rather narrow band stayed on the upper part of the column. The effluent containing the faster moving band was collected and evaporated to dryness *in vacuo*. The reddish-brown residue was hydrolyzed in 5.7 N HCl at 105°C for 16 h in a sealed capillary. The hydrolysate was divided into two parts, one of which was oxidized with performic acid; both were subjected to high voltage (1500–1600 V) ionophoresis on paper (Whatman 3MM, 24 × 40) using a pyridine-acetic acid buffer of pH 4.6. No basic amino acids were present. The following acidic amino acids were detected: glutamic, aspartic, and, in the oxidized sample, cysteic acid, the relative amounts being: Glu ⟩⟩ Asp ⟩ Cys-SO_3H. The neutral amino acids were eluted from the paper and subjected to descending paper chromatography, using the solvent system of butanol, glacial acetic acid and water (4 : 1 : 5). The following amino acids were detected: leucine, phenylalanine, proline and small amounts of serine and threonine.

RESULTS AND DISCUSSION

When protoporphyrin was reduced with sodium amalgam, subsequently acidified and illuminated with white light in 1 N H_3PO_4 and in the presence of a large excess of cysteine, porphyrin *c* was formed in small quantities. The yield of porphyrin *c* was remarkably increased by carrying out the reaction between pH 6.5 and 7.5. A further increase in the yield of porphyrin *c* was observed when reduced porphyrin and cysteine were allowed to react in the absence of air. The effect of illumination was negligible; the yields of porphyrin *c* did not change significantly when the reaction was performed under exclusion of light. The reaction time was varied between 1 and 3 h, a 2 h period generally being satisfactory.

Bovine serum albumin is known to have approx. 0.65 mole of free –SH groups/molecule[10] and was therefore used as a model for proteins containing available –SH groups. The reaction between serum albumin and protoporphyrin reduced by sodium amalgam was carried out under conditions similar to those in the case of cysteine, but only a slight molar excess of porphyrin over serum albumin was used. In neutral or almost neutral solutions, protoporphyrin is known to be strongly adsorbed on serum albumin[11]. The removal of adsorbed porphyrin from the protein was effected by gel-filtration on columns of Sephadex G-50 in a slightly basic (pH 9.1) borate buffer containing a small amount of sodium dodecyl sulfate detergent.

The reaction between reduced protoporphyrin and serum albumin resulted in a 3 mμ shift to lower wavelengths in the absorption maximum of band III of the reaction product relative to protoporphyrin. The formation of porphyrin *c* was characterized by a 5 mμ shift. The difference between the two changes can be explained by assuming that with excess cysteine both vinyl groups of the porphyrin reacted, while with serum albumin only one of the vinyl groups was involved.

The protein fraction obtained after the gel-filtration of the reaction product of reduced porphyrin and serum albumin contained, beside the porphyrin adduct of serum albumin, large amounts of unreacted protein, but was entirely free of adsorptively bound porphyrin. This mixture was subjected to digestion with pepsin in order to confirm the covalent nature of the protoporphyrin-protein linkage. Porphyrin-containing peptides were obtained and were separated from colorless peptides by adsorption on talc and partition chromatography. The fraction containing the colored peptides was hydrolyzed in 5.7 N hydrochloric acid. High voltage ionophoresis and paper chromatography of the hydrolysate showed the presence of glutamic acid, aspartic acid, cysteine, phenylalanine, leucine, proline, and, in minute quantities, serine and threonine. The six major amino acids are the same amino acids present in the yellow nonapeptide obtained by Witter and Tuppy[9] from serum albumin treated with N-(4-dimethylamino-3,5-dinitrophenyl)-maleimide (DDPM) and subjected to proteolytic digestion. A close examination of the peptide sequence or sequences attached to the porphyrin in the porphyrin-containing peptide fraction has yet to be carried out.

It has not been established which reduction stage of protoporphyrin is the molecular species most readily reacting with sulfhydryl compounds. Sodium amalgam has been shown to reduce porphyrins to porphyrinogens eventually, given sufficient time[12]. In the reductions carried out here, however, relatively short reaction periods were used in order to prevent reduction of the vinyl groups. It is possible that tetrahydro- or even dihydro-protoporphyrin present in the reduction-product may react with sulfhydryl compounds. It can be stated definitely that porphyrin reduced with titanous chloride, which according to Mauzerall[12] is the tetrahydro stage, combines readily with cysteine.

The rather facile reaction of cysteine or an —SH containing protein with reduced protoporphyrin under near-physiological conditions emphasizes the possibility suggested by Sano and Granick[5] that a reaction of the —SH groups of apocytochrome *c* with reduced protoporphyrin may be involved in the formation of cytochrome *c*.

Acknowledgement. We are indebted to the *National Institutes of Health* for a postdoctoral fellowship to T. L. P.

REFERENCES

1. Theorell, H. *Biochem. Z.* **298** (1938) 242.
2. Theorell, H. *Biochem. Z.* **301** (1939) 201.
3. Zeile, K. and Meyer, H. *Z. Physiol. Chem.* **262** (1939/40) 178.
4. Neilands, J. B. and Tuppy, H. *Biochim. Biophys. Acta* **38** (1960) 351.
5. Sano, S. and Granick, S. *J. Biol. Chem.* **236** (1961) 1173.
6. Ramsey, V. G. *Biochem. Prep.* **3** (1953) 39.
7. Tuppy, H. and Paléus, S. *Acta Chem. Scand.* **9** (1955) 353.
8. Eriksen, L. *Scand. J. Clin. Lab. Invest.* **5** (1953) 155.
9. Witter, A. and Tuppy, H. *Biochim. Biophys. Acta* **45** (1960) 429.
10. Boyer, P. D. *J. Am. Chem. Soc.* **76** (1954) 4331.
11. Stenhagen, E. and Rideal, E. K. *Biochem. J.* **33** (1939) 1591.
12. Mauzerall, D. *J. Am. Chem. Soc.* **84** (1962) 2437.

Received March 30, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S51–S54

Amides of Aliphatic Selena-carboxylic Acids

ARNE FREDGA

Chemical Institute, University of Uppsala, Uppsala, Sweden

Thirteen amides of fatty acids, having a selenium atom incorporated in the chain, have been prepared. The methods of preparation are discussed.

In the course of current work on organoselenium compounds of potential biological interest, the author and his collaborators have prepared a number of long-chain selena-carboxylic acids of the general formula I[1–3]. The acids are beautifully crystallising compounds, very similar to ordinary fatty acids. Their biological and physico-chemical properties are being investigated. Most of the acids have rather good Factor 3 activity, but the effect varies in an interesting way with the position of the selenium in the chain[4]. The presence of the heavy selenium atom in the molecule is favourable for X-ray studies of the crystal structure.

C_mH_{2m+1}–Se–$(CH_2)_n$–COOH (I) CH_3–CH_2–Se–CH_2–$CONH_2$ (II)

The work has now been extended to amides of such acids and in the present paper thirteen amides of moderate chain length are described. The 3-selena-valeramide (ethylseleno-acetamide) (II) has earlier been prepared by Bergson, who obtained it in the conventional way from the corresponding acid chloride and ammonia[5]. As ω-bromo esters and ω-bromo nitriles are often the best starting materials for preparing selenosubstituted acids, the author has tried to find a more direct way to the selena amides without isolating the acids.

Starting from the ω-bromo-nitriles, the following sequence of reactions was found convenient:

$$Br\text{–}(CH_2)_n\text{–}CN \xrightarrow[\text{Water}]{Na_2Se_2} [\text{–}Se\text{–}(CH_2)_n\text{–}CN]_2 \xrightarrow[\text{Ether}]{HCl,\ H_2O}$$

$$[\text{–}Se\text{–}(CH_2)_n\text{–}CONH_2]_2 \xrightarrow[NH_3,\ aq]{\text{Rongalite}} HSe\text{–}(CH_2)_n\text{–}CONH_2 \xrightarrow{C_mH_{2m+1}Br}$$

$$C_mH_{2m+1}\text{–}Se\text{–}(CH_2)_n\text{–}CONH_2$$

A solution of sodium diselenide is obtained by shaking calculated amounts of sodium hydroxide, Rongalite and elemental selenium with water in a closed bottle. The ω-bromo nitrile is then added and the mixture shaken until the deep red-brown colour has disappeared and the nitrile layer is purely yellow. The diseleno-dinitrile is then taken up in ether and the ether solution, which contains the necessary amount of water, is saturated with hydrogen chloride. On standing, the amide separates as a yellow crystalline precipitate. After 4–5 days, it is filtered off, washed with ether and treated with sodium carbonate solution to remove all hydrogen chloride. In most cases, the alkaline water layer remains colourless, indicating that no carboxylic acid has been formed. Finally the amide is recrystallised several times from methanol.

The crude product is often contaminated with monoseleno and polyseleno diamides. Probably the sodium diselenide solution, the stoichiometric composition of which corresponds to the formula Na_2Se_2, contains an equilibrium mixture of HSe-ion and various polyselenide ions. According to Schwarzenbach and Fischer, such complex equilibria exist in the solutions of alkali polysulphides[6].

The diseleno diamide is suspended in aqueous ammonia and shaken with Rongalite in a closed bottle until a colourless solution is obtained (the selenol amide formed is soluble in ammonia owing to the acid properties of the selenol group). Finally, alkyl bromide, dissolved in a little ethanol, is added and the shaking is continued for some hours.

In most cases, the selena-amide separates as a crystalline precipitate during the shaking. If the yield of crude amide is not satisfactory, a second crop may be obtained by concentrating the mother liquor. The lowest homologues are, however, rather soluble in water and do not separate. They can be isolated by exhaustive extraction with ether or by evaporating the reaction mixture to dryness and subsequent extraction of the residue with boiling carbon tetrachloride. The product is recrystallised two or three times from carbon tetrachloride (once with charcoal).

Starting from the ω-bromo esters, the synthesis may be performed as follows:

$$Br{-}(CH_2)_n{-}COOC_2H_5 \xrightarrow[NH_3,\ aq]{(NH_4)_2Se_2} [{-}Se{-}(CH_2)_n{-}COOC_2H_5]_2 \xrightarrow[NH_3,\ aq]{Rongalite}$$

$$HSe{-}(CH_2)_n{-}COOC_2H_5 \xrightarrow{NH_3,\ aq} HSe{-}(CH_2)_n{-}CONH_2 \xrightarrow{C_mH_{2m+1}Br}$$

$$C_mH_{2m+1}{-}Se{-}(CH_2)_n{-}CONH_2$$

A solution of ammonium diselenide is prepared by shaking calculated amounts of selenium and Rongalite with concentrated ammonia in a closed bottle. ω-Bromo ester is added and the shaking continued until the colour is purely yellow. More Rongalite and more concentrated ammonia (which should be present in considerable excess) are then added and the mixture is shaken until the ester layer has dissolved and the yellow colour of the diselenide has dis-

Table 1. 4-Selena-amides.

	m. p. °C	Se calc. %	Se found %
4-Selena-valeramide	87–88	47.53	47.37
4-Selena-capronamide	77–79	43.84	43.63
4-Selena-oenanthamide	88.5–89.5	40.68	40.56
4-Selena-caprylamide	88–89	37.93	38.04
4-Selena-pelargonamide	89.5–90.5	35.53	35.48
4-Selena-capramide	90.5–91.5	33.43	33.18
4-Selena-undecanamide	93–94	31.55	31.40

appeared. On standing for 4–6 days, the selenol ester is converted into the selenol amide, which stays in solution. Alkyl bromide is then added and the reaction is performed as described above.

In some cases, the diseleno ester is contaminated by monoseleno ester, which is of course not reduced to selenol ester. Instead it is converted into monoseleno diamide, which may separate as a crystalline precipitate. This should be filtered off before adding the alkyl bromide. If the solution turns yellow during the filtration (oxidation to diselenide), it is decolourised by adding some Rongalite.

It may also be desired to prepare and purify the diseleno diamide. The oxidation of the selenol amide is easily performed by evaporating the solution by a current of air. The diselenide separates as a yellow crystalline precipitate (sometimes discoloured by elemental selenium) and is purified by recrystallisation from methanol.

Direct treatment of esters of diseleno-dicarboxylic acids or selena-carboxylic acids with concentrated ammonia is practicable only for the lowest homologues. The diseleno-dipropionic amide could thus be prepared from the corresponding methyl ester. The higher members of the series react very slowly at room temperature. On heating, undesired side reactions take place.

Table 2. Selena-capramides.

	m. p. °C	Se calc. %	Se found %
3-Selena-capramide	66.5–68	33.43	33.20
4- „ „	90.5–91.5	„	33.18
5- „ „	81–82	„	33.26
6- „ „	89.5–90.5	„	33.22
7- „ „	95.5–96.5	„	33.23
8- „ „	85–86	„	33.25
9- „ „	86.5–88.5	„	33.21

If a halogenosubstituted amide is accessible, it may of course be used as starting material. Thus the 3-selena-capramide was prepared from chloroacetamide via the diseleno diamide.

Two series of amides have been prepared, one with the selenium in position 4 and various chain lengths and one with constant chain length and the selenium atom in various positions. The data are given in Tables 1 and 2 (the 4-selena-capramide is common to both series). The compounds crystallise very readily, the 3-selena-capramide in needles and the others in glistening scales or flakes.

Acknowledgement. A grant from *AB Astra* is gratefully acknowledged.

REFERENCES

1. Fredga, A. and Lindgren, A. *Acta Chem. Scand.* **15** (1961) 938.
2. Rebane, E. *Arkiv Kemi* **20** (1962) 205.
3. Fredga, A. *11 Nord. Kemistmötet, Helsingfors 1962, Berättelse och föredrag,* Helsingfors 1963.
4. Schwarz, K. and Fredga, A. *To be published.*
5. Bergson, G. and Delin, A.-L. *Arkiv Kemi* **18** (1961) 441.
6. Schwarzenbach, G. and Fischer, A. *Helv. Chim. Acta* **43** (1960) 1365.

Received March 27, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S55—S58

The Respiration and Aerobic Glycolysis of Mouse Embryo Cell Cultures Infected with the SE Polyoma Virus

B. SKARZYNSKI†, M. GUMINSKA and Z. PORWIT-BÓBR

Department of Physiological Chemistry and Department of Medical Microbiology, Medical Academy, Kraków, Poland

In mouse embryo cell cultures infected with SE polyoma virus the following changes were observed: decrease in endogenous respiration, increase in aerobic glycolysis, and increase of the Crabtree effect. Just before the cytopathic effect appeared the decrease of oxygen uptake reached its maximum, and the increase of glycolysis was arrested. Selected cells, surviving the cytopathic changes, showed a greater oxygen consumption and lactic acid production.

The polyoma virus described in 1953 by Stewart *et al.*[1] multiplies easily in cultures of mouse embryo cells. The metabolic effects of animal cells infected by oncogenic viruses have not yet been studied. The possible changes in respiration and glycolysis might be of particular value, since the transformation of the normal into the neoplastic cell is associated with changes in the metabolism of glucose described in the classical work of Warburg[2]. Therefore we have undertaken a study of the respiratory metabolism of cell cultures infected with SE polyoma virus.

EXPERIMENTAL

Tissue cultures. Embryos of the Porton strain mice were used for the experiments. The embryo cell suspension was prepared in 0.25 % trypsin solution (Difco), centrifuged, washed twice and resuspended with a sufficient volume of medium to obtain 10^5 cells per ml. The growth medium consisted of Earle's salt solution, 0.5 % lactalbumin hydrolysate (N. B. C. Cleveland, Ohio) and 10 % calf serum.

Virus and infection of cultures. The original SE polyoma virus was obtained from the G. Roussy Institute in Villejuif by courtesy of Dr. G. Barski, and a strain derived from the cell culture passage was used for the experiments. In 48-h cultures of mouse embryo cells the medium with 10 % calf serum was replaced by one containing 3 % serum. Immediately afterwards 1 ml of virus, infectious titre 10^5 TCID (tissue culture infectious dose) per ml, was added to each Roux flask, containing on the average 70×10^5 cells. TCID was calculated by Kärber's method[3], studying the cytopathic effect.

Biochemical studies. Cultures of uninfected cells (controls) and infected cells were prepared simultaneously, and were examined before infection and 1, 3, 5, 6, 7, and 9 days after infection. The cells growing as a monolayer on the walls of Roux flasks were detached by

† Deceased March 17, 1963.

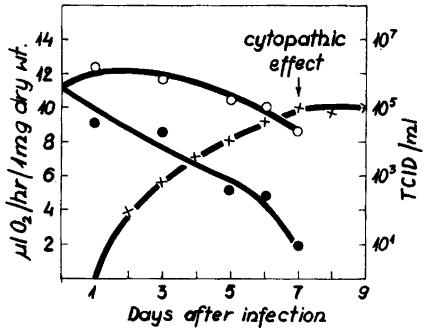


Fig. 1. Endogenous respiration (first 15 min) of mouse embryo cell culture normal o, and infected with SE polyoma virus •. The SE polyoma virus infectivity titre in mouse embryo cell culture medium ×.

scrubbing, washed twice, centrifuged, and suspended in a small volume of Krebs-Ringer buffer, pH 7.4, without glucose. The amount of cells in the suspensions was estimated by determining their dry weight. Endogenous respiration, and respiration in the presence of 10 mM glucose were measured by the method of Warburg[4] in Krebs-Ringer phosphate buffer, pH 7.4. Lactic acid was assayed by the Barker-Summerson method[5] after incubation of the cells with Krebs-Ringer solution containing 10 mM of added glucose.

RESULTS

The endogenous oxygen uptake by mouse embryo cell culture infected or uninfected with polyoma virus is shown in Fig. 1. The infected cells displayed diminished oxygen uptake when compared with the uninfected cells.

The results of determination of the increase in the virus titre presented in Fig. 1, indicate that the first release of the virus into the medium was preceded by the reduction of oxygen uptake, observed after 24 h. The lowest oxygen uptake on the sixth day coincided in time with the presence in the medium of the largest amount of virus and with the appearance of necrotic changes in the cells. Endogenous respiration of the selected cells, after the detached necrotic cells had been discarded with the medium, was much higher on the ninth day after infection than just before the appearance of cytopathic changes in the whole material.

Oxygen uptake in the presence of glucose both by infected and uninfected mouse embryo cells, was lower than the endogenous oxygen consumption (Table 1). Inhibition of oxygen uptake by glucose, *i. e.* the Crabtree effect, was always greater in the cells infected with polyoma virus.

Infection augmented aerobic glycolysis (Fig. 2). The increase of lactic acid production was proportional to the increase in virus formation. In the uninfected culture aerobic glycolysis also increased with time, but this increase was slower and probably due to the long period of cultivation in an unchanged medium. The reduction in lactic acid formation on the seventh day after infection was correlated in time with the decrease in oxygen uptake, with the detachment of the cells from the glass, and with the appearance of cytopathic changes.

Table 1. The effect of 10 mM glucose on oxygen uptake by mouse embryo cell cultures uninfected and infected with polyoma virus. Mean values of 3 experiments are given.

Days	Cells	Mean values of oxygen uptake during 1st hour of experiment μl O_2/1 h/1 mg dry wt		Decrease in oxygen uptake %
		endogenous	in presence of glucose	
Before infection		11.3	8.3	19.8
After infection				
1	Uninfected	11.5	9.8	14.8
	Infected	6.2	4.1	33.9
2	Uninfected	8.9	6.6	25.8
	Infected	6.3	4.1	34.9
5	Uninfected	6.5	5.2	20.0
	Infected	5.7	1.7	54.0
6	Uninfected	10.4	6.2	40.3
	Infected	8.1	4.5	44.4
7	Uninfected	7.6	5.1	32.8
	Infected	3.6	2.0	44.5
9	Uninfected	15.2	12.2	19.7
	Infected	11.2	9.4	16.1

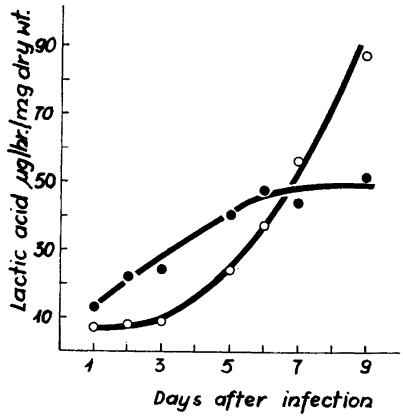


Fig. 2. Aerobic glycolysis in mouse embryo cell culture normal o, and infected with SE polyoma virus •.

DISCUSSION

The decrease in endogenous oxygen uptake and the increase in aerobic glycolysis already observed 24 h after infection, *i. e.* before the appearance of virus in the medium, might constitute evidence for alteration in the metabolism of the cells. When necrotic changes in the cells were first observed, the amount of virus reached its maximum and did not rise further, either because of the exhaustion of the cells necessary for the synthesis of virus, or as the result of development of defence mechanisms in the still undamaged cells. The surviving selected cells, investigated on the ninth day, were more resistant, or able to develop defence mechanisms, possibly leading to transformation into neoplastic cells. These cells may correspond to the fibroblast-type cells described by Negroni[6], more resistant to the virus and capable of surviving the period of cytopathic changes manifested mainly in sensitive embryo cells of the epithelial type.

The Crabtree effect observed in aerobically glycolysing neoplastic cells[7], as well as in those cultivated *in vitro*[8] was much higher in cells infected with oncogenic polyoma virus than in those uninfected. According to current views the Crabtree effect is due to a decrease of the phosphorus compounds which are essential for activation of the respiratory chain[9]. The increasing Crabtree effect in infected cells may be due to a shunt of the nucleotides and P_i which activate the respiration to the synthesis of viral nucleic acids. Further studies have been undertaken on these assumptions.

REFERENCES

1. Stewart, S. E., Eddy, B. E. and Stanton, M. F. In Homburger, F. and Karger, S. *Progr. Exptl. Tumor Res.*, Basel, 1960, vol. I, p. 67.
2. Warburg, O. *Naturwissenschaften* **42** (1955) 401.
3. Irwin, J. O. and Cheeseman, E. A. *J. Hyg.* **39** (1939) 574.
4. Umbreit, W. W., Burris, R. H. and Stauffer, J. P. *Manometric Techniques and Tissue Metabolism*, Burgess Publ. Co., Minneapolis, 1951, p. 7.
5. Barker, S. A. and Summerson, W. H. *J. Biol. Chem.* **138** (1941) 535.
6. Negroni, G. A. *Ciba Found. Symp. on Tumor Viruses of Murine Origin*, Churchill Ltd., London, 1962, p. 332.
7. Crabtree, H. G., *Biochem. J.* **23** (1929) 536.
8. Guminska, M. and Borysiewicz, J. *Acta Biochim. Polon.* **9** (1962) 71.
9. Chance, B. and Hess, B. *J. Biol. Chem.* **234** (1959) 2421.

Received March 28, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S59–S66

The Disulphide-Reducing Capacity of Liver Mitochondria

L. ELDJARN and J. BREMER*

Institute of Clinical Biochemistry, Rikshospitalet, University of Oslo, Oslo, Norway

1. Isolated mammalian mitochondria have been shown to reduce a number of small molecular disulphides in the presence of citric acid cycle intermediates.
2. The physiologically occurring CSSC, GSSG and homocystine are not reduced.
3. The α-keto acids of the citric acid cycle are the most efficient reducing substrates.
4. The reduction is stimulated by AMP, ADP, magnesium ions and by oxygen.
5. On the basis of these results it is suggested that the disulphides are reduced *via* exchange reactions with the thioctic acid.

Glutathione reductase is the only well characterized disulphide-reducing system of mammalian cells. This system requires $NADPH_2$ as coenzyme and is only active with GSSG as substrate. Even mixed disulphides containing glutathione are inactive as substrates[1]. Upon disintegration of rat liver cells, the glutathione reductase is found in the particle-free supernatant fraction[2].

Most mammalian cells demonstrate the ability to reduce a variety of low molecular disulphides[3]. This reduction may be brought about by the glutathione reductase *via* spontaneous exchange reactions between glutathione and the disulphide in question[1].

The reduction of homocystine[4] and of insuline disulphide groups[5] observed in rat liver extracts, are also ultimately caused by glutathione and glutathione reductase.

Romano and Nickerson[6] have described a GSH-independent but $NADH_2$-dependent cystine reductase in extracts of hog liver. A similar cystine-reductase activity has been found in acetone powder preparations of liver[7]. These systems have a relatively low activity, but closer characterization is lacking.

In the present investigation we report on a disulphide reducing system present in mammalian mitochondria. Isolated mitochondria rapidly reduce a number

* Research Fellow, Norwegian Cancer Society, Oslo, Norway.

of low molecular disulphides, provided substrates for the citric acid cycle are present. This reduction does not require glutathione as a cofactor, and data are presented which indicate that the disulphides are reduced at the thioctic acid level of the α-keto acid oxidation.

MATERIALS AND METHODS

Cystamine and cystamine derivatives were obtained and prepared as previously described[3]. All other chemicals used were commercial products of highest purity.

Mithochondria were prepared from grown rats of mixed breed. Both female and male rats were used. The rats were sacrificed by a blow to the neck and bled from the neck vessels. The isolated organs were cooled on crushed ice, weighed and homogenized in ice cold 10 % sucrose in a teflon-glass Potter-Elvehjem homogenizer. After removal of cell debris and nuclei by centrifugation at $800 \times g$ for five min the mitochondria were isolated by centrifugation at $20\,000 \times g$ for 15 min, washed once in the same volume of homogenizing medium, and resuspended in 10 % sucrose in a concentration corresponding to about 1 g of fresh tissue per ml.

Incubations were performed at 30°C with air as the gas phase. Where not otherwise stated, the following additions were used (expressed as μeqv. per 3 ml): glycylglycine buffer of pH 7.6, 40; potassium phosphate, 40; AMP, 10; magnesium chloride, 20. The appropriate disulphide was added as stated under the individual experiments. All additions were added as 0.1 M solutions. Mitochondria were added in amounts corresponding to approximately one gram of liver tissue (1 ml mitochondria suspension in sucrose) per 3 ml of incubation mixture. The volume of the incubation mixture was adjusted to 3 ml with 0.15 M potassium chloride.

Sulphhydryl groups were determined by titration with 1 mM iodine after precipitation of the proteins with trichloroacetic acid. Soluble starch was used as indicator.

To check the titration method, incubations were performed with ^{35}S-labelled cystamine. After acidification of the reaction mixture cystamine and cysteamine were separated by electrophoresis as previously described[8]. This method also would have revealed a number of possible cystamine metabolites such as hypotaurine, taurine and sulphate. The only metabolite found was cysteamine, and in amounts corresponding closely to the iodine titration values.

Some variations in the amounts of SH formed could be observed from one experiment to the other. The reason for this is not entirely clear, but small variations in pH could in part account for these differences.

Preincubation experiments also showed that a rapid inactivation of the mitochondria took place in the presence of disulphides and in the absence of a reducing substrate. Furthermore, it must be stressed that the reported iodine titration data represent minimum values for the disulphide reduction, since cysteamine and cysteamine derivatives undergo rapid spontaneous oxidation by atmospheric oxygen.

RESULTS

Fig. 1 A shows the time course for the reduction of cystamine and for glutathione by rat liver mitochondria with α-ketoglutarate as the substrate. The maximum reduction of the disulphide was usually obtained after 30 to 40 min. In the experiments presented in Fig. 1, 35 and 50 % of the disulphide was reduced. With higher concentrations of mitochondria (Fig. 2), a complete reduction was generally obtained. It is evident that oxidized glutathione is not reduced to any significant extent by rat liver mitochondria (see also Table 2). No reduction of cystamine was observed when α-ketoglutarate was omitted. Anaerobiosis also impaired the efficiency of the reduction (Fig. 1 B) in spite of the fact that the spontaneous oxidation of cysteamine is slowed down by this treatment.

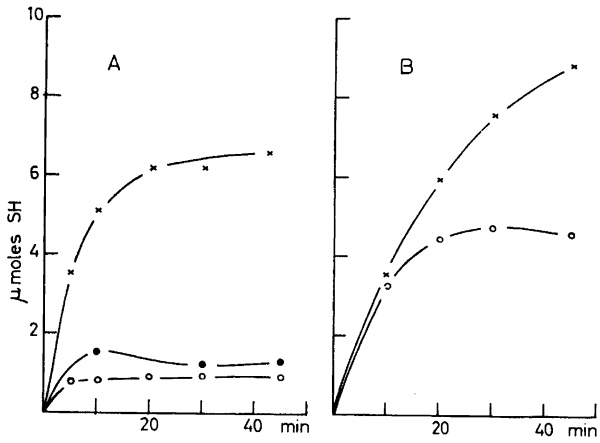


Fig. 1. Time curves for the reduction of cystamine (3 mM) and glutathione (3 mM). Mitochondria from 1 g of rat liver was incubated with the disulphides in the presence of α-ketoglutarate (6.7 mM). A: x, cystamine; o, glutathione; ●, cystamine without α-ketoglutarate. B: x, cystamine in the presence of oxygen; o, cystamine in the absence of oxygen.

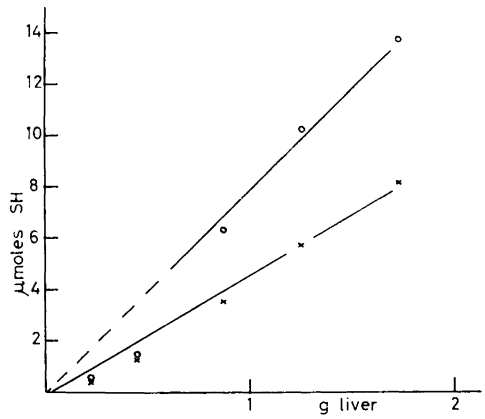


Fig. 2. The effect of mitochondria concentration on the reduction velocity of cystamine (3 mM). Mitochondria from the given amounts of rat liver tissue was incubated for 10 min (x) and 20 min (o) with α-ketoglutarate (6.7 mM) as substrate.

Fig. 2 demonstrates that except for the lowest concentrations the reduction velocity under the described experimental conditions is proportinal to the mitochondrial concentration for at least 20 min. At low mitochondrial concentrations a more rapid inactivation of the reduction was observed.

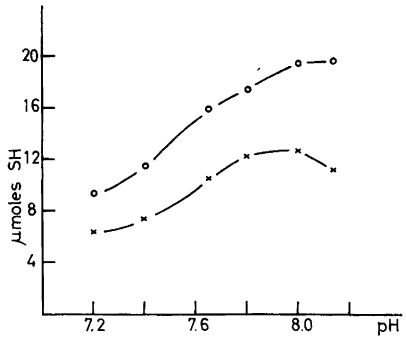


Fig. 3. The effect of pH on the reduction velocity of cystamine (3 mM). Mitochondria from 1 g of rat liver was incubated for 10 min (x) and 30 min (o) with α-ketoglutarate (6.7 mM) as substrate.

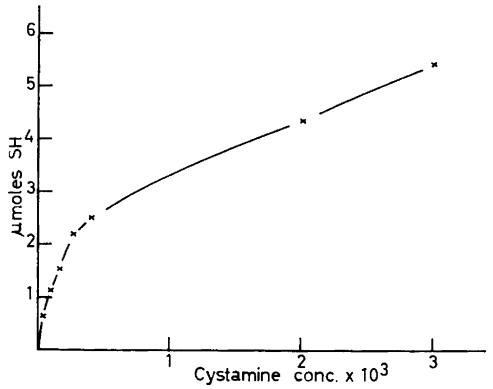


Fig. 4. The effect of cystamine concentration on the reduction velocity. Mitochondria from 1 g of rat liver was incubated for 10 min in the presence of α-ketoglutarate (6.7 mM) with the given cystamine concentrations in equilibrium with a glutathione containing "SS buffer system" of total SS concentration 10 mM.

Fig. 3 shows that an increase in pH up to about 7.8 causes a rapid rise in the reduction velocity. This effect must be even greater than here observed, since the increase in pH also is known to increase the spontaneous oxidation of the thiol formed. Increase of the pH above 8 did not give further rise in the reduction rate.

Fig. 4 shows the effect of cystamine concentration on the reduction velocity. In these experiments "disulphide buffer systems"[9] were used in order to obtain significant amounts of SH formation even with the 10^{-5} M concentrations of cystamine. These SS buffers consisted of equilibrium mixtures of varying amounts

Table 1. The effect of cofactors on the reduction of cystamine (3 mM) by rat liver mitochondria with α-ketoglutarate as substrate. The complete system contained (μeqv. per 3 ml.): glycylglycine buffer of pH 7.6, 40; potassium phosphate, 40; AMP, 10; magnesium chloride, 20.

Cofactor omitted	μmoles SH produced by mitochondria from 1 g of tissue. Incubation time		
	10 min	20 min	45 min
None	5.4	6.6	6.8
Phosphate	4.5	6.8	7.5
Mg++	4.6	4.8	4.7
AMP	3.8	3.8	4.1
Cystamine	0.7	0.7	0.8

of cystamine with oxidized glutathione and with a total SS concentration of 10 mM. It could be deduced from the relationship between concentration and reduction velocity that of the three disulphides present in these mixtures (GSSG, cystamine and their mixed disulphide) only cystamine was reduced by mitochondria, whereas the other two served as disulphide stores which replaced cystamine as it became reduced. The initial equilibrium concentration of cystamine as calculated from the known equilibrium constants[8] is plotted on the abscissa of Fig 4. In the course of the experiment these values became lowered with less than 10 % of the initial values owing to the buffering capacity of the system. It appears that with rising cystamine concentrations there is a rapid increase in the reduction velocity until in the mM range the reduction rate levels off.

Table 1 shows that no cofactor was found which had any significant effect on the initial reduction rate, but both magnesium and AMP (as well as ADP) were repeatedly observed to maintain the reduction for a longer period of time. The reduction was also found to proceed for a longer period of time under aerobic conditions (Fig. 1 B). The omission of phosphate had no significant effect. In other experiments, AMP was found on a molar basis to have approximately twice the effect of ADP. ATP had far less effect than AMP and ADP. The addition of NAD, NADP or nicotinamide had no effect on the reducing velocity. Of particular significance is the finding that NADP plus citrate had no effect on the reduction of GSSG, thus excluding contamination of the mitochondria with the extramitochondrial isocitric dehydrogenase and the soluble glutathione reductase of rat liver[2].

Table 2 demonstrates that cystamine, its N-alkyl derivatives and cystine diethylester are all efficiently reduced. NN′-diacetylcystamine and bis(hydroxyethyl) disulphide are reduced to some extent, whereas with GSSG, homocystine, tetrathionate and NNN′N′-cystamine tetraacetic acid no significant reduction was observed. Table 3 shows the substrate requirements for the reduction of cystamine by rat liver mitochondria. Choline effected no reduction, although

Table 2. The reduction of different disulphides by rat liver mitochondria with α-ketoglutarate as substrate (6.7 mM).

	Disulphide	μmoles SH produced by mitachondria from 1 g of tissue Incubation time 10 min	20 min	45 min
Exp. I	None	0.8		0.9
	Cystamine	7.8		12.9
	NN'-Tetraethylcystamine	6.9		10.0
	NN'-Tetramethylcystamine	6.4		11.4
	Bis(hydroxyethyl)disulphide	1.8		4.2
	GSSG	1.0		1.3
	NNN'N'-Cystaminetetraacetic acid	0.9		1.1
Exp. II	None	0.7	0.9	
	Cystamine	8.9	10.5	
	Cystine diethyl ester	6.4	5.4	
	NN'-Diacetylcystamine	2.4	3.3	
	Sodium tetrathionate	1.4	1.5	
	L-Homocystine	1.3	1.5	
	CSSC	1.2	1.3	

oxygen uptake showed that the compound was rapidly metabolized by the mitochondria. All the intermediates of the citric acid cycle could serve as reducing substrates. Although the differences were not great, it was constantly observed that the α-keto acids were superior to the other substrates. Citrate showed an intermediary efficiency, whereas malic and succinic acid were found to be the poorest substrates.

DISCUSSION

The mitochondrial system for disulphide reduction reported on in the present paper, most likely is different from the previously known disulphide reducing systems of mammalian tissues. Thus, the finding that GSSG is not reduced, even in the presence of citrate and NADP, strongly indicates that the glutathione reductase is not involved in the mitochondrial system. This conclusion is further supported by the finding that the latter enzyme is lacking in rat liver mitochondria[2].

Succinate belongs to the relatively poor substrates, and choline could not bring about any reduction at all (Table 3). These substrates are oxidized directly by flavoproteins and the electrons are fed directly into the electron transport chain[10]. The electrons required for the reduction of disulphides by mitochondria, therefore, most likely are not derived from this respiratory chain.

The participation of $NADH_2$ in the mitochondrial reduction cannot be exclu-

Table 3. Reducing substrate requirement for the reduction of cystamine by rat liver mitochondria. Substrate concentration 6.7 mM, cystamine concentration 3 mM.

Substrate	μmoles SH produced by mitochondria from 1 g of tissue Incubation time	
	10 min	20 min
None	2.1	2.1
Choline	2.4	2.2
Fumaric acid	7.2	10.8
Malic acid	7.6	11.2
Succinic acid	7.8	11.3
Citric acid	8.7	12.6
α-Ketoglutaric acid	9.7	15.5
Glutamic acid	10.8	15.2
Oxaloacetic acid	9.6	14.4
Pyruvic acid	10.2	15.6
Oxaloacetic + pyruvic acids	11.3	16.5

ded with certainty, since this coenzyme is known to be retained by mitochondria even after washing. However, the finding that malate, which is oxidized in the mitochondria by a $NADH_2$ dependent dehydrogenase, is a relatively poor substrate for the reduction, indicates that $NADH_2$ is not involved. It should be recalled that the slow acting "cystine reductase" of Romano and Nickerson[6] is $NADH_2$-dependent.

The α-keto acids of the citric acid cycle were repeatedly found to be the best reducing substrates. This fact points to the thioctic acid step of the α-keto-acid oxidation to be the immediate donor of electrons for the reduction of disulphides. Thioctic acid is known to be reduced at the aldehyde oxidation step, and it is claimed to be reoxidized by transfer of electrons to a protein disulphide group[11]. The added small molecular disulphides may become reduced by spontaneous interaction with these groups in the thiol form. If such a view is correct, higher concentrations of the foreign disulphide would be expected to bring about a "disulphide poisoning" of the α-keto acid oxidation of mitochondria. That this is indeed the case, will be shown in a forthcoming communication. The observed effect of pH on the reduction velocity is also in accordance with the proposed reduction mechanism. It is known that the ionized thiol is the reactive species in the exchange reactions between SH and SS compounds[8]. Since the pK_{SH} of the thiols in question most likely are above 8, an increase in pH will be expected to speed up the reaction rate.

The effects of AMP, of magnesium ions and of oxygen in maintaining the mitochondrial reduction for a longer period of time is difficult to explain. Most

probably these effects are connected with the preservation of the integrity of the mitochondrial structure and with the reformation of α-keto acids of the citric acid cycle.

The results shown in Table 2 demonstrate that rat liver mitochondria do not reduce the physiologically occurring GSSG, CSSC and homocystine to any significant extent. This can not be explained by differences in the oxidation-reduction potentials of these disulphides[8]. Most probably these compounds do not reach the reducing site in the mitochondria (the thioctic acid SH groups), and impermeability of the mitochondrial membrane appears to be the most likely explanation.

REFERENCES

1. Pihl, A., Eldjarn, L. and Bremer, J. *J. Biol. Chem.* **227** (1957) 339.
2. Rall, T. W. and Lehninger, A. L. *J. Biol. Chem.* **194** (1952) 119.
3. Eldjarn, L., Bremer, J. and Börresen, H. C. *Biochem. J.* **82** (1962) 192.
4. Racker, E. *J. Biol. Chem.* **217** (1955) 867.
5. Narahara, H. T. and Williams, R. H. *J. Biol. Chem.* **234** (1959) 71.
6. Romano, A. H. and Nickerson, W. J. *J. Biol. Chem.* **208** (1954) 409.
7. Patrick, A. D. *Biochem. J.* **83** (1962) 248.
8. Eldjarn, L. and Pihl, A. *J. Am. Chem. Soc.* **79** (1957) 4589.
9. Börresen, H. C. and Eldjarn, L. *Acta Chem. Scand.* **17** (1963) 884.
10. Rendina, G. and Singer, T. P. *J. Biol. Chem.* **234** (1959) 1605.
11. Fluharty, A. and Sanadi, D. R. *Proc. Natl. Acad. Sci. U. S.* **46** (1960) 608.

Received April 1, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S67–S73

Activation and Inhibition of NADH Oxidation by Brain Mitochondrial Fragments

HAROLD J. STRECKER* and GUIDO DI PRISCO**

Department of Biochemistry, Albert Einstein College of Medicine, Yeshiva University, New York, U. S. A.

The NADH oxidase activity of brain mitochondrial fragments is considerably greater when determined in salt solutions than in water or in 0.25 M sucrose solutions. Inhibition by dicumarol and amytal but not by chlorpromazine also depended on the addition of electrolytes in the incubation medium. Using phosphate as a representative ion it was found that retention of dicumarol and chlorpromazine by the mitochondrial fragments was correlated with the degree of inhibition. However, there was no retention of amytal. Dicumarol also inhibited succinate oxidation and to a greater degree in an incubation medium containing phosphate. It is proposed that an interaction of added electrolytes with lipids in the fragments is the underlying phenomenon responsible for the increased permeability to some solutes and an increase in mutual accessibility of components of the electron transport chain.

Previous work in this laboratory has demonstrated that the antimycin and amytal sensitive pathway of NADH oxidation in brain mitochondria undergoes a great increase of activity on fragmentation of the mitochondrial fraction and that a major part of this increased activity is only apparent when the assay is conducted in a medium containing salts[1]. Further investigation of this phenomenon has now revealed that a relationship may exist between the activation brought about by added electrolytes and the extent of inhibition observed with substances which inhibit electron transport from NADH *via* the antimycin sensitive respiratory chain. These effects have been studied using three classes of inhibitors, represented by dicumarol, amytal and chlorpromazine. A comparison has also been made of the effects of added electrolytes and inhibitors on succinate oxidation. The results presented in this paper suggest that added electrolytes including ATP and ADP alter the membrane permeability so that

* Research Career Development Awardee GM-K3-2487-C5 of the U. S. Public Health Service.
** On leave of absence from the International Laboratory of Genetics and Biophysics, Naples, Italy.

certain inhibitors and perhaps also substrates can more readily gain access to the organized respiratory chain embedded within the mitochondrial membrane.

MATERIALS AND METHODS

The chemical preparations used, the preparation of rat brain mitochondria and of mitochondrial fragments by sonic irradiation, the assays for NADH oxidase, NADH-cytochrome *c* reductase and succinate-cytochrome *c* reductase have been previously described[1,2]. In those experiments in which buffer was not added, all solutions were adjusted to pH 7.3–7.5. The pH (glass electrode) did not change beyond these limits during any experiment. Recording of the absorbancy spectrum of dicumarol, amytal and chlorpromazine, as described in the experimental results, was carried out with the Bausch & Lomb, model 505, spectrophotometer.

In the experiments of Figures 1–3, 1 ml of enzyme suspension catalyzed the oxidation of 940 mμmoles of NADH per minute in 0.1 M phosphate buffer, pH 7.3.

RESULTS

Stimulation of NADH oxidase by added electrolytes. The NADH oxidase activity of the mitochondrial fraction of rat brain homogenates prepared with 0.25 M sucrose is usually very low, ranging from 100 to 250 mμmoles per minute per gram (wet weight). The poor capacity of intact mitochondria to oxidize NADH has been reported also for other tissues[3–5]. Fragmentation of the brain mitochondrial fraction by sonic irradiation results in an increase of NADH oxidase activity up to 6 000 mμmoles per minute per gram. Much of this increased rate of oxidation depends on the addition of electrolytes in the incubation medium; in water or in 0.25 M sucrose solutions, NADH is oxidized by the mitochondrial fragments at only 6- to 8-fold as compared to intact mitochondria. Activating electrolytes previously reported include amino acids, neutral salts, ethylenediamine tetraacetate and various buffer solutions[1]. Extension of these studies to the adenosine polyphosphates as representative of cellular electrolytes demonstrated that ATP was also effective, ADP to a lesser degree and AMP had no effect. The data of Table 1, column 1, compare the NADH oxidase activity in water and in sucrose incubation media with the activity in some ionic media. The added electrolytes are at previously determined optimal concentrations. These experiments were carried out without added cytochrome *c*. Addition of cytochrome *c* stimulated NADH oxidation 2- to 3-fold in all experiments. Comparable data were obtained when NADH oxidase activity was measured by the change in absorbancy at 340 mμ, or cytochrome *c* reduction measured by the change in absorbancy at 550 mμ in the presence of KCN. The results to be described below, while referred to NADH oxidase without cytochrome *c* present, in general also apply to experiments with added cytochrome *c* whether measured at 340 or 550 mμ.

Effect of inhibitors. NADH oxidation in all incubation media was inhibited more than 95 per cent by antimycin A (1.72 μg/ml). During the course of our studies on the soluble dicumarol sensitive NADH oxido-reductases[6,7] *, it was observed that concentrations of dicumarol considerably in excess of that required to inhibit the soluble oxido-reductases were also inhibitory to the respiratory chain with either NADH or succinate as substrates. At a concentration of 1×10^{-4} M dicumarol, which is close to the solubility limit at the pH used,

* These enzymes have been referred to as diaphorases in previous publications. They are listed as menadione reductases (EC1.6.5.2) by the Enzyme Commission.

Table 1. Ion stimulation of NADH oxidation by fragmented mitochondria, and inhibitory effect by dicumarol, amytal and chlorpromazine. The incubation mixture contained 1×10^{-4} M NADH and activator or/and inhibitor at the final concentrations indicated in the table. Activity units are calculated as μmoles per min per g (wet weight) of brain.

Activator	Activity	Inhibition in % with		
		5×10^{-5} M dicumarol	2×10^{-4} M amytal	3.3×10^{-5} M chlorpromazine
none	800	0	0	52
0.1 M phosphate buffer	6 250	43	68	40
5×10^{-3} M ATP	2 200	15	10	45
5×10^{-3} M ADP	960	8	6	29
5×10^{-3} M AMP	755	2	0	34
5×10^{-2} M EDTA	5 750	50	60	55
0.25 M sucrose	800	0	0	32

inhibition of about 50 to 60 % was obtained. However, this inhibition was completely dependent on the addition of electrolytes to the incubation media, and there appeared to be a correlation between the degree of activation by the added ion and the degree of inhibition exerted by dicumarol in each ionic solution (Table 1, column 2). Similar experiments carried out with two other representative compounds, which are inhibitory to the respiratory chain, amytal[8–10] and chlorpromazine[11–13], demonstrated that amytal behaved like dicumarol, but that chlorpromazine was inhibitory in all incubation media, although the degree of inhibition varied (Table 1, columns 3, 4). In these experiments suboptimal concentrations of inhibitor were used, so as to permit the effect of the various added electrolytes to be seen more clearly. Sucrose media appeared to behave qualitatively like water; the addition of electrolytes to sucrose gave the same results as the addition of electrolytes to water.

Retention of inhibitors. The data in the literature indicating that phosphate promotes mitochondrial swelling by increasing the permeability of the membrane[14] and the evidence that sonic irradiation of mitochondria results in the formation of small, membrane-surrounded particles[15] suggested a study of the penetration of inhibitors into the mitochondrial fragments under the influence of phosphate and other added electrolytes. A feasible experimental approach was to compare the retention of the inhibitors by the mitochondrial fragments in water or sucrose with the retention in phosphate solutions. The characteristic optical absorption of each of these compounds in the ultraviolet region, permitted these experiments to be conducted readily. Suspensions of mitochondrial fragments were incubated in water, in 0.25 M sucrose or in 0.1 M phosphate solution, at room temperature for 10–15 minutes, with the inhibitor. The suspension was centrifuged at $105\,000 \times g$ for one hour and absorbancy of the supernatant determined in the region 230–350 mμ against a blank prepared in the same way without inhibitor present. Fig. 1 A compares the spectra of a dicumarol solution

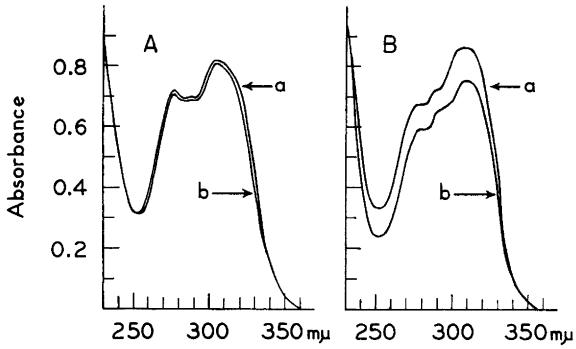


Fig. 1. Retention of dicumarol by sonicated mitochondria. Experimental details are given in the text. A: spectrum of dicumarol in water; *curve a* before incubation, *curve b* after incubation with mitochondrial fragments. B: same as A in phosphate buffer. Spectra recorded against blanks obtained after centrifuging off equal amounts of particles previously incubated with water and phosphate, respectively.

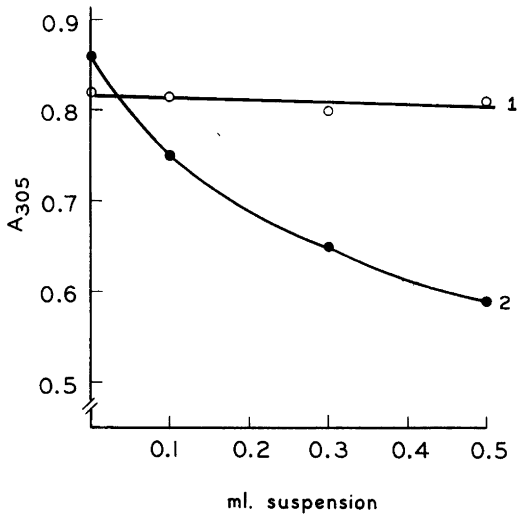


Fig. 2. Retention of dicumarol as a function of concentration of sonicated mitochondria. *Curve 1,* in water; *curve 2,* in phosphate buffer. Blanks as in Fig. 1.

(5×10^{-5} M) before and after incubation with fragmented mitochondria in water. No retention of dicumarol was observed under these conditions. By contrast, the incubation in 0.1 M phosphate resulted in the removal of about 13 % of the dicumarol by the particles (Fig. 1 B). Determination of the absorbancy changes at 305 mμ, where dicumarol absorbs maximally, was used to obtain the data presented in Fig. 2, which demonstrates that in water little or no dicumarol was removed with increasing concentrations of particles whereas in phosphate the removal of dicumarol depended on the concentration of fragments.

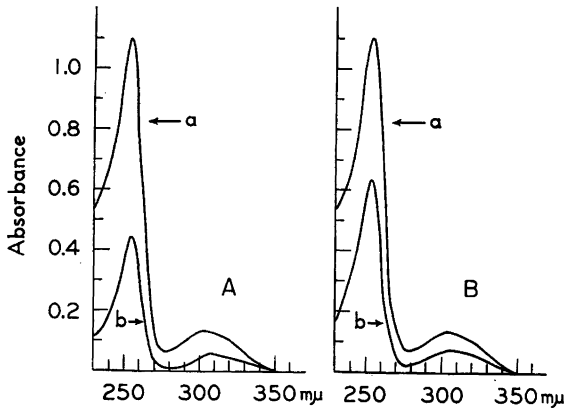


Fig. 3. Retention of chlorpromazine by sonicated mitochondria. A: spectrum of chlorpromazine in water; *curve a* before incubation, *curve b* after incubation with mitochondrial fragments. B: same as A in phosphate buffer. Blanks as in Fig. 1.

The same experiments carried out with chlorpromazine (3.3×10^{-5} M) revealed that under the same conditions this inhibitor was retained by the fragments when incubated in water, 0.25 M sucrose or phosphate (Fig. 3) and, indeed, that greatest retention was observed in the water incubation, and that a rough correlation existed between the retention of chlorpromazine and the degree of inhibition seen in Table 1.

In contrast to the results with chlorpromazine and dicumarol, which indicated a correlation between retention of the inhibitor by the fragments and degree of inhibition, amytal (2×10^{-4} M) was not retained when incubated with mitochondrial fragments in either water or sucrose on the one hand or in phosphate solution on the other.

Inhibition of succinate oxidation. Since succinate oxidation by heart and kidney preparations has been shown to be stimulated by phosphate and other added electrolytes[16–18], it was of interest to examine the effect of dicumarol and chlorpromazine on succinate oxidation with fragmented brain mitochondria. However, since saturating concentrations of succinate provided an ion concentration sufficiently high to activate the enzymatic system, it was necessary to carry out these experiments at substrate concentrations lower than optimal. At two different concentrations of succinate (2×10^{-3} M and 6×10^{-3} M) dicumarol inhibited the reduction of cytochrome *c* to the extent of 35 % in 0.1 M phosphate solutions and about 10 % in water. Chlorpromazine, as previously reported[12] and confirmed[13], did not inhibit succinate-cytochrome *c* reductase.

DISCUSSION

Dicumarol interferes with reduced nicotinamide adenine nucleotide oxidizing systems at different levels of concentration. The NADH and NADPH oxidoreductases are inhibited at a concentration of 10^{-8} M, and phosphorylation is uncoupled at a concentration of 10^{-5} M[6,7,19,20]. As shown in the experiments re-

ported here, 10^{-4} M dicumarol inhibits both NADH and succinate oxidation by the respiratory chain of fragmented brain mitochondria. Chlorpromazine has also been shown to have a similar spectrum of action. It inhibits brain NADH and NADPH oxido-reductases, uncouples oxidative phosphorylation and inhibits electron transport from NADH, succinate and NAD-linked dehydrogenases[7,11–14]. Amytal inhibits NADH oxidation but does not inhibit the oxidation of succinate. The effect of 0.1 M phosphate, as a representative ion, on each of these inhibitors is different. Thus phosphate, which is required for dicumarol inhibition, also promotes the retention of this compound by the mitochondrial fragments. With chlorpromazine, which inhibits in water or ionic media, retention is obtained in either media. Amytal, however, which also requires an ionic medium for inhibition, is not retained by the fragments. The assumption that phosphate increases the permeability to solutes of the membrane of the fragments, as has been shown for intact mitochondria, apparently is not true for chlorpromazine, although it could account for the effect on dicumarol and amytal. The latter two substances are acids, whereas chlorpromazine is a base. The undissociated form of amytal has been shown to be the active form in the inhibition of cell division of sea urchin eggs[21], and in the inhibition of NADH oxidation by brain mitochondrial fragments[22]. It is conceivable that the permeability to the unionized forms of acidic substances is promoted by the active electrolytes, whereas bases are easily permeable in either electrolyte or non-electrolyte media. In order to account for the retention of dicumarol and chlorpromazine compared to the non-retention of amytal, it would seem necessary to postulate that the former two substances after penetrating the membrane barrier are bound to some internal structure in sufficiently high concentration to be measured, whereas amytal is only bound to an active site of an enzyme involved in NADH oxidation, which enzyme is present in relatively low concentrations.

Chlorpromazine inhibition differs from that obtained with dicumarol in only one aspect, the lack of effect on the succinate-cytochrome *c* portion of the respiratory chain[12,13]. The data for the most part are in accord with the hypothesis that the inhibitory effects of dicumarol and chlorpromazine, in contrast to amytal, are the result of an interference with the physical structure containing the respiratory chain which causes a decrease in the mutual accessibility of some of the components of the chain.

The nature of this physical change is unknown. The inclusion of salts in the incubation medium results in a visible increase in turbidity of the suspension, perhaps indicating a contraction or agglutination of the particles. A similar increase in turbidity is observed when salts are added to a fine suspension of lecithin. The addition of dicumarol to the turbid suspension of mitochondrial fragments does not alter to any measurable extent the optical absorbancy of the suspension. It is conceivable that the increase in permeability, presumably brought about by added electrolytes, the hypothetical alteration of component accessibility and the visible increase in turbidity all reflect the same underlying phenomenon, an interaction of electrolytes with lipids in the particle structure.

The phenomenon described may be the same as or closely related to the activation of succinate and NADH oxidation, previously studied in heart and kidney preparations[16–18]. If further study indicates the two phenomena to be

indeed related, it would appear that the elucidation of the difference in inhibitory effect between dicumarol and chlorpromazine on the succinate-cytochrome *c* portion of the respiratory chain might aid in understanding the relationship of the common cytochrome *c* oxidase pathway to the convergent NADH-cytochrome *c* reductase and succinate-cytochrome *c* reductase pathways.

Acknowledgement. This work was suported by Grants No. RG 9904 from the *U. S. Public Health Service* and No. 235 from the *National Multiple Sclerosis Society.* Thanks are due to the *International Laboratory of Genetics and Biophysics* for travel funds to Dr. Guido di Prisco.

REFERENCES

1. Giuditta, A. and Strecker, H. J. *J. Neurochem.* **5** (1959) 50.
2. Levine, W., Giuditta, A., Englard, S. and Strecker, H. J. *J. Neurochem.* **6** (1960) 28.
3. Lehninger, A. L. *J. Biol. Chem.* **190** (1951) 345.
4. Sacktor, B. *Ann. Rev. Entomol.* **6** (1961) 103.
5. Boxer, G. E. and Devlin, T. M. *Science* **134** (1961) 1495.
6. Giuditta, A. and Strecker, H. J. *Biochim. Biophys. Acta* **48** (1961) 10.
7. Harper, E. and Strecker, H. J. *J. Neurochem.* **9** (1962) 125.
8. Davies, D. R. and Quastel, J. H. *Biochem. J.* **26** (1932) 1672.
9. Greig, M. F. *J. Pharmacol. Exptl. Therap.* **87** (1946) 185.
10. Ernster, L., Jalling, O., Löw, H. and Lindberg, O. *Exptl. Cell Res. Suppl.* **3** (1955) 124.
11. Berger, M., Strecker, H. J. and Waelsch, H. *Nature* **177** (1956) 1234.
12. Strecker, H. J. In Pennes, H. H. *Progress in Neurobiology: III. Psychopharmacol.*, Hoeber-Harper, New York, 1957, p. 23.
13. Dawkins, M. J. R., Judah, J. D. and Rees, K. R. *Biochem. J.* **73** (1959) 16.
14. Recknagel, R. O. and Malamed, S. *J. Biol. Chem.* **232** (1958) 705.
15. Green, D. E. *Advan. Enzymol.* **21** (1959) 73.
16. Keilin, D. and Hartree, E. F. *Biochem. J.* **44** (1949) 205.
17. Slater, E. C. *Biochem. J.* **45** (1949) 8.
18. Bonner, W. D. *Biochem. J.* **56** (1954) 274.
19. Martius, C. and Nitz-Litzow, D. *Biochim. Biophys. Acta* **12** (1953) 134.
20. Ernster, L., Danielson, L. and Ljunggren, M. *Biochim. Biophys. Acta* **58** (1962) 171.
21. Clowes, G. H. A., Keltch, A. K. and Krahl, M. E. *J. Pharmacol. Exptl. Therap.* **68** (1945) 312.
22. Levine, W. and Strecker, H. J. *Unpublished results.*

Received March 29, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S74–S78

A Method for Estimating Benzyl Isothiocyanate, Benzyl Thiocyanate, and Benzyl Nitrile in the Crushed, Moistened Seeds of *Lepidium sativum*

MAIJA SAARIVIRTA and ARTTURI I. VIRTANEN

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

Methods for the quantitative estimation of benzyl isothiocyanate, benzyl thiocyanate, and benzyl nitrile are described. Using these methods the enzymic formation of the compounds in question could be followed. Benzyl thiocyanate was shown to be formed from benzyl isothiocyanate, whereas benzyl nitrile as well as benzyl isothiocyanate were found to be primary products of the enzymic splitting of benzyl mustard oil glucoside.

When the natural enzymic cleavage of the thioglucoside, glucotropaeolin, in the seeds of *Lepidium sativum* was investigated, it was observed that besides the known fission product benzyl isothiocyanate (BITC) two other compounds are formed, namely benzyl nitrile (BCN) and benzyl thiocyanate (BTC). At a low temperature (+1 to +2°C) and within short reaction times (5 min or less) the glucoside is completely split, BITC and BCN being the primary reaction products, while BTC is formed secondarily from BITC by way of some isomerase mechanism[1,2] (Fig. 1 and 2). This concept of the formation of BCN presupposes that it is formed from the same glucoside as BITC.

Since the investigation was made on a microscale, a method suitable for determining minute amounts of the three components in the same reaction mixture had to be developed.

Whole *Lepidium* seeds were ground in a mill, defatted with petrol ether, air-dried, and ground once more to a very fine powder. About 2/3rds of the husk was removed in this operation.

250 mg of the seeds were placed in a Pozzi-Escot microdistillation apparatus[3], 20 ml of water was added, and after an appropriate reaction time the mixture was steam-distilled for about 40 min so that the distillate amounted to 150 ml. The distillate was made up to 160 ml, of which 50 ml was taken for BCN estimation. 15 ml of saturated NaCl solution was added to the other part of the distil-

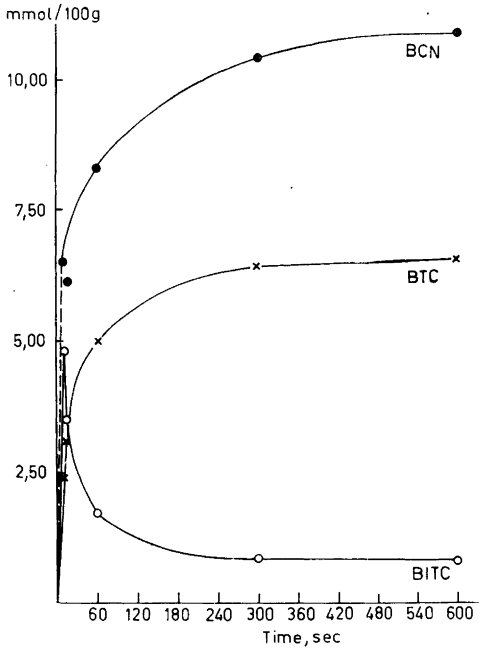


Fig. 1. Formation of benzyl nitrile (BCN), benzyl isothiocyanate (BITC), and benzyl thiocyanate (BTC) in finely ground *Lepidium sativum* seeds. Reaction temperature about +1°C.

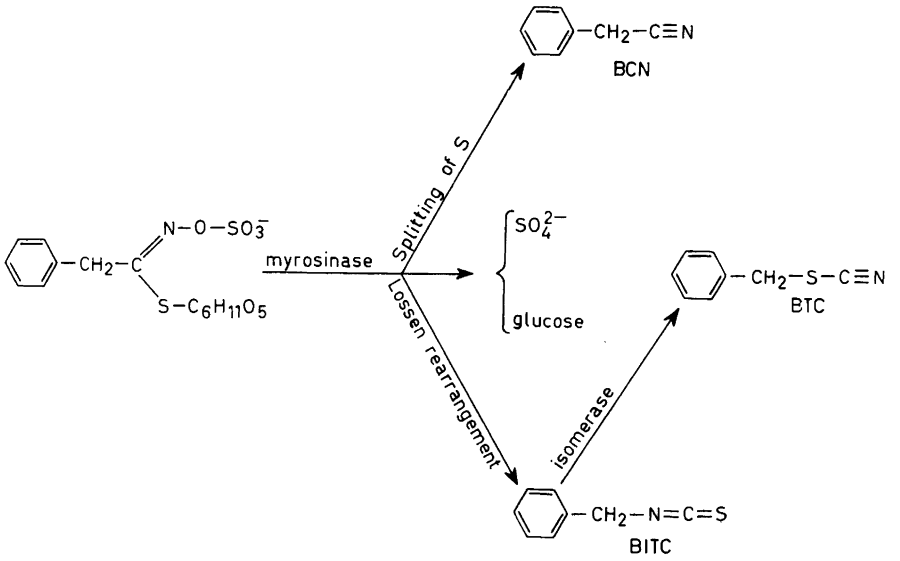


Fig. 2. Enzymic splitting of benzyl mustard oil glucoside in finely ground *Lepidium sativum* seeds.

late, the volatile compounds were extracted four times with about 25 ml of ether, and the ether was divided into two 50 ml portions from which BTC and BITC estimations were made.

Estimation of benzyl isothiocyanate

BITC was converted to thiourea in the usual manner with ammonia. 15 ml of ethanol and 10 ml of concentrated ammonia were added to one of the 50 ml portions of ether. The mixture was kept overnight at room temperature, distilled to dryness under a vacuum (motor pump), and the remaining benzyl thiourea (BTU) was taken up in 50 to 250 ml of methanol. The maximum absorption at 243 mμ was measured in a Beckman Spectrophotometer DK 2. The amount of BITC in the sample was calculated from a standard curve prepared from pure BTU.

Estimation of benzyl thiocyanate

The method is based on the usual method of estimating inorganic thiocyanate as a coloured ferric complex. In this case the usual method did not give reproducible values.

1 ml of 1 % Na_2S in water and 15 ml of 96 % ethanol (to ensure that the Na_2S is evenly dispersed throughout the ether solution) were added to the 50 ml of ether. Subsequently the bottle was kept in a water-bath until completely dry. During the ether evaporation the water-bath was kept at about 60–80°C, after which it was allowed to boil. 10 ml of acetone, 5 ml of 10 % hyamine* in ethanol, and 5 ml of ferric reagent** were added to the dry residue. The bottle was made up to 25 ml with acetone and the red colour measured in a Klett-Summerson colorimeter, with a green filter (No. 50). The amount of BTC was calculated by comparison with a standard curve prepared from pure BTC in the same manner. The error in concentrations ranging from 0.2 to 1.5 mg BTC/25 ml was about ± 5 %. It made no difference whether the standard was made directly from pure BTC or was treated in exactly the same manner as the sample. It is very important that the solutions to be added (acetone, Hyamine, and ferric reagent) after the water-bath treatment are added in the right proportions, because otherwise the final solution is cloudy and unsuited for colorimetric measurement.

Estimation of benzyl nitrile

Attempts to estimate BCN have presented some difficulties because of the fact that all three components, BITC, BTC, and BCN, were present in the test solutions and the estimation of BCN was disturbed in one way or another by the other two. However, it was observed that sodium 1,2-naphthoquinone-4-sulphonate (NQS), a usual reagent for reactive NH_2 and CH_2 groups, gives a brown-red colour with BCN, whereas BTC and BITC do not. The colour is proportional to the concentration of BCN within certain limits. On this basis a colorimetric micromethod for estimating BCN was developed. Although the NQS is by no means a specific reagent for BCN, it was concluded that under our reaction conditions – and in the absence of other nitriles – it is responsible for the colour developed. The method is as follows: 10 to 100 μl of standard solution (200 mg of BCN/10 ml ethanol) and 20 ml of water or 20 ml of the test solution, which has to be neutral, were placed into a 50 ml test-tube (190 × 18 mm, glass stopper), to which exactly 2 ml of 0.3 % NQS in water was then added. The tubes were placed in a smoothly boiling water-bath and kept there for exactly 4 min. Precisely 2 ml of 0.5 M NaOH solution in water was added, and the tubes were left to stand for 20 min at room temperature, and finally put to cool in an ice-bath. The contents were poured into 25 ml calibrated flasks and filled with water up to the mark. A solution prepared in the same way with NQS and NaOH served as a blank. The brown-red colour was measured in a Klett-

* "Hyamine", Rohm & Haas Co., Philadelphia, Pennsylvania, 50 % solution in water, active ingredients: methyldodecylbenzyltrimethyl ammonium hydroxide and methyldodecylxylene bis (trimethyl ammonium hydroxide).

** Ferric nitrate 9 H_2O 5.0 g
Nitric acid conc. 2.5 ml
Aq. dest. to 100.0 ml

Summerson colorimeter, with a green filter (No. 50). The blank itself was quite dark and was always measured against distilled water first. If the Klett-Summerson values of the blank were higher than 370, it was impossible to adjust the blank to zero. The amount of BCN was calculated by comparison with a standard curve. The error in concentrations ranging from 0.1 to 1.0 mg of BCN/25 ml was at most ± 6 %.

When the colour formed in the reaction was more brown than red, it was reasonable to assume that it might have been formed with some compound other than BCN. In such a case an additional test for nitriles was made. 3 ml of 0.5 M NaOH and 10 ml of ethylmethylketone were added to the reaction mixture. The bottle was shaken. When the layers separated, the upper layer had a clear violet colour if nitriles were present. The upper layer with BTC, BITC, and some other compounds tested, was pale yellow in colour.

DISCUSSION

The methods for estimating BITC and BTC need little comment. In the case of BTC the most important thing is to evaporate the solution to complete dryness, otherwise the moisture causes cloudiness when the reagents are added. When estimating BITC as thiourea, some other volatile compounds may cause error. Kjær *et al.*[4] have presented a method for correcting such error. However, in our experiments Kjær's correction factor for BITC was not used because the main foreign compounds were known and probably different from those in his experiments. We therefore treated the three compounds alone and in different relative concentrations together with ammonia and measured the absorbances. BCN caused no disturbance even at reasonably high concentrations, whereas if the concentration of BTC was very high in regard to BITC the curve could sometimes be flattened and lifted. However, this happened very seldom in our work, and the results were then discarded.

When estimating BCN, the sources of error were greater. In the method the point of importance was the heating time and the temperature. The tubes were kept for exactly 4 min in a water-bath, the temperature of which was 97–98°C. If the water-bath was boiling vigorously, the blank became too dark and dilution did not give true values. The other source of error could be caused by volatile compounds present in the test solution. In our experiments the compounds probably present in the distillate were some sulphides or mercaptans, ammonia, hydrogen sulphide, carbon disulphide, steamvolatile amines or amines produced as decomposition products after boiling BITC and BCN with alkali. Some of these compounds gave a faint brown colour with NQS and alkali, but when treated with more alkali and ethylmethylketone they gave no violet colour, and hence uncertain cases were easy to detect.

As a control, 300 g of crushed *Lepidium* seeds in water were steam-distilled when the enzymic reactions were completed. The oil recovered was separated into fractions in a counter-current apparatus (methanol:H_2O:petrol ether 8:2:5). One of these fractions gave an infrared spectrum completely identical with pure commercial BCN.

It ought, perhaps, to be pointed out that the handling of the fine seed-powder of *Lepidium*, which in aqueous solution forms a very tough slime, needs some experience before reproducible values can be achieved.

Some of our results concerning the enzymic reaction products of crushed *Lepidium sativum* seeds and factors influencing these reactions have been published in preliminary reports[1,2]. The analytical methods described in the present

paper were not included in these reports. A more detailed paper will be published in the near future.

Acknowledgement. This research has been financed in part by a grant made by the *United States Department of Agriculture, Agricultural Research Service.*

REFERENCES

1. Virtanen, A. I. and Saarivirta, M. *Suomen Kemistilehti B* **35** (1962) 102.
2. Virtanen, A. I. and Saarivirta, M. *Suomen Kemistilehti B* **35** (1962) 428.
3. Houben-Weyl, *Methoden der organischen Chemie,* G. Thieme Verlag, Stuttgart 1959, Vol. 2, p. 434.
4. Kjær, A., Conti, J. and Larsen, I. *Acta Chem. Scand.* **7** (1953) 1276.

Received February 21, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S79–S83

X-Ray Ultramicroanalysis of Sulfur in Calf Costochondral Plates*

DAVID S. HOWELL** and LEON CARLSON

Institute for Cell Research and Genetics, Karolinska Institutet, Stockholm, Sweden

1. X-Ray absorption spectrophotometry has been applied to ultramicroanalysis of sulfur. Model systems with S*** concentrations in the range 0.7–2.5 % were used to test the method.

2. Regional distribution of S concentration was determined in cartilage sections subsequent to extraction by various techniques.

3. Evidence was obtained that formalin fixation and incubation of histologic slices in 0.5 molar EDTA*** for 16 h resulted in a significant loss of S compounds from the distal hypertrophic cell zone of calf epiphyseal plates.

Dentine, bone and cartilage have been intensively studied by a variety of histologic and histochemical techniques, which have provided qualitative data supporting a role of acid mucopolysaccharides in the development of calcifying matrix[1–3]. Biochemical studies of demineralized bones, fracture callus[4,5], and epiphyseal plates reveal significant concentrations of acid mucopolysaccharides. In studies of calf bones, chondroitin 4-sulfate, chondroitin 6-sulfate, as well as unidentified sulfate fractions have been isolated[5]. Slices of calf epiphyseal cartilage incubated *in vitro* have shown that 90 % or more of labeled S when provided as $Na_2{}^{35}SO_4$ appears within SMPS*** fractions as ester sulfate[6,7], and similar results occur if the isotope is given *in vivo*. Based on biochemical evidence ^{35}S radioautography has been employed extensively to examine SMPS metabolism of hard tissues[8]. However, information yielded, even with grain counts, is only of partially quantitative value. In those tissues such as calf rib plate, in which less than 10 % total S has been isolated in fractions other than SMPS,

* This investigation was supported by grants from the *Wallenberg Foundation* and by Grants C-4716 and A-1155, from *U. S. Public Health Service.*

** Scholar of the John and Mary Markle Foundation on leave from the University of Miami, School of Medicine, Miami, Florida.

*** Abbreviations: EDTA, ethylenediamine tetraacetic acid; SMPS, sulfated mucopolysaccharides; S, sulfur.

micro-regional variations in total S would provide additional information on the fate of these compounds.

The present report utilizes a method of monochromatic X-ray absorption spectrophotometry for analysis of total S in tissue in amounts of 10^{-11} g and demonstrates its use in respect to extractive procedures for SMPS. The theoretical foundation of this method of microanalysis has been described in detail by Engström[9] and Lindström[10].

MATERIALS AND METHODS

The spectrophotometric analysis for S was principally performed according to the procedure described by Lindström[10]. Some modifications of apparatus and measuring technique were introduced and will be published elsewhere. The densitometric evaluation of the microradiograms was performed with an evenly illuminated measuring spot 20 μ in diameter. The total dry mass per unit area was determined from one of the microradiograms using a value of the mass absorption coefficient calculated for the elementary composition of collagen. This simplified calculation of the mass absorption coefficient may introduce an error if the elements Na, Mg, P, K and Ca are present in appreciable amounts. An estimate of this error has been calculated for the case of cartilage using the concentration values of these elements given by Howell *et al.*[11]. The error is about 15 % for the region of provisional calcification and adjacent hypertrophic cells and 6 % for the region of resting cells, giving too high values of total mass. Another possible source of error is the effect of re-radiation[12] but this will introduce an error of only circa 2 %. The data of Table 2 are presented uncorrected because of the approximative nature of these corrections.

Models for use as testing objects were prepared as follows: To aqueous solutions containing 3–5 % gelatin (Difco) at 90°C, crude chondroitin sulfate (Sigma) was added in varying proportions with constant stirring for ½ h. Portions were saved for gravimetric analysis of dried ashed samples for S by the method of Andersen[13]. The remainder of each gelatin preparation was spread as a thin film on glass slides. After cooling, microscopic strips were sliced from these films, placed between two parlodion foils, and pressed on grids for X-ray analysis.

Tissue analysis. Samples were dissected from costochondral junctions of normal calves within ½ h of sacrifice and were frozen in isopentane surrounded by liquid nitrogen. Materials were sectioned in a cryostat at 10–40 μ thickness and freeze-dried. Appropriate regions were removed under a dissecting microscope and placed on grids for X-ray exposures. Eight samples could be analyzed simultaneously on a single grid. A standard gelatin model was included on each grid.

For the investigation of extractability of S compounds (Table 2) adjacent slices of cartilage were treated as follows: Group 1, no treatment. Group 2, incubation at 37°C for 24 h in unbuffered baths of distilled water to which was added testicular hyaluronidase (20 000 international units/ml) and penicillin (250 units/ml)[14]. Group 3, methylation and desulfation by the method of Kantor and Schubert[15] with incubation at 58°C for 24 h[16]. Group 4, fixation in neutral formalin an extraction with 0.5 molar EDTA at pH 7.4 at 20°C for 16 h. Group 5, extraction with water after heating briefly to 100°C. Adjacent slices of some of the tissues were appraised visually for region and intensity of staining by aqueous toluidine blue[17] and Astrablau[18].

RESULTS

Data obtained by X-ray elemental analysis of gelatin models (Table 1) reveal good agreement between X-ray and chemical determinations on samples containing S in concentrations ranging from 0.7 to 2.5 %. The discrepancy between mean values by the two methods did not exceed 6 % of the gravimetric value of S. The accuracy of the chemical determination is about $\pm$ 5 %.

The X-ray analysis results for untreated cartilage can be compared with previously published data gravimetrically measured in pooled slices of 4 to 6 weeks

Table 1. Results of X-ray S analysis upon models varied in respect to S concentration.

Model	Sulfur concentration		Number of densitometric points	Range of sulfur mass per unit area 10^{-5} g cm^{-2}
	Gravimetric analysis %	X-ray analysis %		
A	0.69	0.73 ± 0.04[a]	25	1–4
B	0.88	0.91 ± 0.04	15	1–5
C	1.09	1.15 ± 0.07	10	1–2
D	1.44	1.49 ± 0.06	14	2–8
E	2.45	2.60 ± 0.04	25	5–8

[a] Standard error of the mean.

old calves in the costal epiphyseal plate[11]. The mean value of X-ray determinations at 6 mm from the bone marrow surface (Group 1, Table 2) 1.33 %, agreed well with the mean value at this site in similar samples estimated by the gravimetric procedure, 1.26 %. The 5-fold reduction in S content caused by hyaluronidase treatment (Group 2) or methylation and desulfation of the current samples (Group 3) measured by X-ray analysis was in accord with histologic observations of Fisher and Lillie[14]. Loss of metachromasia from methylated sections was observed by them for cartilage stained with toluidine blue. Also, methylation of potassium chondroitin sulfate by Kantor and Schubert[15] gave a high yield of potassium methyl sulfate, soluble in methanol hydrochloric acid mixtures which bathed the current samples. A finding not anticipated from previous studies[16]

Table 2. Effect of various extractive procedures upon S content of normal calf costal plates.

Group number	Treatment	Distal hypertrophic zone and provisional calcification			Resting cell zone		
		Sulfur concentration %	No. densitometric points	Range of S per unit area 10^{-5} g cm^{-2}	Sulfur concentration %	No. densitometric points	Range of S per unit area 10^{-5} g cm^{-2}
1	None	2.48 ± 0.15[a]	26	1 –4	1.33 ± 0.10[a]	15	0.2–2
2	Hyaluronidase	0.52 ± 0.07	18	0.2–3	0.41 ± 0.05	12	0.3–1
3	Methylation and desulfation	0.52 ± 0.09	17	0.3–1	0.35 ± 0.07	14	0.2–1
4	EDTA extraction	1.68 ± 0.24	14	1 –4	1.24 ± 0.15	14	1 –3
5	Aqueous extraction	1.17 ± 0.19	14	1 –4	0.90 ± 0.10	8	0.4–1

[a] Standard error of the mean.

was the reduction of S by about one third in the distal hypertrophic zone after formalin fixation and treatment with EDTA for 16 h (Group 4). Less than 10 % of total S was extracted from points in the resting cell layer. Approximately one half the S at points within the distal hypertrophic zone was removed by 96 h water extraction of fresh untreated freezedried cartilage, and approximately one third was removed from the resting cell zone (Group 5). Large standard errors in S analysis of the distal hypertrophic zone corresponded to increased microheterogeneity of dye distribution judged from toluidine blue and Astrablau staining of regions in adjacent slices. The effect was the natural consequence of a large cell size and steep gradient of S concentration between matrix and cell columns. This problem may be overcome by use of differently shaped densitometer beams, by which cells and matrix regions can be separately analyzed.

Visual inspection of stained models showed increasing intensity of staining by Astrablau and greater metachromasia by toluidine blue with increasing S content. Similarly, staining of untreated cartilage by the former dye was shown to increase in intensity progressing from the resting cell zone to the zone of provisional calcification. A weak reaction to Astrablau and slight metachromasia remained in the distal hypertrophic zone of both hyaluronidase-treated and methylated cartilages, while remainder of the tissue registered no stain. Considerable positive staining by Astrablau, heterogeneously located, remained in the tissues after water extraction.

DISCUSSION

Ultramicroanalysis by the present method is particularly advantageous for study of essentially unaltered tissues, using frozen sectioned, freeze-dried material. Demonstrated here (Table 1) is the fact that sulfur concentration can be accurately assessed in homogeneous models and that it is possible to measure wide distributional differences of S content among various histologic areas of cartilage. This was performed in a region representing 3000 μ^3 of tissue containing from 1 to 8×10^{-11} g of S.

In spite of the bulk extraction of S following treatment of cartilage slices with hyaluronidase or the methylating mixture, residual levels of S were high. At a theoretical concentration of 0.15 – 0.20 % S in mammalian collagen[19] only 0.05 – – 0.10 % S would be expected in the current tissues based on levels of hydroxyproline measured in calf costal cartilage slices pooled according to histologic zone[11]. However, in the desulfated and hyaluronidase-treated tissues an average residual of 0.3 – 0.4 % of S was obtained. Since previous studies of calf costal cartilage indicate that at least 90 % of total S in the pooled tissue can be accounted for by SMPS[5], the present discrepancy between theoretical and actual S content following extraction is most likely explained by incomplete extraction of SMPS as well as reduction of total mass by extraction of components lacking S. Loss of metachromasia invoked by the procedures could result from the presence of a variety of degradative products and, of course, provide no index of the total sulfated compounds remaining in the tissue. Astrablau staining at pH 0.2 should theoretically react only with anionic groups which remain dissociated at that hydrogen ion concentration, primarily sulfate[18]. The results of the persistent

staining reaction with Astrablau after water extraction indicate partial desulfation of the matrix and incomplete extraction of sulfated compounds.

Previous absence of quantitative ultramicro methods have left in doubt the integrity of SMPS within histologic slices of cartilage fixed and demineralized by various techniques[20]. The present results prove conclusively that with the use of EDTA under the conditions of the present experiments there were losses of S compounds, probably including SMPS, from certain microscopic areas. These losses could only be detected to a slight and variable degree with the staining procedures and visual inspection. S extraction might be minimized with shorter periods of incubation and reduced pH.

Acknowledgements. The authors are indebted to Professor Hugo Theorell, Professor T. Caspersson, Dr. Gunnar Bloom, and Dr. Nils Ringertz for helpful suggestions and laboratory facilities.

REFERENCES

1. Rubin, P. S. and Howard, J. E. In Reifenstein, E. C. Jr. *Transactions Fourth Conference on Metabolic Interrelations.* Josiah Macy Jr. Foundation, New York, 1950, p. 74.
2. Belanger, L. F. *Can. J. Biochem. Physiol.* **32** (1954) 161.
3. Dziewiatkoski, D. D., Bronner, F., Di Ferrante, N. and Archibald, R. M. *J. Biophys. Biochem. Cytol.* **3** (1957) 151.
4. Maurer, P. H. and Hudak, S. S. *Arch. Biochem.* **38** (1952) 49.
5. Meyer, K., Davidson, E., Linker, A. and Hoffman, P. *Biochem. Biophys. Acta* **21** (1956) 506.
6. Boström, H. and Månsson, B. *Arkiv Kemi* **6** (1953) 23.
7. Boström, H. *J. Biol. Chem.* **196** (1952) 477.
8. Dziewiatkoski, D. D. In LaCroix, P. and Budy, A. M. *Radioisotopes and Bone,* Blackwell Scientific Publications, Oxford, 1962, p. 277.
9. Engström, A. *Acta Radiol. Suppl.* **63** (1946).
10. Lindström, B. *Acta Radiol. Suppl.* **125** (1955).
11. Howell, D. S., Delchamps, E., Reimer, W. and Kiem, I. *J. Clin. Invest.* **39** (1960) 919.
12. Hoh, F. C. and Lindström, B. *J. Ultrastruct. Res.* **2** (1959) 512.
13. Andersen, L. *Acta Chem. Scand.* **7** (1953) 689.
14. Fisher, E. R., and Lillie, R. D. *J. Histochem. Cytochem.* **2** (1954) 81.
15. Kantor, T. G. and Schubert, M. *J. Am. Chem. Soc.* **79** (1957) 152.
16. Greulich, R. C. and Friberg, U. *Exptl. Cell Res.* **12** (1957) 685.
17. Irving, J. T. *Clin. Orthoped.* **17** (1960) 92.
18. Bloom, G. and Kelly, J. W. *Histochemie* **2** (1960) 48.
19. Gustavson, K. H. *The Chemistry and Reactivity of Collagen,* Academic Press, New York, 1956, p. 42.
20. Pearse, A. G. E. *Histochemistry Theoretical and Applied,* Little, Brown and Co., Boston, 1960, p. 778.

Received February 22, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S84–S90

A Hypothesis Involving Competitive Inhibitions in the Metabolism of Polyunsaturated Fatty Acids*

RALPH T. HOLMAN and HANS MOHRHAUER**

The Hormel Institute, University of Minnesota, Austin, Minnesota, USA

The inhibition of metabolism of oleate to eicosatrienoate and of linoleate to higher unsaturated fatty acids by dietary linolenate has been demonstrated. Under some conditions the metabolism of linolenate to docosahexaenoate is inhibited by linoleate or arachidonate. The influence of dietary linolenate upon the several product/precursor ratios for steps in linolenate metabolism has been examined and found to be different for different steps. Competitive inhibition seems to operate between polyunsaturated acids of the oleate, linoleate and linolenate families so that the pattern of polyunsaturated fatty acids is controlled by the concentrations of the precursors of each family.

In recent studies of metabolism of polyunsaturated fatty acids (PUFA), the changes in fatty acid composition of the total lipids of liver[1], heart[2], brain[3], erythrocytes and adipose tissue[4] were recorded as functions of the level of intake of single supplements to a fat-free diet: linoleate, arachidonate and linolenate. These studies revealed that increasing dietary content of linoleate or arachidonate causes an increasing tissue content of 5,8,11,14-eicosatetraenoate (20 : 4) and 4,7,10,13,16-docosapentaenoate (22 : 5ω6)***, both of which have a terminal structure equivalent to linoleate. Likewise, increasing dietary linolenate increases the tissue content of 5,8,11,14,17-eicosapentaenoate (20 : 5), 7,10,13,16,19-docosapentaenoate (22 : 5ω3) and 4,7,10,13,16,19-docosahexaenoate, all of which have terminal structures equivalent to linolenate. These results confirmed in detail the conclusions of Mead and his co-workers[5,6], and demonstrated that there is no metabolic crossover between the families of PUFA.

* Supported in part by a grant (AM-04524-03) from the *National Institutes of Health*, Bethesda, Maryland.

** Permanent address: Bundesministerium für wissenschaftliche Forschung, Luisenstr. 46, Bad Godesberg, Germany.

*** This shorthand formula indicates 22 carbon atoms and 5 double bonds, the nearest of which lies 6 carbon atoms from the terminal methyl group. This notation is necessary to avoid confusion between isomeric metabolites of oleate, linoleate and linolenate.

In our studies certain unexpected anomalies were observed. When esters of one of these families of PUFA were fed in an otherwise fat-free diet, the concentrations of members of the other family in tissue lipids were depressed. This was particularly consistent and strikingly evident in a study in which linolenate was fed at six different levels together with linoleate at three different dietary intakes[7]. These results suggested metabolic competition between the two families of PUFA.

Such depressions of one PUFA by increasing another have been observed before. The content of hexaenoic acids in liver and brain lipids was less when corn oil was fed than when a fat-free diet was fed to rats[8]. Similarly, when the only dietary fatty acid was linoleate, the hexaenoic acid content of heart, liver, muscle and intestine lipid was less than when a fat-free diet was fed[9]. Hexaenoic acid content of rat liver, kidney, muscle, intestine, skin and adipose tissue was depressed by dietary arachidonate[10]. Privett *et al.*[11] observed a depression in total body arachidonate when linolenate was administered to rats. Klenk and Oette[12] also found that the arachidonate content of liver phospholipids of rats fed linolenate was less than when rats were fed a fat-free diet. These observations were given additional meaning when Marco *et al.*[13] and Machlin[14] reported that when linseed oil (containing linolenate) was fed, the linoleate content of chick liver was higher and the arachidonate content was lower than when linoleate was fed at a level equal to that in the linseed oil. They called attention to the probable inhibition of linoleate metabolism by linolenate. Dhopeshwarkar and Mead[15] have postulated a similar competition between oleic and linoleic acids for enzymes concerned with their metabolism.

The purpose of this communication is to describe the phenomena of competitive inhibition as we have observed them in PUFA metabolism of rats which received only purified fatty acid esters as dietary fat and PUFA precursors.

EXPERIMENTAL

The nutritional and analytical details of the studies of PUFA metabolism appear elsewhere[1,7], and will be repeated here only in general outline. Male weanling rats were fed a fat-free diet, and group-wise were administered orally different amounts of linoleate, arachidonate or linolenate. In one study linoleate was fed at three levels and linolenate at six levels to provide both precursors at different ratios. The food consumption of the rats was measured so that the proportion of calories provided by the supplements could be calculated and controlled. After 100 or 87 days, respectively, the rats were killed and the organs dissected. Lipids were extracted and methyl esters of the total fatty acids were prepared. These were analyzed by gas-liquid chromatography on ethylene glycol succinate columns.

RESULTS AND DISCUSSION

When linoleate was fed alone in increasing levels of the diet[1–3], the only PUFA which was significantly or consistently depressed was the 5,8,11-eicosatrienoic acid, which is elevated in essential fatty acid deficiency and which is synthesized from oleic acid[15]. In these animals (which were fed no linolenate) the levels of linolenate metabolites in the tissue lipids were so low that in many cases they could not be measured. Moreover, the levels of linoleate fed were not sufficiently high to effectively compete with linolenate metabolism. When arachidonate was fed as the sole fatty acid supplement in a fat-free diet, the 22 : 6 content of liver and brain lipids diminished as the arachidonate supplement increased. Thus,

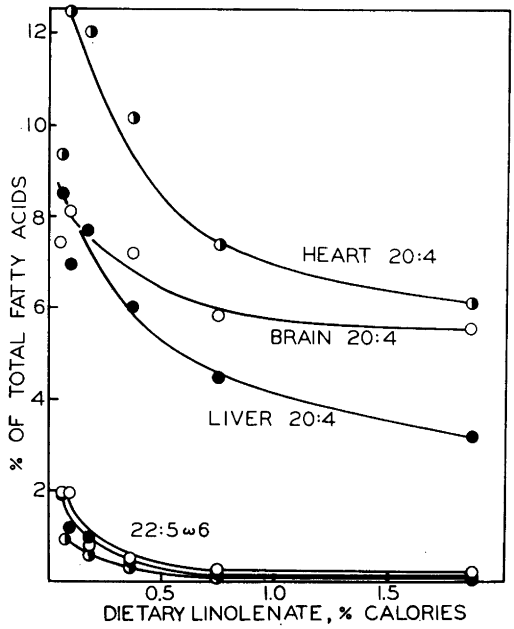


Fig. 1. The effect of increasing levels of dietary linolenate upon the content of two linoleate metabolites in liver and heart lipids of rats fed linoleate at 0.6 % of calories.

arachidonate was able to compete more effectively with linolenate metabolism than could linoleate.

When linolenate was the sole fatty acid supplement to the fat-free diet, several inhibitions of linoleate metabolism were observed. As dietary linolenate increased, 20 : 4 and 20 : 5ω6 contents of liver, heart and brain lipids decreased regularly. The 20 : 4 content of erythrocytes was likewise diminished significantly.

When either linoleate, arachidonate or linolenate was fed, the most striking effect was the depression of 5,8,11-eicosatrienoate, the oleate metabolite prominent in essential fatty acid deficiency. All the dietary supplements depressed this metabolite in all tissues measured, and dietary 20 : 4 was more efficient in depressing it than was dietary linoleate (18 : 2). Linolenate (18 : 3) was similar to arachidonate in this regard.

In the foregoing experiments the contents of tissue PUFA, except those synthesized from the dietary supplement, were low and subject to the animals' dietary history prior to the experiment. In the second group of experiments[7] the animals were fed both linoleate and linolenate to provide at least a minimal constant supply of each precursor in several proportions. In this experimental situation the same competitions and inhibitions were observed more clearly.

At all three levels of linoleate (0.1, 0.3 and 0.6 % of calories) increasing levels of dietary linolenate suppressed the content of 20 : 3ω9 (oleate metabolite), 20 : 3ω6, 20 : 4 and 22 : 5ω6 (linoleate metabolites) in liver and heart lipids. In

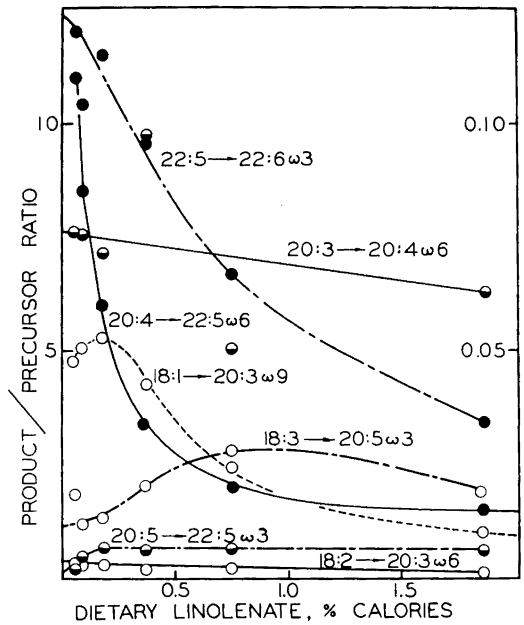


Fig. 2. The effects of increasing level of dietary linolenate upon the product/precursor ratios for several steps of oleate, linoleate and linolenate metabolism. The linoleate content of the diet was held at 0.6 % of calories for all groups. Notice that the right hand scale applies to $20:4 \rightarrow 22:5\omega6$ and $18:1 \rightarrow 20:3\omega9$.

brain lipids $20:4$ and $22:5\omega6$ were likewise depressed. Some typical responses are shown in Fig. 1. The curves indicate that as the precursor to one family of PUFA is increased in the metabolic system, the synthesis of the higher members of the other principal family of PUFA is inhibited.

Conversely, the synthesis of linolenate metabolites was seemingly unaffected by increases in dietary linoleate. However, the three levels of linoleate were quite low and the competitive equilibrium appears to be strongly in favor of the linolenate metabolites. When the ratios of principal product to its precursor are calculated for the conversion $18:1 \rightarrow 20:3$, $18:2 \rightarrow 20:4$, and $18:3 \rightarrow 22:6$ in rats provided a minimal amount of linolenate (0.7 % of calories), these ratios are 0.45, 1.2 and 12.3, respectively. In these rats whose liver lipids contain the respective precursors, more than 30 % 18 : 1, 1.4 to 3.7 % 18 : 2, and 0.4 to 0.8 % 18 : 3, the metabolic pathway of chain lengthening and dehydrogenation obviously favors the substrates in the order linolenate > linoleate > oleate despite the wide differences in precursor concentrations favoring a reverse order.

We consider that in rats which have been fed a constant diet for 87 days the composition of their lipid fatty acids represents a steady state. We assume also that the total fatty acids of liver lipid reflect substrates and products of metabolic conversions of PUFA. If a steady state exists in these lipids, differences in ratio between products and precursors might reflect competitive inhibitions of

specific steps in the metabolic pathway as a competitive metabolite is increased in concentration. Some such relationships are shown in Fig. 2 using data from rats fed a constant level of linoleate of 0.6 % of calories and differing levels of linolenate.

Consider first the product/precursor ratios for the metabolites of linolenate itself, which is the dietary variable. The ratio of 20 : 5/18 : 3, representing the first two steps of its metabolic chain, varies erratically and minimally as the available 18 : 3 is increased. The ratio 22 : 5ω3/20 : 5 rises slightly but consistently through a low narrow range. Thus, these two steps in linolenate metabolism are largely uninhibited by increased linolenate substrate. However, the ratio for the last step of linolenate metabolism 22 : 6/22 : 5ω3 is depressed with increasing dietary 18 : 3, indicating that this step is self-limiting and that 22 : 6 accumulates as the metabolite in principal amount followed by accumulation of 22 : 5ω3.

The conversion of 18 : 1 to 20 : 3 is inhibited distinctly by increasing its competitive inhibitor, linolenate. Under the conditions of this experiment in which linoleate is fed at constant level, the content of 20 : 3 is already depressed by linoleate, but increasing linolenate depresses it even farther.

Within the linoleate family the product/precursor ratio for 18 : 2 $\rightarrow$ 20 : 3ω6 is very low indicating that the latter does not accumulate in high concentration. The low ratios are depressed somewhat by increasing linolenate. The product /precursor ratio for 20 : 3ω6 $\rightarrow$ 20 : 4 is considerably higher but varies erratically as linolenate is increased. The high ratio reflects the accumulation of 20 : 4 which is the metabolite in principal abundance, and its erratic variability through a narrow range indicates that competition from linolenate metabolites does not play a dominant role at this step. However, the last step of the metabolic chain 20 : 4 $\rightarrow$ 22 : 5ω6 is profoundly inhibited by linolenate metabolites. The effect of strong inhibition of this last step is to cause the accumulation of arachidonate as the most abundant metabolite of linoleate. This phenomenon probably operates in natural circumstances, for linolenate metabolites occur widely in foodstuffs.

Let us assume what seems evident, that three metabolic chains of reaction are in competition for a common metabolic system of enzymes:

$$18:1 \rightarrow 18:2 \rightarrow 20:2 \rightarrow 20:3 \qquad \text{(all } \omega 9\text{)}$$

$$18:2 \rightarrow 18:3 \rightarrow 20:3 \rightarrow 20:4 \rightarrow 22:4 \rightarrow 22:5 \qquad \text{(all } \omega 6\text{)}$$

$$18:3 \rightarrow 18:4 \rightarrow 20:4 \rightarrow 20:5 \rightarrow 22:5 \rightarrow 22:6 \qquad \text{(all } \omega 3\text{)}$$

Assume also that enzyme substrate affinities increase with unsaturation. At each chain length stage of metabolism, the metabolites of linolenate would then preferentially occupy the enzyme sites to the inhibition of linoleate and oleate metabolism. Linolenate would be metabolized principally to 22 : 6, and the accumulation of the latter would inhibit the formation of 22 : 5ω6. Arachidonate would accumulate as principal metabolite in its family, all members of which would be depressed by competitive inhibition exerted by linolenate metabolites of comparable chain length. These suppositions in the main agree with the experimental data given in Fig. 2 and other observations mentioned.

The assumption of a common metabolic pathway for the three families of PUFA is not mandatory. Each family could be metabolized *via* a separate path-

way parallel to the others. Competitive inhibition by the other substrates could lead to the same phenomena. Competitive inhibition is known to operate in a similar situation, in the lipoxidase oxidation of linoleate. This reaction is competitively inhibited by oleate, octanoate, conjugated linoleate and *trans,trans,trans*-linolenate, none of which are oxidized by the enzyme[17]. Thus, each separate metabolic pathway of the three fatty acid families could be inhibited competitively by the substrates of the other two families. A third mechanism of inhibition is also possible. If the metabolism of linolenate proceeds much more rapidly than the parallel metabolisms of oleate and linoleate, a common required co-factor, such as coenzyme A, may be preferentially involved in the linolenate metabolic chain, reducing its concentration or availability for the other two metabolic sequences.

It should be pointed out that in considering changes in product/precursor ratios, all metabolites arising from oleate and linoleate are depressed by increasing linolenate, and that we are considering only those increases or decreases which are not proportional to the whole, and are, therefore, specifically involved in competitive inhibition to a disproportionate degree. The observations that individual metabolites are influenced to differing degrees by linolenate precludes the possibility that the inhibitions or depressions observed are mere artifacts of dilution. If, for example, inclusion of linolenate in a diet caused accumulation of it and its metabolites without inhibiting oleate or linoleate metabolism, all of their metabolites would decrease by the same proportion due to dilution. This is clearly not the case, for 5,8,11-eicosatrienoic acid is dramatically reduced, arachidonate is moderately depressed, and saturated and monoenoic acids are essentially unaffected by the same level of linolenate.

CONCLUSION

The changes in composition of tissue PUFA as a consequence of changes in dietary fat have heretofore presented a complex and confused picture. The changes in PUFA involve principally the metabolites of oleate, linoleate and linolenate, although other minor families of unsaturated acids are present. The principal PUFA in fat-deficient animals is an oleate metabolite normally found in low concentrations in tissues. The highly unsaturated fatty acids of normal tissues are metabolites of linoleate and linolenate, and the pattern of these varies with the kind and amounts of these precursors. We offer a unified hypothesis to explain the regulation of all these metabolites and the variations in pattern of PUFA resulting from deprivation or supplementation of essential or polyunsaturated fatty acids. Observations to date indicate that the families of PUFA competitively inhibit each other's metabolism. Competitive inhibition requires that the metabolism of the three families of PUFA have some enzymatic pathway or co-factor in common. Enzyme-substrate affinities of the three families of PUFA are in the order linolenate $>$ linoleate $>$ oleate. When linolenate is present in the substrate pool, its conversion to higher unsaturated acids takes precedence over the metabolism of linoleate by a factor near tenfold. Linoleate metabolism proceeds in preference to oleate metabolism. Oleate metabolism to higher unsaturated acids can take place only when linoleate and linolenate are in very low concentration. Thus, in simplification, the pattern of PUFA in tissue

lipids is controlled by the concentrations of competing substrates in a common metabolic pathway.

The observations reported here and the speculations inspired by them have led to a working hypothesis which fits the experimental observations to date. Undoubtedly, as presented here, it is incomplete and subject to considerable change by future experimentation. The hypothesis is presented in the hope that it will provoke investigations to test its validity. Nutritional experiments and compositional studies perhaps cannot yield more precise data concerning specific enzyme mechanisms, but the insights provided by such experiments can lead to studies of the same phenomena on a cellular or enzymatic level of observation.

The authors wish to acknowledge René Maier and Joseph Rahm for fruitful discussions during the preparation of this manuscript.

REFERENCES

1. Mohrhauer, H. and Holman, R. T. *J. Lipid Res. In press.*
2. Mohrhauer, H. and Holman, R. T. *Proc. Intern. Conf. Biochem. Probl. Lipids,* Birmingham, July, 1962. *In press.*
3. Mohrhauer, H. and Holman, R. T. *J. Neurochem. In press.*
4. Mohrhauer, H. and Holman, R. T. *J. Lipid Res. In press.*
5. Howton, D. R. and Mead, J. F. *J. Biol. Chem.* **235** (1960) 3385.
6. Mead, J. F. *Am. J. Clin. Nutr.* **8** (1960) 55.
7. Mohrhauer, H. and Holman, R. T. *J. Nutr. In press.*
8. Rieckehoff, I. G., Holman, R. T. and Burr, G. O. *Arch. Biochem.* **20** (1949) 331.
9. Widmer, C. and Holman, R. T. *Arch. Biochem.* **25** (1950) 1.
10. Holman, R. T. and Taylor, T. S. *Arch. Biochem.* **29** (1950) 295.
11. Privett, O. S., Pusch, F. J. and Holman, R. T. *Arch. Biochem. Biophys.* **57** (1955) 156.
12. Klenk, E. and Oette, K., *Z. Physiol. Chem.* **318** (1960) 86.
13. Marco, G. J., Machlin, L. J., Emery, E. and Gordon, R. S. *Arch. Biochem. Biophys.* **94** (1961) 115.
14. Machlin, L. J. *Nature* **194** (1962) 868.
15. Dhopeshwarkar, G. A. and Mead, J. F. *J. Am. Oil Chem. Soc.* **38** (1961) 297.
16. Fulco, A. J. and Mead, J. F. *J. Biol. Chem.* **234** (1959) 1411.
17. Holman, R. T. *Arch. Biochem.* **15** (1947) 403.

Received April 1, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S91–S95

Spectral Observations Concerning the Binding of Zinc in Liver Alcohol Dehydrogenase

ROBERT A. PLANE and THOMAS V. LONG

Department of Chemistry, Cornell University, Ithaca, New York, USA

The ultraviolet absorption spectrum of 1,10-phenanthroline when bound to liver alcohol dehydrogenase has been measured and compared with measurements made of the spectrum of 1,10-phenanthroline bound to zinc in various complex ions. In all cases the spectra are qualitatively similar, but difference spectra reveal quantitative differences in extinction coefficients and slight frequency shifts. These changes were studied as a technique for determining the nature of the binding of zinc in the enzyme by finding the model complex having the same spectral effects on 1,10-phenanthroline as does the enzyme. As a means of checking the method, a thorough study of the spectra of the mono-, bis-, and tris-1,10-phenanthroline-Zn(II) complexes was made. From these, three independent spectral criteria were adopted for distinguishing between the spectra of 1,10-phenanthroline in the three complexes. Two of these criteria showed that the enzyme-1,10-phenanthroline complex was spectrally identical to the tris-1,10-phenanthroline-Zn(II) complex but different from either the mono, or bis complex. All three spectral criteria showed the enzyme-phenanthroline complex to be indistinguishable from phenanthroline bound to $(imidazole)_{3\text{ or }4}Zn^{++}$. When the ligands glycine, L-cysteine or L-histidine were studied, the complexes formed differed significantly from those formed with the enzyme. It is concluded that on the basis of the spectral criteria developed, three or four imidazole ligands provide the best model for the environment of the zinc atom in liver alcohol dehydrogenase.

An important but difficult problem has been the elucidation of the active site of an enzyme. For metalloenzymes it is hoped that the presence of a metal atom might provide the means necessary to learn about the nature of the active site. To date all experimental evidence concerning liver alcohol dehydrogenase (LADH) is consistent with the belief that a zinc atom is included at the active site. Unfortunately such zinc atoms have neither spectral nor magnetic properties which allow direct study. The present research is directed toward a spectral study by indirect means of the nature of the environment of the zinc in the enzyme.

Vallee and Coombs[1] have shown that it is possible to measure the binding of

the ligand 1,10-phenanthroline (OP) to the enzyme by measuring changes in the ultraviolet absorption spectrum of the ligand. The spectral changes which occur on binding are quite similar, although not identical, to the spectral changes which a molecule of 1,10-phenanthroline undergoes when bound to Zn^{++} in aqueous solution. We have found that as subsequent phenanthroline molecules are added to the zinc ion there are small spectral shifts and intensity changes. Thus, the spectrum of the phenanthroline molecule when bound to a Zn atom is influenced by the nature of the other ligands which are also bound to the zinc – *i. e.*, it matters whether the zinc is bound to other phenanthrolines or whether it is bound only to water molecules of the solvent. By studying the spectrum of 1,10-phenanthroline when bound to liver alcohol dehydrogenase and comparing the results with those for 1,10-phenanthroline bound to Zn^{++} in various complexes, it should be possible to learn about the environment of the zinc ion in the enzyme. Specifically, it might be learned whether the zinc is octahedrally or tetrahedrally bound, how many points of attachment there are between zinc and the enzyme, and something about the chemical nature of these binding groups. In hope of gaining information on these points, the present research was undertaken. As model complexes for comparison with the enzyme, zinc ion was bound to the following ligands, in addition to 1,10-phenanthroline: glycine, L-cysteine, L-histidine, and imidazole.

EXPERIMENTAL

Reagents: The following chemicals were used without further purification: 1,10-phenanthroline (OP) and zinc perchlorate $Zn(ClO_4)_2 \cdot 6H_2O$, G. Frederick Smith Chemical Co.; L-cysteine (free base) and L-histidine (free base), Nutritional Biochemical Corp.; glycine (ammonia-free) and imidazole, Eastman Kodak Co. Horse liver alcohol dehydrogenase (LADH) was obtained from Worthington Enzyme Co. Water used in preparation of the enzyme solutions and in all the reagents added to the enzymes was distilled and then passed through a Barnstead demineralizer and organic material remover.

Instruments and instrumental conditions: All solutions were buffered at $pH = 6.50 \pm 0.02$ with a KH_2PO_4-NaOH buffer. All spectral measurements with the exception of those measuring the concentration of OP were made with a Cary 14 Recording Spectrophotometer. In the enzyme solution measurements, an expanded scale attachment was used that permitted recorder full scale measurements of the optical density (O. D.) in the 0.00 – 0.10 range, and the cell compartment was thermostated at $25.0 \pm 0.2^{\circ}C$. All other measurements were made at room temperature. Determinations of the OP concentrations were made on a Beckman Model DU Spectrophotometer. Matched quartz cells of 1.0 mm, 1.0 cm, 5.0 cm, and 10.0 cm path length were used to allow optical density measurements in the range 0.20 – 0.80.

Solution preparation: A standard solution of *ca.* 3.0×10^{-2} M Zn^{++} was prepared gravimetrically. The exact molarity of the Zn^{++} was determined by a ethylenediamine-NNN'N'-tetraacetic acid titration[2]. Solutions of glycine, OP, L-cysteine, L-histidine and imidazole were prepared by standard gravimetric and volumetric technique. The concentrations of the OP solutions were checked by measuring the optical densities at 2925 Å and using the recorded value $E_{2925A} = 6800$ cm²mole^{-1} liter^{-1} [3].

Procedure for ZnOP complexes: One liter OP solutions were prepared with concentrations varying at 0.5×10^{-4} M intervals from 0.5×10^{-4} to 18×10^{-4} in OP. 5.0 ml of distilled water were added to one 500 ml flask and 5.0 ml of the standard zinc solution were added to another. These were diluted to the 500 ml mark with one of the OP solutions. A difference spectrum for the region 2600 Å – 3700 Å was obtained by measuring the ZnOP complex solution versus the OP-H_2O solution of identical OP concentration. This was repeated for each OP concentration. As a check against hidden errors from continuously increasing the concentration of OP, which itself absorbs in part of the spectral region observed, a series of experiments was

Table 1. Spectral results. For the first fourteen entries in the table the $[Zn^{++}]=3.02\times10^{-4}$ M. For all other entries (exclusive of enzyme) the $[Zn^{++}]=3.08\times10^{-4}$ M. OP means 1,10-phenanthroline.

$OP/_{Zn^{++}}$	Other $lig/_{Zn^{++}}$	Position *ca.* 2940 Å peak	Position *ca.* 2725 Å peak	$\frac{O.D._{3275}}{O.D._{3440}}$
0.346		2930	2713	2.58
0.663		2930	2712	1.88
1.040		2932	2714	1.60
1.330		2935	2717	1.54
1.66		2938	2721	1.42
1.960		2937	2720	1.30
2.26		2939	2721	1.29
2.60		2942	2721	1.21
2.75		2941	2724	1.17
3.23		2943	2725	1.10
3.55		2944	2726	1.17
3.78		2943	2725	1.13
4.17		2944	2727	1.16
4.49		2944	2727	1.13
4.81		2942	2727	1.13
5.20		2943	2726	1.11
5.85		2943	2729	1.11
0.975	1.95 His	2934	2713	1.65
0.975	1.95 Cys	2932	2714	1.71
0.975	1.95 Gly	2932	2715	1.68
0.975	2.90 Imid.	2935	2715	1.68
0.975	31.4 Imid.	2936	2716	1.58
0.975	31.4 Imid.	2943	2723	1.34
Excess	Enzyme	2943	2725	1.36

done in which a constant concentration of OP was maintained and the concentration of Zn^{++} was varied. The two methods gave equivalent spectra.

Procedure for enzyme solutions: Two 10 mg samples of lyophilized LADH were placed in two 10 ml flasks. One of the samples was dissolved in cold, buffered 1.5×10^{-4} M OP and its spectrum was taken versus an OP reference solution of the same concentration. The other sample was dissolved in buffer and measured with the buffer as a reference. The spectrum of the second sample was subtracted from that of the first with proper linear adjustment for slightly differing enzyme concentrations.

Procedure for mixed ligand solutions: One liter solutions which were 3.0×10^{-4} M in OP and contained either L-cysteine, L-histidine, glycine or imidazole as the second ligand were prepared by standard gravimetric and volumetric techniques. The zinc complexes and their spectra were prepared in the same way as the ZnOP solutions.

RESULTS

Representative results of these experiments are summarized in Table 1. About half of the total observations made are quoted in the table; omitted are duplicates and points in the ZnOP system at various intermediate ratios. In all cases results are in good agreement with those quoted. The ZnOP spectrum has absorp-

tion peaks at *ca.* 2725 Å, *ca.* 2940 Å, 3275 Å, 3425 Å and shoulders at *ca.* 2990 Å, 3125 Å, 3192 Å, and 3340 Å. The *ca.* 2725 Å peak and the *ca.* 2990 Å, 3192 Å, and 3340 Å shoulders are previously unreported. Three definitive changes in this spectrum were noted on increasing the [OP]/[Zn^{++}] ratio. These were bathochromic shifts of the *ca.* 2725 Å and *ca.* 2940 Å peaks and a decrease in the ratio $O.D._{3275A}/O.D._{3425A}$. The *ca.* 2725 Å peak shifted by 16 Å and the *ca.* 2940 Å peak shifted by 14 Å. For an individual measurement the possible error in the peak position introduced by the recorder drive was ± 2 Å. A much smaller error in the peak position is anticipated in the series of ZnOP measurements. The error in the ratio of the optical densities is seen from the tabulated data to be at most 3 %. From Table 1 it is noted that the spectrum of the LADH-OP solution corresponded to an OP/Zn^{++} ratio greater than or equal to three for both of the peak shift criteria, but to an OP/Zn^{++} approximately equal to two for the third criterion, the ratio of the optical densities. Spectra of solutions which had ligand/Zn^{++} ratios of 2:1 (where the ligand is L-cysteine, L-histidine, glycine or imidazole) and OP/Zn^{++} ratios of 1:1 were identical to spectra of solutions which contained only OP in a 1:1 ratio with the Zn^{++}. All the ligands with the exception of the imidazole have stability constants of approximately equal or greater magnitudes than OP[4]. Since the spectra showed that the 1,10-phenanthroline was bound, it seems most likely that a mixed complex must be formed containing both ligands. Because of the smaller stability constants of the zinc-imidazole complexes it was necessary to prepare solutions having imidazole/Zn^{++} ratios of 31.4 and 314 also containing OP in a 1:1 ratio with the zinc. This was in order to insure the formation of mixed complexes containing at least three imidazoles and one OP. The spectrum of the mixed complex that had a 31.4 imidazole/Zn^{++} ratio corresponded to that of the 4/3 OP/Zn^{++} complex. The spectrum of the solution with an imidazole/Zn^{++} ratio of 314 was identical to that of the LADH-OP spectrum on the basis of all three criteria.

CONCLUSIONS

The subject of ligand-ligand interactions is one which has not been studied sufficiently, in the past, to allow a decision as to why the spectrum of a phenanthroline molecule bound to a zinc ion is a function of the nature of other ligands bound to the zinc. At least two explanations seem possible. Zinc is an atom which can assume either coordination number 4 or 6. Since its actual coordination number depends upon the ligand to which it is bound and the nature of the ligand, changing the coordination number would be expected to change the absorption spectrum of a bound ligand. Even if introduction of a new ligand did not cause a coordination number change, the presence of the new ligand could cause a spectral change in other ligands by the following mechanism. The new ligand would differ from the ligand it replaced in terms of the amount of electron charge displaced from it to the Zn. This in turn would influence the charge distribution between the Zn^{++} and all the other ligands. Hence the spectrum of each ligand should be a function of all the ligands present.

A comparison of the spectrum of phenanthroline bound to LADH to that of the mono-, bis-, and tris-phenanthroline Zn^{++} complexes shows that the LADH-

OP spectrum is most like that of the tris complex. This observation could be taken to indicate that the zinc in the enzyme is most probably bound octahedrally and by at least three points of attachment to the enzyme. This picture is in agreement with that proposed by Theorell based on kinetic results[5].

In order to learn more about the identity of the groups binding the zinc atom to the enzyme, the spectra of the other model complexes are helpful. Because the stability constants for zinc complexes with glycine, histidine and cysteine exceed or are approximately equal to those for phenanthroline and since they were present in a concentration double that of the phenanthroline, it seems unlikely that the phenanthroline would be able to displace any of these ligands from the zinc. If in fact it does not, then the mixed complex should be octahedral, and the spectral results indicate that none of these ligands furnishes an environment like that in the enzyme. The result with cysteine is especially interesting in this regard since it has been postulated that the strong binding of zinc in the enzyme is due to its being bound to sulfur[5]. The only model complex to exactly match spectrum with that furnished by the enzyme is the imidazole complex. This result along with the close agreement with the tris-phenanthroline complex might indicate that the zinc in liver alcohol dehydrogenase is bound to three or four imidazole residues.

REFERENCES

1. Vallee, B. L. and Coombs, T. L. *J. Biol. Chem.* **234** (1959) 2615; *J. Am. Chem. Soc.* **80** (1958) 397.
2. Schwarzenbach, G. *Complexometric Titrations,* Methuen Company, London, 1957.
3. McClure, J. H. and Banks, C. V. *U. S. Atomic Energy Commission Report* **ISC-164** (1951).
4. *Stability Constants, Part I, Organic Ligands,* Chem. Soc., London, 1957, Special Publication No. 6.
5. Theorell, H. *Federation Proc.* **20** (1961) 967.
6. Wallenfels, K. and Sund, H. *Biochem. Z.* **329** (1957) 59.

Received March 26, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S96–S101

Studies on Liver Alcohol Dehydrogenase Complexes

I. The Coenzyme-Binding Sites and Effects of Adenosine Diphosphate Ribose and *o*-Phenanthroline

TAKASHI YONETANI

Department of Biochemistry, Nobel Medical Institute, Stockholm, Sweden

ADPR*, a potent inhibitor of LADH, competes strictly with NAD+ and NADH for LADH. The LADH–ADPR interaction is independent of substrates and PHL. LADH, ADPR, and PHL seem to form a mosaic complex (PhEA), in which ADPR and PHL are independently attached to LADH at their own binding sites of the enzyme. It is concluded that the ADPR moiety of the coenzyme is the site of the enzyme-coenzyme binding common to NAD+ and NADH, that Zn of LADH is not involved in the binding between LADH and the ADPR moiety of the coenzyme, and that the interaction of LADH with substrate-analogue-inhibitors and the dihydronicotinamide- or nicotiniumamide** -moiety of the coenzyme occurs at or near Zn.

Over the past decade, a number of studies have been reported in order to elucidate the mode of interaction between LADH and the coenzyme (NAD+ and NADH)[1–11]. It is generally agreed that the dissociation step of the enzyme-coenzyme complexes (EO and ER) is a rate-limiting one of the LADH reactions[1]. The formation of ER is characterized by a shift in the absorption maximum of NADH[2] and by an intensification and a band shift of the NADH-fluorescence[3]. NAD+ and NADH compete with one another for LADH[4]. These observations made it possible to determine the dissociation constants of ER ($K_{E,R}$) and EO ($K_{E,O}$) by spectrophotometric and spectrophotofluorometric methods[1,4,5]. Analyses of pH-dependence of $K_{E,R}$ and $K_{E,O}$ have revealed the nature of the ionic groups involved in the enzyme-coenzyme binding[4–6]. Studies of the inhibition of LADH by PHL[7–9] and of the complex formation between LADH and PHL[10,11] led to an assumption that Zn of LADH is the site of the enzyme-coenzyme binding[5,7–11].

* Abbreviations: LADH (E = ½ LADH), liver alcohol dehydrogenase; NAD+ (O), nicotiniumamide-adenine dinucleotide; NADH (R), dihydronicotinamide-adenine dinucleotide; ADPR (A), adenosine diphosphate ribose; PHL (Ph), *o*-phenanthroline; (I), isobutyramide; (Py), pyrazole; TN, turnover number. Letters in parentheses are simplified abbreviations used in expressing dissociation constants and forms of LADH complexes.

** 3-Carbamyl pyridinium is referred to as nicotiniumamide group[11a].

The marked protection of LADH by NADH and by NADH + isobutyramide against inactivation indicates that LADH and NADH interact at more than one site[12]. Inhibitors structurally analogous to the coenzyme will be useful in the study of the enzyme-coenzyme interaction. ADPR is one of potent LADH-inhibitors of this type[13,14].

This paper deals with a study of the interaction between LADH and ADPR. The competition between ADPR and the coenzyme for LADH as well as the interaction of LADH with ADPR and PHL have been studied by kinetic and equilibrium measurements.

EXPERIMENTAL

Materials. Crystalline LADH was prepared according to a modification of Dalziel's method[15]. The enzyme concentration was expressed as N, the normality of NADH-binding sites per l and was determined spectrophoto-fluorometrically[12]. The purity of the enzyme preparation was found to be 100 % on the basis of an absorbancy index at 280 mμ of 0.42 ml/mg × cm^{-1} [15] and a molecular weight = 84 000 per two binding sites[16]. NADH ($\varepsilon_{340\ m\mu} = 6.22\ mM^{-1} \times cm^{-1}$), ADPR ($\varepsilon_{259\ m\mu} = 16.0\ mM^{-1} \times cm^{-1}$), adenine, and adenosine were purchased from Pabst Laboratories. NAD+ ($\varepsilon_{259\ m\mu} = 18.0\ mM^{-1} \times cm^{-1}$), 1-methyl nicotiniumamide iodide ($\varepsilon_{265\ m\mu} = 4.2\ mM^{-1} \times cm^{-1}$), nicotiniumamide mononucleotide ($\varepsilon_{266\ m\mu} = 4.6\ mM^{-1} \times cm^{-1}$), AMP, ADP, and ATP were obtained from Sigma Chemical Co. Isobutyramide and *o*-phenanthroline were products of Eastman Organic Chemicals. Pyrazole was kindly supplied by Dr. B. Sjögren, AB. Astra, Sweden. Most of the experiments were performed in sodium phosphate buffer, pH 7.0, ionic strength 0.1. The pH-dependence of $K_{E,A}$ and K_i was determined in sodium phosphate buffer, ionic strength 0.1 (pH 6.0, 7.0, and 8.0) and in 0.1 M sodium glycine buffer (pH 9.0 and 10.0).

Methods. The inhibition of LADH by ADPR was studied fluorometrically by measuring the initial rates of the forward and reverse reactions of LADH with varied concentrations of NADH, acetyldehyde, NAD+, and ethanol at fixed concentrations of their reaction partners (Cases 1 to 4, respectively)[5,6] in the presence of ADPR. The competitive inhibitor constant of ADPR (K_i) was calculated from the slopes of Lineweaver-Burk plots[17]. The LADH-ADPR binding was studied spectrophotofluorometrically by titrating LADH with NADH[4,5] in the presence of ADPR. This is based on the strict competition between ADPR and NADH for the coenzyme-binding sites of LADH. The data from the titration were fed into a Wegematic 1000 digital computer which was programmed to calculate $K_{E,R}$ and apparent $K_{E,R}$ according to Theorell-Winer equations[4]. The dissociation constant of EA ($K_{E,A}$) was calculated from the apparent $K_{E,R}$ in the presence of ADPR and [ADPR] by analogy to the $K_{E,O}$ calculation[4,5]. No quenching of the NADH-fluorescence by ADPR was observed under the experimental conditions. The interaction between ADPR and LADH-complexes was studied by *double difference spectrophotometry*[18]. In this method, two pairs of cuvettes were used in order to subtract unnecessary light-absorption of the complexes and reductants and to observe small light-absorption changes in strongly light-absorbing backgrounds. A recording fluorometer[6], a recording spectrophotofluorometer[19], a Beckman DK-2 recording spectrophotometer, and a Radiometer pH-meter model 25 were used. All experiments were performed at 23.5°C.

RESULTS AND DISCUSSION

Lineweaver-Burk plots of initial rates of forward and reverse reactions of LADH in the presence and absence of ADPR revealed that ADPR is strictly competitive with both NAD+ and NADH, and is non- and un-competitive with ethanol and acetaldehyde, respectively. K_i values of ADPR are given in Table 1, together with $K_{E,A}$ values which were determined by the equilibrium titration in the absence of substrates. Three independently determined constants of ADPR, *i. e.*, K_i's for forward and reverse reactions and $K_{E,A}$, are in good agreement with each other at any pH values tested within the limits of experimental

Table 1. Competitive inhibitor constants (K_i) and dissociation constant ($K_{E,A}$) of ADPR.

	Kinetic constants						Equilibrium constants		
	NAD+ reduction [a]			NADH oxidation [b]					
pH	TN_{max} sec^{-1}	$K_{m(O)}$ [c] μM	K_i μM	TN_{max} sec^{-1}	$K_{m(R)}$ [c] μM	K_i μM	$K_{E,A}$ [d] μM	$K_{(E,R)}$ [e] μM	$K_{E,O}$ [e] μM
6.0	1.18	16	9.3	58	5.9	12	8.7	0.23	266
7.0	2.93	14	20	66	8.3	17	16	0.31	160
8.0	3.70	11	31	48	9.1	21	21	0.41	51
9.0	4.44	13	64	11	3.3	40	36	0.65	12
10.0	4.65	29	128	1.0	3.0	100	145	5.0 [f]	10 [f]

[a] 2.7×10^{-8} N LADH, 6.2 mM ethanol and $\pm$ 50μM ADPR with varied NAD+ (10, 20, 50, 100, 200, and 500 μM).
[b] 3.0×10^{-9} N LADH, 1.1 mM acetaldehyde and $\pm$ 50 μM ADPR with varied NADH (2, 5, 10, and 20 μM).
[c] In the absence of ADPR.
[d] 1.10 μN LADH was titrated with 8 successive additions of 0.487 μM NADH in the presence and absence of ADPR (5, 10, 20, 50, 100, 200, or 400 μM).
[e] From Theorell and McKee[5].
[f] From Theorell and Winer[4].

error. These observations indicate that the *LADH-ADPR interaction is independent of substrates,* and that *the ADPR moiety of the coenzyme is the site of the enzyme-coenzyme binding common to NAD+ and NADH.* This conclusion was further supported by the following experiments. The 325-mμ absorption maximum of ER was shifted toward 340 mμ upon an addition of excess ADPR. Similarly, the intensity of the 290-mμ absorption maximum of PyEO[20] decreased appreciably upon the addition of excess ADPR. Nicotinamide, 1-methyl nicotiniumamide iodide, nicotiniumamide mononucleotide, adenine, adenosine, AMP, ADP, and ATP were inactive as a potent inhibitor of LADH at pH 7.0. A combination of ADPR and each one of these substances did not affect the inhibitory effect of ADPR. Thus *the ADPR moiety seems to be a minimal structure required for the unique firm binding of the coenzyme with LADH.*

Although $K_{E,A}$ values are considerably higher than $K_{E,R}$ values, the effect of pH on $K_{E,A}$ and $K_{E,R}$ are remarkably similar: both are relatively pH-independent at pH 6 to pH 8 and rise suddenly above pH 9[4–6]. Therefore, *the general features of pH-dependence of $K_{E,R}$ should be attributed to the interaction between LADH and the ADPR moiety of NADH. The additional interaction between LADH and the dihydronicotinamide moiety of NADH may be responsible for the further stabilization of the fundamental binding between LADH and the ADPR moiety of the coenzyme.* The positively-charged nicotiniumamide moiety of NAD+, therefore, is responsible for the considerable difference in pH-dependence between $K_{E,A}$ and $K_{E,O}$. The fact that EO dissociates more at lower pH values suggests that *the ionic groups which become more positively-charged at lower pH values, such as Zn-OH^-, are involved in the interaction between LADH and the*

functional group of the coenzyme, i. e., the nicotiniumamide- or dihydronicotinamide-moiety, as suggested previously by Theorell *et al.*[4–6]

PHL, a metal-chelating agent, is a potent inhibitor of LADH, competing with the coenzyme[7–9] and with substrates[8,9], and acting on Zn of the enzyme[10,11]. A kinetic study of the LADH inhibition by a mixture of ADPR and PHL revealed that the inhibitory effects of ADPR and PHL are strictly independent of one another. Thus, these inhibitors seem to interact with LADH at different sites, though both are competitive with the same substance, the coenzyme.

Interactions of LADH with the coenzyme, substrate-analogue-inhibitors, ADPR, and PHL were studied by means of double difference spectrophotometry as described in details elsewhere[18]. The LADH-PHL interaction was previously studied by ordinary difference spectrophotometry[10,11], in which extra absorption maxima due to the Zn-PHL chelation were observed above strong absorption of the enzyme protein. Because of the interference of strong absorption at around 280 mμ due to aromatic amino acids, the 297-mμ absorption maximum of the Zn-PHL chelate was not observed in the case of Zn-containing enzymes[10]. By double difference spectrophotometry, however, extra absorption maxima at 297, 329, and 345 mμ due to the chelation between LADH-bound Zn and PHL were

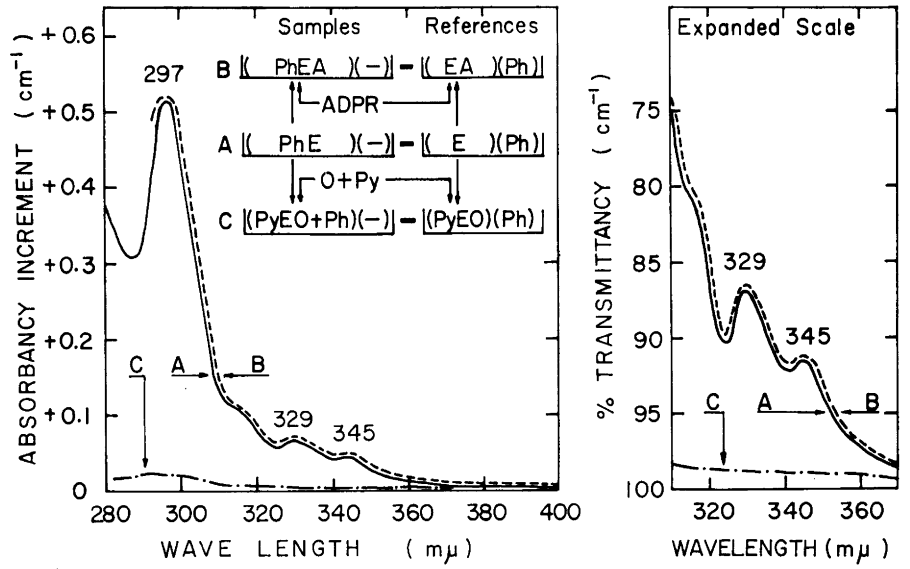


Fig. 1. Interactions of PHL-LADH complex with ADPR and with NAD+ + pyrazole. Measurements are performed at pH 7.0 with a Beckman DK-2 spectrophotometer which is set at 0–1.0 absorbancy or 75–125 % transmittancy scales. Reactant concentrations are 100 μN LADH (E), 100 μM PHL (Ph), 100 μM NAD+ (0), 500 μM ADPR (A), and 1 mM pyrazole (Py). A pair of sample cuvettes and a pair of reference cuvettes are used: the contents of each cuvette are indicated in parentheses in the figure. A, PhE (or Zn-PHL) absorption spectrum. B, PhEA absorption spectrum: ADPR has no effect on the Zn-PHL absorption. The spectrum below 295 mμ was not measurable because of too strong absorption of 500 μM ADPR. C, the Zn-PHL absorption disappears upon the addition of NAD+ + pyrazole. A similar effect is also observed with NADH + isobutyramide.

distinctly observable, because strong absorptions of both protein and PHL were simultaneously subtracted by the references (see details Fig. 1, A). Since values of $K_{E,A}$ and $K_{E,Ph}$ (the dissociation constant of PhE) are in the same order of magnitude, *i. e.*, $K_{E.A} = 16\ \mu M$ (pH 7.0, Table 1) and $K_{E,Ph} = 8.0\ \mu M$ (pH 7.0)[18], one can expect a partial disappearance of the Zn-PHL absorption upon additions of ADPR to PhE, provided ADPR and PHL compete for the same sites of LADH. However, upon the addition of ADPR, even in a large excess, to PhE, no change in the Zn-PHL absorption was observed (Fig. 1, B). This result further confirms the above-mentioned assumption that *ADPR and PHL independently interact with LADH at their own binding sites.* Judging from $K_{E,A}$ and $K_{E,Ph}$ values it seems likely that ADPR and PHL can attach simultaneously to the same molecule of LADH to form a ternary complex, PhEA, in which ADPR and PHL have no direct interaction between them. Thus this ternary complex, PhEA, is definitely different from ternary complexes of LADH, substrate-analogue-inhibitors, and the coenzyme, such as IER[5,12,21] and PyEO[20], in which three components are assumed to be bound with each other. *The complex, PhEA,* can be considered *to be a mosaic or hybrid complex of two binary complexes, PhE and EA.* Therefore, *the interaction between Zn of LADH and the adenine moiety of the coenzyme in the LADH-coenzyme complexes*[5,22,23] seems unlikely.

Because of the coenzyme-competitive inhibition[7–9] and the bound-Zn specific chelation[10,11] of PHL, Zn of LADH has been inductively assumed to be the site of the coenzyme-enzyme binding. Now it is possible to present more direct evidence for this assumption. Upon the addition of NADH, a partial disappearance of the Zn-PHL absorption of PhE was observed. The Zn-PHL absorption of PhE disappeared completely upon additions of either NADH + isobutyramide or NAD^+ + pyrazole (Fig. 1, C). This suggests that *the interaction of LADH with substrate-analogue-inhibitors and the nicotiniumamide- or dihydronicotinamide-moiety of the coenzyme occurs at or near Zn of the enzyme.* This is not only *direct evidence for the identification of Zn of LADH as the coenzyme-binding site,* but also provides *a convenient method to distinguish the enzymically essential Zn from extraneous Zn in LADH preparations.*

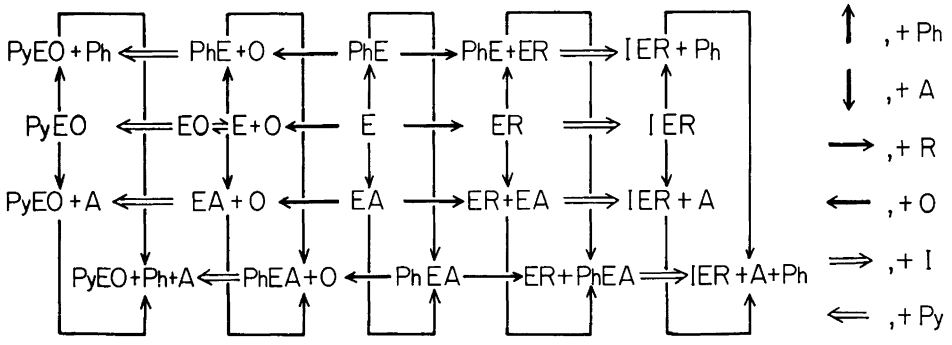


Fig. 2. Enzymically inactive equilibria of LADH (E), NAD+ (0), NADH (R) ADPR (A), PHL (Ph), pyrazole (Py), and isobutyramide (I). Although they are indicated by one-directioned arrows, all the reactions are fully reversible.

Further studies of interactions of LADH with coenzyme and inhibitors[18] have revealed that enzymically inactive equilibria, as shown in Fig. 2, exist among these multiple components. These equilibria are controlled by dissociation constants of the corresponding binary and ternary complexes. Since all of these constants have been determined by extensive kinetic- and equilibrium experiments[1,2,4–6,11,18–21], it is possible to calculate the amount of a complex present in a particular equilibrium mixture from the reactant concentrations.

In despite of many hypotheses[5,6,22–26], there has been no experimental evidence to show which part of the nicotiniumamide- or dihydronicotinamide-moiety of the coenzyme interacts with LADH and substrates at or near Zn of the enzyme. Further experiments are needed before presenting a convincing picture of the mode of their interaction. The nicotiniumamide modified analogues of the coenzyme, such as 3-acetyl-, 3-aldehyde-, and 3-thiocarbamyl-pyridinium-adenine dinucleotides[26], may be useful for this purpose.

The formation of the hypothetical mosaic-complex, PhEA, has been proved by the crystallization of PhEA as well as EA and PhE[18].

Acknowledgements. The author is indebted to Professor Hugo Theorell for his introduction to and guidance in the LADH study. This work was supported by grants from *Statens Medicinska Forskningsråd, Knut och Alice Wallenbergs Stiftelse, Institutet för Maltdrycksforskning,* and the *Rockefeller Foundation* to Professor Hugo Theorell.

REFERENCES

1. Theorell, H. and Chance, B. *Acta Chem. Scand.* **5** (1951) 1127.
2. Theorell, H. and Bonnichsen, R. *Acta Chem. Scand.* **5** (1951) 1105.
3. Boyer, P. D. and Theorell, H. *Acta Chem. Scand.* **10** (1955) 447.
4. Theorell, H. and Winer, A. D. *Arch. Biochem. Biophys.* **83** (1959) 291.
5. Theorell, H. and McKinley McKee, J. S. *Acta Chem. Scand.* **15** (1961) 1797, 1811, 1834.
6. Theorell, H., Nygaard, A. P. and Bonnichsen, R. *Acta Chem. Scand.* **9** (1955) 1148.
7. Vallee, B. L., Williams, R. J. P. and Hoch, F. L. *J. Biol. Chem.* **234** (1959) 2621.
8. Plane, R. A. and Theorell, H. *Acta Chem. Scand.* **15** (1961) 1866.
9. Dalziel, K. *Nature* **197** (1963) 462.
10. Vallee, B. L., Coombs, T. L. and Williams, R. J. P. *J. Am. Chem. Soc.* **80** (1958) 397.
11. Vallee, B. L. and Coombs, T. L. *J. Biol. Chem.* **234** (1959) 2615.
11a. Yonetani, T. *Acta Chem. Scand.* **17** (1963) 855.
12. Yonetani, T. and Theorell, H. *Arch. Biochem. Biophys.* **99** (1962) 433.
13. Dalziel, K. *Nature* **191** (1961) 1098.
14. Dalziel, K. *J. Biol. Chem.* **237** (1963) 1538.
15. Dalziel, K. *Acta Chem. Scand.* **12** (1958) 459.
16. Ehrenberg, A. and Dalziel, K. *Acta Chem. Scand.* **12** (1958) 465.
17. Lineweaver, H. and Burk, D. *J. Am. Chem. Soc.* **56** (1934) 658.
18. Yonetani, T. *Biochem. Z.* (1963). *In press.*
19. Ehrenberg, A. and Theorell, H. In Florkin, M. and Stotz, E. H. *Comprehensive Biochemistry,* Elsevier Publishing Co., Amsterdam, 1962, Vol. III, p. 187.
20. Theorell, H. and Yonetani, T. *Biochem. Z.* (1963). *In press.*
21. Winer, A. D. and Theorell, H. *Acta Chem. Scand.* **14** (1960) 1729.
22. Wallenfels, K. and Sund, H. *Biochem. Z.* **329** (1957) 59.
23. Mahler, H. R. and Douglas, J. *J. Am. Chem. Soc.* **79** (1957) 1159.
24. Rabin, B. R. and Whitehead, E. P. *Nature* **196** (1962) 658.
25. Dixon, M. and Webb, E. C. *Enzymes,* Longmans Green Co., London, 1958, p. 351.
26. Kaplan, N. O. *The Enzymes,* 2nd Ed., Academic Press, New York, 1960, Vol III, p. 105.

Received March 28, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 102 – S 106

Struktur und Wirkungsweise der Glutaminsäuredehydrogenase

I. Grösse und Gestalt der Glu-DH aus Rinderleber*

HORST SUND

Chemisches Laboratorium der Universität Freiburg i. Br., Deutschland

In the aggregated state the particle weight of Glu-DH from beef liver is 1.99 millions. This value is derived from $s_{20,w}^{\circ} = 31.19$ S, $D_{20,w}^{\circ} = 1.52$ F, and a partial specific volume of 0.75. From the frictional ratio (1.67), the intrinsic viscosity ($[\eta] = 14.89$ ml/g), and the parameter β according to Scheraga and Mandelkern it was concluded that Glu-DH possesses a prolate shape with an axial ratio of 13–14.

Aus Sedimentations- und Diffusionsmessungen hatte man geschlossen, dass Glu-DH** aus Rinderleber ein Molekulargewicht von 1 Million besitzt und bei Konzentrationen unterhalb 4 mg/ml in kleinere Teilchen dissoziiert[1]. Bei unseren Untersuchungen über die Einwirkung von Sulfonylharnstoffderivaten, Harnstoff und Schwermetallionen auf die molekulare Struktur der Glu-DH erhielten wir Ergebnisse[2,3], die darauf schliessen liessen, dass dieses Enzym im aggregierten Zustand ein höheres Teilchengewicht besitzt, als es bisher angenommen wurde. Um weitere Informationen über Grösse und Gestalt dieses Enzymmoleküls zu erhalten, haben wir seine hydrodynamischen Eigenschaften eingehend untersucht.

EXPERIMENTELLER TEIL

Die untersuchten Präparate von kristallisierter Glu-DH aus Rinderleber entsprechen in ihren Eigenschaften den früher verwendeten[4]. Vor den Untersuchungen wurde das Protein 4 Tage gegen M/15 K-Na-Phosphat-Puffer, pH 7,6 ($\mu = 0{,}17$), dialysiert, nach jeweils 24 und 48 Stunden wurde die Aussenflüssigkeit gewechselt. Zur Methodik und Auswertung der Diffusions-, Sedimentations- und Viskositätsmessungen siehe[5]. Die Proteinbestimmung erfolgte auf Grund des Kjeldahl-Stickstoffs (15,50 %) bzw. der UV-Absorption bei 280 mμ ($\varepsilon_{280} = 0.95$ cm$^2 \cdot$ mg^{-1}). Weitere Einzelheiten werden demnächst in der Biochem. Z. veröffentlicht.

---

* Ein Teil der hier mitgeteilten Versuche wurde in den Jahren 1960 und 1961 in Stockholm während eines Aufenthaltes in der biochemischen Abteilung des Medizinischen Nobelinstitutes durchgeführt. Herrn Professor Hugo Theorell danke ich sehr für seine Unterstützung und die mir gewährte Gastfreundschaft.

** Abkürzungen: Glu-DH: Glutaminsäuredehydrogenase; Tris: Tri-(hydroxymethyl)-aminomethan.

ERGEBNISSE

Sedimentation. Infolge des Assoziations-Dissoziations-Gleichgewichtes beobachtet man bei Glu-DH aus Rinderleber einen anomalen Verlauf der Konzentrationsabhängigkeit des Sedimentationskoeffizienten und unsymmetrische Konzentrationsgradienten in der analytischen Ultrazentrifuge[1]. Unsere Sedimentationsmessungen (Abb. 1) haben prinzipiell die Werte von Olson und Anfinsen bestätigt. Diese Autoren fanden für $s_{20,w}°$ 26,6 S, unser Wert liegt um etwa 15 % höher. Die Kurve A der Abb. 1 ($s_{20,w}° = 29{,}04$ S) wurde durch Versuche in einer Normalzelle erhalten, Kurve B durch Versuche in einer Überschichtungszelle ($s_{20,w}° = 31{,}19$ S). Bei diesen Versuchen wurde aber nicht mit Pufferlösung überschichtet, sondern mit einer Enzymlösung niederer Konzentration. Der Grund hierfür war folgender: Während der Sedimentation bildet sich in der Ultrazentrifugenzelle eine Grenzschicht Lösung-Lösungsmittel aus. In dieser Grenzschicht fällt die Konzentration der Glu-DH entsprechend dem Konzentrationsverlauf auf null ab, so dass das Molekül in kleinere Teilchen dissoziieren kann. Für den Fall, dass die Geschwindigkeit der Dissoziation gross, diejenige der Assoziation dagegen klein ist, würde man bei einer bestimmten Enzymkonzentration nicht den Sedimentationskoeffizienten des bei dieser Konzentration vorliegenden Teilchens bestimmen, sondern denjenigen eines Dissoziationsproduktes.

Im Gegensatz zu den Versuchen in der Normalzelle beobachtet man nach Überschichten mit Glu-DH-Lösung symmetrische Konzentrationsgradienten auch

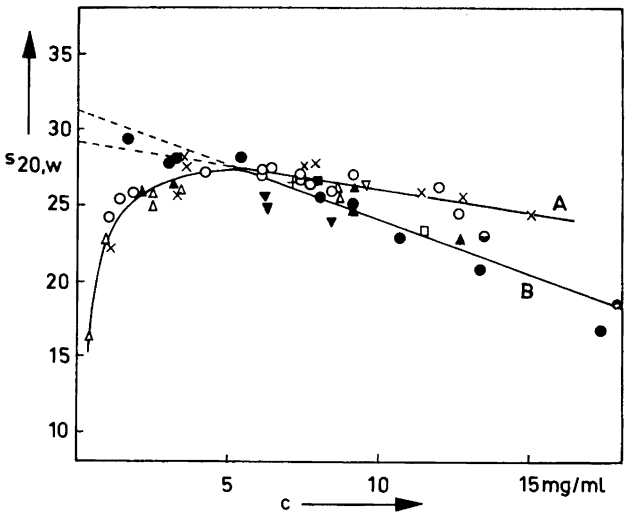


Abb. 1. Abhängigkeit des Sedimentationskoeffizienten $s_{20,w}$ (in Svedberg-Einheiten) von der Proteinkonzentration c bei Glu-DH aus Rinderleber. Messungen in 0,05 M Tris-Puffer pH 7,6 (×) bzw. M/15 K-Na-Phosphat-Puffer pH 7,6 (alle anderen) bei 20°C in der Spinco-Ultrazentrifuge bei 4 197 (■), 12 590 (●, ◒, ◓, □, +), 42 040 (▽), 50 740 (△, ▼) und 59 780 (▲, o, x) UpM. A: Versuche in der Normalzelle. B: Versuche in der Überschichtungszelle. Überschichtung mit Glu-DH-Lösung: $c = 45$ % (◒), 48 % (◓), 53 % (□), 67 % (■, ●, ▲, ▼) bzw. 86 % (+) der Konzentration der Enzymlösung in der Zelle.

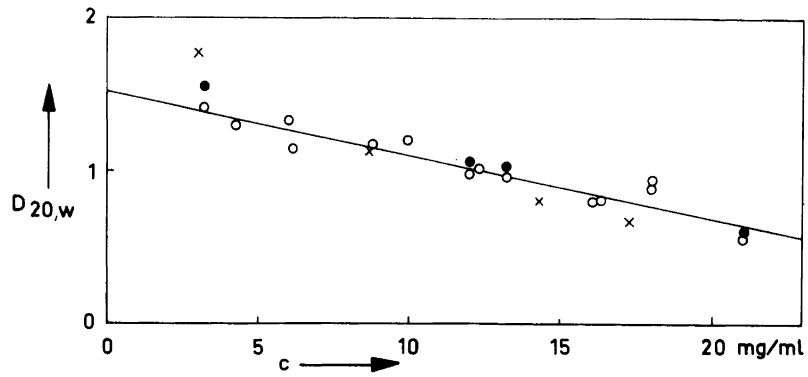


Abb. 2. Abhängigkeit des Diffusionskoeffizienten $D_{20,w}$ (in Fick-Einheiten) von der Proteinkonzentration c bei Glu-DH aus Rinderleber. Messungen bei 20°C in M/15 K-Na-Phosphat-Puffer pH 7,6. x, o: Nach der Flächenmethode berechnet. •: Nach der Momentmethode berechnet. Versuche in der Meyerhoff-Zelle[6] (o, •) bzw. in der Ultrazentrifuge bei 4 197 UpM (x).

bei solchen Konzentrationen, bei denen in der Normalzelle unsymmetrische auftreten. In diesem Fall ist die sonst bei der Diffusion des Proteins in das Lösungsmittel erfolgende Dissoziation nicht zu beobachten. Abb. 1 zeigt, dass nach Extrapolation auf die Konzentration null für die Versuche bei Konzentrationen oberhalb 5 mg/ml nach beiden Methoden ähnliche Sedimentationskoeffizienten erhalten werden. Das bedeutet, dass auch die in der Normalzelle bestimmten Sedimentationskoeffizienten richtige Resultate liefern.

Diffusion. Den Diffusionskoeffizienten untersuchten wir im Bereich von 3 bis 21 mg/ml (Abb. 2). Bei Konzentrationen oberhalb 4 mg/ml gehorcht die Konzentrationsabhängigkeit des Diffusionskoeffizienten der Gleichung

$$D_{20,w} = 1{,}52 - 0{,}0417\ c$$

in der c die Enzymkonzentration in mg/ml bedeutet und 1,52 den auf die Konzentration null extrapolierten Diffusionskoeffizienten ($D_{20,w}°$) darstellt. Die Bestimmungen in der Diffusionsschieberzelle sowie in der Ultrazentrifuge ergaben übereinstimmende Werte. Ebenso lieferte die Berechnung nach der Momentmethode[7] die gleichen Ergebnisse wie diejenige nach der Flächenmethode. Unterhalb einer Konzentration von 4 mg/ml treten Abweichungen – wahrscheinlich infolge der Dissoziation – von der oben angegebenen Gleichung auf.

Viskosität. Die Viskositätsmessungen sind der Abb. 3 zu entnehmen. Für Konzentrationen oberhalb 6 mg/ml besitzt $[\eta]$ einen Wert von 14,89 ml/g, für Konzentrationen von 1 bis 6 mg/ml etwa 7,2 ml/g. Für das Viskositätsinkrement ν errechnen sich daraus Werte von 19,85 bzw. 9.6.

DISKUSSION

Nach der Svedberg-Formel[8] errechnet sich mit einem partiellen spezifischen Volumen von 0,75[1] aus $s_{20,w}° = 31{,}19$ S und $D_{20,w}° = 1{,}52$ F das Teilchengewicht der Glu-DH zu 1,99 Millionen (verschiedene Versuchsergebnisse lassen vermu-

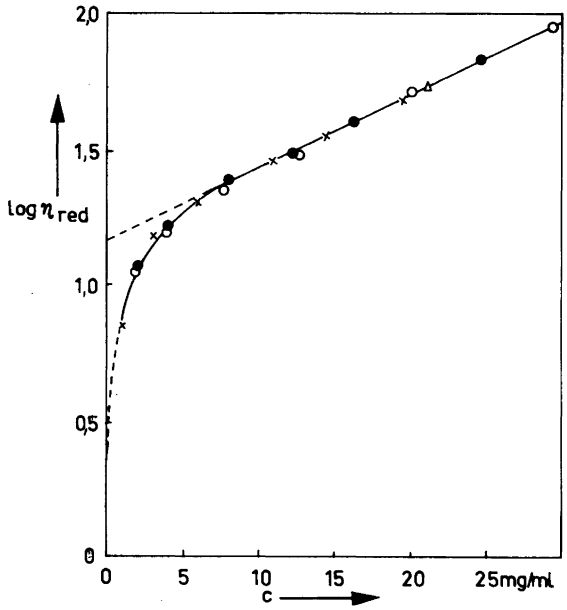


Abb. 3. Abhängigkeit der reduzierten Viskosität η_{red} ($= \eta_{sp}/c$, c in g/ml) von der Proteinkonzentration c bei Glu-DH aus Rinderleber. Messungen bei 20°C in M/15 K-Na-Phosphat-Puffer pH 7,6.

ten, dass auch Teilchen mit noch höheren Teilchengewichten auftreten). Für das Reibungsverhältnis f/f_0[8] erhält man aus $s°$ und $D°$ 1,67 und aus $[\eta]$ 1,69. Berechnet man unter Einbeziehung der Viskositätsdaten aus $s°$ und $D°$ den von Scheraga und Mandelkern[9] aus der Theorie für starre Rotationsellipsoide entwickelten Gestaltsparameter β

$$\beta_s = \frac{N_L\, s°\, [\eta]^{1/3}\, \eta_0}{M^{2/3}\, (1\text{-}V_Q)} \qquad \text{bzw. } \beta_D = \frac{D°\, [\eta]^{1/3}\, M^{1/3}\, \eta_0}{k\, T}$$

so ergibt sich sowohl β_s als auch β_D zu $2{,}51 \cdot 10^6$ Dieser Wert überschreitet den charakteristisch engen Variationsbereich von $2{,}12 - 2{,}15 \cdot 10^6$ für ein abgeplattetes Rotationsellipsoid, und es darf daher aus diesen Werten abgeleitet werden, dass das Glu-DH-Molekül eine gestreckte Form besitzt. Aus dem Reibungsverhältnis und den β-Werten erhalten wir für das Achsenverhältnis des Glu-DH-Moleküls 12,5 aus $s°$ und $D°$; 13,7 aus β_s und β_D und 12,9 aus $[\eta]$. Im aggregierten Zustand, bei Konzentrationen oberhalb 6 mg/ml, liegt Glu-DH als stäbchenförmiges Teilchen mit einem Achsenverhältnis von 13 bis 14 vor. Für Konzentrationen unterhalb 6 mg/ml ergibt sich aus $[\eta]$ von etwa 7,2 ml/g ein Achsenverhältnis von 7,7. Bei diesen Konzentrationen tritt Dissoziation ein[1,10,11], die zu symmetrischer aufgebauten Teilchen führt[12].

Das nach der Svedberg-Formel berechnete Teilchengewicht von 2 Millionen ist doppelt so hoch wie das bisher angenommene von 1 Million. Die Differenz

beruht in der Hauptsache in den unterschiedlichen Werten für den Diffusionskoeffizienten $D_{20,w}^{\circ}$, der früher zu 2,54 F[1], jetzt dagegen zu 1,52 F bestimmt wurde. Der Grund ist neben den unterschiedlichen Versuchstemperaturen wohl in der Hauptsache in den differierenden Versuchszeiten (4 Tage gegenüber einigen Stunden bei den vorliegenden Messungen) zu suchen, die einen wesentlichen Einfluss auf den Gehalt an den durch Verdünnung und anschliessende Dissoziation entstehenden kleineren Teilchen ausüben. Bei Konzentrationen oberhalb von 4 mg/ml erhalten wir für die nach der Flächen- sowie Momentmethode berechneten Diffusionskoeffizienten gleiche Werte (Abb. 2). Dies lässt darauf schliessen, dass sich bei diesen Konzentrationen die Dissoziation noch nicht bemerkbar macht (bei Uneinheitlichkeit $D_m \rangle D_A$[7]).

Im reversiblen Assoziations-Dissoziations-Gleichgewicht der Glu-DH aus Rinderleber werden als kleinste Teilchen solche mit Teilchengewichten von 250 000 gebildet[11], die – nach bisher vorliegenden Ergebnissen nur irreversibel – in 4 bis 8 Teilchen aufgespalten werden können[11,13,14]. Im aggregierten Zustand liegt das Enzym mit einem Teilchengewicht von 2 Millionen demnach als Oktameres der Einheit eines Teilchengewichtes von 250 000 vor, das im reversiblen Gleichgewicht mit den tetrameren, dimeren und monomeren Formen steht.

Herrn Professor Dr. Kurt Wallenfels danke ich sehr für seine grosszügige Unterstützung. Der *Deutschen Forschungsgemeinschaft* danke ich für ein Stipendium und für Sachbeihilfen.

LITERATUR

1. Olson, J. A. and Anfinsen, C. B. *J. Biol. Chem.* **197** (1952) 67.
2. Sund, H. *Verh. 4e Congrès de la Fédération intern. du Diabète* (publ. par M. Demole), Éditions Médecine et Hygiène, Genf, 1961, Vol. I, p. 726.
3. Sund, H. *Biochem. Z.* **333** (1960) 205.
4. Sund, H. *Acta Chem. Scand.* **15** (1961) 940.
5. Sund, H. und Weber, K. *Biochem. Z.* **337** (1963) 24.
6. Meyerhoff, G. *Makromol. Chem.* **6** (1951) 197.
7. Gralén, N. Dissertation, Uppsala, 1944.
8. Svedberg, T. und Pedersen, K. O. *Die Ultrazentrifuge,* Theodor Steinkopff, Dresden und Leipzig, 1940.
9. Scheraga, H. A. and Mandelkern, L. *J. Am. Chem. Soc.* **75** (1953) 179.
10. Kubo, H., Yamano, T., Iwatsubo, M., Watari, H., Soyama, T., Shiraishi, J., Sawada, S., Kawashima, N., Mitani, S. et Ito, K. *Bull. Soc. Chim. Biol.* **40** (1958) 431.
11. Frieden, C. *J. Biol. Chem.* **237** (1962) 2396.
12. Sund, H. In *Mechanismen enzymatischer Reaktionen,* Springer-Verlag, Berlin-Göttingen-Heidelberg, 1963. *Im Druck.*
13. Jirgensons, B. *J. Am. Chem. Soc.* **83** (1961) 3161.
14. Fisher, H. F., McGregor, L. L. and Power, U. *Biochem. Biophys. Res. Commun.* **8** (1962) 402.

Eingegangen am 2. April 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 107–S 109

On the Reaction between *p*-Chloromercuribenzoate and Rhodanese

BO SÖRBO

Nobel Medical Institute, Biochemical Department, Stockholm, Sweden

Spectrophotometric measurements show that *p*-chloromercuribenzoate reacts slowly with crystalline rhodanese and a simultaneous inhibition of the enzyme activity is observed. In the presence of a detergent (dodecyl sulfate) a very rapid reaction is obtained, which allows the number of sulfhydryl groups in the enzyme to be determined. Both with PCMB and by amperometric titration with silver ions a value of 4 sulfhydryl groups per molecule rhodanese was obtained.

The enzyme rhodanese is inhibited by certain sulfhydryl reagents[1,2] and the presence of sulfhydryl groups in the crystalline enzyme has been demonstrated[3] by the nitroprusside reaction. However, as the inhibition experiments so far published have been carried out with dilute solutions of impure rhodanese and inhibitions have been obtained only at fairly high concentrations of inhibitor, the role of sulfhydryl groups in the enzymic reaction is very uncertain. One of the sulfhydryl reagents found to inhibit rhodanese, *p*-chloromercuribenzoate, has the advantage that its reaction with sulfhydryl groups can easily be followed spectrophotometrically. The present communication is concerned with the reaction between this compound and crystalline rhodanese.

MATERIALS AND METHODS

Crystalline rhodanese was prepared from beef liver as previously[3] described. A molecular weight of 37 100[3] has been used in the calculations. *p*-chloromercuribenzoic acid (PCMB) was a commercial product, which was dissolved in sodium hydroxide and then precipitated with hydrochloric acid. This purification step was repeated and the product then washed with distilled water and dried *in vacuo*. Other compounds were commercial products which were used without further purification. Sulfhydryl groups were determined spectrophotometrically with PCMB according to Boyer[4] and by amperometric titration with silver ions in tris buffer according to Benesch *et al.*[5]. Rhodanese activity was determined by a modification of a test system previously[3] described. The volume of enzyme solution added to the assay system was reduced by a factor of 10 (to 0.05 ml) and the time of reaction by a factor of 5 (to 1 min). These changes were made in order to avoid the otherwise necessary dilution steps and also in order to minimize any reactivation of the PCMB-inhibited enzyme in the assay system. Such a

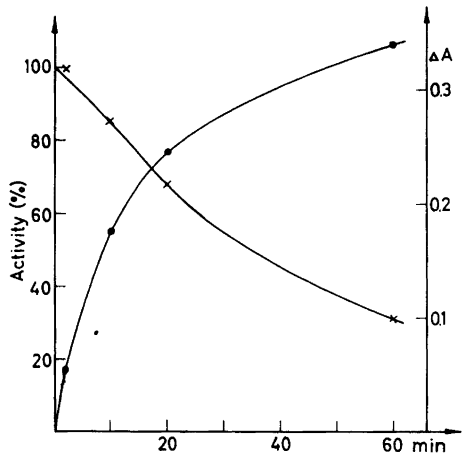


Fig. 1. Reaction between PCMB and rhodanese. Reaction conditions were PCMB 8.6×10^{-5} M, rhodanese 1.0×10^{-5} M, phosphate 0.05 M, pH 7.0 and temp. 23°C. • Increase in absorbancy at 250 mμ. X Rhodanese activity.

reactivation could be expected, as spectrophotometric measurements demonstrated that the rhodanese substrates, thiosulfate and cyanide, both reacted with an appreciable velocity with PCMB.

RESULTS

When native rhodanese was treated with PCMB at pH 7.0 a slow increase of absorbancy at 250 mμ was found (indicating a formation of PCMB-mercaptides) and at the same time the enzyme was inhibited (Fig. 1). The reaction was so slow, however, that a quantitative evaluation of the number of sulfhydryl groups present in the enzyme was impossible, as PCMB was not stable under the prolonged time periods necessary for complete reaction. At pH 4.6 an immediate turbidity developed which prevented any quantitative determinations at this pH. In the presence of 2 % dodecyl sulfate, however, a very rapid reaction (completed in less than 1 min) ensued at pH 7.0, which allowed the number of sulfhydryl groups in rhodanese to be determined according to the principles described by Boyer[4]. The unusual high molar absorbancy increment of 9.4×10^3 was obtained for rhodanese under these conditions. Determinations on two different samples of crystalline rhodanese gave values of 4.01 and 4.25 SH-groups per molecule of rhodanese.

The number of SH-groups in rhodanese was also determined by amperometric titration with silver ions. In the absence of any detergent only a change of slope of the titration curves without any detectable endpoint was observed. In the presence of 0.5 % dodecyl sulfate however, sharp and reproducible endpoints were obtained, corresponding to 4.35 and 4.57 SH-groups per molecule in satisfactory agreement with the values obtained in the experiments with PCMB. The fact that somewhat higher values are obtained by silver titration may be due to a binding of silver ions to other sites than sulfhydryl groups[6] in the protein.

DISCUSSION

Rhodanese shows in its reaction with PCMB a behaviour similar to that of other enzymes[7,8] which contain "masked" sulfhydryl groups. The fact that rhodanese activity decreases, when the sulfhydryl groups in the enzyme are blocked with PCMB, seems to support the interpretation that the sulfhydryl groups are "active sites" in the enzyme and participate in the enzymatic reaction. However, it has recently been shown[9–11] by measurements of optical rotatory dispersion that PCMB may induce changes in the tertiary structure of enzymes equivalent to denaturation, and such an effect could as well explain the results obtained with rhodanese. Of interest in this connection is the fact that crystalline rhodanese contains labile sulfur[12,13] and evidence has recently been obtained[13], which suggests that the labile sulfur is bound to a sulfhydryl group in the enzyme. This sulfhydryl group may be the only one, which participates in the enzymatic reaction, and may be protected from reaction with PCMB (and other sulfhydryl reagents) by the sulfur atom attached to it. PCMB could however react with the three other sulfhydryl groups present in the enzyme and the latter may then be inactivated by a secondary denaturation. It would be of interest to study the reaction between rhodanese and PCMB by optical dispersion measurements in order to further elucidate this point.

Acknowledgement. This investigation has been supported by a grant from *The Swedish Medical Research Council.*

REFERENCES

1. Saunders, J. P. and Himwich, W. A. *Am. J. Physiol.* **163** (1960) 404.
2. Sörbo, B. *Acta Chem. Scand.* **5** (1951) 724.
3. Sörbo, B. *Acta Chem. Scand.* **7** (1953) 1128.
4. Boyer, P. D. *J. Am. Chem. Soc.* **76** (1954) 4331.
5. Benesch, R. E., Lardy, H. A. and Benesch, R. *J. Biol. Chem.* **216** (1955) 663.
6. Brown, W. D., Martinez, M. and Olcott, H. S. *J. Biol. Chem.* **236** (1961) 92.
7. Tanaka, Y. and Schwert, G. W. *J. Biol. Chem.* **223** (1956) 157.
8. Pfleiderer, G., Hohnholz-Merz, E. and Gerlach, D. *Biochem. Z.* **336** (1962) 371.
9. Elöidi, P. *Biochim. Biophys. Acta* **40** (1960) 272.
10. Snodgrass, P. J., Vallee, B. L. and Hoch, F. L. *J. Biol. Chem.* **235** (1960) 504.
11. Li, T. K., Ulmer, D. D. and Vallee, B. L. *Biochemistry* **1** (1962) 114.
12. Green, J. R. and Westley, J. *J. Biol. Chem.* **236** (1961) 3047.
13. Sörbo, B. *Acta Chem. Scand.* **16** (1962) 2455.

Received April 2, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 110–S 116

Investigation of a Fraction Separated from Beef-Heart Cytochrome *c*

R. W. HENDERSON* and S. PALÉUS

Medicinska Nobelinstitutet, Biokemiska avdelningen, Karolinska Institutet, Stockholm, Sweden

Amino-acid analysis of the non-pigmented fraction (X) obtained from 0.3 % Fe content beef-heart cytochrome *c* by electrophoretic separation (Theorell and Åkeson[1]) has been carried out. Fraction (X) has been found to have a nitrogen content of 8.9 %. The high non-protein content has been partly explained by the presence of carbohydrate. There appears to be no identity between fraction (X) and globin from either myoglobin or haemoglobin.

Cytochrome *c* of 0.34 % iron content was first prepared from heart-muscle by Theorell[2,3] using the technique of acid extraction followed by one of several alternative fractionation procedures. A modification involving the use of trichloroacetic acid to obtain a product of similar iron content was introduced by Keilin and Hartree[4,5].

Theorell and Åkeson[1] separated electrophoretically a colourless fraction from the pigment so obtained and by this means increased the iron content to 0.43 %. Resin column chromatography of cytochrome *c* (Paléus and Neilands[6]) may also be used for this purpose. The colourless fraction (X) so removed has been generally considered to be protein in nature and the view was early expressed (see *e. g.* Lemberg and Legge[7]) that it was likely to be associated with cytochrome *c in vivo*.

Margoliash[8] obtained evidence that globin from myoglobin and also globin from haemoglobin would combine with resin column purified cytochrome *c*. This along with their similarity in behaviour during paper electrophoresis led Margoliash to the view that the fraction (X) was globin from myoglobin present as an artifact of the acid-extraction procedure. Evidence has however been obtained by Henderson and Rawlinson[9] that fraction (X) is present in cytochrome

* Present address: Russell Grimwade School of Biochemistry, University of Melbourne, Victoria, Australia.

c obtained by neutral extraction and zinc precipitation of myoglobin and haemoglobin.

It was clear that amino-acid analysis would assist in the identification or otherwise of the fraction in question with globin. This was accordingly carried out.

MATERIALS AND METHODS

Cytochrome c was prepared from minced beef-hearts by the method of Keilin and Hartree[4]. After the final precipitation with trichloroacetic acid the preparation was dialyzed free from sulphate against 0.5 % sodium chloride. The precipitated material was filtered off; the cytochrome was then dialyzed until chloride free against 0.005 M ammonium hydroxide and freezedried. Iron content of many preparations were 0.3 % ± 0.04 %. The two column resin treatment of Margoliash[10] was used to obtain cytochrome of 0.43 % Fe content from part of one of the above preparations.

Fraction (X). In order to minimize denaturation with consequent insolubility resulting from resin-column procedures this fraction was separated using (i) a Spinco Model H Electrophoresis instrument*. 1 % (w/v) cytochrome *c* solutions in phosphate buffer pH 7.2 and 0.1 ionic strength were run for several hours according to the size of cell used. At the conclusion of the run the colourless boundaries (Fig. 1) which had separated toward the anode were removed together by suction constituting fraction (X). This material was then either dialyzed until salt-free and freeze-dried or stored at −10°C. (ii) A Perkin-Elmer Model 38 Electrophoresis instrument was also used. In this apparatus 2 % (w/v) cytochrome *c* in 0.02 M phosphate buffer pH 7.3 and 0.15 M KCl, 0.2 ionic strength was used. In all cases the cytochrome *c* was in the oxidized condition.

Amino-acid analysis. About 2.5 mg samples of salt-free freezedried fraction (X) were weighed and hydrolyzed 46 hr at 110°C in a sealed and evacuated tube. Isoluble humin formed was removed by centrifugation and washed twice with dist. water. The washings were added to the hydrolysate which after evaporation to dryness over KOH was analysed according to Moore *et al.*[11]

Nitrogen content of fraction (X) was determined by a modified Dumas method[12].

Carbohydrate estimation was carried out by a modification due to Shetlar and Masters[13] of the thymol-sulphuric acid reaction method which it was reported was not significantly influenced by the presence of protein. Recovery of glucose added to resin treated cytochrome *c* was found to be 96 %.

Spectrophotometry. A Beckman Model DK2 and a Zeiss Model PMQII Spectrophotometer were used.

RESULTS AND DISCUSSION

Electrophoresis. A typical electrophoretic pattern obtained with 0.3 % Fe content beef-heart cytochrome *c* is shown in Fig. 1. The non-pigmented material (X) formed three boundaries (a, b and c) which moved toward the anode at a rate equal to or greater than that at which the cytochrome moved toward the cathode (Table 1).

Fractions (a) and (b) represent 90–95 % (by determination of areas under the curves – means of values from each limb) of the total non-pigmented material (X) separated. In some preparations there was more equality in the amounts of (a) and (b) present. From the similarity of the sum of the amounts of these two fractions present, however, in different cytochrome *c* samples it seemed likely that (b) was split from (a) during preparation. The ratio of cytochrome *c* to the

* We wish to thank Docent A. Åkeson for his assistance in the use of this apparatus and particularly for making available to us the cell assembly which he has devised and which enables long runs to be carried out with minimal trouble due to gassing at the electrodes.

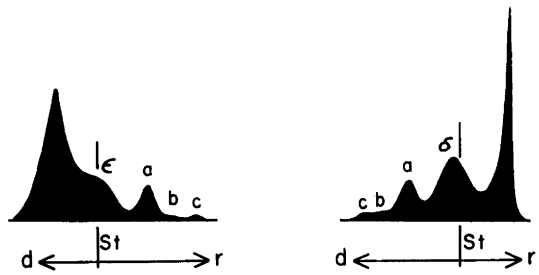


Fig. 1. Electrophoresis patterns of 0.3 % beef-heart cytochrome *c*. 2 % (w/v) cytochrome *c* in 0.02 M PO_4 buffer pH 7.3 and 0.15 M KCl, 0.2 ionic strength. Run 120 min, potential gradient 4.82 V. cm^{-1}. (St.), starting points; (ε) and (δ), stationary salt boundaries on sides where cytochrome *c* descending (d) and rising (r), respectively; (a), (b) and (c), the three non-pigmented boundaries which together constituted fraction (X).

total non-pigmented fraction (X) was estimated to be 100 : 25–33. Fraction (X) thus represents 20–25 % of 0.3 % Fe content cytochrome *c*, which is consistent with the value expected from a comparison of iron-contents before and after electrophoresis.

The electrophoretic mobilities determined for the cytochrome *c* are within the range of values already reported by Theorell and Åkeson[1] and Tint and Reiss[14]. Differences in shape and mobility of cytochrome *c* boundaries between the two limbs of the cell were reported by Theorell and Åkeson[1]. Tint and Reiss[14] considered that these differences were due to the use of solutions of 0.1 ionic strength and could be obviated by increasing the ionic strength to 0.2. The boundaries shown in Fig. 1 are typical of many obtained in solutions of 0.2 ionic strength. Considerable differences are apparent in the patterns from opposite limbs of the cell. Furthermore the mobilities (Table 1) of the rising cytochrome *c*

Table 1. Electrophoretic mobilities (cm^2 $volt^{-1}$ sec^{-1} $\times$ 10^{-5}) of fractions separated from 0.3 % beef-heart cytochrome *c*, 0.02 M phosphate buffer pH 7.3 ionic strength 0.2 (including 0.15 M KCl). Means of a number of determinations except in the case of electrophoretically separated cytochrome *c* where the values are from a single run. Lettering as in Fig. 1.

Fraction	Limb of cell in which cytochrome *c* descending	Limb of cell in which cytochrome *c* rising
Cytochrome *c*	2,4	2,8
Fraction (a)	3,0	3,0
Fraction (b)	4,9	4,6
Fraction (c)	5,9	5,7
Electrophoretically separated cytochrome *c* re-run	2,3	2,8

show small but consistently higher values than those for the descending pigment. There was some evidence in the fractions (a), (b) and (c) also of higher mobilities where the components were rising and out of contact with oppositely moving protein. In the case of the already electrophoretically separated cytochrome *c* run in 0.2 ionic strength buffer (not shown) only one peak was apparent in each limb of the cell and the patterns were much more similar than those obtained when fraction (X) was present. The rising cytochrome *c* still showed, however, a higher value than that of the descending pigment (Table 1).

It would seem likely therefore that the observed differences are due in some measure both to the formation of dissociable salt complexes between the various fractions present as suggested by Theorell and Åkeson[1] plus salt concentration and pH changes which may occur throughout the cell during electrophoresis (see *e. g.* Longsworth[15]).

No attempt was made to separate the three fractions (a), (b) and (c) and they were removed from the cell together. There was no absorption in the visible region by fraction (X) either before or after addition of dithionite. Protohaem was not present in detectable amounts under conditions of pyridine ferrohaemochromogen formation.

Amino-acid analysis. Mean values of a number of amino-acid analyses of fraction (X) are listed in Table 2. The nitrogen content (8.9 %) of fraction (X) indicates a protein content of about 60 % which is consistent with the recovery of 62 g amino-acid/100 g unhydrolyzed sample.

The amino-acid values (Table 2) have therefore been multiplied in one column by the factor $\frac{100}{60}$ in order to facilitate comparison with reported analyses of beef myoglobin, haemoglobin and cytochrome *c*. The results do not agree closely with any of the comparison proteins listed and in particular fraction (X) was found to have a markedly lower histidine content than either beef myoglobin or haemoglobin.

Carbohydrate was detected qualitatively in 0.3 % beef-heart cytochrome *c* by the micro aniline acetate test as outlined by Feigl[18].

Light absorption curves obtained by application of a modified thymol-sulphuric acid reaction method of carbohydrate estimation (Shetlar and Masters[13]) to a number of samples are shown in Fig. 2.

It was found that the absorption peak position of 0.3 % Fe content beef-heart cytochrome *c* (507–509 mμ) was practically identical with that for glucose alone and also for resin-column treated cytochrome *c* of 0.43 % Fe content to which glucose had been added. Fructose under the same conditions gave an absorption maximum at 510–511 mμ. Other carbohydrates listed by the above authors have maxima at wavelengths still further removed from that for glucose.

A curve (not shown) was obtained with fraction (X) which was almost identical in shape with that for glucose. As expected the absorption peaks at 550 mμ and 596–597 mμ, due almost certainly to the haem prosthetic group of the cytochrome *c*, were completely absent.

The carbohydrate content of fraction (X) calculated as glucose was found to be 8 %. This compared with 1.5 % for 0.3 % Fe content cytochrome *c*.

Table 2. Results of amino-acid analysis of fraction (X) from beef-heart cytochrome *c* compared with similar analyses of the apo-proteins of several other pigments. (Figures in parentheses in first column refer to the number of determinations from which the values were obtained.)

	g amino-acid/ 100 g fraction (X) from 0.3 % Fe content beef-heart cytochrome *c*	Results from first column $\times \frac{100}{60}$ *i. e.* g amino-acid/ 100 g protein	Adult beef myoglobin g/100 g protein[16]	Adult beef haemoglobin g/100 g protein[16]	Beef-heart cytochrome *c* 0.43 % Fe content g/100 g protein[17]
Cysteic	Trace		–	–	2.6
Aspartic	6.9 (3)	11.5	10.0	11.9	9.7
Threonine	3.3 (3)	5.1	3.3	4.8	7.4
Serine	1.8 (3)	3.0	3.1	5.2	
Glutamic	10.2 (3)	17.0	13.9	7.9	13.6
Proline	2.7 (2)	4.5	2.5	3.8	3.5
Glycine	2.5 (3)	4.2	5.1	3.8	9.0
Alanine	4.0 (3)	6.6	8.1	10.3	4.6
Valine	3.5 (3)	5.8	6.1	10.4	3.0
Methionine	Trace		1.9	1.4	2.3
Iso-Leucine	3.7 (3)	6.2	3.9	0	6.3
Leucine	4.9 (3)	8.2	12.8	15.2	6.3
Tyrosine	1.1 (3)	1.8	2.3	2.1	5.3
Phenylalanine	3.5 (3)	5.8	5.9	6.6	4.2
Lysine	8.4 (2)	14.0	15.4	11.5	20.7
Histidine	2.0 (2)	3.3	11.5	8.1	3.7
Arginine	3.4 (2)	5.7	2.5	4.2	4.6

It is of course obvious that this estimation takes no account of non-protein which is inert in the reaction. The above values compared with the high non-protein content of about 40 % calculated from the nitrogen content, indicate the presence of non-reactive material.

It is clear from the results that resin-column treatment and electrophoresis remove much of the carbohydrate. Even, however, in the case of the product from the former treatment which usually has a slightly higher Fe content than the latter there remained (Fig. 2) an absorption at 507–509 mμ which in terms of glucose amounted to 0.5 %.

It is of interest that about 3 % reducing sugar was found after acid hydrolysis in one of the early 0.3 % Fe content cytochrome *c* preparations of Theorell[3].

Preliminary results with 0.3 % Fe content horse-heart cytochrome *c* have established that this material also contains carbohydrate but it appears to be present in lesser amount than in the corresponding beef-heart preparations.

Although it is not known yet whether the carbohydrate or non-protein material is distributed between the three fractions (a), (b) and (c) or associated with any one exclusively, its relatively high content makes it appear unlikely that it would

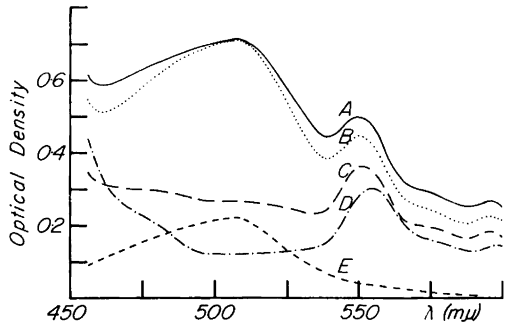


Fig. 2. Light absorption curves obtained from the complex formed by application under standard conditions, of the thymol-sulphuric acid method of carbohydrate estimation (Shetlar and Masters[13]) to the following samples: *A.* 0.3 % Fe content beef-heart cytochrome *c* (4.5 mg); *B.* 0.43 % Fe content resin-column treated beef-heart cytochrome *c* (prepared from part of the same batch used to obtain curve *A*) 2.5 mg, + 40 μg glucose; *C.* As for curve *B* without glucose addition; *D.* As for curve *C* without thymol addition; *E.* 40 μg glucose.

be restricted to the minor fraction (c). Furthermore there is some evidence that fraction (b) arises from fraction (a) during preparation. In this event the non-protein (carbohydrate) moiety would be a constituent of fraction (a) plus (b). In any case and irrespective of consideration of distribution of the non-protein (carbohydrate) component, the amino-acid analysis shows that globin from either myoglobin or haemoglobin if present at all could only be a very minor constituent of 0.3 % Fe content beef-heart cytochrome *c* or of fraction (X), which may be separated from it.

Addendum May 8, 1963: The electrophoretic mobility of beef apo-myoglobin I has recently been determined under similar conditions to those given in Table 1 above. The major apo-Mb fraction (comprising more than 70 % of the preparation) showed an electrophoretic mobility of 5.1×10^{-5} cm^2 $volt^{-1}$ sec^{-1}, thus moving at a rate approaching twice that of what is usually the main component of fraction (X).

Acknowledgements. The authors are indebted to Prof. H. Theorell for his advice and encouragement. One of us (R. W. H.) is especially grateful to Prof Theorell and his staff for their kindness during his stay at the Medicinska Nobelinstitutet when part of this work was carried out; also to the *Rockefeller Foundation* and the *National Health and Medical Research Council* (Commonwealth of Australia) for travel grants. It is a pleasure to thank Ing. A.-M. Fridén for performing quantitative aminoacid analyses. The authors wish to express their thanks for the support of this work to *Magnus Bergvalls Stiftelse* and *Statens Medicinska och Naturvetenskapliga Forskningsråd.*

REFERENCES

1. Theorell, H. and Åkeson, Å. *J. Am. Chem. Soc.* **63** (1941) 1804.
2. Theorell, H. *Biochem. Z.* **279** (1935) 463.
3. Theorell, H. *Ibid.* **285** (1936) 207.
4. Keilin, D. and Hartree, E. F. *Proc. Roy. Soc. (London)* **B 122** (1937) 298.
5. Keilin, D. and Hartree, E. F. *Biochem. J.* **39** (1945) 289.

6. Paléus, S. and Neilands, J. B. *Acta Chem. Scand.* **4** (1950) 1024.
7. Lemberg, R. and Legge, J. W. *Hematin Compounds and Bile Pigments*, Interscience, New York and London, 1949, p. 348.
8. Margoliash, E. *Biochem. J.* **56** (1954) 529.
9. Henderson, R. W. and Rawlinson, W. A. In Falk, J. E. Lemberg, M. R. and Morton, R. K. *Proceedings of the Haematin Enzyme Symposium, Canberra*, Pergamon Press, London, 1961, Pt 2, p. 370.
10. Margoliash, E. *Biochem. J.* **56** (1954) 535.
11. Moore, S., Spackman, D. H. and Stein, W. H. *Anal. Chem.* **30** (1958) 1185.
12. Kirsten, W. In Wilson, C. L. and Wilson, D. W. *Comprehensive Analytical Chemistry*, Elsevier Publishing Company, Amsterdam, 1960, Vol. 1 B, p. 486.
13. Shetlar, M. R. and Masters, Y. F. *Anal. Chem.* **29** (1957) 402.
14. Tint, H. and Reiss, W. *J. Biol. Chem.* **182** (1950) 385.
15. Longsworth, L. G. In Bier, M. *Electrophoresis Theory, Methods and Applications*, Academic Press, New York, 1959, p. 91.
16. Timmer, R., van der Helm, H. J. and Huisman, T. H. J. *Nature* **180** (1957) 239.
17. Ehrenberg, A. and Theorell, H. *Acta Chem. Scand.* **9** (1955) 1193.
18. Feigl, F. *Spot Tests in Organic Analysis*, Elsevier, Amsterdam, 1960, p. 426.

Received March 27, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 117–S 122

Species Specificity of Transfer RNA

ROBERT B. LOFTFIELD and ELIZABETH ANN EIGNER

John Collins Warren Laboratory of the Collis P. Huntington Memorial Hospital of Harvard University, Massachusetts General Hospital, Boston, Massachusets, USA

New techniques have permitted the study of the kinetics of the aminoacylation of transfer ribonucleic acid (S-RNA) by valine and isoleucine. It is observed that a single amino acid activating enzyme catalyzes the aminoacylation of S-RNA from different species at greatly different rates, but that the Michaelis constants are quite similar. This observation is confirmed by the demonstration of competition between different valine specific S-RNA's for the valine activating enzyme of *E. coli*.

We conclude that the enzyme recognition site and the code reading site of S-RNA may well be the same site and identical throughout all species. From these data there is no need to postulate separate sites or different codes. There is also no evidence of degeneracy of the code in these systems.

It is widely believed that the biosynthesis of protein proceeds through the following steps[1]:

$$AA_i + Ezy_i + ATP \rightleftharpoons Ezy_i\,(AA_i \sim AMP) + PP \quad (1)$$

$$Ezy_i\,(AA_i \sim AMP) + S\text{-}RNA_i \rightleftharpoons Ezy_i + AMP + AA_i \sim S\text{-}RNA_i \quad (2)$$

$$AA_i \sim S\text{-}RNA_i + AA_j \sim S\text{-}RNA_j \rightarrow AA_i\text{–}AA_j + S\text{-}RNA_i + S\text{-}RNA_j \text{ etc.} \quad (3)$$

where AA_i is a particular natural L-amino acid, Ezy_i is an enzyme specific for the activation of AA_i, ATP is adenosine triphosphate, $S\text{-}RNA_i$ is a molecule of transfer ribonucleic acid specific for AA_i, and AA_i–AA_j– represents the growing peptide chain. Reaction (3) is catalyzed by guanosine triphosphate[2] and microsomes together with enzymes which appear to show species specificity with regard to the microsomes but not with regard to the S-RNA[3,4]. On the other hand, Reaction (2) appears to show quite variable species specificity. Mammalian enzymes catalyze the synthesis of yeast valyl S-RNA[5] but not *E. coli* leucyl S-RNA[6]. Yeast or hog enzymes yield either yeast or hog tyrosyl S-RNA but not *E. coli* tyrosyl S-RNA while the *E. coli* enzyme cannot synthesize either yeast or hog tyrosyl S-RNA[7]. Yeast enzymes can react with only 40 % of the methionine

specific S-RNA of *E. coli*[8]. Leucine enzymes and leucine S-RNA's from various bacteria cross-react with each other but cannot cross-react with the leucine S-RNA's and leucine enzymes of yeast or mammals[4]. Finally, there is little or no cross-reaction between yeast and *E. coli* in the formation of tyrosyl or arginyl S-RNA, but there is cross-reaction in the formation of lysyl S-RNA[9].

The development of a very sensitive and accurate micro-assay for aminoacyl S-RNA in these laboratories[10] has permitted us to examine the kinetics of the synthesis of yeast and *E. coli* valyl S-RNA. We find that the heterologous enzymes and S-RNA's which superficially appear not to cross-react do, in fact, react almost as well as the homologous pairs as measured by Michaelis constants and inhibition constants.

EXPERIMENTAL

L-^{14}C-valine and L-^{14}C-isoleucine were prepared from $Ba^{14}CO_3$ by way of the Bucherer hydantoin synthesis[11]. Each was at least 99.95 % pure. ATP and CTP were purchased from Sigma Chemical Company. S-RNA was isolated from baker's yeast or *E. coli* by the method of Monier *et al.*[5] Treatment at pH 10 for 30 minutes at 37°C (to remove bound amino acids) followed by dialysis increased the acceptor ability of the S-RNA by 2- to 10-fold. Mouse ascites "S_4 fraction" was prepared according to Hecht *et al.*[12] and *E. coli* activating enzymes were prepared by modifications of the method of Bergmann *et al.*[13] The enzymes were assayed by the hydroxamate method of Loftfield and Eigner[14], one unit of activity being the ability to form 1 $\mu\mu$mole of hydroxamate per hour under saturation conditions.

Technique. Typically 150 μl 0.1 M ATP-Mg, 150 μl 1.0 M tris buffer pH 7.6, 150 μl 0.84 mM ^{14}C-valine (11.2×10^6 cpm per μmole), 50 μl 2 % bovine albumin and 25 μl enzyme solution (900 units) are combined. One hundred μl of this mixture and appropriate amounts of water are brought to 26°C in a water bath and various amounts of S-RNA solution are added to give a total volume of 325 μl. Four aliquots of 75 μl are removed at intervals from 1 to 30 min and immediately pipetted into 5 ml of cold 5 % trichloroacetic acid (TCA). After 5 min the nearly clear suspension is filtered through a No. AAWG 02400 Millipore filter (Millipore Filter Corporation, Bedford, Mass., U. S. A.). The filter disk is washed twice with 5 % TCA and dried in a 60°C oven for 15 min before being assayed in a low background end window Geiger counter (Nuclear Chicago Corporation). Under these conditions S-RNA is quantitatively recovered over a range of 1 to 200 μg and there is no significant self absorption of β-rays if the weight of the combined RNA and protein is less than 1 mg[10].

RESULTS

Fig. 1 shows that per unit of enzyme, *E. coli* valine enzyme acylates *E. coli* S-RNA about 500 times more rapidly than it does yeast S-RNA while S_4 enzyme is about 120 times more active toward yeast S-RNA than *E. coli* S-RNA. Curves E and F show that there is no measurable "fixation" of ^{14}C-valine into the *E. coli* enzyme preparation and that the amount of such "fixation" into the S_4 preparation was only 3 % of that in Curve C. Curves C and D did not achieve the level of acylation shown by Curves A and B. If S_4 enzyme is added to the incubation of Curve C at 0 or 15 min the incorporation of ^{14}C-valine quickly rises to the level of Curve B indicating that both enzymes acylate the same site of yeast S-RNA and that there has been no deterioration of the S-RNA. On the other hand, after 1 h of incubation with *E. coli* enzyme, only half of the yeast S-RNA is acylatable. This suggests that incubation with the *E. coli* enzyme results in some deterioration of yeast S-RNA which accounts for our failure to achieve maximum acylation of S-RNA by heterologous enzymes.

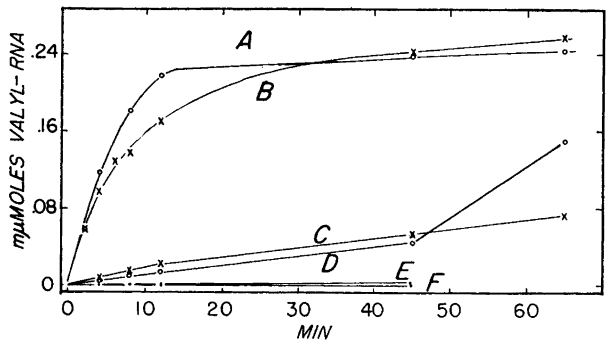


Fig. 1. Rate of formation of yeast or *E. coli* valine RNA by S_4 or *E. coli* activating enzymes. Curve A, 260 μg *E. coli* S-RNA and 190 units *E. coli* valine enzyme. Curve B, 300 μg yeast S-RNA and 610 units S_4 enzyme. Curve C, 300 μg yeast S-RNA and 3800 units of *E. coli* valine enzyme. Curve D, 260 μg *E. coli* S-RNA and 3050 units of S_4 enzyme. Curve E, 3050 units S_4 enzyme, no added RNA. Curve F, 3800 units *E. coli* valine enzyme, no added RNA. Total volume, 325 μl. At 45 min additional enzyme was added in each case. Other conditions as described under Experimental.

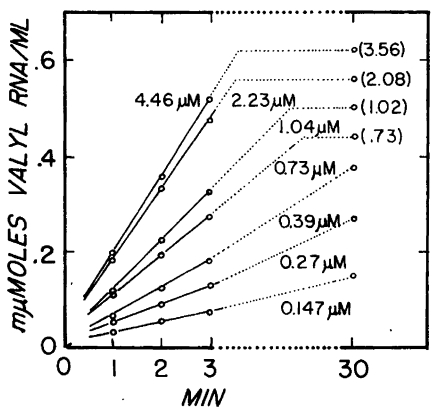


Fig. 2.

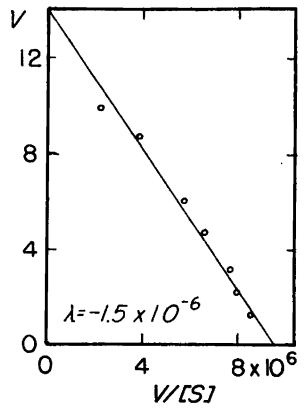


Fig. 3.

Fig. 2. Typical experiment showing RNA concentration dependence for aminoacylation of RNA. 570 units of *E. coli* valine enzyme were used, other conditions as described in the text except that the concnteration of *E. coli* S-RNA was varied from 0.103 mg/ml to 3.10 mg/ml. Based on the maximal incorporation of ^{14}C-valine observed at 50 min, this corresponded to a molarity of valine specific S-RNA from 147 mμM to 4.46 μM.

Fig. 3. Eadie plot of rate data of Fig. 2. V is expressed in mμmoles valyl S-RNA formed per h per ml.

Combinations A, C and D were inhibited by between 20 % and 60 % by the presence of CTP, phosphoenol pyruvate and pyruvate kinase, so these substances were omitted from all the subsequent experiments.

By choosing appropriate concentrations of enzyme and S-RNA, it is possible to get quite good estimates of initial rates of reaction as shown in Fig. 2 for the

reaction of *E. coli* valine S-RNA with *E. coli* valine enzyme. From the specific activity of the ^{14}C-valine (11.2 cpm per $\mu\mu$mole) and the maximal incorporation of valine into this S-RNA, one calculates that this S-RNA reacts with 1.41 mμ-moles of valine per mg. In turn, this permits the estimation of the concentration of valine specific S-RNA. Plotting the velocity of the reaction against the velocity divided by substrate concentration[15], we find in Fig. 3 a typical Michaelis-Menten relationship with a K_m of 1.5×10^{-6}. Comparable experiments indicate that *E. coli* valine S-RNA and yeast valine S-RNA react with S_4 enzyme with K_m's of about 1.3×10^{-6} and 2.5×10^{-7} respectively. Yeast valine S-RNA reacts very slowly with *E. coli* valine enzyme but a rough approximation to the K_m is 2.5×10^{-6}. The K_m for *E. coli* isoleucine S-RNA on *E. coli* isoleucine enzyme is 1.2×10^{-6}.

In every case we noted that the rate of aminoacylation decreases more rapidly than would be predicted from the progressive decrease in the amount of substrate. This is probably due to inhibition of the enzyme by the product. The rate of ^{14}C-valine addition to *E. coli* S-RNA is reduced by 20 % in the presence of a 6-fold greater concentration of ^{12}C-valine saturated S-RNA. A 10-fold excess of ^{12}C-isoleucine saturated S-RNA suppressed the reaction of ^{14}C-isoleucine by 75 %. Since similar effects were not observed for unacylated S-RNA, we consider these to be due to product inhibition.

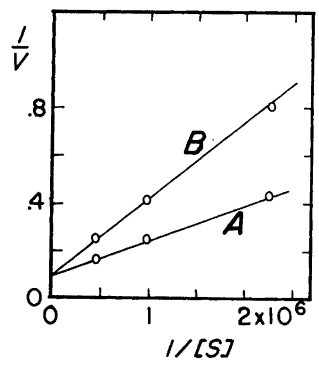


Fig. 4.

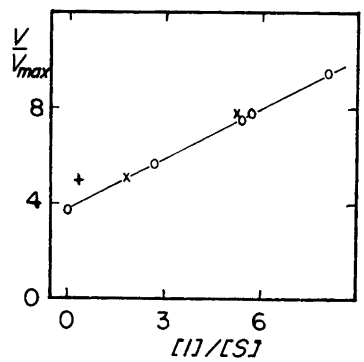


Fig. 5.

Fig. 4. Competition between yeast S-RNA and *E. coli* S-RNA for *E. coli* valine activating enzyme. Each inhibited reaction contained 600 μg of yeast S-RNA (2.78 μM of valine specific S-RNA) and all reactions included 238 units of *E. coli* valine enzyme in 325 μl of solution. Other conditions and components as described in the experimental section except that the *E. coli* S-RNA concentration was varied from 315 μg/ml to 1.57 mg/ml (0.44 μM to 2.23 μM of valine specific S-RNA). Curve A uninhibited. Curve B inhibited. $K_m = 1.5 \times 10^{-6}$, $K_i = = 2.1 \times 10^{-6}$. Units of V are mμmoles of valyl S-RNA formed per h per ml.

Fig. 5. Dependence of yeast S-RNA inhibiting ability on concentration of valine specific yeast S-RNA. Each tube contained 361 μg *E. coli* S-RNA (0.51 μM of valine specific S-RNA), 190 units of *E. coli* valine enzyme, other components as described in the text and the following yeast S-RNA's: O, 0 to 2.76 mg/ml of ordinary yeast S-RNA (0 to 4.2 μM valine acceptor S-RNA); ×, 152 and 412 μg/ml of enriched valine acceptor yeast S-RNA[16] (0.84 or 2.68 μM valine acceptor sites); or +, 3.2 mg/ml of enriched tyrosine and serine acceptor yeast S-RNA containing less than 1 % of the usual valine acceptor ability (0.08 μM valine acceptor sites).

If yeast S-RNA is capable of being aminoacylated by *E. coli* valine enzyme at a very slow rate, it would be expected that yeast S-RNA would be inhibitory to the aminoacylation of *E. coli* S-RNA. This effect is demonstrated in Fig. 4. In this plot the slope is equal to $(1 + [I]/K_i)K_s$. If the inhibitor, I, is considered to be only that part of the yeast S-RNA that will accept valine from S_4 enzyme, we may calculate K_i to be 2.1×10^{-6} in excellent agreement with the previously observed K_s (2.5×10^{-6}) for reaction of yeast valine S-RNA with *E. coli* valine enzyme.

If the usual equation for competitive inhibition is rewritten as $V_{max}/v = K_s/[S] + 1 + K_s [I]/K_i [S]$ a plot of V_{max}/v *vs.* $[I]/[S]$ at constant substrate concentration should give a straight line of slope K_s/K_i. Fig. 5 shows that several concentrations of crude yeast S-RNA conform to this equation. The evidence of Fig. 4 and 5 is consistent with valine specific yeast S-RNA being a specific competitive inhibitor of valine specific *E. coli* S-RNA. It does not exclude the possibility that several or all components of yeast S-RNA are inhibitory. In order to test this possibility, yeast S-RNA enriched 5-fold in valine acceptor capability was prepared[16] and tested for inhibitor activity. It can be seen in Fig. 5 that the inhibitory activity was exactly proportional to the extent of enrichment in valine acceptor capability. It should however be noted that a massive concentration (3 mg/ml) of yeast S-RNA enriched in tyrosine and serine acceptor capability and depleted in valine activity[17] showed more inhibition than would have been predicted. At this high concentration of S-RNA there may be non-specific effects. Had the inhibition been due equally to all S-RNA molecules it should have been about 70 % at this concentration instead of only 25 %.

DISCUSSION

If previously available methods had been used, we would surely have concluded that *E. coli* valine enzyme was inactive toward yeast S-RNA. From this, according to the absence of species specificity in Reaction (3), we might have concluded that the "code reading" area of the S-RNA is universal while the "activating enzyme recognition" site varies from species to species, hence that the two functions of S-RNA are distinct.

The present data argue to the contrary. From the relative Michaelis constants, the inhibition constants, and the data of Fig. 5, it is clear that valine specific yeast S-RNA associates with *E. coli* valine enzyme very nearly as well as *E. coli* S-RNA does. The great difference between these two S-RNA's is in the rate of reaction of the enzyme-substrate complex to yield valyl S-RNA. This difference in reaction rates is unquestionably a consequence of structural differences, but these are not necessarily or even likely in the enzyme recognition area. It is therefore not necessary to postulate that the "code reading" and "enzyme recognition" sites are distinct in S-RNA.

It should be observed that there is no evidence for degeneracy of the S-RNA in these experiments. Our techniques would have disclosed the presence of two different yeast or *E. coli* valine S-RNA's if they were present in similar amounts and had K_m's differing by only a factor of two.

Acknowledgments. We are indebted to Paul Zamecnik and Mary Louise Stephenson for a sample of valine-specific yeast S-RNA, to Jesse Scott and Peter Bergquist for a sample of

tyrosine + serine specific yeast S-RNA and to Marvin Lamborg for samples of *E. coli* S-RNA and for assistance in culturing *E. coli*. The work has been supported by United States Public Health Grant CAO2387(09). This is publication No. 1111 of the Cancer Commission of Harvard University.

Många, många goda år tillönskas Professor och fru Theorell av Robert Loftfield, som hade livets bästa år hos Professor Theorell året 1952–1953.

REFERENCES

1. Zamecnik, P. C. *Biochem. J.* **85** (1962) 257.
2. Keller, E. B. and Zamecnik, P. C. *J. Biol. Chem.* **221** (1956) 45.
3. Nathans, D. and Lipmann, F. *Proc. Natl. Acad. Sci. U. S.* **47** (1961) 497.
4. Rendi, R. and Ochoa, S. *J. Biol. Chem.* **237** (1962) 3707.
5. Monier, R., Stephenson, M. L. and Zamecnik, P. C. *Biochim. Biophys. Acta* **43** (1960) 1.
6. Zillig, W., Schachtschnabel, D. and Krone, W. Z. *Physiol. Chem.* **318** (1960) 100.
7. Clark, J. M. and Eyzaguirre, J. P. *J. Biol. Chem.* **237** (1962) 3698.
8. Berg, P., Bergmann, F. H., Offengand, E. J. and Dieckmann, M. *J. Biol. Chem.* **236** (1961) 1726.
9. Benzer, S. and Weisblum, B. *Proc. Natl. Acad. Sci. U. S.* **47** (1961) 1149.
10. Scott, J. F. *To be published.*
11. Loftfield, R. B. *Submitted to Biochemistry* **2** (1963).
12. Hecht, L. I., Zamecnik, P. C., Stephenson, M. L. and Scott, J. F. *J. Biol. Chem.* **233** (1958) 954.
13. Bergmann, F. H., Berg, P. and Dieckmann, M. *J. Biol. Chem.* **236** (1961) 1735.
14. Loftfield, R. B. and Eigner, E. A. *Biochim. Biophys. Acta. In press.*
15. Eadie, G. S. *Science* **116** (1952) 688.
16. Stephenson, M. L. and Zamecnik, P. C. *Biochem. Biophys. Res. Commun* **7** (1962) 91.
17. Tanaka, K., Richards, H. H. and Cantoni, G. L. *Biochim. Biophys. Acta* **61** (1962) 846.

Received March 30, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 123–S 128

Purification and Charaterization of Succinyl CoA Synthetase from *Rhodopseudomonas spheroides**

BRUCE F. BURNHAM

Charles F. Kettering Research Laboratory, Yellow Springs, Ohio, USA

Succinyl CoA synthetase has been purified 200 fold from the photosynthetic bacterium *Rhodopseudomonas spheroides*. Some of the properties of the purified enzyme are described. The relative ease of preparation of this enzyme and the high activity obtained suggest that succinyl CoA synthetase may have some value in coupled enzyme reactions as a generator of succinyl CoA.

The reversible, substrate level phosphorylation of nucleoside diphosphate is catalyzed by the enzyme succinyl CoA synthetase as shown in eqn. 1.**

$$\text{SUCCINATE} + \text{NTP} + \text{CoA} \longleftrightarrow \text{SUCCINYL} - \text{CoA} + \text{NDP} + \text{PO}_4 \quad (1)$$

The enzyme catalyzing this reaction has been called the phosphorylating enzyme (P enzyme)[1], succinic thiokinase[2], and succinyl CoA synthetase[3].

Previous work on this enzyme has centered largely on the cofactor requirements, and upon the mechanism of the phosphorylation reaction. Sanadi *et al.*[4] demonstrated that the enzyme from mammalian sources required either GTP or ITP for activity and that ATP could not replace either of these nucleotides. Kaufman and Alivisatos[5], however, found that the enzyme from spinach was specific for ATP. Kaufman[6], Hager[7], Gunsalus and Smith[8], and Mazumder *et al.*[2] have examined the mechanism of the reaction but have not yet shown definitive evidence for only one of the several postulated reaction mechanisms.

The present paper describes a simple procedure for the purification of this enzyme from *Rhodopseudomonas spheroides*, which is a fairly rich source of the enzyme. The specific activity of the crude bacterial extract is 0.18 μmoles/min/mg protein compared to 7.8×10^{-3} μmoles/min/mg protein in extracts of spinach.

---

* Contribution No. 106 of the Charles F. Kettering Research Laboratory.

** Abbreviations used: NTP, nucleoside triphosphate; ATP, Adenosine triphosphate; GTP, Guanosine triphosphate; ITP, Inosine triphosphate; UTP, Uridine triphosphate; CTP, Cytosine triphosphate; CoA, Coenzyme A; DEAE, Diethylaminoethylcellulose.

The enzyme from *R. spheroides* is relatively stable both in the crude extracts and in its most highly purified form. Because of the relative abundance of the enzyme, its stability, and its ease of preparation, it offers some promise as a method for producing succinyl CoA in reaction systems requiring this compound as a substrate. In studies on the enzyme δ-aminolevulinic acid synthetase, which requires succinyl CoA and glycine as substrates, Burnham and Lascelles[9] used succinyl CoA synthetase to generate succinyl CoA in the ALA synthetase assay mixture using catalytic amounts of CoA. Rowbury also used succinyl CoA synthetase as a generator of succinyl CoA in his studies on cystathionine synthesis by *E. coli* extracts (R. J. Rowbury personal communication).

EXPERIMENTAL

Chemicals: Nucleoside di and tri phosphates, Coenzyme A, and DEAE cellulose were purchased from Sigma Chemical Company, St. Louis Missouri. The DEAE cellulose was treated as described by Peterson and Sober[10] before use. Calcium phosphate gel was prepared according to the method of Keilin and Hartree[11].

Organism: Rhodopseudomonas spheroides was obtained from June Lascelles (Oxford). The organism was cultured anaerobically in the light on medium S as described by Lacelles[12]. After growth for 24 hours, the cells were harvested by centrifugation. They were resuspended in distilled water and stored at − 20°C until used.

Determinations: Enzyme activity was determined by incubating the enzyme for 30 min at 37°C in the following mixture: (μmoles) succinate 100, $MgCl_2$ 10, ATP 4, CoA 0.27, tris buffer (pH 7.4) 50, NH_2OH (freshly adjusted to pH 7.4) 200, enzyme and water to 1.0 ml. The incubation was terminated by adding 1 ml of $FeCl_3$ reagent[13]. The ferric hydroxamine acid was determined by measuring the optical density at 540 mμ. Succinic hydroxamate formation was proportional to enzyme concentration only at very low enzyme levels, *e. g.*, only at concentrations that produced 40 mμmoles of succinic hydroxamate/min or less. Protein was determined by the method of Lowery[14] except following DEAE chromatography. The method of Warburg and Christian[15] was used on fractions from the DEAE column.

Purification of enzyme: Three hundred eighty ml of resuspended cells (100 mg dry wt cells/ml) were thawed and allowed to remain at 2°C for 24 hrs. This suspension was then passed once through a French pressure cell (American Instrument Co., Silver Spring, Md.). Distilled water was added to the disrupted cells to bring the volume up to the equivalent of 50 mg dry wt cells/ml. This viscous preparation was sonicated in 50 ml batches for 1 to 2 min to denature the nucleic acid. The pooled crude cell extract was carefully adjusted to pH 6.2 with molar acetic acid and was then centrifuged at 34 000 × g for 30 min in a Servall Model RC-2 refrigerated centrifuge at 2°C. The total volume of crude supernatant was 670 ml with a protein concentration of 22.4 mg/ml.

Step 1. Protamine sulfate treatment: Freshly prepared 2 % protamine sulfate (maintained at room temperature) was slowly added with thorough mixing to the crude enzyme supernatant. At intervals, (after adding 60 ml samples) the precipitate was removed by centrifugation. Addition of protamine sulfate was continued until the supernatant was clear and free of all pigmented material except cytochrome *c*. Two hundred ml of 2 % protamine sulfate were required for this step. The volume following the final protamine sulfate addition and centrifugation was 730 ml, protein concentration 5 mg/ml. This solution of enzyme was dialyzed with stirring against 4 l of distilled water for 2 hrs, and then overnight against 4 l of 0.015 M PO_4-succinate buffer pH 6.2. The precipitate which formed during dialysis was removed by centrifugation; final volume 750 ml, protein 4.2 mg/ml.

Step 2. Calcium phosphate gel adsorption: Calcium phosphate gel, 27 mg dry wt/ml, was added batchwise to the dialyzed enzyme preparation in the following amounts: 400 mg, 400 mg, 1 350 mg, 675 mg. After each addition the gel was equilibrated 10 min and removed by centrifugation. The first two additions of gel did not result in a significant decrease in the enzyme in the supernatant. The bulk of the enzyme was removed by the third addition of gel. This gel fraction, containing about 90 % of the total enzyme was washed two times with distilled water, and was then equilibrated for 15 min with the following series of eluents: (1) 20 ml 0.015 M PO_4 − succinate buffer pH 6.2, (2) 20 ml 0.03 M PO_4 − succinate buffer

Table 1. Summary of purification.

Step	Volume ml	Protein mg/ml	Specific activity μmole/min/mg protein	Total activity
Uncentrifuged sonicate	720	34	0.180	4 500
Centrifuged sonicate	670	22.4	0.28	4 250
Protamine sulfate treated extract	730	5	1.0	3 750
Combined active $CaPO_4$ gel eluents	73	5.5	3.75	1 490
Off DEAE column	27	0.45	36.2	1 570
Off DEAE column	126	ca 0.4	16.5	

pH 6.2, (3) 17 ml 0.03 M PO_4 – succinate pH 6.2 + 2 ml M KCl + 1 ml substrate mixture (see methods), (4) repeat of number 3, (5) through (10) – successive 15 ml aliquots of 0.2 M PO_4 buffer pH 6.8. The gel was resuspended in the eluting buffers by 15 seconds sonication with a probe sonicator. Elution was continued as long as enzyme continued to be desorbed. Those fractions with a specific activity above 3 μmoles/min/mg protein were pooled and dialyzed against 3 changes of 0.01 M tris buffer pH 7.4 for 24 hrs. The precipitate which formed upon dialysis was removed by centrifugation.

Step 3. DEAE Chromatography: A 2 × 16 cm DEAE column was prepared from washed and sized DEAE, equilibrated with 0.01 M tris buffer pH 7.4. The clear pale pink supernatant, (86 ml, protein concentration 3.1 mg/ml) was placed on the column which was then washed with 100 ml of additional 0.01 M tris buffer pH 7.4. The enzyme was eluted from the column with a linear gradient of KCl by increasing the concentration from zero to 0.25 M. The enzyme appeared as a broad band when the KCl concentration reached 0.14 M. All fractions with a specific activity of over 16 μmoles/min/mg were frozen and stored at –20°C for use in the studies on enzyme properties described below. The purification procedure is summarized in Table 1.

RESULTS

Requirements for activity: The enzyme was incubated with the standard assay mixture in a series where single components were omitted. All components of

Table 2. Requirements for enzyme activity. Enzyme was incubated under standard conditions in a series of reactions where a single component was omitted.

Compound omitted	Succinic hydroxamate formed μmoles/min/mg protein
None	36.2
Succinate	0
CoA	0
ATP	0
NH_2OH	0
Mg	0
Minus Mg plus Mn*	27.4
Minus Mg plus Zn**	0

* $MnSO_4$ – Concentration in reaction 5 μmoles/ml.
** $ZnSO_4$ – Concentration in reaction 10 μmoles/ml.

Table 3. Substrate specificity. Succinyl COA synthetase was incubated with 0.1 M acids as shown with the standard assay system.

Substrate	Hydroxamic acid μmoles/min/mg protein
Succinate	33
Malonate	0
Acetate	0
Fumarate	Trace
DL-Malate	0
L-Aspartate	0

the reaction mixture were necessary for activity (Table 2). Manganese, but not zinc could replace magnesium.

Substrate specificity: The enzyme was tested for activity with a number of acids similar in structure to succinate. These compounds were tested (100 μmoles/ml) by substituting them for succinate in the standard assay mixture and incubating them with 4 times the usual amount of enzyme. Succinate was the only compound tested that showed measurable activity (Table 3). The trace of activity observed with fumarate, too low to measure accurately, could very likely have been due to a slight contamination with succinate.

Cofactor requirements: The nucleoside triphosphate requirement of succinyl CoA synthetase was examined using a modification of the usual assay mixture. The results (Table 4) showed that the purine nucleotides were all active, while the two pyrimidine compounds were not active. The activity of the nucleotides was measured at a series of concentrations, so that activity due to contamination

Table 4. Nucleotide specificity. Succinyl CoA synthetase was incubated in standard assay system with the various nucleotides indicated.

Nucleotide	Concentration μmoles/ml	Activity μmoles/min/mg protein	Relative activity
ATP	4	19	100
GTP	4	16	84
UTP	4	0	
ITP	4	14.5	76
CTP	4	0	
ADP	4	0	
ATP	1	11.2	100
GTP	1	10	89
ITP	1	8	72

Table 5. Stability at 37°C at various pH values. Enzyme with a specific activity of 20 μmoles/min/mg protein was added to 0.2 M imidazole buffer at the pH values shown. Volume of each sample = 0.4 ml, protein concentration 0.114 mg/ml. Incubation was at 37°C and at the times shown 0.02 ml samples were withdrawn for activity determination.

pH of incubation	Specific activity μmoles/min/mg protein 0 hrs incubation	4 hrs incubation	7 hrs incubation
6.2	20	14.4	10.6
6.6	20	14.8	14.3
7.0	20	18	18
7.4	20	16.8	11.7
7.8	20	12.7	9.5

by one of the other nucleotides would be apparent. In the case of all of the active compounds, the ratios of activity at saturating concentrations of nucleotide were close to the ratios at concentrations less than K_m (Table 6). The activity of the 3 purine nucleotides was also examined at two stages during purification. The relative rates following the calcium phosphate gel step were: ATP 100, GTP 71, and ITP 76. These values compare favorably with those obtained using enzyme that had been chromatographed on DEAE cellulose (Table 4). Succinyl CoA synthetase from *R. spheroides* is apparently less specific in its nucleotide requirement than succinyl CoA synthetase from spinach[5] or pig liver[2]. The nucleotide specificity of succinyl CoA synthetase from *E. coli* has not been reported[3].

The comparison of relative activities of the different nucleotides at two stages in purification, indicates that the activity of the nucleotides is not due to the presence of a nucleotide transphosphorylase.

pH Stability: The stability of the enzyme was examined following DEAE chromatography. For this purpose, the enzyme solution was diluted to a con-

Table 6. Michaelis constants. Activity was measured in the standard assay system while the concentration of a single component was varied. K_m values were calculated using the double reciprocal plot of Lineweaver and Burk[16].

Substrate	K_m moles/l
Succinate	1.9×10^{-3}
CoA	0.4×10^{-4}
ATP	1.4×10^{-3}
Mg++	6.4×10^{-3}

centration of 0.114 mg/ml in 0.2 M imidazole buffer at several pH values between 6.2 and 7.8. The tubes containing the diluted enzyme were incubated at 37°C. At intervals, samples were removed and tested for activity. As shown in Table 5 the enzyme is comparatively stable for several hours under these conditions.

Determination of Michaelis constants: In measurements of activity for the purpose of K_m determination, standard assay concentrations were used with the exception of the compound being tested. The values of K_m were obtained using the double reciprocal plot of Lineweaver and Burk[16]. Straight lines were obtained in all cases except with Mg^{++}. The skewed curve observed at high Mg^{++} (2×10^{-2} M) concentrations suggested substrate inhibition. The value given in Table 6 for Mg^{++} was obtained by extrapolation of the "best straight line" obtained at low Mg^{++} concentrations. Half maximum activity was observed with 2×10^{-3} M Mg^{++}.

REFERENCES

1. Kaufman, S., Gilvarg, C., Cori, O. and Ochoa, S. *J. Biol. Chem.* **203** (1953) 869.
2. Mazumder, R., Sanadi, D. R. and Rodwell, V. W. *J. Biol. Chem.* **235** (1960) 2546.
3. Hager, L. P. In Boyer, P. D., Lardy, H. and Myrbäck, K. *The Enzymes*, 2nd Ed., Academic Press, New York, 1962.
4. Sanadi, D. R., Gibson, D. M., Ayengar, P. and Jacob, M. *J. Biol. Chem.* **218** (1956) 505.
5. Kaufman, S. and Alivisatos, S. G. A. *J. Biol. Chem.* **216** (1955) 141.
6. Kaufman, S. *J. Biol. Chem.* **216** (1955) 153.
7. Hager, L. P. *J. Am. Chem. Soc.* **79** (1957) 4864.
8. Gunsalus, I. C. and Smith, R. A. *Proceedings of the International Symposium on Enzyme Chemistry, Tokyo and Kyoto, 1957.* Academic Press, New York, p. 77.
9. Burnham, B. F. and Lascelles, J. *Biochem. J.* **87** (1963). *In press.*
10. Peterson, E. A. and Sober, H. A. In Colowick, S. P. and Kaplan, N. O. *Methods in Enzymology.* Academic Press, New York, 1962, Vol. V, p. 3.
11. Keilin, D. and Hartree, E. F. *Proc. Roy. Soc., London* **B 124** (1938) 397.
12. Lascelles, J. *Biochem. J.* **62** (1956) 78.
13. Lipmann, F. and Tuttle, L. C. *J. Biol. Chem.* **159** (1945) 21.
14. Lowry, O. H., Rosenbrough, N. J., Farr, R. L. and Randall, R. J. *J. Biol. Chem.* **193** (1951) 265.
15. Warburg, O. and Christian, W. *Biochem. Z.* **310** (1941) 384.
16. Lineweaver, H. and Burk, D. *J. Am. Chem. Soc.* **56** (1934) 658.

Received April 2, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 129–S 134

The Citrate Condensing Enzyme of Pigeon Breast Muscle and Moth Flight Muscle*

PAUL A. SRERE, HAROLD BRAZIL and LEA GONEN

Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan, USA

Citrate condensing enzyme has been purified from pigeon breast muscle and from moth (*Cecropia* and *Cynthia*) flight muscle. The preparations were homogeneous proteins as judged by ultracentrifugal data and starch gel electrophoresis. The kinetic behavior of the two preparations was studied using a newly developed assay for citrate condensing enzyme.

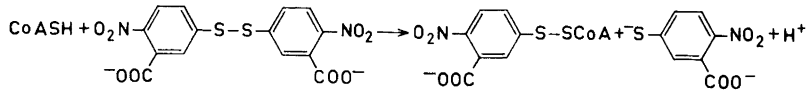
The study of the activity-structure relationship of a number of proteins has been aided by the isolation from various sources, of pure proteins having apparently identical functions. Theorell, his colleagues, and others[1] have studied the structure of cytochrome *c* from several sources and have found similarities in the structure of the peptide surrounding the point of attachment of the heme residue. The amino acid sequences for the same hormone from a number of sources have been determined and compared. From such studies the structure of the active site of these hormones has been deduced[2,3]. More information is now available for some hormones because chemical variants can be synthesized and their activities compared.

Aldolase[4], malate dehydrogenase[5] and phosphorylase[6] are examples of enzymes which have been purified from a number of sources and whose behavior and structure have been rather extensively studied. Though the citrate condensing enzyme occurs in high concentrations in a number of biological materials it has been highly purified only from pig heart tissue[7]. In our recent studies on citrate condensing enzyme[8] we had cause to compare the enzyme from a number of sources. This paper presents the procedures for the purification of citrate condensing enzyme from pigeon breast muscle and moth (*Cecropia* and *Cynthia*) flight muscle. We will report, in addition, on the sedimentation behavior and kinetic behavior of these proteins and a new method for the assay of citrate condensing enzyme.

* Supported by a grant from the *United States Public Health Service* (HE-05328).

METHODS AND MATERIALS

The assay in which citrate condensing enzyme is coupled to malate dehydrogenase, as described by Ochoa[9], was used as the routine assay during purification. This assay measures the appearance of NADH at 340 mμ. In the assay of the moth muscle enzyme it was necessary to include 0.5 μmole of NaCN to inhibit a NADH oxidase that is present in early fractions. In addition to assays that have already been described[10], a new assay for citrate condensing enzyme has been developed. It is based on the reaction of CoASH with Ellman's[11] reagent 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). When a sulfhydryl compound such as CoASH reacts with this compound the following reaction occurs:



The mercaptide ion absorbs light at 412 mμ and has a molar absorbancy index of 13 600. Thus if acetyl CoA and oxalacetate react in the presence of citrate condensing enzyme and DTNB the rate of CoASH formation can be followed spectrophotometrically at 412 mμ. This assay has several advantages over earlier ones in that it is more sensitive, can be followed in a region where the other components do not absorb light and a wider concentration range of substrates can thus be tested in kinetic experiments on the enzyme. At low enzyme concentrations a slight lag period is observed (15 sec) in this reaction.

Our results (Table 1) show that under the conditions of the assay the rate of change of absorption at 412 mμ is proportional to enzyme concentration and

Table 1. Characteristics of the DTNB assay for citrate condensing enzyme. Except where that component is the variable, reaction mixtures were 0.2 M Tris-HCl pH 8.1, 1×10^{-4} M DTNB, 4.7×10^{-5} M acetyl CoA, 2.3×10^{-4} M oxalacetate and pig heart enzyme sufficient to give the indicated rates. Total volume was 1.0 ml in 1.0 cm light path cells. All rates are corrected to 25°C.

Condensing enzyme μg	ΔA/min 412 mμ
0.4	0.057
0.8	0.111
1.2	0.155
1.6	0.203
2.0	0.254

DTNB M × 10⁵	ΔA/min 412 mμ
1	0.061
2	0.119
3	0.153
5	0.178
8	0.177
10	0.178
200	0.178

Acetyl CoA determination μmoles/ml

μl Solution assayed	412 mμ	ml Solution assayed	233 mμ
20	4.5	0.1	5.7
30	4.7	0.2	5.3
50	4.7	0.4	4.9
100	4.7		

Oxalacetate determination μmoles/ml

412 mμ	Malate dehydrogenase 340 mμ
103	105

that change of DTNB concentration from 5×10^{-5} M to 2×10^{-3} M does not affect the rate of reaction. If the coupled enzyme assay of Ochoa *et al.*[9] is followed at 355 mμ (an isosbestic point for the DTNB change) no effect of the rate of the reaction is observed. If enzyme is titrated with DTNB or with other SH binding reagents, no effect on the rate of reaction is observed. Neither DTNB or 2-nitro-5-mercaptide benzoate reacts with acetyl CoA under the conditions of the assay. However, the initial velocities measured with the DTNB assay are from 10–30 % lower than assays which measure the disappearance of acetyl CoA and oxalacetate at 233 mμ when only the reaction components are present. The reaction rate at 233 mμ is non-linear for the whole reaction course while the DTNB assay gives a relatively long period of linear reaction rate. No explanation can be given at present for these discrepancies.

Kinetic measurements were performed using a Beckman DU monochromator, a Gilford optical density converter and a Minneapolis Honeywell recorder with a chart speed of 8 in/min. The cell compartments were thermostated and triplicate determinations were run at all concentrations. Acetyl CoA and oxalacetate concentrations were determined using the DTNB assay with limiting amounts of the component being assayed and an excess of other reaction components. Enzyme concentration was determined each day by assaying the preparation at high concentrations of acetyl CoA and oxalacetate. Temperature of the reaction mixture was determined at the end of each assay and corrections were applied assuming that the temperature dependence of the moth muscle and pigeon breast enzymes were the same as that for the pig heart enzyme[10]. Temperature corrections were routinely less than ± 10 %.

Sedimentation studies were carried out in a Spinco Model E centrifuge. Starch gel electrophoresis was performed as described earlier[12]. Protein was determined using the method of Warburg[13] or Lowry *et al.*[14]

Thoraces of the moths (*Cynthia* and *Cecropia*) were kindly supplied by Dr. David Shappirio, Department of Zoology, The University of Michigan. These were assumed to be essentially all flight muscle and no further disections were performed.

Purification of pigeon breast muscle citrate condensing enzyme. The method used for the purification of this enzyme was identical to the method we have described previously for the pig heart enzyme. The only difference in behavior that was observed was during the

Table 2. Purification of citrate condensing enzyme from pigeon breast muscle. The top half of the table represents a typical purification from 100 g of pigeon breast muscle. The bottom half of the table represents a purification where the elutes from three separate 100 g runs were combined and purified together. One unit of activity is one μmole of NADH formed per min in the coupled malate dehydrogenase assay. Specific activity are units per mg protein.

	Total activity (units)	Specific activity
KCl-EtOH Extract	2 900	0.08
50–70 % Ammonium sulfate	2 600	0.58
DEAE Eluate	1 540	–
Combined calcium phosphate eluate	4 750	8.9
70 % Ammonium sulfate ppt.	4 250	16.1
1st X's	2 420	30
Residue	900	10
2nd X's	1 750	50

Table 3. Purification of citrate condensing enzyme from moth *(Cynthia)* flight muscle. See Table 1 for units.

	Total Activity (units)	Specific Activity
H_2O Extract	4 200	0.83*
DEAE Eluate	3 500	2.1*
Calcium phosphate eluate	3 850	8.9
80 % Ammonium sulfate ppt.	2 580	15
Crystals		40†

† The crystals reported here represent the reprecipitation of a number of ammonium sulfate precipitates. A 30–50 % yield in the crystallization step is obtained.
* These extracts have materials which absorb strongly at 260 mμ so that Lowry's method is used for protein determination at these steps.

DEAE cellulose step. The pigeon breast enzyme is eluted with 0.008 M potassium phosphate buffer (pH 7.4), while the pig heart enzyme is not eluted until 0.018 M potassium phosphate buffer (pH 7.4). A summary of a typical purification is shown in Table 2.

Purification of moth flight muscle citrate condensing enzyme. Fifty grams of thoraces are homogenized for 2 min in 500 ml of ice cold water using a Waring blendor. The homogenate is centrifuged for 15 min at 20 000 g. The supernatant fluid is poured through cheese cloth. 200 gm (wet weight) of DEAE cellulose (equilibrated with 0.002 M potassium phosphate pH 7.4) are taken up in 500 ml H_2O and added to the extract. The purification of condensing enzyme then follows the procedure outlined for the pig heart enzyme except in the last ammonium sulfate step. Where the pig heart enzyme is recovered with 70 % ammonium sulfate saturation it is necessary to have 80 % ammonium sulfate saturation at pH 6.5 in order to precipitate all of the moth enzyme. A summary of the purification procedure is shown in Table 3.

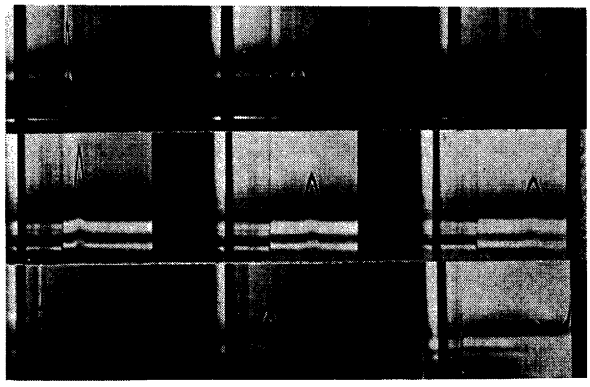


Fig. 1. Sedimentation of pig heart (top 2, 31 and 71 minutes), pigeon breast (middle 20, 52 and 68 minutes) and moth flight muscle (bottom 1, 17 and 76 minutes) citrate condensing enzymes. 59 780 RPM, 20°C, 0.05 M potassium phosphate pH 7.4.

RESULTS

Purity of the enzymes. Fig. 1 shows photographs of the sedimentation of the three citrate condensing enzymes. Only one symmetrical peak is seen in each case. The sedimentation coefficient ($s_{20,w}$) in Svedberg units, at infinite dilution was 4.5 for the moth enzyme, 6.2 for the pigeon enzyme and 6.1 for the pig enzyme. The final ammonium sulfate precipitation of these enzymes look "crystalline"; that is as the protein precipitates the solutions take on a characteristic silkiness or sheen that has been associated in the crystallization of other enzymes. We have examined these proteins by starch gel electrophoresis and when one-half of the gel is developed for protein staining material only a single band is visible in both cases. When the region of starch corresponding to the protein band is eluted from the other half of the starch gel, enzyme activity could be demonstrated. No difference was noted in the purification of the enzyme from the species *Cecropia* and *Cynthia.*

Kinetic studies. Figures 2 and 3 show Lineweaver-Burk plots for acetyl CoA at several oxalacetate concentrations for each enzyme. The apparent K_m of acetyl CoA in the case of the pigeon breast enzyme is 1.8×10^{-4} M and that for acetyl CoA for the moth flight muscle is 2.0×10^{-4} M. Insufficient data is available at present for an estimate of the apparent K_m's of oxalacetate. However, they seem to be of the magnitude of 10^{-6} M for the pigeon breast enzyme and 10^{-5} M for the moth muscle enzyme. The nature of the kinetics with the pigeon breast enzyme indicates that K_m and the association constant (K_s) for acetyl CoA may be equal[15], whereas this it not the case with the moth enzyme or the pig heart enzyme.

The kinetic behaviour of the pig heart enzyme was also determined using the new assay described here. The slopes of lines for $1/V_0$ *vs* 1/acetyl CoA at several

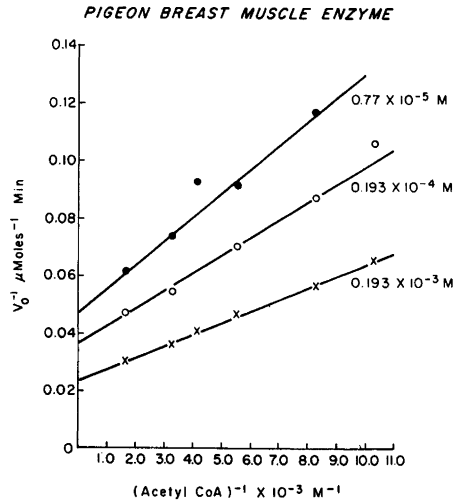


Fig. 2. Reaction mixtures contained acetyl CoA and oxalacetate at the concentrations noted and 0.2 M Tris-HCl pH 8.1 and 1×10^{-4} M DTNB. The reaction was initiated by the addition of enzyme and all velocities are reported on the basis of 0.34 mg of pure enzyme.

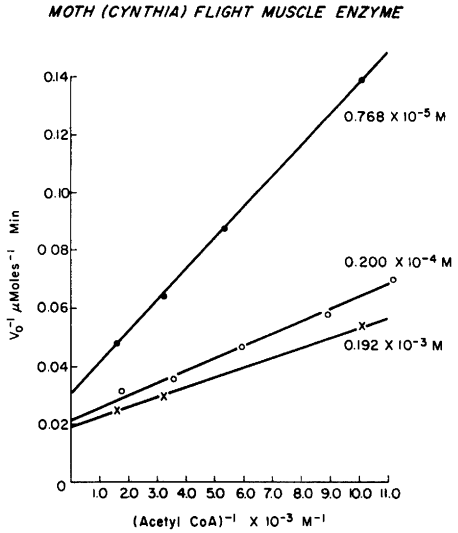


Fig. 3. See legend to Fig. 2.

oxalacetate concentrations were similar to our previous results in that they were almost equal. However, in the 233 mμ assay where the slopes decreased slightly with decreasing oxalacetate concentration, our results with the 412 mμ assay showed a very slight increase in slope with decreasing oxalacetate concentration. Since the 233 mμ assay is a less accurate one at low reaction rates we are inclined to place more reliance on our present results. In any case, the kinetic analysis for each of the three enzymes seems to be sufficiently different so that a common kinetic pathway seems unlikely.

REFERENCES

1. Paul, K. G. In Boyer, P. D., Lardy, H. and Myrbäck, K. *The Enzymes*, Academic Press Inc., New York, 1960, Vol. 3, p. 277.
2. Lerner, A. B. and Lee, T. H. *Vitamines and Hormones* **20** (1962) 337.
3. Li, C. H. *Vitamines and Hormones* **19** (1961) 313.
4. Rutter, W. In Boyer, P. D., Lardy, H. and Myrbäck, K. *The Enzymes*, Academic Press Inc., New York, 1961, Vol. 5, p. 341.
5. Thorne, C. J. R. *Biochim. Biophys. Acta* **59** (1962) 624.
6. Brown, D. H. and Cori, C. F. In Boyer, P. D., Lardy, H. and Myrbäck, K. *The Enzymes*, Academic Press Inc., New York, 1961, Vol. 5, p. 207.
7. Ochoa, S., Stern, J. R. and Schneider, M. C. *J. Biol. Chem.* **193** (1951) 691.
8. Broder, I. and Srere, P. A. *Biochim. Biophys. Acta* **67** (1963) 617.
9. Ochoa, S. In Shemin, D. (Ed.) *Biochemical Preparations*, John Wiley and Sons, Inc., New York, 1957, Vol. 5, p. 19.
10. Srere, P. A. and Kosicki, G. W. *J. Biol. Chem.* **236** (1961) 2559.
11. Ellman, G. *Arch. Biochem. Biophys.* **82** (1959) 70.
12. Broder, I. and Srere, P. A. *Biochim. Biophys. Acta* **67** (1963) 626.
13. Warburg, O. and Christian, W. *Biochem. Z.* **310** (1941–42) 384.
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* **193** (1951) 265.
15. Frieden, C. *J. Am. Chem. Soc.* **79** (1957) 1894.

Received March 26, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 135 – S 139

Site of Action of Plastoquinone in the Electron Transport Chain of Photosynthesis

DANIEL I. ARNON* and A. A. HORTON**

Department of Cell Physiology, University of California, Berkeley, USA

Plastoquinone (2,3-dimethyl-5-solanesyl-benzoquinone), a natural component of chloroplasts, was found to be required for the photoreduction of triphosphopyridine nucleotide (TPN) by isolated chloroplasts but only when this photoreduction was accompanied by oxygen evolution, *i. e.* when water (OH^-) was the electron donor. When experimental conditions were so arranged that ascorbate plus 2,6-dichlorophenol indophenol replaced OH^- as the electron donor system, the photoreduction of TPN was no longer dependent on the presence of plastoquinone in chloroplasts.

The requirement for plastoquinone was established when its extraction from chloroplasts resulted in a loss, and its addition to extracted chloroplasts resulted in a recovery, of photochemical activity. Ubiquinone, tocopherylquinone and menadione were not substitutes for plastoquinone.

The role of plastoquinone as an electron carrier in the photochemical reactions of photosynthesis is discussed.

Attention to a possible role of quinones in photosynthesis was first directed by the finding of Dam[1,2] that a substituted naphthoquinone, vitamin K_1, is localized in chloroplasts – a finding which was recently confirmed by Kegel and Crane[3]. In addition to naphtoquinones, green tissues also contain substituted benzoquinones, the first of which, 2,3-dimethyl-5-solanesyl-benzoquinone, was isolated by Kofler[4], named plastoquinone by Crane[5] and shown by him to be localized in chloroplasts.

Bishop[6] and Krogmann[7] have shown that plastoquinone ("Q-255") is required for the photoreduction of ferricyanide or 2,6-dichlorophenol indophenol by isolated chloroplasts. This article presents evidence that plastoquinone is required for the photoreduction of triphosphopyridine nucleotide (TPN) by isolated chloroplasts, but only when this photoreduction is accompanied by oxygen evolution, *i. e.* when OH^- is the electron donor***. The requirement for plastoquinone in the photoreduction of TPN was no longer apparent when ascorbate plus indo-

* Aided by grants from the *National Institutes of Health* and the *Office of Naval Research.*

** Present address: Department of Biochemistry, University of Leicester, Leicester, England.

*** OH^- represents here the hydroxyl ion at neutral pH as in the reaction: $4\ OH^-\ (10^{-7}\ M) \rightarrow O_2 + 2H_2O + 4e^-$; $E_0' = 0.815$ V (pH 7).

phenol dye[8] replaced OH^- as the electron donor system. Plastoquinone thus appears to be required in that portion of the photosynthetic electron transport chain that is concerned with the photooxidation of OH^- and which has been identified by Losada, Whatley and Arnon[9] as the first of the two light reactions that jointly bring about the transfer of electrons from OH^- to TPN.

A role for plastoquinone in the photosynthetic electron transport chain is consistent with the observations of Crane *et al.*[10] and Redfearn and Friend[11] that plastoquinone in chloroplasts is reduced by illumination and reoxidized in darkness. The role of plastoquinone in photophosphorylation is discussed in the succeeding article[12].

METHODS

Chloroplasts were isolated by differential centrifugation from deribbed, fresh spinach leaves which had been blended[13] for 1.5 min. in ice-cold 0.5 M sucrose containing 1 % NaCl. The chloroplasts were lyophilized, extracted under N_2 at room temperature, in a glass homogenizer, with a spectral grade heptane[7] and dried in a current of N_2. The quinone to be added was first dissolved in spectral grade isooctane and mixed under N_2 with the extracted chloroplasts; the solvent was quickly removed *in vacuo*. Prior to use, the chloroplasts were suspended in cold 0.25 M sucrose containing 1 % NaCl. O_2 evolution was measured manometrically and TPN reduction spectrophotometrically by observing changes in optical density at 340 mμ.

The plastoquinone used in these experiments was a gift of Dr. O. Isler; the ubiquinone was a gift of Dr. D. P. Hackett.

RESULTS AND DISCUSSION

Fig. 1 shows that, on extraction of plastoquinone, chloroplasts simultaneously lost the capacity to photoreduce TPN and to photoevolve oxygen. Both were restored almost completely by adding plastoquinone to the extracted chloroplasts. (Similar results, omitted here for the sake of brevity, were obtained when TPN was replaced by ferricyanide). However, as shown in Fig. 2, plastoquinone

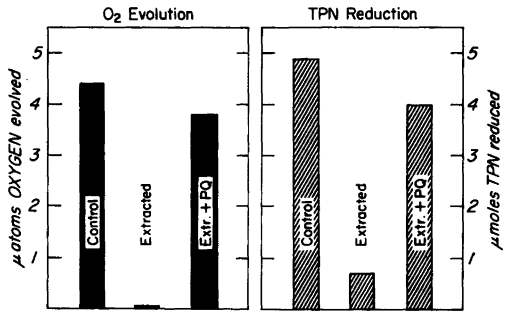


Fig. 1. Plastoquinone requirement for oxygen evolution and TPN reduction by illuminated chloroplasts. The "control" vessel received lyophilized chloroplasts, the "extracted" vessel received lyophilized chloroplasts extracted with heptane, and the "extracted + PQ" vessel received extracted lyophilized chloroplasts to which plastoquinone (PQ) had been added (0.05 mg/0.5 mg chlorophyll). The reaction mixture contained, in a total volume of 3 ml, the following, in μmoles: tris (hydroxymethyl) aminomethane buffer, pH 7.8, 80; TPN, pH 7.8, 6; $MgCl_2$, 10; ADP, 10; K_2HPO_4, 10; and partially purified chloroplast ferredoxin from spinach. Each vessel received chloroplasts containing 0.5 mg chlorophyll. The experiment was carried out in Warburg manometer vessels at 15°C in a nitrogen atmosphere. The vessels were illuminated at 25,000 lux for 15 minutes.

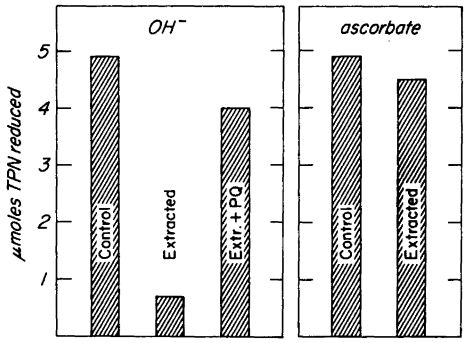


Fig. 2. Effect of plastoquinone on the photoreduction of TPN with either OH^- or ascorbate + dye as the electron donor system. The experimental conditions were as described in the legend of Fig. 1. The reaction mixture for the "OH^-" system was as described in Fig. 1; in the "ascorbate" system the following were also added (μmoles): Na ascorbate, 20; 2,6-dichlorophenolindophenol, 0.2; and *p*-chlorophenyldimethylurea (CMU), 0.2.

was not essential for the photoreduction of TPN *per se*. When oxygen evolution was inhibited by *p*-chlorophenyldimethylurea (CMU) and the ascorbate-indophenol dye couple was added as a hydrogen donor system[8,14], the photoreduction of TPN was not significantly decreased by the extraction of plastoquinone from chloroplasts.

These results are in harmony with the separation of the noncyclic electron transport in chloroplasts into two component photochemical reactions[9]: (1) a photooxidation of OH^- – a reaction which seems to be catalyzed by chlorophyll *b*[15] and which is obligatorily coupled with oxygen evolution, and (2) the photoreduction of TPN – a reaction which seems to be catalyzed by chlorophyll *a*[15] and which, under physiological conditions, depends on reaction (1) as a source of electrons. However, when reaction (1) is experimentally suppressed by the use of an effective inhibitor of oxygen evolution such as CMU, reaction (2) can proceed with an alternative electron donor, the ascorbate-indophenol dye couple.

According to this interpretation, plastoquinone acts as a link between light reactions (1) and (2) in a manner diagrammatically represented in Fig. 3. The ascorbate-indophenol dye couple reacts with the photosynthetic electron transport chain at a point subsequent to the plastoquinone site and can therefore supply electrons to TPN by a pathway (heavy line in Fig. 3) from which plastoquinone is excluded.

When TPN is the terminal electron acceptor, the photoproduction of O_2 by chloroplasts always involves the participation of both light reactions (1) and (2) and hence requires plastoquinone. But Losada, Whatley and Arnon[9] have shown that when ferricyanide replaces TPN as the terminal electron acceptor in the noncyclic electron transport, photoproduction of oxygen by isolated chloroplasts may proceed by one of two pathways: either by a "long" route, *i. e.* a collaboration between light reactions (1) and (2) or by a "short" route which involves only light reaction (1). The demonstration of light reaction (1), the photooxidation of

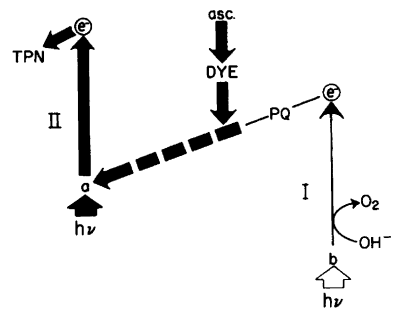


Fig. 3. A scheme for the photoreduction of TPN by ascorbate (photoreaction II). When the passage of electrons from OH^- *via* plastopuinone (PQ) to TPN is blocker (thin line), electrons from ascorbate enter the photosynthetic electron transport chain (heavy line) *via* the dye at a point past the plastoquinone site. The electrons pass to chlorophyll *a* and are then raised at the expense of light energy to a redox potential sufficient to reduce TPN. The participation of OH^- as the electron donor is prevented by the presence of CMU, a powerful inhibitor of oxygen evolution.

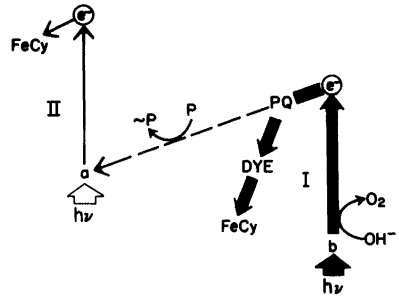


Fig. 4. A scheme to show the mechanism of photooxidation of water (photoreaction I). Electrons are donated from OH^- to the accessory pigment, chlorophyll *b*, where they are raised by absorbed light energy to a potential sufficient to reduce plastoquinone. The photooxidation of OH^- ion liberates oxygen. The reduced plastoquinone reacts, *via* the dye, with ferricyanide.

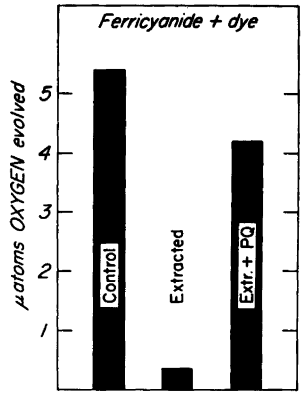


Fig. 5. Plastoquinone requirement for the photooxidation of water by isolated chloroplasts (photoreaction I). The experimental conditions were as described in the legend to Fig. 1. The reaction mixture was the same as described in Fig. 1, except that TPN and ferredoxin were omitted and 15 μmoles $K_3Fe(CN)_6$ and 0.2 μmoles 2,6-dichlorophenolindophenol were added. The vessels were illuminated for 17 min.

water, was accomplished by adding catalytic amounts of 2,6-dichlorophenol indophenol jointly with substrate amounts of ferricyanide (see Table 2 in Ref. 9).

In the light of present results, this special effect of the indophenol dye on electron transport to ferricyanide can be represented diagrammatically by Fig. 4. It is envisaged that the indophenol dye intercepts the electrons which reach plasto-

Table 1. Specificity of Plastoquinone requirement for the photoproduction of oxygen by isolated chloroplasts, with either TPN or ferricyanide-dye as the electron acceptor system. Measurement of photoreaction I was carried out as in legend to Fig. 5; measurement of photoreactions I and II as in legend to Fig. 1.

Chloroplast treatment	μ atoms oxygen evolved	
	TPN (photoreactions I and II)	dye/FeCy (photoreaction I)
Lyophilized	4.8	3.6
Extracted	1.8	0.7
Extracted + Plastoquinone	4.4	5.2
Extracted + $Ubiquinone_{50}$	1.0	1.1
Extracted + Tocopherylquinone	1.8	0.9
Extracted + Vitamin K_3	1.3	1.4

quinone from OH^- *via* the first light reaction and transfers them directly to ferricyanide by a nonenzymic reaction. This would result in a bypass of that portion of the electron transport chain which leads to the phosphorylation step and to the second light reaction (Fig. 4).

If this interpretation is correct, it would be expected that oxygen evolution in the absence of TPN but in the presence of ferricyanide plus indophenol dye (the first light reaction[9]) would also require the presence of plastoquinone. This has been experimentally verified (Fig. 5). Chloroplasts from which plastoquinone was extracted were unable to accomplish a transfer of electrons from OH^- to ferricyanide via 2,6-dichlorophenol indophenol dye but regained this ability when plastoquinone was added.

As shown in Table 1, the requirement for plastoquinone for oxygen evolution with either TPN or ferricyanide as the electron acceptor, was specific and was not replaced by vitamin K_3 (menadione), ubiquinone or tocopheryl quinone.

REFERENCES

1. Dam, H. *Adv. Enzymol.* **2** (1942) 317.
2. Dam, H., Hjorth, E. and Kruse, I. *Physiologia Plantarum* **1** (1948) 379.
3. Kegel, L. P. and Crane, F. L. *Nature* **194** (1962) 1282.
4. Kofler, M. *Festschrift E. C. Barell*, Hoffman-La Roche and Co., Basel 1946, p. 199.
5. Crane, F. L. *Plant Physiol.* **34** (1959) 128.
6. Bishop, N. I. *Proc. Natl. Acad. Sci. U. S.* **45** (1959) 1696.
7. Krogmann, D. W. *Biochem. Biophys. Res. Comm.* **4** (1961) 275.
8. Vernon, L. P. and Zaugg, W. S. *J. Biol. Chem.* **235** (1960) 2728.
9. Losada, M., Whatley, F. R. and Arnon, D. I. *Nature* **190** (1961) 606.
10. Crane, F. L., Ehrlich, B. and Kegel, L. P. *Biochem. Biophys. Res. Comm.* **3** (1960) 37.
11. Redfearn, E. R. and Friend, J. *Nature* **191** (1961) 806.
12. Whatley, F. R. and Horton, A. A. *Acta Chem. Scand.* **17** (1963) Suppl. 1, p. 140.
13. Arnon, D. I. and Whatley, F. R. *Arch. Biochem.* **23** (1949) 141.
14. Jagendorf, A. T. and Margulies, M. *Arch. Biochem. Biophys.* **90** (1960) 184.
15. Arnon, D. I., Losada, M., Whatley, F. R., Tsujimoto, H. Y., Hall, D. O. and Horton, A. A. *Proc. Natl. Acad. Sci. U. S.* **47** (1961) 1314.

Received March 29, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 140–S 143

The Function of Plastoquinone in Photosynthetic Phosphorylation

F. R. WHATLEY and A. A. HORTON*

Department of Cell Physiology, University of California, Berkeley, California

The effect of plastoquinone on the cyclic photophosphorylations catalyzed by phenazine methosulfate, vitamin K_3 and flavin mononucleotide was examined. The extraction of plastoquinone from lyophilized chloroplasts by heptane treatment resulted in an almost complete loss of phosphorylating ability; the readdition of plastoquinone restored cyclic photophosphorylation in all three systems. The significance of this plastoquinone requirement for the mechanism of photosynthetic phosphorylation is discussed.

The preceding article[1] discussed the role of plastoquinone in the noncyclic electron transport of photosynthesis. Krogmann[2] has shown that the extraction of plastoquinone impairs a type of light-induced ATP formation in chloroplasts (cyclic photophosphorylation) that is catalyzed by phenazine methosulfate (PMS). The present communication extends the investigation of the effect of plastoquinone to cyclic photophosphorylation of the types catalyzed by vitamin K_3 (menadione) and flavin mononucleotide (FMN).

There is evidence that details of the electron flow pathway involved in cyclic photophosphorylation catalyzed by vitamin K_3 and FMN differ not only from that of noncyclic electron flow[3] but also from the electron flow pathway in cyclic photophosphorylation catalyzed by PMS. This investigation (a preliminary report of which has already been made[4]) has shown that plastoquinone is needed for the cyclic photophosphorylation catalyzed by vitamin K_3 and FMN, as well as that catalyzed by PMS.

METHODS

The chloroplast fragments were prepared, and plastoquinone was extracted and added back by the methods described in the preceding paper[1]. ATP formation was measured with ^{32}P as described previously[5,6]. The plastoquinone used in these experiments was a gift from Dr. O. Isler.

* Present address: Department of Biochemistry, Leicester University, Leicester, England.

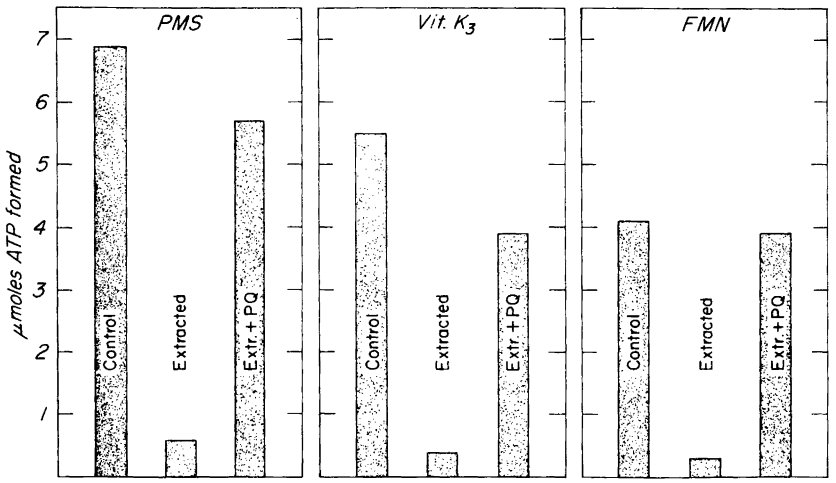


Fig. 1. Requirement of plastoquinone for cyclic photophosphorylation. The "control" vessel received lyophilized chloroplasts, the "extracted" vessel received lyophilized chloroplasts extracted with heptane, and the "extracted + PQ" vessel received extracted lyophilized chloroplasts to which plastoquinone (PQ) had been added (.05 mg/.5 mg chlorophyll). The reaction mixture contained, in a total volume of 3 ml., the following in micromoles: tris (hydroxymethyl) aminomethane buffer, pH 7.8, 80; $MgCl_2$, 10; ADP, 10; $K_2H^{32}PO_4$, 10; and one of the following cofactor combinations, as indicated (1) *PMS,* phenazine methosulfate, 0.1 (2) *Vit. K*$_3$, menadione, 0.3; and ascorbate, 10; (3) *FMN,* flavin mononucleotide, 0.3; and ascorbate, 10. Each vessel received chloroplasts containing 0.5 mg. chlorophyll. The experiment was carried out in Warburg manometer vessels at 15°C in a nitrogen atmosphere. Illumination at 25,000 lux was for 10 min. in the *PMS* series, 20 min. in the *Vit. K*$_3$ series and 30 min. in the *FMN* series. ATP formation was measured as described earlier[4,5].

RESULTS AND DISCUSSION

As shown in Fig. 1, all three types of cyclic photophosphorylation responded similarly to the experimental treatment. The thorough extraction of plastoquinone from the lyophilized chloroplast fragments by heptane resulted in an almost complete loss of phosphorylating ability, and the readdition of pure plastoquinone (by evaporation of isooctane from a solution of plastoquinone in isooctane) restored the phosphorylating ability in each case. Similar results were also recently reported by Krogmann and Olivero[7]. In individual experiments the degree of restoration by adding back plastoquinone was variable; sometimes the "restored" rate was low compared with the unextracted chloroplasts (only 50 %), but in several experiments the "restored" rate was greater (up to 150 %) than that of the unextracted chloroplasts. In any one experiment all three systems behaved similarly. The data shown in Fig. 1 demonstrate a requirement for plastoquinone in all three types of cyclic photophosphorylation, but these data do not by themselves distinguish between a requirement for plastoquinone (a) for the photophosphorylation steps proper and (b) for the cyclic electron transport with which the photophosphorylation steps are coupled. However, in view of the evidence for the participation of plastoquinone in the noncyclic electron flow, as discussed

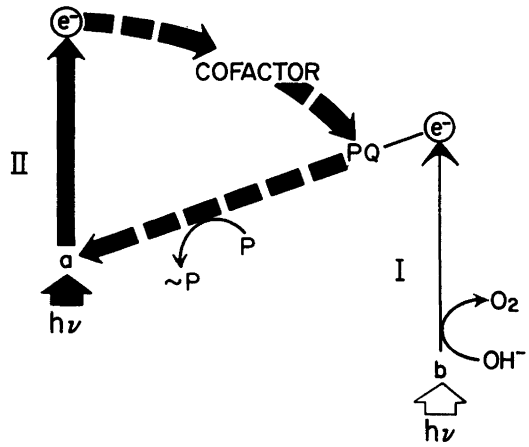


Fig. 2. A scheme for electron flow in cyclic photophosphorylation. Chlorophyll *a* (bound to a protein in the chloroplast) becomes excited by the absorption of a photon. The excited chlorophyll donates an electron, via the cofactor, to plastoquinone. The cycle is completed when the plastoquinone donates an electron (via cytochrome) to chlorophyll *a*. The phosphorylation step is shown to be linked with the transfer of the electron between plastoquinone and chlorophyll *a*.

in the preceding paper[1], the simplest interpretation is to assign plastoquinone to a site in the photosynthetic electron transport chain that is common to both cyclic and noncyclic electron flow (Fig. 2).

This interpretation, although stressing the similarities in the different photosynthetic electron transport pathways, is not in conflict with some important differences which have been observed among the three cases of cyclic photophosphorylation discussed here. These differences in cyclic photophosphorylation were first observed in their strikingly different responses to temperature[8] and light intensity[3]. Hall and Arnon[8], using a low light intensity (which at 15°C was rate-limiting), showed that the PMS-catalyzed system was not temperature-sensitive down to −5°C, whereas the FMN- and vitamin K_3-catalyzed systems were markedly temperature sensitive. In addition, as shown in Table 1, at 9 000 lux (white light) the amount of ATP formed was much less with PMS than with

Table 1. Effect of light intensity on cyclic photophosphorylation with various cofactors. (Tsujimoto, Hall and Arnon[3])

Light intensity lux	Cofactor system PMS	Vit. K_3	FMN
	micromoles ATP formed		
9 000	1.3	3.5	3.9
25 000	4.5	4.6	4.2
50 000	9.8	5.0	5.1

vitamin K_3 or FMN, at 25 000 lux there was no significant difference between the three systems, but at 50 000 lux the vitamin K_3- and FMN-catalyzed systems gave no additional phosphorylation (light saturation), while in the PMS-catalyzed system ATP formation continued to increase in proportion to the light intensity.

These differences have been explained [3,8] on the premise that the vitamin K_3- and FMN-catalyzed systems differ from the PMS system in having an additional phosphorylation site which, however, becomes rate-limiting at high light intensity. Since we have concluded that electron flow in all three systems proceeds through plastoquinone it would appear that the additional phosphorylating site envisaged for the vit. K_3- and FMN-catalyzed systems must be located in that segment of the cyclic electron pathway which is shown (by the heavy line) between "e⁻" and "PQ" in Fig. 2.

Although plastoquinone seems to be more probably a component of the electron transport chain rather than of the phosphorylation reaction proper, this conclusion must still remain tentative[4] because of the experimental difficulties, encountered also by other investigators[7,9], in extracting and restoring a fat-soluble constituent to chloroplasts, and in maintaining a high level of phosphorylating activity. It is still possible, as was suggested earlier[4], that plastoquinone may play a dual role in photosynthesis, both as an electron carrier and as a catalyst of the phosphorylation reaction proper.

REFERENCES

1. Arnon, D. I. and Horton, A. A. *Acta Chem. Scand.* **17** (1963) Suppl. 1, p. 135.
2. Krogmann, D. W. *Biochem. Biophys, Res. Comm.* **4** (1961) 275.
3. Arnon, D. I. In McElroy, W. D. and Glass, B. *Light and Life,* The Johns Hopkins Press, Baltimore, Maryland, 1961, p. 489.
4. Arnon, D. I., Whatley, F. R. and Horton, A. A. *Federation Proc.* **21** (1962) 91.
5. Arnon, D. I., Allen, M. B. and Whatley, F. R. *Biochem. Biophys. Acta* **20** (1956) 449.
6. Arnon, D. I., Whatley, F. R. and Allen, M. B. *J. Am. Chem. Soc.* **76** (1954) 6324.
7. Krogmann, D. W. and Olivero, E. *J. Biol. Chem.* **237** (1962) 3292.
8. Hall, D. O. and Arnon, D. I. *Proc. Natl. Acad. Sci. U. S.* **48** (1962) 833.
9. Bishop, N. I. *Proc. Natl. Acad. Sci. U. S.* **45** (1959) 1696.

Received March 29, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 144–S 149

Inhibition of the Respiration of *Escherichia coli* by Carbonyl Cyanide *m*-Chlorophenylhydrazone

YORAM AVI-DOR

Israel Institute for Biological Research, Ness-Ziona, Israel

The effect of carbonyl cyanide *m*-chlorophenylhydrazone (*m*-Cl-CCP) on the oxidation of various substrates by resting cells of *E. coli* has been investigated. $5 \cdot 10^{-7}$ M *m*-Cl-CCP inhibited the oxidation of succinate by 65 % while only fifty times higher concentration inhibited significantly the oxidation of glucose. Anaerobic CO_2 production from glucose was not inhibited. *m*-Cl-CCP had no inhibitory effect on the oxidation of succinate when the bacterial cells were preincubated with either succinate, pyruvate or glucose before the addition of the inhibitor. Glucose suppressed the inhibition of succinate oxidation also when added after *m*-Cl-CCP. The possible significante of the above findings is discussed.

It has been recently shown by Heytler and coworkers[1–3] that certain derivatives of carbonyl cyanide phenylhydrazone (CCP) uncouple oxidative phosphorylation in mitochondria and photophosphorylation in chloroplasts. This new class of uncouplers has, however, no appreciable effect on respiration in the above systems. During a study of the factors which control respiration in bacteria it was noted that low concentrations of carbonyl cyanide *m*-chlorophenylhydrazone (*m*-Cl-CCP) inhibited oxidative processes in resting cells of *Escherichia coli*, strain B. It was thought that a study of the mechanism of this inhibition may further the understanding of the differences which exist between the bacterial and the mammalian respiratory systems (See Ref. 4).

EXPERIMENTAL

Materials. *m*-Cl-CCP was the generous gift of Dr. P. G. Heytler (E. I. du Pont de Nemours and Company). Uniformly labeled D-glucose-^{14}C, succinic acid-1,4-^{14}C and $Na_2H^{32}PO_4$ were purchased from The Radiochemical Centre, Amersham, England. Other chemicals were of analytical grade.

Escherichia coli, Strain B, was grown on nutrient agar (Difco). The cultures were incubated at 37°C for 24 hours. The bacterial cells were collected in 0.25 M sucrose and washed three times with isotonic sucrose solution. Manometric experiments were carried out by the conventional Warburg technique. The nitrogen used in anaerobic experiments was purified by passing it through Fieser's solution and water. In most experiments the assay mixture contained $2 \cdot 10^{-2}$ M Tris buffer, pH 7.4, $5 \cdot 10^{-3}$ M NaCl, $5 \cdot 10^{-3}$ M KCl, bacterial cells in a con-

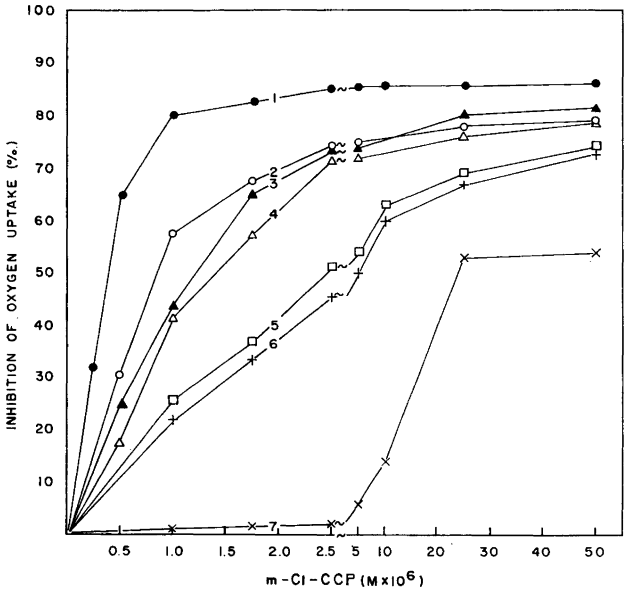


Fig. 1. Effect of *m*-Cl-CCP on the oxidation of various substrates. The main compartment of the Warburg flask contained the standard assay mixture and *m*-Cl-CCP in a concentration as indicated. After 10 min of thermoequilibration 3×10^{-3} M of the respective substrate was added from the side arm. Duration of the experiment 60 min. Designation of curves: 1, Succinate; 2, Pyruvate; 3, Acetate; 4, L-Glutamate; 5, DL-Glyceraldehyde; 6, D-Fructose; 7, D-Glucose.

centration corresponding approx. to 2.0 mg of protein/ml and water to 3.0 ml (Standard assay mixture). The incubation was carried out at 30°C.

Radioactivity was measured in a Tri-Carb Model 314F liquid scintillation counter (Packard Instrument Company). The respiratory $^{14}CO_2$ was trapped on KOH-moistened paper in the Warburg center well and the counting was performed in toluene-ethanol scintillator solution as described by Buhler[5].

Protein was assayed according to Lowry *et al.*[6] after solubilizing the bacterial cells by boiling with 1 N NaOH for 20 minutes.

RESULTS

Effect of m-*Cl-CCP on the oxidation of various substrates.* A survey was made of the effect of *m*-Cl-CCP on the oxidation of various substrates. NaCl and KCl were added to the assay mixture since the rate of oxidation of some of the substrates tested was accelerated by Na^+ and K^+ ions[7]. The Q_{O_2} (N) values measured for glucose varied between 700 and 900 and for the other substrates tested between 300 and 500. As shown in Fig. 1, when succinate was used as the substrate, preincubation of the bacterial cells with $5 \cdot 10^{-7}$ M *m*-Cl-CCP caused 65 % inhibition of the oxygen uptake; maximum inhibition of 85 % was reached at a concentration of $2.5 \cdot 10^{-6}$ M. In contrast to succinate the respiration in the presence of glucose was not inhibited by *m*-Cl-CCP concentrations below $2.5 \cdot 10^{-6}$ M and the maximum inhibition which could be obtained ($2.5 \cdot 10^{-5}$ M *m*-Cl-CCP) was only 53 %. The sensitivity to the inhibition of oxidation of the other substrates

Table 1. Effect of m-Cl-CCP on anaerobic CO_2 production from glucose. *m*-Cl-CCP pre-incubated with bacterial cells for 10 min at 30°C before the addition of 0.15 μC randomly labeled glucose-^{14}C diluted by 3.3×10^{-3} M carrier glucose.

	m-Cl-CCP ($M \times 10^6$)			
	None	1.0	2.5	25.0
$^{14}CO_2$ produced (c.p.m.) in 60 min	1535	1425	1380	1483

tested was intermediate between that of succinate and that of glucose; pyruvate, acetate and glutamate were closer to succinate in this respect while fructose and glyceraldehyde were more similar to glucose. In some experiments ^{14}C-labeled substrates were used and $^{14}CO_2$ production was measured in addition to oxygen uptake. Since the two methods revealed the same inhibition pattern these experiments will not be described in detail.

The effect of *m*-Cl-CCP on CO_2 production from glucose was determined also under anaerobic conditions. Randomly labeled glucose-^{14}C was used in this experiment and the radioactivity in the collected CO_2 was measured. As seen from Table 1, *m*-Cl-CCP had no inhibitory effect in this case.

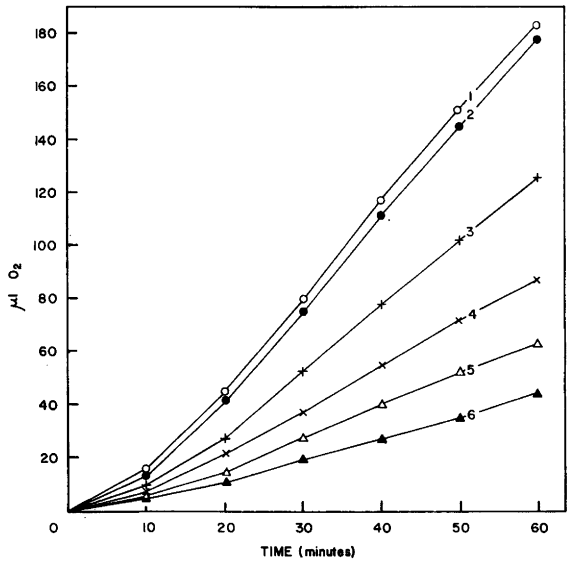


Fig. 2. Oxygen uptake as a function of the sequence of addition of substrate and inhibitor. The main compartment of the Warburg flask contained the standard assay mixture. Succinate (3.3×10^{-3} M) and *m*-Cl-CCP (1×10^{-6} M) were added as indicated. In all experiments the gas phase was air with the exception of the experiment shown in curve 5, where nitrogen was the gas phase during preincubations and it was changed to air at zero time. Curve 1: Succinate added at zero time; Curve 2: Succinate added at zero time, *m*-Cl-CCP five min after succinate; Curve 3: Succinate added at zero time, *m*-Cl-CCP one min after succinate; Curve 4: Succinate and *m*-Cl-CCP added simultaneously; Curve 5: 20 min of anaerobic pre-incubation with succinate, *m*-Cl-CCP added 10 min after succinate; Curve 6: *m*-Cl-CCP added 10 min before succinate.

Table 2. Effect of various substrates on the inhibition of succinate oxidation.

Composition of assay mixture in main compartment	Added from side arm		$^{14}CO_2$ produced (c.p.m.) in 60 min
	After 10 min	After 20 min	
S.M.[a]	–	Succinate[b], Succinate-^{14}C[c]	2505
S.M. + *m*-Cl-CCP[d]	–	Succinate, Succinate-^{14}C	600
S.M. + Succinate	*m*-Cl-CCP	Succinate-^{14}C	2450
S.M. + *m*-Cl-CCP	Pyruvate[b]	Succinate, Succinate-^{14}C	866
S.M. + Pyruvate	*m*-Cl-CCP	Succinate, Succinate-^{14}C	1847
S.M. + *m*-Cl-CCP	Glucose[b]	Succinate, Succinate-^{14}C	2366
S.M. + Glucose	*m*-Cl-CCP	Succinate, Succinate-^{14}C	2150
S.M. + *m*-Cl-CCP	Fructose[b]	Succinate, Succinate-^{14}C	1350

a Standard assay mixture.
b 3.3×10^{-3} M.
c 0.016 μC Succinate-1,4-^{14}C.
d 1×10^{-6} M.

The effect, on inhibition, of preincubation with substrate. During these experiments it was noted that the inhibition of the respiration by *m*-Cl-CCP depends on the sequence in which substrate and inhibitor were added. In Curve 6 of Fig. 2 we see the striking inhibition of succinate oxidation by $1 \cdot 10^{-6}$ M *m*-Cl-CCP when the inhibitor was added 10 min before succinate. However, the inhibition decreased when inhibitor and substrate were added simultaneously (Fig. 2; Curve 4). When the substrate was added one minute before the inhibitor, the inhibition by *m*-Cl-CCP decreased still further (Fig. 2; Curve 3) and when the preincubation with the substrate was extended to five min (Fig. 2; Curve 2) the rate of oxygen uptake was the same as in the control experiment (Fig. 2; Curve 1). The time of preincubation with succinate which was necessary to obtain full protection varied from batch to batch of bacteria 2—10 min). It was essential for the protection that the preincubation with succinate should be carried out under aerobic conditions. If the preincubation with succinate was carried out under anaerobic conditions as in the experiment shown by Curve 5 of Fig. 2, no diminution in the inhibition was observed.

Effect of various substrates on the inhibition of succinate oxidation. An experiment was carried out in order to determine whether the preincubation with succinate is specifically required to prevent the inhibition of succinate oxidation

by *m*-Cl-CCP. Therefore, the bacteria were preincubated with other respiratory substrates before the addition of the inhibitor and only then was succinate added. Interference by the added substrates in measurement of succinate oxidation was avoided by using ^{14}C-labeled succinate and determining $^{14}CO_2$ production instead of oxygen uptake. It can be seen from Table 2 that preincubation with (unlabeled) succinate abolished the decrease in counts which was observed in a control experiment in which *m*-Cl-CCP was added to bacteria without previous preincubation with the substrate. Moreover, it is apparent that when the cells were preincubated with pyruvate or glucose instead of succinate the drop in the counts due to the effect of the inhibitor was largely reversed. Pyruvate, the oxidation of which is inhibited by *m*-Cl-CCP, was found to be effective only when added before the inhibitor. In contrast, glucose which itself is not affected by an $1 \cdot 10^{-6}$ M *m*-Cl-CCP protected succinate oxidation whether it was added before or after the inhibitor. Similarly since fructose oxidation is less sensitive to inhibition than that of pyruvate it was more effective than the latter in the reversal of inhibition of succinate oxidation when added after the inhibitor.

Effect of preincubation with succinate on the bacterial cells. In order to elucidate further the nature of possible changes which take place in the bacterial cell on preincubation with an oxidizable substrate and which make the cell more resistent to *m*-Cl-CCP, the experiment shown in Table 3 was carried out. A larger sample of bacterial cells was incubated for 30 min under the conditions of the experiment described in Fig. 1, using $1 \cdot 10^{-2}$ M succinate. In a control experiment succinate was omitted from the medium. The cells were harvested by centrifugation, washed once with 0.15 M NaCl and resuspended in the same medium. The two suspensions obtained designated *Coli* (Succinate) and *Coli* (Control) were added to an assay medium which contained succinate ($3.3 \cdot 10^{-3}$ M) and *m*-Cl-CCP as indicated. Their sensitivity to *m*-Cl-CCP was compared with that of untreated *E. coli*. As seen from the table, incubation with succinate caused some change in the cells, since after washing, the *Coli* (Succinate) cells proved to be significantly less sensitive to *m*-Cl-CCP than either *Coli* (Control) or the untreated cells.

DISCUSSION

The inhibitory effect of *m*-Cl-CCP on the respiration of *E. Coli* is comparable to that of the most potent inhibitors reported in the literature[8]. The extent of inhibition depended, however, to a large measure on the sequence of the addition of the components of the reaction mixture. It became apparent that the

Table 3. Sensitivity of *Coli* (Succinate) and *Coli* (Control) to *m*-Cl-CCP.

	Coli (Succinate)[a]		*Coli* (Control)[a] *m*-Cl-CCP ($M \times 10^6$)		*Coli*[a]	
	none	1.0	none	1.0	none	1.0
μl O_2/h[b]	240	190	260	90	247	105

a 2.0 mg protein per Warburg flask.
b average from three experiments.

addition of various substrates to the reaction mixture whose oxidation, under the conditions of the test, was not inhibited by *m*-Cl-CCP, abolished also the effect of this inhibitor on the oxidation of succinate, which would have been inhibited most pronouncedly by *m*-Cl-CCP in the absence of the added oxidizable substrate.

This raises the question of the identity of the substance which could have been formed during the oxidation of the various substrates in the bacterial cell and which would make it resistant to the *m*-Cl-CCP. In the present state of this investigation we can offer no definite answer to this question. Considering, that in all systems in which the effect of *m*-Cl-CCP has been studied heretofore, this inhibitor acted on ATP formation and not on respiration, it is likely that in bacteria too its primary site of action is oxidative phosphorylation. The formation of the protective factor might then depend on ATP synthesis. In this context it is noteworthy that succinate oxidation, which in mammalian mitochondria is connected with two phosphorylation steps, was most sensitive to inhibition, whilst that of the pyridine nucleotide-linked substrates with one more phosphorylation step was less affected. The most resistant to inhibition was the oxidation of the glycolytic substances, in particular glucose, which can generate ATP also by substrate-level phosphorylation. Although very little is yet known on the phosphorylation steps in bacteria[8], a difference in the sensitivity of the various sites of phosphorylation to inhibition could account for the differences observed in the extent of respiratory inhibition.

Acknowledgements. The author is much indebted to Dr. P. G. Heytler for a sample of *m*-Cl-CCP. The technical assistance of Miss Tamar Sari is gratefully acknowledged.

REFERENCES

1. Heytler, P. G. and Prickard, W. W. *Biochem. Biophys. Res. Commun.* **7** (1962) 272.
2. Heytler, P. G., Prickard, W. W. and Goldsby, R. A. *Federation Proc.* **21** (1962) A–54 c.
3. Heytler, P. G. *Biochemistry. In press.*
4. Dolin, M. I. In Gunsalus, I. C. and Stanier, R. Y. *The Bacteria,* Academic Press, New York, 1961, Vol. II, p. 319.
5. Buhler, D. R. *Anal. Biochem.* **4** (1962) 349.
6. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* **193** (1951) 265.
7. Miller, Sh. and Avi-Dor, Y. *J. Gen. Microbiol.* **18** (1958) 221.
8. Smith, L. In Gunsalus, I. C. and Stanier, R. Y. *The Bacteria,* Academic Press, New York, 1961, Vol. II, p. 365.

Received March 26, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 150 – S 153

A New Abnormal Serum Globulin α_1-Antitrypsin

STEN ERIKSSON and CARL-BERTIL LAURELL

Department of Clinical Chemistry, General Hospital, Malmö, University of Lund, Sweden

A serum has been discovered which contains two α_1-antitrypsin globulins, which are immunologically indistinguishable with conventional antisera but differ in electrophoretic mobility. The anomaly gives rise to an atypical paper electrophoretic pattern and is easily visualized in agar gel electrophoresis.

Schultze *et al.*[1] recently identified Schmid's[2] and Schultze *et al.'s*[3] 3.5 S α_1-glycoprotein (α_1 A-globulin of Burtin[4]) with the α_1-antitrypsin in human serum. Bromphenol blue stains this globulin more intensely than it does the other main constituents of the α_1-globulin fraction obtained on zone electrophoresis in agar or on paper with barbital buffer pH 8–9 and ionic strength 0.1–0.2. In conventional zone electrophoresis the concentration of this globulin is roughly proportional to the colour intensity of the α_1-band (more sharply demarcated in agar than on paper).

In an earlier paper[5] we reported a series of human sera with paper electrophoretic patterns suggesting α_1-antitrypsin deficiency (no visible α_1-band). Further analysis revealed that a subnormal serum content of α_1-antitrypsin (<10 % of normal) may be a manifestation of an inherited predisposition to emphysema.

This paper is concerned with another type of abnormality of the α_1-antitrypsin globulin. The serum was subjected to close analysis because the normally demarcated α_1-antitrypsin band appeared broader and less intense than normally.

METHODS

Serum protein concentration was determined with the Biuret method of Kingsley[6], *quantitative paper electrophoresis* in accordance with Laurell *et al.*[7], *agar gel electrophoresis* in 2 % agar (purified) on cooled (+2°C) glass plates (4 × 40 cm) with calcium containing barbital buffer, a modified Wieme[8] procedure, and *immunoelectrophoresis* as described under agar gel electrophoresis. Rabbit antihuman serum (R.A.H.S.) from Behringwerke was used before and after adsorption with human serum from a patient with only traces of α_1-antitrypsin in her serum.

α_1-antitryptic activity was calculated from the loss in antitryptic activity of serum (or fractions) when treated at 60°C for 20 min after adjustment of pH to 5.1 by addition of 0.5 M acetic acid. The method for estimation of tryptic activity with BAPA (benzoyl DL-arginine p-nitroanilide hydrochloride) as substrate was based on the principles given by Erlanger *et al.*[9].

The electrophoretic distribution of the antitryptic activity in sera was determined after separation in agar (see above). The gel was cut into 0.2 cm segments. The slices were eluted, by agitation over night, in 0.05 M barbital buffer, pH 8.6, at +5°C.

MATERIAL

Serum was prepared from blood drawn from the patient on two occasions and from her three adult children.

Case history. The patient was a Para-III, born in 1900. She had undergone cholecystectomy in 1948. Afterwards she felt well until 1962 when she suffered from dizziness, fatigue and headache. She was admitted to hospital in Nov. 1962, since when her E.S.R. has been continuously elevated (about 50 mm/1 h). The woman was fairly fat but physical examination on admission revealed no signs of a pathological condition. B.P. 220/120 mm Hg. Roentgen examination of the heart and chest showed normal appearances. Intravenous urography gave normal findings. Hgb. 81 %. Red blood cell count 4.3 mill./mm^3. E.S.R. 30–50 mm/1 h and the serum creatinin was 0.72 mg/100 ml.

RESULTS

The paper electrophoretic patterns of serum from the patient and from a healthy subject are presented in Fig. 1 for comparison of the colour distribution in the α_1-zone. In the patient's pattern the normal α_1-band was replaced by a broader, less intensely stained band. Fig. 2 gives the results of agar gel electrophoresis and immunoelectrophoresis of sera from the patient and a control. It is clear from the illustration that the patient's pattern shows a relatively faint band at the site corresponding to the α_1-antitrypsin in the control pattern. However, in contrast to the control serum the patient's serum contains a distinct band in the α_1—α_2 interzone. These two bands of the patient's pattern are of about the same colour intensity. From the immunoelectrophoretic pattern it is apparent that the globulins corresponding to these two bands in the patient's serum share the antigenic determinants normally responsible for the development of the α_1 A precipitation line. The same result was obtained on development of the precipitation lines with the horse antihuman serum. The precipitation line was nearest to the antiserum well at the α_1—α_2 interzone.

The trypsin inhibiting capacity of the patient's serum (990 μg/ml) was normal. That both antigenically related α-globulins had antitryptic activity is clear from Fig. 3, which shows the electrophoretic distribution of the antitryptic activity in the patient's serum in relation to that in a control serum. The antitryptic

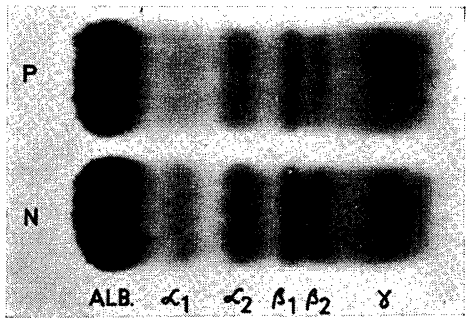


Fig. 1. Paper electrophoretic protein pattern of patient's serum (P) and of normal serum (N).

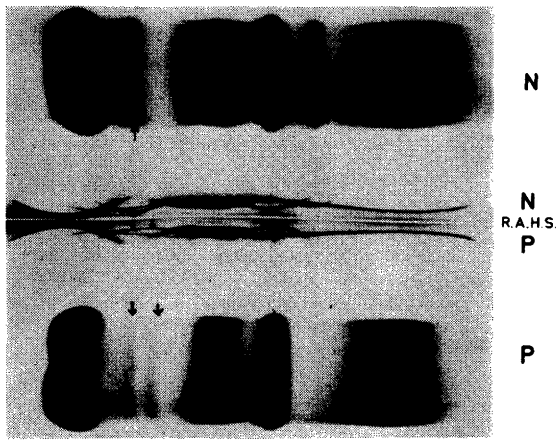


Fig. 2. Protein pattern of patient's serum (P) and of normal serum (N) in agar gel. Immunoelectrophoretic pattern obtained with rabbit antihuman serum (R.A.H.S.) in the well. The arrows indicate the position of the α_1-antitrypsin globulins.

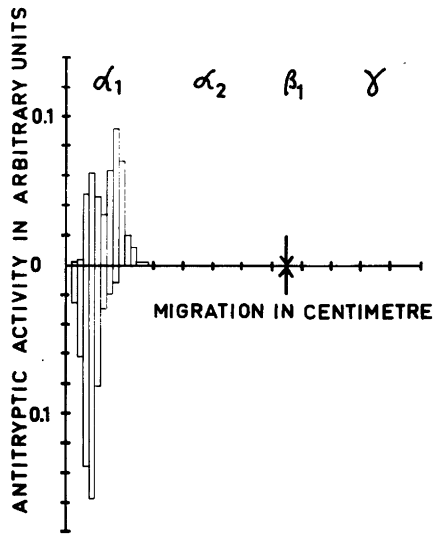


Fig. 3. Distribution of the antitryptic activity after electrophoresis in agar gel. Patient's serum above the abscissa and a normal serum on mirrored scale below the abscissa. Arrow indicates point of application.

activity in the patient's serum caused by the globulins in the electrophoretic α_1- and inter-α-zone disappeared after heating the serum at 60°C for 20 min at pH 5.1, as did the α_1-antitrypsin in the control.

After treatment of the serum with neuraminidase purified from pneumococci the electrophoretic mobility of both α_1-antitrypsin globulins decreased compared with that of the albumin.

DISCUSSION

A serum has been found which, on paper electrophoresis, gives rise to an atypical α_1-globulin pattern. Further analysis revealed that the serum contains two globulins with roughly the same antitryptic activity and the same antigenic properties as the normal α_1-antitrypsin. Patterns of sera from blood samples collected at about one month's interval gave the same atypical pattern, and the patient's disease presumably has nothing to do with the abnormality.

Schultze[10] has shown that the 3.5 S α_1-globulin has much neuraminic acid bound to its surface and that it is rapidly released by neuraminidase. During such treatment the whole α_1-antitrypsin fraction loses in charge and one retarded fraction appears on electrophoresis, but not two globulins, as in our case. That both globulins are synthesized with a different amount of neuraminic acid has not been excluded yet. Neither of the globulins showed increased thermal stability, for both were completely inactivated when heated at 60°C for 20 min at pH 5.1. In our experiments with chromatography of serum and α_1-globulins on DEAE-cellulose in the pH range 5–8 it has repeatedly been found that the α_1-antitrypsin spontaneously loses in charge and somewhat in electrophoretic mobility. Schultze *et al.*[1] have recently suggested that the α_1-antitrypsin during purification has a tendency to dimerization, which may result in a decreased charge.

The most probable explanation for the cleavage of the α_1-antitrypsin in our case is an inborn error of the synthesis of one peptide chain of this globulin. Sera from the patient's three children (all female) have been examined and all showed a normal α_1-antitrypsin band with normal biological activity. The anomaly is probably of no physiological importance since the total antitryptic activity is normal.

Acknowledgements. This investigation was supported by grants from the *Swedish Medical Research Council* and from *U. S. Public Health Service* (Grant AI-04703-02).

We thank Dr. Lennart Arin, chief physician, Nässjö Central Hospital, for kind co-operation and for placing patient's records at our disposal.

REFERENCES

1. Schultze, H. E., Heide, K. and Haupt, A. *Klin. Wochschr.* **40** (1962) 427.
2. Schmid, K. *J. Am. Chem. Soc.* **75** (1953) 60.
3. Schultze, H. E., Göllner, K., Heide, K., Schönenberger, M. and Schwick, G. *Z. Naturforsch.* **10 b** (1955) 463.
4. Burtin, P. *Analyse Immuno-electrophorétique*, Masson, Paris, 1960, p. 93.
5. Laurell, C.-B. and Eriksson, S. *Scand. J. Clin. Lab. Invest.* **15** (1963). *In press.*
6. Kingsley, C. R. *J. Lab. Clin. Med.* **27** (1942) 840.
7. Laurell, C.-B., Laurell, S. and Skoog, N. *Clin. Chem.* **2** (1956) 99.
8. Wieme, R. J. *Studies on agar gel electrophoresis.* Arscia, Brussels, 1959.
9. Erlanger, B., Kokowsky, N. and Cohen, W. *Arch. Biochem. Biophys.* **95** (1961) 271.
10. Schultze, H. E. *Scand. J. Clin. Lab. Invest.* **10** (1957) 135.

Received April 1, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 154–S 160

Some Properties of Myeloma Proteins and their Papain Produced Subunits

N. O. THORPE, M. R. MACKENZIE and H. F. DEUTSCH

Department of Physiological Chemistry, University of Wisconsin, Madison, Wisconsin, USA

The 7 S myeloma proteins resolve into a number of components when subjected to electrophoresis in low ionic strength media. Their 3.5 S papain digest fractions likewise contain multiple electrophoretic components when studied under similar conditions. The fraction of lowest mobility in alkaline buffers contains the same number of components as the parent molecules. The factors responsible for this electrophoretic heterogeneity have not been ascertained.

A single myeloma protein component appears to possess most or all of the 3.5 S fragments found in papain digests of the unresolved parent system.

The 3.5 S subunit fractions of myeloma proteins produced by the actions of (1) papain, (2) pepsin and reducing agents, and (3) 8 M urea on reduced and alkylated protein, show distinct differences when subjected to electrophoresis in starch gel.

The normal 7 S γ-globulins and the hyperglobulinemic proteins from myeloma sera are being extensively employed in studies on the subunit composition of antibodies. Myeloma proteins display greater electrical homogeneity than normal γ-globulins as shown by moving boundary electrophoretic techniques[1]. It has been also previously noted that myeloma proteins may resolve into several distinct components upon electrophoresis in starch gel[2,3]. The present work indicates a relationship between the electrophoretic heterogeneity of myeloma proteins and certain of their papain produced subunits. Some of the properties of these subunits have been investigated and preliminary comparisons made between them and those produced by other methods.

EXPERIMENTAL

Moving boundary electrophoretic studies utilized the Spinco Model H instrument. Vertical starch gel electrophoretic experiments were carried out by the method of Smithies[4] and employed a pH 8.6 Tris (0.045 M)-Versene (0.001 M)-Borate (0.025 M) buffer except when noted.

The myeloma (MM) proteins and their subunits were subjected to various treatments. They were reduced with 0.05 M 2-mercaptoethanol in 0.2 ionic strength, pH 8 Tris-HCl buffer for

one hour at 37°C and then alkylated by addition of iodoacetate or iodoacetamide to a concentration of 0.1 M. Reduced and alkylated proteins were separated from other reactants by dialysis or by passage through Sephadex G-25.

Conversion of MM proteins to 3.5 S fragments was effected by treatment with sulfhydryl activated papain[5], by the action of pepsin followed by treatment with mercaptan[6], and by treatment of the reduced and alkylated proteins with 8 M urea[7]. Removal of the activating sulfhydryl compound from the papain prior to the digestion of the MM proteins did not appear to influence the splitting reaction. Ultracentrifugal analyses were carried out with the Spinco Model E instrument to determine whether conversion to 3.5 S subunits was complete. The papain digestion products were separated by chromatography on CM- or DEAE-cellulose in a manner similar to that previously described[5].

RESULTS

Starch gel electrophoretograms of seven different 7 S MM proteins are shown in Fig. 1. The initials designate the patient source. It can be seen that each contains a series of components closely related with respect to mobility. The components are designated in order of decreasing cathodic mobility as MM-1, MM-2, etc. Similar patterns are obtained on electrophoresis in starch gel in pH 8.5, 0.01 M sodium diethylbarbiturate buffer. These MM proteins all migrate as relatively homogeneous peaks on moving boundary electrophoresis in 0.1 ionic strength buffers, but at low ionic strength a heterogeneity qualitatively similar to that observed in starch gel is seen. The results of moving boundary electrophoretic experiments on two of the MM proteins are shown in Fig. 2. One (Hu) is relatively acidic while the other (CO) has a much higher isoelectric point. They have electrophoretic mobilities in the pH 8.5, 0.1 ionic strength Na diethylbarbiturate buffer of -2.28 and -0.64×10^{-5} cm^2 volt^{-1} sec^{-1}, respectively.

The electrophoretic patterns of the papain digested (PD) proteins are included with the data of Fig. 2. When a MM protein is converted into 3.5 S units by this enzyme, two electrophoretic components, one more acidic and the other more basic than the parent protein, are seen. These fractions have been designated B and C respectively[5] and are analagous to the fast (F) and slow (S) subunits of other investigators[8].

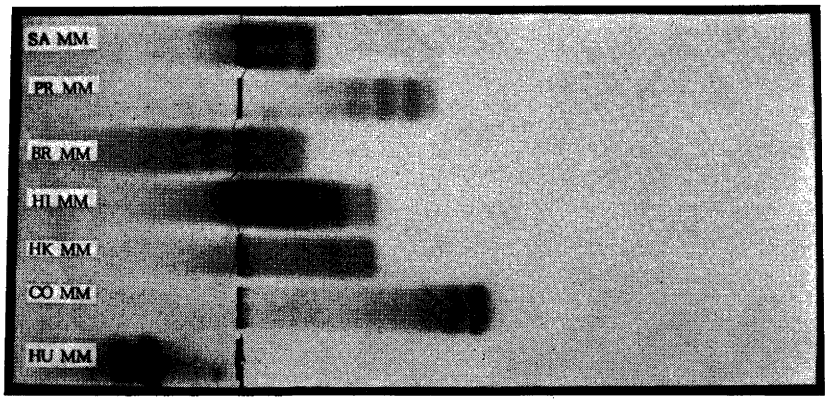


Fig. 1. Starch gel electrophoretograms of myeloma globulins. Migration to the right is cathodic.

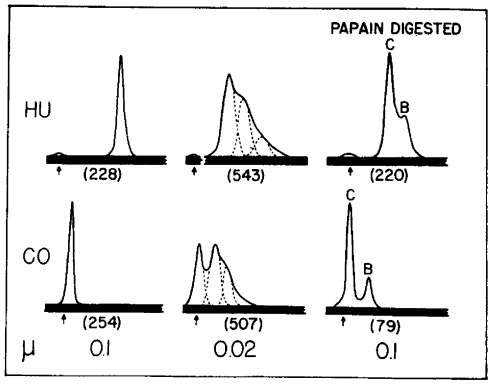


Fig. 2. Moving boundary electrophoretic diagrams of MM proteins HU and CO and their papain digestion products in pH 8.5, Na diethylbarbiturate buffer. The position of the starting boundary is indicated by an arrow and the duration of the experiment in minutes is given by the number in parenthesis. The potential gradient of the 0.1 ionic strength (μ) experiments was near 5.7 volts cm^{-1}; for the 0.02 μ experiment it was 7.6 and 9.5 for HU and CO respectively.

Some separation of the electrophoretic components of certain MM systems has been achieved by chromatography on DEAE-cellulose, particularly when low ionic strength solutions have been employed in the early stages. An example of this is shown for the HI system in Fig. 3. A resolution qualitatively paralleling that observed on starch gel and on free electrophoresis at low ionic strength is noted.

A starch gel electrophoretogram of the papain digests of nine MM proteins, a pool of these papain digests (MM-PD's) and of papain digested normal γ_2-globulin (γ_2-N-PD) are shown in Fig. 4. As previously indicated[9], the anodically migrating B fractions from various MM globulins are quite similar whereas the C-fractions are different. The number of cathodic C-components seen in a given

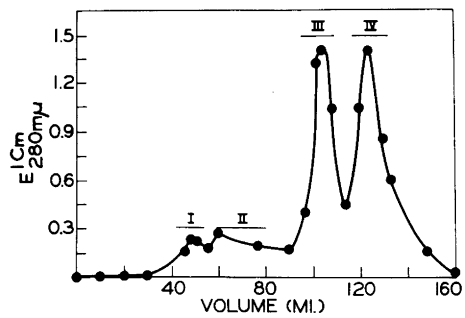


Fig. 3. Chromatography of 50 mg of myeloma protein HI on a 1.2 × 30 cm. column of DEAE-cellulose. A pH 9.0, 0.02 M Tris-HCl buffer and a continuous salt gradient to 0.15 M NaCl was used to elute the protein.

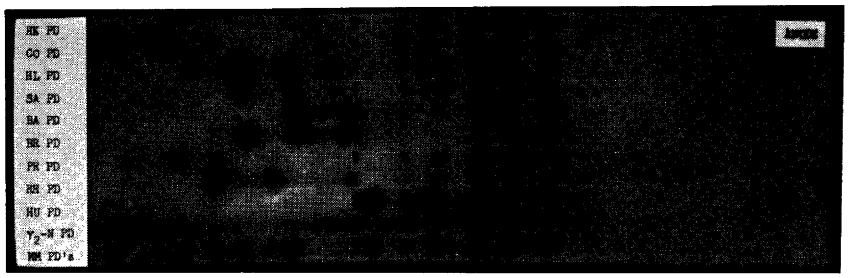


Fig. 4. Starch gel electrophoretograms of papain digests of myeloma and normal γ-globulins.

papain digest agreees with the number of components noted in the parent MM protein (see Fig. 1). The pooled MM papain digests (MM-PD's) possess components distributed throughout the mobility range of those of the γ_2-N-PD system. It is apparent that if a larger number of individual MM-PD's had been pooled a starch gel electrophoresis result similar to that for γ_2-N-PD would result. This reiterates a previous finding that the various γ_2-type MM proteins possess properties within the range of the proteins making up normal γ_2-globulins[1].

The structural features of the various components in the acidic and basic fractions of the papain digests which are responsible for their electrophoretic mobility differences are not known. When the parent myeloma proteins and their papain digest products are reduced and then alkylated with iodoacetate, a decreased cathodic mobility for each system is noted (see Fig. 5). The position of each of the reduced and alkylated C-fraction components appears to be shifted to that of a more acidic component of the untreated fraction. These results indicate that each of the starch gel components of the C-fraction has the same number of readily cleaved, *i. e.*, labile, disulfide bonds. Papain digestion of reduced and alkylated MM protein gives a C-fraction having the same starch gel electrophoretic composition as C-fractions which have been reduced and alkylated following their isolation. Reduction and alkylation of the B-fraction likewise increases the anodic mobility of its components. However, in this case an increased number of components is also seen.

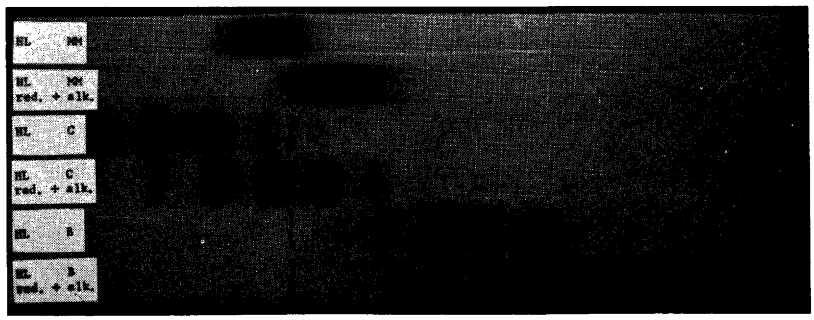


Fig. 5. Starch gel electrophoretograms of a myeloma protein and its papain digest products before and after reduction and alkylation with iodoacetic acid.

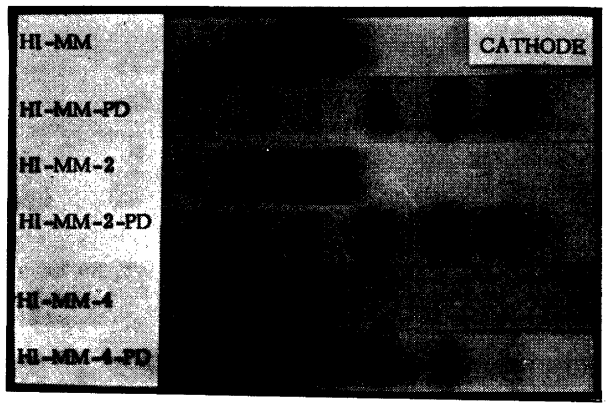


Fig. 6. Starch gel electrophoretograms of some HI-MM components and their papain digest products.

Preliminary amino acid analyses, cysteine and cystine determinations and immunochemical studies of these individual components suggest an identity with respect to these parameters. Treatment with neuraminidase produces no changes in their starch gel electrophoretic properties which indicates that differences in sialic acid content do not occur. Further studies on these systems are in progress to elucidate the basis of the electrophoretic heterogeneity noted in low ionic strength media.

A study of the effect of papain on the individual MM components resolved on electrophoresis has been hampered by the difficulty in purifying them in adequate amounts. The utilization of fractions separated by chromatographic methods of the type used to provide the data of Fig. 3, has given a partial answer to the obvious question of whether papain digestion of a single MM component will produce only one C-fraction component. The results shown in Fig. 6 indicate that the same number of basic components seen in papain digests of the whole MM protein is produced on digestion of one or two MM components. However, differences in relative amounts are indicated. It appears that the higher isoelectric point MM components yield larger amounts of similarly charged C-fraction components.

Starch gel electrophoretic studies of the 3.5 S material produced from MM proteins by the action of pepsin followed by reduction indicate differences from the papain digested systems. These results are shown in Fig. 7 A. The action of papain on the pepsin digest produces cathodically migrating components similar to those of the papain digest C-fraction. Material with the electrophoretic properties of the papain B-fraction is not produced from MM proteins by pepsin, and indeed, this fraction is cleaved by pepsin into dialyzable fragments.

Fig. 7 B shows the electrophoretic properties in a urea-formate starch gel[10] of the 3.5 S fragments of MM proteins produced by treatment with (1) pepsin and 2-mercaptoethanol, (2) papain, and (3) reduction and alkylation in 8 M urea. It is apparent that different subunits are produced by these methods. Reduction

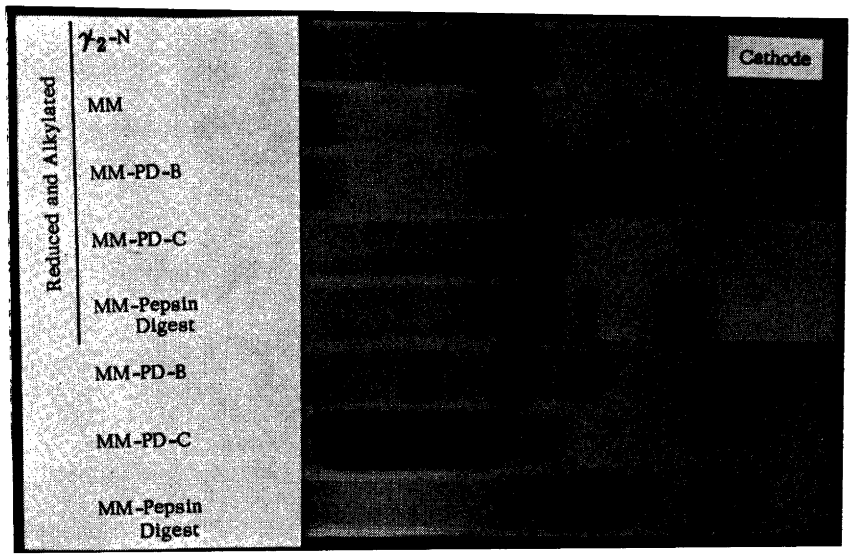


Fig. 7 A. Starch gel electrophoretograms in pH 8.5, Tris-Versene-Borate buffer of the HI-MM proteins digested with papain, with pepsin and with pepsin followed by papain.

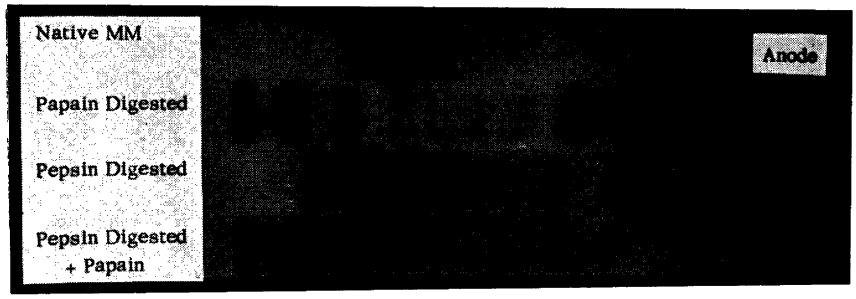


Fig. 7 B. Electrophoretograms in urea-formate starch gel of normal γ_2-globulins and various products of myeloma protein HI. The upper five reservoirs contain proteins that were reduced and alkylated prior to treatment with urea.

and alkylation is seen to markedly affect the 3.5 S products produced by pepsin and papain. The papain digestion C-fraction appears to have at least two subunits in common with the pepsin digest. Further relationships of the 3.5 S subunits produced by the three methods described remain to be explored.

DISCUSSION

Myeloma proteins appear to be composed of a family of molecules which show small differences in electrophoretic mobility in alkaline buffers of low ionic strength. Preliminary study of these components suggests that they posses many chemical and biological properties in common and differ only by slight

variations in charge. The acidic and basic fractions of the papain digests of myeloma proteins also show a number of starch gel electrophoretic components. Those of the basic fraction appear to reflect the differently charged entities in the parent protein. Unpublished data indicate that the individual components have a similar amino acid composition and immunochemical properties.

The myeloma proteins of mol. wt. near 160 000 separate into a series of components when subjected to electrophoresis in alkaline buffers of low ionic strength. Each of these components appears to consist of four 3.5 to 4 S subunits. One of them is acidic and is common to every 7 S myeloma protein. The remainder are relatively more basic and appear to be closely related but differentiable from each other on the basis of charge. The number of the latter in a given myeloma protein appears to be the same as the number of parent 7 S components which can be resolved on electrophoresis. It would appear that three such different basic subunits should be found in each parent myeloma protein component along with one of the above discussed acidic units. Concepts of the unit composition of the 7 S molecules will be dependent on a better understanding of these subunits. This knowledge must await more adequate separation of the individual 7 S MM components.

Each of the electrophoretic components of the papain digest fractions possesses the biological activity characteristic of the parent molecules. The genetic Inv factors are present in each of the C-fraction components while those of the B-fraction possess Gm-1 genetic and anticomplementary activity[9].

Further more searching experimentation will be required to explain the noted interesting electrophoretic polymorphism. The results of such studies should provide insight into the properties of normal γ_2-globulins.

Acknowledgement. This work was supported in part by a grant (No. C-1786) from the *United States Public Health Service.* One of us (N. O. T.) is the recipient of a Pre-doctoral fellowship from the *Division of General Medical Sciences, U. S. Public Health Service.*

REFERENCES

1. Deutsch, H. F., Morton, J. I. and Kratochvil, C. H. *J. Biol. Chem.* **222** (1956) 39.
2. Binette, J. P. and Schmid, K. *Nature* **192** (1961) 732.
3. Fahey, J. L. *J. Clin. Invest.* **42** (1963) 111.
4. Smithies, O. *Biochem. J.* **7** (1959) 585.
5. Deutsch, H. F., Stiehm, E. R. and Morton, J. I. *J. Biol. Chem.* **236** (1961) 2216.
6. Nisonoff, A., Wissler, F., Lipman, L. and Woernly, D. *Arch. Biochem. Biophys.* **89** (1960) 230.
7. Edelman, G. M. and Gally, J. A. *J. Exptl. Med.* **116** (1962) 207.
8. Edelman, G., Heremans, J., Heremans, M. and Kunkel, H. *J. Exptl. Med.* **112** (1960) 203.
9. Deutsch, H. F., Thorpe, N. O. and Fudenberg, H. H. *Immunology. In press.*
10. Smithies, O., Connel, G., and Dixon, G. *Am. J. Hum. Genet.* **14** (1962).

Received March 25, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 161 – S 164

Nicotinamide-Adenine Dinucleotide Nucleosidase of *Mycobacterium butyricum*

I. TOIDA

Research Institute, Japan Anti-Tuberculosis Association, Kiyose Machi, Tokyo, Japan

NAD-ase was extracted and purified from *Mycobacterium butyricum* by repeated ammonium sulfate fractionation and DEAE-cellulose ion-exchanger chromatography. Enzymological studies were carried out.

Many works have been done on NAD-ase*, an enzyme which hydrolyzes NAD* at the nicotinamide-ribose linkage, but very little was known about this enzyme of tubercle bacilli. Kern and Natale[1] observed that an extract of *Mycobacterium butyricum* contained rather heat-stable enzyme as well as a heat-labile inhibitor of this enzyme. Except this report, only scattered data, whereby the absence of NAD-ase activity was reported by human or bovine type of tubercle bacilli, can be found in literature[2,3].

On the other hand, NAD-ase exchange reaction, whereby isonicotinoyl hydrazide can replace nicotinamide in the NAD molecule[3–5], was postulated by Oka *et al.*[6] and Eda *et al.*[7] as a reaction providing a possible explanation for the anti-tuberculous activity of isonicotinoyl hydrazide (isoniazid).

Considering the importance of isoniazid in tuberculosis treatment, it is of significance to investigate mycobacterial NAD-ase in more details. The purpose of this paper is to describe the purification method and to report some properties of *Mycobacterium butyricum* NAD-ase.

MATERIALS AND METHODS

NAD (β-type) of 98 per cent purity was obtained from the Sigma Chemicals Company.

Mycobacterium butyricum was grown on Sauton synthetic medium, which was composed of 4.0 g of asparagine, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of K_2HPO_4, 2.0 g of citric acid, 0.05 g of ferri-ammonium citrate, 60 ml of glycerol per liter and adjusted to pH 7.0. After incubation at 37°C for 5 or 7 days, the cells were harvested on filter paper and washed thoroughly with distilled water. The purification procedures are described below.

*The following abbreviations will be used: NAD, nicotinamide-adenine dinucleotide; NAD-ase, nicotinamide-adenine dinucleotide nucleosidase; DEAE cellulose, diethylaminoethyl cellulose.

NAD-ase activity was measured in a reaction mixture containing 0.1 ml of NAD (10 μmoles per ml), enzyme preparation, 0.4 ml of M/15 phosphate buffer at pH 7.2, water to 1.0 ml of the total volume. The reaction mixture was incubated at 38°C for 30 or 60 min and the reaction was terminated by the addition of 5.0 ml of 1.0 M potassium cyanide. Cyanide was added to a control tube at zero time. From the change of extinction value at 325 mμ before and after the incubation, the amount of NAD decomposed was determined. A unit of enzyme is that amount which causes the hydrolysis of 0.01 μmole of NAD per hour under the conditions. Protein content of preparation was determined from extinction value at 260 mμ and 280 mμ according to the following equation: Protein mg per ml $= 1.45 \times E_{280} - 0.74 \times E_{260}$.

RESULTS

I. Procedure for NAD-ase purification

Step 1. Preparation of crude extract. Washed cells were suspended in five volumes of distilled water with minimal grinding in a mortar and the suspension in an Erlenmeyer flask was placed in a boiling water bath for 5 min and immediately cooled down in water. After being cooled and centrifuged at 10 000 r.p.m. for 30 min, the precipitated cell pellet was homogenized with original volume of distilled water. The suspension was placed in a Kubota's sonic disintegrator for 30 min. The resulting turbid solution was centrifuged for 30 min at 10 000 r.p.m. The supernatant was saved and the precipitated pellet was subjected once more to sonic oscillation and centrifugation as before. The combined supernatant served as the starting material.

Step 2. First ammonium sulfate fractionation. To each 100 ml of the above solution 17.6 g of solid $(NH_4)_2SO_4$ were added. After standing for three or four hours and centrifuged at 10 000 r.p.m. for 30 min, the precipitate was discarded and to each 100 ml of the supernatant 19.8 g of solid $(NH_4)_2SO_4$ were added. After three or four hours the solution was centrifuged at 10 000 r.p.m. for 30 min and the supernatant was discarded. The precipitate was dissolved in a minimal quantity of distilled water and dialyzed overnight against distilled water.

Step 3. Second ammonium sulfate fractionation. To each 100 ml of the above solution 27.7 g of solid $(NH_4)_2SO_4$ were added. After three or four hours the solution was centrifuged at 10 000 r.p.m. for 30 min and the precipitate was discarded. To each 100 ml of the supernatant 9.9 g of solid $(NH_4)_2SO_4$ were added. After standing for three or four hours and centrifuged for 30 min at 10 000 r.p.m., the supernatant was discarded. The precipitate was dissolved in a minimal amount of distilled water and dialyzed against distilled water overnight.

Step 4. DEAE-cellulose ion-exchanger chromatography. The above solution was subjected to the column of DEAE-cellulose ion-exchanger buffered with M/40 phosphate at pH 7.2. The column was washed with the same buffer to elute out inactive proteins and then the active material was eluted with M/15 phosphate buffer at pH 7.2.

Step 5. Concentration with ammonium sulfate. To 100 ml of the above eluate 39.0 g of solid $(NH_4)_2SO_4$ were added. After three or four hours the solution was centrifuged at 10 000 r.p.m. for 30 min and the supernatant was discarded. The precipitate was dissolved in a minimal amount of M/45 phosphate buffer at pH 7.2 and dialyzed against the same buffer overnight.

A typical result of the purification procedures is summarized in Table 1.

Table 1. Purification of NAD-ase.

Preparation step	Volume ml	Total protein mg	Total units	Units per mg	Purity
Cell-suspension	105	5 722.5	250.95	0.0438	
Suspension of heated cells	110	4 994.0	26 011.70	5.209	
Crude extract	200	1 414.0	176 372.00	124.73	1
1st $(NH_4)_2SO_4$ fractionation	50	113.5	47 658.50	419.90	3.37
2nd $(NH_4)_2SO_4$ fractionation	40	52.8	35 956.40	680.99	5.46
DEAE-cellulose chromatography	80	1.680	24 272.80	14 448.10	115.84
$(NH_4)_2SO_4$ concentration	10	0.573	8 991.70	15 692.32	125.81

II. Properties of *Mycobacterium butyricum* NAD-ase

Ultra-violet absorption spectrum of the preparation after Step 5 had a single peak at 280 mμ due to the presence of aromatic amino acids. No positive evidence for the presence of any prosthetic group was obtained by this method.

Enzyme activity was maximal at pH 6.2, but it was essentially unchanged between pH 5.5 and 7.2. Approximately 70 per cent of maximal activity was retained at pH 8.0. Initial velocity of NAD hydrolysis was proportional to the amount of the enzyme used. Under the conditions the reaction proceeded until all NAD was consumed up completely. Apparent Michaelis constant was calculated from Lineweaver-Burk plot as $K_M = 1.88 \times 10^{-3}$ M.

Inhibition of the enzyme activity by some of pyridine carboxylic acid derivatives was tested. As shown in Table 2, the hydrolysis of NAD was inhibited

Table 2. Inhibition by Pyridine-carboxylic Acid Derivatives. Reaction mixtures consisted of 0.1 ml of NAD (1 μmole), 0.4 ml (for Exp. 1) or 0.3 ml (for Exp. 2) of the enzyme (0.0449 mg protein per ml), 0.4 ml of inhibitor in M/15 phosphate buffer at pH 7.2, water to 1.0 ml. Incubated at 38°C for 1 hour.

Exp. No.	Addition	E_{325}	Inhibition %
1	None	0.3619	0
	Nicotinamide 5×10^{-2}M	0.2436	32.7
	Nicotinic acid 5×10^{-2}M	0.2410	33.4
2	None	0.2757	0
	Isonicotinamide 5×10^{-2}M	0.2442	11.3
	Isonicotinic acid 1.25×10^{-2}M	0.2308	16.2

only in the presence of rather high concentration of inhibitor. Nicotinoyl and isonicotinoyl hydrazides were also tested as for their inhibition, but very intensive absorption at 325 mμ, which developed by the addition of cyanide to these hydrazides, interferred with the NAD determination and made it impossible to get any definite result.

DISCUSSION

As already reported by Kern and Natale[1], NAD-ase activity of *Mycobacterium butyricum* was only detectable after boiling of the cell-suspension or of the extract. Starting from the extract of heat-treated cells 150 times purification was achieved by the repeated ammonium sulfate fractionation and DEAE-cellulose ion-exchanger chromatography, while Kern and Natale had got 15 times purification starting from boiled extract of untreated cells.

The properties of the enzyme thus prepared were essentially identical to what was reported by Kern and Natale. But NAD hydrolysis by the enzyme preparation was inhibited in the presence of 5×10^{-2}M nicotinamide, though the lack of the inhibition by 10^{-2}M nicitinamide had been reported.

Studying the inhibition by isonicotinoyl hydrazide, the formation of isonicotinoyl hydrazide analogue of NAD was suggested by the developement of yellow color after addition of potassium cyanide, which is strong alkaline, to the reaction mixture containing isonicotinoyl hydrazide, NAD and NAD-ase. But by the control tube, which contained the same components, but where NAD was added after incubation and addition of cyanide, the yellow color developed in almost the same degree as by the experimental tube. The role of NAD-ase in this exchange reaction is now under investigation.

In the preliminary work for the extraction of NAD-ase from *Mycobacterium butyricum* cells, it was noticed that cell-free extract prepared by mechanical grinding of cells with sea-sand in mortar did not possess any NAD-hydrolyzing activity before or after boiling, but unheated extract thus prepared inhibited the NAD hydrolysis by purified *Mycobacterium butyricum* NAD-ase. This suggests that the enzyme and its inhibitor locate in different subcellular units but not as a complex.

The distribution of NAD-ase and its inhibitor in *Mycobacterium* and the specificity of the inhibitor will be published elsewhere.

REFERENCES

1. Kern, M. and Natale, R. *J. Biol. Chem.* **231** (1958) 41.
2. Artman, M. and Bekierkunst, A. *Proc. Soc. Exptl. Biol. Med.* **106** (1961) 610.
3. Zatman, L. J., Kaplan, N. O., Colowick, S. P. and Ciotti, M. M. *J. Biol. Chem.* **209** (1954) 453.
4. Zatman, L. J., Colowick, S. P., Kaplan, N. O. and Ciotti, M. M. *Bull. Johns Hopkins Hosp.* **91** (1952) 211.
5. Zatman, L. J., Kaplan, N. O., Colowick, S. P. and Ciotti, M. M. *J. Biol. Chem.* **209** (1954) 467.
6. Oka, S., Konno, A. and Nagayama, H. *Kekkaku* **36** (1961) 552.
7. Eda, T., Yokota, K. and Akiba, H. *Kekkaku* **37** (1962) 440.

Received March 26, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 165–S 169

The Effect of Hypophysectomy on the Concentration in Serum of an Inhibitor of Hypophyseal Proteinases

U. J. LEWIS, J. N. FISHER and W. P. VANDERLAAN

Division of Endocrinology, Scripps Clinic and Research Foundation, La Jolla, California, USA

The fragmentation of growth hormone by hypophyseal proteinases is inhibited by rat serum. Hypophysectomy causes an initial two-fold rise in the amount of inhibitor in the serum of the rats. Administration of bovine growth hormone or bovine thyrotropin does not prevent the increase. The inhibitor from human serum was partially purified by chromatography on DEAE-cellulose and by preparative disc-electrophoresis.

The serum of normal rats has been found[1] to contain a substance which inhibits the fragmentation[2] of growth hormone by hypophyseal proteinases. The inhibitor was shown to be heat-labile and non-dialyzable. The concentration of the inhibitory substance was also found to be influenced by the function of the thyroid gland[1]. The inhibitor was high in early stages of induced hypothyroidism and abnormally low in rats made hyperthyroid. In this communication we report that hypophysectomy causes an initial increase in the concentration of the inhibitor in serum. A method for partially purifying the inhibitory substance in human serum is also described.

MATERIALS AND METHODS

Electrophoretic analysis. The fragmentation of growth hormone was observed by electrophoretic analysis on columns of polyacrylamide. The method has been named "disc-electrophoresis" by its inventors, Ornstein and Davis[3]. The apparatus is available commercially from Canal Industrial Corporation, Bethesda, Maryland. The use of the technique in the study of pituitary hormones has been described[1,2,4].

Preparative disc-electrophoresis. Initial attempts to isolate the inhibitor from whole serum were unsuccessful because of instability of the substance. We found that a commercially available fraction of human serum (α-globulins, Fraction IV, California Corporation for Biochemical Research, Los Angeles, California) was rich in the inhibitor and at this stage of purification the substance was not inactivated during subsequent processes of isolation.

Disc-electrophoresis can be used as a preparative method by cutting out sections of unstained columns and eluting the protein. Usually an identical column is stained for the purpose of locating the components that are resolved by the electrophoresis. This was not necessary

for the α-globulin-fraction. By adding bromphenolblue to the sample, the albumin was stained blue and since transferrin has a reddish-brown color, we had two markers for determining where to cut sections from the columns of gel.

Columns 1 cm in diameter and 7 cm in length (small pore gel[3]) were used. To each column 2 mg of the α-globulin-fraction was added. Electrophoresis was carried out exactly as described by Ornstein and Davis[3]. When the buffer-front was within 1 cm of the end of the column, the polyacrylamide gel was removed from its glass tube and 4 sections were cut from the gel (Fig. 1). The protein in the sections of gel was eluted electrophoretically into dialysis sacking as described previously[4]. The recovered protein was dialyzed overnight against water and then lyophilized. All processes were carried out at 5°C.

Chromatography on DEAE-cellulose. The inhibitor in the α-globulin-fraction was adsorbed by DEAE-cellulose that had been equilibrated with 0.005 M phosphate buffer, pH 7. Many electrophoretically slowly migrating components, including transferrin, were eluted from the column with 0.04 M K_2HPO_4. The inhibitor together with albumin were then removed with 0.2 M K_2HPO_4. A 3-fold purification was achieved by this procedure.

Bovine growth hormone. This hormone has been found[2] to be contaminated with varying amounts of proteinases depending on the procedure used in its isolation. The preparation used in these studies was isolated by a combination of the methods of Campbell and Davidson[5] and Wilhelmi *et al.*[6] as suggested by Hays and Steelman[7]. The kind and amount of contaminating proteinases make a convenient system for studying the fragmentation of the hormone.

Incubation procedure. Bovine growth hormone readily undergoes fragmentation[2] by contaminating proteinases when allowed to stand at neutrality or at weakly alkaline conditions and the degradation is inhibited by the addition of serum[1]. These observations form the basis of the test used to detect the inhibitor in serum. The test is carried out by first thoroughly suspending the hormone in 0.05 M phosphate buffer, pH 7, to give a concentration of 5 mg/ml. Then to 0.2 ml of this suspension is added 2 μl of a 1:10 dilution of whole serum prepared in the same phosphate buffer. A drop of toluene is added, and the tubes are stoppered and placed at 37°C for 8 hours. After incubation 25 μl-aliquots are analyzed by disc-electrophoresis to determine the degree of degradation of the growth hormone.

Maintenance of rats and collection of serum. Mature, male rats weighing about 200 g were used. They were fed a diet of Purina mink chow. Hypophysectomy was performed either by

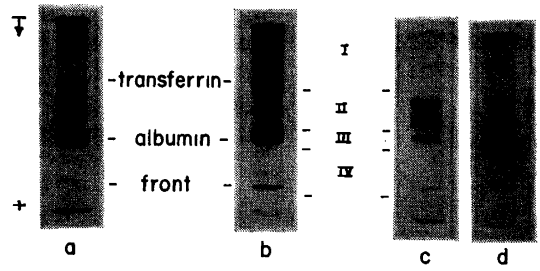


Fig. 1. Disc-electrophoretic patterns of human serum and fractions obtained from serum. *a*, a sample of whole serum; *b*, the α-globulin-fraction used as starting material for isolation of the inhibitor; *c*, the proteins that were isolated by preparative electrophoresis from section II of the column shown in *b*; *d*, albumin isolated from section III.

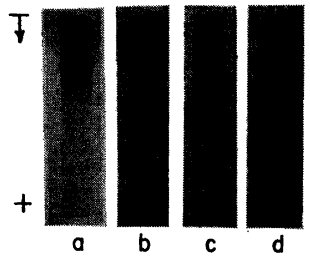


Fig. 2. Effect of rat serum on the fragmentation of bovine growth hormone. *a*, electrophoretic pattern of unincubated hormone; *b*, the same material after incubation at 37°C for 8 hours; *c*, the hormone incubated with serum from a normal rat; *d*, the hormone incubated with serum from a hypophysectomized rat.

the parapharyngeal or by the transaural[8] route. At sacrifice the rats were anesthetized with ether, and blood was removed from the left renal vein, and the sella turcica was examined for completeness of hypophysectomy. After the blood had clotted, the serum was collected by centrifugation. The inhibitor is inactivated upon prolonged standing in whole serum; therefore, the serum was used as soon after collection as was practical. Rats that were treated with growth hormone or thyrotropin received intraperitoneal injections daily. Dosage of growth hormone was 0.5 mg/day and of thyrotropin, 25 milliunits/day.

RESULTS

Inhibition by serum from hypophysectomized rats. We have found[1] that bovine growth hormone degrades in a stepwise manner when allowed to stand at room temperature, whereas an overall decrease in intensity of the bands is observed when the hormone is incubated at 37°C. Serum inhibits both reactions. In these studies we have used the higher temperature because of the shorter time required to observe fragmentation. Prevention of the decrease in intensity of the bands of growth hormone was used as the criterion for inhibition.

Fig. 2 illustrates how serum inhibits the overall decrease in the intensity of the bands of growth hormone and also how much more strongly the serum from hypophysectomized rats inhibits as compared to serum from normal rats. The rats had been hypophysectomized two weeks before sacrifice. The normals were the same age. The increase in the amount of inhibitor in the serum of hypophysectomized animals was estimated to be at least two-fold. The estimate was made by diluting the serum of hypophysectomized rats until the degree of inhibition matched that of the normal animals. Administration of bovine growth hormone or bovine thyrotropin during the two weeks following hypophysectomy did not prevent the rise in the amount of inhibitor. Normal human serum inhibited the degradation process in the same manner as did rat serum.

The protein-bound iodine[9] in the serum of normal rats was 3.2 μg/100 ml of serum; hypophysectomy reduced the value to 0.8 μg/100 ml. Administration of bovine growth hormone or thyrotropin did not restore the amount to normal limits.

Essentially the same rise in inhibitor was noted in the serum of rats that had been hypophysectomized either by the parapharyngeal or aural route. There is, perhaps, a greater possibility of damage to the hypothalamus when the rats are operated upon through the otic canal, but apparently if any such damage did occur, it did not prevent the rise in the amount of inhibitor in the serum. No increase in the inhibitor was noted after a sham operation by the transaural approach which left the pituitary gland undisturbed.

The increase in inhibitory properties of the serum was not due to a rise in total serum-protein since no more than a 5 % increase in total protein was noted in certain animals and the higher values of protein did not always correlate with increased inhibitory action. Heat-inactivated serum was not inhibitory indicating that the inhibition was not caused by an increased amount of substrate for the proteinases.

Fig. 1*a* shows the disc-electrophoretic pattern of a sample of human serum. Photograph *b* is the pattern obtained with the α-globulin-fraction of human serum used as starting material for the isolation of the inhibitor. As can be seen,

the material does not appear much different from normal serum except for the transferrin. The section designated as II had the highest concentration of the inhibitor. The disc-electrophoretic pattern of the proteins of this section after they were isolated by preparative disc-electrophoresis is shown in *c*. Either albumin was not completely separated from this section when the columns were cut, or perhaps minute quantities of albumin were adsorbed by the polyacrylamide as the albumin passed through the column. This albumin would then have been eluted and concentrated during the isolation of the protein in section II.

2 μg of protein from section II, when incubated with 1 mg of growth hormone, produced approximately the same inhibition as 22 μg of the starting material. 14 μg of material from section III gave an inhibition equivalent to 2 μg of section II.

Fig 1*d* represents the pattern obtained when the albumin-band (section III) was analyzed after isolation by preparative disc-electrophoresis. Unexpectedly, two slowly migrating bands were observed. Since these components could not have migrated to the area of albumin before the column was cut into sections, we assume that they are peptides or proteins that had been adsorbed upon the albumin and which became dissociated during the process of isolation.

DISCUSSION

The increase in the amount of inhibitor after hypophysectomy is similar to the rise seen when rats are made hypothyroid by feeding propylthiouracil[1]. These observations suggest that when the concentration of thyroxine in serum is lowered, either by loss of thyrotropin after hypophysectomy or by suppression of thyroid activity by propylthiouracil, one of the physiologic responses is the production of an increased amount of the inhibitor. If this suggestion proves to be correct, the failure of bovine thyrotropin to prevent the rise in inhibitor could be explained by the fact that the protein-bound iodine values were uneffected by administration of the thyrotropin. Related to this is the observation of VanderLaan and Greer[10] that thyrotropin does not cause a decrease in iodine content of the thyroid gland of the rat after hypophysectomy. It will be of interest to determine if thyroxine prevents the rise in the inhibitor in hypophysectomized animals. In any case we feel that the data presented here definitely indicate that the proteolytic inhibitor is under endocrine control, and this in turn suggests that control of the enzymatic fragmentation of growth hormone has physiologic significance.

The presence of some inhibitor in section III may be a result of imperfect sectioning of the columns. Another possibility is that there is more than one inhibitor and that one migrates in section II while the other travels in the area of albumin. Martin[11] has also made this suggestion to explain results he obtained during the isolation of an inhibitor of trypsin in ovine serum. It is quite logical to assume that a number of different proteolytic inhibitors are needed to control the many types of proteinases in the body.

Acknowledgments. This work was supported by Grants A-1328 and 2A-5249 from the *United States Public Health Service* and by a grant from the *Timken-Sturgis Foundation.* One of us (U. J. L.) acknowledges the receipt of a Career Development Award from the *National Institutes of Health.*

REFERENCES

1. Lewis, U. J. and VanderLaan, W. P. *J. Biol. Chem. In press.*
2. Lewis, U. J. *J. Biol. Chem.* **237** (1962) 3141.
3. Ornstein, L. and Davis, B. J. *Disc Electrophoresis,* preprinted by Distillation Products Industries, Rochester, New York, 1962.
4. Lewis, U. J. and Clark, M. O. *Anal. Biochem. In press.*
5. Campbell, J. and Davidson, I. W. F. *Abstracts of the 19th International Physiological Congress,* Thérien Frères, 1953, p. 257.
6. Wilhelmi, A. E., Fishman, J. B. and Russel, J. A. *J. Biol. Chem.* **176** (1948) 735.
7. Hays, E. E. and Steelman, S. L. In Pincus, G. and Thimann, K. V. *The Hormones,* Academic Press, Inc., New York, 1955, Vol. III, p. 203.
8. Tanaka, A. *Annual Report of Shinonogi Research Laboratory,* 1955, p. 5.
9. Zak, B., Willard, H. H., Myers, G. B. and Boyle, A. J. *Anal. Chem.* **24** (1952) 1345.
10. VanderLaan, W. P. and Greer, M. A. *Endocrinology* **47** (1950) 36.
11. Martin, C. J. *J. Biol. Chem.* **236** (1961) 2672.

Received March 18, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 170–S 173

Experimental Infectious Arteritis and Ulcers of the Gastric Mucosa

GUNNAR NORLIN

Chemistry Department II, Karolinska Institutet and Chemistry Department, Statens Bakteriologiska Laboratorium, Stockholm, Sweden

Rabbits were injected repeatedly with β-hemolytic streptococci. Besides lesions of other organs superficial ulcers were observed in the gastric mucosa of the animals.

As the arteries in the submucosa showed degenerative and proliferative inflammation the cause of the lesions was likely to be insufficient blood supply.

During experimental work on rheumatic diseases Norlin[1] observed black spots on the inner surface of the stomach wall. These spots were examined histologically.

MATERIAL AND METHODS

Experimental procedure. Rabbits were divided into three groups. Group 1 contained 10 animals, which were injected intravenously first with heat killed β-hemolytic streptococci for three weeks. Then living streptococci were injected for four months. The injections were given with intervals of 3 to 6 days. The doses were always 2 ml of a 20 hours' streptococcal culture grown on Todd-Hewitt[2] broth. The strain used was the type 2 strain Rockefeller T-2/44/RB4. By mixing avirulent and virulent mutants of the strain the infection was so arranged that the rabbits got bacteria of continuously rising average virulence. The food in this group was hay, oats and Swedish turnips.

Group 2 consisted of 26 rabbits which were given 175 ml of a mixture of equal parts of oats and rabbit pellets per day. To this food 0.5 g cholesterol and 5 g margarine was added to the daily ration.

Group 3 included 16 rabbits and they got the same "atherogenic diet" as those of group 2 and also streptococcal infections after the scheme described for group 1.

Histological technique. Necropsies were performed as soon after death as possible. The tissues were fixed in 10 % formalin solution, and sections cut from paraffin blocks were stained by Weigert's hematoxylin and eosin and by van Gieson's method.

RESULTS

In the respective groups 2, 2, and 6 rabbits had ulcers in the gastric mucosa. This makes only 19 % (10/52) on the whole material.

As a control there were used: 4 normal rabbits, 10 rabbits injected for 3 weeks with *E. coli*, 12 infected intracutaneously with vaccinia, 7 immunized

Fig. 1. Rabbit 183. Ulcer in gastric mucosa with infiltrated and necrotic margins.

Fig. 2. Rabbit 183. Obliterating arteritis in the submucosa beneath an ulcer.

Fig. 3. Rabbit 206. Obliterating endarteritis in the bottom of a shallow ulcer.

Fig. 4. Rabbit 218. Degenerated artery with "lipoid cells" beneath a large shallow ulcer.

with fungi *(Epidermophyton floccosum, Keratomyces ajellii, Trichophyton equinum, Trichophyton Schoenleini or Tricophyton butonrougei)* for 6 weeks and 7 others with the same fungal strains during 8–13 months. Of these 40 animals 5 had suspected superficial dark spots in the gastric mucosa, four of which were hardly visible and without microscopical lesions. Only one had a 1 mm greyish erosion that was seen in the microscope as a slight defect in the most superficial layer of the mucosa.

In the animals suffering from the sequelae of an infection or of atherogenic diet or both there were distinct ulcers 1–6 mm in diameter with more or less severe hemorrage, granulations or fibrous tissue and degeneration or necrosis of the cells of the mucosal glands. In no case a chronic ulcus proceeding through the *lamina muscularis mucosae* was seen.

In group 1 both ulcers had sharp margins and necrosis and hemorrage (Fig. 1) was distinctly separated from undamaged tissue. The arteries beneath the ulcers were the seats of obliterating endarteritis (Fig. 2).

In the two latter groups of rabbits where atherogenic diet was given the mucosal ulcers were shallow and with less distinct limits between unaffected and damaged tissue. When the ulceration went down to the bottom of the glandular layer the sloping edge surrounded a digested center that was several millimeters in diameter. Whether streptococci were given or not the supplying arteries were swollen and contained abundant "lipoid cells" (Figs. 3 and 4).

Further work is needed to find out if there is any real difference between the morphology of the ulcers in the different groups.

DISCUSSION

Experimental peptic ulcers have been produced by surgical operations as in the Shay rat, where the pylorus was closed or in the Mann-Williamson dog where the pylorus was sutured to the jejunum and the duodenum to the ilium. Later investigators[5] definitely ascribe these mostly marginal ulcers to impaired blood supply of the sutured areas.

Excessive doses of histamine[6] also give rise to gastric ulcers but as the amounts of histamine were far beyond what can be reached physiologically[7,8] these experiments prove the importance of peptic digestion but have only partial interest for the etiology of ulcers.

A considerable experience definitely points at nervous influence and stress as important ulcerogenic factors[9]. The experimental "restraint ulcers" in animals enclosed in narrow tubes or boxes are, although definitely unphysiological, most likely explained as being caused by stress.

The stress reaction seems to be effected *via* hypothalamus by ACTH and adrenal cortex hormones which increase the gastric secretion and may impair the circulation[10].

It is quite instructive to read the classic text book by Boyd[11] where it is suggested that the common cause of peptic ulcer might be a hematogenous infection of the stomach wall and if the ulcer healed the patient might become reinfected and a fresh ulcer would form.

Several papers read at the 2nd World Congress for Gastroenterology last year accentuate the importance of vascular lesions for the origin of peptic ulcer. Especially Hoffman[12] definitely states that peptic ulcer was the result of a primary infarction.

Other recent papers[13,14] also point at the vascular factors as the most important cause for the rise and chronicity of gastric ulcers. De La Fuente-Chaas *et al.*[15] were able to show by perfusion with india ink that there were occluded blood vessels and a zone of intense ischemia in peptic ulcer. These results are in good agreement with the experimental findings of this paper.

Jahiel *et al.*[16] realizing the infectious and vascular components of the ulcerogenic process found it likely that hypersensitivity of the Arthus type could be a possible reaction mechanism. They studied their hypothesis experimentally and sensitized rabbits with horse serum directly into the stomach wall. After 1½–9 weeks an intravenous "shocking" injection of horse serum gave 50 % ventricular

damages from small infiltrates and erosions to necroses and bleeding ulcers.

Several authors[17,18] have observed Arthus reactions and ulcers in the stomach after general active or passive sensitization.

Norlin[19] has tried to explain the rheumatic lesions, including the arteritis, as caused by antigen-antibody complexes in antigen excess acting on an immunologically paralyzed host. This explanation is also valid for peptic ulcer as a result of a focal arteritis.

Acknowledgement. This work was supported by grants from *Svenska Nationalföreningen mot Hjärt- och Lungsjukdomar* and *Stiftelsen Therese och Johan Anderssons minne.*

REFERENCES

1. Norlin, G. *Acta Rheumatol. Scand.* **8** (1962) 297.
2. Todd, E. W. and Hewitt, L. F. *J. Pathol. and Bacteriol.* **35** (1932) 973.
3. Shay, H., Komarov, S. A., Fels, S. S., Meranze, D., Gruenstein, M. and Siplet, H. *Gastroenterology* **5** (1954) 43.
4. Mann, F. C. and Williamson, C. S. *Annal. Surg.* **77** (1923) 409.
5. Kuroyanagi, Y., Chiles, Th. and Necheles, H. *Am. J. Gastroenterol.* **38** (1962) 567.
6. Schmid, J. *Wien. klin. Wochenschr.* **1951** 377.
7. Levin, E., Kisner, J. B. and Palmer, W. L. *Gastroenterology* **12** (1949) 561.
8. Ihre, B. R. Boller, *Der Magen und seine Krankheiten,* Urban und Schwarzenberg, Wien und Insbruck 1954, p. 25.
9. Woldman, E. E. *J. Am. Med. Assoc.* **149** (1952) 984.
10. Gray, S. J., Ramsey, C., Reifenstein, R. W. and Benson Jr., J. A. *Gastroenterology* **25** (1953) 156.
11. Boyd, W. *A Text Book of Pathology,* Lea and Febiger, Philadelphia 1938, p. 526.
12. Hoffman, W. *Münch. Med. Wochenschr.* **104** (1962) 1679.
13. Polet, F., Conte-Marti, M., Conte, M. and Lambling, A. *Arch. Maladies Appareil Digest.* **50** (1961) 1038.
14. Modena, H. G. *Gastroenterology* **41** (1961) 174.
15. De La Fuente-Chaas, A. and Puente Dominguez, A. *Intern. Abstr. Surg.* **104** (1957) 354.
16. Jahiel, R., Jahiel, R. and Krakauer, J. *Proc Soc. Exper. Biol. and Med.* **79** (1952) 54.
17. Shapiro, P. F. and Ivy, A. C. *Arch. Int. Med.* **38** (1926) 237.
18. de Gara, P. F. and Angevine, P. M. *J. Exper. Med.* **78** (1943) 135.
19. Norlin, G. *Acta Rheumatol. Scand.* **8** (1962 141.

Received April 1, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 174–S 179

Fermentation as a Means of Preserving Organic Materials*

RAGNAR NILSSON and CONRAD RYDIN

Mikrobiologiska Institutionen, Lantbrukshögskolan, Uppsala, Sweden

In ensiling both vegetable and animal material, first-class silage is obtained if sufficient quantities of a meal mixture consisting of enzyme-rich malt and cereal are added to the fresh, finely divided fodder. This meal mixture supplies the organic material with lactic-acid bacteria and furthermore with the amino acids and vitamins which these bacteria require for their growth, and above all, with carbohydrates (starch), which are hydrolysed by the enzymes in the malt to sugar fermentable by the lactic-acid bacteria.

In ensiling forage crops with malt-cereal meal, it was found that the effluent can safely be kept in the silo by absorption in chopped straw mixed with the green fodder, thus avoiding large losses of nutrients from the fodder and rather heavy contamination of the surrounding water recipients.

Dried silage of fish, beef and blood, ensiled with malt-cereal meal, proved to be unobjectionable from a sanitary point of view.

Hens were fed for 18 months with silages made from rumen contents, fish and pigs' blood. Their state of health and egg production were good; the eggs and carcasses were of good quality and did not give any unpleasant taste or smell.

This new means of preserving organic matter is so simple that it can be practised at sea, using absolutely fresh fish which is directly ground together with the malt-cereal meal. There also appears to be a possibility of producing, after the necessary modifications, a cheap protein of biologically full value for human consumption.

In silage-making by purely biological processes the basis of positive conservation of the organic material is a lactic-acid fermentation which sets in so rapidly and is so intense that it extinguishes all other, undesirable, microbial or enzymatic activities. In order to make possible such a lactic-acid fermentation, relatively large quantities of carbohydrates fermentable by lactic-acid bacteria are required. In many types of feed- and foodstuffs the lack of such carbohydrates often limits the production of an efficient lactic-acid fermentation. Examples of such materials are lucerne, clover, brewer's grains, potatoes and, above all, animal products such as meat, fish, blood and slaughterhouse waste.

* United Nations Conference on the Application of Science and Technology for the Benefit of the Less Developed Areas, Genève 4–20 Feb. 1963. Agenda Item: D.4.2.

The types of lactic-acid bacteria, which appear to be responsible for most of the lactic-acid production, ferment different types of sugar, including certain pentoses (xylose, arabinose) as well as hexoses (glucose, fructose, galactose) and disaccharides (sucrose, maltose), producing chiefly lactic acid but also smaller quantities of acetic acid. Polysaccharides such as dextrin and starch are consider ably more difficult to ferment than these sugars.

In ensiling experiments with green fodder, in which different enzyme preparations with high amylase and cellulase activities, either alone or together with cereal meal, were added to material of low sugar content, it was possible to hydrolyse to a high degree polysaccharides not fermentable by lactic acid bacteria into fermentable sugar, so that a sufficient lactic-acid fermentation was produced to preserve the fodder properly. A meal of enzyme-rich malt produced by special methods from six-rowed barley proved to be particularly suitable for this purpose. Of particular interest is the ability of the enzymes in malt to attack and hydrolyse the *raw* native starch. In this hydrolysis α-amylase is an active enzyme. This enzyme does not exist in ungerminated grain but is formed during germination.

Besides the effect of the carbohydrases in the malt, the addition of the mixture of enzyme-rich malt-cereal meal (usually mixed in the ratio of 1 : 5) promotes the lactic-acid fermentation in the silage, owing to the fact that the meal mixture has a high content of various *Lactobacillus* species and therefore the addition of meal is accompanied by substantial inoculation with desirable microorganisms. The ensiling malt has a high content of certain free amino acids (valine, leucine, isoleucine, threonine, methionine, glutamic acid, tryptophane, phenylalanine) and certain B vitamins (riboflavine, B_6, pantothenic acid, biotin), which the heterotrophic lactic-acid bacteria require for their growth. After the addition of this malt-cereal mixture the lactic-acid bacteria in the silage seem to have optimal conditions as regards the substrate, which leads to rapid and substantial lactic-acid fermentation and thereby a good result in the ensiling. We would point out that, even without the addition of malt, meal of various kinds of cereal contains large amounts of lactic-acid bacteria and in addition smaller amounts of carbohydrates fermentable by lactic-acid bacteria and also growth factors for these bacteria. The addition of *large* quantities of cereal meal, without the simultaneous addition of malt, may therefore yield good results. However, the *large* quantities of cereal which are required in this connection and the uncertainty involved in the use of this supplement limit to a great extent the use of cereal meal *alone* as a supplement in ensiling.

Good-quality silage is characterized by low figures for ammonia nitrogen as a percentage of total nitrogen (NH_3-N of total N); this figure should be less than about 12 and furthermore butyric acid must only occur in insignificant quantities.

The losses during fermentation are greatly limited if vigorous lactic-acid fermentation is started *rapidly* and also if the effluent is not drawn off but allowed to remain in the silo, thereby being utilized as fodder. Silage appetizing to cattle has a high content of lactic acid and often some sugar also.

The results of some ensiling experiments with the addition of malt-cereal meal to various kinds of fodder will now be presented. The ensilings were carried out on a laboratory scale in silos of stainless steel containing 12–16 kg green fodder

Table 1. The effect of barley meal and malt-barley meal on the fermentation process in ensiling a crop of lucerne-grass. Ensiling time, 8 weeks. Temperature, 28°C.

Supplements	pH	Dry matter %	Sugar %	Lactic acid %	Butyric acid %	NH_3-N of tot. N %
Control: No supplements	5.6	17.1	0.09	0.7	0.82	30.5
Barley meal 3.0 %	5.3	17.8	0.08	0.6	0.89	23.9
„ „ 4.5 „	5.8	14.7	0.08	0.5	1.17	24.8
„ „ 6.0 „	5.0	19.8	0.14	1.9	0.46	13.4
„ „ 9.0 „	5.1	19.7	0.05	1.1	0.66	15.5
Malt meal 0.5 % + barley meal 2.5 %	4.3	19.1	0.23	2.7	0.07	9.3
„ „ 0.5 „ + „ „ 4.0 „	4.2	18.5	0.26	3.3	0.01	9.3
„ „ 0.75 „ + „ „ 2.25 „	4.2	19.6	0.20	2.7	0.01	8.7
„ „ 0.75 „ + „ „ 3.75 „	4.3	18.2	0.46	3.0	0.04	9.0

and in smaller silos of glass containing 2–3 kg fish, meat, blood, rumen contents and rumen wall. Effluent was not drawn off.

Table 1 shows analyses of lucerne-grass silage, partly without supplement and partly with the addition of various amounts of barley meal or mixtures of malt- and barley meal. It is clear from these analyses that the silages without supplement and with 3 % or 4.5 % of barley meal were a complete failure; 6 % or 9 % of barley meal improved the quality, though not to such an extent that the silage could be regarded as satisfactory. The supply of sugar fermentable by the lactic-acid bacteria was not sufficient to achieve sufficiently intense lactic-acid fermentation. The addition of 3 % or 4.5 % of mixtures of malt- and barley meal resulted in first-class silage. The enzymes in the malt attacked and sufficiently hydrolysed the starch in the meal mixtures, so that an adequate quantity of sugar was available for the lactic-acid bacteria. In the silages with a malt supplement lactic-acid fermentation greatly predominated, thus largely precluding the processes of decomposition and butyric-acid fermentation. Silages with low contents of butyric acid often have low contents of spores of butyric-acid bacteria, a fact which may be of importance when silage is fed to cows whose milk is used in the production of certain kinds of cheese.

Animal material such as fish (cod and herring), meat, rumen contents, rumen wall, blood and the like have been successfully ensiled by admixture of malt-cereal meal to the finely ground, fresh material. Within the temperature range of 20–28°C the material can safely be kept for 4 weeks. After that lower storage temperatures should be aimed at. As far as is known up to the present, however, there is no objection to using the material even before complete fermentation. The lactic-acid fermentation is essential when it is a question of *conserving* the material, but does not seem to be necessary to make it suitable as a feed-stuff, though it seems possible to foresee that this fermentation may be of great im-

Table 2. The effect of oats meal and malt-oats meal on the fermentation process in ensiling small Baltic herring. Ensiling time, 4 weeks. Temperature, 28°C.

Supplements	pH	Dry matter %	Lactic acid %	Butyric acid %	NH_3-N of tot. N %
Control: No supplements	7.3	14.9	0.6	2.91	55.4
Oats meal 20 %	6.8	32.3	0.5	3.18	38.8
„ „ 25 „	5.5	35.8	3.3	1.05	25.4
„ „ 30 „	4.9	46.2	4.6	0.04	15.9
Malt meal 2 % + oats meal 18 %	4.5	34.6	5.7	0.01	14.2
„ „ 2 „ + „ „ 23 „	4.4	34.2	5.7	0.01	13.3
„ „ 2 „ + „ „ 28 „	4.4	45.3	4.9	0.01	11.3
Malt meal 4 % + oats meal 16 %	4.6	34.4	5.4	0.01	12.6
„ „ 4 „ + „ „ 21 „	4.5	37.7	5.4	0.01	12.3
„ „ 4 „ + „ „ 26 „	4.5	45.4	5.0	0.01	10.8

portance as regards material infected with pathogenic germs. Silage made with the kinds of fodder mentioned is easy to dry. The result of ensiling experiments with animal material will now be presented.

Table 2 shows analyses of silage of small Baltic herring, which, after having been ground in an ordinary meat-mincer were ensiled partly without and partly with an addition of either oats meal or malt- and oats meal in various mixing ratios and quantities. When sufficient amounts of the mixture of malt- and oats meal were added to the material, the quality of the silage was first-class. Silage with this supplement was incomparably better than silage with a similar supplement of oats meal alone.

Table 3. The effect of malt-barley meal on the fermentation process in ensiling small Baltic herring. Ensiling time, 4 weeks. Temperature 20, 28 or 37°C.

Temp. °C	Supplements	pH	Sugar %	Lactic acid %	Butyric acid %	NH_3-N of tot. N %
28	Control: No supplements	7.1	–	0.6	1.98	43.9
20	Malt meal 4 % + barley meal 20 %	4.3	3.5	5.2	0.01	8.0
	„ „ 6 „ + „ „ 30 „	4.4	5.4	4.8	0.01	7.4
	„ „ 8 „ + „ „ 40 „	4.4	6.5	4.7	0.01	7.6
28	Malt meal 4 % + barley meal 20 %	4.3	3.4	5.0	0.01	8.9
	„ „ 6 „ + „ „ 30 „	4.4	5.1	4.8	0.01	7.7
	„ „ 8 „ + „ „ 40 „	4.3	6.9	4.5	0.01	7.7
37	Malt meal 4 % + barley meal 20 %	4.2	1.0	5.9	0.23	12.8
	„ „ 6 „ + „ „ 30 „	4.1	2.3	5.5	0.12	10.3
	„ „ 8 „ + „ „ 40 „	4.1	4.4	5.0	0.04	9.9

Table 4. The effect of malt meal and cereal (oats-barley) meal (1 : 5) on the fermentation process in ensiling cod-fish, beef and pigs' blood. Ensiling time, 7 – 10 days. Temperature, 28°C.

Material (ground)	Malt-cereal meal (1 : 5)	pH	Sugar %	Lactic acid %	Butyric acid %	NH_3-N of tot. N %
Cod fish (with skin and bone)	50 %	4.5	4.68	3.8	0.0	6.1
„ „ – „ –	50 % + NaCl 2.25 %	4.5	5.54	3.8	0.0	6.3
Cod fish (filet)	33 %	4.2	5.46	3.4	0.0	6.7
„ „	50 „	4.6	6.06	4.0	0.0	8.5
Beef meat	33 %	4.2	4.10	2.3	0.0	1.8
„ „	33 % + NaCl 1.25 %	4.2	4.40	3.2	0.0	2.3
Blood (pigs)	21 %	4.0	2.26	3.9	0.01	2.0
„	50 „	4.1	6.50	3.5	0.0	1.8

Table 3 shows analyses of silage of small Baltic herring with malt-barley meal (1 : 5) in various quantities and ensiled at different temperatures. Fermentation temperatures of 20–28°C throughout yielded first-class silage. At 37°C the quality was poorer. It is nevertheless obvious that there is a possibility of utilizing sensitive organic material by this method even under tropical conditions.

The usefulness of the method, as concerns animal material, may be illustrated by Table 4. This table shows silage analyses of animal material which may possibly be used as food for human consumption. As is evident, very large quantities of the malt-cereal mixture were used. This was in order to guarantee a very rapid lactic-acid fermentation and thereby an effective inhibition of the processes of decomposition and consequently toxin formation. The very high contents of sugar in these silages indicate that probably unnecessarily large quantities of the meal mixture were used.

Dried silages made from cod-fish, meat and blood, using our method, have been investigated bacteriologically by the Swedish National Institute of Public Health and found to be unobjectionable as regards human consumption. Further ensiling experiments with animal products, including infection experiments with pathogenic bacteria, must certainly be carried out before such silage can be recommended for human consumption.

Table 5 shows the results of ensiling the contents of the rumens of slaughtered cows. As the table shows, malt-cereal meal (1 : 5), together with straw meal, was used as a supplement.

The rumen contents of slaugtered cows vary in weight between 60 and 90 kg per animal. The dry-matter content is low but the content of vitamins (thiamine, riboflavin, nicotinic acid, B_{12}) and valuable protein from micro-organisms is relatively high.

It is clear from the table that the non-fermented rumen contents held a few tenths per cent of lactic acid and butyric acid. By the addition of malt-cereal meal a satisfactory silage for animal feeding is obtained.

Silage made from the contents of the rumens of slaughtered cows which had

Table 5. The effect of malt meal and cereal (oats-barley) meal (1 : 5) and straw meal on the fermentation process in ensiling rumen contents from slaughtered cows. Ensiling time, 10 days. Temperature, 28°C.

Supplements	pH	Dry matter %	Sugar %	Lactic acid %	Butyric acid %	NH_3-N of tot. N %
Control: No supplements, not fermented	6.9	11.3	0.08	0.2	0.15	5.4
Control: No supplements, fermented	5.6	9.6	trace	0.2	0.32	25.3
Malt-cereal-meal (1 : 5) 6 % + straw meal 6 %	4.3	16.5	0.11	1.8	0.36	13.6
Malt-cereal-meal 9% + straw meal 9 %	4.2	19.1	0.19	2.1	0.36	12.7
Malt-cereal-meal 12 % + straw meal 12 %	4.0	22.4	0.88	2.3	0.34	11.6

been at pasture was fed fresh to hens. Dried and ground silage made from the contents of the rumens of slaughtered cows which had been stable-fed was fed to pigs. Both these kinds of animals consumed the silages with avidity.

Small-scale feeding experiments have been going on for 18 months at the Department of Microbiology with hens (2×24), which are fed with fresh or dried silage made from clover-grass, rumen contents, rumen wall, fish and blood. The laying percentage is 60–70 %. It has not been possible to demonstrate any fishy taste in the eggs or in the carcases. The quality of the egg shell is good.

Our ensiling method, using vegetable crops, has been applied full-scale under practical conditions on Swedish farms in altogether about 100 ensilings during the past 5 years with good results.

Received April 2, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 180–S 183

Reactions of Thiosulphate with Proteins

TADEUSZ W. SZCZEPKOWSKI

Department of Physiological Chemistry, Academy of Medicine, Craców, Poland

It has been shown that serum proteins corresponding electrophoretically to α_1-globulin bind the whole molecule of thiosulphate, whereas albumin, α_2- and β-globulins bind only the outer sulphur of thiosulphate. Oxytocine and papain bind the whole molecule of thiosulphate presumably to the disulphide group. In view of the possibility that thiosulphate reacts with the disulphide grouping, the rate of reduction of cytochrome *c* by thiosulphate in the presence of either cystine or cystamine was investigated. An acceleration of reduction rate was found in both cases.

Sulphur compounds are known to form a variety of mixed derivatives, but little information is available in the literature as to their possible role in biological systems, except for mixed disulphides. In the present paper experiments with thiosulphate as one of the intermediates of sulphur metabolism are reported. The studies involved some reactions of thiosulphate with proteins. Attention was also paid to the reduction of cytochrome *c* by thiosulphate in the presence of disulphide.

EXPERIMENTAL

Materials and methods

Radioactive thiosulphate labelled with ^{35}S in the inner or outer position (Sp. activity 10.6 mc/mM) were provided by the Radiochemical Centre, Amersham, England. Cytochrome *c*, pharmaceutical product of Biomed, Craców, was obtained from beef hearts by the adsorption method and proved to be spectrally pure according to Paléus[1]. Papain was purified from Light's preparation by the method of Kimmel and Smith[2]. The oxytocine used was a pharmaceutical product of Sandoz, Basel (10 IU/ml). All reagents used were either analytical grade or proved to be pure. Electrophoresis and chromatography were carried out on Whatman No. 1 filter paper. Reduction of cytochrome *c* was estimated spectrophotometrically with Hilger's "Uvispec". The estimation of radioactivity on paper strips was made with the automatic scanning device of Frieseke and Hoepfner.

RESULTS

1. Reactions of thiosulphate with serum proteins were investigated in fresh human sera. Thiosulphate labelled in the outer or in inner position was added in the proportion of 1 mg to 0.5 ml of serum. The added radioactivity was in the

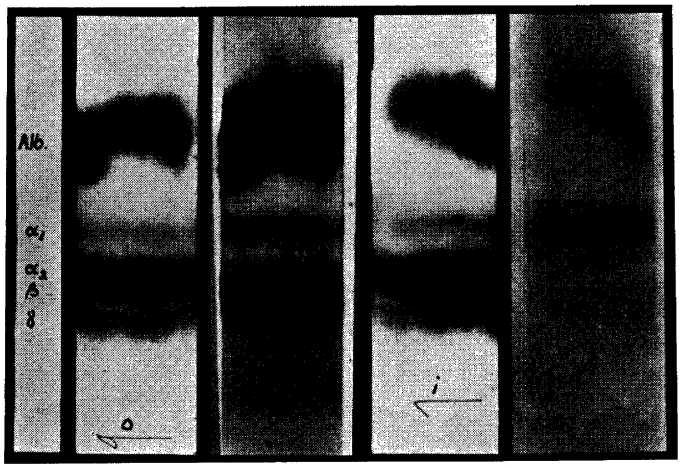


Fig. 1. Electrophorograms (o, i) and corresponding autoradiograms (O, I) of serum proteins incubated with thiosulphate labelled with ^{35}S : o, O — outer sulphur radioactive, i, I — inner sulphur radioactive.

range of 40–60 μC. After incubation for 30 min the serum was applied to paper strips and submitted to electrophoresis in veronal buffer, pH 8.6, ionic strength 0.05. Under experimental conditions free thiosulphate migrated beyond the paper strips. Autoradiograms were prepared from the electrophorograms and then the proteins were located with bromothymol blue. Typical results are represented in Fig. 1. It can be seen that radioactive sulphur has been bound to proteins in a different way depending on whether it occupied the inner or outer position in the thiosulphate. Radioactivity of the inner sulphur of thiosulphate occurred only in the fraction corresponding to α_1-globulin, whereas radioactivity of the outer sulphur atom was bound to albumin, α_1-, α_2- and β-globulins. This observation was supported by radioactivity measurements of the electrophorograms (before staining) and equal values were found for the α_1-globulin band regardless of the position of radioactive sulphur in the thiosulphate. These results are interpreted to mean that α_1-globulin combined with whole molecules of thiosulphate, whereas the other protein fractions were acceptors for outer sulphur only.

2. In view of the possibility that thiosulphate reacts with the disulphide grouping, the products formed with oxytocine were investigated. To a neutralized oxytocine solution of 0.5 ml volume was added 0.1 mg of differently labelled thiosulphate, and the mixture incubated for 30 min. The aliquots were then chromatographed on filter paper using butanol-acetone-water (2:2:1) as solvent, or submitted to electrophoresis. The corresponding autoradiograms revealed minor spots perhaps due to some impurities but the main pictures were similar for the two kinds of thiosulphate. Thus it may be concluded that the whole thiosulphate molecule is bound by the oxytocine moiety.

3. Similar experiments with papain and thiosulphate labelled in the outer or

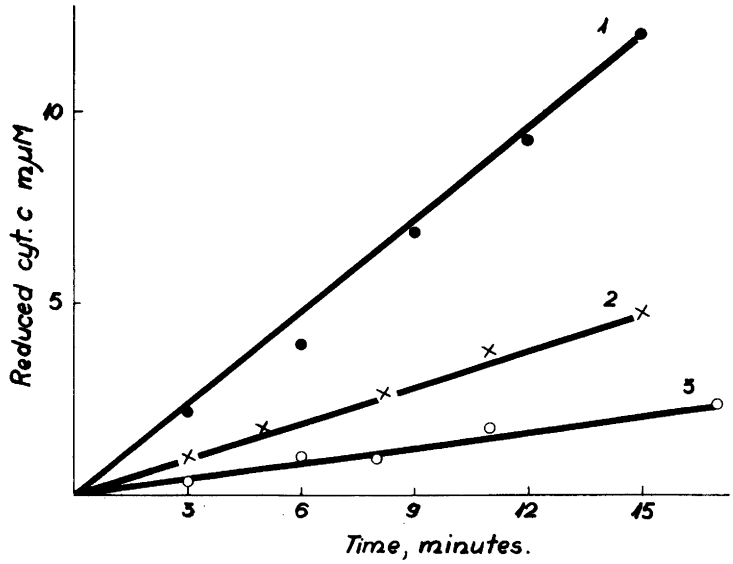


Fig. 2. The rate of reduction of 0.1 μmoles of cytochrome *c* by 10 μmoles of thiosulphate in the presence of: (1) 1 μmole cystamine; (2) 0.1 μmoles cystine; (3) 0.2 ml serum. Medium: 0.1 M phosphate buffer, pH 7. Final volume 2.5 ml. Blank values for the reduction due to thiosulphate alone was about 0.4 mμmoles and that for serum alone about 0.8 mμmoles of cytochrome *c* after 15 min. Temp. 14°C.

inner position showed that in either case radioactivity was bound to the protein, as estimated on the basis of electrophorograms and autoradiograms. It appears then that papain as well as oxytocine is capable of binding the whole molecule of thiosulphate.

4. As shown by the experiments with oxytocine and by earlier work on the reaction of thiosulphate with cystine[3], thiosulphate can react with disulphide linkage. Thus it seemed probable that thiol groups formed in the course of reaction could alter the reductive properties of the solution. In order to prove such an assumption, the rate of reduction of cytochrome *c* by solutions of thiosulphate in a mixture with disulphide, *e. g.* cystine or cystamine, was studied. Neutral and dilute solutions of thiosulphate were found to reduce cytochrome *c* only very slowly. However, as can be seen in Fig. 2, the rate of reduction increased in the presence of disulphides. Serum also stimulated the reduction, though very slightly, and this effect might perhaps be related to the results of a preceding experiment.

DISCUSSION

Thiosulphate is known to be formed in sulphur metabolism as an intermediate[4,5], quickly undergoing oxidation in the animal organism[6]. It therefore seemed desirable to examine the possibility that thiosulphate reacts in biological systems. Two atoms of sulphur may be distinguished in the thiosulphate mole-

cule, and so two kinds of labelled thiosulphate are available, one labelled with ^{35}S in the outer position ($^*S \cdot SO_3^=$) and the other labelled in the inner position ($S \cdot {}^*SO_3^=$). The experiments described in this paper have shown that thiosulphate is not an inert compound and can in fact react with proteins as well as with disulphides. It also appears to be involved in the reductive processes. The experiments with serum proteins indicate that in its reactions with different electrophoretic fractions, thiosulphate can be bound in the form of the whole molecule or give off outer sulphur only. The combination of outer sulphur with the protein moiety has so far only been reported in the case of rhodanese[7,8]. The present experiments show that this kind of binding is more common.

REFERENCES

1. Paléus, S. *Acta Chem. Scand.* **14** (1960) 1743.
2. Kimmel, J. R. and Smith, E. L. *J. Biol. Chem.* **207** (1954) 515.
3. Szczepkowski, T. W. *Nature* **182** (1958) 934.
4. Sörbo, B. *Biochim. Biophys. Acta* **24** (1957) 324.
5. Szczepkowski, T. W., Skarzyński, B. and Weber Miroslawa. *Nature* **189** (1961) 1007.
6. Trachtenberg, *Dissertation,* Dorpat 1861; cited after Pirie, N. W. *Biochem. J.* **28** (1934) 1063.
7. Szczepkowski, T. W. *Acta Biochim. Polon.* **8** (1961) 265.
8. Westley, J. and Nakamoto, T. *J. Biol. Chem.* **237** (1962) 547.

Received March 26, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 184–S 189

Studies on Flavin Semiquinone

YASUYUKI OGURA, SATOSHI NAKAMURA
and TAKAO NAKAMURA

Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Tokyo, Japan

The nature of reddish compound arising in oxido-reduction of flavins was investigated by measuring its spectrophotometric properties and electron spin resonance, and it was proven to be a semiquinone. Descriptions were made of the procedures for determining the absorption spectrum of the semiquinone in the presence of other oxidoreductive forms of flavins and for evaluating the equilibrium constants between these various forms. Using the values of the equilibrium constant, the spectra of the intermediate semiquinones, both of RF and FMN, were determined in the range from 230 to 580 mμ.

The reddish intermediate occurring in the course of oxido-reduction of flavins in strongly acidic solutions has been thought to be the semiquinone form of flavins[1–6]. In their previous work the present authors[7] have determined, by analysing graphically the spectra of equilibrium mixtures of the oxidized and reduced forms of FMN*, the absorption spectrum and the extinction coefficients in the visible region of the intermediate semiquinone. In the present work, the measurement was extended to the ultraviolet region using not only FMN but also RF. With both forms of flavins the semiquinone nature of the red-colored intermediates was definitely proven by measuring their ESR.

EXPERIMENTAL

The samples of FMN and RF used were those commercially obtained, which have been confirmed to be chromatographically pure. The concentrations of both flavins in their test solutions were determined spectrophotometrically by taking their molecular extinction coefficient as 12.2×10^3 $M^{-1}cm^{-1}$ at 450 mμ and pH 7.0[8]. Spectrophotometric measurements were performed using a Hitachi photoelectric spectrophotometer model EPU-2A. The pH's of test solutions were measured after each experiment with a glass electrode pH-meter model HM-5A of the Toa Electronic Co. The ESR spectrometer used was a model JES-3B X-band instrument of Japan Electron Optics Laboratory Co. which was combined with a 30 cm magnet and a proton resonance fluxmeter. The microwave frequency was measured by a microwave frequency meter model NJM-133B of the Nihon Musen Co.

The procedure of treating the test solutions in the spectrophotometric measurements was

* Abbreviations used are: FMN, flavin mononucleotide; RF, riboflavin; ESR, electron spin resonance.

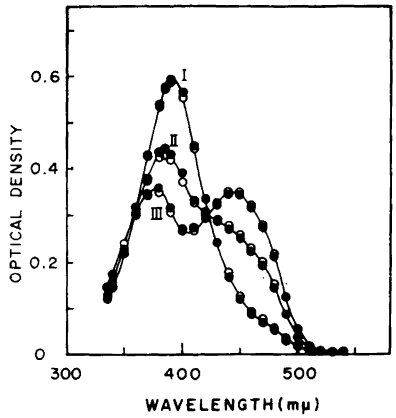


Fig. 1. Absorption spectra of oxidized RF in different concentrations of hydrochloric acid and in presence and absence of zinc chloride. RF concentration was 3.0×10^{-5} M. Curves I, II and III were obtained at the hydrochloric acid concentrations of 2.12, 0.69 and 0.19 N, with pH values of < 0, 0.2 and 0.7, respectively. The values shown by solid and open circles are those obtained in presence and absence, respectively, of 0.2 M $ZnCl_2$.

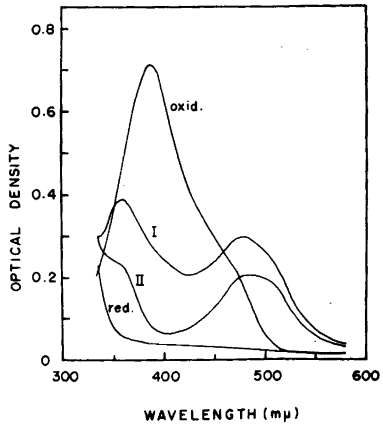


Fig. 2. Absorption spectra in the visible region of oxidized, reduced and intermediary oxidation levels of RF. RF concentration, 4.5×10^{-5} M; pH, −0.1. In curve I, 58.9 % of oxidized and 41.1 % of reduced forms were mixed in test solution, and in curve II, 25.5 % of oxidized and 74.5 % of reduced forms were mixed.

the same as that previously reported; namely the oxidized flavin was titrated under the anaerobic condition with the reduced form keeping the concentrations of acid and total flavin concentrations constant. In the ESR measurements, the test solution was introduced into a quartz capillary, 0.5 mm in inner diameter and 150 mm in length, by using an injection syringe, and the capillary, after being sealed with vaseline, was inserted into the microwave cavity. Diffusion of molecular oxygen into the test solution was found to be negligible even after one hour. The concentration of free spins in the test solution was calculated by double integration of the derivative spectra, and by comparison with the value found for standard solution which contained 1 mM $CuSO_4$. The g-value of the ESR spectrum was determined by measuring the field strength and microwave frequency at the center of the resonance spectrum. All the measurements were made at room temperature.

RESULTS

Spectrophotometric measurements. The absorption spectrum of the oxidized FMN, as it changes with pH, has been measured in detail in our previous paper[7]. The corresponding measurement was made with RF in the pH range which was of interest in the present study. The measurements were made in the presence and absence of zinc ion to see whether or not the absorption spectrum of the oxidized form of RF is affected by the presence of zinc ion, since zinc powder was used in preparation of the reduced RF. The results obtained are presented in Fig. 1. As may be seen, the absorption spectrum of RF changed markedly with pH, and was not affected by the addition of zinc ion. Isosbestic points were found at 355 and 423 mμ, and the extinction coefficient at the latter wavelength was estimated to be 9.8×10^3 $M^{-1}cm^{-1}$. The results coincides with those obtained earlier with FMN[7]. In Fig. 2 are shown the absorption spectra of RF

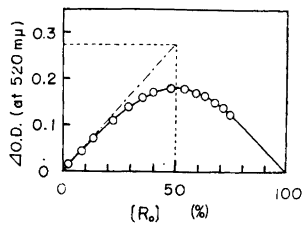


Fig. 3. Plot of $\triangle$O.D.$_{520}$ against relative values of [R_0]. The maximum amount of semiquinone was found to be 66 % of the total flavin. Straight line represents tangent of the curve at [R_0] = 0. RF concentration used was 4.5×10^{-5} M; pH, −0.1. Solid curve represents the theoretical values calculated by applying the experimental value ($K = 15$) obtained for the equilibrium constant of effective semiquinone formation.

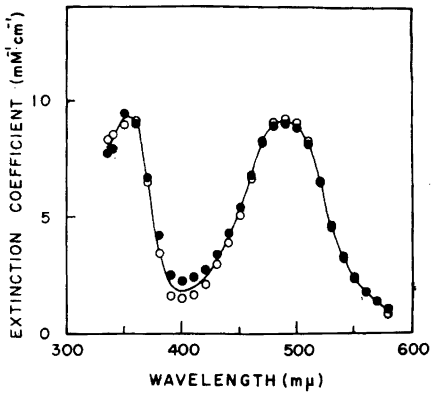


Fig. 4. Spectrum of semiquinone of RF at pH −0.1. The curve was obtained by calculation using the K value obtained in Fig. 3. The values shown by solid and open circles are those calculated from curve I and curve II, respectively, of Fig. 2.

in its oxidized, reduced and two intermediate oxidation levels at pH −0.1*. Worth noticing is the existence at 520 mμ of an isosbestic point of the oxidized and reduced RF. At this wavelength, the increment of optical density ($\triangle$O.D.$_{520}$) may be assumed to be proportional to the amount of semiquinone formed; namely,

$$\triangle \text{O.D.}_{520} = (\varepsilon_S - \varepsilon_T)[S]$$

where ε_S and ε_T are, respectively, molecular extinction coefficients of semiquinone and the oxidized form of RF at 520 mμ, and [S] the concentration of the semiquinone. In Fig. 3, $\triangle$O.D.$_{520}$ is plotted against the relative value of the initial concentration of the reduced form [R_0]. Since the semiquinone is formed by the reversible reaction Oxidized + Reduced $\rightleftharpoons$ 2Semiquinone, and in our experiments the total concentration of RF has been kept constant, we may deduce[7] that the tangent of the curve in Fig. 3 at [R_0] = 0 should be given by the expression:

$$\triangle \text{O.D.}_{520} = 2(\varepsilon_S - \varepsilon_T)[R_0]$$

The value of $(\varepsilon_S - \varepsilon_T)$ and therefore the value of ε_S can thus be obtained graphically. Consequently, the values of [S] at different R_0 values can also be calculated. Experimental results showed that at pH −0.1 the amount of semiquinone was maximal at [R_0] = 50 %, being 66 % of the total RF concentration. The equilibrium constant of effective formation of semiquinone[9], *i. e.* K = [Semiquinone]^2/[Oxidized] × [Reduced], was calculated to be 15. The solid curve in Fig. 3 is that drawn theoretically by using the K-value mentioned above which

* Under the said condition, the exact value of hydrogen ion concentration could not be determined titrimetrically owing to the presence of zinc ion in the mixture. This may be checked by referring to the experimental data presented in Fig. 1. The hydrochloric acid concentration in the experiment shown in Fig. 2 was estimated to be 1.1 N.

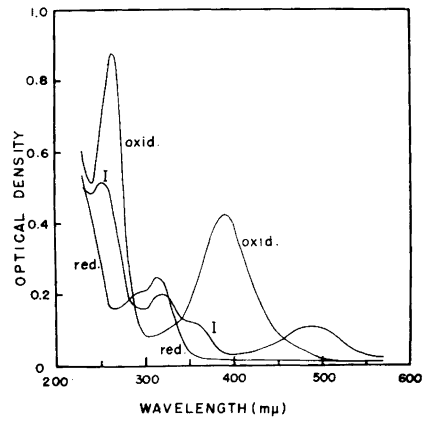


Fig. 5. Absorption spectra of oxidized, reduced and intermediary oxidation levels of RF in the ultraviolet and visible region. RF concentration, 2.2×10^{-5} M; pH, −0.4. The curve for intermediary level was obtained with a mixture containing 25.7 % of oxidized and 74.5 % of reduced forms.

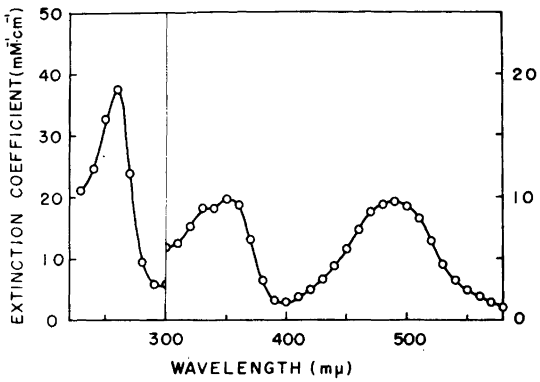


Fig. 6. Spectrum of semiquinone of RF at pH −0.4.

is in good agreement with the experimental results. Calculations made, on the basis of these considerations, with the data presented in Fig. 2, led to the conclusion that the spectrum of the semiquinone in question would be such as given in Fig. 4. The curves computed for $[R_0] = 41.1$ % and 74.5 % coincided with each other almost perfectly, showing two absorption maxima at 350 and 490 mμ with the extinction coefficients of 9.2 and 9.1×10^3 $M^{-1}cm^{-1}$, respectively. Determination of the absorption spectrum of RF semiquinone was made at various pH's, extending, in some cases, the measurement to the ultraviolet region. In Fig. 5 are presented the spectra thus obtained at pH −0.4 for the oxidized, reduced and intermediate states of RF. The whole spectrum of RF semiquinone at pH −0.4 is shown in Fig. 6. Almost the same spectrum was obtained by the similar measurements performed with FMN at pH −0.4. It may be noticed that also in this case the semiquinone spectra has peaks at 260, 350 and 490 mμ and a shoul-

Table 1. Spectrophotometric characteristics of RF and FMN semiquinones and equilibrium constants for their effective formation at different pH values.

Semiquinone	pH	$[S]_{max}$ %	K	λ_{max} mμ	Extinction coefficient 10^3 $M^{-1}cm^{-1}$	Total flavin concentration 10^{-5} M
RF	−0.4	69	20	260	37	2.2
				(330)		
				350	9.8	
				490	9.6	
	−0.1*	66	15	350	9.2	4.5
				490	9.1	
	0.3*	51	4.3	350	8.9	5.9
				490	9.1	
FMN	−0.4	70	22	260	45	2.6
				(330)		
				350	10	
				490	9.5	

* Measurements were made only in the spectral range from 340 to 580 mμ.

der at 330 mμ. The *K*-value calculated for FMN in this case was 22 in comparison with 20 for RF obtained from the data presented in Fig. 5. All these results are summarized in Table 1.

ESR measurements. The ESR measurements with RF and FMN were performed at pH −0.4. As expected, no significant signals were recorded with the oxidized and reduced forms of both flavins. When, however, the oxidized and reduced forms were mixed, there appeared a distinct signal indicating the formation of an intermediate being a free radical. Relevant data are shown in Fig. 7. The g-values of the signals obtained from the intermediates of RF and FMN were identical, being in both cases 2.003. The relationship between the concentration of free spin and $[R_0]$ in the case of FMN is presented in Fig. 8, which shows a satisfactory agreement with the corresponding curve obtained for semiquinone determined by spectrophotometry.

DISCUSSION

According to Beinert[4], there are two forms of flavin semiquinone; one which is reddish in color arises in strongly acidic solution, and the other, greenish in color, is observed at neutral pH when the flavin concentration is rather high. The latter was characterized by absorption bands at 565 and 880 mμ, which were thought to be due to the existence of the monomer and dimer of the semiquinone, respectively. However, Gibson *et al.*[10] and Massey *et. al.*[11] have recently suggested that the compound observed in the intermediary mixture showing a broad absorption band in the infrared region is a charge transfer complex of the oxidized and reduced forms, and that the compound showing an absorption at 570 mμ is a complex of free radical and the reduced form of flavin. The flavin concentrations used in the present study were of the order of 1/100 to 1/1000 of those used by Gibson *et al.* so that the possibility of formation of a complex molecule, if any, seems to be slim. Since the semiquinone spectra obtained by using various total concentrations of flavin were identical, the formation of a dimer form

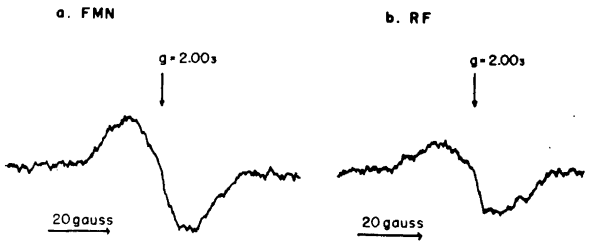


Fig. 7. ESR spectra of FMN and RF semiquinones at pH −0.4. Spectrum *a* was obtained with 3.8×10^{-5} M FMN solution, in which 57 % of oxidized and 43 % of reduced forms were mixed. The estimated concentration of semiquinone was $2.7 \times \times 10^{-5}$ M. Spectrum *b* was obtained with 3.8×10^{-5} M RF solution, in which 13 % of oxidized and 87 % of reduced forms were mixed. The estimated concentration of semiquinone in this case was 2×10^{-5} M. The field strength decreases from left to right and the arrows indicate $g = 2.003$. Measurements of spectra *a* and *b* were performed using different capillaries.

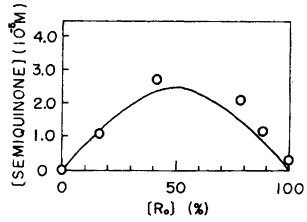


Fig. 8. Plot of FMN semiquinone concentration against relative values of $[R_0]$. Open circles show the concentrations estimated by ESR measurements, and the curve represents the concentration measured by spectrophotometry. FMN concentrations was 3.8×10^{-5} M; pH, −0.4.

is unlikely in our experiments. It may, therefore, be justifiable to assume that under our experimental conditions the equilibrium mixture contained only three molecular forms; namely, oxidized, reduced and semiquinone forms. The g-values of ESR signals obtained with FMN and RF agreed well with that reported elsewhere[12], and they are no doubt ascribable to free radicals. That the red intermediate compound here studied was a semiquinone is evidenced by the fact that the free spin concentration obtained by the ESR measurement and the concentration of semiquinone determined by spectrophotometry coincided with each other quantitatively.

Acknowledgements. The authors wish to express their gratitude to Prof. Hiroshi Tamiya for his valuable criticism in this work. This paper is dedicated to Prof. Hugo Theorell on the occasion of his 60th birthday.

REFERENCES

1. Kuhn, R. and Wagner-Jauregg, T. *Ber.* **67** (1934) 361.
2. Michaelis, L., Schubert, M. P. and Smythe, C. V. *J. Biol. Chem.* **116** (1936) 587.
3. Swallow, A. J. *Nature* **176** (1955) 793.
4. Beinert, H. *J. Am. Chem. Soc.* **78** (1956) 5323.
5. Commoner, B. and Lippincott, B. *Proc. Natl. Acad. Sci. U. S.* **44** (1958) 1110.
6. Ehrenberg, A. *Acta Chem. Scand.* **14** (1960) 766.
7. Nakamura, S., Nakamura, T. and Ogura, Y. *J. Biochem.* **53** (1963) 143.
8. Whitby, L. G. *Biochem. J.* **54** (1953) 437.
9. Michaelis, L. *J. Biol. Chem.* **96** (1932) 703.
10. Gibson, Q. H., Massey, V. and Atherton, N. M. *Biochem. J.* **85** (1962) 369.
11. Massey, V. and Palmer, G. *J. Biol. Chem.* **237** (1962) 2347.
12. Beinert, H. In Blois, M. S. *et al. Free Radicals in Biological Systems,* Academic Press, New York, 1961, p. 17.

Received March 3, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 190 – S 194

Synthesis and Reactions of the α-N-Hydroxyamino Acids

J. B. NEILANDS and PARVIZ AZARI

Department of Biochemistry, University of California, Berkeley, California, USA

The reduction of α-nitro esters with zinc dust in aqueous acetic acid has been employed as a general synthesis for an important group of naturally occurring substances, the α-N-hydroxyamino acids. The latter are instantly cleaved by periodic acid to CO_2, nitrous oxide and aldehydes. The acyl-N-hydroxamido ester derivatives react with periodic acid to yield the acyl moiety and the corresponding α-oximino ester.

The colored coordination compound, ferric-hydroxamate, has found favor in analytical chemistry for several decades[1,2]; living cells, as master analytical chemists, have apparently employed this complex in iron metabolism for many millions of years[3,4].

$$n\ \begin{matrix} R' - N - OH \\ | \\ R - C = O \end{matrix} + Fe^{III} \rightleftharpoons \left[\begin{matrix} R' - N - O \\ | \\ R - C = O \end{matrix}\right]_n + Fe^{III} + n\,H^+$$

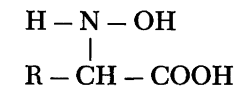
Yeast suspended in nitrite solution have been shown[5] to contain acethydroxamic acid (R = CH_3, R′ = H). Here the substance probably arises by reaction of the formed hydroxylamine with active acyl compounds such as acetyl coenzyme A. All naturally occurring hydroxamic acids apparently belong to the secondary series (R′ = alkyl) and in many instances the carbon skeleton bears a striking resemblance to that of the familiar amino acids. Thus the α-amino-ω-hydroxyamino acid corresponding to L-ornithinine occurs in the ferrichrome compounds[6] and in albomycin[7] whilst the lysine analogue is found in mycobactin[8]. The decarboxylation product of the latter analogue, 1-amino-5-hydroxyamino pentane, is a principal constituent of nocardamin[9]. Similarly, the α-N-hydroxyamino acids appear in hadacidin[10] (glycine), mycelianamide[11] (alanine) and the aspergillic[12] acid family (isoleucine).

The chemical synthesis of the DL-α-amino-ω-hydroxyamino acids has already been reported[13]. Recently, in connection with a projected study on the biosynthe-

sis and structure of proteins, it became necessary to obtain a number of synthetic α-N-hydroxyamino acids and to elucidate the mechanism of periodic acid attack on these compounds and their derivatives.

Synthesis of the α-N-hydroxyamino acids

In the present communication we wish to describe certain synthetic methods and reactions for the α-N-hydroxyamino acids. These compounds,

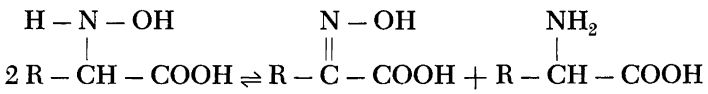


which were known in the last century[14], have been prepared through a variety of routes including the addition of HCN to aldoximes followed by hydrolysis[15], the treatment of α-bromo acids with hydroxylamine[16], the addition of hydroxylamine to α-β unsaturated acids[17], and the reaction of nitrogen oxide with a substituted malonic ester[15].

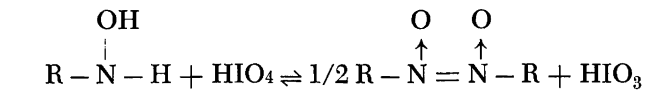
The only general method for introduction of the hydroxyamino group is the reduction of the parent nitro compound with zinc dust. However, in the case of the α-N-hydroxyamino acids, this approach has been used sparingly and in one reported application the product was not extensively characterized[18]. Since α-nitroesters are now quite readily available[19,20], we undertook to investigate the general applicability of this reaction. It was discovered that yields of 15–25 % could be obtained by carrying out the reaction at 0–5°C in glacial acetic-water solution. In spite of the modest yields, the method, which was proven by preparation of a known (N-hydroxyphenylalanine) and a new (N-hydroxyisoleucine) α-N-hydroxyamino acid, is recommended in those instances where the parent nitro compound is readily available.

Reactions of the α-N-hydroxyamino acids

The hydroxyamino group is readily oxidized or reduced, thus affording disproportionation reactions[21] of the type:



Two useful color tests for the hydroxyamino function are the instant reduction of tetrazolium in alkaline solution in the cold[8], and the formation of ferric hydroxamate after acylation and addition of ferric chloride. For simple alkyl-substituted hydroxylamines, treatment with performic acid apparently causes the carbon bearing the hydroxyamino group to be degraded to carboxyl[7]. A notable reaction of the alkyl-hydroxylamines, that with periodic acid[22,23], gives rise to the corresponding *cis*-nitroso dimer:



This reaction appears to be instantaneous and, in view of the substantial absorption of the dimer in the ultraviolet, it has possibilities for analytical application. On oxidation with periodic acid, free hydroxylamine is converted to nitrous oxide, possibly via a nitroxyl dimer intermediate[23].

$$H-\overset{\uparrow}{O}-N=N-\overset{\uparrow}{O}-H \rightleftharpoons N_2O + H_2O$$

Snow[8] reported that α-N-hydroxyamino acids react with periodic acid to give CO_2, aldehydes and possibly "oxides of nitrogen". The experiments to be reported here confirm and extend these observations. Under the conditions used, the following equations probably account for the observed reaction of periodic acid with the α-N-hydroxyamino acids and their derivatives:

$$R-\underset{|}{\overset{H-N-OH}{CH}}\,COOH + 2\ HIO_4 \rightleftharpoons 1/2\ N_2O + 3/2\,H_2O + CO_2 + R-\overset{O}{\overset{\|}{C}}-H + 2\,HIO_3$$

$$C_6H_5-\underset{|}{\overset{O}{\overset{\|}{C}}}=N-OH \qquad\qquad N-OH$$
$$R-\underset{|}{CH}-COOC_2H_5 + HIO_4 \rightleftharpoons C_6H_5COOH + R-\overset{\|}{C}-COOC_2H_5 + HIO_3$$

Thus the periodic acid oxidation of a peptide chain containing an α-N-hydroxyamino acid residue should afford the oxime if the residue is either N-terminal or internal. At the C-terminal position, the aldehyde, CO_2 and nitrous oxide should result.

EXPERIMENTAL

Synthesis of α-N-hydroxyamino acids

α-N-hydroxy-β-phenyl propionic acid. This N-hydroxyamino acid was obtained by reduction and hydrolysis of ethyl-α-nitro-β-phenyl propionate[24].

Diethylnitromalonate (40 g, 195 mmoles) was added with stirring to 120 ml of ethanol containing 4.5 g (195 mmoles) sodium and 34.2 g (200 mmoles) of benzyl bromide was added in small portions to the hot solution. The mixture was refluxed for 48 hours (exclusion of moisture), the ethanol was removed under reduced pressure, the residue was suspended in 200 ml of water and thrice extracted with 100 ml portions of toluene. The combined toluene extract was dried over $MgSO_4$ to yield 42 g of viscous oil. The latter was dissolved in 100 ml ether, the resulting solution was chilled in an ice bath and to it was added, with vigourous stirring, 100 ml of ethanol containing 3.26 g (142 mmoles) sodium. Stirring was continued for five hours in the cold, the solution evaporated to a reddish-brown oil and the latter suspended in 300 ml of water and twice extracted with 100 ml portions of ether. The yellow aqueous phase was chilled to 1°C and acidified to Congo red. The ether extract of this solution was dried over $MgSO_4$ and evaporated to yield 20 g (89 mmoles) of substance with properties corresponding to those of ethyl-α-nitro-β-phenyl propionate prepared by an alternate procedure[24].

The yellow oil (20 g, 89 mmoles) was added to mixture of 100 ml glacial acetic acid and 5 ml water in a 250 ml flask. The flask was immersed in an ice-salt mixture, and to the solution zinc dust (30 g, 460 mmoles) was added in small portions with vigorous stirring over a period of one hour. The temperature was maintained at 5° ± 2°C. The mixture was then filtered and the pH was adjusted to 7 with slow addition of cold concentrated NaOH solution. The reaction

mixture was immediately twice extracted with 250 ml portions of chloroform. The combined chloroform extracts were extracted three times with 100 ml water followed by two extractions with 250 ml of 3 N HCl. The combined aqueous extracts were then refluxed for 7 hours, the volume of the solution was reduced to 50 ml, decolorized with charcoal and filtered. The pH of the clear filtrate was adjusted to 4 with cold concentrated NH_4OH. A white crystalline mass formed upon leaving the mixture in the ice box for 18 hours. The precipitate was filtered, dissolved in 1 N HCl and recrystallized by the addition of concentrated NH_4OH to pH 4, yielding 3 g (16.6 mmoles, 19 %) of white powder.

The product gave a strong tetrazolium test and a neutral equivalent of 182.8 (theoretical, 181.2). This hydroxyamino acid was obtained previously in a series of steps beginning with the treatment of an alkylacetoacetic ester with N_2O_2[15].

Ethyl-α-benzoylhydroxamido-β-phenylpropionate. The solution obtained by filtering off the zinc oxide from the reduction of 20 g (89 mmoles) of ethyl-α-nitro-β-phenylpropionate (see above) was treated with 9.8 ml (12.3, 89 mmoles) of benzoyl chloride. Several volumes of water were added and the hydroxamic acid extracted into ethyl acetate. The latter solution was washed twice each with 100 ml of 0.1 N HCl and cold 0.1 M phosphate buffer pH 7. The ethyl acetate solution was then washed twice with 0.1 N HCl and twice with water. The evaporation of the organic layer under reduced pressure left an oily residue which was twice recrystallized from ethanol-water yielding 7 g (22 mmoles, 25 %) of colorless needles, m.p. 115–118°C (uncorr.). Titration of a sample in 50 % ethanol gave a neutral equivalent of 321.0 (theoretical, 313.3) with a pK_a' of 10.

α-N-Hydroxy-β-methyl valeric acid. This hydroxyamino acid, which apparently has not previously been reported, was obtained by reduction and hydrolysis of the corresponding α-nitroester[24] via the above procedure. The overall yield was 15 % of material with a pK_{a_2}' of 5.9, a strongly positive tetrazolium test and a neutral equivalent of 148.8 (theoretical, 147.2). (Found: C 49.2; H 8.7; N 9.2. Calc. for C_6HNO_3: C 48.9; H 8.9; N 9.5.)

Ethyl-α-benzoylhydroxamido-propionate. This compound was obtained by acylation (see above) of the zinc reduction product of ethyl-α-nitropropionate[19]. The yield was 25 % of almost colorless needles, m.p. 88°C (uncorr.). The pK_a' in 10 % ethanol was 9.5 with neutral equivalent of 242.1 (theoretical, 236.2). An 8.4 mg (35.5 μmole) sample treated with excess ferric chloride in 100 ml of aqueous solution at pH 2.5 displayed an absorption maximum of 500 mμ with an extinction coefficient of 1.07×10^3.

Periodic acid oxidation of free (A) and substituted (B) α-N-hydroxyamino acids

A. Free N-hydroxyamino acids

(1) Hydroxyamino moiety. A 147 mg (1 mmole) quantity of DL-α-hydroxyamino-γ-methyl valeric acid[15] was dissolved in 10 ml of water contained in a manometer. The introduction of 5 ml of aqueous solution containing 912 mg (4 mmoles) H_5IO_6 from a side arm gave rise to instantaneous evolution of gas. The latter was collected and analyzed at room temperature by gas chromatography on a column of silica gel[25] using helium carrier and an Aerograph Model A-100 detector. The column was fitted with a caroxite CO_2-trap and a calibration curve prepared by injecting various volumes of pure nitrous oxide. Injection of an aliquot of the sample demonstrated the presence of a component with the same retention volume as authentic nitrous oxide. The yield, 80 % of theory, was regarded as satisfactory in view of the known substantial water-solubility of nitrous oxide.

(2) Carboxyl moiety. A 33 mg (0.25 mmole) quantity of DL-α-hydroxyamino-β-methyl butyric acid was dissolved in 10 ml of water in a closed system. Excess 0.2 N periodic acid solution was introduced via a dropping funnel and the evolved gas was trapped in a barium hydroxide solution. The precipitate was collected, washed and dried to yield 50.8 mg of $BaCO_3$ (theoretical, 49.4 mg; 102 %).

(3) Carbon skeleton. Aqueous solutions of several different α-N-hydroxyamino acids containing paraffinic side chains were treated with periodic acid and the resulting solution extracted with ether. The ether extracts were analysed by gas chromatography on a firebrick column at 125°C. These experiments showed the presence of components with the same retention volume as authentic samples of the expected[8] aldehydes.

B. Substituted N-hydroxyamino acid

(1) N-Benzoyl-hydroxyamino-alanine ethyl ester. Exactly 240 mg (1 mmole) of ethyl-DL-α-benzoylhydroxamidopropionate was dissolved in 30 ml of warm water and allowed to react with 912 mg (4 mmoles) H_5IO_6 added dropwise in 5 ml of water. There was no obvious evolution of gas and hence the reaction mixture was worked up in the following way. The solution was exhaustively extracted with ether and the aqueous phase discarded. The ether extract was evaporated, the residue dissolved in aqueous alcohol and the solution carefully neutralized with N NaOH. The neutral solution was again exhaustively extracted with ether and the combined ether extracts set aside as Fraction I. The aqueous phase was acidified with dilute HCl and the ether extract of this solution designated as Fraction II.

Fraction I was evaporated and the residue recrystallized from ethanolpetroleum ether to yield needles with m.p. 92–93°C, not depressed on admixture with authentic[26] α-oximinoethyl-propionate. The pK_a' (9.8) and infrared spectrum (KBr pellet) were identical with those of the authentic specimen. The yield was 68 mg (52 %).

Fraction II was evaporated and the residue crystallized from ethanol-water yielding 77 mg (63 %) of benzoic acid, the latter characterized by m.p. (119°C), pK_a' (4.2), neutral equivalent (120), all of which were identical with those of a known sample.

Acknowledgement. Thanks are due to the *Office of Naval Research* (Grant 222-39) and the *United States Public Health Service* (Grant E-4156) for financial assistance. One of us (P.A.) holds post doctoral fellowship No. CPD – 12,355 – C 2 (1961 – 63) from the *U.S. Public Health Service.*

REFERENCES

1. Feigl, F. *Spot Tests in Organic Analysis,* 5th Ed., Elsevier, Amsterdam, 1956.
2. Lipmann, F. and Tuttle, L. C. *J. Biol. Chem.* **159** (1945) 21.
3. Neilands, J. B. *Bact. Rev.* **21** (1957) 101.
4. Zähner, H., Bachmann, E., Hütter, R., and Nüesch, J. *Path. Microbiol.* **25** (1962) 708.
5. Virtanen, A. I. and Saris, N. E. *Acta Chem. Scand.* **10** (1956) 483.
6. Emery, T. and Neilands, J. B. *J. Am. Chem. Soc.* **83** (1961) 1626.
7. Turkova, J., Mikes, O. and Sorm, F. *Czech. Chem. Com.* **27** (1962) 591.
8. Snow, G. A. *J. Chem. Soc.* **1954** 2588.
9. Bickel, H., Fechtig, B., Hall, G. E., Keller-Schlierlein, W., Prelog, V. and Vischer, E. *Helv. Chim. Acta* **43** (1960) 901.
10. Gaczka, E. A., Gitterman, C. O., Dulaney, E. L. and Folkers, K. *Biochemistry* **1** (1962) 340.
11. Birch, A. J. *Proc. Chem. Soc.* **1957** 233.
12. Dutcher, J. D. *J. Biol. Chem.* **232** (1958) 785.
13. Rogers, S. and Neilands, J. B. *Biochemistry* **2** (1963) 6.
14. Traube, W. *Ber.* **28** (1895) 2300.
15. Neelakantan, L. and Hartung, W. H. *J. Org. Chem.* **23** (1958) 964.
16. Cook, A. H. and Slater, C. A. *J. Chem. Soc.* **1956** 4130.
17. Posner, T. *Ber.* **36** (1903) 4305.
18. Weisblat, D. I. and Lyttle, D. A. *J. Am. Chem. Soc.* **71** (1949) 3079.
19. Kornblum, N. *Organic Reactions* **12** (1962) 101.
20. Finkbeiner, H. L. and Stiles, M. *J. Am. Chem. Soc.* **85** (1963) 616.
21. Spenser, I. D. and Ahmad, A. *Proc. Chem. Soc.* **1961** 375.
22. Emery, T. and Neilands, J. B. *J. Am. Chem. Soc.* **82** (1960) 4903.
23. Emery, T. and Neilands, J. B. *J. Org. Chem.* **27** (1962) 1075.
24. Emmons, W. D. and Freeman, J. P. *J. Am. Chem. Soc.* **77** (1955) 4391.
25. Smith, D. H. and Clark, F. E. *Soil Sc. Soc. Am. Proc.* **24** (1960) 111.
26. Kornblum, N., Blackwood, R. K. and Powers, J. W. *J. Am. Chem. Soc.* **79** (1953) 2507.

Received April 1, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 195 – S 202

Equilibrium Reaction Rates and Enzyme Mechanisms*

P. D. BOYER** and E. SILVERSTEIN***

Department of Biochemistry, University of Minnesota Medical School, Minneapolis 14, Minnesota, USA

General relationships are developed for rates of substrate interchange at equilibrium for enzyme reactions of the type $A + B \rightleftharpoons C + D$ and $A + B \rightleftharpoons C$ proceeding through interconversion of ternary complexes and with compulsory or with alternative orders of addition of substrates. Usefulness of such measurements for determination of substrate binding order and of ternary complex interconversion as a possible rate-limiting step, for setting minimum values for dissociation constants, and for detection of other aspects of catalytic mechanisms are presented and discussed.

Many approaches are essential for study of enzyme mechanisms. For example, measurements of initial reaction velocities and of substrate and inhibitor binding under various conditions have been powerful tools in the skilful hands of Professor Theorell and his associates, and it is with pleasure that this paper is dedicated to Professor Theorell. A new approach which may deserve considerably more attention is the measurement of reaction rates at equilibrium, as made possible by isotopic techniques. Biochemists have made frequent and valuable use of measurements of exchange reactions, but without consideration of the fundamental relationships governing such exchange rates in enzyme systems. Rate measurements at equilibrium, although technically somewhat difficult, have the theoretical advantage that all reaction intermediates are at equilibrium concentration, thus considerably simplifying derivation and interpretation.

Some theoretical bases for equilibrium reaction rate studies in enzyme systems were first presented by Boyer in 1959[1], and have been correlated with experimental observations in subsequent studies from his laboratory[2–4]. The reports have stimulated a generalized treatment by Morales and co-workers[5] of transient states of tracer distribution in enzyme systems where the amount of labeled substrate combined with enzyme may be appreciable, and of considerations by Alberty *et al.*[6] of alternative means of deriving the appropriate relationships for simplified linear sequences.

* Supported in part by grants from the *U. S. Public Health Service* and the *Hill Family Foundation.*

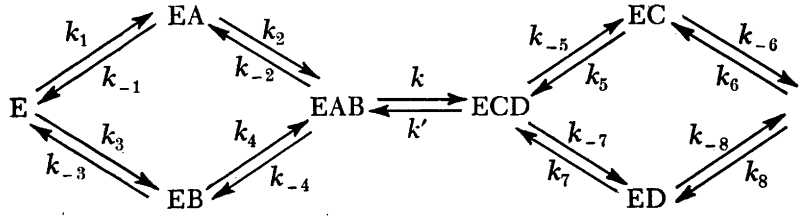
** Present address: Department of Chemistry, University of California, Los Angeles 24, California.

*** American Cancer Society Post-Doctoral Fellow.

Previous considerations have not included enzyme reactions proceeding through interconversion of two ternary complexes and with either alternative or compulsory order of addition of substrates. The purpose of this paper is to present some appropriate equations and their implications. These serve as the basis for studies of equilibrium reaction rates with liver and yeast alcohol dehydrogenase, with heart and skeletal muscle lactate dehydrogenases, and with hexokinase which have quite recently been completed in our laboratory[4].

DERIVATION OF BASIC RELATIONSHIPS

The approach previously used[1] leads readily to general equations governing rates of interchange of reactants in enzyme systems as described by eqn. (1).*



(1)

For example, for the rate, R, of interconversion of A with C at equilibrium, let

$EA = p + q$, where $p =$ EA derived most recently from A**
and $q =$ " " " " " C;
$EAB = w + x$, where $w =$ EAB " " " " A
and $x =$ " " " " " C;
$ECD = y + z$, where $y =$ ECD " " " " A
and $z =$ " " " " " C;
$EC = m + n$, where $m =$ EC " " " " A
and $n =$ " " " " " C.

The enzyme conservation equation and the various equilibria involved lead to

$$E_t = (y+z)\left[1 + \frac{k}{k'}\left(1 + \frac{K_2}{B} + \frac{K_4}{A} + \frac{K_1K_2}{AB}\right) + \frac{K_5}{D} + \frac{K_7}{C}\right] \qquad (2)$$

where K_1, K_2, etc. are the respective dissociation constants and A, B, etc. refer to reactant concentrations. In the system given by eqn. (1), $R = k_{-7}y + k_{-6}m$, and from this and the relation $dm/dt = O = k_{-5}y - k_5mD - k_{-6}m$, one obtains $y = R(k_{-6} + k_5D)/(k_{-5}k_{-6} + k_{-6}k_{-7} + k_5k_{-7}D)$. Similarly, R also $= k_{-4}x + k_{-1}q$, and from this and the relation $dq/dt = O = k_{-2}x - k_2qB - k_{-1}q$ one obtains

* Relationships for rates of isotopic interchange in linear systems or systems where only one ternary complex is formed, whether at equilibrium or considerably displaced from equilibrium, may be obtained from initial velocity equations as developed by Cleland[7] (W. W. Cleland, *personal communication*). The initial velocity equation for a scheme such as given by eqn. (1) is extremely complex, and the simple derivation given herein is preferable for the equilibrium situation.

** The chemical properties of the various enzyme-substrate combinations are obviously independent of their route of formation. The useful concept given here can also be visualized in terms of specific activities. For example, if isotope is added as A and negligible isotope is present in C, the ratio $p/q \times$ specific activity of A = specific activity of EA.

$x = \mathrm{R}(k_{-1} + k_2\mathrm{B})/(k_{-1}k_{-2} + k_{-1}k_{-4} + k_2k_{-4}\mathrm{B})$. In addition, $\mathrm{R} = k'z - kx$, which allows solving for z in terms of x and R. Substitution of the values for z and y into eqn. (2) leads to general eqn. (3).*

$$\mathrm{R} = \mathrm{E}_t \Bigg/ \left\{ \left[1 + \frac{k'(k_{-6} + k_5\mathrm{D})}{k_{-5}k_{-6} + k_{-7}(k_{-6} + k_5\mathrm{D})} + \frac{k(k_{-1} + k_2\mathrm{B})}{k_{-1}k_{-2} + k_{-4}(k_{-1} + k_2\mathrm{B})} \right] \times \right.$$
$$\left. \times \left[\frac{1}{k'}\left(1 + \frac{K_5}{\mathrm{D}} + \frac{K_7}{\mathrm{C}} \right) + \frac{1}{k}\left(1 + \frac{K_2}{\mathrm{B}} + \frac{K_4}{\mathrm{A}} + \frac{K_1K_2}{\mathrm{AB}} \right) \right] \right\} \quad (3)$$

The rate of $\mathrm{B} \rightleftharpoons \mathrm{D}$ interconversion, R′, is given by a similar expression as follows:

$$\mathrm{R}' = \mathrm{E}_t \Bigg/ \left\{ \left[1 + \frac{k'(k_{-8} + k_7\mathrm{C})}{k_{-7}k_{-8} + k_{-5}(k_{-8} + k_7\mathrm{C})} + \frac{k(k_{-3} + k_4\mathrm{A})}{k_{-3}k_{-4} + k_{-2}(k_{-3} + k_4\mathrm{A})} \right] \times \right.$$
$$\left. \times \left[\frac{1}{k'}\left(1 + \frac{K_5}{\mathrm{D}} + \frac{K_7}{\mathrm{C}} \right) + \frac{1}{k}\left(1 + \frac{K_2}{\mathrm{B}} + \frac{K_4}{\mathrm{A}} + \frac{K_1K_2}{\mathrm{AB}} \right) \right] \right\} \quad (4)$$

Relationships for compulsory pathways (linear sequences) are readily derived, as limiting cases of general relationships, such as eqns. (3) and (4). For example, for the pathway involving only the upper part of eqn. (1), the terms K_4, K_7, k_{-4} and k_{-7} are set equal to 0 to give:

$$\mathrm{R} = \mathrm{E}_t \Bigg/ \left\{ \left[1 + \frac{k'(k_{-6} + k_5\mathrm{D})}{k_{-5}k_{-6}} + \frac{k(k_{-1} + k_2\mathrm{B})}{k_{-1}k_{-2}} \right] \times \right.$$
$$\left. \times \left[\frac{1}{k'}\left(1 + \frac{K_5}{\mathrm{D}} \right) + \frac{1}{k}\left(1 + \frac{K_2}{\mathrm{B}} + \frac{K_1K_2}{\mathrm{AB}} \right) \right] \right\} \quad (5)$$

$$\mathrm{R}' = \mathrm{E}_t \Bigg/ \left[1 + \frac{k'}{k_{-5}} + \frac{k}{k_{-2}} \right] \left[\frac{1}{k'}\left(1 + \frac{K_5}{\mathrm{D}} \right) + \frac{1}{k}\left(1 + \frac{K_2}{\mathrm{B}} + \frac{K_1K_2}{\mathrm{AB}} \right) \right] \quad (6)$$

Unwarranted confusion and apparent discrepancy may arise because of lack of clarity in definition rather than experimental result. For reaction proceeding through complexes between an enzyme and two or more substrates, the following definitions apply to terms as used in this paper and are suggested for general usage:

1. *Random* order or sequence of substrate addition. The addition of substrates to an enzyme or their dissociation from enzyme-substrate complexes occurs at equal rate whether or not another substrate or other substrates are bound to the enzyme. In terms of eqn. (1), this means that $k_1 = k_4$, $k_{-1} = k_{-4}$, $k_2 = k_3$, etc.

2. *Alternative* order or sequence of substrate addition. Complexes of enzyme with more than one substrate may be formed by prior addition of any of the substrates, and similarly, dissociation of substrates may occur in any order. Alternative order of addition thus includes the random pathway, but also includes enzymes for which the rate constant for binding of a substrate to the enzyme is greater or less than the rate constant for binding when another substrate is already present on the enzyme. Alternative orders with differences in rate constants

* The equalities $K_1K_2/k\mathrm{AB} = K_3K_4/k\mathrm{AB} = K_5K_6/k'\mathrm{CD} = K_7K_8/k'\mathrm{CD}$ obviously obtain, and these terms may be used interchangeably in the denominator.

For many purposes, the form of eqn. (3) as given here is preferable to the form of equations given previously for simpler systems. For example, eqn. (45) of Boyer[1] may be written:

$$\mathrm{R} = \mathrm{E}_t \Bigg/ \left\{ \left[\frac{k_{-8} + k_7\mathrm{D}}{k_{-5}k_{-8} + k_{-7}(k_{-8} + k_7\mathrm{D})} + \frac{k_{-1} + k_2\mathrm{B}}{k_{-1}k_{-2} + k_{-4}(k_{-1} + k_2\mathrm{B})} \right] \times \right.$$
$$\left. \times \left[1 + \frac{K_4}{\mathrm{A}} + \frac{K_2}{\mathrm{B}} + \frac{K_1K_2}{\mathrm{AB}} + \frac{K_5}{\mathrm{C}} + \frac{K_7}{\mathrm{D}} \right] \right\}$$

for binding must also have differences in some rate constants for dissociation for equilibrium to be maintained.

The designation *branched* sequence is synonymous with alternative order of substrate addition, and, similarly, includes but is not limited to random addition.

Designations such as "partially compulsory" or "partially random" may be appropriate for patterns of behavior of certain enzymes with branched sequences.

3. *Compulsory* order or sequence substrate binding. The addition of one specific substrate must occur before another substrate can be bound, and similarly, dissociation of a certain substrate must occur before another substrate can dissociate.

The designations *linear, ordered,* or *obligatory* sequences are synonymous with a compulsory sequence.

Mechanisms of appropriate enzymes are best described in relation to possible orders of substrate addition as defined above, and not in relation to the order that may exist under prescribed conditions of substrate concentration, pH, etc. Thus an enzyme with a branched mechanism may, under appropriate conditions, catalyze net reaction almost entirely through ordered addition of substrates. This may be appropriately described as kinetically ordered under prescribed conditions, but the enzyme should not be described as having a branched or ordered mechanism.

When one pair of substrates is at high concentration, simplified relationships are also obtaiend. Thus if B and D are increased until they no longer influence R, general eqn. (3) for the alternative pathway becomes

$$R = E_t \Big/ \left[1 + \frac{k'}{k_{-7}} + \frac{k}{k_{-4}}\right]\left[\frac{1}{k'}\left(1 + \frac{K_7}{C}\right) + \frac{1}{k}\left(1 + \frac{K_4}{A}\right)\right] \quad (7)$$

When all reactants are at high concentration, eqn. (3) becomes

$$R = E_t \Big/ \left(1 + \frac{k'}{k_{-7}} + \frac{k}{k_{-4}}\right)\left(\frac{1}{k'} + \frac{1}{k}\right) \quad (8)$$

and eqn. (4) becomes $R' = E_t \Big/ \left(1 + \frac{k'}{k_{-5}} + \frac{k}{k_{-2}}\right)\left(\frac{1}{k'} + \frac{1}{k}\right)$ (9)

Relationships for three component systems, such as $A + B \rightleftharpoons C$, are readily obtained from eqns. (3) or (4) by dropping appropriate terms. For example, if eqn. (1) is modified so that $EAB \underset{k'}{\overset{k}{\rightleftharpoons}} EC \underset{k_5}{\overset{k_{-5}}{\rightleftharpoons}} E + C$, the eqn. (3) terms k_{-6}, k_{-7}, k_{-8}, K_7/C, k_5D, K_1K_2/kAB $(= K_7K_8/k'CD)$ would be dropped with K_5/C replacing K_5/D to give eqn. (10) for $A \rightleftharpoons C$.

$$R = E_t \Big/ \left[1 + \frac{k'}{k_{-5}} + \frac{k(k_{-1} + k_2B)}{k_{-1}k_{-2} + k_{-4}(k_{-1} + k_2B)}\right]\left[\frac{1}{k'}\left(1 + \frac{K_5}{C}\right) + \frac{1}{k}\left(1 + \frac{K_4}{A} + \frac{K_2}{B}\right)\right] \quad (10)$$

APPLICATIONS OF RELATIONSHIPS

Compulsory pathways – If a compulsory pathway exists such that either the top or the bottom of eqn. (1) applies, increase in concentration of all reactants or of one or both of the last reactants to add in formation of the ternary complexes will give a predictable and distinguishable rate behavior. For example, as shown by eqn. (5), increase in B and D while maintaining equilibrium will result first in an increase, then in progressive inhibition of the $A \rightleftharpoons C$ interchange until complete inhibition occurs; in contrast, the $B \rightleftharpoons D$ interchange will increase

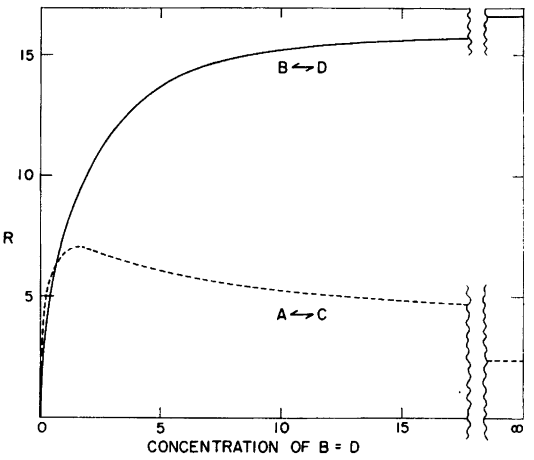


Fig. 1. Theoretical exchange at equilibrium showing decrease in the rate A ⇌ C when dissociation is limited to ternary complexes. The curves are calculated from eqns. (3) and (4) assuming that $E_t = 100$, all K's k', k, k_2, k_{-2}, k_5 and $k_{-5} = 1$; k_4, k_{-4}, k_7, $k_{-7} = 0.1$; and k_1, k_{-1}, $k_6 = 2$; $A \gg K_4$, $B \gg K_2$, $k_4A \gg k_{-3}$, $k_{-7}k_{-8}$; and $k_7C \gg k_{-8}$, $k_{-3}k_{-4}$.

to a maximum. Appropriate experiments will thus readily identify the order of reactant combination in any linear sequence.

If partially compulsory pathways occur, these may be revealed in some circumstances by incomplete inhibition in the interchange rates of one substrate pair as the concentrations of another substrate pair is increased. As B and D are increased from 0 to ∞ the left-bracketed term of the denominator of eqn. (3) increases from $1/(k_{-2} + k_{-4})$ to $1/k_{-4}$, thus tending to decrease R. Conversely, the right-bracketed term decreases, thus tending to increase in R. When certain relationships between rate constants hold, these effects may occur relatively independently of one another. Such a possibility is shown in Fig. 1. The conditions for this plot are such that the rate of dissociation of A from EAB is less than the rate from EA, and that of C from EAC less than that from EC. Thus at high concentration of B and D, the interchange A ⇌ C is effectively limited to the slower dissociation steps.

Results obtained with glutamine synthetase[3], in which a "hump" in rate plats at equilibrium was observed, may perhaps be ascribed to relationships such as those discussed above.

Ternary complex interconversion as a rate-limiting step. When the rate of ternary complex interconversion, governed by k and k' is very small compared to the rate of dissociation of substrates from the ternary complexes, equality of A ⇌ C and B ⇌ D interchanges will result at all levels of A, B, C, and D for the alternative addition sequence. This may be illustrated, for example, by further examination of eqns. (3) and (4). If $k' \ll k_{-7}$ and $k \ll k_{-4}$, the leftbracketed denominator term of eqn. (3) becomes equal to 1. A similar situation holds for eqn. (4) if $k' \ll k_{-5}$ and $k \ll k_{-2}$. When these limitations on rate constants apply, eqn. (3) becomes identical to (4). These limitations are the requirements for the ternary complex interconversion as the rate-limiting step. *Thus for the alternative addition of substrates, when* $k' \ll k_{-5},k_{-7}$ *and* $k \ll k_{-2},k_{-4}$, *equality of* A ⇌ B *and* C ⇌ D *will be observed.*

Alberty *et al.*[6] have regarded previous deductions[1,2,8] that equilibrium exchange rates may provide information about rate-limiting steps as being without foundation. In their develop-

ment they consider only linear sequences, and incorrectly infer that the previous considerations of Boyer *et al.*[1,2,8] were similarly limited to linear or compulsory sequences. As noted in this section, and in agreement with earlier deductions[1,2,8], measurements of exchange rates can give information about rate-limiting steps in branched sequences.

Even with linear sequences inferences about rate-limiting steps may be derived from exchange rate measurements. For example, if ternary complex interconversion was extremely slow, equality of exchanges in a linear mechanism would occur until inordinately high concentrations of the last substrates to add were reached. Also, irrespective of the rate of ternary-complex interconversion, if the first pair of substrates to add equilibrate very rapidly between free and bound forms, equality of exchanges would be observed until sufficiently high concentrations of the last substrates to add were reached so as to reform the ternary complexes before rapid equilibration with the first substrates could ensue. Thus eqns. (5) and (6) for R and R′, compulsory pathways, are identical as long as $k_{-1} \gg k_2\mathrm{B}$ and $k_{-6} \gg k_5\mathrm{D}$. Further, if exchanges such as those of oxygen among substrates of glutamine synthetase are observed[3], additional inferences are possible about rate-limiting steps for linear as well as for branched sequences.

Equality of the interchanges $\mathrm{A} \rightleftharpoons \mathrm{B}$ and $\mathrm{C} \rightleftharpoons \mathrm{D}$ might also be observed even when $k' \ll k_{-5}, k_{-7}$ and $k \ll k_{-2}, k_{-4}$ if a fortuitious equality existed in the left-bracketed denominator terms of eqns. (3) and (4) at certain substrate concentrations, or with excess substrates present and if $k_{-2} = k_{-4}$ and $k_{-7} = k_{-5}$. Such situations would, however, give inequality of interchanges with variations of B and D or A and C at less than saturation levels and thus could readily be detected.

Equalities of reaction rates at low substrate concentrations. When the reaction proceeds by a compulsory sequence and substrate concentrations are high, inequality of $\mathrm{A} \rightleftharpoons \mathrm{C}$ and $\mathrm{B} \rightleftharpoons \mathrm{D}$ must be observed, as noted before, even if the ternary complex interconversion is slow. However, irrespective of the rate of ternary complex interconversion, equalities of exchange will appear as substrate concentrations approach low levels for both linear and branched sequences. For linear sequences, eqns. (5) and (6) become identical when D and B are decreased sufficiently so that $k_5\mathrm{D} \ll k_{-6}$ and $k_2\mathrm{B} \ll k_{-1}$. Thus the concentration range at which exchange rates in a compulsory sequence approach equality, as B and D are varied and lowered, gives information about the relative magnitudes of k_2 and k_{-1}, and of k_5 and k_{-6}.

With branched sequences, eqns. (3) and (4) become identical when $k_5\mathrm{D} \ll k_{-6}$, $k_2\mathrm{B} \ll k_{-1}$, $k_7\mathrm{C} \ll k_{-8}$, and $k_4\mathrm{A} \ll k_{-3}$. Conditions for equality of exchange with decrease in substrate concentration for branched sequences are thus somewhat more stringent than those for linear sequences.

Relationships to dissociation constants. From eqn. (7) for $\mathrm{A} \rightleftharpoons \mathrm{C}$, random pathway, at high B and D concentrations, R will be a function of K_7/C or K_4/A or both. Under some circumstances it may be possible to adjust equilibria concentrations so that B, D, and C remain high so that eqn. (7) becomes of the form

$$\mathrm{R} = \frac{\text{a constant}}{1 + \dfrac{k}{k'} + \dfrac{K_4}{A}} \tag{11}$$

A limitation in obtaining eqn. (11) is that B must be sufficiently high so that not only is K_2/B negligible, but K_1K_2/AB must remain negligible throughout the concentration range studied. If eqn. (11) applies, the ratio of the slope to intercept of the 1/R *vs.* 1/A plot will give $K_4/(1 + k/k')$, or a minimum value for K_4.

Similarly, if A can be maintained high, a minimum value for K_7 may be obtained. Values obtained from such approaches will give K_2 and K_4 each divided by $(1 + k/k')$, but K_5 and K_7 each divided by $(1 + k'/k)$. If $k = k'$, the ratios of slopes to intercepts will give ½ the values of dissociation constants. If $k \rangle\rangle k'$, actual values for K_5 and K_7 but far too small values for K_2 and K_4 will be obtained, or vice versa if $k' \rangle\rangle k$. Equality of k and k' would be unusual fortuity, and thus actual values for dissociation constants of some substrates are likely to be approached. If values of dissociation constants are known from independent studies, the equilibrium rate studies may allow evaluation of the ratio k/k'.

Similar approaches are applicable to linear sequences, but with dissociation constants given by the concentration dependency of R'. For example, when concentrations of A, B, and C are held high, the equilibrium rate dependencies will give a minimal value for the dissociation constant of D.

Effects of abortive ternary complex formation. The formation of abortive ternary complexes, or of combinations of enzyme with more than one substrate molecule might have definite effects on the rate relationships. For example, the addition of reactions for formation of the complexes EAC and EBD to eqn. (1) leads to relationships like eqns. (3) and (4) but with additional terms involving ratios of A/C and of B/D together with appropriate dissociation constants in the second bracketed term of the denominator. Thus if the ratios of A/C and B/D are held constant, the general form of the equation will not vary. But if, for example, concentrations of A and D are increased, the term involving B/D will drop out but that involving A/C will continue to increase with continued fall in R.

Relationships between the chemical transformation rate and equilibrium reaction rate. The rate of interconversion of the ternary complexes, or the actual chemical transformation rate, R'', is equal to $k(\mathrm{EAB}) = k'(\mathrm{ECD})$.* For alternative addition sequences, and at high substrate concentrations eqn. (2) becomes $\mathrm{E}_t = (\mathrm{ECD})\,(1 + k/k')$. Thus it follows that $\mathrm{R}'' = \mathrm{E}_t kk'/(k + k')$. From this, and eqn. (8), the ratio of the equilibrium reaction rate to the rate of chemical transformation would be:

$$\mathrm{R}/\mathrm{R}'' = 1 \Big/ (1 + k'/k_{-7} + k/k_{-4}) \qquad (12)$$

When $k_{-7} \rangle\rangle k'$, and $k_{-4} \rangle\rangle k$, R will $=$ R''. Similarly, when $k_{-5} \rangle\rangle k'$ and $k_{-2} \rangle\rangle k$, R' will $=$ R'' at high substrate concentrations. These are the same conditions as for ternary complex interconversion being rate limiting with alternative orders of substrate addition. Thus under these circumstances, the equilibrium interconversion rate would equal the chemical transformation rate.

DISCUSSION

The relationships developed in this paper for an enzyme system as described by eqn. (1) show that measurement of equilibrium rates may be a powerful tool for detection of compulsory pathways of substrate binding, for discerning whether or not ternary complex interconversion is rate-limiting, for finding mini-

* The rate of actual chemical transformation in any enzymic reaction whether or not at equilibrium must equal but may far exceed the rate of net reaction or the highest equilibrium interconversion rate of any substrate pair or atoms of substrates. Rapid chemical transformation of enzyme-bound reactants accounts for a rapid transfer of oxygen from the glutamate carboxyl to the terminal phosphoryl group of ATP without formation of free orthophosphate[3].

mal values of dissociation constants, and for gaining information about relative velocities of certain steps. These considerations have been applied to results from liver and yeast alcohol dehydrogenases and to skeletal and heart muscle lactate dehydrogenase as presented elsewhere[4]. With liver alcohol dehydrogenase alternative order addition of substrates is found, with dissociation of coenzymes as rate-limiting steps. This is in agreement with the extensive studies of Theorell and associates[9] but not with recent conclusions of Dalziel[10]. A similar situation holds with yeast alcohol dehydrogenase, in contrast to the conclusions of Nygaard and Theorell[11] and of Mahler and Douglas[12]. Compulsory order of binding is found with lactate dehydrogenase, particularly with bovine heart lactate dehydrogenase at pH 7.9. These results are in harmony with data of Takenaka and Schwert[13]. With hexokinase, in agreement with Zewe and Fromm[14] but not with Hammes and Kochavi[15], an alternative order of substrate addition is found. In addition, particularly with the alcohol dehydrogenase, inhibitory effects on alcohol and acetaldehyde interconversions and information about the chemical transformation steps were obtainable.

The equations as given are based upon rates of interconversion of substrates at equilibrium, and are exact for the systems described. Isotopes provide the only known method for measurement of such rates; in their use certain assumptions must be made, as well as appropriate corrections for approach to isotope equilibrium used in evaluation of rates[1].

Equations are presented in this paper only for interconversion of A with C and of B with D. Appropriate relationships may, of course, be readily derived for other interconversions such as A with B or A with D, as well as for a wide variety of other enzyme reaction schemes. Such extensions can perhaps best be done in connection with appropriate experimental data.

Methodology limitations and restriction of the equilibrium set some obvious limits to the applications of equilibrium rate measurements, but the potentialities, as well as the results obtained to date, would appear to warrant much broader application.

REFERENCES

1. Boyer, P. D. *Arch. Biochem. Biophys.* **82** (1959) 387.
2. Boyer, P. D. and Graves, D. J. *Proc. Symp. IV, 4th Intern. Congr. Biochem., Moscow,* August 1961. Pergamon Press. *In press.*
3. Graves, D. J. and Boyer, P. D. *Biochem.* **1** (1962) 739.
4. Silverstein, E. *Ph. D. Thesis,* Univ. of Minnesota, 1963.
5. Morales, M. F., Horowitz, M. and Botts, J. *Arch. Biochem. Biophys.* **99** (1962) 258.
6. Alberty, R. A., Bloomfield, V., Peller, L. and King, E. L. *J. Am. Chem. Soc.* **84** (1962) 4381.
7. Cleland, W. W. *Biochim. Biophys. Acta* **67** (1963) 104.
8. Boyer, P. D., Mills, R. C. and Fromm, H. J. *Arch. Biochem. Biophys.* **81** (1959) 242.
9. See Sund, H. and Theorell, H. In Boyer, P. D., Lardy, H. A. and Myrbäck, K. *The Enzymes,* 2nd Ed., Academic Press, N. Y., 1963, Vol. VII, p. 25.
10. Dalziel, K. *Biochem. J.* **84** (1962) 244; *Nature* **197** (1963) 462.
11. Nygaard, A. P. and Theorell, H. *Acta Chem. Scand.* **9** (1955) 1300, 1551.
12. Mahler, H. R. and Douglas, J. *J. Am. Chem. Soc.* **79** (1957) 1159.
13. Takenaka, Y. and Schwert, G. W. *J. Biol. Chem.* **223** (1956) 157.
14. Zewe, V. and Fromm, H. J. *J. Biol. Chem.* **237** (1962) 1668.
15. Hammes, G. G. and Kochavi, D. *J. Am. Chem. Soc.* **84** (1962) 2069, 2073, 2076.

Received April 1, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 203–S 209

Dissociation Constants of Bovine Heart Lactate Dehydrogenase-Reduced Coenzyme Compounds*

ALFRED D. WINER**

Department of Biochemistry, University of Kentucky Medical School, Lexington, Kentucky, USA

Dissociation constants of LDH · $NADH_2$*** over a wide range of pH and experimental conditions were determined by fluorescence titration equilibrium measurements. Fair agreement was observed over the pH range 6 to about 8 in Tris-KCl buffer, $\mu = 0.2$, 28.5°C with constants calculated from kinetic parameters assuming a compulsory ternary compound mechanism. At higher pH values, the fluorimetrically determined constants were 3 – 4 times smaller than the kinetically determined constants. The binary compound LDH · NAD as well as the ternary inactive compound LDH · NAD · pyruvate have been observed by fluorescence and absorbancy measurements and it is suggested that the latter compound contributes significantly to the reaction mechanism, particularly in alkaline solution.

The evaluation of enzyme-coenzyme dissociation constants by the fluorescence equilibrium method, first exploited by Theorell and co-workers[1,2] with horse liver alcohol dehydrogenase, permits a comparison of these constants with those calculated from kinetic parameters as derived from any proposed reaction mechanism. Discrepancies between the kinetically determined and directly determined dissociation constants indicate either that the proposed reaction

* Presented in part at the 141st and 142nd Meetings of the American Chemical Society, April 1962, Washington, D. C. and September 1962, Atlantic City, N. J.

** Research Career Development Awardee of the U. S. Public Health Service.

*** Abbreviations:
LDH, bovine heart lactate dehydrogenase; E, ¼ LDH (since each molecule of LDH has 4 independent binding sites per 135 000 g[3]); ADH, horse liver alcohol dehydrogenase; $NADH_2$ (R), reduced nicotinamide adenine dinucleotide; NAD (O), oxidized nicotinamide adenine dinucleotide; APNAD, oxidized 3-acetyl pyridine analog of NAD; $K_{E,R}$, apparent dissociation constant of the binary compound, LDH · $NADH_2$; $K_{E,O}$, apparent dissociation constant of the binary compound, LDH · NAD; *p*MB, *p*-mercuribenzoate (written to indicate the uncertainty of the associated anion in solutions of the mercurial); Tris, tris (hydroxymethyl) aminomethane; EDTA, ethylenediaminetetraacetate.

mechanism needs further consideration, that fluorescence measurements do not represent quantities which can be compared to the specifically defined dissociation constants, or that the rate measurements are in error. In the case of liver alcohol dehydrogenase, Theorell *et al.*[1,2] found good agreement between the values for the dissociation constants of the binary compounds ER and EO ($K_{E,R}$ and $K_{E,O}$) as determined from fluorescence equilibrium measurements in absence of substrate, and those calculated from the relation between the kinetic "off" and "on" rate constants, as determined in kinetic studies in the presence of substrate. In these calculations, the Theorell-Chance mechanism, in which only binary compounds of EO and ER are kinetically significant, was assumed valid over the pH range investigated.

For bovine heart LDH, a reaction sequence was formulated in 1956 by Takenaka and Schwert[3] in which there is a compulsory pathway such that substrates can combine only with the enzyme-coenzyme compounds to form one or more kinetically indistinguishable but significant ternary compounds. Kinetic data obtained over a wide range of pH and experimental conditions, coupled with ultracentrifuge equilibrium binding experiments[4] have supported the ternary compound mechanism. Similar mechanisms in which ternary compounds have kinetic significance have been suggested for other dehydrogenases such as ribitol[5], pig heart malic[6], yeast alcohol[7], rabbit muscle lactate[8] and rat liver lactate[9]. Recently, Alberty and co-workers[10] and Baker[11] have suggested that even for liver ADH the Theorell-Chance mechanism is unlikely to provide a satisfactory description of the reaction mechanism as a result of the omission of kinetically significant ternary compounds.

Since kinetic data on bovine heart LDH was available over a wide range of pH[12] it was considered of interest to compare the apparent dissociation constants of the LDH · $NADH_2$ compound obtained fluorimetrically in the absence of substrate with those constants calculated from kinetic parameters assuming the intermediary ternary compound mechanism to be valid under the experimental conditions used. This paper reports on $K_{E,R}$ at various pH values and experimental conditions. A comparison of these constants with the dissociation constants as derived from kinetic measurements indicates fairly good agreement over the pH range 6–8 but differs considerably at higher pH values, perhaps indicating that the reaction mechanism needs further consideration. It is suggested that the presence of the inactive compound LDH · NAD · pyruvate, the formation of which is favored in alkaline solution, contributes significantly to the reaction mechanism. Preliminary spectrophotometric and fluorimetric evidence for the formation of the compounds LDH · NAD and LDH · NAD · pyruvate is also presented.

EXPERIMENTAL

Enzyme. Bovine heart LDH was prepared according to the method of Schwert *et al.*[13] and component "A" isolated by column chromatography on hydroxyapatite. The chromatographed enzyme was recrystallized from ammonium sulfate 2–8 times and all final titrations with coenzyme were performed with 8× recrystallized enzyme. To ensure that no L-lactate was present on the enzyme, solutions of LDH in 0.05 M phosphate, pH 6.9 + 0.001 M EDTA were treated with a few mg of NAD/ml of enzyme and the solution dialyzed 4–5 times (2–3 days) against 0.05 M phosphate buffer, pH 6.9. However, treatment of the enzyme in this way

gave identical fluorescent titration curves with $NADH_2$ as did untreated enzyme. Diluted enzyme solutions from either the crystals in 0.6 saturated ammonium sulfate or from the NAD treated enzyme, were prepared fresh daily. Activity measurements were made before and after each titration in a number of experiments and no loss of activity was found during this time over the pH range 6 to 10. The enzyme was assayed either in the presence of excess L-lactate and NAD at pH 10 in glycine-phosphate buffer[13] or by determining the concentration of binding sites by titration with $NADH_2$ in the presence of excess oxalate (0.3 M) as was done previously with liver ADH, $NADH_2$ and isobutyramide[2]. During the early phase of this work, protein concentration was estimated from absorbancy measurements at 280 mμ. A value of 19.3×10^4 cm^{-1} M^{-1} was used as the absorbancy index of the crystalline enzyme[3] and the molecular weight was taken as 135 000. The enzyme was assumed to be pure fraction "A" when stock solutions had an E_{280} which in relation to the activity determined either from rate measurements or from titration in the presence of oxalate, had an absorbancy index of $19 \pm 0.5 \times 10^4$ cm^{-1} M^{-1}.

Coenzymes and reagents. β-$NADH_2$ was purchased from the Sigma Chemical Co. and assayed with yeast alcohol dehydrogenase. The β-$NADH_2$ used was 72 % by weight using an absorbancy index of 6.22×10^3 cm^{-1} M^{-1} at 340 mμ. The residual fluorescence after enzymatic oxidation was 5 % which was corrected for in all calculations. Concentrations of β-$NADH_2$ were used which showed no fluorescence quenching as described previously[14]. Stock solutions of $NADH_2$ were prepared in Tris-KCl buffer, ionic strength 0.2, pH 10 and working solutions made in the desired buffer directly before use and stored in the dark at 3°C. β-NAD was purchased from the Sigma Chemical Co. and was assayed according to the method of Dalziel[15]. It was found to be 97 % by weight using an absorbancy index at 260 mμ of 18.3×10^3 cm^{-1} M^{-1}. The 3-acetyl pyridine analog of NAD (APNAD) (from Sigma) was 95 % by weight assuming an absorbancy index at 260 mμ of 16.4×10^3 cm^{-1} M^{-1}. Stock solutions of coenzymes were prepared in water and titrated to pH 6 with sodium hydroxide. These solutions were used directly in both the fluorescence titrations and absorbancy measurements and were stable for days in the cold at 3°C. All buffers contained 0.001 M EDTA and were prepared in 2× quartz distilled water. Potassium pyruvate, L-lactate and oxalate were prepared as described earlier[3].

Methods. Fluorescence measurements were performed on a Farrand Model A recording fluorimeter equipped with a water jacketted cell compartment. A Corning primary filter No. c. s. 7–60 was used at the excitation entrance and a secondary filter No. c. s. 3–74 at the fluorescence emission exit. All fluorescence measurements were made at 28.5°C in either 0.2 ionic strength phosphate or Tris-KCl buffers unless otherwise stated. The temperature in the cells was measured with a probe electronic thermometer and pH measurements were made with a Beckman combination probe electrode. The excitation wave length was 340 mμ and the fluorescence intensity measured at 420 mμ. Corrections were made on both the activation and emission spectra. Although the apparent maximum fluorescence emission of $NADH_2$ is at 465–470 mμ on this instrument and at the settings employed, 420 mμ was used for recording fluorescence intensity since the deflection ratio of bound to free coenzyme is considerably higher at these wave length settings *i. e.* Q (see Ref. 1) is 7.6 and independent of pH from 6 to 10. A final volume of 2.00 ml in 1×1 cm quartz cuvettes was used in all titrations with addition of the coenzymes from a microtitrator. Spectrophotometric measurements were made on a Cary Model 11 equipped with a constant temperature sample cell compartment.*

Evaluation of $K_{E,R}$. As has been reported with liver ADH[1], the apparent dissociation constants of EDH · $NADH_2$ were evaluated either by single additions of $NADH_2$ to a constant enzyme concentration (about twice $K_{E,R}$) or in experiments where Q is to be determined, additions of enzyme to a constant amount of $NADH_2$.

The first method leads to the compound E · $(NADH_2)_4$ at infinite $NADH_2$ concentration and the second method leads to the formation of the compound E · $NADH_2$ at infinite enzyme concentration. Q was independently determined over the whole pH range investigated and found to be 7.6 ± 0.5. Values of $K_{E,R}$ as determined by titration were averaged from 5–6 points and the absence of any systematic drift gives credence to the equivalence of the four coenzyme binding sites per 135 000 g of protein.

* Constructed by Mr. Thomas Orr of the Research Machine Shop.

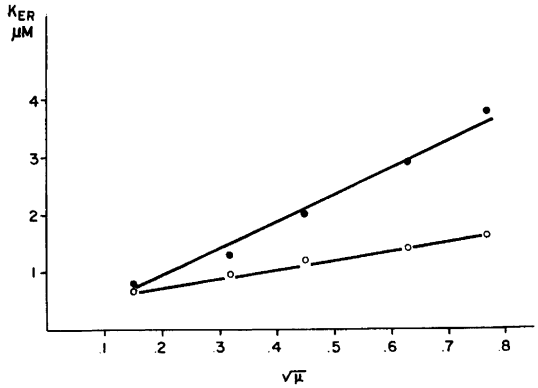


Fig. 1. Variation of $K_{E,R}$ with ionic strength of phosphate (O) and Tris-KCl (●) buffer, at 28.5°C.

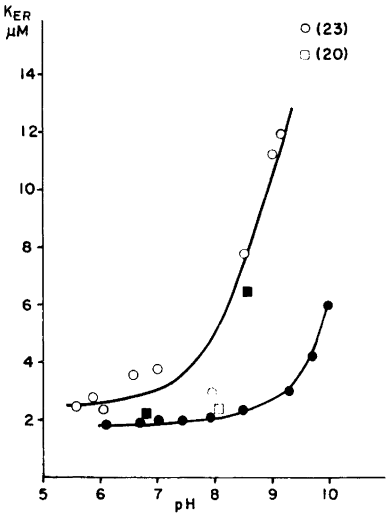


Fig. 2. Comparison of $K_{E,R}$ with pH in Tris KCl buffer, $\mu = 0.2$, at 28.5°C as determined fluorometrically (●), kinetically from K_{RP}/K_P (O), from *p*MB inhibition experiments (□), and from competitive inhibition experiments (■). For definitions of the Michaelis and rate constants see Ref[12].

RESULTS

It has been reported that the bovine heart LDH system is very susceptible to anion effects[16]* and the magnitude of the kinetic constants depends markedly on the buffer system used. For example, the Michaelis constants are approximately ten times greater in Tris-KCl buffer than in phosphate buffer of identical pH and ionic strength. Earlier kinetic studies[17] had indicated that $K_{E,R}$ in 0.1 M phosphate buffer was about half as large as it was later shown to be in Tris-KCl buffer of the same concentration[12]. The dependence of $K_{E,R}$ on the ionic strength of phosphate and Tris-KCl buffers is shown in Fig. 1. The value of $K_{E,R}$ at an ionic strength of 0.2 is about twice as great in Tris-KCl as in phosphae, and at an ionic strength of 0.125, the values of $K_{E,R}$ approach one another to about 0.8 μM. As seen in the lower curve of Fig. 2 values of $K_{E,R}$ as determined in the present fluorescence studies range from 2.0 μM at pH 6 to 6.3 μM at pH 10. The upper curve shows the variation of $K_{E,R}$ with pH as calculated from a variety of kinetic studies, as defined in the legend, and the values range from 2.2 μM at pH 5.8 to

* Buffers such as Tris-acetate have been used to evaluate $K_{E,R}$[18] and these values are undoubtedly low since acetate (I), as an inhibitor, would have the effect of lowering the apparent dissociation constant, thus $K_{ER,I} \ll K_{E,R}$.

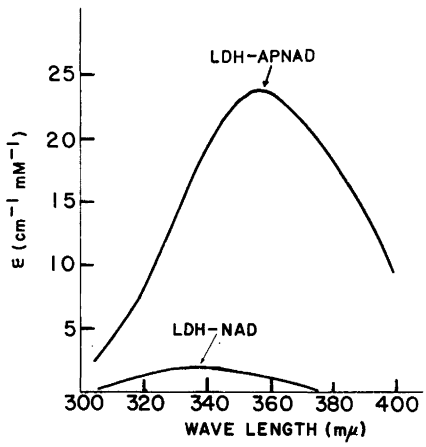


Fig. 3. Difference spectra of the LDH·NAD and LDH·APNAD compounds in Tris-KCl buffer, pH 8.15, μ=0.2, 28.5°C. The spectra have been corrected for absorbancy due only to enzyme or to coenzyme and represent enzyme saturated with coenzyme.

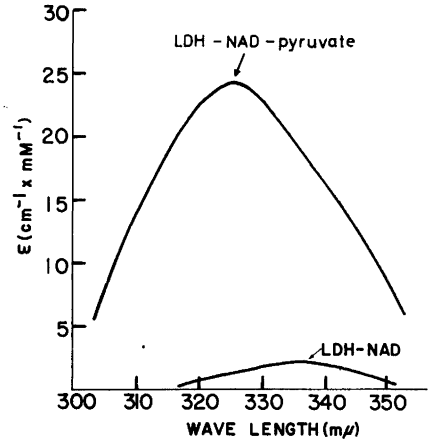


Fig. 4. Difference spectra of the LDH·NAD and LDH·NAD·pyruvate compounds in Tris-KCl buffer, pH 8.15, μ=0.2, 28.5°C. The spectra have been corrected for absorbancy due only to enzyme, pyruvate or NAD and represent enzyme saturated with NAD or NAD and pyruvate.

23 at pH 9.8. Independent determinations of $K_{E,R}$ from kinetic experiments in which LDH is protected from *p*MB inactivation in the presence of $NADH_2$ gave values of 2.2 μM at pH 8.03 and 20 μM at pH 10.02, while values obtained from experiments in which NAD and $NADH_2$ compete for the same site on the enzyme were 2.2 μM at pH 6.8 and 6.3 μM at pH 8.5.

When an attempt was made to determine $K_{E,O}$ by the addition of NAD to the enzyme prior to $NADH_2$ additions, as described earlier with alcohol dehydrogenase[1], it was observed that solutions of enzyme and oxidized coenzyme, when activated by 340 mμ light, exhibit a fluorescence emission spectrum with a maximum at about 450 mμ*. A spectrophotometric maximum was also observed at 335 mμ as was the compound LDH · APNAD with a wave length maximum of 355 mμ as shown in Fig. 3. The millimolar absorbancy index of the LDH · APNAD compound is about 10 times greater than that of the LDH · NAD compound. Formation of both binary compounds is pH dependent, and, at equilibrium, a greater amount of fluorescence or absorbancy is observed at higher than at lower pH values. Determination of the apparent dissociation constants from the concentrations of added coenzyme required to obtain half maximal fluorescence or optical density gave values in Tris-KCl buffer, pH 8.15 of 8.0×10^{-5} M for LDH · NAD and 0.9×10^{-5} M for LDH · APNAD. Titrations with APNAD, in which the equilibrium is very much more favorable than NAD, indicated that 3.7 moles of coenzyme analog were bound per mole of enzyme. When pyruvate

* A complete report on the properties of the binary LDH · NAD and ternary LDH · NAD · pyruvate compounds is in preparation and will be reported elsewhere.

is added to the LDH · NAD compound, a new absorbancy maximum is seen at 325 mμ as shown in Fig. 4. The millimolar absorbancy index of the LDH · NAD · pyruvate compound is 24 and the apparent dissociation constant 5×10^{-4} M under the conditions of the experiment as indicated in the legend of Fig. 4. Formation of the LDH · NAD · pyruvate compound at equilibrium is pH dependent and favored in alkaline solution. This compound is probably identical to the one recently reported by Fromm[19] with rabbit muscle LDH.

DISCUSSION

The fluorometric titration measurements of the apparent dissociation constants of LDH · $NADH_2$ from pH 6 to 8 in 0.2 ionic strength Tris-KCl buffer appear to be in fairly good agreement with those constants calculated from kinetic parameters as derived from a reaction mechanism including significant ternary compounds. That such equilibrium constants do indeed represent quantities which can be compared to the specifically defined dissociation constants may be questioned. The fact that these constants show rather good agreement from pH 6 to 8 with the independent kinetic evaluations of $K_{E,R}$ from *p*MB or competitive coenzyme inhibition experiments may be fortuitous. However, the stoichiometry of coenzyme binding by fluorescence techniques is in agreement with the stoichiometry as determined by ultracentrifuge binding studies[3]. Also oxamate and oxalate, inhibitors of the enzymatic reaction, in that they form ternary compounds E · NADH · oxamate and LDH · NAD · oxalate, respectively, show similar dissociation constants whether estimated from fluorescence[20] or ultracentrifuge binding data[4]. The existence of the LDH · NAD compound has previously been assumed from competitive binding experiments[12] and ultracentrifuge separation data[3] but this is the first direct fluorometric and spectrophotometric demonstration of this binary compound. The single value of $K_{E,O}$ determined by absorbancy measurements is about 2× lower than the corresponding value as calculated from kinetic parameters[12]. A more complete determination of $K_{E,O}$ over a wide range of pH will permit a comparison of these constants with those calculated from kinetic parameters and this work is currently in progress.

The fluorometric detection of the ternary compound LDH · NADH · L-lactate has been reported at low pH values[21] and the compound LDH · NAD · pyruvate with rabbit muscle enzyme has recently been reported at pH 7.6 in Tris-chloride buffer by Fromm[19] using difference spectrophotometry. The concentration of pyruvate used to detect this compound, however, was sufficient to produce complete inhibition of the enzymatic reaction at the pH employed[12]. The concentrations of pyruvate used in the present studies were of the order of the Michaelis constant and the equilibrium constant for the dissociation of the LDH · NAD · pyruvate compound to give pyruvate and LDH · NAD is also of the order of the Michaelis constant for pyruvate.

Recently, the kinetic importance of ternary compounds in the mechanism of dehydrogenases was shown experimentally by the product inhibition method first proposed by Alberty[22]. Fromm and co-workers[5,7] have found inhibition data for ribitol and muscle lactate dehydrogenases to be consistent with the formation of inactive ternary compounds between enzyme, coenzyme, and the wrong substrate, but only when relatively high concentrations of the product inhibitor

were employed. However, whether the formation of the inactive compound of LDH · NAD · pyruvate can account fully for the difference in $K_{E,R}$ observed fluorimetrically and kinetically in the present studies at higher pH values cannot be answered at this time.

Acknowledgements. This investigation was supported by grants No. GM 07578-03 and 9015 from the *U. S. Public Health Service.* The author wishes to thank Prof. George W. Schwert for the many helpful suggestions offered during the course of this investigation. Skilful technical assistance was given by Mrs. Linda Noble.

REFERENCES

1. Theorell, H. and Winer, A. D. *Arch. Biochem. Biophys.* **83** (1959) 291.
2. Theorell, H. and McKinley-McKee, J. S. *Acta Chem. Scand.* **15** (1961) 1811.
3. Takenaka, Y. and Schwert, G. W. *J. Biol. Chem.* **223** (1956) 157.
4. Novoa, W. B. and Schwert, G. W. *J. Biol. Chem.* **236** (1961) 2150.
5. Fromm, H. J. and Nelson, D. R. *J. Biol. Chem.* **237** (1962) 215.
6. Raval, D. N. and Wolfe, R. G. *Biochemistry* **1** (1962) 215.
7. Nygaard, A. P. and Theorell, H. *Acta Chem. Scand.* **9** (1955) 1300.
8. Zewe, V. and Fromm, H. J. *J. Biol. Chem.* **237** (1962) 1668.
9. Vestling, C. S. *Methods of Biochem. Analysis,* Interscience, New York, 1962, Vol. X, p. 137.
10. Bloomfield, V., Peller, L. and Alberty, R. A. *J. Am. Chem. Soc.* **84** (1962) 4367.
11. Baker, R. H., Jr. *Biochemistry* **1** (1962) 41.
12. Winer, A. D. and Schwert, G. W. *J. Biol. Chem.* **231** (1958) 1065.
13. Schwert, G. W., Millar, D. B. S. and Takenaka, Y. *J. Biol. Chem.* **237** (1962) 2131.
14. Winer, A. D., Schwert, G. W. and Millar, D. B. S. *J. Biol. Chem.* **234** (1959) 1149.
15. Dalziel, K. *Biochem. J.* **84** (1962) 244.
16. Winer, A. D. and Schwert, G. W. *J. Biol. Chem.* **234** (1959) 1155.
17. Hakala, M. T., Glaid, A. J. and Schwert, G. W. *J. Biol. Chem.* **221** (1956) 191.
18. Velick, S. F. *J. Biol. Chem.* **233** (1958) 1455.
19. Fromm, H. J. *Biochim. Biophys. Acta* **52** (1961) 199.
20. Novoa, W. B., Winer, A. D., Glaid, A. J. and Schwert, G. W. *J. Biol. Chem.* **234** (1959) 1143.
21. Winer, A. D., Novoa, W. B. and Schwert, G. W. *J. Am. Chem. Soc.* **79** (1957) 6571.
22. Alberty, R. A. *J. Am. Chem. Soc.* **80** (1958) 1777.

Received April 5, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 210–S 215

Intracellular Localization of Catalase in Rat Liver

CHRISTIAN de DUVE, HENRY BEAUFAY* and PIERRE BAUDHUIN**

Laboratoire de Chimie Physiologique, Université de Louvain, Louvain, Belgique

Catalase is associated with cytoplasmic particles to the extent of about 70 % in homogenates of rat liver tissue. Fractionations performed under a variety of conditions indicate that the particles which contain this enzyme are different from mitochondria and from lysosomes, and are closely similar to, or identical with, those bearing urate oxidase. Catalase is partly latent in intact particles; it can be simultaneously unmasked and solubilized by several treatments, though not by exposure to distilled water.

Catalase is one of the numerous haemoproteins to the study of which Hugo Theorell has devoted some of his most inspiring research activities. The intracellular localization of this enzyme has been investigated by a number of authors and with a variety of techniques[1–11]. In general, it has been recovered partly in cytoplasmic particles and partly in the final supernatant, the ratio of soluble to particulate activity depending on the species and sex of the animal and on the fractionation method employed. The particles which contain the enzyme sediment largely with the mitochondrial fraction and are believed by most workers to be mitochondria. However, this view has been questioned by Thomson and Klipfel[8], who have found that the sedimentation pattern of particulate mouse liver catalase is very similar to that of urate oxidase. Feinstein[12] has quoted this finding, together with the observation by Adams and Burgess[9] that the catalase activity of the particles is enhanced by small amounts of Triton X-100, in support of the hypothesis that the enzyme may be associated with the lysosomes. Since it has now been found that urate oxidase is associated with a special group of cytoplasmic particles, different both from the mitochondria and from the lysosomes[13–15], it seemed of interest to investigate further the localization of catalase in liver tissue. The results of these investigations are described in the present paper. Some of them have already been mentioned briefly in preliminary reports[14,15] and at a recent symposium[16].

* Associé du F. N. R. S.
** Aspirant du F. N. R. S.

EXPERIMENTAL

Rat liver was homogenized in 0.25 M sucrose and fractionated as described by de Duve *et al.*[17]. The scheme followed differs from the classical one in that two separate mitochondrial fractions are isolated: a heavy one (M), containing the larger part of the mitochondrial nitrogen and cytochrome oxidase activity and relatively poor in lysosomal enzymes and in urate oxidase; and a light one (L), containing the residual mitochondria and some microsomes, together with a particularly high proportion of lysosomes and of bodies containing urate oxidase. When density gradient experiments were performed, these two fractions were first isolated together, washed and then centrifuged in suitable density gradients until they had reached a position in the gradient corresponding to their own density. The techniques used in these experiments were those described by de Duve *et al.*[18] and Beaufay *et al.*[13].

Catalase was assayed at 0°C according to a modification of the method described by Chantrenne[19]. For measurements of total activity, the preparations were pretreated with 1 % Triton X-100 to release the enzyme. The reaction was found to be of first order with respect to hydrogen peroxide concentration under the conditions of the assay and the observed activities were expressed in units, one unit being the amount of enzyme causing the disappearance of 90 % of the substrate in one min in a volume of 50 ml at 0°C. Nitrogen, protein and reference enzymes were measured as described before[13,17].

RESULTS

Intracellular distribution pattern

In Table 1 are summarized the results obtained in 7 fractionation experiments. Only the data for catalase are shown, but each experiment included measurements of cytochrome oxidase, glucose-6-phosphatase, acid phosphatase and urate oxidase. The distributions observed for the latter enzymes were similar to those reported by de Duve *et al.*[17]. As indicated in Table 1, catalase exhibits a distinct peak of specific activity in fraction L, thus resembling urate oxidase and the lysosomal hydrolases, rather than the mitochondrial cytochrome oxidase which has its highest specific activity in fraction M. Approximately 30 % of the total catalase activity was recovered in the final supernatant.

Subfractionation in density gradients

In Figs. 1 and 2 are shown the enzymatic distributions found after isopycnic centrifugation of mitochondrial fractions in linear density gradients made up respectively with sucrose in D_2O and with glycogen in 0.5 M sucrose. In the

Table 1. Intracellular distribution of catalase (total activity of liver = 60.5 ± 6.0 U/g). Means of 7 experiments ± S.E.M.

Fraction	Total activity % of total	Specific activity % activity/% nitrogen
Nuclear (N)	7.4 ± 2.1	0.37 ± 0.08
Heavy mitochondrial (M)	21.7 ± 4.0	1.09 ± 0.17
Light mitochondrial (L)	21.7 ± 3.6	6.64 ± 0.82
Microsomal (P)	9.0 ± 2.3	0.44 ± 0.11
Supernatant (S)	31.2 ± 2.6	0.98 ± 0.14
Recovery	91.0 ± 2.3	–

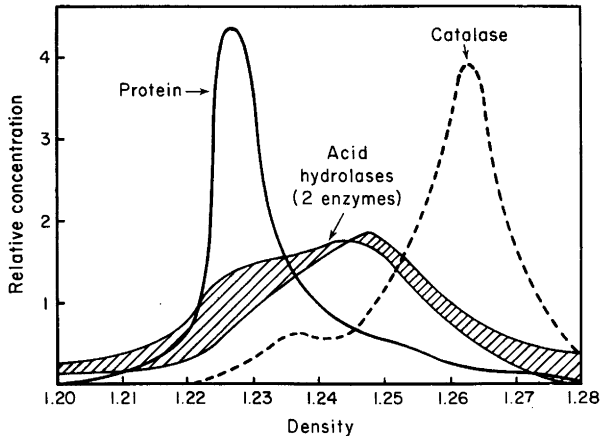


Fig. 1. Mitochondrial fraction (M + L) from rat liver, equilibrated in linear gradient of sucrose in D_2O (0.85 to 1.58 M over 4.1 cm). The particles were included homogeneously in the gradient and the curves indicate the ratio of the final to the initial concentration of the measured components. The two acid hydrolases are acid phosphatase (left) and acid deoxyribonuclease (right).

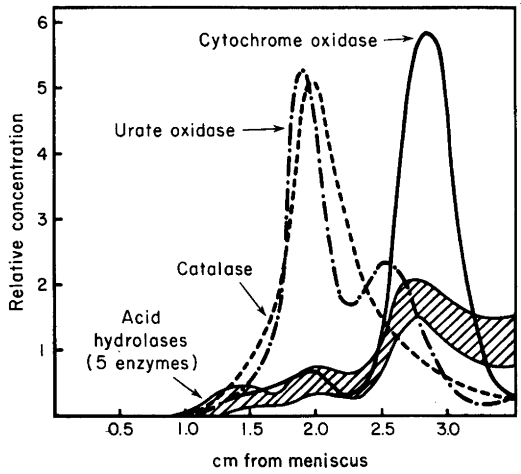


Fig. 2. Mitochondrial fraction (M + L) from rat liver, equilibrated in gradient of glycogen in 0.5 M sucrose (0 to 0.23 g/ml over 4.1 cm). The gradient was initially linear but became distorted in the course of centrifugation. The results are expressed as in Fig. 2, but as a function of the position in the tube. The five acid hydrolases are acid phosphatase, β-glucuronidase, cathepsin, acid ribonuclease and acid deoxyribonuclease. Their distribution curves were all included within the shaded area.

experiment of Fig. 1, the distribution of catalase resembles closely that observed previously for urate oxidase in a similar gradient[13] and differs significantly both from that of the proteins, which belong mostly to the mitochondria, and from that of the lysosomal hydrolases. The similarity between catalase and urate

oxidase shows up in an even more striking fashion in the glycogen gradient, which allows an almost complete separation of these two enzymes from the lysosomes (Fig. 2).

Several other experiments of this type were performed, in gradients of sucrose in water and of glycogen in sucrose solutions of various concentrations. In all cases, the distribution of catalase was similar to that of urate oxidase, though slightly shifted towards the region of lower density. These results have shown that the median density of the two particulate enzymes increases linearly with the density of the sucrose solution to which they are exposed. On the other hand, mitochondria and lysosomes exhibit a different behaviour, obeying a more complex function.

Activation experiments

The observation[9,12] that particulate catalase is partly latent has been confirmed. As shown in Table 2, the free activity measured on homogenates is essentially equal to the activity which is recovered in the supernatant after high-speed centrifugation, suggesting that under these conditions the activity associated with particles is entirely masked. However, some activity, corresponding to 10 – 20 % of the total activity present, is always released from the particles when the latter are further isolated. Complete release can be accomplished by the addition of Triton X-100 or digitonin to the suspensions. The concentration of digitonin necessary to unmask the catalase activity quantitatively is distinctly higher than that sufficient for complete release of the lysosomal hydrolases. Catalase also differs from the latter enzymes by the fact that exposure of the particles to media of low tonicity (down to 5 mM sucrose) causes almost no release of activity. In contrast with catalase, urate oxidase is fully accessible to its substrate under the conditions of its assay, which, however, is carried out at 37°C.

Table 2. Latency of catalase in rat-liver homogenates. Total activity refers to measurements made on whole homogenate with addition of Triton X-100. Free activity refers to similar measurements carried out without the addition of Triton X-100 and in the presence of 0.25 M sucrose. Soluble activity is total activity of supernatant isolated after centrifuging the homogenate for 30 min at 100 000 × *g*.

Expt.	% of total activity	
	Free activity	Soluble activity
1	23.6	21.7
2	19.8	16.3
3	20.9	21.9
4	30.5	31.9

DISCUSSION

In its centrifugal behaviour, particulate catalase differs clearly from mitochondria in all the systems investigated and it may safely be concluded that these particles have little or no catalase activity. The enzyme sediments together with the lysosomes in conventional centrifugal fractionation experiments, but can be largely separated from the latter particles by means of density gradient isopycnic centrifugation. Also, while resembling the lysosomal hydrolases in being largely latent in preparations of intact particles, it is not released as easily as these enzymes by means of digitonin and is not liberated by exposure of the particles to hypotonic media. Therefore, a major localization of catalase in the lysosomes can also be ruled out.

On the other hand, catalase shows a striking correlation with urate oxidase in a number of different fractionation systems. In particular, it shares with this enzyme the property of accumulating in the denser regions in aqueous sucrose gradients and in the lighter regions in glycogen – 0.5 M sucrose gradients, a consequence of the unique linear relationship linking the density of their host-particles to that of the sucrose solution in the medium. As shown before[16,18], this relationship indicates that the particles are permeable to sucrose and do not respond osmotically to changes in sucrose concentration. In the case of catalase, this feature is confirmed by the lack of osmotic release of the enzyme.

In view of these observations, it seems very likely that catalase is associated with the urate oxidase-containing particles, which have been recently identified with the so-called "microbodies"[15]. However, it must be pointed out that, in contrast with urate oxidase, catalase is always found in soluble form to the extent of at least 20 % in hepatic homogenates and that the density distribution curve of the particulate enzyme is shifted slightly but significantly in the direction of lower densities, with respect to that of urate oxidase[16]. It must also be remembered that structure-linked latency has not been demonstrated so far for the latter enzyme.

One explanation could account for the distribution differences without introducing the assumption that the enzymes belong to different particles or that they are associated together, but in varying proportion, in the same particles. It is possible that homogenization causes the rupture of a number of microbodies and the consequent dissociation of catalase, which is released in soluble form. from urate oxidase which remains attached to an insoluble residue of higher density than the intact particle. Supporting this hypothesis is the fact that the denser fractions, which are very rich in urate oxidase and relatively poor in catalase, contain, in addition to intact microbodies, a large number of the multilamellar or multicanalicular cores, which characterize these bodies, in free form[15]. This observation suggests that urate oxidase may be part of this core, whereas catalase would be present in the soluble phase of the microbodies.

This interpretation is apparently contradicted by the fact that catalase displays structure-linked latency, whereas urate oxidase does not. However, catalase, owing to its very high activity, was measured at 0°C, whereas urate oxidase has always been assayed at 37°C for the opposite reason. It remains to be seen whether differences in latency would be observed if the enzymes could be determined under identical conditions. It must also be pointed out that if the acces-

sibility of two enzymes to their respective substrate is restricted by the same structural barrier, diffusion of the substrate may be limiting for a very active enzyme and not for one of low activity.

Accordingly, it is provisionally concluded that catalase is largely, and perhaps completely, associated with urate oxidase in the microbodies of rat liver. The same is probably true for D-amino acid oxidase, whose distribution in density gradients is identical with that of catalase[14,16]. The physiological role of these bodies raises intriguing problems. The only known link between the three enzymes so far identified in them is hydrogen peroxide, which is formed by two of them and utilized by the third one. However, it is difficult to imagine how such a tenuous link could form the basis of a physiological function.

Acknowledgement. These investigations have been supported by the *Fonds National de la Recherche Scientifique*, the *Centre National de Recherches Enzymologiques*, the *Fonds de la Recherche Scientifique Fondamentale Collective*, the *Rockefeller Foundation* and the *U. S. Public Health Service* (Grant No. RG-8705).

REFERENCES

1. von Euler, H. and Heller, L. *Z. Krebsforsch.* **56** (1949) 393.
2. Ludewig, S. and Chanutin, A. *Arch. Biochem.* **29** (1950) 441.
3. Nyberg, A., Schuberth, J. and Änggård, L. *Acta Chem. Scand.* **7** (1953) 1170.
4. Feinstein, R. N., Hampton, M. and Cotter, G. J. *Enzymologia* **16** (1953) 219.
5. Thomson, J. F. and Mikuta, E. T. *Arch. Biochem. Biophys.* **51** (1954) 487.
6. Klein, P. D. and Thomson, J. F. *Am. J. Physiol.* **187** (1956) 259.
7. Greenfield, R. E. and Price, V. E. *J. Biol. Chem.* **220** (1956) 607.
8. Thomson, J. F. and Klipfel, F. J. *Arch. Biochem. Biophys.* **70** (1957) 224.
9. Adams, D. H. and Burgess, E. A. *Brit. J. Cancer* **11** (1957) 310.
10. Adams, D. H. and Burgess, E. A. *Biochem. J.* **71** (1959) 340.
11. Adams, D. H. *Biochem. J.* **74** (1960) 141.
12. Feinstein, R. N. *Arch. Biochem. Biophys.* **79** (1959) 399.
13. Beaufay, H., Bendall, D. S., Baudhuin, P., Wattiaux, R. and de Duve, C. *Biochem. J.* **73** (1959) 628.
14. de Duve, C., Beaufay, H., Jacques, P., Rahman-Li, Y., Sellinger, O. Z., Wattiaux, R. and De Coninck, S. *Biochim. Biophys. Acta* **40** (1960) 186.
15. Baudhuin, P. and Beaufay, H. *Arch. Intern. Physiol. Biochim.* **71** (1963) 119.
16. Beaufay, H. and Berthet, J. *Biochem. Soc. Symp. (Cambridge, Engl.)* **23** (1963) 66.
17. de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. and Appelmans, F. *Biochem. J.* **60** (1955) 604.
18. de Duve, C., Berthet, J. and Beaufay, H. *Progr. Biophys.* **9** (1959) 325.
19. Chantrenne, H. *Biochim. Biophys. Acta* **16** (1955) 410.

Received April 1, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 216–S 221

Effect of Electrolytes on the Absorption Spectrum of Alkaline Ferriheme

G. BLAUER and H. ROTTENBERG

Weizmann Institute of Science, Rehovot, and Department of Biological Chemistry, Hebrew University, Jerusalem, Israel

Spectral changes observed in the region 270–650 mμ upon addition of various electrolytes to $10^{-3} - 10^{-5}$ M alkaline ferriheme (ferriprotoporphyrin IX) are mainly characterized by the appearance of a broad band around 580 mμ, a minimum near 540 mμ and by a considerable diminution of the Soret band maximum at 385 mμ. In the visible range, these changes were found to depend on both the ionic strength of the added electrolyte and the total ferriheme concentration. In the system 5×10^{-5} M ferriheme – 1.9 M NaCl, the ratio $\varepsilon_{580}/\varepsilon_{540}$ decreased significantly from pH 9 to 5.5 indicating a transition with an estimated midpoint of pH 7.1 at 27°C. Both spectral and sedimentation-diffusion data suggest that ferriheme molecules, at high ionic strength, form micelles of considerable size in which optical interactions may occur. This aggregation is considered to be favored by screening of electrostatic interactions between porphyrin carboxylates and also by changes in the structure of water around the hydrophobic regions of the protoporphyrin.

The absorption spectra of the prosthetic group iron-protoporphyrin IX as they appear in various hemoproteins and their derivatives have been classified by Theorell[1] many years ago on the basis of extensive research on these substances. A recent treatment of the absorption bands of ferric porphyrin complexes[2] suggests two charge-transfer bands to high-spin ferric ions and three porphyrin absorption bands which appear in low-spin complexes.

A spectral type not found in native systems has been observed in ferriheme solutions of pH > 9 containing high concentrations of pyridine (1 – 6 M)[3–6] and, more recently, in the presence of random or highly ionized polybases such as poly-L-lysine at pH 8 – 9[7] or poly-4-vinylpiperidine[8]. These "green complexes" appeared whenever the regular helical structure of poly-L-lysine-ferrihemochromes seemed to be disturbed *e. g.* by ionization, copolymerization or increase in temperature[8]. It has now been found that simple electrolytes, at sufficiently high concentrations, also caused the appearance of analogous spectra. The present investigation is concerned with the formation of the "green complexes" produced by salts, in particular as measured by the visible-range absorption. These complexes probably constitute higher aggregates. According to the con-

ditions for their formation, similar aggregates will not be expected to be produced in native proteins under normal conditions.

EXPERIMENTAL

Materials. Sigma Bovine Hemin (2 × recrystallized) was again recrystallized. Salts used were analytical-grade.

Spectra. Beckman DU and Zeiss PMQ II spectrophotometers were used. Constant temperature (usually 26–27°C) was maintained by circulation around the cell compartment of water from a temperature-controlled bath.

Sedimentation and diffusion were measured in a Spinco Model E Ultracentrifuge using Schlieren optics with Kodak plates type 103a-E and a red Ilford filter 205.

Stability and reproducibility. The absorbancy of three different solutions of 0.4, 0.7 and 2 M NaCl, respectively, all containing 5.1×10^{-5} M ferriheme at pH 10.5 and 26°C, was checked at 385, 540 and 580 mμ at constant time intervals up to 24 h and in one case (2.0 M NaCl) up to one week. Maximal changes in ε were of the order of 10 % but in most cases the deviations were smaller. The Soret-bandabsorption was generally less stable with time than the visible-range absorption. Parallel preparations of identical composition occasionally disagreed by 5 – 10 %, in particular at pH $\approx$ 6 which is close to the precipitation limit of the ferriheme-salt system (Fig. 4). CO_2 was not excluded rigorously from the solutions since the effect of Na_2CO_3, which could be formed by CO_2-absorption from the air, was considered negligible.

The light absorption of solutions diluted from higher concentration was in reasonable agreement with values obtained directly, though in some cases there were indications of partial irreversibility. The order of mixing of salt and ferriheme solutions had no effect on the spectrum. ε_mM is the decadic millimolar extinction coefficient in $mM^{-1} \times cm^{-1}$ calculated per 1 Fe. Concentrations of polymer and ferriheme are in monomolar units.

RESULTS AND DISCUSSION

The effect of 1.9 M NaCl on the spectrum of alkaline ferriheme in the range 270–650 mμ is given by curves 1 and 2 of Fig. 1. Under the conditions of Fig. 1,

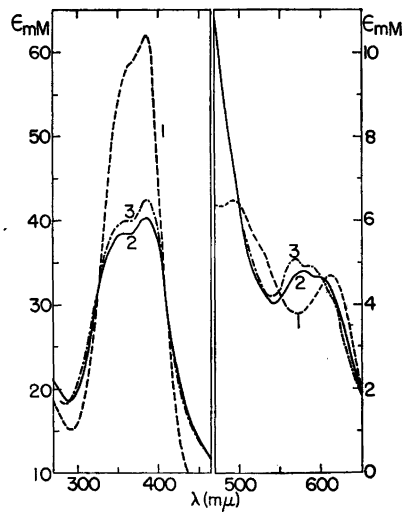


Fig. 1. Absorption spectra at 26°C. Curve 1: Ferriheme 5.1×10^{-5} M; pH 11. Curve 2: Ferriheme 5.1×10^{-5} M; NaCl 1.9 M; pH 11. Curve 3: Ferriheme 5.1×10^{-5} M; poly-L-lysine 5.0×10^{-4} M; pH 8.9.

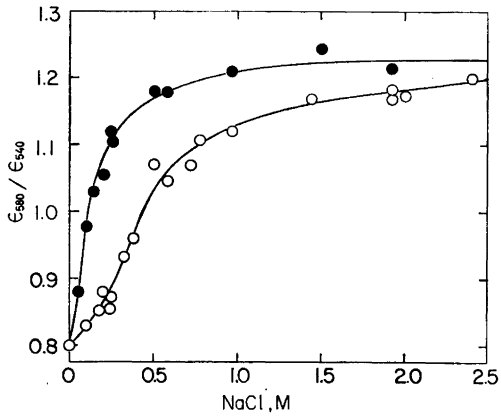


Fig. 2.

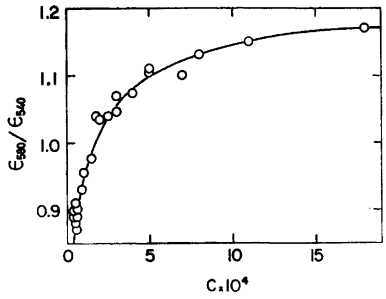


Fig. 3.

Fig. 2. Dependence of the ratio $\varepsilon_{580}/\varepsilon_{540}$ on salt concentration at 27 ± 1°C. Open Circles: Total ferriheme 5×10^{-5} M; pH 11. Dots: Total ferriheme 5×10^{-4} M; pH 11 – 12.

Fig. 3. Dependence of the ratio $\varepsilon_{580}/\varepsilon_{540}$ on the total ferriheme concentration at constant NaCl concentration (0.25 M), 27 ± 2°C and pH 11.

the Soret peak at 385 mμ is diminished to $\varepsilon_{mM} = 41$ in 1.9 M NaCl. An adjacent and pronounced shoulder near 360 mμ also decreases. In the visible range, the main band maximum is at 580 mμ ($\varepsilon_{mM} = 5$) with a shoulder near 600 mμ. Minima are at 540–545 and 290 mμ, respectively. The regular ferriheme peak near 490 mμ is absent. In spectra intermediate between curves 1 and 2, isosbestic points were not always well defined. The spectrum of the green poly-L-lysine complex at pH 9 which is added for comparison (curve 3) is very similar in the UV-region to the system ferriheme-1.9 M salt with a slight shift to the red (max. at 385–390 mμ). In the visible range, the bands appear shifted by about 10 mμ to the blue (570 and 590 mμ) relative to curve 2. The shoulder at 590–600 mμ persists at excess electrolyte or polypeptide and is therefore considered to constitute a band belonging to the green complexes. For characterization of the ferriheme-salt system the ratio $\varepsilon_{580}/\varepsilon_{540}$ is used below since it was found experimentally to give more reproducible values than either ε_{580} or ε_{385}. This may be due to optical effects occurring in systems close to precipitation.

According to Fig. 2, the formation of the green species induced by salt depends on both the salt and ferriheme concentrations. At higher ferriheme concentrations less electrolyte is required to produce an equal spectral effect. From the levelling off of the upper curve at an absorption ratio of about 1.2 it appears that 2 M NaCl converts most of the 5×10^{-5} M ferriheme into the green form. Fig. 3 demonstrates the effect of ferriheme concentration at a single salt concentration.

The dependence of the ratio $\varepsilon_{580}/\varepsilon_{540}$ on pH (conditions of Fig. 4) cannot be followed to pH $\langle$ 5.5 because of immediate coagulation of ferriheme under these conditions. This makes it difficult to estimate the midpoint of transition. Assuming $\varepsilon_{580}/\varepsilon_{540}$ to remain constant between pH 5.5 – 6.0 (between pH 10 – 13, $\varepsilon_{580}/\varepsilon_{540}$ is practically independent of pH), this pK is 7.1 ± 0.15 at 27°C. Similar

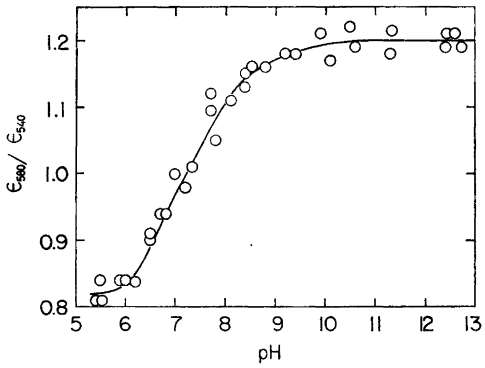


Fig. 4. pH-dependence of the spectral ratios in the presence of 1.9 M NaCl. Ferriheme 5.1×10^{-5} M; temperature 27 ± 1°C. In most experiments 0.01 M NaH_2PO_4 was included.

behavior with pH was observed by us for ferriheme in the absence of external salt. (Kajita *et al.*[9] found a p*K* of 7.3 at 20 – 22°C). Shack and Clark[6] found a p*K* of 7.4 – 7.6 for ferriheme and attributed the change to neutralization of an OH^- attached to the central iron. This could also explain the pH-dependence of the ferriheme-salt system which is fairly close to a simple titration curve. The state of aggregation of this neutralized ferriheme was not investigated. Reduction of 5×10^{-5} M ferriheme in 2 M NaCl at both pH 10 and 6 by $Na_2S_2O_4$ produced the typical ferroheme spectrum observed in the absence of salt. (The absorption was, however, lower in salt solution.) Again, this indicates participation of the OH^- in forming the green species.

Above pH 8 spectral changes with temperature of ferriheme-NaCl remained within the limits of experimental error in the range 5–40°C.

The effect of salts other than NaCl on ferriheme was also tested. At pH 11, 0.25 M Na_2SO_4 or K_2CO_3 and 0.6 M NaCl caused similar spectral effects in the visible range, under otherwise identical conditions, which demonstrates the effect of ionic charge. 0.2 M Na_2HPO_4 gave $\varepsilon_{580}/\varepsilon_{540} = 1.0$ (for 5×10^{-5} M ferriheme at pH 9). 2 M NaCl and NaBr exhibited similar visible range spectra. Comparing equal concentrations of LiBr and NaCl up to about 1 M, the former showed a much larger spectral effect. (At 5×10^{-5} M ferriheme the values for LiBr were closer to the upper curve of Fig. 2). It appears that the present three-component system conforms to salting-out relations and that both ionic strength and radii are important. 0.6 M $CaCl_2$ or $MgSO_4$, and 2 M LiBr caused rapid coagulation.

Evidence for aggreggation between ferriheme molecules at various salt concentrations has been presented by Shack and Clark[6] by measurement of sedimentation and diffusion (see also references cited therein). We have observed that 4×10^{-4} M ferriheme at pH 10.5 in the presence of 0.74 M NaCl sedimented broadly with an average of about 3 S at 20°C. Similar *s*-values were obtained with 3.6×10^{-3} M poly-DL-lysine in the presence of 4×10^{-4} M ferriheme while the pure polypeptide sedimented more slowly. On the other hand, 4×10^{-4} M

ferriheme alone at pH 11 – 12 did not produce any detectable sedimentation pattern at 50 740 rpm. The diffusion coefficient was about twice as large for ferriheme (D of the order 40×10^{-7} cm^2sec^{-1}) in the absence of NaCl as compared to ferriheme in 0.7 M NaCl. Very fast moving colored peaks ($\approx$ 45 S) were observed in the system ferriheme: poly-L-lysine (polymer 4.5×10^{-3} M; mol. wt. $\approx$ 50 000; NaCl 0.74 M; ferriheme 4×10^{-4} M; pH 9). Most of the polypeptide sedimented more slowly (1.1 S) and remained colorless indicating that it is unbound[10]. The spectrum of this system was similar to curve 3, Fig. 1. It therefore seems that the highly ionized poly-L-lysine forms high aggregates of ferriheme which also exhibit blue shifts in the visible range relative to ferriheme-salt. The number of lysine residues necessary to produce the green complex appears to be less than 4–5. Using an excess of highly ionized di- and trilysine at pH 9, precipitates were formed with ferriheme. The supernatant gave spectra intermediate between curves 1 and 3 of Fig. 1, while tetralysine showed a full green spectrum at pH 9 but not at low degrees of ionization (pH 11).

The similar spectral changes in ferriheme brought about either by interaction with (random) polybases or by high electrolyte concentrations also suggest that higher aggregates are formed in both cases. Aggregated dye systems have been investigated extensively in recent years (*e. g.* Ref. 11). For example in acridine orange binding to a polyelectrolyte and salting-out will both produce similar spectral effects[12]. In the present case the main band in the visible range is in the α-band region. However, upon aggregation, one may consider possible interactions between identical transition moments of neighboring and suitably oriented ferriheme molecules and changes in the polarizability of the medium which may cause splitting and shifts[13]. Hypochromism is not apparent in the present system, not even in the Soret band (Fig. 1) where the ratio of the oscillator strengths f of salt-free and highly aggregated ferriheme was estimated to 1.1:

$$f_1/f_2 = \int \varepsilon_1 d(1/\lambda) / \int \varepsilon_2 d(1/\lambda) \qquad (1)$$

The described spectral data combined with previous[6] and present sedimentation experiments establish the link between the visible spectrum of ferriheme and its state of aggregation. With polylysine or other polybases, aggregation is supposed to be conditioned by electrostatic interactions between the positively charged basic side groups and the porphyrin carboxylates. The ferriheme molecules may then interact optically by being stacked. The low-spin values obtained at high ratios of ε-amino groups of polylysine to ferriheme at pH 8–9 have previously been accounted for[14] by coordination of one or two ε-amino groups to the iron despite the high degree of ionization of the polypeptide. The magnetic moment has not yet been measured under the present conditions. Salting-out by electrolytes may lead to several types of micelles in which the ionized carboxyl groups point outwards so as to minimize their electrostatic interaction[15]. The present data did not permit an exact analysis of the equilibria involved. Most of the results seem to indicate that the free energy of formation $\triangle F_f$ of the reversible aggregates by electrolytes is mainly determined by screening of the repulsive potential between ferriheme carboxylates, by the entropy changes due

to differences in the structure of water, in particular around hydrophobic regions, and also by the various enthalpies of interaction:

$$\triangle F_f^0 = \triangle F_{el}^0 - T\triangle S_w^0 + \sum_i \triangle H_i^0 \quad (2)$$

Other terms are assumed to be relatively smaller. Further work is in progress to evaluate these quantities.

The green complex-type of spectrum observed in alkaline pyridine or dimethyl formamide[16] may indicate aggregation, although sedimentation experiments on the former system did not reveal any aggregated species.

According to Lemberg & Legge[17] "It is still a matter of controversy whether hematin in alkaline solutions is to be considered polymeric, dimeric or monomeric". The present data show that both salt and ferriheme concentration promote aggregation of the latter and that significant spectral changes occur also in the visible region upon aggregation. It may therefore now be possible to resolve some earlier discrepancies. The spectral data given for ferriheme at high pH or in borate buffer of pH 10 (quoted in Ref. 17, p. 173) which include a band maximum at 580 – 590 mμ apparently relate to the higher aggregates. The ferriheme spectrum obtained at pH 11 – 12 in the absence of external salt is possibly that of a ferriheme dimer[6] or oligomer. Recent experiments in the Soret band region at very high dilution[18] seem to confirm these relations.

The present data also emphasize the importance of evaluating the effect of buffer ions and other electrolytes as well as that of the ferriheme concentration whenever physical or catalytic properties of ferriheme are being investigated.

Acknowledgments. Thanks are due to Dr. A. Ehrenberg for valuable discussion and to Mrs. T. Robinson for skilful technical assistance.

REFERENCES

1. Theorell, H. *Arkiv Kemi* **16A** (1943) No. 3.
2. Brill, A. S. and Williams, R. J. P. *Biochem. J.* **78** (1961) 246.
3. Barron, E. G. S. *J. Biol. Chem.* **121** (1937) 285.
4. Drabkin, D. L. *J. Biol. Chem.* **142** (1942) 855.
5. Drabkin, D. L. In Falk, J. E., Lemberg, R. and Morton, R. K. *Haematin Enzymes*, Pergamon Press, Oxford, 1961, p. 162.
6. Shack, J. and Clark, W. M. *J. Biol. Chem.* **171** (1947) 143.
7. Blauer, G. *Nature* **189** (1961) 396.
8. Blauer, G. *To be published.*
9. Kajita, A., Uchimura, F., Mizutani, H., Kikuchi, G. and Kaziro, K. *J. Biochem. (Tokyo)* **46** (1959) 593.
10. Ehrenberg, A. and Blauer, G. *In preparation.*
11. Bradley, D. F. *Trans. N. Y. Acad. Sci.* **24** (1961) 64.
12. Blauer, G. *J. Phys. Chem.* **65** (1961) 1457.
13. Tinoco, I., Jr., Halpern, A. and Simpson, W. T. In Stahmann, M. A. *Polyamino Acids, Polypeptides and Proteins*, Univ. of Wisconsin Press, 1962, p. 147.
14. Blauer, G. and Ehrenberg, A. *Acta Chem. Scand.* **17** (1963) 8.
15. Stauff, J. *Kolloidchemie*, Springer-Verlag, Berlin, 1960, Sections 75–80.
16. Scheler, W. *Biochem. Z.* **332** (1960) 344.
17. Lemberg, R. and Legge, J. W. *Hematin Compounds and Bile Pigments*, Interscience, New York, 1949, p. 172.
18. Inada, Y. and Shibata, K. *Biochem. Biophys. Res. Commun.* **9** (1962) 323.

Received April 1, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 222 – S 225

Some Hybrids of Deoxygenated Sea Lamprey Hemoglobins (*Petromyzon marinus*)

NEVENKA M. RUMEN and WARNER E. LOVE

Department of Biophysics, Johns Hopkins University, Baltimore, Md., USA

Oxy-hemoglobin from the sea lamprey is a single chain, single heme molecule (Mol. wt. 18 400). Even though there are six different lamprey hemoglobins they only associate when deoxygenated. Upon mixing the six hemoglobins with each other, and deoxygenation it was possible to form several hybrids. It appears that the various monomers have preferential affinity for each other. The electrophoretic mobility of the hybrid was always governed by that of the faster component. Despite the wide variety of hybrids possible, only two bands appear on gel electrophoresis; however, it is believed these are not homogeneous but consist of several hybrids with nearly similar mobilities.

In a previous paper, the isolation of the six lamprey hemoglobins was reported[1]. It was also previously found that deoxygenated lamprey hemoglobin forms dimers and tetramers[2]. Although these experiments had been done in the analytic ultracentrifuge where hybrid dimers and tetramers would be indistinguishable from self-dimers and self-tetramers, it was possible to show the formation of hybrids (Hb_1Hb_2), (Hb_1Hb_4), (Hb_1Hb_5), ($Hb_3Hb_4)_2$ and ($Hb_3Hb_5)_2$[2]. Thus a preferential affinity of the various deoxygenated monomers for each other is indicated.

This paper reports some further experiments on the formation of hybrid polymers, as revealed by anaerobic electrophoresis on a gel of cyanogum 41.

PREPARATION OF HYBRID HEMOGLOBINS

The six lamprey hemoglobins were separated and purified by continuous flow paper electrophoresis[1]. The homogeneity of each component was checked by free boundary electrophoresis at three different values of pH[1]. The homogeneous components were dialyzed salt-free against distilled water plus a drop of ammonia to keep the pH between 7.0 and 7.5. After determination of the concentration by dry weight, the salt-free material was divided into portions of 0.3 ml and kept frozen for a few days, until needed.

The individual hemoglobins were thawed, mixed in depression slides, refrozen and introduced into a glove box. This was flushed with a mixture of 99 % prepurified N_2 (99.97 % pure) and 1 % H_2 which was passed over hot copper. The glove box also had a piece of hot copper

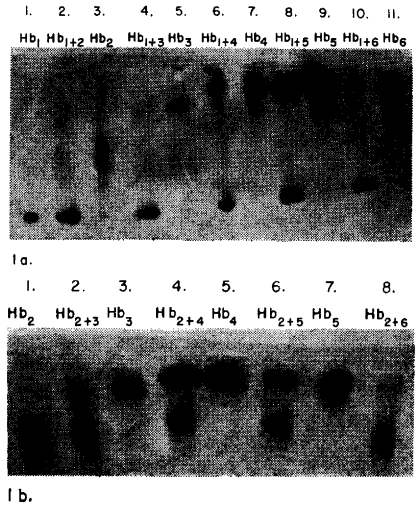


Fig. 1. a) Cyanogum 41 gel electrophoresis of deoxygenated hemoglobin 1 and equimolar mixtures of Hb_1 with the other five Hb's. b) The same for hemoglobin 2.

in it, and the atmosphere inside was stirred with a fan so that any oxygen coming in would be eliminated.

The cyanogum 41 gel was made by mixing 300 ml of tris buffer[3] pH = 7.6, after it had been degassed with a water pump for 15 min, with 30 g of cyanogum 41 (American Cyanamid Co.) and 56 ml of 10 % DMAPN (dimethylaminopropionitrile) which was also degassed. Finally, 3.6 ml of 10 % ammonium persulphate and 300 mg of sodium dithionite were mixed in, and the solution was poured into a lucite frame 37 × 14.5 × 0.4 cm. The degassing seems to inhibit gel formation and it was found necessary to add 1.5 ml more of the ammonium persulphate solution to produce gelation.

The slits were made in the gel with a razor blade and pieces of Whatman 3 MM filter paper which had been dipped into the tris buffer and dithionite were inserted into the slits. The gel block was then covered with saran film and placed in the glove box on a cooling plate circulated with water at 4°C, and left there for a few hours.

Inside the glove box the ferri-hemoglobin mixtures were reduced with sodium dithionite and were transferred into the pre-cut gel with pieces of filter paper. Cloth wicks were used to connect the gel block to the electrode vessels which contained 0.3 M borate buffer[3,4]. About 160 V and 105 mA were applied for about six hours. After the run the gel block was removed from the glove box and developed with buffalo black. The excess dye was washed off with Smithie's solution[3].

Alkaline pyrogallol was used to check for oxygen in the glove box.

RESULTS

Despite the above precautions, it is not certain that completely anaerobic conditions were obtained. Nevertheless some preliminary observations on the formation of hybrids are worthy of note. Figs. 1a and b show the gel electrophoresis of hemoglobins 1 and 2 in all possible equimolar combinations with the other four hemoglobins. Figure 2 shows combinations of hemoglobins 3, 4, 5 and 6. Figure 3 shows unpurified ferri-hemoglobin (aerobic conditions), the same sample, reduced (anaerobic), the same sample enriched with hemoglobins

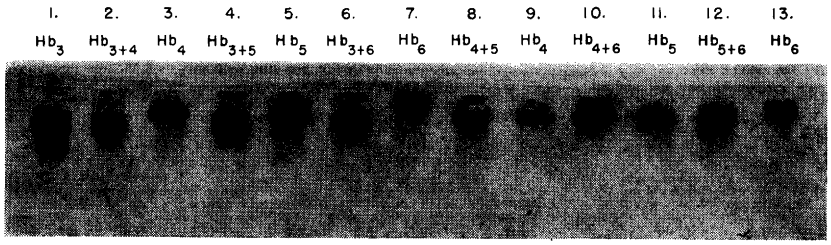


Fig. 2. Cyanogum 41 gel electrophoresis of deoxygenated hemoglobins 3, 4, 5 and 6 and their equimolar mixtures.

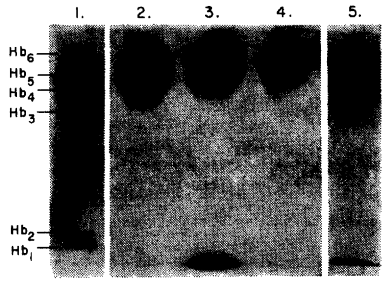


Fig. 3. Cyanogum 41 gel electrophoresis. 1) Unpurified ferri hemoglobins (aerobic). Hemoglobins 4 and 5 were not resolved under these conditions. 2) Same mixture as 1) but reduced and anaerobic. 3) Same as 2) but enriched with hemoglobins 1 and 2. 4) Same as 2) but enriched with hemoglobin 3. 5) Equimolar mixture of all six hemoglobins, reduced and anaerobic.

1 and 2, reduced (anaerobic), the same sample enriched with hemoglobin 3, reduced (anaerobic), and an equimolar mixture of all six hemoglobins, reduced (anaerobic).

DISCUSSION

From the results obtained thus far it is believed that hemoglobins 1 and 2 only form dimers in equimolar mixtures with the other hemoglobins. It appears that the hybrids of hemoglobins 1 and 2 with the others are easily dissociated, and therefore frequently show two electrophoretic bands in these mixtures. Indeed, in the gel experiment it is not at all clear that hemoglobin 2 forms hybrids, but the ultracentrifuge clearly shows a hybrid (Hb_1Hb_2) dimer[2].

The experiments with unpurified hemoglobin and with the equimolar mixture of all six show only two electrophoretic bands. We suspect that the slow band is not homogeneous, but consists of several hybrid tetramers which did not separate due to their very similar mobilities. The fast band, which in the standard mixture is very faint, probably consists of hybrid dimers of hemoglobins 1 and 2 with the others. Further work is necessary to identify the components present and their ratios. In the case of the equimolar mixture, it is quite clear that all possible hybrids are not formed and therefore the affinity of the deoxygenated monomers for each other is preferential. On the basis of the above experiments, ultracentrifuge experiments[2] and some speculation we believe equimolar mixtures will behave as shown in Figure 4.

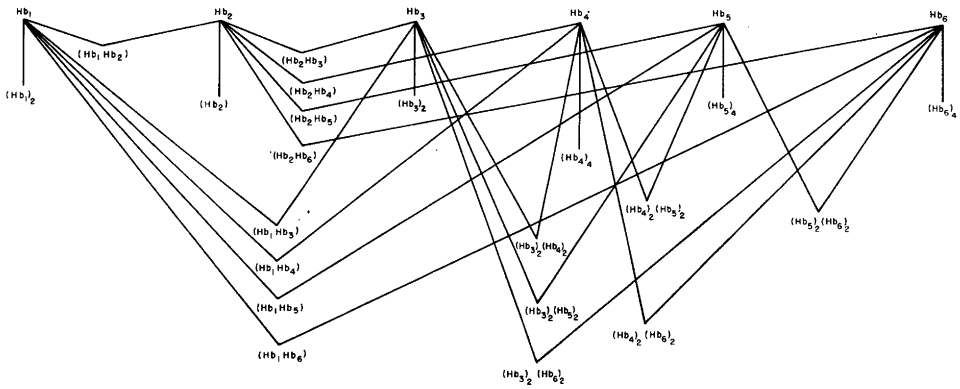


Fig. 4. The proposed behavior of the six deoxygenated lamprey hemoglobins when mixed 1 to 1.

It may be that hemoglobins 1 and 2 are evolutionary relics, and that the development of a tetramer-forming capacity conferred a physiological advantage upon the lamprey. It is certainly true that the oxygenation of a dimer or tetramer, whether or not it dissociates afterward, presents the possibility for heme-heme interaction, or sigmoid dissociation curves with their well-known advantages.

This investigation was supported by a grant from the *National Institute for Arthritis and Metabolic Diseases, U. S. P. H. S.* (A2528).

REFERENCES

1. Rumen, N. M. and Love, W. E. *Submitted to Arch. Biochem. Biophys.* (1963).
2. Rumen, N. M. and Love, W. E. *Submitted to Arch. Biochem. Biophys.* (1963).
3. Smithies, O. *Biochem. J.* **61** (1955) 629.
4. Poulik, M. D. *Nature* **180** (1957) 1477.

Received April 1, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 226 – S 232

Enzymic Homology

Structural and Catalytic Differentiation of Fructose Diphosphate Aldolase*,**

WILLIAM J. RUTTER, BEULAH M. WOODFIN and RHODA E. BLOSTEIN

Department of Chemistry and Chemical Engineering, Biochemistry Division, University of Illinois, Urbana, Illinois, USA

The catalytic and structural properties of aldolase isolated from muscle (aldolase A) and two aldolases isolated from liver (aldolases B_1 and B_2) have been summarized. The immunochemical properties as well as analysis of fingerprint patterns of peptides have indicated that aldolases B_1 and B_2 have a closely similar structure but that aldolase A has a different primary structure. It is thus presumed that the synthesis of aldolase A is at least partially defined by separate regions of the genome than that of aldolases B_1 and B_2. In spite of the specific differences reported, the general molecular structure and certain properties of the catalytically active site of aldolases A, B_1 and B_2 are reminiscent of each other. It is proposed that these two species of aldolase thus are related phylogenetically.

The biological significance of these enzyme homologs may be in the regulation of metabolic processes in specific cells.

Metabolic pathways may be defined as a sequence of enzyme-catalyzed reactions. It has been frequently assumed that a single enzymatic species is associated with a particular reaction. The credence of this concept has been strengthened by the demonstration that certain metabolic pathways can be blocked by a genetic mutation affecting the structure of a single enzymatic entity. The availability in recent years of more discriminating procedures for resolution of protein mixtures has resulted in the demonstration of heterogeneity in enzymatic populations catalyzing a single reaction. These multiple molecular forms associated with one enzyme activity have been termed "isozymes" by Markert and Møller[1]. Isozymes need not have an independent genetic origin,

* This investigation was supported in part by a grant from the *National Institutes of Health, United States Public Health Service.*

** Part of the data are taken from a dissertation submitted by Beulah M. Woodfin to the Graduate College of the University of Illinois in partial fulfilment of the requirements for the degree of Doctor of Philosophy, 1963.

since multiple forms may also represent 1) heterogeneity in a genetic product, 2) multiple, stable conformations of the same structure, or 3) the modification of a given gene product by other enzymatic systems or by the chemical environment.

The existence and biological significance of structurally unique enzymes catalyzing the reversible aldol cleavage of fructose-1,6-diphosphate (FDP)* is considered here. It has been previously demonstrated[2–4] that the aldolase found in muscle differs from the enzyme found in liver tissue, both catalytically (as evidenced by differences in the relative specific activity toward the substrates FDP and F1P) and structurally (as evidenced by the different immunochemical properties of the molecules against anti-rabbit muscle aldolase). Two proteins exhibiting aldolase activity have now been isolated from rabbit liver in crystalline form. The catalytic and structural properties of liver aldolases (hereafter called aldolases B_1 and B_2) are very similar, but they are significantly different from the primary structure and catalytic activity of muscle aldolase (hereafter called aldolase A).

MATERIALS AND METHODS

Enzymes. The isolation and assay of the enzymes are described in detail elsewhere[5]. The enzymatic assays were performed using the coupled α-glycerophosphate dehydrogenase system. One unit of enzymatic activity is defined as the amount of enzyme facilitating the aldol cleavage of 1 μmole of substrate/min. The specific activity is defined in terms of units of activity/mg of protein. Protein was estimated by the absorption at 280 mμ (O.D. of .91 = 1 mg protein/ml).

Structural studies. Immunochemical procedures are detailed elsewhere[4]. Antibodies to crystalline rabbit muscle aldolase (anti-aldolase A) and to crystalline rabbit liver aldolase 2 (anti-aldolase B_2) were prepared in chickens. Studies of the molecular properties have included: electrophoretic and ultracentrifugal analysis, molecular weight determinations, amino acid analysis, two-dimensional chromatographic-electrophoretic (fingerprint) analysis, and amino acid terminal group estimations; detailed procedures are described elsewhere[5].

RESULTS

Catalytic activity. The comparative catalytic properties of aldolases A, B_1 and B_2 are presented in Table 1. The activities of aldolases B_1 and B_2 are similar but not identical. The basic difference in catalytic properties of aldolases A and B, originally suggested by studies of rabbit muscle and bovine liver aldolases[2], is clearly indicated by the data obtained on enzymes from the same species. The catalytic activity of aldolase A is an order of magnitude greater than aldolases B with respect to FDP cleavage. In contrast, aldolases B exhibit a greater activity toward F1P than aldolase A. Aldolases B are also relatively more effective in catalyzing the synthesis of FDP from triose phosphates under the experimental conditions employed. Aldolases B have lower K_m values for FDP and F1P and the triose phosphates.

The pH optima of the aldolases A and B are different. The acidic portions of the pH profile are similar but the activity of aldolase A is unaltered from pH 6.5 to 9.0, while the activity of aldolases B declines above pH 8.0 giving a maximum at approximately pH 7.5. A difference of at least one dissociable group at the

* The following abbreviations are employed in this paper: FDP, fructose-1,6-diphosphate; FIP, fructose-1-phosphate.

Table 1. Catalytic properties of aldolases.

Aldolase	Substrate	K_m(M)	V_{max}*	pH optimum	% activity remaining after carboxypeptidase treatment
A	FDP	6.1×10^{-5}	5 300	7–9	5
	F1P	1.2×10^{-2}	105	7.0	50
	DHAP	2.1×10^{-3}	9 450	–	–
	GA3P	1.1×10^{-3}	10 400	–	–
B_1	FDP	1.2×10^{-6}	230	7.7	48
	F1P	8.7×10^{-4}	460	7.6	50
	DHAP	4.2×10^{-4}	2 500	–	–
	GA3P	2.8×10^{-4}	2 600	–	–
B_2	FDP	2.3×10^{-6}	460	7.8	49
	F1P	8.3×10^{-4}	460	7.7	48
	DHAP	3.7×10^{-4}	2 800	–	–
	GA3P	3.0×10^{-4}	3 000	–	–

* Moles of hexose cleaved or synthesized per min per mole of enzyme at 30°C.

active site of the two enzymes is necessary to explain the difference observed in the basic regions of the profile.

It has been shown that carboxypeptidase treatment of muscle aldolase lowers the FDP cleavage activity to 5 % and that of aldolases B to approximately 50 % of the activity of the native enzyme[5,11]. Three, one to two, and no tyrosines are released from aldolases A, B_1 and B_2, respectively, by this treatment[5]. The substrate specificity and specific catalytic activity of carboxypeptidase-degraded aldolases A and B are similar.

Molecular properties. The general molecular properties of the aldolases are presented in Table 2. A single symmetrical boundary was observed in both

Table 2. Molecular properties of aldolases. Conditions of sedimentation constant determinations: for the native aldolases, 8–10 mg/ml enzyme in 0.015 M sodium phosphate, pH 7.4 at 20°C; for acidified aldolases, 1.4 mg/ml enzyme in 0.05 M citrate, pH 2.2 – 2.6, at 5°C.

Aldolase	Molecular weight	$S_{20,w}$ sec $\times 10^{-13}$	$D_{20,w}$ cm^2 sec$^{-1} \times 10^{-7}$	$\frac{f}{f_o}$	V_{20} ml g^{-1}	μ(pH 7.5) cm^2 v^{-1} sec$^{-1} \times 10^{-5}$	$S_{20,w}$ (pH < 3.0) sec $\times 10^{-13}$
A	147 000	7.35	4.70	1.27	.742	–1.50	2.13
B_1	152 000	7.49	4.55	1.32	.735	–0.83	2.54*
B_2	154 000	7.56	4.35	1.35	.730	–0.91	2.08*

* Rapidly sedimenting material (10 S – 22 S) also appeared in acidified solutions of aldolases B.

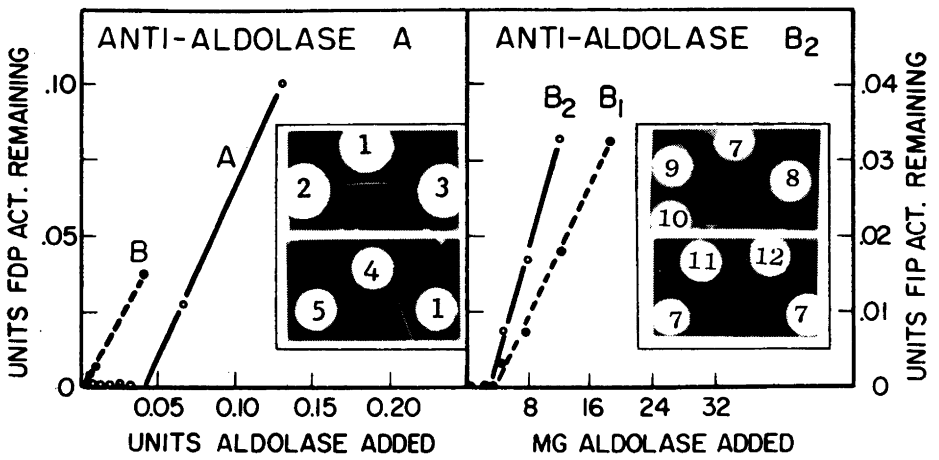


Fig. 1. Immunochemical analysis of aldolases. Increasing amounts of the aldolases A, B_1, B_2 or B (crude liver extract), as indicated, were incubated with .025 ml anti-aldolase A or .05 ml anti-aldolase B_2 in a final volume of 1.0 ml or 0.5 ml, respectively, centrifuged and assayed. For the double diffusion analysis, immune sera were placed in center wells and aldolases (mg/ml A, B_1 or B_2) in numbered wells as follows: (1) .10, A; (2) 1.0, B_2; (3) 1.7, B_2; (4) .34, B_1; (5) .68, B_1; (7) .06, B_2; (8) .14 units/ml liver extract; (9) .5, A; (10) 1.0, A; (11) .25 A; (12) .06, B_1.

electrophoretic and sedimentation analysis suggesting homogeneity of the preparations. The electrophoretic mobility and sedimentation constants of the three enzymes are in the same general range, but there are significant differences, especially between aldolases A and B. The molecular weight of aldolase A, as determined from hydrodynamic data, is significantly lower than that of aldolases B.

Recently it has been shown that aldolase A may be dissociated in acid to subunits having about one-third the molecular weight of the original molecule[7,8]. These subunits reassociate on reneutralization to form a product with the catalytic and molecular properties of the native molecule. Under appropriate conditions, essentially complete recovery of catalytic activity has been achieved. In acid solution, aldolases B_1 and B_2 both yield materials having low sedimentation constants like the sub-units derived from aldolase A and, in addition, aggregates of high sedimentation constant. Aldolase A exhibits similar behavior at higher protein concentrations, or at somewhat higher pH[7]. On reneutralization, recovery of only 30–50 % of the initial activity of aldolases B has been achieved. The general behavior in acid, the demonstration of three C-terminal tyrosines in aldolase A by Drechsler *et al.*[9], together with the finding of three N-terminal residues for aldolases A, B_1 and B_2 in the present work are in agreement with the postulate that these aldolases are each composed of three subunits.

An immunochemical analysis of the aldolases was performed using anti-aldolase A and anti-aldolase B_2. The results of double diffusion on agar plates as well as quantitative precipitin tests are presented in Fig. 1. Anti-aldolase A quantitatively precipitates aldolase A. Moreover, there is no detectable catalytic activity in the

Table 3. Total amino acid analysis of aldolases.

Aldolase	Ala	Arg	Asp	Cys	Glu	Gly	His	Ileu	Leu	Lys	Met	Phe	Pro	Ser	Thr	Try	Tyr	Val
A	143	62	104	29	147	108	42	77	129	112	12	27	72	69	74	11	41	83
B_1	139	63	136	(30)	167	113	39	73	130	108	19	42	59	62	75	14	37	91
B_2	131	63	141	(19)	159	107	34	71	137	112	17	47	64	66	74	12	37	88

antigen-antibody complex[4]. In contrast, this antibody cross-reacts only very slightly with aldolases B_1 and B_2. Anti-aldolase B_2 quantitatively precipitates aldolases B_1 and B_2. There is no detectable cross-reaction of anti-aldolase B_2 with aldolase A. A slight cross-reaction between anti-aldolase A and aldolases B suggests the presence of (a) common immunological determinant(s) in the two proteins. The method of isolation precludes contamination of aldolases B_1 and B_2 with traces of native aldolase A, but it is, of course, possible that traces of aldolase A have been degraded in such a way that they migrate in the various chromatographic procedures and co-crystallize with aldolases B. These results indicate a structural distinction between aldolases A and B and suggest a close structural relationship between aldolases B_1 and B_2. These conclusions are confirmed and amplified by studies of the specific composition of the proteins[5]. The relative amino acid contents of the three proteins are broadly similar. The compositions of aldolases B_1 and B_2 appear almost identical (Table 3), but the composition of aldolase A is significantly different. A fingerprint analysis of the peptides produced from aldolases A and B is presented in Fig. 2. Separate analyses of increasing concentrations of partially-degraded enzymes allow the resolution of 74, 67, and 78 peptides for aldolases A, B_1 and B_2, respectively. Aldolases B_1 and B_2 give very similar patterns, but the distribution of the peptides on the fingerprint of aldolase A is distinctly different.

DISCUSSION

From the experimental evidence presented, it is concluded that aldolases A and B are distinct entities. The immunochemical analyses indicate that few immunological determinants are shared by these enzymes. The marked contrast in the fingerprint patterns obtained from these enzymes together with the demonstration that there are probably three polypeptide subunits in the molecules, indicate that there are qualitative differences in the amino acid sequence of these enzymes. On the other hand, aldolases B_1 and B_2 resemble each other so strongly in overall structure that the differences reported may be a result of modification of a single protein. The possibility of a hybrid of similar polypeptide chains or even of independent genetic derivation is not excluded.

The difference in primary structure of aldolases A and B implies that the synthesis of at least some portions is controlled by separate regions on the genome. Since the proportion of the two types varies in different tissues and changes markedly in the liver during embryological development[3], it is presumed that the synthesis of these two types of aldolases is controlled separately.

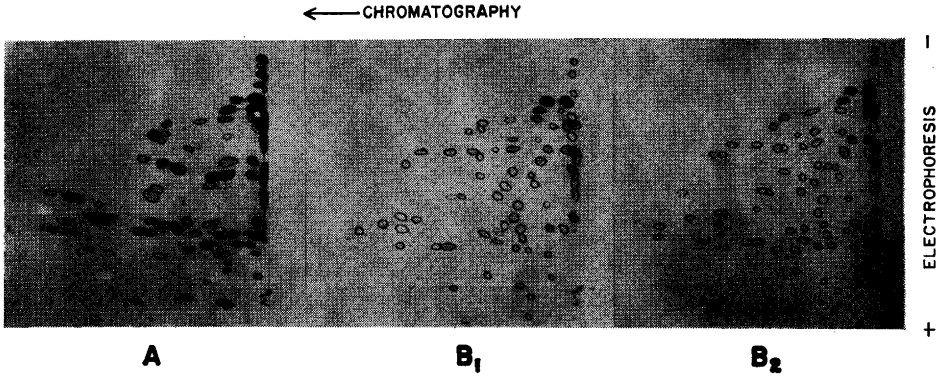


Fig. 2. Qualitative analysis of peptides produced by partial degradation of aldolases A, B_1 and B_2. 20 mg of each protein were subjected to performic acid oxidation at −10°C. The lyophilized proteins were dissolved in 3.0 ml of 0.1 M ammonium carbonate, pH 8.2, and incubated for 2.5 h with 0.2 mg trypsin at 37°C, followed by incubation with 0.2 mg α-chymotrypsin for 2.5 h at 37°C. Hydrolysate corresponding to 3 mg protein was placed at the origin (×). The chromatograms were run in butanol: acetic acid: water, 4 : 1 : 5 (upper phase) for 16 h, descending. This was followed by electrophoresis for 90 min in pyridine: acetic acid: water, 1 : 10 : 289, pH 3.2. Peptide maps were developed with 0.2 % ninhydrin in 95 % ethanol, and outlined before photographic recording.

Hers[10] has pointed out that certain cases of fructose intolerance in humans may be a result of the absence of liver type aldolase (aldolase B) and its partial replacement by muscle type aldolase (aldolase A) in the liver of the patients.

In spite of the conclusion of the independent genetic origin of these enzymes, it is clear from the data available that the molecular features of all three enzymes are similar. The molecular size, the reversible dissociation of the molecule in acid, the probable number of polypeptide chains, the general amino acid composition, as well as the noticeable resemblance in the fingerprint patterns, all indicate that the general molecular architecture, and to some degree the specific structure of the molecules must be related. The specific alteration in the catalytic properties of aldolases A and B by removal of C-terminal residues with carboxypeptidase reflects a basic similarity in the active sites of these enzymes. Previous comparative studies of bovine liver aldolase and rabbit muscle aldolase present evidence for a basic catalytic similarity[11].

These facts thus suggest that even though these molecules are genetically distinct, they may be phylogenetically related. A similar conclusion has been drawn by Ingram for fetal, adult and the various hemoglobin variants[12]. In this instance, hybridization of the different polypeptide chains in the complex hemoglobin molecule has been clearly demonstrated. The interesting and informative studies of Markert and Appella[13] and Cahn *et al.*[14] on lactic dehydrogenase (LDH) in mammalian tissue are relevant. Of the five forms of this enzyme present in mammalian tissues, two are apparently distinct molecules. It has been proposed[14] that the other three, which exhibit intermediate properties, are hybrid combinations of the two different polypeptide chains. The fingerprint analysis

of aldolases A and B tend to rule out the possibility that these enzymes are simply hybrid variants, since in this instance the number and distribution of peptides in the analysis would be similar. The possibility that aldolases B_1 and B_2 are hybrid variants must be resolved by a more discerning analysis of these proteins.

It is proposed that the term "enzyme homologs" be employed for the fraction of isozymes which have distinct primary structure and therefore are presumably of independent genetic origin but nevertheless have strong similarities in structure and thus may be phylogenetically related. "Enzyme analogs" may then refer to enzymes of independent genetic origin and with apparently independent phylogenetic origin. An example of the latter case is found in *Euglena gracilis* where two structurally and catalytically unrelated forms of aldolases have been discovered[15]. Multiple forms of a single genetic product can appropriately be termed "enzyme derivatives", or simply enzyme variants. On this basis, aldolases A and B and probably the LDH isozymes would be termed enzyme homologs.

Whereas it is not yet possible to define the biological significance of all isozymes, the properties of the enzyme homologs may be related to specific control of metabolic processes. The catalytic properties of aldolase A are tailored primarily to the aldol cleavage of FDP in glycolysis and those of aldolases B to the broadened substrate specificity required for the metabolism of F1P and also for the synthesis of FDP from triose phosphates in the process of glyconeogenesis. A major catalytic difference between LDH variants is the sensitivity of the active site to substrate inhibition. The desirability of this feature in skeletal muscle and the lack of desirability of it in heart muscle has been argued by Cahn *et al*[14].

A more comprehensive analysis of enzymatic populations facilitating the same reaction may elucidate hitherto unappreciated aspects of the control of metabolic processes and provide the basis for definition at the molecular level of one aspect of cellular differentiation.

REFERENCES

1. Markert, C. L. and Møller, F. *Proc. Natl. Acad. Sci, U. S.* **45** (1959) 753.
2. Rutter, W. J. *The Enzymes*, Academic Press, New York and London, 1961, Vol. V, p. 341.
3. Rutter, W. J., Blostein, R. E., Woodfin, B. M. and Weber, C. S. In *Symposium on Regulation of Enzyme Activity and Synthesis in Normal and Neoplastic Liver*, Indianapolis, Indiana, 1962, Pergamon Press. *In press.*
4. Blostein, R. E. and Rutter, W. J. *J. Biol. Chem.* (1963). *In press.*
5. Woodfin, B. M. and Rutter, W. J. *J. Biol. Chem. In preparation.*
6. Richards, O. C. and Rutter, W. J. *J. Biol. Chem.* **236** (1961) 3177.
7. Deal, W. C., Jr., Rutter, W. J. and Van Holde, K. E. *Biochemistry* **2** (1963) 246.
8. Stellwagen, E. and Schachman, H. K. *Biochemistry.* **1** (1962) 1056.
9. Drechsler, E. R., Boyer, P. D. and Kowalsky, A. G. *J. Biol. Chem.* **234** (1959) 2627.
10. Hers, H. G. *Chemisch Weekblad* **57** (1961) 437.
11. Rutter, W. J., Richards, O. C. and Woodfin, B. M. *J. Biol. Chem.* **236** (1961) 3193.
12. Ingram, V. M. In *The Molecular Control of Cellular Activity*, McGraw-Hill Company, New York, 1962, p. 181.
13. Markert, C. L. and Appella, E. *Ann. N. Y. Acad. Sci.* **94** (1961) 678.
14. Cahn, R. D., Kaplan, N. O., Levine, L. and Zwilling, E. *Science* **136** (1962) 962.
15. Rutter, W. J. and Groves, W. E. In *International Conference on Taxonomic Biochemistry, Physiology and Serology*, University of Kansas, Lawrence, Kansas, September, 1962.

Received March 30, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 233–S 237

Radiochemical Determination of Choline Acetyltransferase

JAN SCHUBERTH

Research Institute of National Defence, Department 1, Sundbyberg, Sweden

A simple and sensitive radiochemical method for analysis of choline acetyltransferase in small samples of tissue extracts is described. In the system used for enzymatic synthesis of acetylcholine, ^{14}C-acetate, in the presence of an acetyl-coenzyme A generating system, is activated to ^{14}C-acetyl-coenzyme A. Acetyl-coenzyme A then reacts with choline, through choline acetyltransferase, to form ^{14}C-acetylcholine. ^{14}C-acetate is completely volatile as acetic acid when the acid sample is evaporated to dryness, whereas the ^{14}C-acetylcholine formed is stable under the same conditions and thus obtainable for radiochemical determination. By this method it is possible to analyze choline acetyltransferase, producing as little as 10 mμmoles of acetylcholine with good accuracy and without time-consuming manipulations.

Choline acetyltransferase catalyzses the formation of acetylcholine from choline[1] and acetyl-coenzyme A[2] (acetyl-CoA). Previously described methods for the assay of choline acetyltransferase activity are based on the determination of acetylcholine formed after incubation of the enzyme with both substrates. The amount of acetylcholine thus formed is determined either by a rapid but insensitive photometric method[3] or by a sensitive but rather time-consuming and laborious biological method[4].

The use of an acetyl-CoA donor system is preferred to the use of stoichiometric amounts of preformed acetyl-CoA because, according to Berman *et al.*[5] and Smallman[6], there is good evidence that the latter method does not give as high a rate of acetylcholine synthesis as is obtainable with the donor system. Acetate activating enzyme (AAE) has been used by Hebb[7] and by Berry and Whittaker[8], as an acetyl-CoA generating enzyme during acetylcholine synthesis.

The aim of the present paper is to describe a new method for the analysis of choline acetyltransferase in small amounts of crude tissue extracts with good accuracy and without time-consuming manipulations. The complete system used by us for enzymatic synthesis of acetylcholine is described by the following reaction scheme:

$$^{14}\text{C-acetate} + \text{ATP} + \text{CoA} \xrightleftharpoons{\text{AAE}} {}^{14}\text{C-acetyl-CoA} + \text{AMP} + \text{PP}$$

$$^{14}\text{C-acetyl-CoA} + \text{choline} \xrightleftharpoons[\text{acetyltransferase}]{\text{choline}} {}^{14}\text{C-acetylcholine} + \text{CoA}$$

The assay method is based on the fact that free acetate is completely volatile when the acid sample is evaporated to dryness, while acetylcholine is stable[9] and non-volatile and therefore obtainable for radiochemical determination.

EXPERIMENTAL

Material

AAE was prepared from pressed baker's yeast. Disruption of the yeast cells was performed by the quick-freeze method as described by Jones *et al.*[10] and the AAE was fractioned from the crude extract with ammonium sulfate at + 4°C and at pH 7.0. The precipitate formed in 35 % ammonium sulfate was discarded and the active material was obtained as a precipitate at 45 % saturation. The AAE was stable for several months when stored either in saturated ammonium sulfate or in 0.02 M phosphate buffer pH 7.0 at − 25°C.

Choline acetyltransferase. Full term human placenta was a convenient source of choline acetyltransferase[11]. The placenta, trimmed from membranes, large vessels and necrotic areas, was cut in small pieces and homogenized in dry acetone at − 15°C (100 × tissue volume). The acetone was filtered, the filter cake washed with ether at − 15°C and then left to dry in vacuum for 3–4 h. The enzyme was extracted from the dry powder in 0.02 M phosphate buffer at pH 7.0 (50 mg powder per 1 ml buffer) and fractioned from the clear extract with ammonium sulfate at + 4°C. The precipitate obtained at 33 % saturation was discarded and choline acetyltransferase was precipitated at 40 %. The active material was stable for several months when stored in saturated ammonium sulfate. When the enzyme activity was to be assayed the precipitate in ammonium sulfate was sedimented by centrifugation and dissolved in 0.02 M phosphate buffer at pH 7.0.

Reagents. CoA was obtained from Sigma, USA, and sodium acetate-1-^{14}C from the Radiochemical Centre, England. Only chemicals of Analytical grade were used.

Methods

Assay[12] of AAE. The rate of activation was measured with neutral hydroxylamine as a trapping agent for formed acetyl-CoA; the acetylhydroxamic acid formed was assayed photometrically as a complex with ferric ion.

Photometric assay of choline acetyltransferase. The acetylcholine formed after incubation at 37.0°C was determined by the method of Hestrin[3] in 1.0 ml samples of the reaction mixture which in addition to the choline acetyltransferase extract contained in 1.0 ml: potassium phosphate buffer pH 7.0 (50 μmoles), potassium acetate (10 μmoles), prostigmine bromide (0.1 μmole), KBH_4 (1 μmole), CoA (70 % pure, 0.25 μmoles), ATP (10 μmoles), KF (30 μmoles), $MgCl_2$ (3 μmoles), AAE (1.5 units[12]), choline (16 μmoles). Under these conditions, we found that the rate of acetyl-CoA formation and the choline concentration did not limit the rate of acetylcholine synthesis. In the blank choline was replaced with water.

Radiochemical assay of choline acetyltransferase. Ten or twenty μl of enzyme extract were incubated in the same incubation mixture as previously described for photometric analysis of choline acetyltransferase, with the addition of 0.3 μC sodium ^{14}C-acetate. The final incubation volume amounted to 100 μl. After incubation for one h at 37.0°C the reaction was stopped by the addition of 100 μl of 1 % trichloroacetic acid (TCA) in 1 M HCl. To minimize the adsorption of ^{14}C-acetylcholine on the precipitate formed, the TCA-HCl mixture contained 20 mM unlabelled acetylcholine. After 5–10 min 2.0 ml 0.2 M HCl was added and the precipitate was sedimented by centrifugation. 0.1 ml of the clear supernatant was uniformely spread out on a plate of stainless steel over an area of 2.25 cm^2 and evaporated at room temperature in a vacuum desiccator containing solid sodium hydroxide. When the sample had been evaporated to dryness, which usually occurred after 15 min, the radioactivity was measured with a flow counter (Frieseke-Hoepfner). Selfabsorbtion of the sample was less than 0.01 % of the total radioactivity. The time for 10^4 counts was measured. The determination of the standard sample, *i. e.* the radioactivity corresponding to 100 % conversion of acetate to acetylcholine, was performed by adding to the incubation mixture 2.1 ml 0.01 M NaOH instead of 2.1 ml of the above mentioned acid solutions. The radioactivity was then determined in the same manner as previously described. Selfabsorbtion of the standard sample was also less

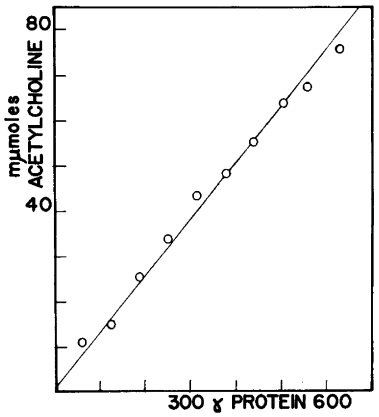


Fig. 1. Relation between the acetylcholine formation and the relative concentration of choline acetyltransferase.

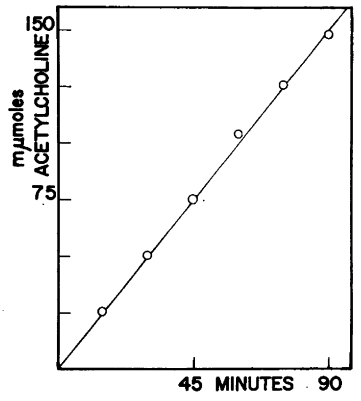


Fig. 2. Relation between acetylcholine formation and the time of incubation.

than 0.01 % of the total radioactivity. The amount of formed acetylcholine is obtained by calculating the radioactivity of the specimen corrected for the blank value divided by the standard radioactivity and by multiplying this quotient by the total amount of acetate in the reaction mixture.

RESULTS

Relation between activity and concentration. Different dilutions from a stock solution of choline acetyltransferase were prepared and the protein concentration of each sample was determined according to Warburg and Christian[14]. Fig. 1 illustrates the relation between the choline acetyltransferase activity as determined by the radiochemical method described in "methods" and the relative concentration of choline acetyltransferase expressed by the total amount of added protein.

Comparison of photometric and radiochemical method. The choline acetyltransferase activity of the stock solution described above in "experimental and results" was assayed by the photometric method[3] and the protein concentration determined[14]. Five independent determinations on each 0.2 ml of the stock solution gave a mean value of 796 mμmoles of synthesized acetylcholine after one hour's incubation. The specific activity of choline acetyltransferase as defined by Berman *et al.*[5] was calculated to be 0.0125 ± 0.0002 (± standard error). The mean specific activity[5] calculated from the values in Fig. 1 was 0.0126 ± 0.0003 (± standard error of estimate) which value is not significantly different from the specific activity assayed spectrophotometrically.

Kinetics of reaction. Choline acetyltransferase was incubated in a total of 1.0 ml of the incubation mixture as described in methods for "radiochemical assay of choline acetyltransferase". 0.1 ml of the mixture was withdrawn at subsequent times and radioassayed for choline acetyltransferase activity according to the describtion in methods. The result is illustrated in Fig. 2.

Precision of method. The coefficient of variation (standard deviation as percentage of the mean) was obtained as 4.3 % when determined on 12 replicate determinations of choline acetyltransferase activity.

Choline acetyltransferase activity in the brain of rat. Three albino rats were killed by decapitation and the whole brains were quickly removed. The brains were separately treated with acetone, ether and extracted as described in "material" for preparation of choline acetyltransferase from placenta. The crude extracts were radioassayed for choline acetyltransferase and the mean value was found to be 22.8 μmoles of synthesized acetylcholine per gram of powder.

Chromatographic identification of acetylcholine: To verify acetylcholine as the radioactive product after the incubation of choline acetyltransferase, AAE system, ^{14}C-acetate and choline, paper chromatographic identification was carried out. Acid specimens, containing enzymatically formed ^{14}C-acetylcholine as described in "radiochemical assay of choline acetyltransferase", were pooled, evaporated to dryness in vacuum and dissolved in 0.5 ml of water. 40 μl of the clear solution was concentrated in small spots on each three filter papers (Whatman no 1) and chromatographed by descending method in three solvent systems[13]: butanol saturated with water, propanol-H_2O (9:1) and propanol-formic acid-H_2O (8:1:1). The spots containing radioactive material, identified by scanning the paper strips with a flow counter, overlapped perfectly the spots containing unlabelled acetylcholine identified by spraying the paper with hydroxylamine and $FeCl_3$[13]. The following R_F values of the added acetylcholine as well as of the ^{14}C-acetylcholine were obtained: butanol saturated with water 0.11, propanol-H_2O 0.33 and propanol-formic acid-H_2O 0.45.

DISCUSSION

The radiometric method described has been worked out by measuring the enzyme activity of partially purified choline acetyltransferase from human placenta. The author has thus shown that by the radiochemical determination of choline acetyltransferase there is a strict linear relationship between the enzyme concentration and the amount of formed acetylcholine in the range of 10 to 80 mμmoles of acetylcholine. The radiochemical method is thus about 50 times more sensitive than the photometric method[3] but about 5 times less sensitive than the biological method[4]. By the radiometric method about 15 double determinations per person per day can be performed with an accuracy of 4.3 % (coefficient of variation).

It has also been shown that the formation of acetylcholine in the system used by the author follows a zero order kinetics in the range of zero to 90 min of incubation time and zero to 150 mμmoles of synthesized acetylcholine.

The metabolism of acetate to nonvolatile substances different from acetylcholine can evidently occur, and this has been corrected for by subtracting the blank value from the specimen value. The validity of this correction, however, requires that the enzyme is free from choline which might not be the case when using crude extracts or homogenates from brain tissue. K_m for choline is 5×10^{-4} [15] which means that fairly small amounts of choline in the enzyme extract might give too high blank values and thus too low choline acetyltransferase activity. To obtain full choline acetyltransferase activity from brain tissue, the homogenate

must, before extraction, be treated with an organic solvent[16], *e. g.* ether or acetone. The advantage of using acetone to ether is, in accordance with the above discussed demand for choline free enzyme extracts, that choline is fairly soluble in acetone and therefore removed from the tissue powder before the enzyme is extracted. To test the radioassay method on a crude extract of brain tissue the determination of choline acetyltransferase activity in the brain of rat was performed as described in experimental and results. The value thus obtained was in good agreement with that obtained by Berry and Whittaker[8].

Acknowledgements. The author wishes to thank Dr Bo Sörbo and Dr Lennart Larsson for valuable discussions and miss Ulla Persson for able technical assistance.

REFERENCES

1. Nachmansohn, D. and Machado, A. L. *Neurophysiol.* **6** (1943) 397.
2. Lipmann, F. *Science* **120** (1954) 855.
3. Hestrin, S. *J. Biol. Chem.* **180** (1949) 249.
4. Chang, H. C. and Gaddum, J. H. *J. Physiol.* **79** (1933) 255.
5. Berman, R., Wilson, J. B. and Nachmansohn, D. *Biochim. Biophys. Acta* **12** (1953) 315.
6. Smallman, B. N. *J. Neurochem.* **2** (1958) 119.
7. Hebb, C. *Quart. J. Exptl. Physiol.* **40** (1955) 176.
8. Berry, J. F. and Whittaker, V. P. *Biochem. J.* **73** (1959) 447.
9. Nilsson, R. *Svensk Kem. Tidskr.* **65** (1953) 213.
10. Jones, M. E., Black, F. R. M. and Lipmann, F. *Biochim. Biophys. Acta* **12** (1953) 141.
11. Bull, G., Hebb, C. and Ratković, D. *Nature* **190** (1961) 1202.
12. Berg, P. *Methods in Enzymology,* Academic Press, New York and London, 1962, Vol. V, p. 461.
13. Whittaker, V. P. and Wijesundera, S. *Biochem. J.* **51** (1952) 348.
14. Warburg, O. and Christian, W. *Biochem. Z.* **310** (1941) 384.
15. Berman-Reisberg, R. *Yale J. Biol. Med.* **29** (1957) 414.
16. Stedman, E. and Stedman, E. *Biochem. J.* **31** (1937) 817.

Received March 30, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 238–S 240

The Iodine Content of Swedish Dairy Milk

G. WIDSTRÖM, C.-B. THORELL and B. ÅBERG

Department of Clinical Chemistry, Stockholms läns Centrallasarett, Danderyd, and Department of Clinical Biochemistry, Royal Veterinary College, Stockholm, Sweden

The Swedish dairy milk iodine-127 content was determined and evaluated with regard to its protective effects against thyroidal uptake of iodine-131 from fall-out.

During the first years of life milk dominates as food and to a considerable extent this milk comes from cows. For the synthesis of thyroid hormones children depend on their iodine intake and thus on the iodine content of dairy milk. The iodine content of the milk is also of interest because milk is more or less contaminated with radio-iodines formed by fission when nuclear weapons are tested, in case of reactor accidents *etc.* The iodine isotopes 129–136 might occur but due to physical half-life and atom per cent formed, only two radio-isotopes are of importance *i. e.* iodine-131 and iodine-132. Iodine-132 is formed by decay of tellurium-132 but its half-life is only 2 h. Iodine-131 has a half-life of 8 days.

In this article the variations of stable iodine in dairy milk from some Swedish dairies will be discussed together with the contamination with iodine-131 observed in 1962. The 1961–1962 series of nuclear tests gave rise to contamination of milk with iodine-131.

MATERIAL AND METHODS

From April 1962 to March 1963 milk samples were taken from six Swedish dairies once or twice monthly. The content of inactive iodine was determined according to *Barker et al.*[1] as total iodine. All analyses were made in duplicate. The standard deviation (S. D.) for a single determination was $\pm$ 2.5 μg per l milk.

RESULTS

In Table 1 the monthly mean values for the six dairies are given as μg of iodine per l milk.

DISCUSSION

It is obvious from the values in Table 1 that there is a considerable monthly variation of iodine content in the milk. The lowest values occur during the sum-

Table 1. Total iodine in milk from six dairies in μg/l, April 1962 to March 1963.

Dairy	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Febr.	March	Mean ± S.D.
Malmö	107	53	31	42	41	58	45	41	65	63	50	43	53 ± 20
Kalmar	44	31	13	18	18	21	29	22	30	52	56	30	30 ± 18
Skara		74	22	21	24	33	64	109	64	52	76	65	55 ± 29
Eskilstuna	66	48	14	20	17	25	37	63	66	62	78	64	47 ± 21
Östersund	56	60	50	28	33	29	31	41	54	40	42	28	41 ± 11
Boden	22	27	16	22	56	54	68	33	39	26	15	51	36 ± 25
Average	59	49	24	25	32	37	45	52	53	49	53	47	

mer months, which could be due to dietary changes. The average monthly values vary between 24 and 59 μg/l whereas in single determinations values have been found varying between 11 and 116 μg/l. The radio-iodine (^{131}I) content of Swedish dairy milk between August 28th and December 3rd, 1962 varied between 0 and 406 pC/l. The measurable values in connection with fall-out from nuclear tests varied between 15 and 406 pC/l[2]. Determinations of radio-iodine in milk from single farms during the autumn 1962 gave the maximal value of 3 000 pC/l[2]. The mean intake of iodine-131 in Sweden during 1962 was about 30 000 pC[2].

At an expert meeting in Oslo in September 1962 it was decided that an accumulated yearly intake of 200 000 pC of iodine-131 warrants actions from the health point of view[3]. The maximal allowable initial concentration of iodine-131 in milk after a reactor accident in Sweden would be 70 000 pC/l corresponding to a yearly intake of 800 000 pC. The highest observed radio-iodine content in milk from a Swedish farm (3 000 pC/l) corresponds to a yearly accumulated intake of 100 000 pC[2].

If the observations on stable iodine and radio-iodine of Swedish dairy milk during 1962 is used for determination of the specific avtivity (C of ^{131}I/g iodine) in milk the variation is $0 - 17 \times 10^{-6}$. For single determinations (in single farms and single dairy samples respectively) the maximal value will be 27×10^{-5}. It is well known expecially since radio-iodine has been in general use for diagnosis of thyroidal disorders that inactive iodine greatly reduces the radio-iodine uptake by the thyroid gland. It is thus from a hygienic point of view important that the milk contains as much inactive iodine as possible without causing allergic conditions in disposed milk drinkers. Furthermore, in cases of expected radio-iodine fall-out, it is helpful if this content is as constant as possible. It does not matter, however, if the milk iodine comes from the cow's udder or from iodine-containing sterilizing agents used in the farms and/or dairies.

The daily intake of inactive iodine for adults is stated to be about 50 μg[4]. Determination of urinary excretion of iodine in 89 persons from around Stockholm who were not taking iodinated medicines gave a mean daily excretion value of 104 ± 49 μg which obviously corresponds to a considerably higher mean

intake of iodine. The daily requirement for children should be proportional to the caloric needs and thus be in the order of 25–50 μg. With a milk consumption of one liter per day the milk iodine content should be at least 25, and preferably 50 μg/l. From the values in Table 1 it is clear that the milk examined contains just this amount. With the present iodine content however no depressing action on the thyroidal uptake of radio-iodine can be expected. This is especially true for the summer months. It seems therefore that it should be of value if the iodine content of dairy milk was increased. The best way to achieve this would no doubt be to increase the iodine in the minerals given to the cows.

Acknowledgements. The investigation was aided by grants from *The Research Institute of National Defense, The Swedish Agricultural Research Council* and *The Atomic Energy Research Council.*

REFERENCES

1. Barker, F. B., Humphrey, M. J. and Soley, M. H. *J. clin. Invest.* **30** (1951) 55.
2. Edvarson, K., Fredriksson, L., Lindell, B. and Åberg, B. *Radioaktivitetsmätningar på livsmedel 1962,* Medicinalstyrelsens strålskyddsnämnd 1962, Annex A., Stockholm, 1963. *Stenc.*
3. *Rapport till de nordiska ländernas strålskyddsmyndigheter från nordiskt möte mellan strålskyddsexperter från Danmark, Finland, Norge och Sverige,* Oslo, September 1962. *Stenc.*
4. Bodansky, M. and Bodansky, O. *Biochemistry of Disease,* 2nd edition, The Macmillan Co., New York, 1952.

Received April 3, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 241 – S 245

The Riboflavin Flavoprotein from Egg Yolk

WLODZIMIERZ OSTROWSKI and ADAM KRAWCZYK

Department of Physiological Chemistry, Medical Academy, Cracow, Poland

A flavoprotein with riboflavin as the prosthetic group has been isolated from the soluble fraction of egg yolk. The complex is a glycoprotein with a molecular weight of 37 000. It proved to contain one molecule of riboflavin per one molecule of protein. Absorption maxima of the flavoprotein were found to lie at 276, 375 and 458 mμ. The protein-bound riboflavin is uncapable of reducing on the dropping electrode. The dissociation constant of the complex as established on the basis of fluorometric measurements equals 2.65×10^{-9} M.

Recently, flavoproteins with riboflavin as the prosthetic group, have been reported to occur in avian egg white[1] and yolk[2]. The riboflavin proteins are uncapable of oxidizing reduced pyridine nucleotides[1]. They resemble the avidin-biotin system and probably constitute a storage form of vitamin B_2 for developing embryo. In this paper some properties of a flavoprotein as isolated from egg yolk have been described. A preliminary account on the subject was published earlier[2].

EXPERIMENTAL

Preparation procedures and properties

Partially saturated flavoprotein was prepared from fresh hen eggs according to the method described previously[2]. To completely saturate apoprotein with riboflavin, the original preparation was dialyzed in 0.05 M Na-phosphate buffer, pH 7.0, containing 39.5 μg riboflavin per ml, followed by dialysis against several changes of distilled water. The dialysis residue was then lyophilized and the resulting product was used for analysis.

The flavoprotein proved to comprise about 10 μg riboflavin per mg protein as determined both microbiologically[3] and spectophotometrically[4]. It gave a single peak in the ultracentrifuge (Fig. 1.). The molecular weight calculated by Ehrenberg's modification of the Archibald approach to sedimentation equilibrium method[5] amounted to 37 400. The minimum molecular weight calculated on the basis of the riboflavin content gave approximately the same result. The preparation proved to be homogeneous also when checked with starch gel electrophoresis in the range of pH from 4.5 to 8.5 and density gradient electrophoresis

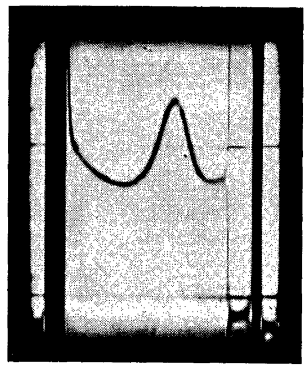


Fig. 1. Ultracentrifuge pattern of egg yolk flavoprotein. The sedimentation measurements were made with a Spinco model E ultracentrifuge at a rotor speed of 59 780 rpm at 20°C. 1 % flavoprotein in Na-phosphate buffer, pH 7.0, $\mu = 0.2$ was used. The exposure was made after 92 min of sedimentation with a phase plate angle of 30°.

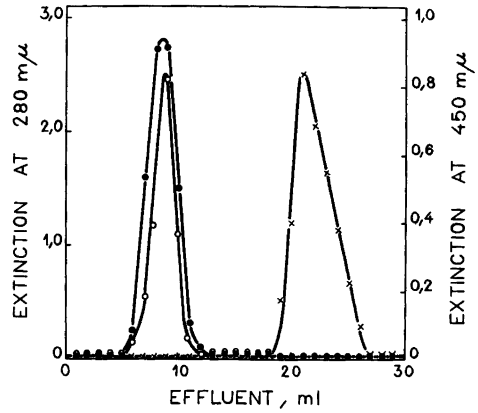


Fig. 2. Gel filtration of the flavoprotein on Sephadex G-25. 10 mg in one ml was applied to the column (1.4 × 18 cm) and eluted with 0.1 M citrate buffer, pH 3.0 at a rate of 5 ml/hr. O—O absorbancy at 280 mμ, ×—× at 450 mμ, •—• absorbancy at 450 mμ when flavoprotein was filtrated at pH 7.0 (0.05 M phosphate buffer); 3°C.

at pH 5.0. The pure substance is readily soluble in water and in salt solutions in a wide range of pH. It contains 13 % N, 0.2 % P and about 8 % hexoses. Neutral flavoprotein solution is very resistant to heat as it neither precipitates nor dissociates flavin on boiling for 30 minutes.

The riboflavin-free protein was prepared by passing the flavoprotein through a Sephadex G-25 column (Pharmacia, Uppsala). The column was equilibrated and eluted with 0.1 M citrate buffer of pH 3.0. At this pH riboflavin undergoes dissociation and entirely separates from the protein component while in the range of pH 3.5 to 8.0 the complex appears in one peak as the excluded solute (Fig. 2.). Fractions containing riboflavin were pooled together, extracted with butanol and dried in vacuo. Further purification of riboflavin involved paper chromatography with the use of saturated water isoamyl alcohol[6], as a solvent. The yellow spot which migrated in parallel to the reference spot of authentic riboflavin was then eluted from the chromatograms with water and resulting solution evaporated in *vacuo*.

The so obtained flavin was further identified by paper electrophoresis at pH 5.1 and 8.0[7] and microbiologically[3]. Moreover, the purified material was analyzed in the Unicam SP-100 IR-spectrophotometer (Cambridge, England) using a KBr pellet. The spectrum was found essentially identical with synthetic riboflavin, showing characteristic absorption bands at frequencies 1248, 1181, 1080 and 851 cm^{-1} [8]. All these results have demonstrated that free riboflavin is the prosthetic group.

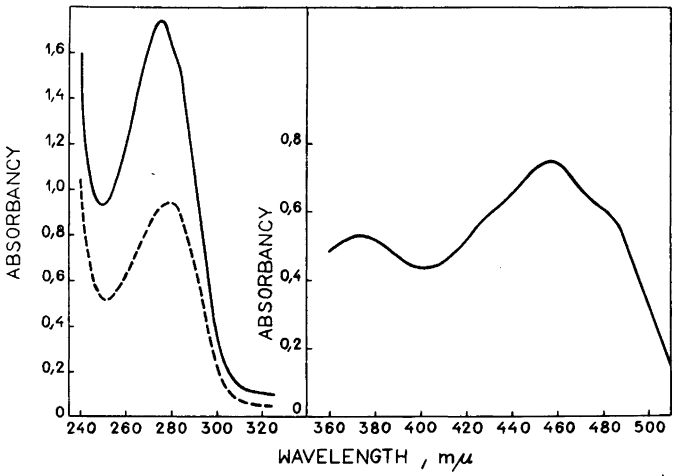


Fig. 3. Absorption spectra of the pure flavoprotein in 0.05 M Na-phosphate buffer at pH 7.0. Protein concentrations were 3.0 and 1.0 mg per ml, respectively, for obtaining visible (360 to 510 mμ) and ultraviolet (240 to 320 mμ) spectra. Dotted line depicts the UV spectrum of apoprotein prepared by gel filtration (one mg per ml, pH 7.0).

The absorption spectrum of the flavoprotein at pH 7.0 is shown in Fig. 3. There occur three maxima: at 276, 375, and 457–459 mμ with the shoulder at about 485 mμ. The ratio of extinction at 276 mμ to that at 458 is 6.5. The molar extinction coefficient as determined from the absorption at 276 mμ and the molecular weight is 6×10^7 cm²/M. The apoprotein reveals only one peak at 280 mμ with an extinction coefficient by about 45 % lower than that of the flavoprotein.

Polarographic titrations

Titrations were performed in the polarograph type PO3 (Radiometer, Copenhagen) in a 2 ml cell adapted for deaeration with N_2. The potential of the dropping electrode was referred to a saturated calomel reference electrode. The riboflavin solution in 0.05 M Na-phosphate buffer, pH 7.0 was polarographed in the range from –0.2 to –0.8 V and the diffusion current was measured by the height of polarographic waves[9].

Lingane and Davis[10] have shown that riboflavin being reduced at neutral pH gives a well defined polarographic wave with $\pi/2$-potential of –0.47 V. In our studies we have observed that protein-bound riboflavin is no longer able to reduce on the dropping electrode. Since the cathodic diffusion current was proportional to concentrations of the free riboflavin undergoing reduction, the stoichiometry of the reaction: riboflavin–protein could readily be followed in the polarograph. Quantitative studies were made by titrating the apoprotein solution with riboflavin under standard conditions. A typical experiment is shown in Fig. 4. Curve A represents a blank titration in which increasing amounts of riboflavin were added in a final volume of 2 ml to the polarographic cell, Curve B illustrates the course of titration of 0.016 μM apoprotein under the same

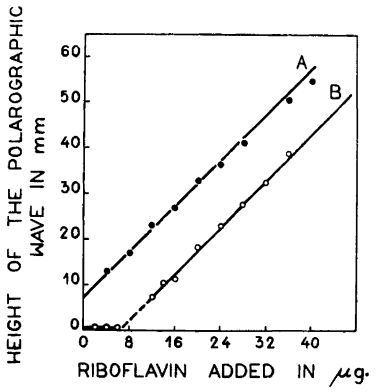


Fig. 4. Polarographic titrations of apoprotein with riboflavin solution. A – blank titration, B – titration of 0.016 μM of apoprotein. The solution of the riboflavin was added to the polarographic cell by means of an "Agla" microburette. Trace of silicone (Dow Corning Co., USA) was added to the solution as the antifoam agent.

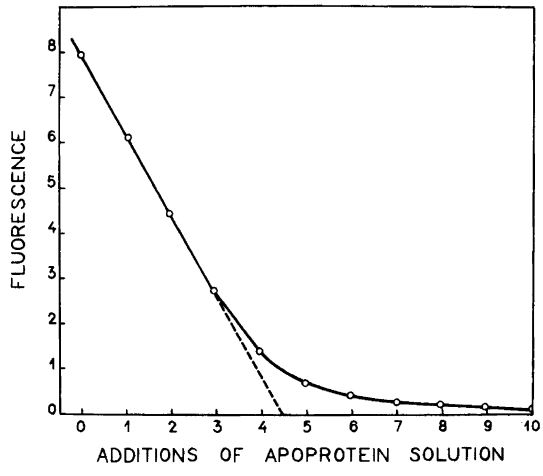


Fig. 5. Fluorometric titrations of riboflavin with partially saturated flavoprotein. To the 2 ml volume of 0.05 M Na-phosphate buffer, pH 7.0, containing 4.06×10^{-4} μM of riboflavin, 2 μl portions of flavoprotein solution were added. Each addition represents 3.46 μg of free apoprotein. Excited at 373 mμ, fluorescence measured at 530 mμ.

conditions. From the breaking point of latter curve the amount of riboflavin binding sites could be derived. It was found, that 0.016 μM apoprotein combined with 6.3 μg riboflavin which gives the molar ratio of the two components as 1 : 1.

Fluorometric titrations

In this set of experiments the riboflavin was titrated with a solution of partially saturated flavoprotein at neutral pH by following the disappearance of fluorescence. Fluorometric titrations were carried out with a spectrofluorometer constructed by Theorell[11]. All determinations were done at 23.5°C in 0.05 M Na-phosphate buffer of pH 7.0. Fig. 5 shows the corresponding titration curve. Since the apoprotein solution was saturated with riboflavin in 69 % of its maximal binding capacity, 4.4 additions were necessary to bind 4.06×10^{-4} μM riboflavin present in the spectrophotometric cell. Admitting the molecular weight to be 37 000 one gets one molecule riboflavin per one molecule flavoprotein. Identical results were obtained by reversed titration, *e. g.* when a pure apoprotein solution was titrated with riboflavin.

From the fluorometric titration the dissociation constant of the complex can be calculated according to the formula:

$$K_{AR} = [A_F]\,[R_F]/[AR]$$

where AR indicates flavoprotein, A_F and R_F indicate free apoprotein and free

riboflavin, respectively. Provided the complex did not fluoresce and the solution of riboflavin was devoid of any fluorescing impurities, the values of dissociation constant as established on the basis of fluorometrical measurements for several points of a curve varied between 2.06 and 2.94 $\times$ 10^{-9} M with a mean value of 2.65 $\times$ 10^{-9} M, at pH 7.0 and 23.5°C. This figure is of the order of magnitude reported for dissociation constants of numerous flavin enzymes and suggests that riboflavin is very strongly bound to the protein moiety.

DISCUSSION

Following the publication of Rhodes *et al.*[1], the present paper provides further evidence for the occurrence of natural flavoproteins in which riboflavin rather than a riboflavin nucleotide is the prosthetic group. In fact, attempts to conjugate the isolated apoprotein with either FMN or FAD were negative in as much as the resulting complex could readily be displaced by free riboflavin[1].

Like other flavoproteins, that from egg yolk completely quenches the fluorescence of riboflavin, shifting its absorption bands at 373 and 445 mμ toward greater wave length. As anticipated, the protein-bound riboflavin was unable to reduce on the dropping electrode. This property may be taken advantage of for further polarographic studies. The behaviour of the yolk flavoprotein on the dropping electrode points to the concomittant reduction of riboflavin at both N (1) and N (10) of the isoalloxazine ring. The quenching effect is probably due to the formation of a linkage between N (3) and tyrosine residue of the protein[12,13]. According to Rhodes *et al.*[1] the ribitol group at N (9) also is essential for the formation of a flavoprotein complex. It appears then, that the conjugation of a flavin with protein involves several groups of the isoalloxazine ring, the structure of which specifically conforms to the binding sites of the protein moiety.

Acknowledgement. We are indebted to Professor H. Theorell for the financial help in the initial stages of the work, to Dr. A. Ehrenberg for the ultracentrifugal analyses and to Dr. Ch. L. Woronick for the fluorometric determinations.

REFERENCES

1. Rhodes, M. B., Bennett, N. and Feeney, R. E. *J. Biol. Chem.* **234** (1959) 2054.
2. Ostrowski, W., Skarzynski, B. and Zak, Z. *Biochim. Biophys. Acta* **59** (1962) 515.
3. Roberts, E. G. and Snell, E. E. *J. Biol. Chem.* **163** (1946) 499.
4. Whitby, L. G. *Biochem. J.* **54** (1953) 437.
5. Ehrenberg, A. *Acta Chem. Scand.* **11** (1957) 1257.
6. Hais, I. M. and Macek, K. *Papirova Chromatografie,* Ceskoslovenské Akademie Vêd, Praha, 1959, p. 632.
7. Cerletti, P. and Siliprandi, N. *Biochem. J.* **61** (1955) 324.
8. Rosenkrantz, H. *Methods Biochem. Anal.* **5** (1957) 407, p. 419.
9. Kolthoff, I. M. and Lingane, J. J. *Chem. Rev.* **24** (1939) 1.
10. Lingane, J. J. and Davis, O. L. *J. Biol. Chem.* **137** (1941) 567.
11. Theorell, H. *Advan. Enzymol.* **20** (1958) 31.
12. Theorell, H. and Nygaard, A. P. *Acta Chem. Scand.* **8** (1954) 1649.
13. Weber, G. *Biochem. J.* **47** (1950) 114.

Received March 25, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 246 – S 249

Fluorometric Analysis of Enzyme-Substrate Complex Formation

KUNIO YAGI and TAKAYUKI OZAWA

Institute of Biochemistry, Faculty of Medicine, University of Nagoya, Nagoya, Japan

Theorell's method for recording fluorescence was applied to follow the change in fluorescence intensity of D-amino acid oxidase mixed with the substrate. Bringing the conditions for the enzyme catalysis apart from their optima, it was demonstrated that a lag time (t_1) appears before the rapid decrease in the fluorescence intensity (t_2). t_1 may correspond to the time for the complex formation between the enzyme and the substrate, and t_2 to that for the enzyme in the complex to convert into its semiquinoid form. As the functions of t_1 and t_2 obey the equation of Arrhenius, it can be postulated that the enzyme passes through two transitionary activated complexes to reach its semiquinoid form. Similarity of the pH – $1/t_1$ curve to the pH-activity curve indicates that $1/t_1$ determines the reaction rate.

According to Michaelis and Menten[1], enzyme catalysis involves two steps, *viz.* (1) the formation of a complex of enzyme (E) and substrate (S) followed by (2) its breakdown to enzyme and product of the reaction,

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E \cdot S \xrightarrow{k_{+2}} E + P \qquad (1)$$

and the rate of the second step determines the rate of the reaction.

In the series of our study on D-amino acid oxidase (D-amino acid : O_2 oxidoreductase (deaminating) EC 1.4.3.3), we succeeded in crystallizing a complex of the semiquinoid form of the holo-enzyme and the partially modified substrate as an intermediate complex ($E' \cdot S'$) during the enzymic reaction[2,3]. A complex of the holo-enzyme and a reaction product, pyruvate, ($E \cdot P$) was also isolated in the crystalline form[4]. Besides these intermediate complexes, it may be supposed that there exists a complex of the holo-enzyme and the substrate without modification ($E \cdot S$). Thus, the minimum number of steps involved in the reaction of the enzyme catalysis could be represented as follows:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E \cdot S \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E' \cdot S' \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E \cdot P \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} E + P \quad (2)$$

Now, we intended to study the E · S formation. For this purpose, we measured the dynamic change in fluorescence intensity of the holo-enzyme when mixed with the substrate.

MATERIALS AND METHODS

D-Amino acid oxidase holo-enzyme was prepared[6] as follows: The crystal suspension of the Michaelis complex (E′ · S′) was gradually oxidized by dialyzing against distilled water at 5°C overnight. The yellow-coloured holo-enzyme crystals were collected by centrifugation and dissolved in distilled water.

The substrate, D-alanine, was obtained from Nutritional Biochemicals Corporation.

The recording fluorometer was essentially the same as that of Theorell and Nygaard[5]. 1.5 ml of the solution of the holo-enzyme crystals (20 μM) mixed with an equal volume of pyrophosphate buffer (0.03 M) was transferred to a cuvette (10 × 10 mm) in the fluorometer. Under anaerobic condition, 0.1 ml of D-alanine solution was mixed and the change in fluorescence intensity of the holo-enzyme recorded (chart speed, 6 cm per min; response time, 0.3 sec).

RESULTS

Dynamic change in fluorescence intensity. At the optimum pH, 8.3, for the enzyme catalysis, the fluorescence intensity of the enzyme began to drop immediately after mixing with an excess of substrate, as shown by Curve I in Fig. 1. However, it was found that a lag time appeared when the substrate concentration was decreased (Curve II and III), and when the pH (Curve IV), or the temperature (Curve V) was lowered. As can be seen in these curves, the lag time (t_1) could be easily distinguished from the time of rapid decrease in fluorescence intensity (t_2).

At the same time, the change in the absorbancy of the holo-enzyme at 453 mμ (A_{453})[7] and that of the semiquinoid form of the enzyme at 550 mμ (A_{550})[8] were

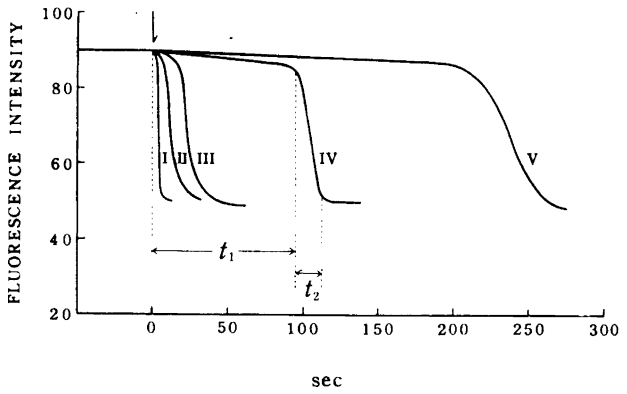


Fig. 1. Dynamic change of the relative fluorescence intensity of the holo-enzyme with added substrate. Holo-enzyme was 10 μM in all cases. Curve I: D-alanine 16 mM, pH 8.3, 20°C; Curve II: D-alanine 0.5 mM, pH 8.3, 20°C; Curve III: D-alanine 0.2 mM, pH 8.3, 20°C; Curve IV: D-alanine 0.2 mM, pH 7.2, 20°C; Curve V: D-alanine 0.2 mM, pH 7.2, 15°C. The arrow shows the addition of the substrate.

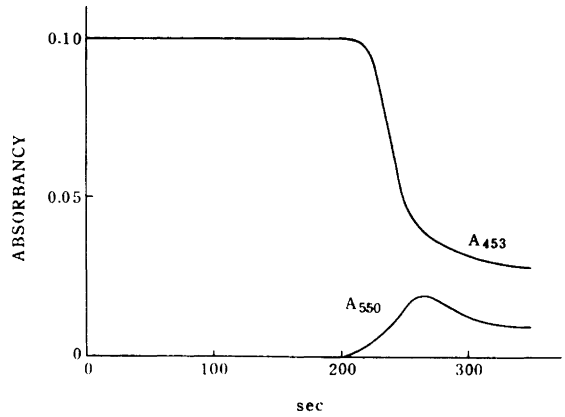


Fig. 2. Dynamic change of the absorbancy of the holo-enzyme. Absorbancies at 453 mμ (A_{453}) and at 550 mμ (A_{550}) of the holo-enzyme, 10 μM, were recorded after addition of the substrate, 0.2 mM, at pH 7.2, 15°C.

traced on a recording spectrophotometer. The dynamic changes in absorbancies of the sample of Curve V in Fig. 1 are shown in Fig. 2. The curves show that the decrease in fluorescence of the enzyme was accompanied by a decrease of A_{453} and an increase of A_{550}. This indicates that t_2 corresponds to the time for the enzyme in the complex to pass into its semiquinoid form.

pH-dependence of t_1 and t_2. t_1 and t_2 were measured with a constant substrate concentration (0.2 mM) and temperature (18°C) at different pH.

The plots of t_1 and t_2 against pH are shown in Fig. 3. As seen from the curves, the pH-dependence of t_1 is remarkable, whereas t_2 shows a very small pH-dependence. It should be noted that the shape of the plot of $1/t_1$ against pH is nearly the same as that of the pH-activity curve of the enzyme[9].

Temperature-dependence of t_1 and t_2. The relation between temperature and t_1 or t_2 was examined by measuring t_1 *and* t_2 at constant pH and at different temperatures.

It was found that log $1/t_1$ or log $1/t_2$ is directly proportional to negative reciprocal of the absolute temperature at any pH measured. Two typical results are shown in Fig. 4.

DISCUSSION

The fluorometric and spectrophotometric results (Figs. 1 and 2) show that t_2 is the time required for the second step of the enzyme catalysis, *viz.* $E \cdot S \rightleftharpoons E' \cdot S'$, and t_1 for the first step, *viz.* $E + S \rightleftharpoons E \cdot S$. Under optimum conditions, the lag time, t_1, is masked by the second step and can not be demonstrated.

The pH-dependence of t_1 shown in Fig. 3 presents an evidence that the enzyme combines with the substrate through some ionized residues. As it is considered that the plot of $1/t_1$ against pH is essentially a titration curve of $E \cdot S$, the $pK_a = 8.1$ suggests that some weak acidic groups are involved in $E \cdot S$ formation. It is also noted that the shape of the pH – $1/t_1$ curve is similar to that of the pH-activity curve[9].

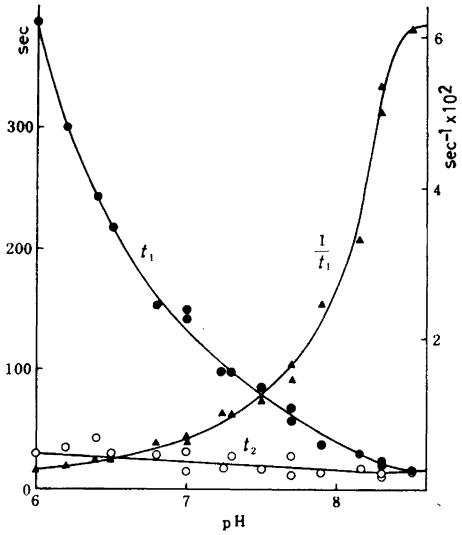


Fig. 3. Plots of t_1, t_2 and $1/t_1$ against pH. t_1 and t_2 were measured by mixing the holo-enzyme, 10 μM, with D-alanine, 0.2 mM, at 18°C. The left ordinate scale is for t_1 and t_2, and the right for $1/t_1$.

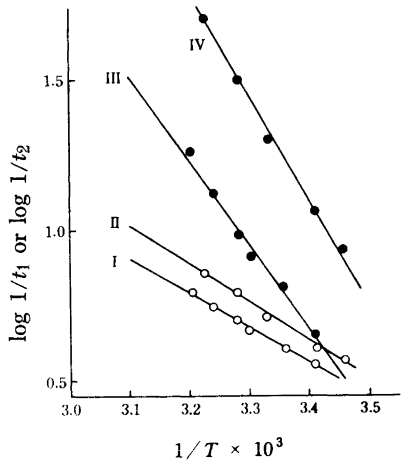


Fig. 4. Plots of log $1/t_1$ and log $1/t_2$ against $1/T$ (T in °K). t_1 and t_2 were measured by mixing the holo-enzyme, 10 μM, with D-alanine, 0.2 mM. Curve IV: $2.5 + \log 1/t_1$ at pH 7.9, Curve III: $2.5 + \log 1/t_1$ at pH 7.0, Curve II: $1.8 + \log 1/t_2$ at pH 7.9, Curve I: $1.8 + \log 1/t_2$ at pH 7.0.

On the assumption that both the rate constant of the first step (k_{+1}) and that of the second step (k_{+2}) in Reaction (2) are proportional to $1/t_1$ and $1/t_2$, respectively, it was demonstrated that both k_{+1} and k_{+2} obey the equation of Arrhenius,

$$\ln k = -\mathrm{E_a}/RT + \ln \mathrm{A}$$

and it can thus be postulated that the enzyme passes through two transitionary activated complexes to reach E′ · S′.

From these results, it is considered that the E · S formation is an important factor in determining the rate of the enzymic reaction.

REFERENCES

1. Michaelis, L. and Menten, M. L. *Biochem. Z.* **49** (1913) 333.
2. Yagi, K. and Ozawa, T. *Biochim. Biophys. Acta* **60** (1962) 200.
3. Yagi, K. and Ozawa, T. *Biochim. Biophys. Acta. In press.*
4. Yagi, K. and Ozawa, T. *Unpublished results.*
5. Theorell, H. and Nygaard, A. P. *Acta Chem. Scand.* **8** (1954) 877.
6. Yagi, K. and Ozawa, T. *Unpublished results.*
7. Yagi, K. and Ozawa, T. *Biochim. Biophys. Acta* **56** (1962) 413.
8. Yagi, K. and Ozawa, T. *Biochim. Biophys. Acta* **67** (1963) 685.
9. Yagi, K. and Ozawa, T. *Biochim. Biophys. Acta* **56** (1962) 420.

Received March 30, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 250 – S 256

A Comparison of the Amino Acid Sequences of the Cytochromes *c* of Several Vertebrates

E. MARGOLIASH, S. B. NEEDLEMAN and J. W. STEWART

Biochemical Research Department, Abbott Laboratories, North Chicago, Illinois, USA

The amino acid sequences of four vertebrate cytochromes *c* (horse, human, pig and chicken) show a remarkable degree of similarity. All are 104 residues long, are acylated at the amino-terminal residue, the two cysteinyl residues binding the heme are in positions 14 and 17, and two long sequences (residues 16 to 43 and 63 to 82) are identical in all four proteins. Comparing each cytochrome *c* in turn with the others, 28 amino acid exchanges are obtained. These occur at only 15 positions which are grouped into three areas along the chain. The variations do not disrupt the groupings of basic and hydrophobic residues, even though such residues represent a large proportion of the entire protein. Some positions are particularly prone to change. At residue 89 four different amino acids are observed. Residues in certain positions appear to be characteristic of particular species and known phylogenetic relations are in conformity with the observed variations. The relations of the amino acid sequence of cytochrome *c* to the possible secondary and tertiary structures of the protein are discussed. The consistence of the observed amino acid exchanges with known triplet nucleotide sequences in ribonucleic acid, coding for particular amino acids, is examined.

Complete amino acid sequences of the cytochromes *c* prepared from horse[1–4], man[5], chicken[6] and pig[6] have been determined. The purpose of the present article is to compare these structures and to examine the implications of the observed amino acid replacements.

Table 1 gives the amino acid sequence of horse heart cytochrome *c*, while Table 2 lists the differences between the amino acid sequences of the horse protein and the corresponding sequences of the other proteins examined.

COMMON FEATURES IN THE STRUCTURE OF VERTEBRATE CYTOCHROMES *c*

The most striking single fact revealed by an examination of Tables 1 and 2 is the remarkable degree of similarity of the amino acid sequences of the four cytochromes *c* listed, even though the species examined derive from phylogenetic origins relatively as divergent as chicken and man. This fundamental homology

Table 1. Amino acid sequence of horse heart cytochrome c[1].

Acetyl – Gly – Asp – Val* – Glu – ***Lys*** – Gly – ***Lys*** – ***Lys*** – **Ileu*** – **Phe** – Val* – $GluNH_2$ – ***Lys*** – CyS* – Ala – $GluNH_2$ – CyS*
(10; HEME)

– ***His*** – Thr* – Val* – [Glu – ***Lys*** – Gly – Gly – ***Lys*** – ***His*** – ***Lys***] – Thr* – Gly – Pro* – [$AspNH_2$ – **Leu** – ***His*** – Gly –
(20; 30)

Leu – **Phe** – Gly – ***Arg*** – ***Lys***] – Thr* – Gly – $GluNH_2$ – **Ala** – **Pro*** – Gly – **Phe** – **Thr*** – **Tyr** – Thr* – [Asp – Ala – $AspNH_2$
(40; 50)

– ***Lys*** – $AspNH_2$ – ***Lys*** – Gly] – **Ileu*** – Thr* – **Try** – ***Lys*** – Glu – Glu – Thr* – [**Leu** – **Met** – Glu – **Tyr** – **Leu** – Glu –
(60)

$AspNH_2$] – Pro* – ***Lys*** – ***Lys*** – **Tyr** – **Ileu*** – Pro* – Gly – Thr* – ***Lys*** – **Met** – **Ileu*** – **Phe** – Ala – Gly – **Ileu*** – ***Lys*** – ***Lys*** –
(70; 80)

Lys – Thr* – Glu – ***Arg*** – Glu – Asp – **Leu** – **Ileu*** – [Ala – **Tyr** – **Leu** – ***Lys*** – ***Lys*** – Ala] – Thr* – $AspNH_2$ – GluCOOH
(90; 100; 104)

Sequences of six or more helix forming residues are **boxed**. Non-helix forming residues are marked **with an** asterisk. Basic residues are in *italics* and hydrophobic residues **in bold type.**

Table 2. Differences in the amino acid sequences of horse[1], pig[6], human[5] and chicken[6] cytochromes *c*. A blank space indicates that the amino acid is identical to the one found in the horse protein.

	Residue position in peptide chain														
Species	3	11	12	15	44	46	47	50	58	60	62	83	89	92	104
Horse	Val	Val	$GluNH_2$	Ala	Pro	Phe	Thr	Asp	Thr	Lys	Glu	Ala	Thr	Glu	Glu
Pig							Ser			Gly			Gly		
Human		Ileu	Met	Ser		Tyr	Ser	Ala	Ileu	Gly	Asp	Val	Glu	Ala	
Chicken	Ileu			Ser	Glu		Ser			Gly	Asp		Ser	Val	Ser

presumably stems from the genetic derivation of these proteins from a common primordial cytochrome *c*, amino acid variations being limited to those consistent with structures able to carry out the necessary specific electron transfer and energy conserving functions.

All four vertebrate cytochromes *c* under consideration are single chains 104 residues long, have an amino-terminal residue acylated at the α-amino group and the heme attached through its two vinyl side chains by thio-ether linkages to cysteinyl residues in positions 14 and 17. The structural similarities around the attachment of the prosthetic group to the peptide chain, first observed by Tuppy and collaborators[7,8] for a number of different cytochromes of the *c* group, have been fully borne out by the amino acid sequences discussed here. These similarities consist of two cysteinyl residues, separated by two other residues, preceded by a basic amino acid, lysine or arginine, and followed by the sequence His-Thr. This pattern has, however, also been found in cytochromes functionally entirely different from vertebrate cytochrome *c*, such as the protein from *Rhodospirillum rubrum*[9] and more recently in the cytochromoid of *Chromatium*[10]. These features therefore most probably represent requirements for the attachment of the heme moiety and the provision of at least part of its immediate environment, irrespective of the specificity of the overall protein for particular enzyme systems.

The 28 amino acid substitutions observed by comparing, in turn, each of the cytochromes *c* with the other three, occur at only 15 positions along the peptide chain, leaving two long sequences, those from residue 16 to 43 and residue 63 to 82, completely unchanged. It is probable that the first of these sequences, which contains the three histidyl residues in the molecule as well as the unreactive lysyl residue[11], is part of the "crevice"[12] structure which enfolds the prosthetic group and may contain one[13] or both[14,15] of the hemochrome-forming groups. The lack of variation in this area could thus be ascribed to specific amino acid sequence requirements for the formation of a "crevice" which is functionally efficient.

The second large constant part of the sequence (residues 63 to 82) contains one of the major groupings of basic residues and three of the eight groupings of hydrophobic residues[3]. It is tempting to speculate that it represents the area of the protein responsible for the inhibition of cytochrome oxidase[16] and cytochrome *c* peroxidase[17]. Both basic[16–18] and non-cationic groups[19] have been implicated in the protein-enzyme bond.

Basic amino acids (lysine, arginine and histidine) constitute 22 to 23 % of the

entire protein. Except for one variation, that of residue 60 in the horse protein which is exchanged for a glycine in all the other proteins, none of the basic residues vary. Similarly, except for an isoleucine in position 3 in chicken cytochrome *c*, where the other proteins carry a valyl residue, the eight groups of hydrophobic residues[3] are not disturbed, even though such residues represent 22 to 25 % of the peptide chain. The only other increase in the number of such residues is found in human cytochrome *c* (residues 11, 12 and 58, isoleucine, methionine and isoleucine, respectively). The effect of this change is to increase the size or continuity of two hydrophobic areas already present in the other proteins. This remarkable stability of the basic residues, which are largely grouped together, and of the eight clusters of hydrophobic amino acids, increases the probability that these groupings do indeed represent, as previously noted[3], definite functional or structural necessities.

COMMENTS ON THE SPATIAL CONFIGURATION OF THE CYTOCHROME *c* CHAIN

A recent electron microscopic study of horse heart cytochrome *c* by the negative contrasting technique[20] gives an estimate of the dimensions of the molecule of 38 to 40 Å × 28 Å. The protein is visualized as consisting of three segments of about equal length, running parallel to each other and connected by two turns[20]. The thickness of the straight portions identify them as α-helices. The model looks like a letter e collapsed in the vertical direction[20]. This structure might be considered to be consistent with the amino acid sequence of the protein. The chain carries four prolyl residues at position 30, 44, 71 and 76, respectively. Since helices are interrupted at such residues, this situation may provide for a wide 180° turn in the vicinity of the two first prolines and a similar tight turn at the last two prolines. The three straight sections left would contain 29, 26 and 28 residues, respectively, which could provide α-helices of about 43.5, 39 and 42 Å in length, in reasonable agreement with the dimensions of the straight portions of the above model[20].

Such a model, however, implies a very high helical content, calculated by Levin[20] to be about 80 %. This does not appear to be in accord with the distribution of helix-forming and non-helix forming residues as identified by Blout[21] from the conformation of synthetic polyamino acids. The non-helix forming residues are marked in Table 1 by an asterisk. The sequences of six or more helix-forming amino acids are boxed, since the very smallest polypeptides which show properties indicative of a tendency towards a helical conformation are six residues long[22]. There are only five sections which, on this basis, could form helices and these encompass no more than 34.6 % of the entire chain. In particular it is notable that the area near the attachment of the heme contains 6 non-helix forming residues in three of the cytochromes and 7 such amino acids in the human protein, out of a total of 12 residues, making it improbable that this region is helical as has been assumed[14].

With regard to the proline residues, all four are not essential since in chicken cytochrome *c* the proline in position 44 is replaced by a glutamyl residue. Such a substitution does not necessarily change the number of non-helical portions of the molecule. Indeed as was shown by Kendrew and collaborators[23] for myoglobin, non-helical sections can occur without the presence of a proline.

DIFFERENCES IN THE AMINO ACID SEQUENCES OF VERTEBRATE CYTOCHROMES *c*

As noted above the amino acid exchanges occur at only 15 sites along the chain and are grouped in three regions located between residues 3 and 15, 44 and 62, 83 and 104. Some sites appear to be particularly prone to change. Among the four proteins examined four different residues appear in position 89, while three are found in position 92. This indicates loci at which mutations appear to be favored, similar to the genetic "hot-spots" observed by Benzer[24] in bacteriophage.

Residues in certain positions are probably characteristic of particular species or groups of closely related species. Thus horse cytochrome *c* has a threonine in position 47 and a lysine in position 60, where the other proteins carry seryl and glycyl residues, respectively, in these locations. The horse protein is the only one of the present set containing 19 lysyl residues as compared to the 18 present in the other cytochromes *c*. This extra basic group must account for the relatively easy separation of the horse protein from other cytochromes *c* by cation exchanger chromatography[11]. Horse cytochrome *c* also differs from the other proteins by its total lack of serine. Similarly chicken cytochrome *c* is unique in having an isoleucine in position 3 and a serine at the carboxyl-terminal end of the chain, positions in which all the other proteins have valine and glutamic acid respectively. Human cytochrome *c* is distinguished by an Ileu-Met sequence in place of the Val-GluNH_2 found in the other proteins in positions 11 and 12.

As expected from known phylogenetic relations there are only three amino acid differences between the cytochromes of horse and pig. Chicken cytochrome *c*, the only bird protein represented in the present series, differs by as many as 9 residues from horse, 10 from human and 7 from pig cytochrome *c*. Differences of the same magnitude occur among the mammalian proteins. For example, the human protein differs from horse cytochrome *c* by 11 residues and by 9 residues from the pig protein. Clearly, more precise phylogenetic correlations will be possible only when the amino acid sequences of many more cytochromes *c* from suitably chosen species have been worked out.

The finding that triplets of nucleotides in ribonucleic acid "code" for individual residues in proteins, makes it possible to examine whether the exchanges of amino acids in a series of related proteins result from changes of single nucleotides in the corresponding triplets, or a more extensive variation. For this purpose the composition of the coding triplets, obtained by experimental observations with synthetic polynucleotides[25–27], is not sufficient and it is necessary to consider the actual sequence of nucleotides in each triplet. From known amino acid exchanges in various proteins Smith[28] has proposed six alternative sets of coding triplets and Jukes[29] has proposed a single such set. Table 3 lists the amino acid exchanges found in the four vertebrate cytochromes *c* together with their corresponding nucleotide triplets according to Smith's [28] code 1. Of the 18 types of amino acid exchanges observed 8 involve the change of one nucleotide in the coding triplet and of these, four result from changes of a purine for a pyrimidine base while four are due to exchanges of like bases only. The remaining 10 residue variations appear to be due to more complex changes, presumably through intermediate stages of evolution involving other amino acids. The four residues observed in

Table 3. Nucleotide triplet coding sequences corresponding to amino acid exchanges observed in four vertebrate cytochromes *c*. The nucleotide sequences are those proposed by Smith[28] (code No. 1).

Position of residues in peptide chain	Amino acid interchange	Corresponding nucleotide triplet sequences
3, 11	**Val/Ileu**	**UUG/UUA**
12	**$GluNH_2$/Met**	**UCG/GUA**
15	**Ala/Ser**	**CUG/CUU**
44	**Pro/Glu**	**CCU/UAG**
46	**Phe/Tyr**	**UUU/UAU**
47, 89	**Thr/Ser**	**CUA/CUU**
50	**Asp/Ala**	**UGA/CUG**
58	**Thr/Ileu**	**CUA/UUA**
60	**Lys/Gly**	**UAA/UGG**
62	**Glu/Asp**	**UAG/UGA**
83, 92	**Ala/Val**	**CUG/UUG**
89, 104	**Glu/Ser**	**UAG/CUU**
89	**Thr/Gly**	**CUA/UGG**
89	**Thr/Glu**	**CUA/UAG**
89	**Glu/Gly**	**UAG/UGG**
89	**Ser/Gly**	**CUU/UGG**
92	**Glu/Val**	**UAG/UUG**
92	**Glu/Ala**	**UAG/CUG**

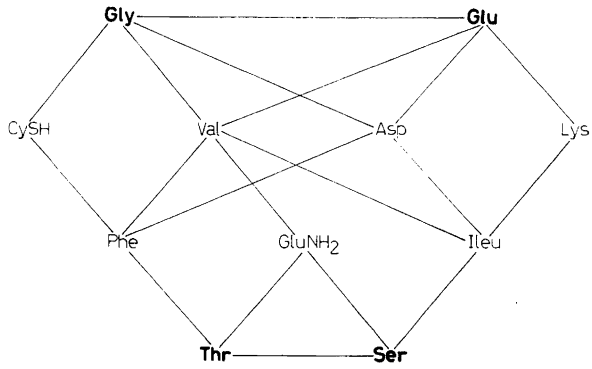


Fig. 1. Possible intermediates in the amino acid exchanges at position 89 of the peptide chain of vertebrate cytochromes *c*. The nucleotide triplet coding sequences used were those proposed by Smith[28] (code No. 1). The residues in **bold type** are the ones so far observed in four vertebrate cytochromes *c*, the others represent possible intermediates. Residues joined by a line can transform one into the other as a result of a single nucleotide change in the coding triplet.

position 89, threonine, glycine, glutamic acid and serine, furnish a good example of such a possibility. These residues can be arranged in two pairs, glycine and glutamic acid on the one hand, and threonine and serine on the other. Transformation within each pair can be obtained by a single base change, while to

proceed from one pair to the other several alternative pathways, involving cysteine, valine, aspartic acid, lysine, phenylalanine, glutamine and isoleucine as intermediates, may be envisaged. The pathways are summarized in Fig. 1. Similar though less complex pathways may be considered for the exchanges occurring at positions 12, 44, 50, 60, 62 and 92. It should however be strongly emphasized that since the above discussion is based on a triplet nucleotide code which is incomplete, as evidenced by the recent discovery of coding triplets not containing uridylic acid[25,26], it is impossible to decide at present whether the apparent complexities of some residue exchanges will not be simplified by a fuller knowledge of coding triplets, or whether the protein did in fact evolve through numerous intermediate forms. The finding of cytochromes *c* carrying the postulated intermediate residues may not always be possible since some may have been eliminated in evolution. Nevertheless the establishment of a large series of amino acid sequences for a set of homologous proteins will help, as already indicated[28,29], in determining the sequence of nucleotides in coding triplets.

REFERENCES

1. Margoliash, E., Smith, E. L., Kreil, G. and Tuppy, H. Nature **192** (1960) 1125.
2. Margoliash, E. and Smith, E. L. *J. Biol. Chem.* **237** (1962) 2151.
3. Margoliash, E. *J. Biol. Chem.* **237** (1962) 2161.
4. Tuppy, H. and Kreil, G. *Monatsh. Chem.* **92** (1962) 780.
5. Matsubara, H. and Smith, E. L. *J. Biol. Chem.* **237** (1962) PC 3575.
6. Chan, S. K., Needleman, S. B., Stewart, J. W., Walasek, O. F. and Margoliash, E. *Federation Proc.* **22** (1963) 658.
7. Tuppy, H. and Bodo, G. *Monatsh. Chem.* **85** (1954) 1024.
8. Tuppy, H. and Paléus, S. *Acta Chem. Scand.* **9** (1955) 353.
9. Paléus, S. and Tuppy, H. *Acta Chem. Scand.* **13** (1959) 641.
10. Dus, K., Bartsch, R. G. and Kamen, M. *J. Biol. Chem.* **237** (1962) 3083.
11. Margoliash, E. *Brookhaven Symp. Biol.* **15** (1962) 266.
12. George, P. and Lyster, R. L. J. *Proc. Natl. Acad. Sci. U. S.* **44** (1958) 1013.
13. Margoliash, E., Frohwirt, N. and Wiener, E. *Biochem. J.* **71** (1959) 559.
14. Ehrenberg, A. and Theorell, H. *Acta Chem. Scand.* **9** (1955) 1193.
15. Paléus, S., Ehrenberg, A. and Tuppy, H. *Acta Chem. Scand.* **9** (1955) 365.
16. Smith, L. and Conrad, H. in Falk, J. E., Lemberg, R. and Morton, R. K. (Eds.) *Haematin Enzymes,* Pergamon Press, London, 1961, p. 260.
17. Beetlestone, J. *Arch. Biochem. Biophys.* **89** (1960) 35.
18. Estabrook, R. W. in Falk, J. E., Lemberg, R. and Morton, R. K. (Eds.) *Haematin Enzymes,* Pergamon Press, London, 1961, p. 276.
19. Conrad, H. and Wasserman, A. R. *Federation Proc.* **20** (1961) 42.
20. Levin, Ö. *Arch. Biochem. Biophys.* **Suppl. 1** (1962) 301.
21. Blout, E. R. in Stahmann, M. A. (Ed.) *Polyamino Acids, Polypeptides and Proteins,* The University of Wisconsin Press, Madison, 1962, p. 275.
22. Mitchell, J. C., Woodward, A. E. and Doty, P. *J. Am. Chem. Soc.* **79** (1957) 3955.
23. Kendrew, J. C., Watson, H. C., Strandberg, B. E., Dickerson, R. E., Phillips, D. C. and Shore, V. C. *Nature* **190** (1961) 666.
24. Benzer, S. *Proc. Natl. Acad. Sci. U. S.* **47** (1961) 403.
25. Jones, O. W., Jr., and Nirenberg, M. W. *Proc. Natl. Acad. Sci. U. S.* **48** (1962) 2115.
26. Wahba, A. G., Gardner, R. S., Basilio, C., Miller, R. S., Speyer, J. F. and Lengyel, P. *Proc. Natl. Acad. Sci. U. S.* **49** (1963) 116.
27. Speyer, J. F., Lengyel, P., Basilio, C. and Ochoa, S. *Proc. Natl. Acad. Sci. U. S.* **48** (1962) 63.
28. Smith, E. L. *Proc. Natl. Acad. Sci. U. S.* **48** (1962) 677, 859.
29. Jukes, T. H. *Proc. Natl. Acad. Sci. U. S.* **48** (1962) 1809.

Received April 2, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 257–S 262

Fine Structure in the Low Temperature Spectra of Catalase Complexes

BRITTON CHANCE and GREGORY R. SCHONBAUM*

Johnson Research Foundation, University of Pennsylvania, Philadelphia 4, Pennsylvania, USA

Application of rapid freezing and low temperature examination of the absorption bands of catalase Complex I reveals fine structure that is not explained by the presence of Complex II. Such fine structure predominates the region 500–600 mμ, and is not observed in the region of the Soret band.

Knowledge of the chemical nature of the enzyme-substrate compounds of hemoproteins depends critically upon the accuracy of the available physical data. Absorption spectra represent the most precise data[1–3] and any improvement in the accuracy of recording the spectra of these intermediates is of general interest. Here we have achieved an improvement in precision and resolution by rapid freezing of the intermediates and recording of the spectra representing differences between the enzyme-substrate intermediate and the free enzyme.

EXPERIMENTAL

Technique. A particular advantage of observing spectra at liquid nitrogen temperatures[4] is that the proportions of the chemical species are constant over the recording interval. A second advantage is that the recording interval may be as prolonged as desired. A third advantage is that the hemoprotein bands are more distinct at the low temperatures. Thus we have adopted the trapped steady state method, employed for scanning the spectra of the frozen steady state of the cytochromes of the respiratory chain[4–6]. The technique has been improved somewhat over that used previously by increasing the heat capacity of the cuvette (changing aluminium to copper) and by decreasing the volume to 0.25 ml. Thus freezing occurs upon contact between the injected intermediate and the pre-cooled chamber. The center of the chamber cools at a rate of 40° K/sec, as indicated in Fig. 1; it should be noted, however, that the injection of water at 300° K into the chilled cuvette gives a temperature rise approaching the freezing point and rapid cooling occurs thereafter. It is probable that the drop to the freezing point occurs very rapidly in the catalase samples. Experiments with glycerol coating for the cuvette seemed to increase the cooling time only slightly; most of the cooling was from the heat capacity of the copper block itself.

* Present address: University of Alberta, Edmonton, Alberta, Canada.

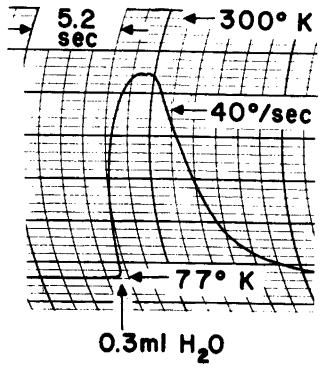


Fig. 1. Thermocouple recording of the time course of cooling of a 0.3 ml liquid sample in the chilled cuvette of the spectroscopic apparatus.

The quick freezing method of Bray[7] has been employed in preliminary tests, but the packing of the frozen material in the cuvettes is not yet sufficiently uniform for precise differential spectrophotometry. Thus we have preferred to use the rigid geometry obtained by rapid freezing in a chilled cuvette. However, further development of this interesting method for precision spectrophotometry is well worthwhile.

Preparations of enzymes and enzyme-substrate compounds. Catalase in the range of 1–4 μM was employed in these experiments in a 2 mm optical path cuvette. The R.Z. values for the three preparations used are 0.77, 1.2, and 1.3 for beef liver (Boehringer), *Micrococcus lysodeicticus* and *Rhodopseudomonas spheroides* catalase. The latter was donated by Dr. R. Clayton. Preparations were diluted in 0.1 M phosphate buffer, pH 6, at 26°C. Ethyl hydrogen peroxide (600 μM) or methyl hydrogen peroxide (60 μM) was used to form the Complex I in less than 1 sec[8,9]. The secondary complex was formed by two successive additions of 600 μM methyl hydrogen peroxide in a 10 min interval[7,8].

The conditions for obtaining complete conversion of catalase to Complex I inevitably led to the formation of small concentrations of Complex II[8,9]. Thus the comparison of the properties of Complex I and II depends upon a comparison of the intensity and position of the bands under conditions which favor maximal conversion of the enzyme to I or II. This procedure was employed in the figures that follow. It is also apparent that the amount of Complex II in our preparation of Complex I was smaller than in Brill and Williams' [10], who employed 6.32 μM catalase and 295 μM ethyl hydrogen peroxide, but required a longer time for measurement.

Procedure. A portion of the diluted catalase solution was injected into the reference portion of the pre-cooled cuvette. The sample was then immersed in liquid nitrogen. After the sample came to thermal equilibrium the cuvette was raised slightly above the surface of the liquid. An appropriate concentration of substrate (between 60 and 600 μM) was blown into the remainder of the catalase solution and the mixture was rapidly sucked up into a 0.5 ml syringe and discharged via a polyethylene catheter into the chilled cuvette. Immediately after filling, the cuvette was plunged beneath the surface of the liquid nitrogen.

Difference spectra were then plotted by a split-beam spectrophotometer covering full scale span ranging from 10 per cent transmission to 0.5 in optical density[11,12]. The original data were replotted against a baseline obtained with catalase in both cuvettes. However, in this paper our attention has been focused upon the location of the peaks of the difference spectra rather than the isosbestic points.

It should be noted that two sensitivities are used in recording the spectra; the vertical dividing line indicates the wavelength at which the sensitivity is altered (usually 470–480 mμ).

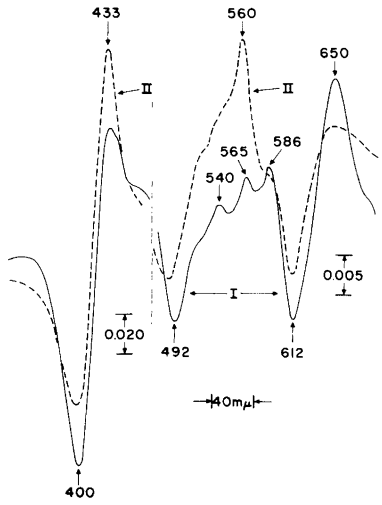


Fig. 2. Difference spectra of catalase complexes under conditions where Complex I (I) or Complex II (II) predominates. 4.7 μM beef liver catalase, 630 μM C_2H_5OOH (I), 1200 μM CH_3OOH (II) (Expt. 775a-2,5).

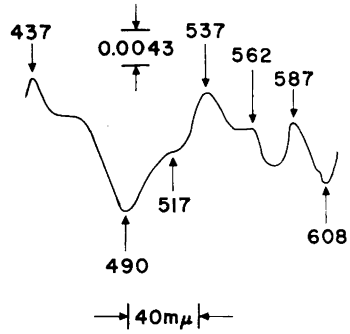


Fig. 3. Difference spectrum similar to Fig. 2, but with 60 μM CH_3OOH (Expt. 775a-3).

RESULTS

Beef liver catalase. Fig. 2 illustrates two low temperature difference spectra obtained at conditions favoring the formation of the primary complex (trace I) or the secondary complex (trace II). Under both conditions the absorption bands of free catalase at 400, 485, and 612 mμ disappear. This corresponds closely to the positions of three of the four absorption bands at 403, 500, 540, and 629 mμ at 300° K[13]. The most prominent band that appears on addition of the low concentration of ethyl hydrogen peroxide is the 650 mμ band. In addition there is a triplet of bands at 540, 566, and 586 mμ which may be considered a part of the Complex I spectrum. A band at 435 mμ, obtained under conditions in which Complex I predominated, was nevertheless difficult to distinguish from that of Complex II, obtained at the higher concentrations of peroxide. However, before considering Complex II, Fig. 3 indicates in more detail the fine structure of the absorption bands obtained at a low concentration of methyl hydrogen peroxide (60 μM or 12 equivalents). Here one can identify distinctive troughs at 490 and 608 mμ and peaks at 437, 537, 562, and 587 mμ. It is of interest that the trough at 608 mμ is not nearly as distinct where a larger conversion of catalase to the intermediate form is observed (Fig. 2).

Considering now the absorption bands of the secondary complex in Fig. 2, it is seen that the predominating bands are at 433 and 560 mμ. In fact, the latter so dominates the "fine structure region" observed at low concentrations of peroxide that they can only be seen as shoulders on the larger band. We would

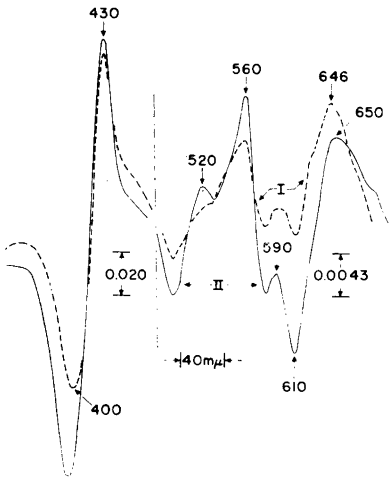


Fig. 4. Difference spectra of catalase complexes as in Fig 2, but using 1.6 μM *M. lysodeicticus* catalase, 120 μM CH_3OOH (I), and 1200 μM CH_3OOH (II) (Expt. 775b-2,3).

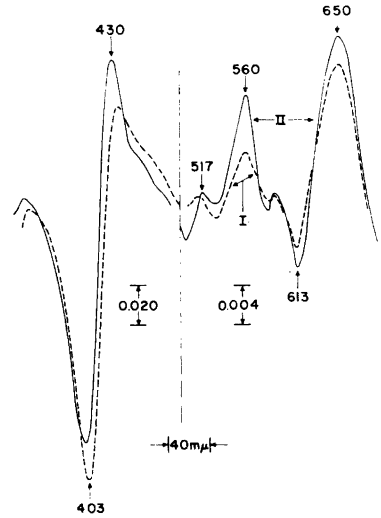


Fig. 5. Difference spectra of catalase complexes as in Fig. 2, but using 1.6 μM *Rhodopseudomonas spheroides* catalase and 60 μM CH_3OOH (I) and 1200 μM CH_3OOH (II) (Expt. 775c-2,3).

therefore tend to identify the band at 650 mμ and the fine structure bands (except that at 560 mμ) with the primary intermediate.

Catalase from M. lysodeicticus. Fig. 4 shows the absorbancy changes resulting on addition of 120 μM methyl hydrogen peroxide; a characteristic absorption band at 650 mμ and the triplet of bands in the region 500–590 mμ again was observed. On repeating the experiment with two additions of 600 μM methyl hydrogen peroxide, the principal increase in intensity is in the bands at 430 and 560 mμ; a much smaller change was observed in the bands at 517, 590, and 650 mμ. Here we associate the bands at 517 and 590 mμ with the primary complex.

Catalase from Rhodopseudomonas spheroides. Fig. 5 shows absorbancy changes due to the addition of low (60 μM) and high (1200 μM) methyl hydrogen peroxide to 1.6 μM *R. spheroides* catalase. The results are similar to those obtained with the other catalases except that the addition of the lower concentration of methyl hydrogen peroxide has led to less than complete development of the 650 mμ band of Complex I. Nevertheless, the triplet of bands shows clearly in the region of 500–600 mμ and the 590 mμ band is not greatly increased at the higher concentration of peroxide; in this case, however, the band at 519 mμ is somewhat more intense at the higher concentration of peroxide.

In Table I we have summarized the band positions obtained at low and high peroxide concentrations for the three types of catalases and it is seen that there is considerable regularity in the positions of all the bands.

Recordings in the ultraviolet region. By inserting a Wratten 39A filter in the optical system, it has been possible to record relatively accurately to 350 mμ.

Table 1. Low temperature spectra of catalase complexes (Expt. 175).

Source of catalase	[E] μM	[S] μM	Type	Test	Predominating complex	Sign of changes +	−	+	+	+	+	−
Beef liver	4.7	630	EtOOH	2a	I	652	610	585	566	540	435	400
	4.7	60	MeOOH	3	I	646	608	587	562	537	437	400
	4.7	1200	MeOOH	5	II	652	610	587	560		433	400
M. lysodeicticus	1.6	600	EtOOH	2	I	650	613	590	560	517	431	399
	1.6	120	MeOOH	3	I	651	610	590	560	516	433	403
	1.6	1200	MeOOH	5	II	652	610		561		430	
R. spheroides	1.6	120	MeOOH	1	I	650	611	592	559	519	431	404
	1.6	1200	MeOOH	3	II	650	610	590	560	517	430	403

In no case were we able to observe an absorption band in this region. It will be noted in Figs. 2, 4, and 5 that the absorption at the shortest wavelengths scarcely rises to the values of absorption above 440 mμ. In short, there seems to be no distinctive absorption in this region comparable to the 650 mμ absorption band.

DISCUSSION

Techniques for the study of labile intermediates. The discovery of the labile primary green intermediates of catalase and peroxide and their subsequent study have been marked by attempts to obtain more and more accurate data. The first description of the difference spectrum of this compound, a point by point evaluation of absorbancy differences between the free enzyme and the labile intermediate (catalase H_2O_2-I), was made at various wavelengths by means of a rapid flow apparatus attached to a double grating monochromator[1]. The absorbancy differences measured at wavelengths in the region 370–450 mμ were recorded and applied to the catalase spectrum. The rapid flow apparatus also has been used to determine the spectra of the primary compounds of catalase and peroxidase with alkyl hydrogen peroxide[2,3]. These methods have attained their ultimate development by the combination of a wavelength scanning spectrophotometer with the rapid flow apparatus[14], which allows the scanning of a difference spectrum over a span of 60 mμ within 9 m sec after mixing. This is, of course, the optimal method for room temperature studies but has a rather large catalase requirement.

Thus the quick freezing method finds a clear application for the accurate examination of spectra of enzymes available in insufficient amounts for the combined flow apparatus and scanning spectrophotometer.

Commercially available instruments have also been used for recording the spectra of these stabilized intermediates of catalase and peroxide; Brill and Williams[10] have employed the Cary spectrophotometer for studies of the spectrum of the primary ethyl hydrogen peroxide complex of bacterial catalase. Even

though they employed the early "single point" method, the recording could not be started until about 10 sec after the addition of the substrate.

Structure of Complex I. Deep interest in the nature of the absorbancy changes accompanying the formation of catalase Complex I arises from the hypothesis presented some time ago[2]: that ring oxidation occurs and that the disappearance of the bands is due to a verdo-hemin structure. This suggestion has been examined in detail by Brill and Williams, who identify the hypothesis by the configuration "ROX". Although their proposal accounts for the retention of the elements of peroxide in the primary complex[15,16], no direct evidence has been presented to date which would permit identification of Complex I as a tautomeric mixture of ROX and POR. It is interesting to note that Brill and Williams have attempted to break down the spectrum of Complex I into three components, having maxima at 340–360, 405–415, and 450–460 mμ. Since it is obvious that these three components, if they exist at all, are inadequately resolved at room temperature, it occurred to us that they might be adequately resolved at low temperatures. The experimental results appear to be strongly against the possibility that low temperatures would resolve these three components of Complex I. Since the low temperatures do show a variety of characteristic bands of hemoproteins in the visible region, it is likely that fine structure in the region 350–450 mμ would have been revealed by this method, if it existed.

If, indeed, catalase Complex I is a mixture of intermediates of different values of spin, the data presented here indicate that the visible region of the spectrum (500–600 mμ) is a much preferable region in which to attempt kinetic or other studies to identify their separate existences.

The existence of the fine structure in the spectra of the primary catalase complexes is of especial interest in view of the increasing evidence that they consist of a peroxide complex of trivalent iron[15,16].

This research has been supported by the *National Science Foundation G 10813.*

REFERENCES

1. Chance, B. *Acta Chem. Scand.* **1** (1947) 237.
2. Chance, B. *J. Biol. Chem.* **179** (1949) 1331.
3. Chance, B. *Arch. Biochem. Biophys.* **21** (1949) 416.
4. Estabrook, R. W. In Falk, J. E., Lemberg, R and Morton, R. K. *Haematin Enzymes,* Pergamon Press, Oxford, 1961, p. 436.
5. Chance, B. and Spencer, E. L., Jr. *Discussions Faraday Soc.* **27** (1959) 200.
6. Chance, B. In Falk, J. E., Lemberg, R. and Morton, R. K. *Haematin Enzymes,* Pergamon Press, Oxford, 1961, p. 254.
7. Bray, R. C. *Biochem. J.* **81** (1961) 189.
8. Chance, B. *J. Biol. Chem.* **179** (1949) 1341.
9. Chance, B. and Herbert, D. *Biochem. J.* **46** (1950) 402.
10. Brill, A. J. and Williams, R. J. P. *Biochem. J.* **78** (1961) 253.
11. Chance, B. *Science* **120** (1954) 767.
12. Yang, C. C. and Legallais, V. *Rev. Sci. Instr.* **25** (1954) 801.
13. Theorell, H. In Summer, J. and Myrbäck, K. *The Enzymes,* Academic Press, New York, 1951, Vol. I, Part II, p. 423.
14. Chance, B. *Discussions Faraday Soc.* **20** (1955) 205.
15. Chance, B. and Schonbaum, G. R. *J. Biol. Chem.* **237** (1962) 2391.
16. Schonbaum, G. R. *Federation Proc.* **21** (1962) 234d.

Received April 8, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 263–S 269

The influence of Catecholamines on the Visible Absorption and on the Electron Spin Resonance Absorption Spectrum of Ceruloplasmin

OTTO WALAAS, EVA WALAAS, THORMOD HENRIKSEN and ROLF LÖVSTAD

Institute of Medical Biochemistry, University of Oslo, and Norsk Hydro's Institute for Cancer Research, Oslo, Norway

The interaction of catecholamines with ceruloplasmin has been studied by measurement of the visible absorption spectrum as well as by electron spin resonance spectrometry. By aid of a stopped flow technique the rate constant of the reaction:

$$\text{Cu(II)ceruloplasmin} + \text{catecholamine}_{red} \xrightarrow{k_1} \text{Cu(I)ceruloplasmin} + \text{catecholamine}_{ox}$$

has been determined spectrophotometrically, recording the decrease of the absorbancy at 605 mμ. Decreasing values of the rate constant k_1 were observed : Dopamine > Noradrenaline > Adrenaline > Isopropylnoradrenaline > DOPA. At steady state equilibrium, in the presence of substrate, the absorption band at 605 mμ of ceruloplasmin was decreased by 50–60 %. The interaction of N,N-dimethyl-ppd with ceruloplasmin was somewhat faster and the visible absorption was lower at equilibrium. It has been shown that oxygen accelerates the interaction of catecholamines with ceruloplasmin, k_1 in O_2 being greater than k_1 in N_2.

By ESR spectrometry it has been confirmed that the decrease of the visible absorption band is directly related to the reaction Cu(II) → Cu(I) in the enzyme when substrates are added. In nitrogen the intensity of the ESR signal is reduced to a greater extent by catecholamines than in oxygen atmosphere.

It is concluded that the bonding of catecholamines to ceruloplasmin involves the amine group in the side chain. N-alkyl substitution decreases the velocity of the interaction of the substrate with the enzyme, and the formation of the initial oxidation product of catecholamines during activity occurs at a slower rate.

A variety of polyamines and polyphenolic compounds can serve as substrates for ceruloplasmin. These include p-phenylenediamine, hydroquinone, catechol, adrenaline, DOPA*[1], noradrenaline[2], dopamine, isopropylnoradrenaline[3], 5-hydroxytryptamine[4], and several hydroxyindoles[5]. Ceruloplasmin can also

* Abbreviations used: Cu(II)CP, oxidized ceruloplasmin; Cu(I)CP, reduced ceruloplasmin; $NADH_2$, reduced nicotinamide adenine dinucleotide; DOPA, L-β-3,4-dihydroxyphenylalanine; Dopamine, 3-hydroxytyramine; N,N-dimethyl-ppd, N,N-dimethyl-*p*-phenylenediamine; ESR, electron spin resonance.

oxidize $NADH_2$ in the presence of *p*-phenylendiamines and catecholamines[2]. Oxidation of the substrates by ceruloplasmin is associated with a reversible reduction oxidation of the copper; Cu(II)CP ⇌ Cu(I)CP[6]. The importance of copper for the catalytic activity has been illustrated in studies on the binding and removal of copper[7–9]. Electron spin resonance spectroscopy has indicated that resting ceruloplasmin contains 4 Cu(II) and 4 Cu(I) atoms[10]; further confirmation of this was obtained by magnetic susceptibility data[11]. The very interesting ESR studies of Malmström and Vänngård[12] show that copper in resting ceruloplasmin is coordinated in a unique manner.

The present work is concerned with the properties of ceruloplasmin during activity in the presence of catecholamine substrates. Kinetic measurements on the visible absorption spectrum after addition of substrates have been made by a technique of rapid spectrophotometric recording. The valence state of copper in the enzyme during activity has been further illustrated by electron spin resonance absorption spectra.

EXPERIMENTAL

Material

Ceruloplasmin was obtained from AB Kabi, Stockholm, and purified as described previously[2], on a DEAE cellulose column. After concentration by ultrafiltration (Metal-Drucksfiltrationsgeräte MD, 70–15, Membranfiltergesellschaft GMBH, Göttingen), ceruloplasmin was dialyzed against 0.01 M NaCl. In some experiments the native Kabi enzyme was used. Most of the substrates used were those reported earlier[2]. In addition experiments were made with the following compounds: L-β-3,4-dihydroxyphenylalanine (DOPA), Sigma Chemical Comp; 3-hydroxytyramine · HCl (Dopamine), Sigma Chemical Comp; D,L-N-isopropylarterenol · HCl, California Corporation Biochemical Research.

Spectrophotometric technique

The kinetic measurements were done on a DK Beckman spectrophotometer by aid of a stopped flow technique. The flow apparatus consisted of two 2 ml injection syringes connected with a mixing chamber. The plungers of the syringes were connected to each other by a plexiglass pushing block. The mixing chamber was connected by Tygon tubing to a circulating system in a plexiglass cuvette. The light beam passed through a bore of 1.5 mm diameter in the plexiglass, covered by quartz plates on each side. In front of the cuvette a diaphragm was placed fixed to the front of the cuvette holder. A small hole in the diaphragm confined the light beam to a cross-section of less that 1.5 × 1.5 mm. This slender light beam could pass through the liquid without touching the cuvette walls. A similar arrangement was used for the reference cuvette. The light path through the cuvette was 10 mm.

In flow experiments the plungers were rapidly pushed down and simultaneously the liquid was discharged from the mixing chamber into the circulation system of the cuvette. The solutions were previously saturated with the gas phases used, air, oxygen or nitrogen, according to a procedure previously described[6]. The temperature was kept constant at +5°C by circulating water from a thermostat. The filled syringes, as well as the mixing chamber, and the circulation system in the cuvette, were surrounded by plexiglass water jackets. To obtain a temperature equilibrium the whole system was cooled for one hour at +5°C before mixing. Immediately before the experiment the condensed vapor on the surface of the cuvette was blown away with an air-blower.

Electron spin resonance (ESR) spectroscopy

The spectrometer used has been described in detail elsewhere[13]. It operates at a frequency of 9200 Mc/second. It has a rectangular transmission cavity (H_{102}mode), crystal detection, and a modulation frequency of 110 kc/sec. Phase sensitive detection is used, and the spectra recorded represent the first derivative of the actual absorption curves. The microwave frequency was measured with a wavemeter, and the magnetic field was measured with a proton resonance

field meter. The accuracy of these measurements is about 0.1 %. The samples were placed in quartz tubes of 4 mm diameter, frozen immediately and kept in liquid air during the experiment.

RESULTS

Catecholamines as substrates for ceruloplasmin

It has previously been reported that N-alkyl substitution in the side chain increases the rate of oxidation of catecholamines to the corresponding "chromes"[3]. Oxidation of dopamine and noradrenaline was very slow compared to adrenaline and isopropylnoradrenaline. However, when the activity of ceruloplasmin against these substrates was studied by the indirect oxidation of $NADH_2$, the results were quite different. As shown in Fig. 1 the oxidation of $NADH_2$ by dopamine and noradrenaline in the presence of ceruloplasmin occurs rapidly. The rate is nearly of the same order of magnitude as mediated by N,N-dimethyl-ppd. On the other hand, in the above system the oxidation of $NADH_2$ was very slow in the presence of adrenaline or isopropylnoradrenaline. The activity of ceruloplasmin against the non-decarboxylated DOPA was very slow by the direct as well as by the indirect method. The importance of characterizing ceruloplasmin activity against the different substrates by the direct as well as the indirect method is obvious.

Visible absorption spectrum of ceruloplasmin

The interaction of catecholamines with ceruloplasmin has been studied in experiments involving measurements of rapid reaction kinetics. At room temperature the blue colored ceruloplasmin is bleached in a few seconds after addition of the substrates. In order to study the interaction of catecholamines with ceruloplasmin, it was therefore necessary to perform the experiments at low temperature (+5°C). Under these conditions the rate of alteration of the blue absorption band of ceruloplasmin could be followed by a recording spectrophotometer. With all substrates studied the intensity of the absorption was decreased but without shift of the maximum at 605 mμ. A typical experiment is presented in Fig. 2. The rate of alteration occurs more rapidly with dopamine and noradrenaline than with adrenaline and isopropylnoradrenaline, while DOPA interacts very slowly with the enzyme. However, the interaction of N,N-dimethyl-ppd with ceruloplasmin was the most rapid reaction observed. One can assume that the interaction of catecholamines with ceruloplasmin involves the reactions:

$$\text{Cu(II)CP} + \text{catecholamine}_{\text{red}} \xrightarrow{k_1} \text{Cu(I)CP} + \text{catecholamine}_{\text{ox}} \qquad (1)$$

$$\text{Cu(I)CP} + \tfrac{1}{2}\text{O}_2 \xrightarrow{k_2} \text{Cu(II)CP} + \text{H}_2\text{O} \qquad (2)$$

The $\text{catecholamine}_{\text{ox}}$ may be a free radical or a quinone, which in the presence of oxygen is further dehydrogenated to the red colored "chrome". Reaction (1) could be expected to be of the second order. However, deviations might occur since the association of the catecholamine with the catalytic center may involve coordination with the copper as well as bonding to the enzyme protein. It was therefore of interest to calculate the rate constant k_1 from different points on the experimental curves to see if they came out as true constants. In these experi-

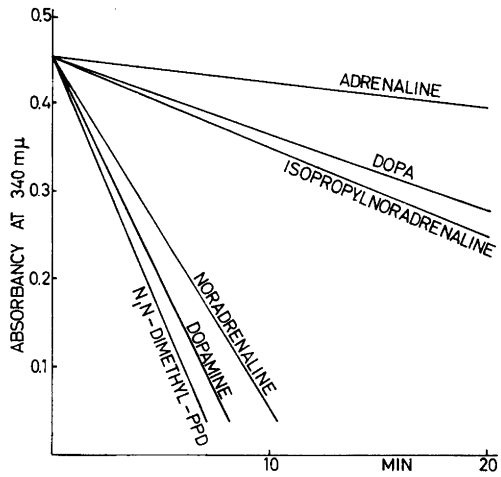


Fig. 1. The rate of oxidation of $NADH_2$ by ceruloplasmin in the presence of different catecholamines. Incubation system: 3.8×10^{-7} M ceruloplasmin, 1.55×10^{-4} M substrate, 1×10^{-4} M $NADH_2$, 0.05 M acetate buffer pH 5.9. Temp. 38°C.

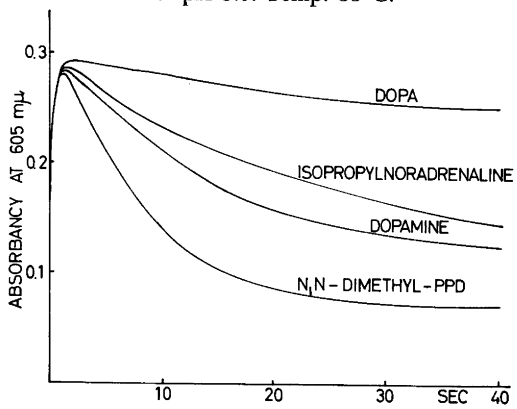


Fig. 2. The change of absorbancy at 605 mμ of ceruloplasmin by addition of catecholamines. The curves for noradrenaline and adrenaline (not recorded on the figure) are intermediate between dopamine and isopropylnoradrenaline. System (initial concentrations): 3.15×10^{-5} M ceruloplasmin, 5×10^{-4} M substrate, 0.03 M acetate buffer pH 5.9. Temp. 5°C. Satured with air.

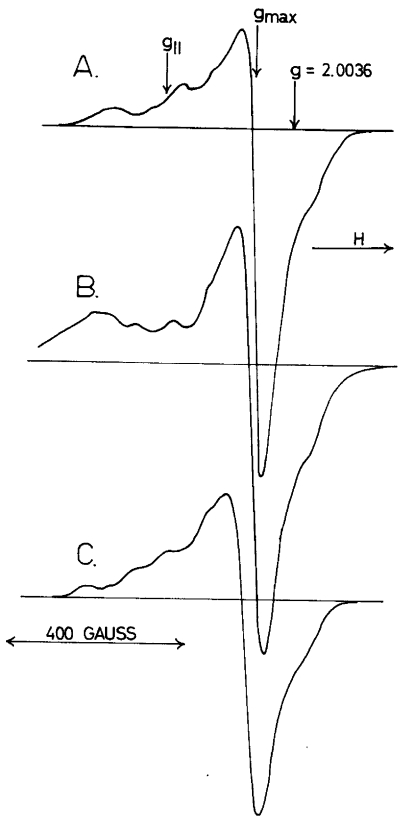


Fig. 3. Electron spin resonance absorption spectra (derivatives) of ceruloplasmin in the absence and in the presence of dopamine. Conditions: 8.2×10^{-5} M ceruloplasmin, 1.67×10^{-4} M Dopamine, 0.01 M acetate buffer pH 5.9. Liquid air temp. A. Resting ceruloplasmin, saturated with 100 % oxygen. B. Ceruloplasmin + Dopamine saturated with 100 % oxygen. C. Ceruloplasmin + Dopamine saturated with 100 % nitrogen. g = 2.0036, resonance field of diphenylpicrylhydrazyl (DPPH).

ments where the substrates had been added in excess, the calculations were made under the assumption that reaction (2) could be neglected during the initial phase of the curve.

Table 1. Values of k_1 for the interaction of substrates with ceruloplasmin calculated at different times from the time course of the absorbancy at 605 mμ (Fig. 2).

Isopropylnor-adrenaline		Dopamine		N,N-dimethyl-ppd	
Time sec	k_1 $M^{-1} \times sec^{-1}$	Time sec	k_1 $M^{-1} \times sec^{-1}$	Time sec	k_1 $M^{-1} \times sec^{-1}$
1.7	89	1.4	124	0.8	250
4.0	95	2.3	145	2.5	314
6.5	83	3.4	148	4.0	407

According to Broman *et al.*[10] it was assumed that ceruloplasmin contained 4 catalytic copper centers. From Table 1 it is seen that the values of k_1 for dopamine and isopropyl noradrenaline were nearly constant within the experimental error, during the initial period of the reaction (1 → 6 sec). The same has been observed for the other catecholamine substrates investigated. However, with N,N-dimethyl-ppd, the values of k_1 varied considerable after a reaction time above 1.5 sec. In Table 2 the mean values of k_1 for the different catecholamines are presented. The initial part of the curves (Fig. 2), where constant values of k_1 are obtained, have been used. It is seen that k_1 for these substrates is decreased by N-alkyl substitution in the side chain, while k_1 DOPA is extremely low. However, the reaction velocity for N,N-dimethyl-ppd was greater than for any catecholamine investigated.

At steady state equilibrium the visible absorbancy at 605 mμ of ceruloplasmin was decreased by 50–60 % in the presence of catecholamines. The alteration in absorbancy by addition of N,N-dimethyl-ppd was even more pronounced (75 %).

Table 2. The influence of catecholamines on the absorbancy of ceruloplasmin at 605 mμ. k_1 = second order rate constant. Average values calculated from different points on the initial part (1 → 5 sec) of the curves in Fig. 2.

Substrate	Decrease of absorbancy at 605 mμ at equilibrium %	Average rate constant k_1 $M^{-1} \times sec^{-1}$
N,N-dimethyl-ppd	74	265*
Dopamine	60	143
Noradrenaline	56	120
Adrenaline	52	99
Isopropylnoradrenaline	50	85
DOPA	23	7

* Average of approximate values calculated from 0.5 → 1.5 sec on the curve. At reaction times ≥ 2 sec deviation of k_1 from the initial value did occur.

In addition, the importance of oxygen for the interaction of catecholamines with ceruloplasmin was studied. The k_1 for dopamine was studied in nitrogen atmosphere after rigorous deoxygenation of the reaction system, and compared with k_1 in 100 % oxygen. In several experiments it was demonstrated that the interaction of dopamine with ceruloplasmin was accelerated by oxygen, k_{1O_2} being 20–30 % higher than k_{1N_2}.

Electron spin resonance absorption spectrum of ceruloplasmin

Malmström and Vänngård[12] observed exceptionally low hyperfine splitting constants in the ESR absorption spectrum of ceruloplasmin. The ESR absorption spectra of frozen ceruloplasmin recorded in this work exhibited the same characteristics for the resting enzyme (Fig. 3 A). They were characterized by $g_{max} = 2.057$, $g_{\parallel} = 2.196$ and a hyperfine splitting constant $A = 0.008$ cm^{-1}.

The addition of substrates decreased the intensity of the ESR signal of ceruloplasmin. By using the same enzyme preparation as in Fig. 2 under same conditions, dopamine decreased the ESR signal by 30 % and N,N-dimethyl-ppd by 45 %. By addition of $Na_2S_2O_4$ the resting enzyme was completely reduced. An extension of these studies will be reported later. Under steady state condition, in the presence of substrates, the intensity of the ESR signal was influenced by oxygen tension. As demonstrated in Fig. 3 B and C dopamine decreased the ESR signal of this enzyme preparation by 6 % in oxygen compared with 30 % in 100 % nitrogen. In some experiments it was observed that the hyperfine structure of the ESR absorption spectrum was somewhat changed after addition of substrates in the presence of oxygen (Fig. 3 B). This effect is now under investigation.

DISCUSSION

The high intensity of the visible blue absorption band in resting ceruloplasmin with molar extinction 1200[1] indicates an unusual type of bonding of copper in the enzyme. It should also be pointed out that the exceptional low hyperfine splitting constants in the ESR signal have not been observed in simple Cu(II)-complexes[12]. However, a similar low hyperfine structure has been observed in laccase[12], pseudomonas copper proteins[14] and in cytochrome oxidase[15]. This suggests that copper is coordinated in a unique manner in ceruloplasmin as well as in other copper oxidases.

In experiments with urea-denaturation of ceruloplasmin Broman *et al.*[10] obtained indication that the strong visible absorption as well as the low hyperfine structure was associated with the oxidase activity. Williams[16] has pointed out that oxime- and hydrazine-copper complexes show visible absorption bands of similar high molar extinctions as the copper oxidases. He has suggested that these complexes as well as ceruloplasmin arise through a high degree of charge transfer where the electrons are associated with the metal ligand complex as a whole, rather than with the copper atom alone.

As shown in the present work the interaction of ceruloplasmin with its substrates greatly change the properties of the enzyme. This can be attributed to reduction of Cu(II) → Cu(I), demonstrated by a decrease in the intensity of the

ESR signal. Simultaneously a decrease of the visible absorbancy by 50 to 75 % takes place.

Orgel[17] has suggested that copper oxidases may be oxygen carriers similar to hemocyanin. In such copper oxygen complexes resonance between different structures of similar energy levels is possible. In the present work it has been shown that at increased oxygen tension the rate constant k_1 for the reaction of ceruloplasmin with catecholamines is increased. In addition, increased oxygen tension influences the steady state level Cu(II)CP $\rightleftharpoons$ Cu(I) CP in favour of Cu(II)CP. However, it has been shown that the catalytic activity of ceruloplasmin as determined by the $NADH_2$ system was inhibited by increased oxygen tension.

The importance of the side chain of catecholamines for the interaction with ceruloplasmin has clearly been demonstrated here. By alkylation at the nitrogen atom (adrenaline, isopropylnoradrenaline) the interaction with ceruloplasmin is retarded and the formation of the initial oxidation product is inhibited. The presence of the COOH group in DOPA, which is a poor substrate, to a great extent hinders interaction with the enzyme. These findings strongly indicate a bonding of the amine group in the catecholamine side chain to the catalytic centers of ceruloplasmin. It has been suggested[18] that the binding of ceruloplasmin to *p*-phenylenediamines may be not through the amine groups but to the π electrons of the aromatic ring. The binding of catecholamines and *p*-phenylendiamines to ceruloplasmin may therefore be different as also indicated by the results of the kinetic studies reported here.

Acknowledgment. We are indebted to AB Kabi for supply of ceruloplasmin. This work has been supported by grants from *The Nordic Insulin Fund* and from *The Norwegian Research Council for Science and the Humanities.*

REFERENCES

1. Holmberg, C. G. and Laurell, C. B. *Acta Chem. Scand.* **5** (1951) 476.
2. Walaas, E. and Walaas, O. *Arch. Biochem. Biophys.* **95** (1961) 151.
3. Walaas, E. and Walaas, O. *Acta Chem. Scand. In press* 1963.
4. Porter, C. G., Titus, D. C., Sanders, B. E. and Smith, E. V. C. *Science* **126** (1957) 1014.
5. Blaschko, H. and Levine, W. *Brit. J. Pharamacol.* **15** (1960) 625.
6. Walaas, E., Walaas, O., Haavaldsen, S. and Pedersen, B. *Arch. Biochem. Biophys.* **100** (1963) 97.
7. Curzon, G. *Biochem. J.* **77** (1960) 66.
8. Scheinberg, I. H. and Morell, A. G. *Science* **127** (1958) 588.
9. Scheinberg, I. H. and Sternlieb, I. *Pharmacol. Rev.* **12** (1960) 355.
10. Broman, L., Malmström, B. G., Aasa, R. and Vänngård, T. *J. Mol. Biol.* **5** (1962) 3.
11. Ehrenberg, A., Malmström, B. G., Broman, L. and Mosbach, R. *J. Mol. Biol.* **5** (1962) 450.
12. Malmström, G. and Vänngård, T. *J. Mol. Biol.* **2** (1960) 118.
13. Henriksen, T. and Pihl, A. *Intern. J. Radiation Biol.* **3** (1961) 351.
14. Mason, H. S. *Biochem. Biophys. Res. Commun.* **10** (1963) 11.
15. Beinert, H., Griffiths, D. E., Wharton, D. C. and Sands, R. H. *J. Biol. Chem.* **237** (1962) 2337.
16. Williams, R. J. P. Vth International Congress of Biochemistry, Moscow, 1961, Symp. No. IV, Reprint No. 40.
17. Orgel, L. E. In Crock, E. M. *Metals and Enzyme Activity,* Biochemical Society, Cambridge, 1958, Symp. No. 15, p. 8.
18. Levine, W. G. and Peisach, J. *Biochim. Biophys. Acta* **63** (1962) 528.

Received April 8, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 270 – S 276

Structural and Enzymatic Properties of the Extracellular Nuclease of *Micrococcus pyogenes*

CHRISTIAN B. ANFINSEN*, MARILYNN K. RUMLEY and HIROSHI TANIUCHI*,**

Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts, USA

The extracellular nuclease produced during the growth of *Micrococcus pyogenes* has been isolated as an essentially homogeneous protein which is capable of hydrolyzing phosphate diester bonds in both yeast RNA and calf thymus DNA. The enzyme has a pH optimum of approximately 8.6 – 8.8 with RNA as substrate. Preliminary studies indicate a sedimentation coefficient of 1.7 S and an isoelectric point at pH 7.0 – 7.5. The results of amino acid analyses permit the calculation of a rough molecular weight of 11 000 – 12 000 based on 2 histidine residues per molecule. On this same basis, the protein contains approximately 100 amino acid residues per molecule, including one residue of tryptophan. The polypeptide chain is free of SH groups and of disulfide bonds suggesting that the calcium ions which are required for activity may be involved in the stabilization of the tertiary structure of the enzyme.

The deoxyribonuclease found in the culture medium of *Micrococcus pyogenes* was originally purified by Cunningham and his colleagues[1–3] and shown to yield mainly 3'-mononucleotides. Further studies by Reddi[4–6] and by Privat de Garilhe *et al.*[7] have confirmed the calcium requirement of the enzyme and have further demonstrated that the partially purified protein preparation exhibits activity towards both RNA and DNA. Alexander *et al.*[8] have purified the enzyme extensively and have presented detailed studies on specificity. These studies, in contrast to some of the data presented by Privat de Garilhe *et al.*[7], strongly suggest that a single enzyme is responsible for both ribonuclease and deoxyribonuclease activities.

The relatively small size of the protein makes it ideally suited for a study of

* Present address: National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland.

** Fellow of the China Medical Board, 1963–64.

covalent structure in relation to activity. Such investigations are of special interest because of the possibility that the catalytic center of the protein cannot distinguish between the ribose and deoxyribose moieties of the substrate molecules. Thus a comparison of the sequence and tertiary structure of the micrococcal nuclease with that of bovine pancreatic ribonuclease, whose structure is known, may lead to useful information on the three-dimensional configurations and mechanisms of action of the respective catalytic sites.

The present studies permit us to state, with some assurance, that the same protein does, indeed, attack both substrates. The enzyme consists of a single polypeptide chain, devoid of disulfide cross linkages. The nuclease has been purified to a point where it appears essentially homogeneous in the ultracentrifuge and during free electrophoresis, and calculations based on amino acid analysis yield a molecular weight of approximately 12 000.

EXPERIMENTAL

Growth of organisms. Micrococcus pyogenes (Staphylococcus aureus, Strain V-8)[9] was grown in 100–150 l lots, with vigorous bottom aeration, of culture medium "CCY"[9]. This medium contains (per l) 30 g oxoid casamino acids, 20 g sodium β-glycerophosphate, 10 g sodium lactate, 2.45 g Na_2HPO_4, 0.4 g KH_2PO_4, 6.4 mg $FeSO_4 \cdot 7H_2O$, 6.4 mg citric acid, 10 mg $MnSO_4 \cdot 4H_2O$, 20 mg $MgSO_4 \cdot 7H_2O$ and 20 % (v/v) of oxoid yeast diffusate (pH 7.2 – 7.4). Growth was carried out at 37°C overnight, with vigorous stirring. We wish to express our gratitude to the Microbiological Research Establishment, Porton, Wilts, England, for growing and harvesting the organisms. The culture medium was cooled to below 10°C, clarified by centrifugation, and the supernatant sterilized by filtration. $(NH_4)_2SO_4$ was stirred into the filtrate until the solution was saturated and the suspension was allowed to stand overnight at room temperature. The bulk of the precipitate rose to the surface and, after the solution below had been siphoned off, was collected by filtration after addition of suitable amounts of Hyflo Supercel. Phosphate buffer (0.05 M, pH 6.8 – 7.0) was added to the filter cake until the ammonium sulfate concentration was reduced to 2.2 M. The suspension was filtered and the residue was re-extracted with 2.2 M ammonium sulfate in the same phosphate buffer. The nuclease was precipitated from the combined extracts by saturation of the solution with ammonium sulfate and, after the addition of Hyflo Supercel, the precipitate collected by filtration. These preliminary steps yielded approximately two-thirds of the total units of nuclease activity in the original culture filtrate. The filter cake from approximately 2000 l of culture medium, containing the precipitated enzyme together with the Hyflo Supercel, was shipped to this laboratory for further purification.

Ribonuclease activity. Enzyme solutions were diluted with sodium borate buffer, 0.1 M, pH 8.8, in such a manner that 5 to 10 μl sufficed for each assay. The aliquot taken was added to a solution containing 0.5 ml 0.4 % yeast RNA, prepared according to Crestfield, Smith and Allen[10], 0.1 ml 0.1 M $CaCl_2$ (calcium ions are an absolute requirement), and 0.4 ml borate buffer, pH 8.8, 0.1 M. The RNA was also made up in the same buffer. Following incubation at 37°C for 30 min, 0.5 ml uranyl acetate (0.75 % in 25 % perchloric acid) were added. The tubes were centrifuged to remove precipitated oligonucleotides and undigested RNA, 0.1 ml of the supernatant were diluted to 3.1 ml with water, and the optical density at 260 mμ was measured. One unit of enzyme activity is defined as that amount which produces, under the above conditions, a change of absorbancy of 1.0 at 260 mμ.

Deoxyribonuclease activity was determined by a modification of the method described by Alexander *et al.*[8]. Five to 10 μl of suitable diluted enzyme solution were added to a solution containing 0.1 ml substrate solution (calf thymus DNA, preparation "Simmons B"[11], 3 mg/ml in a solution which is 0.005 M in NaCl and 0.05 M in sodium borate, pH 8.8). After incubation at 37°C for 30 min, 0.5 ml perchloric acid was added. The tubes were then centrifuged and the supernatant was diluted and read at 260 mμ as described by Alexander *et al.*[8]. Units of DNase activity are defined as for RNase activity above. DNase activity (and, to a smaller extent, RNase activity as well) is strongly influenced by the nature of the buffer with which the enzyme is diluted prior to sampling for assay. Thus citrate buffer may yield much higher

(*ca* 6-fold) activities than phosphate and borate buffers. This effect does not influence the *relative* activities within any single experiment but can cause marked variations in the results obtained from experiment to experiment since the previous history of the samples may have a strong influence on the apparent activities measured. The variations due to ionic environment are being investigated further.

Chromatography was performed at 5°C on CM-cellulose (California Biochemical Institute, Cellex-CM, *ca* 0.7 meqv/g) by a modification of a method described earlier[12] (see below). Relative protein concentrations were estimated by spectrophotometric measurements in the Zeiss spectrophotometer at 280 mμ. Titration of the native enzyme for SH group content was carried out as described by Boyer[13], over a wide range of enzyme concentrations (0.01—0.1 μmoles/2.5 ml).

Samples of protein which appeared to be homogeneous on the basis of physical measurements and estimations of specific enzyme activities of effluent fractions from chromatographic columns were subjected to reduction and alkylation[14]. After removal of urea and mercaptoethanol from the solution of reduced, alkylated protein by dialysis against water, the solutions were lyophilized. Weighed samples of the dried protein were hydrolyzed in constant boiling 6 N HCl in evacuated, sealed tubes at 110°C and the hydrolysates, after removal of HCl, were analyzed for amino acid content in the automatic amino acid analyzer (Beckman-Spinco Model 120B) according to the procedure of Spackman *et al.*[15] Samples were acid hydrolyzed for 20 and 44 h. The values obtained for those amino acids which are unstable, or slowly released, under conditions of acid hydrolysis[16] have not been corrected in the present paper.

Samples of the dried protein, after exposure to conditions of reduction and alkylation as described above, were digested with trypsin to yield peptides for "fingerprinting". In these digestions, 3—4 mg of protein were dissolved in 0.35 ml 0.2 M NH_4HCO_3, pH 8 and treated for 2 h at 37°C with 0.07 mg trypsin which had previously been exposed to limited amounts of diisopropylfluorophosphate to inactivate the bulk of contaminating chymotrypsin-like activity[17]. Peptide maps were prepared by chromatography in butanol-acetic acid-water of aliquots of the digests equivalent to approximately 1 mg of protein, followed by electrophoresis at pH 3.6 in the "High Voltage Electrophorator" (Model D, Gilson Medical Electronics, Middleton, Wisconsin)[18]. The resulting maps were stained either with 0.025 % ninhydrin in absolute ethanol, with Sakaguchi reagent, for the location of arginine residues, or with the Ehrlich stain for tryptophan residues.

Purification. CM-cellulose was successively washed with 1 N NaOH, 3 N HCl, and water. Water washing was continued until a negative test for chloride ions was obtained. The ion exchanger was then equilibrated with 0.05 M Na-PO_4 buffer, pH 6.1. Columns having the dimensions 2.5 × 30 cm were prepared and a solution of the crude ammonium sulfate precipitate, described above, was applied in a small volume after preliminary dialysis against the 0.05 M buffer and removal of Hyflo Supercel. The column was washed with this same buffer until the effluent exhibited an extinction at 280 mμ of less than 0.05. A closed two-compartment gradient was then begun in which potassium phosphate, 0.2 M, pH 8.0, was added to the mixing chamber which contained the 0.05 M buffer at pH 6.1. Fig. 1 shows the typical elution diagram of a preparative run. The nuclease activity emerged last from the column at an effluent pH value of approximately 7.1—7.3. The phosphate concentration at this point was approximately 0.15 M. The tubes containing the active component were pooled and the combined solution diluted with 8 parts of distilled water and 1 volume of 0.1 M $CaCl_2$, in that order. The copious precipitate of calcium phosphate was collected by centrifugation and the supernatant, devoid of all but traces of activity, was discarded. The precipitate was suspended as a slurry in 0.1 M citrate buffer, pH 7.5 and 0.1 M sodium ethylenediaminetetraacetate (EDTA) was added (0.8 ml/40 ml final volume). The suspension was transferred to a dialysis bag and dialyzed over night against two 1 l changes of citrate buffer-EDTA prepared as described above. The resulting slightly turbid solution was centrifuged in the Servall centrifuge for 1 h at 10 000 rpm. The supernatant solution was saturated with ammonium sulfate and allowed to stand for 20 h at 5°C. The precipitate which formed was collected by centrifugation, dissolved in a minimum volume of 0.05 M citrate buffer, pH 7.5 and dialyzed against the same buffer overnight. The purification procedure described above in general permits the recovery of about 50 % of the enzyme activity present in the original crude ammonium sulfate fraction. A more exact appraisal of the recoveries at each stage of purification will be presented in a later communication.

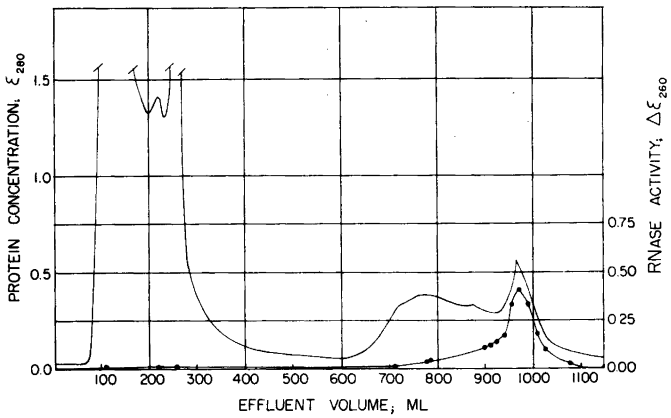
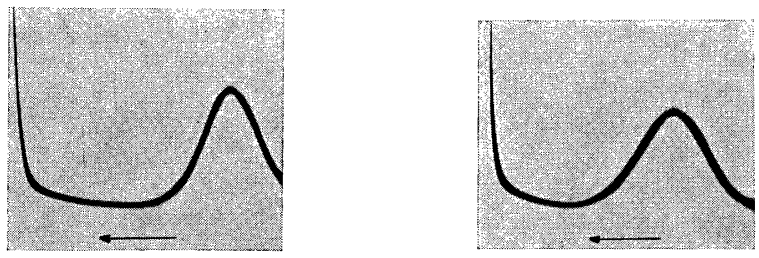


Fig. 1. Elution diagram from a preparative chromatographic purification, on CM-cellulose, of a crude, dialyzed ammonium sulfate precipitate. The precipitate, containing the nuclease activity, was prepared from the culture medium as described in the text, after removal of *Micrococcus pyogenes* cells. The bottom curve represents the levels of ribonuclease activity in the effluent fractions, and the upper curve the protein concentration as estimated by extinction measurements at 280 mμ.

RESULTS AND DISCUSSION

The enzyme, at this point of purification, appears to be essentially homogeneous and suitable for studies of structure and enzymatic specificity. Patterns obtained on such material in the Spinco Ultracentrifuge, Model E, are shown in Fig. 2. No traces of heavy components were seen during the early stages of centrifugation. The same preparation employed in the ultracentrifuge run shown in Fig. 2 was subjected to free boundary electrophoresis and the final pattern obtained after 20 h of migration is shown in Fig. 3. The schlieren diagram shows two small components in a total amount of perhaps 5 % of that of the major peak



a b

Fig. 2. The sedimentation behaviour of purified nuclease: a) 75 min after reaching final rotor speed, bar angle 55°, 21°C, 59 780 rpm; b) 114 min, bar angle 50°, 21°C. The protein was present at a concentration of 7 mg/ml, in a buffer which was 0.025 M sodium citrate, 0.05 M NaCl, pH 7.5. The arrows indicate the direction of sedimentation.

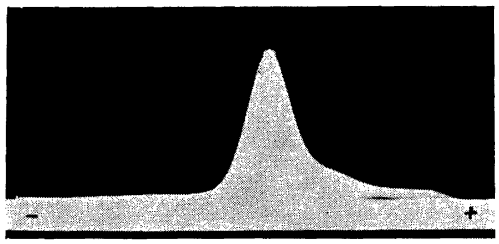


Fig. 3. Electrophoretic pattern obtained on the same preparation employed for the sedimentation experiment shown in Fig. 2. 0.05 M sodium citrate buffer, pH 7.5, 13 mA, 3.4 V/cm, 1200 min, protein concentration 6.5 mg/ml. A sample, withdrawn from the portion of the cell containing the two small components on the cathodic side, was tested for ribonuclease and deoxyribonuclease activity as summarized in Table 1.

and moving slightly toward the cathode. The major peak, itself, migrated only very slightly at the pH employed (citrate buffer, 0.05 M, pH 7.5) and is therefore essentially isoelectric at this pH value. A sample containing the small, more acidic components was removed at the end of the run and analyses for RNase and DNase activity are given in Table 1. This table also includes similar assays on the enzyme at various earlier stages of purification. The data show fairly convincingly that both activities are attributable to the same protein component, confirming the earlier impression of Alexander *et al.*[8]. The purified protein gives an entirely negative test for free SH groups by the method of Boyer[13], even at a level of 0.1 μmole per 2.5 ml. The absence of both cysteine residues and half-cystine residues is also shown by the results of amino acid analysis of hydrolysates of the protein after exposure to conditions of reduction and alkylation. Thus, no trace of S-carboxymethylcysteine was demonstrable nor was cystine itself detected. The amino acid analyses performed on the "reduced-alkylated" protein is summarized in Table 2. Since the protein is free of cysteine residues

Table 1. Relative activities of micrococcal nuclease against RNA and DNA at various levels of purification. RNase and DNase assays were performed on duplicate aliquots of each sample, which had been diluted with phosphate buffer (0.05 M, pH 6.1) to a suitable protein concentration.

	RNase activity $\Delta A_{260m\mu}$	DNase activity $\Delta A_{260m\mu}/15$	Activity ratio RNase/DNase
Dialyzed crude enzyme	0.123	0.032	3.84
After Ca++ precipitation	0.284	0.068	4.18
"Shoulder" from electrophoresis	0.090	0.030	3.00
Main sample from electrophoresis cell	0.314	0.081	3.87
Original sample for electrophoresis (see Fig. 3)	0.322	0.096	3.36

Table 2. Amino acid analyses of purified nuclease.

Amino acid	20 h hydrolysate		44 h hydrolysate		Average % of total μmoles	Assumed number of residues
	μmoles	% of total μmoles	μmoles	% of total μmoles		
Lysine	0.56	14.8	0.65	13.8	14.3	14
Histidine	0.08	2.1	0.08	1.7	1.9	2
Arginine	0.13	3.4	0.12	2.5	3.0	3
Aspartic acid	0.37	9.8	0.47	9.9	9.9	10
Threonine	0.25	6.6	0.30	6.3	6.5	7
Serine	0.12	3.2	0.14	3.0	3.1	3
Glutamic acid	0.47	12.4	0.60	12.7	12.6	13
Proline	0.17	4.5	0.23	4.9	4.7	5
Glycine	0.27	7.1	0.35	7.4	7.3	7
Alanine	0.35	9.2	0.45	9.5	9.4	9
Half Cystine	0		0		0	0
Valine	0.23	6.1	0.27	5.7	5.9	6
Methionine	0.08	2.1	0.06	1.3	(2.1)	(2)[a]
Isoleucine	0.12	3.2	0.17	3.6	3.4	4
Leucine	0.31	8.2	0.46	9.8	9.0	9
Tyrosine	0.19	5.0	0.25	5.3	5.2	5
Phenylalanine	0.09	2.4	0.11	2.3	2.4	2
Tryptophan						(1)[b]
Total μmoles	3.79		4.71			
Total residues						102[c]

[a] Loss of methionine is presumably due to inadequate evacuation of the hydrolysis tubes.
[b] Based on the presence of a single Ehrlich-positive peptide on "fingerprints".
[c] Assuming 2 moles of histidine/mole.

and disulfide bridges, the reduction-alkylation procedure is, in a sense, superfluous. The absence of disulfide bonds suggests that the calcium ions required for enzymatic activity may be involved in the three-dimensional stabilization of the polypeptide chain and experiments to test the role of calcium in the determination of tertiary structure are in progress. The enzyme contains approximately 100 amino acid residues/molecule on the basis of the amino acid analyses if one assumes the presence of two residues of histidine/molecule.

Peptide maps prepared on trypsin digests of the protein as described in the experimental section showed approximately 20 major peptide components and a few much less intensely staining ones. This number is roughly consistent with the analyses for lysine and arginine content. One of the peptides gave a positive test for tryptophan with the Ehrlich stain, and four were strongly positive for arginine, as indicated by spraying the maps with Sakaguchi reagent. Two other minor peptide components gave faint tests for arginine. The excellent separation

of peptides on the "fingerprints" and the absence of material at or near the origin indicate that the trypsin digest contains very few if any large components and should be ideally suited to sequence analysis.

Acknowledgements. The authors are very grateful to Dr. A. Yoshida of the National Institutes of Health for his invaluable assistance in supervising the growth of the organisms and the preliminary fractionations described in this paper, and for making the resulting RNase-rich fraction available to us. Dr. J. L. Bethune very generously performed the ultracentrifugal and electrophoretic analyses. We would like to thank Dr. C. Epstein for his assistance in some of the earlier chromatographic separations and Dr. D. MacD. Green for the sample of calf thymus DNA.

This research was supported in part from *United States Public Health Service* (Grant GM 10373-01).

REFERENCES

1. Cunningham, L., Catlin, B. W. and Privat de Garilhe, M. *J. Am. Chem. Soc.* **78** (1956) 4642.
2. Cunningham, L. *J. Am. Chem. Soc.* **80** (1958) 2546.
3. Cunningham, L. *Ann. N. Y. Acad. Sci.* **81** (1959) 788.
4. Reddi, K. K. *Nature* **182** (1958) 1308.
5. Reddi, K. K. *Biochim. Biophys. Acta* **36** (1959) 132.
6. Reddi, K. K. *Nature* **188** (1960) 60.
7. Privat de Garilhe, M., Fassina, G., Pochon, F. and Pillet, J. *Bull. Soc. Chim. Biol.* **40** (1958) 1905.
8. Alexander, M., Heppel, L. A. and Hurwitz, J. *J. Biol. Chem.* **236** (1961) 3014.
9. Gladstone, G. P. and van Heyningen, W. E. *Brit. J. Exptl. Pathol.* **38** (1957) 123.
10. Crestfield, A. M., Smith, K. C. and Allen, F. W. *J. Biol. Chem.* **216** (1956) 185.
11. Reichmann, M. E., Rice, S. A., Thomas, C. A. and Doty, P. *J. Am. Chem. Soc.* **76** (1954) 3047.
12. Åqvist, S. E. G. and Anfinsen, C. B. *J. Biol. Chem.* **234** (1959) 1112.
13. Boyer, P. E. *J. Am. Chem. Soc.* **76** (1954) 4331.
14. Anfinsen, C. B. and Haber, E. *J. Biol. Chem.* **236** (1961) 1361.
15. Spackman, D. H., Stein, W. H. and Moore, S. *Anal. Chem.* **30** (1958) 1190.
16. Smith, E. L., Stockell, A. and Kimmel, J. R. *J. Biol. Chem.* **207** (1954) 551.
17. Discussion by Neurath, H. in Sanger, F. *J. Polymer Sci.* **49** (1961) 3, p. 18.
18. Katz, A. M., Dreyer, W. J. and Anfinsen, C. B. *J. Biol. Chem.* **234** (1959) 2897.

Received April 5, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 277–S 281

Cross-Linking of Bovine Fibrinogen with Formaldehyde

ELEMER MIHALYI

Laboratory of Biochemistry, Section on Cellular Physiology, National Heart Institute, National Institutes of Health, Bethesda, Maryland, USA

The cross-linking of fibrinogen with formaldehyde has been investigated under a variety of conditions. The reaction proceeds in two steps. First, through an equilibrium reaction, a reactive hydroxymethyl-derivative is formed, which then polymerizes through a condensation reaction. The cross-link is formed between ε-amino and amide groups.

Cross-linking of globular, or fibrous proteins in the moist, semi-solid state has considerable technological importance and has been investigated extensively. Much less attention has been paid to the cross-linking of proteins in solution, with the resulting gel formation. In this paper some observations will be reported on the cross-linking of fibrinogen with formaldehyde in solution. Fibrinogen was chosen because of its gel-forming physiological function, and formaldehyde because of its great reactivity under extremely mild conditions. Some observations on this system have been presented already[1].

MATERIALS AND METHODS

Fibrinogen was purified from Armour's Bovine Fraction I by Laki's procedure[2]. Formaldehyde was Baker's Analyzed Reagent, 37 % solution, containing 10 % methanol as preservative. Gelation time was chosen as the point when flow of the reaction mixture in a test tube ceased. The opacity increase was followed in the Beckman DU-spectrophotometer at 600 mμ. The pH shift was recorded on a Leeds and Northrup Speedomax recorder coupled to a Vibron electrometer, Model 33 B.

Preparation of the formaldehyde-treated samples was performed in the following way. To 10 ml of 1 % fibrinogen in 0.3 M KCl, 1 ml of 1 M phosphate buffer of various pH and 1 ml of 10 % formaldehyde was added. At pH 6 no gelation occurred, at pH 7 and 8 a gel formed in 75 and 5 seconds, respectively. The mixtures were left at room temperature for 30 minutes and then dispersed in 5 volumes of ethanol. After centrifugation, the sediments were transferred into cellophane bags, dialyzed first against running distilled water for one day, and then against stationary water changed every day until no free formaldehyde could be detected in the outside solution with the chromotropic acid reagent (4–5 days)[3]. The protein was then suspended twice in ethanol, once in ether and finally dried in air and pulverized.

Estimation of free ε-amino groups was performed with the Sanger procedure[4] on the protein

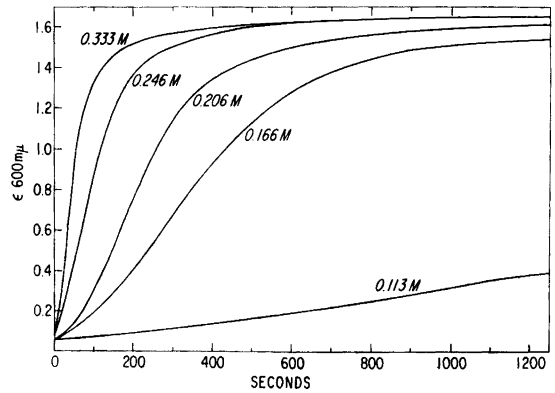


Fig. 1. Increase in optical density of fibrinogen solutions upon formaldehyde addition. Figures indicate formaldehyde molarities in each experiment. Fibrinogen 1.6 %.

powder suspended in water with $NaHCO_3$, or trimethylamine as buffer. The dinitrophenyl protein was hydrolyzed in a sealed vial with 6 N HCl for 18 hours at 115 °C. From the hydrolysate, the ether soluble DNP-amino acids were extracted; then the ε-DNP-lysine shaken out with n-butanol and estimated in the butanol solution at 360 mμ.

The amount of free formaldehyde in the preparation was determined with the chromotropic acid reagent[3], that of reversibly and irrevirsibly bound formaldehyde with the aid of ^{14}C-formaldehyde. The latter, of 99 % radiopurity, 3.8 mC per mM specific activity, was obtained from Isotopes Specialties Company, Burbank, California. In reaction mixtures as described above, a total of approximately 10 million c. p. m. was introduced. Counting was performed with the Packard Tri-Carb Scintillation counter. Reversibly bound formaldehyde was determined as described by Fraenkel-Conrat *et al.*[5]. The acidulated protein solution was distilled over twice in a dimedon solution and, after carrier formaldehyde was added, the precipitate was separated and counted. The total bound formaldehyde was estimated by counting in the scintillation counter, the protein dissolved in methanolic hyamin solution. Corrections for background counts and quenching were performed in the usual way.

RESULTS AND DISCUSSION

The polymerization process follows a sigmoidal curve as shown in Fig. 1. Gelation occurs in approximately twice the time necessary to attain half of the maximal opacity. At the gel point, approximately 80 % of the protein is involved in the network, and can be removed by high-speed centrifugation. Also, the opacity at the gel point is about 80 % of the final value. The reciprocal of the gel time, or the reciprocal of the time necessary to reach half of the final optical density can be used as a convenient measure of the rate of polymerization. In Fig 2, the logarithm of these quantities is plotted against the logarithm of formaldehyde concentration. The reaction mixtures had an ionic-strength of 0.6 and were buffered with 0.2 μ phosphate buffer to pH 7.19, so that the pH drift during the reaction amounted to less than 0.1 units.

Two experiments were performed, one with 0.8 % and the other with 1.6 % fibrinogen. The formaldehyde was in over 30-fold excess over the free amino groups even at its lowest concentration. The half-maximal opacity times are multiplied by two in order to bring the two sets of measurements together. The

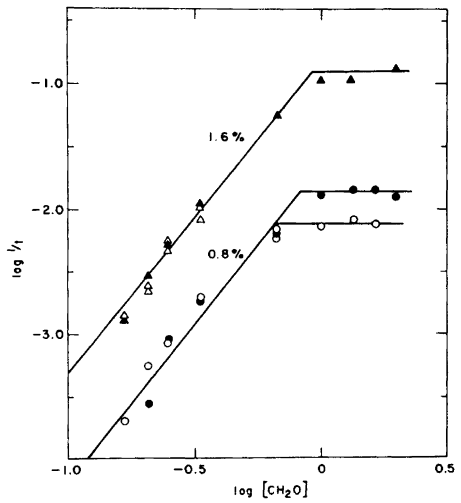


Fig. 2. Log reciprocal reaction time (gel time – solid symbols; 2 × half maximal optical density time – open symbols) plotted against log formaldehyde concentration. The two curves were obtained at the two fibrinogen concentrations indicated.

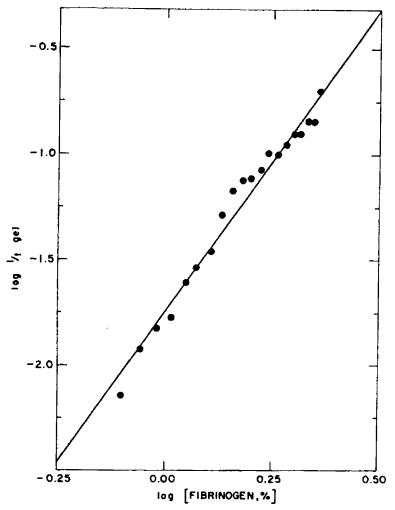


Fig. 3. Log reciprocal gel time plotted against log fibrinogen concentration. pH 7.19, μ 0.6, formaldehyde 0.5 M.

points fall reasonably close to a straight line up to a formaldehyde concentration of approximately 1 M. Above this, the rate seems to be independent of formaldehyde concentration. The slope of both lines, *i. e.*, the order of reaction with respect to formaldehyde, is 2.5 and their separation corresponds to a 7.25 fold increase in rate upon doubling the fibrinogen concentration. A more extended investigation of the dependence of rate on fibrinogen concentration is shown on Fig. 3. From the slope of the line, the reaction appears to be of 2.8 order with respect to fibrinogen in agreement with the value which can be calculated from the previous experiment. The empirical rate equation is therefore, $dx/dt = k' [F]^{2.8} \times [CH_2O]^{2.5}$, where k' is the apparent rate constant, [F] is the concentration of free fibrinogen and $[CH_2O]$ that of free formaldehyde.

The rate of reaction is also very sensitive to ionic strength. Below an ionic-strength of 0.1, polymerization does not occur, above this, the rate increases proportionally with the square-root of the ionic strength (Fig. 4). Such a relationship is expected for the interaction of charged macromolecules.

Formaldehyde reacts in one way or another with nearly all the reactive side-chains of a protein molecule[6]. Undoubtedly, the most important of these reactions is that with the free amino-groups because of its very fast rate and also the large number of groups involved in it. One, or at high formaldehyde concentrations, two moles can combine with one amino group giving a reactive mono- or di-hydroxymethyl derivative. In the concentration range of importance in this study only the monohydroxymethyl compound needs to be considered. The reaction appears to be completely reversible and involves a shift of the p*K* of the amino

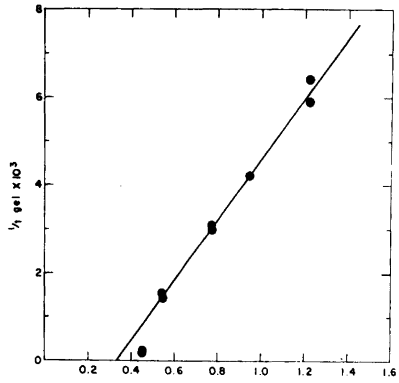


Fig. 4. Reciprocal gel time plotted against square root of ionic-strength. pH 7.19, fibrinogen 0.8 %, formaldehyde 0.33 M.

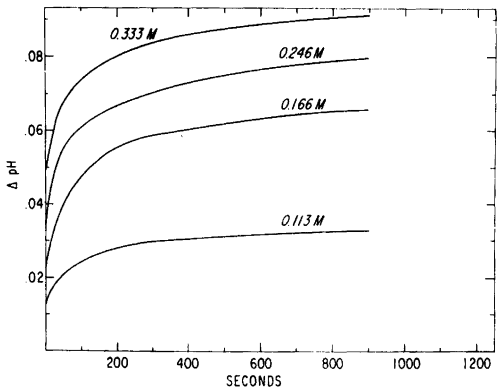


Fig. 5. pH shift on formaldehyde addition. Figures indicate molarities of formaldehyde in each experiment. pH 7.19, μ 0.6, fibrinogen 1.6 %.

group of some 3 units in an acidic direction. Thus, at the proper pH, the reaction can be easily followed by the amount of H^+ liberated, or the pH-shift. Since there are very few data of this nature on proteins, the kinetics of the reaction of formaldehyde with the amino groups of fibrinogen was investigated with the pH shift technique. Reaction with other groups is either too slow, or involves uncharged molecules and thus remains undetected by this method. The pH of the reaction was 7.19 and the ionic strength 0.6, with sufficient phosphate buffer added to limit the pH change to below 0.1 units. Figure 5 shows the recordings of pH shifts with 1.6 % fibrinogen on adding formaldehyde to the indicated molarities. It appears that the rates are not very much affected by the three-fold variation in formaldehyde concentration, but the equilibrium position the reaction finally reaches is very much so.

Comparison of Figs. 1 and 5 reveals that the polymerization process involves two steps. The first is the reversible binding of formaldehyde to the amino group. This leads to an equilibrium which is established fast relative to the polymerization. Thus, as an approximation, the concentration of molecules with bound formaldehyde can be expressed in terms of an association constant K and the concentrations of free fibrinogen and formaldehyde. In the second step, the polymerization of these molecules proceeds as a condensation reaction; consequently this step will be of second order with respect to the fibrinogen-formaldehyde compound. Combining the two steps and denoting by k the bimolecular reaction constant results in: $dx/dt = k \cdot K^2 [F]^2 [CH_2O]^2$. The experimentally found relationship has somewhat higher exponents, but shows nearly equal order with respect to both fibrinogen and formaldehyde, as required by the theory. The difference in the exponents may be explained by the fact that the theoretical equation does not take into account the side reactions which rapidly inactivate the reactive molecules. The effect of these will be more pronounced when the rate of polymerization is slow, than when it is fast, and may

Table 1. Free ε-amino groups and irreversibly bound formaldehyde, expressed as moles per 10^5 g in native and formaldehyde treated fibrinogen.

Material	Free ε-amino groups	Irreversibly bound formaldehyde	Remarks
Native fibrinogen	48.7 ± 1.9		No gel
Treated with CH_2O at pH 6	48.7 ± 0.7	2.7 ± 1.2	No gel
Treated with CH_2O at pH 7	41.2 ± 0.3		Gel
Treated with CH_2O at pH 8	37.3 ± 1.4	11.1 ± 1.8	Gel
Difference: Native – treated at pH 8	11.4 ± 3.3	11.1 ± 1.8	

accentuate in this way the concentration dependence of the rate, making it appear of an order higher than the actual one.

The number and nature of cross-links introduced was inferred from determinations of free ε-amino groups and irreversibly bound formaldehyde. As shown in Table 1, the decrease in the number of free ε-amino groups equals that of the irreversibly bound formaldehyde molecules. The bridge, therefore, cannot be between two ε-amino groups, as proposed earlier[1], but between one amino group and some other group. Estimates of the free tyrosine and tryphophan groups showed no change upon treatment with formaldehyde, but there was a decrease in the number of amide groups. Therefore, the cross-link is probably between the ε-amino and an amide group. Similar suggestions have been made by several authors[7,8]. The number of cross-links introduced is fairly large, up to 38 per molecule of fibrinogen (this may be an overestimate, because some of the links might be intramolecular). The large number suggests an intimate contact between the molecules, which could be achieved only by side-by-side aggregation. Thick bundles may be formed in this way, which could explain the large increase in turbidity which accompanies the cross-linking.

REFERENCES

1. Mihalyi, E. and Lorand, L. *Acta Physiol. Acad. Sci. Hung.* **1** (1948) 218.
2. Laki, K. *Arch. Biochem. Biophys.* **32** (1951) 317.
3. MacFadyen, D. A. *J. Biol. Chem.* **158** (1945) 107.
4. Sanger, F. *Biochem. J.* **39** (1945) 507.
5. Fraenkel-Conrat, H., Cooper, M. and Olcott, H. S. *J. Am. Chem. Soc.* **67** (1945) 950.
6. French, D. and Edsall, J. T. *Advan. Protein Chem.* **2** (1945) 277.
7. Nitschmann, H. and Hadorn, H. *Helv. Chim. Acta* **27** (1944) 299.
8. Fraenkel-Conrat, H. and Olcott, H. S. *J. Am. Chem. Soc.* **70** (1948) 2673.

Received March 23, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 282 – S 287

Isolation of Prostaglandin E_1 from Calf Thymus

Prostaglandins and Related Factors 20

SUNE BERGSTRÖM and BENGT SAMUELSSON

Department of Chemistry, Karolinska Institutet, Stockholm, Sweden

Lipid soluble, acidic material with smooth muscle stimulating activity has been extracted from calf thymus and separated by different chromatographic techniques. Prostaglandin E_1 was identified as the major biologically active component. The concentration of this factor, determined by isotope experiments was found to be 0.8 μg per g tissue.

The presence of a vasodepressor and smooth muscle stimulating activity in seminal plasma and in sheep vesicular gland was first reported by Goldblatt and von Euler[1,2]. The active principle was called prostaglandin by Euler, who demonstrated that it was due to a lipid soluble factor that Theorell with his electrophoresis apparatus showed to be acidic[3–6].

Two crystalline compounds, prostaglandin E_1 and $F_{1\alpha}$*, were later isolated from sheep vesicular gland by Bergström and Sjövall[7]. The structures of these compounds have been reported[8] (Fig. 1). Later, two new biologically active compounds, prostaglandin E_2 and E_3, were isolated from the same source[9]. The structure of prostaglandin E_2 has also been elucidated (Fig. 1). Recently, a biologically active reduction product of prostaglandin E_2, called prostaglandin $F_{2\alpha}$, was isolated in small amounts from lungs of sheep and pig[10] (Fig. 1). Furthermore, the same compound was later identified in extracts of sheep iris[11]. The present report is concerned with isolation of smooth-muscle stimulating lipid soluble material from calf thymus, which has led to the identification of prostaglandin E_1.

EXPERIMENTAL

Extraction. Calf thymus glands (10 kg) were collected immediately after slaughter and kept at −20°C until being processed. The glands were minced in a semi frozen condition and stirred

* Nomenclature: The systematic nomenclature is based on the trivial name prostanoic acid for the parent C_{20} acid numbered as shown in Fig. 1. Prostaglandin E_1 (earlier PGE): 11α, 15-dihydroxy-9-keto-prost-13-enoic acid. Prostaglandin E_2: 11α, 15-dihydroxy-9-keto-prosta-5, 13-dienoic acid. Prostaglandin $F_{1\alpha}$ (earlier PGF_1 of PGF_{1-1}): 9α, 11α, 15-trihydroxy-prost-13-enoic acid. Prostaglandin $F_{2\alpha}$ (earlier PGF_{1-2}): 9α, 11α, 15-trihydroxy-prosta-5, 13-dienoic acid.

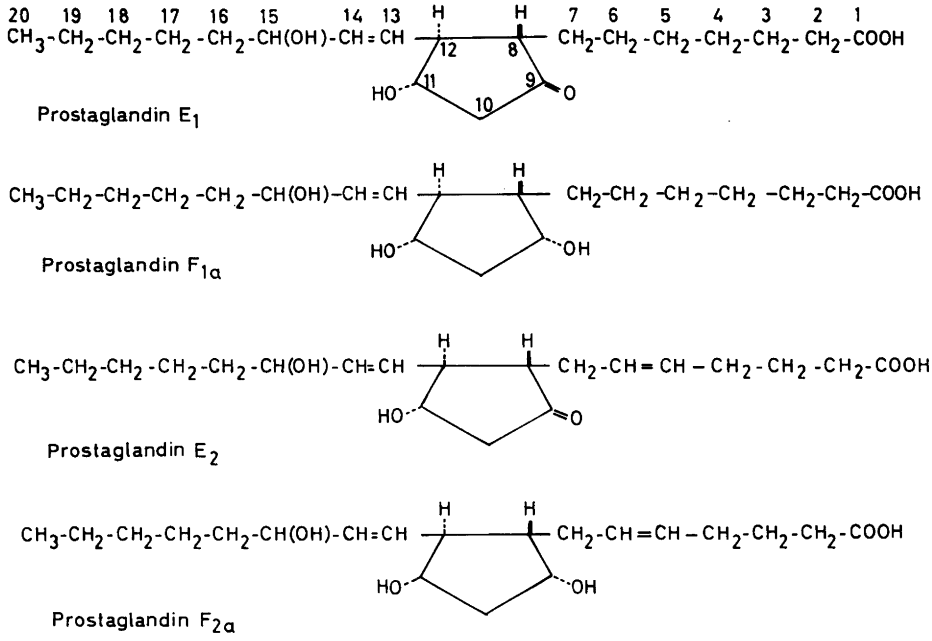


Fig. 1.

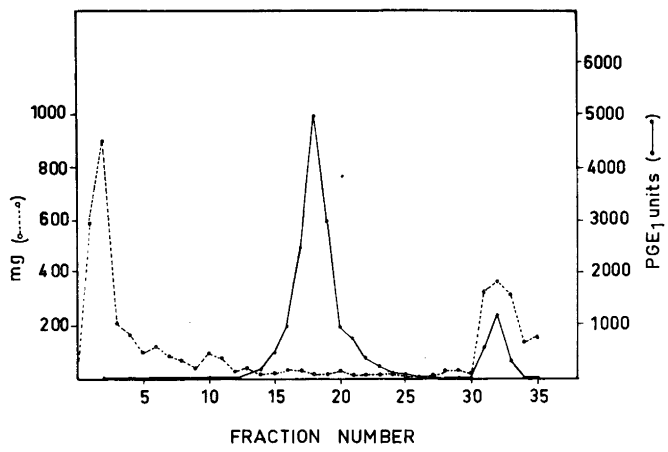


Fig. 2. Silicic acid chromatography of acidic lipids from calf thymus. Column: 550 g silicic acid. Fractions: 1600 ml. 1–10 Ethylacetate-hexane (30 : 70). 11–20 Ethylacetate-hexane (70 : 30). 21–30 Ethylacetate. 31–35 Methanol.

mechanically with 40 l of 96 % ethanol for 4 h. The suspension was filtered and the filtrate and washings concentrated by evaporation *in vacuo*. The resulting aqueous solution was acidified to pH 3 and extracted with ethylacetate. The combined ethyl acetate extracts were washed with a phosphate buffer pH 8 followed by acidification of the buffer to pH 3 and extraction with ethylacetate. The residue obtained after evaporation of the ethylacetate solution was subjected to a three stage distribution between equal volumes of petroleum ether and ethanol-water (2 : 1). The combined aqueous phases were evaporated to dryness.

The biological activities of the extracts were determined on duodenal strips of rabbits against a standard of prostaglandin E_1. (One unit of prostaglandin E_1 is equal to the effect of 1 μg of crystalline prostaglandin E_1).

Silicic acid chromatography. Material present in the aqueous ethanol extract (11 g) was separated on a column of 550 g silicic acid (Mallinckrodt, activated at 120°C (Fig. 2) as described previously[12].

Reversed phase partition chromatography. The residue (231 mg) obtained by evaporation of fractions 14–23 of the chromatography shown in Fig. 2 was separated by reversed phase partition chromatography (Fig. 3) on 45 g of hydrophobic SuperCel using the solvent system described earlier[9].

Thin layer chromatography (TLC). The biologically active material, purified by partition chromatography, was subjected to thin layer chromatography both on an analytical and preparative scale according to the procedure described by Green and Samuelsson[13].

RESULTS

Material obtained from calf thymus by ethanol extraction was shown in preliminary experiments to possess smooth-muscle stimulating activity. The crude extract from 10 kg of glands was subjected to fractionation (see experimental) into acidic and neutral lipids. By following the biological activity in all steps it was demonstrated that the smooth-muscle stimulating material was almost exclusively confined to the acidic fraction. This material could be further purified by distribution between petroleum ether and aqueous ethanol, which resulted in

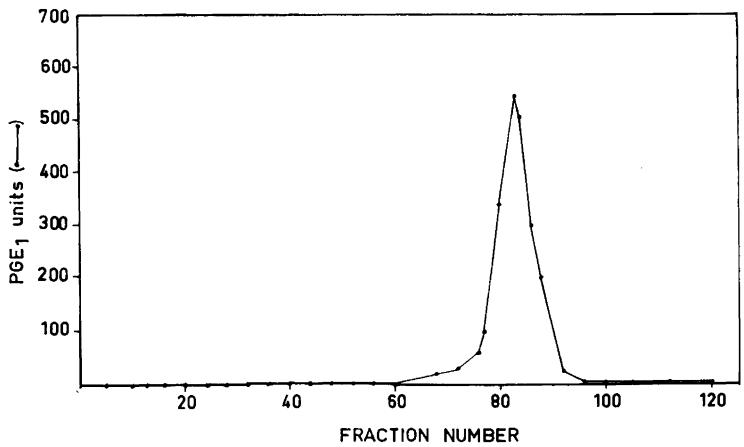


Fig. 3. Reversed phase partition chromatography of material eluted in fractions 14–23 of the chromatography shown in Fig. 2. Column: 45 g Hydrophobic Hyflo SuperCel. Fractions: 17 ml. Stationary phase: 40 ml isooctanol-chloroform (1 : 1). Moving phase: 47.5 % aqueous methanol.

removal of the bulk of relatively non-polar lipids whereas the biologically active material was completely recovered in the aqueous phase.

Material purified in this way was separated by silicic acid chromatography. The elution sequence applied has been worked out earlier for isolation of prostaglandins and offers the possibility of separating prostaglandins of the PGE series from those of the PGF series[12].

Smooth-muscle stimulating material was eluted with ethylacetate-hexane (70 : 30) and with ethylacetate in the same manner as would be expected for prostaglandins of the PGE type on this size of column. No PGF compounds were detected. Elution of the column with methanol gave a second peak with smooth-muscle stimulating material of more polar nature. The latter material has not been further characterized.

Reversed phase partition chromatography of fractions 14–23 from the silicic acid chromatography yielded one peak (Fig. 3) with a retention volume characteristic of prostaglandin E_1. This chromatogram also shows the absence of any of the other known prostaglandins, which would separate from PGE_1 under the conditions employed.

Thin layer chromatography of material present in 1300–1550 ml effluent of the chromatography in Fig. 3 showed one spot with the same R_F-value as PGE_1 and at least two spots with higher R_F-values. Purification by preparative TLC of part of the material from the reversed phase partition chromatography gave a semicrystalline substance, which was pure and identical with prostaglandin E_1 as judged by thin layer chromatography both of the methyl ester and the free acid. Determination of the biological activity showed within the errors of the method the same activity on a weight basis for the isolated compound and for crystalline prostaglandin E_1.

Compounds of the PGE series give rise to an UV absorption with λ_{max} at 278 mμ on treatment with 0.5 N sodium hydroxide at room temperature. This chromophore is due to a dienone formed by dehydration and isomerization of the double bond produced. Treatment of 40 μg of the isolated material with 0.5 N sodium hydroxide solution gave the same absorbancy at 278 mμ as 40 μg prostaglandin E_1 treated under identical conditions.

Further proof of the identity of the isolated compound with prostaglandin E_1 was obtained by mass spectrographic analyses of the methyl ester. This proved practically identical with that of the methyl ester of prostaglandin E_1.

A more accurate estimation of the amounts of prostaglandins in tissue extracts of this type has been greatly hampered by the sensitivity of the compounds and by the rather involved processes, which are necessary for their isolation.

However, by the availability of tritium labelled prostaglandin E_1[14], it was possible to follow the losses occurring during the isolation by isotope dilution experiments. In another experiment, carried out essentially as described above, tritium labelled prostaglandin E_1 (80 μg; 50×10^6 cpm/mg) was added at the beginning of the ethanol extraction. Determination of the total radioactivity coinciding with the peak of biological activity in the reversed phase partition chromatography showed a yield of approximately 42 per cent at this stage of

the purification. The total amount of prostaglandin E_1, based on determination of the biological activity of this material and corrected for the losses during isolation, was found to be 0.8 μg per g tissue (wet weight). Essentially the same figure was obtained when this procedure was repeated on material further purified by thin layer chromatography.

DISCUSSION

The present work demonstrated unequivocally the presence of prostaglandin E_1 in thymus. It is also evident that prostaglandin E_1 is the major prostaglandin present in the material examined, since the other known derivatives would have been detected by the methods applied. The amount of prostaglandin E_1 present in thymus was determined by adding tracer amounts of tritium labelled prostaglandin E_1 at the beginning of the isolation and determining the biological activity and radioactivity of isolated material. This method gave a concentration of 0.8 μg per g tissue (wet weight).

The isolation of prostaglandin E_1 from calf thymus affords a third example of the occurrence of prostaglandins in organs other than those primarily concerned with the genital functions. Recently, prostaglandin $F_{2\alpha}$, a reduction product of prostaglandin E_2 (Fig. 1), was isolated from lungs of sheep and pig[10] and later from sheep iris[11]. The concentration of prostaglandin in sheep lung, determined with the same technique as in these experiments, was found to be about 0.5 μg per g tissue[11]. Judging from the activity in crude fractions similar amounts are present in both male and female lungs of sheep, cattle and horse. As a comparison it might be mentioned that the concentration in human seminal plasma is considerably higher, *i. e.* the sum of the various prostaglandins present exceeded 50 μg per ml[12].

It must also be noticed that two of the species mentioned above in which prostaglandins have been found in lungs and thymus do not appear to have prostaglandins in their sperm or accessory genital glands.

Even if the prostaglandins might have an important function in the genital sphere of humans and sheep these facts indicate that prostaglandins might have a more general physiological function.

This contention is further supported by the very high biological activity shown by the different prostaglandins. Thus, the threshold dose of prostaglandin E_1 on rabbit duodenum is 0.003–0.01 μg per ml[15] and an infusion of 0.2–0.7 μg/kg/min of prostaglandin E_1 in humans causes a fall in blood pressure[16] and an increased heart rate and it can counteract in equimolar amounts the action of catecholamines on both blood pressure and release of free fatty acids[17].

Further work is needed to clarify the physiological role of this new group of hormonally active compounds that seems to be widely distributed in animal tissues.

This work is part of investigations supported by *Statens Medicinska Forskningsråd* and *Stiftelsen Therese och Johan Anderssons Minne.*

REFERENCES

1. Goldblatt, M. W. *Chem. & Ind. (London)* **52** (1933) 1056.
2. v. Euler, U. S. *Arch. Exptl. Pathol. Pharmakol.* **175** (1934) 78.
3. v. Euler, U. S. *J. Physiol. (London)* **84** (1935) 21P.
4. v. Euler, U. S. *J. Physiol. (London)* **88** (1936) 213.
5. v. Euler, U. S. *Skand. Arch. Physiol.* **77** (1937) 96.
6. v. Euler, U. S. *Skand. Arch. Physiol.* **81** (1939) 65.
7. Bergström, S. and Sjövall, J. *Acta Chem. Scand.* **14** (1960) 1693, 1701.
8. Bergström, S., Ryhage, R., Samuelsson, B. and Sjövall, J. *Acta Chem. Scand.* **16** (1962) 501.
9. Bergström, S., Dressler, F., Ryhage, R., Samuelsson, B. and Sjövall, J. *Arkiv Kemi* **19** (1962) 563.
10. Bergström, S., Dressler, F., Krabisch, L., Ryhage, R. and Sjövall, J. *Arkiv Kemi* **20** (1962) 63.
11. Samuelsson, B. and Änggård, E. *To be published.*
12. Samuelsson, B. *J. Biol. Chem. In press.*
13. Green, K. and Samuelsson, B. *To be published.*
14. Samuelsson, B. *To be published.*
15. Bergström, S., Eliasson, R., v. Euler, U. S. and Sjövall, J. *Acta Physiol. Scand.* **45** (1959) 133.
16. Bergström, S., Dunér, H., v. Euler, U. S., Pernow, B. and Sjövall, J. *Acta Physiol. Scand.* **45** (1959) 145.
17. Steinberg, D., Vaughan, M., Nestel, P. J. and Bergström, S. *Biochem. Pharmacol. In press.*

Received April 9, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 288–S 294

Enzymatic Synthesis of the Methyl Group of Methionine*

VI. The Catalytic Role of S-adenosylmethionine in the Enzyme System of *Escherichia coli*

SPENCER ROSENTHAL** and JOHN M. BUCHANAN

Division of Biochemistry, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA

The enzymatic components involved in the transfer of the methyl group of 5-methyltetrahydrofolate to homocysteine to yield tetrahydrofolate and methionine have been studied. An enzyme containing a derivative of vitamin B_{12} as a prosthetic group catalyzes this reaction in the bacterial system isolated from cells of a mutant (113-3) of *Escherichia coli* grown on vitamin B_{12}. The cofactors, reduced flavin adenine dinucleotide and S-adenosylmethionine, are required for this reaction and may be generated from appropriate precursors by enzymatic systems, FAD reductase and S-adenosylmethionine synthetase, present in crude preparations of the B_{12}-enzyme. A method has been described for the further purification of B_{12}-enzyme and its separation from these other enzymes.

S-adenosylmethionine does not act as an intermediate of this reaction but participates in a catalytic manner. S-adenosylhomocysteine does not replace homocysteine as a methyl acceptor in this reaction.

The biosynthesis of the methyl group of methionine from 5-methyltetrahydrofolate (Reaction 1),

5-methyltetrahydrofolate + homocysteine → tetrahydrofolate + methionine (1)

by an enzyme system isolated from *E. coli* strain 113-3 grown on vitamin B_{12} has been shown in Paper IV[1] of this series to require reduced pyridine dinucleotide, ATP, Mg^{++}, FAD, and an enzyme fraction that contains a derivative of vitamin B_{12} as a prosthetic group (B_{12}-enzyme).

The purification of the B_{12}-enzyme and the separation of a protein fraction (D fraction), which contains at least two enzymatic components, have been reported in prelimary communications. One of the components was shown to be an FAD reductase[2]. Further experiments suggested that the D fraction also contained a cofactor that is bound to protein and an enzyme that forms the cofactor from ATP[3]. Mangum and Scrimgeour[4] have studied Reaction 1 with

* Supported by grants-in-aid from the *National Science Foundation* and the *Nutrition Foundation.*

** Postdoctoral Fellow of the National Institutes of Health (1961–62).

the pig liver enzyme and have shown that S-adenosylmethionine may replace ATP in this system.

We have now shown that the cofactor synthesized from ATP by fraction D of the bacterial system is probably identical with S-adenosylmethionine. This paper reports experiments concerned with the role of S-adenosylmethionine in methionine synthesis in the bacterial system and presents evidence that the two enzymatic components referred to collectively as D fraction are in fact an FAD reductase and S-adenosylmethionine synthetase. A procedure for separation of these two enzyme activities from B_{12}enzyme is presented.

EXPERIMENTAL

Materials

Coenzymes, ATP, and S-adenosylmethionine were obtained from California Corporation for Biochemical Research and Pabst Laboratories, Inc. L-methionine labeled in the methyl group with ^{14}C was obtained from New England Nuclear Corporation and S-adenolsylmethionine-methyl-^{14}C was obtained from Tracerlab, Inc. S-adenolsylhomocysteine was kindly provided by Dr. W. Sakami and partially purified pig heart reductase by Dr. Thanos Evangelopoulos.

D,L-5-methyltetrahydrofolate was synthesized by the procedure of Keresztesy and Donaldson[5]. Only one of the stereoisomers is utilized by the enzymatic system. $FADH_2$ was prepared chemically by catalytic hydrogenation with 10 % palladium on charcoal.

Enzyme assays

FAD reductase. The reaction mixture contained in 4 ml: enzyme, FAD, 200 mμmoles, DPNH, 1 μmole, and phosphate buffer, 200 μmoles. The incubation was carried out at 25°C. The decrease in absorbancy at 450 mμ under anaerobic conditions was determined at 30 sec intervals. A unit of FAD reductase activity is defined as that quantity of enzyme that carries out the reduction of 100 mμmoles of FAD per min under the conditions of experiments described above.

S-adenosylmethionine synthetase. Synthesis of S-adenosylmethionine was determined by measurement of the incorporation of ^{14}C into S-adenosylmethionine from methionine-methyl-^{14}C when this substrate was incubated with ATP, 20 μmoles, $MgSO_4$, 100 μmoles, and KCN, 20 μmoles, in a volume of 0.2 ml. The S-adenosylmethionine was isolated by paper chromatography[6]. The spots on the paper corresponding to S-adenosylmethionine were cut out and after elution with 0.1 % acetic acid the radioactivity of the eluate was measured with a Nuclear-Chicago Internal Gas Flow Counter. In control experiments 71 to 73 % of ^{14}C labeled S-adenosylmethionine was recovered by this procedure. The amount of S-adenosylmethionine formed was linear with respect to time and enzyme concentration. A unit of S-adenosylmethionine synthetase activity is defined as that quantity of enzyme that catalyzes at 37°C the synthesis of 1 μμmole of S-adenosylmethionine per h under the conditions described above.

B_{12}-enzyme. The amount of B_{12}-enzyme was estimated by measurement of the radioactivity in the protein fractions isolated from extracts of *E. coli* 113-3 grown on ^{60}Co-labeled vitamin B_{12}. The arbitrary units of B_{12}-enzyme activity are proportional to the radioactivity and are expressed in terms of cpm per ml of enzyme solution. This enzymatic activity was also determined by measurement of the amount of methionine synthesized after a three h incubation with an excess of the necessary components of the system. Methionine synthesis was measured with a microbiological assay as described previously[7]. Details of incubation conditions are given in the legend of Table 2.

RESULTS

Separation of the enzymatic components required for Reaction 1. Purification of the B_{12}-enzyme was carried out to the calcium phosphate gel eluate stage[7]. Separation of the FAD reductase and S-adenosylmethionine synthetase from the B_{12}-enzyme was obtained by chromatography on two successive hydroxylapatite

columns. The protein solution eluted (320 ml, 600 mg protein) from the gel was equilibrated with 0.01 M phosphate buffer at pH 7.5 and applied to the first hydroxylapatite column (4.4 × 13 cm). To elute the protein from the column the phosphate buffer concentration was increased from 0.01 M to 0.02 and 0.03 M in a stepwise manner. A partial separation of the three enzymatic activities was obtained with this column as shown in Fig. 1. Protein fraction I, which elutes with 0.01 M buffer, contains small quantities of FAD reductase activity and S-adenosylmethionine synthetase activity. The first protein fraction that elutes with 0.03 M phosphate buffer (fraction II) contains most of the S-adenosylmethionine synthetase and small amounts of B_{12}-enzyme. The B_{12}-enzyme (cobalt labeled) elutes shortly afterwards as a narrow radioactive band and is contaminated with a small quantity of S-adenosylmethionine synthetase. Peak IV is a yellow fraction, which contains the FAD reductase activity. Each fraction was assayed in two ways, first for its individual activity, which is shown in Fig. 1, and secondly as a component of the methionine synthesizing system as described by Reaction I. Two different procedures were used for this latter assay system. The first contained FAD and DPNH and required FAD reductase for the demonstration of methionine synthesis from 5-methyltetrahydrofolate and homocysteine. In the second assay system $FADH_2$ replaced FAD reductase, FAD and DPNH. Since the FAD reductase activity could be replaced by either pig heart reductase or chemically prepared $FADH_2$, further studies were not made on the reductase fractions. Inspection of Table I shows that the B_{12}-enzyme fraction has no reductase activity, but that it contains some S-adenosylmethionine synthetase. A maximal rate of conversion of substrate to methionine was obtained when aliquots of the fraction corresponding to peak II in Fig. 1 were added to the assay system containing ATP, Mg^{++}, $FADH_2$ and B_{12}-enzyme (Table 1).

Table 1. Requirement for enzyme fractions in Reaction 1. Each vessel contained in 1 ml: phosphate buffer, pH 7.4, 50 μmoles; homocysteine, 5 μmoles; ATP, 0.4 μmoles; $MgSO_4$, 0.8 μmoles; D, L-5-methyltetrahydrofolate, 0.228 μmoles in experiment 1 and 0.456 μmoles in experiment 2; and, where indicated: FAD reductase, 25 μg (peak IV); S-adenolsylmethionine synthetase, 20 μg (peak II); and B_{12}-enzyme, 13 μg. Assay A contained FAD, 0.08 μmoles; DPNH, 1.1 μmoles and was carried out in an atmosphere of N_2. Assay B contained $FADH_2$, 0.2 μmoles, and was performed in an atmosphere of H_2.

Experiment	B_{12}-enzyme*	S-adenosyl-methionine synthetase	FAD reductase	Methionine formed	
				Assay A	Assay B
				mμmoles	
1	+			10	43
		+		4	10
			+	7	9
	+	+		24	100
	+		+	18	56
	+	+	+	107	106
2	+				10
		+			10
	+	+			184

* B_{12}-enzyme in experiment 1 was the effluent of the first chromatography on hydroxylapatite; in experiment 2, the effluent of the second chromatography on hydroxylapatite.

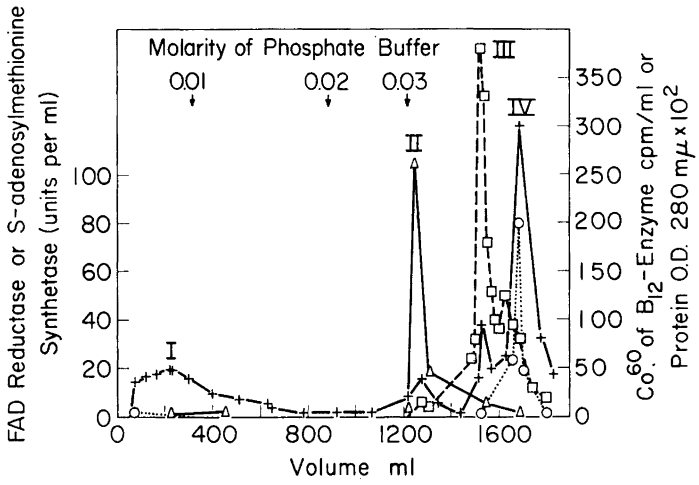


Fig. 1. First hydroxylapatite column (4.4 × 13 cm). 320 ml of protein solution were applied to the column and eluted successively with 600 ml of 0.01 M, 300 ml of 0.02 M, and 600 ml of 0.03 M phosphate buffer, pH 7.5. B_{12}-enzyme □, FAD reductase O, S-adenosylmethionine synthetase △, protein +.

In preliminary studies bacterial extracts were prepared by grinding the cells with alumina and by removal of nucleic acid with protamine sulfate according to Takeyama and Buchanan[8]. Under these conditions a single protein peak containing most of the S-adenosylmethionine synthetase and FAD reductase eluted from the first hydroxylapatite column with 0.01 M phosphate buffer. For this reason preliminary reports refer to D fraction rather than to the individual activities.

B_{12}-enzyme free of S-adenosylmethionine synthetase activity was obtained by rechromatography of fraction III (Fig. 1) on hydroxylapatite under conditions described in Fig. 2. It can be seen that the B_{12}-enzyme eluted from this column has a relatively constant ratio of radioactivity to absorbancy at 280 mμ. This material was free of S-adenosylmethionine synthetase activity.

Function of ATP in methionine biosynthesis. The demonstration that the D fraction contained two separate enzyme activities made it appear likely that one was concerned with ATP utilization. Attempts were made to find the products formed from ATP during methionine biosynthesis.

The crude B_{12}-enzyme (calcium phosphate gel eluate) contained sufficient S-adenosylmethionine synthetase and traces of methionine to catalyze the formation of limited amounts of S-adenosylmethionine now known to be required for the synthesis of methionine from 5-methyltetrahydrofolate and homocysteine.

The observation by Mangum and Scrimgeour[4] that S-adenosylmethionine acts catalytically in the pig liver enzymatic system, was confirmed in the *E. coli* system. The data in Table 2 show that S-adenosylmethionine can replace S-adenosylmethionine synthetase, ATP and Mg^{++}. An absolute requirement for methionine in the reaction was demonstrated by use of material eluted on the

Table 2. Requirement for S-adenosylmethionine in Reaction 1. All vessels contain in 1 ml: B_{12}-enzyme, 1.5 μg; homocysteine, 5 μmoles; D,L-5-methyltetrahydrofolate, 480 mμmoles; $FADH_2$, 0.4 μmoles; phosphate buffer, pH 7.4, 50 μmoles; and, where indicated: S-adenosylmethionine, 20 mμmoles; ATP, 100 mμmoles; Mg^{+2}, 1 μmole, methionine, 20 mμmoles. The vessels were incubated for 3 h under H_2.

Vessel	Additions	Methionine mμmoles
1	None	8
2	S-adenosylmethionine	231
3	ATP + Mg^{+2} + S-adenosylmethionine synthetase	8
4	ATP + Mg^{+2} + S-adenosylmethionine synthetase + methionine	169
5	ATP + Mg^{+2} + methionine	14
6	Methionine + S-adenosylmethionine synthetase	25
7	ATP + Mg^{+2} + S-adenosylmethionine synthetase + methionine	215

extreme right side of peak I of Fig. 1, an area of the chromatogram that contains some S-adenosylmethionine synthetase but is free of methionine.

Role of S-adenosylmethionine in the methyl transfer reaction. Although catalytic amounts of S-adenosylmethionine are required in the methyl transfer reaction, there is no significant conversion of S-adenosylmethionine to methionine either directly or by transfer of the methyl group to homocysteine. The inability of S-adenosylmethionine to serve as a precursor of methionine is best demonstrated in vessel 6 of Table 3 where the substrate, 5-methyltetrahydrofolate, is omitted from the incubation system. On chromatography of incubation mixtures that contained both 5-methyltetrahydrofolate and labeled S-adenosylmethionine (methyl ^{14}C) the primary source of methionine methyl was 5-methyltetrahydrofolate regardless of the ratio of the concentrations of the two substances. These experiments serve to demonstrate that S-adenosylmethionine is not an intermediate of the reactions of methionine biosynthesis.

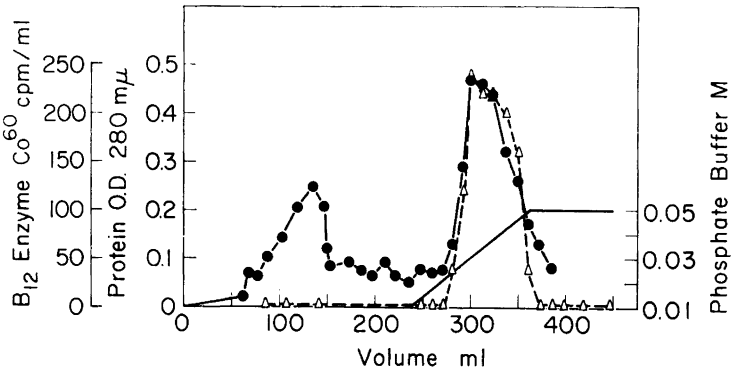


Fig. 2. Second hydroxylapatite column (1.0 × 10 cm). 106 ml of B_{12}-enzyme solution from peak III of Fig. 1, equilibrated with 0.01 M phosphate buffer, pH 7.5, were applied to the column and elution was carried out with 140 ml of 0.01 M phosphate buffer and then more concentrated phosphate buffer was applied as a linear concentration gradient varying from 0.01 M to 0.05 M (70 ml of each). B_{12}-enzyme △, protein •.

Table 3. Non participation of S-adenosylhomocysteine in methionine biosynthesis. All vessels contain in 1 ml: B_{12}-enzyme, 4.6 μg; phosphate buffer, pH 7.4, 50 μmoles; 2-mercaptoethanol, 50 μmoles; and $FADH_2$, 0.2 μmoles. The vessels were incubated for 3 h under H_2.

Vessels	S-adenosyl methionine	Homocysteine	D, L-5-methyl tetrahydrofolate	S-adenosyl homocysteine	Methionine synthesis
	μmoles	μmoles	μmoles	μmoles	mμmoles
1	0.02	5.0	0.73	–	287
2	0.02	5.0	0.73	2.0	264
3	0.02	–	0.73	2.0	0
4	–	5.0	0.73	2.0	2
5	–	5.0	0.73	0.02	6
6	0.50	5.0	–	–	5

Role of S-adenosylhomocysteine. In view of the interest in S-adenosylhomocysteine as a possible acceptor of methyl groups, we have carried out experiments to determine whether it participates in the reactions of our enzymatic system. As shown in Table 3, S-adenosylhomocysteine could not replace homocysteine as an acceptor of methyl groups from 5-methyltetrahydrofolate nor could it substitute for S-adenosylmethionine.

DISCUSSION

The data presented in the foregoing sections have demonstrated that the last step in methionine synthesis (Reaction I) in the bacterial system involves the participation of S-adenosylmethionine and a reducing agent such as $FADH_2$. One of these two essential components of the reaction may be generated from DPNH and FAD in the presence of a flavin reductase and the other from ATP and methionine in the presence of Mg^{++} and S-adenosylmethionine synthetase. Both of these enzymes are present in a protein fraction (D fraction) and may be separated from B_{12}-enzyme by chromatography on hydroxylapatite. We have been able to confirm in the bacterial system the earlier observation of Mangum and Scrimgeour[4] that S-adenosylmethionine is an essential component of the pig liver enzyme system.

In both instances S-adenosylmethionine participates in catalytic quantities. This compound is not an intermediate of the reaction or reactions concerned with the transfer of the methyl group of 5-methyltetrahydrofolate to homocysteine. Furthermore, when S-adenosylmethionine is added to the reaction system in substrate quantities it is not significantly converted to methionine, at least at a rate commensurate with the overall reaction. These experiments thus leave in question the role that S-adenosylmethionine is playing in the reaction. Since this essential agent is required only in catalytic quantities it is possible that it may be functioning by activation of B_{12}-enzyme in some as yet unknown manner. Activation by transfer of a methyl or adenosyl group to the B_{12} prosthetic group of the enzyme are two possibilities currently under investigation in this and other laboratories[9–11].

As a corollary to these experiments with S-adenosylmethionine, we have also found that S-adenosylhomocysteine does not function as a reactant of Reaction 1. Neither does it replace homocysteine as a methyl acceptor nor does it substitute

for S-adenosylmethionine as an "activator" of the enzymatic system. These data thus do not support the scheme of reactions proposed for methionine synthesis by Jaenicke[12] and his collaborators.

Recently a number of papers have appeared describing transfer reactions in microbial systems, involving S-adenosylhomocysteine and S-adenosylmethionine[13–15]. Evidence has been presented for the occurrence of the following reactions:

$$\text{S-adenosylmethionine} + \text{homocysteine} \xrightarrow{\text{methyl transfer}} \text{methionine} + \text{S-adenosylhomocysteine} \qquad (2)$$

$$\text{S-adenosylmethionine} + \text{homocysteine} \xrightarrow{\text{adenosyl transfer}} \text{methionine} + \text{S-adenosylhomocysteine} \qquad (3)$$

Reactions 2 and 3 appear in the same form but the metabolic origin of methionine carbon is different in the two processes. The suggestion has been made that S-adenosylhomocysteine (or a metabolic product) formed in the above reactions acts as a methyl acceptor for a C_1 unit, presumably the methyl group of 5-methyltetrahydrofolate (Reaction 4).

$$\text{S-adenosylhomocysteine} \xrightarrow{C_1 \text{ unit}} \text{S-adenosylmethionine} \qquad (4)$$

Although evidence for the existence of Reactions 2 and 3 is well documented, the connection of these reactions with methyl synthesis *de novo,* has not been established (Reaction 4). In view of the evidence to the contrary there seems to be little ground for the postulation of the participation of S-adenosylmethionine and S-adenosylhomocysteine as intermediates of methionine synthesis *de novo.*

Acknowledgment. The authors wish to acknowledge gratefully the aid of Dr. David Perlman of the Squibb Institute for Medical Research for the supply of *Escherichia coli* cells used in this study.

REFERENCES

1. Larrabee, A. R., Rosenthal, S., Cathou, R. E. and Buchanan, J. M. *J. Biol. Chem.* **238** (1963) 1025.
2. Rosenthal, S. and Buchanan, J. M. *Abstracts of papers of the 140th meeting of the American Chemical Society,* Chicago, 1961, p. 8 c.
3. Rosenthal, S. and Buchanan, J. M. *Federation Proc.* **21** (1962) 470.
4. Mangum, J. M. and Scrimgeour, K. G. *Federation Proc.* **21** (1962) 242.
5. Keresztesy, J. C. and Donaldson, K. O. *Biochem. Biophys. Res. Commun.* **5** (1961) 286.
6. Schlenk, F. and Depalma, R. E. *J. Biol. Chem.* **229** (1957) 1037.
7. Hatch, F. T., Larrabee, A. R., Cathou, R. E. and Buchanan, J. M. *J. Biol. Chem.* **236** (1961) 1095.
8. Takeyama, S. and Buchanan, J. M. *J. Biochem.* (Tokyo) **49** (1961) 578.
9. Guest, J. R., Friedman, S., Woods, D. D. and Smith, E. L. *Nature* **195** (1962) 340.
10. Weissbach, H., Redfield, B. and Peterkofsky, A. *J. Biol. Chem.* **236** (1961) PC 40.
11. Brady, R. O., Castanera, E. G. and Barker, H. A. *J. Biol. Chem.* **237** (1962) 2325.
12. Jaenicke, L. L. In Heinrich, H. C. *Proceedings of the 2nd European Symposium on Vitamin B_{12} and Intrinsic Factor,* F. Enke-Verlag, Stuttgart, 1962.
13. Duerre, J. A. and Schlenk, F. *Arch. Biochem. Biophys.* **69** (1962) 575.
14. Pfeffer, M. and Shapiro, S. K. *Biochem. Biophys. Res. Commun.* **9** (1962) 405.
15. Shapiro, S. K., Lohmar, P. and Hertenstein, M. *Arch. Biochem. Biophys.* **100** (1963) 74.

Received March 30, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 295–S 301

The Role of Sulfhydryl Groups in the Reactivation of Heat Inactivated Milk Alkaline Phosphatase

GORDON C. KRESHECK*

Department of Dairy Technology, The Ohio State University, Columbus, Ohio, USA; and Department of Biochemistry, Nobel Medical Institute, Stockholm, Sweden

A model system was developed for the study of the mechanism of reactivation of heat inactivated milk alkaline phosphatase. Sulfhydryl containing compounds were found to be effective but not compulsory components of the model system. The native and reactivated forms of the enzyme were not susceptible to N-ethylmaleimide (NEM) and *p*-chloromercuriphenylsulfonate (PCMS), whereas these reagents reacted with the heat inactivated enzyme so as to block the reactivation process. It appeared that a sulfhydryl group was activated by the heat treatment, but that this group disappeared during reactivation. This behaviour took place in a manner which was consistent with a mechanism other than reversible oxidation-reduction.

Upon certain conditions of storage, heat inactivated bovine milk alkaline phosphatase has been found to regain a portion of its original activity, and to give rise to an enzyme which cannot be distinguished from the native form. This process has been referred to as reactivation. A summary of the conditions related to this behaviour has been given by Lyster and Aschaffenburg[1]. The early theories of Wright and Tramer[2] do not include a consideration of the role of the sulfhydryl group, which appeared pertinent from the work of Hansson[3].

Therefore, this study was undertaken in an attempt to provide further insight into the mechanism of reactivation with special attention to the role of sulfhydryl. It seemed desirable to employ a model system for these studies, if possible, because the complexity of the whole milk system handicaps the interpretation of data. The model system used was developed independently and without knowledge of the simple system presented by Lyster and Aschaffenburg[1]. This might account for the differences between the composition of the two systems and the conclusions reached. However, many similar experiments were conducted and the results were in qualitative agreement.

* Present address: The Procter & Gamble Company, Miami Valley Laboratories, Cincinnati 39, Ohio.

EXPERIMENTAL

Materials

Milk. The milk used in this study was graciously provided by the Central Laboratory, Mjölkcentralen, Stockholm. The raw milk was of normal commercial grade without attention to source or special handling.

Milk alkaline phosphatase. Purified milk alkaline phosphatase was prepared according to the procedure of Morton[4]. The enzyme routinely used corresponded to that obtained by Morton at stage 6 of his purification, and was of comparable specific activity. Following dialysis against distilled water the material was lyophilized and stored at 4°C over P_2O_5. The brown gummy material which remained in the flask after drying was discarded. Unsuccessful attempts were made to further purify the enzyme by chemical fractionation and ion exchange chromatography. Although fractions were obtained by the latter technique with specific activities about three times greater than Morton's most active preparation, the quantity of material was limited so as to prevent its use for detailed reactivation studies.

Calf intestinal alkaline phosphatase. Highly purified calf intestinal alkaline phosphatase was kindly supplied by Dr. L. Engström, Institute of Medical Chemistry, University of Uppsala. The material had an activity of 172 A_{287}/A_{280}, this unit being previously reported[5].

Substrate. The substrate, *p*-nitrophenylphosphate, was prepared as described by Bessey and Lowe[6].

Procedures

Assay. The assay consisted of incubating 4.0 ml of 0.1 M ethanolamine-HCl buffer, pH 9.8[7], 0.5 ml of 0.016 M *p*-nitrophenylphosphate, and 0.5 ml of the test material at 34 ± 0.1°C for 30 min. The reaction was stopped by the addition of 1.0 ml 25 % trichloroacetic acid (TCA) or saturated sodium tungstate (in cases when turbidity was not removed with TCA) and filtered if one of the components of the system gave rise to a precipitate. To 2.0 ml of the clear solution was added 1.0 ml of 1.9 M Na_2CO_3, and the liberated *p*-nitrophenol was determined in a Beckman DU Spectrophotometer at 400 mμ. The amount of liberated *p*-nitrophenol was evaluated by comparison with a standard curve. Assays were performed in order to be within the limits of Beer's Law and to maintain zero order kinetics. The unit of activity is defined as the μmoles of *p*-nitrophenol released per 30 min incubation.

Heat shocking. The enzyme solutions were routinely heat inactivated by a technique to be referred to as *shocking*. In this case 1.0 ml of the test material was placed into a pyrex test tube (6 × 60 mm) and tightly sealed with a rubber stopper. The tube was placed into an ice bath for at least 60 sec to allow chilling. Then it was transferred to an oil bath maintained at 125 ± 0.5°C, held for 60 sec without shaking, and rapidly returned to the ice bath.

Incubation. The phosphatase was reactivated regularly by placing 0.5 ml of the appropriate mixture into a rubber stoppered test tube and maintaining at 34°C for 30 or 60 min. A control sample consisting of 0.5 ml of the same mixture was assayed directly without the incubation step. In each case, the buffer and substrate were added directly to this tube for the assay. The holding period at 34°C, which permitted reactivation, will be referred to as the *incubation period*, and the process as *incubation*.

RESULTS

The model system. The basic components of the model system consisted of enzyme, a metal salt (normally $MgCl_2$), an organic compound, buffer, and a salt solution. The variables of pH and temperature were optimized, the time of holding at various stages of reactivation investigated, the concentration dependency established, and the order of addition of the various components considered. The amount of enzyme used was 0.06 ml of a 0.2 % solution, and the total volume before heating was 1.0 ml. Normally, 0.2 ml of an appropriate salt solution was added following shocking. After mixing by inversion, two 0.5 ml aliquots were removed for assay and incubation.

pH. The effects of variations of pH were studied with the following buffer systems over the indicated range: 1) 0.1 M NaH_2PO_4 – Na_2HPO_4, pH 5.0 – 7.0, 2) 0.1 M succinate-borax and 0.1 M succinate – NaOH, pH 5.0 – 6.0, 3) 0.1 M HOAc – NaOAc, pH 3.5 – 5.5, 4) 0.1 M HCl – KCl, pH 1.1 – 1.9, and 5) 0.1 M potassium biphtalate – HCl, pH 2.2 – 4.0. Maximum reactivation was obtained with the use of pH 3.5, 0.1 M HOAc-NaOAc buffer. It was learned that the enzyme could be maintained at this low pH for about one hour (either before or after heat treatment) before adding the salt solution without serious loss of reactivation.

Temperature effects. Various intervals of holding at 110, 125, and 140°C were investigated for heat shocking the samples, and reactivation was observed at all three temperatures. However, 60 sec at 125°C was found to be optimum and was used thereafter.

Reactivation in a system consisting of enzyme, cysteine, and magnesium was also observed if the enzyme was inactivated by holding at 63°C for 30 min instead of shocking. Repeated shocking of either milk or model systems prior to incubation resulted in progressive losses in reactivation.

The optimum temperature for incubation was found to be 35 ± 1°C, and 34°C was used throughout this study as recommended by McFarren[8]. Holding simple systems at higher temperatures ranging from 50–95°C for 60 sec after shocking and prior to incubation promoted decreases in reactivation with the greatest losses occurring at the highest temperatures.

Metal. Reactivation was enhanced if the enzyme was incubated in the presence of $MgCl_2$ or $Mg(OAc)_2$ as expected from earlier results[1,8]. It was found that an optimum concentration of magnesium was required and inhibition resulted if this concentration was exceeded. The magnesium was involved in the reactivation process since it was not effective if added after incubation. Also, the magnesium could be removed by dialysis after incubation, without serious loss of reactivation. The concentration of $MgCl_2$ finally employed in the 1.0 ml mixtures was 0.03 ml of a 4.93 M solution.

The role of zinc was investigated in view of recent evidence which suggested that this metal is part of alkaline phosphatase of different origin. The influence of $ZnCl_2$ upon reactivation was studied over a range of 1×10^{-2} M to 1×10^{-8} M. It was found that the amount of reactivation influenced by zinc was related to the presence of cysteine, magnesium, and inorganic phosphate. In the absence of magnesium, zinc could promote reactivation in the presence, but not absence of cysteine. Greater reactivation occurred if inorganic phosphate was included. Optimum reactivation took place with approximately 1×10^{-5} M $ZnCl_2$, 2×10^{-3} M inorganic phosphate, and 6.52×10^{-5} M cysteine. Higher concentrations of all components were inhibitory, and the magnitude of reactivation obtained with magnesium was never obtained. When an inhibitory level of zinc was used, the inhibition could be overcome by adding sufficient amounts of cysteine. Zinc was more effective in promoting reactivation when added to the mixture before rather than after the shock treatment.

Phosphate. The proper concentration of either inorganic sodium phosphate or β-glycerophosphate was found to enhance reactivation. The buffering capacity of these components must be considered when considering their effect, *e. g.* 0.02 M β-glycerophosphate promoted a shift in the pH from pH 3.55 to 4.20 before adding the NaOAc and from 5.80 to 5.90 after adding the salt. For these studies, the enzyme was dissolved in a buffer consisting of 0.01 M $Mg(OAc)_2$ adjusted to pH 7.5 with Tris. In the presence of magnesium, cysteine, and 0.1 M, pH 3.5 HOAc-NaOAc buffer optimum reactivation occurred with 0.002 M phosphate, higher concentrations being inhibitory. The phosphate was more effective when added before rather than after shocking. Optimum reactivation occurred with the same basic system with 0.002 M β-glycerophosphate, higher concentrations up to 0.02 M being inhibitory.

Organic component. In early studies it was learned that β-lactoglobulin at a concentration of 0.25 % would promote reactivation. This component could be replaced by an equivalent concentration of either α-casein or whole casein, but β-casein was only slightly effective. It was observed that an optimum concentration of each component was required. Also, in studies with α-casein in the presence of β-lactoglobulin, optimum concentrations of each component were required when the level of the other was held constant.

Since α-casein but not β-casein was partially effective in replacing β-lactoglobulin, it was decided to try sulfhydryl containing compounds. Bovine serum albumin (0.25 %), glutathione (2.3×10^{-4} M), and cysteine (6.52×10^{-4} M) could all serve as replacements for β-lactoglobulin.

Again, inhibition occurred with higher concentrations when these components were included in the model system. Thus, it appeared that the sulfhydryl group may be a necessary component, especially since N-ethylmaleimide (NEM) was able to inhibit the process. However, it was found that the oxidized glutathione (G-S-S-G), at a concentration of 3.25×10^{-4} M yielded reactivation, and alanine at a concentration of 0.98×10^{-2} M was effective. Ethylenediamine tetraacetic acid (EDTA) at a concentration ranging from 2.69×10^{-4} M to 10.7×10^{-4} M and ascorbic acid (6.52×10^{-4} M) were not able to replace the organic constituents.

Salts. When milk was used as a supplement to the model system it was boiled to destroy the native phosphatase. When studying acid treatment as a means of replacing boiling, it was

Table 1. Comparison of the ability of sodium acetate and sodium propionate to promote reactivation of milk alkaline phosphatase.

Sodium acetate		Sodium propionate	
pH	Units of activity	pH	Units of activity
5.6	0.007	5.8	0.006
5.7	0.026	5.9	0.037
5.75	0.059	6.0	0.048
5.8	0.043	6.1	0.101

learned that NaOAc was an effective activator. Therefore, 0.2 ml of a saturated aqueous solution of the salt at room temperature was added to the 1.0 ml mixture after the 125°C heat shock treatment, and before the incubation period. Saturated aqueous solutions of other salts were tried and $(NH_4)OAc$ and sodium propionate were effective whereas NaCl and $(NH_4)_2SO_4$ were not stimulatory, and sodium formate only slightly so. Sodium propionate was found to be more effective than NaOAc in preliminary experiments, and this effect was studied more closely by adjusting the stock solutions of NaOAc and sodium propionate to various pH values with NaOH to determine if this was a pH effect. These results are presented in Table 1. It is apparent that the difference in the effectiveness of these substances is not entirely related to pH. To further test this possibility, mixtures in the absence of NaOAc were adjusted to various pH values ranging from pH 4.55 to 8.6 with Na_2CO_3, but reactivation was not observed.

Inhibitors. Several components were found to inhibit reactivation when used in the reactivation mixtures. The sulfhydryl blocking agents N-ethylmaleimide (NEM) and *p*-chloromercuriphenylsulfonate (PCMS) were effective when added either before or after shocking. The anionic detergent, sodium dodecyl sulfate (SDS) and non-ionic detergent, Emasol 4130, were used in an attempt to replace the organic constituent, but were inhibitory by themselves and in the presence of β-lactoglobulin. The concentration of detergents ranged from 0.024 to 0.38 %. On one occasion, difficulty was encountered by using excess $CHCl_3$ as a preservative agent in the buffer solution, and it was later found that 2.0 % of $CHCl_3$ was sufficient to completely inhibit reactivation when added to the mixture before shock treatment.

It was found that the inhibitory action of NEM could be overcome by the presence of cysteine, however, cysteine was more effective in this case if added prior to rather than after heating. This suggested that NEM may bbe competing for sulfhydryl originating from the enzyme. This thought was supported when NEM was added to the model system consisting of alanine in lieu of cysteine and reactivation took place in the absence but not presence of NEM. The concentration of NEM used in the presence of alanine, magnesium, and β-glycerophosphate which completely inhibited reactivation was 9.22×10^{-5} M. However, lower concentrations of NEM ranging from $9.22 \times 10^{-6} - 9.22 \times 10^{-8}$ were activating. When the reactivation process was stopped at various intervals up to 60 min by either 1) the addition of NEM (final concentration of 9.22×10^{-5} M) and continued holding at 34°C or 2) by chilling duplicates without NEM to 0°C, parallel increments of reactivation were observed.

Order of addition. The effect of adding the various components of the model system before and after the heat shock treatment was investigated. The concentration of the additives before the addition of 0.2 ml NaOAc was 0.005 M phosphate, 6.52×10^{-4} M cysteine, and 0.143 M $MgCl_2$. A summary of the results obtained is presented in Table 2. If a given component was not included prior to heat shocking, it was added after cooling and prior to adding NaOAc. Apparently, inorganic phosphate was most essential and $MgCl_2$ the least essential component for optimum reactivation.

Also, a comparison was made of the effect of adding NaOAc and $MgCl_2$ before and after heat shocking with a system consisting of enzyme, water, and 50 % skimmilk. With this system, maximum reactivation occourred when the $MgCl_2$ was added prior to shocking and NaOAc after shocking.

Table 2. Relative effectiveness of the components of the model system when included before or after heat shocking.*

Order of decreasing reactivation	Components present before shocking		
	$MgCl_2$	Phosphate	Cysteine
1	+	+	+
2	—	+	+
3	—	+	—
4	+	+	—
5	—	—	+
6	+	—	+
7	+	—	—

*Components missing before heat shocking were added after shocking.

Holding periods. The effect of holding the samples at various stages of reactivation for various times was investigated. It was found that the samples could be maintained at pH 3.5 (either before or after heat shocking) for about one hour, whereas marked losses of reactivation occurred if they were held two hours. After addition of the NaOAc, the samples could be held overnight prior to reactivation with a negligible effect.

Reactivation was found to increase with the time of holding at 34°C after heat shocking up to approximately 10 hr with the milk system and 8 hr with a model system consisting of enzyme, magnesium, β-lactoglobulin, buffer, and NaOAc. Upon longer periods of holding, the amount of reactivation decreased, presumably due to inactivation of the reactivated enzyme.

Comparison of boiled and LTLT treated milk. When the organic component was replaced by either milk which had been placed in boiling water for 5 min or by milk which had been maintained at 63°C for 30 min, reactivation occurred. However, as expected from earlier studies[1], greater reactivation took place with the system containing the boiled milk. When cysteine was added to both systems, the results presented in Table 3 were obtained. In each case, double optima occurred: with tubes 1 and 4 with the boiled samples and tubes 3 and 6 with the 63°C treated samples.

Table 3. Influence of the concentration of cysteine in model system containing boiled and 63°C/30 min (LTLT) treated milk on reactivation.

Tube No.	Added cysteine conc. (M)	% Reactivation with added milk	
		63°C/30 min	Boiled
1	0	51	100
2	3.26×10^{-5}	83	88
3	6.52×10^{-5}	86	98
4	9.78×10^{-5}	74	100
5	1.30×10^{-4}	77	91
6	3.26×10^{-4}	83	91
7	6.52×10^{-4}	74	73
8	9.78×10^{-4}	53	53
9	1.30×10^{-3}	30	32

Reversibility of urea treatment in the presence and absence of NEM. A duplicate series of samples was treated reversibly in urea solutions ranging from zero to 6.4 M urea, in the presence of NEM. The samples were maintained in an ice bath in the presence of urea for 5 min, and then diluted 1:25 with buffer and substrate for assay. No difference between the level of reactivation was noted with these samples, contrary to what might be anticipated if sulfhydryl was liberated by the urea treatment. Only 80 % of the original activity was recovered in the case of the sample treated with 6.4 M urea, whereas only a slight loss of activity was observed with 4.8 M and lower concentrations of urea.

Calf intestinal alkaline phosphatase. Attempts were made to substitute highly purified calf intestinal alkaline phosphatase for milk alkaline phosphatase in the model system, but reactivation did not occur. Nearly five times more intestinal phosphatase was used than the amount of milk phosphatase normally employed (in terms of *p*-nitrophenol liberated per unit time). To insure that the milk phosphatase dit not contain an unidentified activator, both enzymes were treated together, but reactivation occurred to the same extent as when the milk enzyme was used alone.

In a related experiment, the highly purified milk alkaline phosphatase which was obtained from DEAE chromatography, was used in the model system, and reactivation was observed to the same extent as with the partially purified material normally employed.

DISCUSSION

Detailed discussion of the relationship between reactivation and treatment of various milk fractions, will be presented in another publication[9]. The purpose of this presentation was to show how the results obtained with the model system may be related to the behavior of the complex milk system with special reference to the role of sulfhydryl.

From the results of this investigation and of others[1], the lack of specificity of the components of the model system is supported. The role of metals in the mechanism of action of the enzyme has not been elucidated and it is not possible to define their function in the model system. Although zinc has been related to the active site of phosphatase[10], magnesium was a more effective component of the model system. Inorganic phosphate has been found to incorporate into intestinal alkaline phosphatases[11], and its protective action in reactivation could be similar to that of the substrate, β-glycerophosphate. No buffer ion specificity directly related to the reactivation process was observed. It would appear that a specific chelating agent is required for reactivation with the systems employed. Heavy metal contamination could be involved, since it would be expected that enzyme derived sulfhydryl would be susceptible to heavy metals. Apparently, EDTA competes for a metal which is essential for activity, presumably zinc. Although sulfhydryl is an effective agent promoting reactivation, its replacement by alanine indicates that there is not a specific sulfhydryl requirement. The fact that both GSH and G-S-S-G permitted reactivation suggests that the O/R potential of the system is not important for reactivation. Ascorbic acid did not allow reactivation when present in the absence of cysteine, however, reactivation took place if cysteine was included. Also, the low pH of 5.8 used for incubation is not favorable for the oxidation of sulfhydryl. In view of these observations, the release and disappearance of sulfhydryl does not seem to take place by reversible oxidation-reduction in a manner characteristic of other proteins, *e. g.*[12]. The fact that the substrate, β-glycerophosphate, does not protect the heat-inactivated form of the enzyme in the presence of NEM suggests that the liberated sulfhydryl group is removed from the active site. The function of the salt added after heat shocking is not known, and one can only speculate. Apparently, there is some specificity

involved which is related to a function other than pH or ionic-strength and it is tempting to suggest that the salt influences hydrophobic bonding between the enzyme and solvent. The reactivation mechanism may be summarized by the equation:

$$\text{Native enzyme} \xrightarrow[\text{Heat Shock}]{Mg^{2+},\ \text{alanine, pH 3.5, 0.1 M NaOAc–HOAc}} \text{Inactive enzyme-SH} \xrightarrow[34°C/30\ \text{min}]{\text{NaOAc}} \text{Active enzyme}$$

Acknowledgment. This investigation has been aided by a grant from the *U. S. National Institutes of Health.* The advice and support given by Prof. H. Theorell and his staff are gratefully acknowledged.

REFERENCES

1. Lyster, R. L. J. and Aschaffenburg, R. *J. Dairy Res.* **29** (1962) 21.
2. Wright, R. C. and Tramer, J. *J. Dairy Res.* **20** (1953) 258.
3. Hansson, E. *Svenska Mejeritidn.* **53** (1961) 183.
4. Morton, R. K. *Biochem. J.* **55** (1953) 795.
5. Engström, L. *Biochim. Biophys. Acta* **52** (1961) 36.
6. Bessey, O. A. and Love, H. H. *J. Biol. Chem.* **196** (1952) 175.
7. Zittle, C. A. and Della Monica, E. S. *Arch. Biochem.* **26** (1950) 112.
8. McFarren, E. F., Thomas, R. C., Black, L. A. and Campbell, J. E. *J. Assoc. Offic. Agr. Chemists* **43** (1960) 414.
9. Kresheck, G. C. and Harper, W. J. *In preparation.*
10. Plocke, D. J., Levinthal, C. and Vallee, B. L. *Biochem. J.* **1** (1962) 383.
11. Engström, L. *Biochim. et Biophys. Acta* **52** (1961) 49.
12. White, F. H. Jr. *J. Biol. Chem.* **235** (1960) 383.

Received March 25, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 302–S 307

Die Isolierung eines kristallinen Antithiaminwirkstoffes aus Karpfeneingeweide

J. C. SOMOGYI und H. KÜNDIG

Institut für Ernährungsforschung, Rüschlikon-Zürich, Schweiz

Eine Methode zur Isolierung eines kristallinen Antithiaminwirkstoffes aus Karpfeneingeweide wird beschrieben. Die Rohkristalle sind dunkelbraun, prismenförmig und leuchten im polarisierten Licht auf. Sie weisen folgende Zusammensetzung auf: C 60,41 %; H 8,69 %; O 24,52 %; N 7,15 %. Zersetzungspunkt: 250–270°C. Die Rohkristalle sind im Gegensatz zum Ausgangsmaterial (Rohextrakt, Dialysat) thermostabil. Die I. R.- und U. V.-Spektren, sowie andere charakteristische Eigenschaften der Substanz werden mitgeteilt. Auf das Auffinden eines zweiten, noch nicht weiter untersuchten Antithiaminwirkstoffes aus Karpfeneingeweide wird hingewiesen.

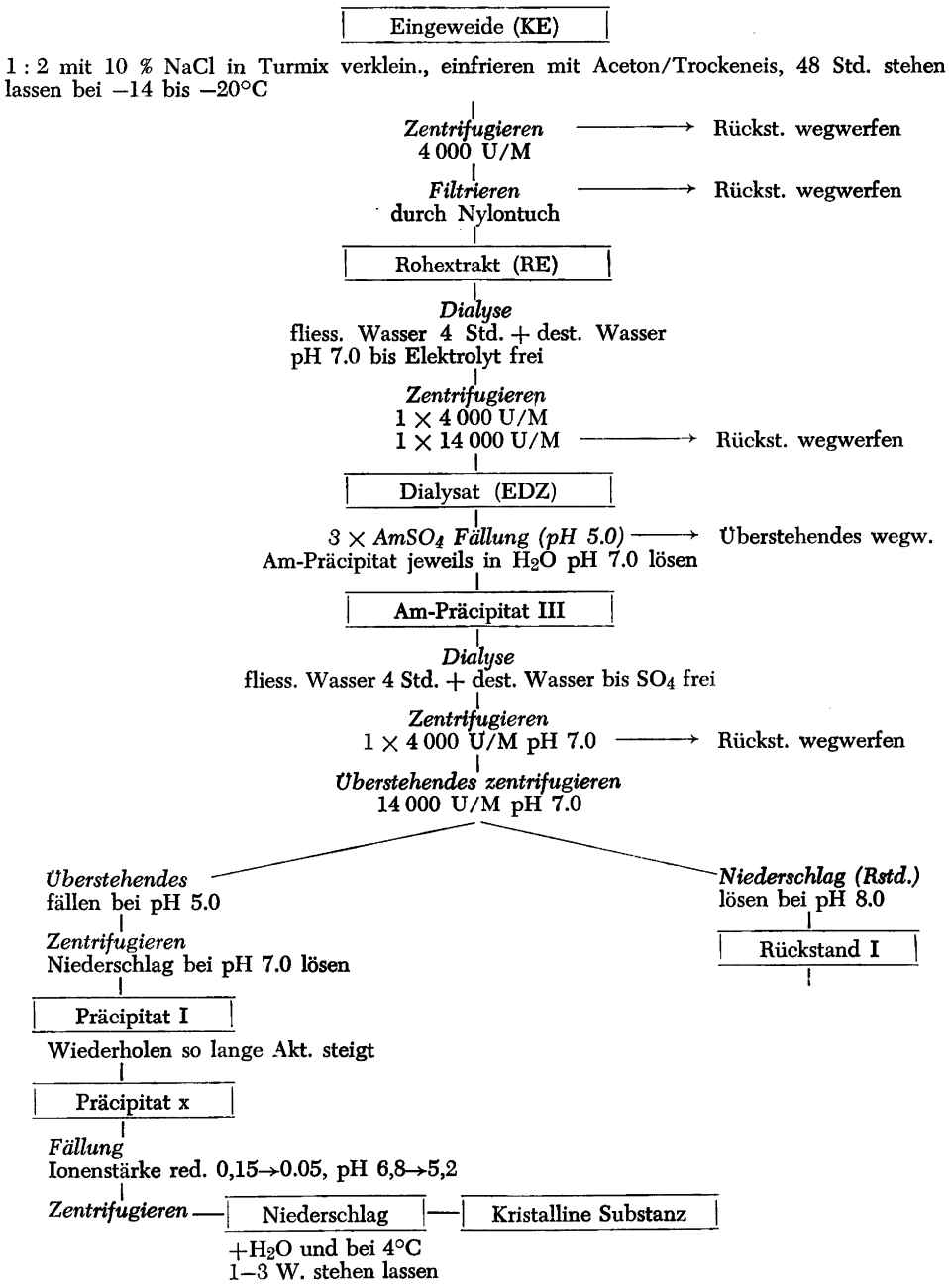
Unsere früheren Versuche[1–3] haben gezeigt, dass die im Rohextrakt und in einem durch Dialyse gereinigten Extrakt enthaltenen Antithiaminwirkstoffe aus Karpfeneingeweide thermolabil und nicht dialysierbar sind. Im elektrischen Feld wandern sie teilweise kathodisch, teilweise verhalten sie sich elektroneutral. Später haben wir[4] eine weitere Reinigung eines Antithiaminwirkstoffes durch wiederholte Fällungen mit Ammoniumsulfat ($AmSO_4$) und beim isoelektrischen Punkt erzielt. In einigen Fällen konnte eine kristalline Substanz isoliert werden. Der genaue Vorgang, welcher zur Bildung dieser Kristalle führt, blieb damals jedoch unabgeklärt.

Wir haben darum vor ca. zwei Jahren diese Versuche wieder aufgenommen. Es gelang, die Reinigungsmethode so weit zu entwickeln, dass der Wirkstoff nunmehr praktisch ausnahmslos als kristalline Substanz erhalten werden kann. Ferner hat sich gezeigt, dass das Karpfeneingeweide, wie früher vermutet, einen zweiten Antithiaminfaktor mit anderen chemischen und physicochemischen Eigenschaften enthält.

METHODIK

Die Bestimmung des Thiamins erfolgte mit der Thiochrommethode und mit Hilfe eines von uns beschriebenen Fluorimeters[5]. Um zu vergleichbaren Ergebnissen zu gelangen, wurde die Antithiaminaktivität verschiedener Reinigungsstufen mit einem modifizierten 50 %-Inaktivierungstest bestimmt und die Aktivität in γ inaktiviertem Thiamin/mg Trockensubstanz bzw. Eiweiss ausgedrückt. Bei dieser Methode wird diejenige Menge eines Antithiaminpräparates

Tabelle 1. Schema der Isolierung eines Antithiaminwirkstoffes aus Karpfeneingeweide. Alle Operationen bei der Reinigung erfolgten, falls nichts anderes vermerkt, bei ca. +4°C.



festgestellt, die bei einer Vorlage von 100 γ Thiamin/20 ml Versuchsgemisch 50 γ zu inaktivieren vermag. Daraus wird berechnet, wieviel γ Thiamin durch 1 mg Substanz inaktiviert wird.

VERSUCHE

Der Reinigungsvorgang ist schematisch in Tab. 1 zusammengefasst. Alle Operationen erfolgten, falls nichts anderes vermerkt, bei ca. + 4°C.

Der Rohextrakt des Karpfeneingeweides (Herz, Niere, Milz und Leber) wird dialysiert, zentrifugiert und anschliessend dreimal mit Ammoniumsulfat gefällt. Das Präparat wird bis zur völligen Freiheit von Sulfat dialysiert; anschliessend erst bei einer Tourenzahl von 4 000 und dann bei 14 000 U/Min. bei 4°C zentrifugiert. Es bildet sich beim hochtourigen Zentrifugieren ein Niederschlag (Bezeichnung: Rückstand), der eine mittlere Antithiaminaktivität, jedoch andere chemische bzw. physicochemische Eigenschaften aufweist als die nachfolgend beschriebene Substanz (Präcipitat).

Der Niederschlag (Rückst.) wandert im elektrischen Feld nämlich bei pH 8.6 kathodisch (das Präcipitat wandert nicht), weist einen N-Gehalt von 4,6 % (Präcipitat 7,1 % N) auf, ist in Wasser erst bei pH 8 und darüber löslich (das Präcipitat löst sich schon bei pH 7,0). Die Isolierung und weitere Aufklärung der chemischen Natur dieses *zweiten Antithiaminwirkstoffes* soll später durchgeführt werden.

Der grössere Teil der Antithiaminaktivität ist in der überstehenden Lösung enthalten. Diese wird nun beim isoelektrischen Punkt (pH 5.0) gefällt. Es entsteht ein Präcipitat, welches nach Zentrifugieren bei pH 7.0 wieder gelöst wird. Dieser Vorgang wird so oft wiederholt bis die Aktivität des Präcipitats nicht mehr steigt. Auf diese Weise gelangt man zu einem Präparat, das eine 60–90 mal grössere Antithiaminaktivität aufweist (auf Trockengewicht bezogen) als der Rohextrakt.

Bei unseren früheren Versuchen ist der Wirkstoff aus der aktivsten Fraktion in 2–3 Fällen auskristallisiert, aber die Bedingungen, die zur Kristallisation der Substanz führten, blieben – wie erwähnt – unbekannt.

Es wurde hierauf auf verschiedene Weise versucht, die Kristalle zu erhalten. So z. B. durch weitere Reinigung des Präcipitates mit Hilfe einer DEAE Ionenaustauscher-Zellulosekolonne gemäss *Sober* und Mitarbeiter[6–8], durch Fällung des aktivsten Präcipitates mit Ammoniumsulfat usw. Keine dieser Methoden hat die gewünschten Ergebnisse gezeitigt.

Zum Erfolg führte eine sog. "Euglobulin-Fällung" der aktivsten Fraktion. Bei der Dialyse fällt im Dialysenschlauch, der das gelöste Präcipitat enthält, durch sukzessive Verringerung der Ionenkonzentration und gleichzeitiges Ansäuren der Aussenflüssigkeit ein Niederschlag aus. Dieser wird zentrifugiert und nach Zugabe von Wasser in einem Uhrglas in hochkonzentrierter Form bei 4°C stehen gelassen. Nach 1–3 Wochen Aufbewahrung im Kühlschrank fallen die Antithiaminwirkstoff-Kristalle aus. Die Ausbeute ist gering. Sie variiert je nach der Aktivität des Präcipitates zwischen 8–25 %.

Die Erhöhung der Antithiaminaktivität während dieses Reinigungsvorgangs ist aus der folgenden Abbildung (Abb. 1) ersichtlich. Wie daraus zu entnehmen ist, weist die kristalline Substanz eine ca. 100 – 200 mal grössere Antithiaminaktivität als der Rohextrakt auf.

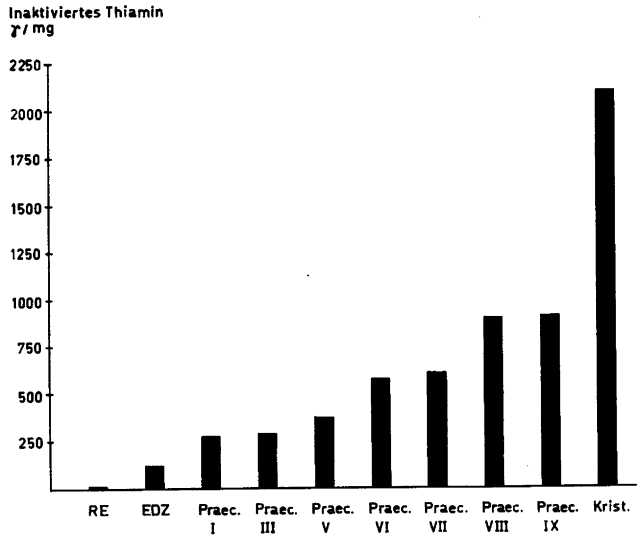


Abb. 1. Die Antithiaminaktivität verschiedener Reinigungsstufen. RE = Rohextrakt, EDZ = Dialysat, Praec. = Praecipitat, Krist. = Rohkristalle (s. auch Tab. 1).

Die Kristalle sind dunkelbraun, prismenförmig und leuchten im polarisierten Licht auf. (Abb. 2). Sie sind im Wasser (bei pH 7 und darüber), in Na_2CO_3, in 10 N Ameisensäure, Pyridin, Aethanolamin usw. löslich, in Aethylalkohol, Aether, Aceton unlöslich. Zersetzungspunkt: 260–270°C.

Die Kristalle sind im Gegensatz zu Ausgangsmaterial (Rohextrakt, Dialysat) *thermostabil.* Erhitzen auf 100°C während 1 Std. verringert die Antithiaminaktivität nicht. Sie sind nicht dialysierbar. Die Mikroanalysen der Rohkristalle ergaben folgendes Resultat: C 60,41 %; H 8,69 %; O 24,52 %; N 7,15 % auf aschenfreie Substanz berechnet. Der Wirkstoff ist frei von Schwefel, Phosphor, Jod, Brom Kobalt, Schwermetallen und von reduzierendem und nicht reduzierendem Zucker. Die Substanz enthält Chlorid und gibt positive Phenolreaktionen.

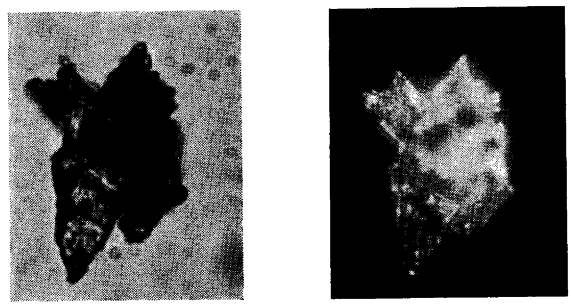


Abb. 2. Kristalle des Antithiaminwirkstoffes links in gewöhnlichem und rechts in polarisiertem Licht.

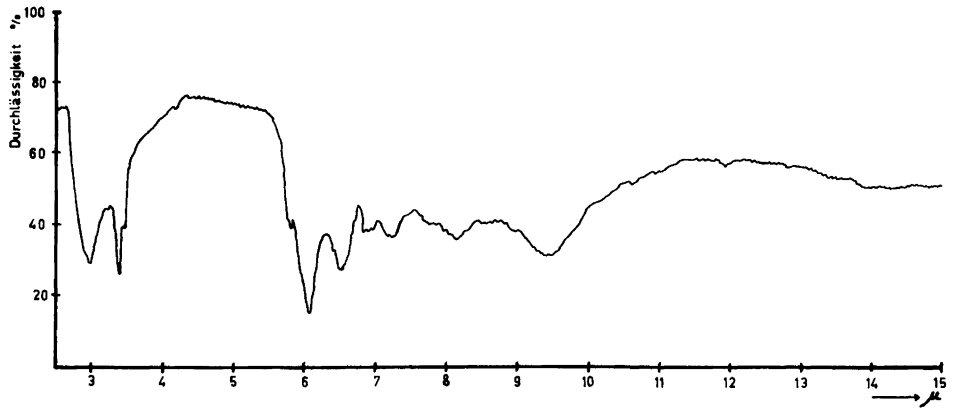


Abb. 3. I. R.-Spektrum des kristallinen Antithiaminwirkstoffes.

Eine Umkristallisation dieser Rohkristalle konnte bis anhin nur in einigen Fällen durchgeführt werden. Die Antithiaminaktivität, Form und Farbe der Kristalle, sowie der Zersetzungspunkt blieb unverändert.

Wie aus der Mikroanalyse hervorgeht, enthält die Substanz nur 7,15 % N. Daraus folgt, dass dieser Antithiaminwirkstoff kein typisches Eiweiss ist. Es kann sich aber auch nicht um ein Glykosid handeln, da weder in der Substanz, noch im Hydrolysat desselben reduzierende bzw. nicht reduzierende Zucker nachweisbar sind. Die Verbindung muss jedoch ein grösseres Molekül sein, da sie die Dialysenmembrane nicht passiert.

Sowohl die Mikroanalysen, wie auch die mehrmals aufgenommenen I.R.-Spektren und die praktisch immer gleich grosse Antithiaminaktivität der Kristalle (2200 – 2400 γ/mg Trockengewicht) zeigen, dass die beschriebene Reinigungsmethode ein reproduzierbares Präparat ergibt.

Gemäss I.R.-Spektrum (Abb. 3) handelt es sich bei den Rohkristallen um eine weitgehend einheitliche Substanz, die eine oder mehrere Amidgruppen, sekundäre und tertiäre OH-Gruppen ($>CH_2$ +– $\underset{|}{CH}$) und eventuell phenolische OH-Gruppen enthält.

Zum Schluss soll noch erwähnt werden, dass das U. V.-Spektrum der Rohkristalle (gelöst im Wasser, pH 7,0) ein deutliches Maximum bei 398 mμ aufweist.

Die weite Aufklärung der chemischen Konstitution dieser Substanz ist in Arbeit.

Dem *Schweizerischen Nationalfonds für wissenschaftliche Forschung* möchten wir für die gewährte Unterstützung unseren besten Dank aussprechen.

Herrn P. D. Dr. A. Dreiding, der die Aufnahmen der I. R.-Spektra im Organisch-Chemischen Institut der Universität Zürich veranlasst hat, danken wir bestens. Die Mikroanalysen wurden von Herrn E. Thommen, Organisch-Chemische Anstalt der Universität Basel, durchgeführt.

LITERATUR

1. Somogyi, J. C. *Helv. Physiol. Pharmacol. Acta* **7** (1949) C 24. *Intern. Z. Vitaminforsch.* **21** (1949) 341.
2. Somogyi, J. C. *Helv. Physiol. Pharmacol. Acta* **9** (1951) C 79.
3. Somogyi, J. C. *Die Antithiaminfaktoren*, Hans Huber Verlag, Bern, 1952.
4. Somogyi, J. C. *Ergebnisse der med. Grundlagenforschung; Metaboliten und Antimetaboliten*, Georg Thieme Verlag, Stuttgart, 1956, S. 157.
5. Somogyi, J. C. und Koller, A. *Bull. Schweiz. Akad. Med. Wiss.* **14** (1958) 172.
6. Peterson, E. A. und Sober, H. A. *J. Am. Chem. Soc.* **78** (1956) 751.
7. Sober, H. A., Gutter, F. J., Wyckoff, M. M. und Peterson, E. A. *J. Am. Chem. Soc.* **78** (1956) 756.
8. Sober, H. A. und Peterson, E. A. *Federation Proc.* **17** (1958) 1116.

Eingegangen am 22. Februar 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 308–S 312

Inhibition of Photophosphorylation in Isolated Spinach Chloroplasts by Lower Aliphatic Straight-Chain Alcohols

HERRICK BALTSCHEFFSKY*

Department of Zoology, University of California, Los Angeles, USA

Photophosphorylation in isolated spinach chloroplasts is strongly inhibited by lower aliphatic, saturated, straight-chain alcohols. The effect of methanol, ethanol, propanol, butanol and pentanol was tested and found to be increasing with the chain-length of the alcohol. In spinach chloroplasts the photophosphorylation was inhibited by lower concentrations of alcohols than in "chromatophores" from the photosynthetic bacterium *Rhodospirillum rubrum*. The inhibition in chloroplasts was found to be reversible in the sense that removal or strong dilution of alcohol after the preincubation restored a high photophosphorylation activity.

The inhibitory effect of aliphatic, straight-chain alcohols on photophosphorylation in spinach chloroplasts has been investigated in a search for suitable methods to inactivate photophosphorylation in a controlled and reproducible manner, in connection with attempts to obtain insight into the ultrastructural requirements for this electron transport-linked process. Aliphatic alcohols were selected for trial as comparatively low concentrations of ethanol (in the range of 1 volume %), which has been used as a solvent for certain inhibitors, have been found to slightly decrease the rate of photophosphorylation in isolated chloroplasts[1]. All the alcohols, which were systematically tested, namely methanol, ethanol, propanol, butanol and pentanol, are completely soluble in water in the concentrations used.

EXPERIMENTAL

The alcohols were of analytical grade. Hexokinase (Type IV), ATP, flavin mononucleotide (FMN)** and phenazine methosulfate (PMS) were from Sigma Chemical Company, St. Louis, Missouri, USA.

Spinach was obtained as whole plants from local growers. Unless otherwise mentioned, the chloroplasts were isolated according to the method of Allen, Whatley and Arnon[2], with a change in the pH of the NaCl-Tris medium from 8.0 to 8.3. One washing was performed in the undiluted isolation medium, to secure a fraction containing so-called "whole" chloroplasts.

* Present address: Wenner-Gren Institute, University of Stockholm, Stockholm, Sweden.

** Abbreviations: P_i, orthophosphate; FMN, flavin mononucleotide; PMS, phenazine methosulfate; Tris, tris(hydroxymethyl)aminomethane.

The medium for the phosphorylation experiments contained in 1.0 ml of 0.8 M Tris of pH 8.3, 12 μmoles $K_2H^{32}PO_4$, 30 μmoles $MgCl_2$, 1 μmole ATP, 60 μmoles glucose and an excess of yeast hexokinase. Either FMN or PMS were added as electron carriers in the phosphorylation experiments, in the following amounts: 0.4 μmoles FMN or 0.6 μmoles PMS. The measurements of phosphorylation and chlorophyll content have been described and the same is true for the light source and its intensity[3]. Aerobic conditions were used throughout. The temperature was kept at 20°C. The reaction volume was 3.0 ml.

In the restoration experiments the concentration of alcohol was brought down by either dilution or centrifugation. In the dilution experiments, concentrated suspensions of chloroplasts were preincubated with alcohol and subsequently diluted 15-fold, according to the principle outlined in the following scheme, where + and − indicate whether or not the preincubation medium (preparation medium) and the dilution medium (phosphorylation medium) contain the alcohol in question and 1, 2 and 3 represent absence, presence and 15-fold dilution of alcohol, respectively, in the suspension which after dilution was tested for photophosphorylation activity:

	Preincubation medium	Dilution medium
1	—	—
2	+	+
3	+	—

In the centrifugation experiments, the preincubation mixtures were centrifuged after the incubation period for 7 min at 2 500 × g in a Refrigerated Serwall centrifuge and the chloroplast pellets were resuspended as in the scheme above, thus with centrifugation and resuspension instead of dilution as the sole difference.

The preincubation time was usually 30 min for convenience, and during this time light was prevented from entering the samples. In control experiments not to be described here it was found that a preincubation time of 5–10 min or less was sufficient to allow the alcohol to exert its inhibitory effect to completion. The temperature during preincubation was 0°C. As will be shown below, a very strong effect may indeed be obtained without any preincubation with alcohol.

The "chromatophores" from the photosynthetic bacterium *Rhodospirillum rubrum* for the comparative experiments were prepared as in Ref.[4], where also growth conditions, harvesting and pertinent details about the photophosphorylation experiments are reported. The "chromatophore" fraction was used and a 30 min preincubation with alcohol in the dark preceded the photophosphorylation experiments.

RESULTS

In Table 1 are given the approximate concentration ranges, where a 50 % inhibition of the photophosphorylation was obtained in isolated spinach chloroplasts under the given conditions*. An increase in the chain-length of the alcohol

Table 1. Approximate alcohol concentration ranges for 50 % inhibition of photophosphorylation.

Alcohol	Concentration in % (v/v)
Methanol	4 – 5
Ethanol	1.5 – 3
Propanol	0.5 – 1
Butanol	0.1 – 0.5
Pentanol	0.04 – 0.1

* In control experiments, performed with the aid of Dr. M. L. Ibanez, Department of Biophysics, University of California, Los Angeles, it was found that neither methanol nor ethanol was metabolized in any significant amount in the studied system.

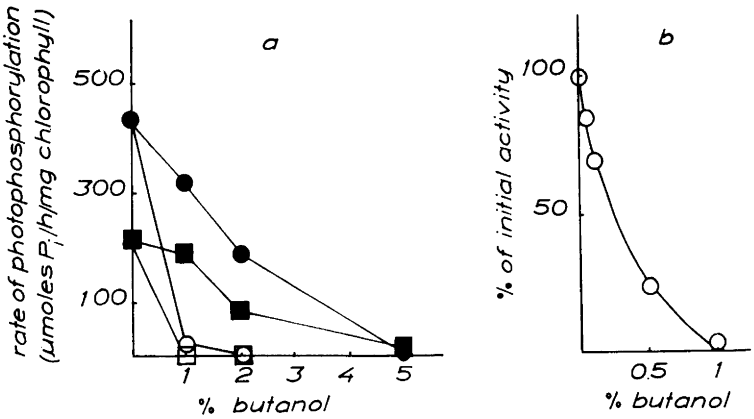


Fig. 1. a) The effect of butanol on photophosphorylation measured as disappearance of P_i. Preincubation with 1 % (v/v) butanol. □ : FMN, undiluted butanol; o : PMS, undiluted butanol; ■ : FMN, 15-fold diluted butanol; • : PMS, 15-fold diluted butanol. Chlorophyll concentration was 0.017 mg/3 ml. b) Inhibition curve for butanol without preincubation of the chloroplasts with the alcohol.

results in a decrease in the concentration which is necessary for inhibition. In similar experiments with isolated "chromatophores" from the photosynthetic bacterium *Rhodospirillum rubrum*, higher alcohol concentrations than with the spinach chloroplasts were found to be required for an inhibition of the photophosphorylation. For example, in this bacterial system a 50 % inhibition was obtained by 7 % ethanol and 0.7 % butanol.

Fig. 1a shows the result of comparisons between photophosphorylation in samples of spinach chloroplasts, where the alcohol concentration in the preincubation medium was retained during a 15-fold dilution of the chloroplasts for the phosphorylation experiment and samples, where a 15-fold dilution of both the chloroplasts and the alcohol was made. Dilution relieved the inhibition within a suitable concentration range of butanol. As was to be expected, this "restoration" of photophosphorylation activity was stronger at lower alcohol concentrations than at higher. A similar picture of reversibility within a certain concentration range was obtained with each of the five alcohols tested.

The curve in Fig. 1b was obtained without preincubation of the chloroplasts with alcohol, also in this case butanol. It is seen that the effect must be a rapid one. Thus no significant structural barriers within the chloroplast prevent the inhibitor from reaching its region or site of action.

Table 2 shows that one may restore photophosphorylation by removing a strongly inhibiting alcohol concentration by centrifugation of the chloroplast suspension and resuspension of the chloroplast pellet in an alcohol-free medium. 1.0 % (v/v) butanol was used in this experiment. An efficient removal of the alcohol and a possibility to virtually completely restore the photophosphorylation after alcohol-treatment of isolated spinach chloroplasts is demonstrated, as the

Table 2. Photophosphorylation in spinach chloroplasts. Its inhibition by addition and restoration by removal of 1 % (v/v) butanol. The final chlorophyll concentration in each tube was roughly 0.01 mg/3.0 ml. In this experiment the medium for chloroplast preparation and preincubation contained a 0.05 M Na_2HPO_4-KH_2PO_4 buffer of pH 7.0 instead of 0.02 M Tris buffer of pH 8.3. The phosphorylation medium was unchanged. Preincubation time: 15 min.

Butanol		Activity μmoles P_i esterified/h/mg chlorophyll	
In incubation medium	In phosphorylation medium	Added co-factor FMN	PMS
—	—	290	570
+	+	30	110
+	—	320	560

phosphorylation rates are, within the experimental error, the same in the samples, where butanol has been added and removed, as in the alcohol-free controls.

DISCUSSION

The fact that an increasing chain-length in the series from methanol to pentanol gives the alcohol a stronger inhibitory capacity against the structure-dependent photophosphorylation in isolated spinach chloroplasts may well be partially or completely dependent on an increasingly lipophilic character of the alcohols in question. As these compounds are asymmetrical due to their possession of a hydrocarbon-part and a polar alcohol-group, they may be expected to become oriented in a determined way in the complex lipid-protein double-membrane matrix of the photosynthetic apparatus, be it localized on both the grana and the stroma membranes within the spinach chloroplast or only on the former. The well-known possibility that the chlorophyll molecules are oriented with their porphyrin-discs at the boundaries between the lipid and protein layers of the grana discs and "anchored" in the lipid phases by means of the lipid-soluble phytyl-chain should be considered in connection with the inhibitory effect of the alcohols on photophosphorylation. If the system for the primary photoreactions and the subsequent dark electron transport and energy transfer reactions for the production of ATP is localized on or in immediate vicinity of lipid-protein interphases, the interference of added alcohols with the photophosphorylation may well be a reflection of surface-active properties of the alcohols at the interphase. Then the enrichment of added alcohol in the region where photophosphorylation occurs may cause inhibition by changing through mass action the three-dimensional geometrical structure of the photosynthetic apparatus.

A comparison of the amounts of alcohol necessary to inhibit photophosphorylation with the amounts of chlorophyll in the inhibited suspensions tends to support the concept that a mass action of alcohol on the gross membrane structure may

occur and cause the effect. From the data in Table 2 one can calculate that the molar ratio butanol/chlorophyll is about 30/1 and the weight ratio about 2.5/1 in this experiment.

What effects can one expect if one goes further in the alcohol series from pentanol to the higher alcohols, which are more lipophilic and more insoluble in water? Practically nothing appears to be known about how such agents may influence sensitive photosynthetic functions. Krogmann and Jagendorf[5] have reported, in connection with a study of fatty acid inhibition of the Hill reaction in spinach chloroplasts, that octadecanol was not inhibitory, but the tested concentration range was not given. On the other hand, we tested in a single experiment whether 0.1 and 3.0 % heptanol affected photophosphorylation in spinach chloroplasts and obtained after a preincubation which included shaking of the material a complete inhibition. It is planned to study the effect of higher alcohols further.

The finding that the inhibition of photophosphorylation may be even completely reversed by removal of the alcohol provides a system where various parameters of photosynthetic structure and function may be investigated in a well-controlled manner. It would appear to be worthwhile to test if the alcohols also influence in isolated chloroplasts more stable reactions than photophosphorylation, such as the Hill reaction and more primary reactions such as the photoreactions, and also if both an effect and its reversal can be demonstrated on the energy transfer-linked electron transport control mechanism and on the carbon dioxide fixation reactions. By such a continuation of this approach it may become possible to obtain further information about what the relationships are between various photosynthetic functions and about their dependency on ultrastructural integrity within the chloroplasts.

Acknowledgements. The author gratefully acknowledges a travel grant from the *Charles F. Kettering Foundation, Ohio, USA.* This work was made possible by a *U. S. National Science Foundation* grant (NSF G-23392) to Professor F. S. Sjöstrand, in whose laboratory at the Zoology Department, University of California, Los Angeles, the experiments were done, and to whom thanks are due for his active support and interest.

REFERENCES

1. Baltscheffsky, H. *Unpublished observations.*
2. Allen, M. B., Whatley, F. R. and Arnon, D. I. *Biochim. Biophys. Acta* **27** (1958) 16.
3. Baltscheffsky, H. *Acta Chem. Scand.* **14** (1960) 264.
4. Baltscheffsky, H. *Biochim. Biophys. Acta* **40** (1960) 1.
5. Krogmann, D. W. and Jagendorf, A. T. *Arch. Biochem. Biophys.* **80** (1959) 421.

Received April 16, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 313–S 318

Formation of a Complex Between a Derivative of the Plant Hormone Indoleacetic Acid and Ribonucleic Acid from Pea Seedlings

N. P. KEFFORD, RAVINDAR KAUR-SAWHNEY and A. W. GALSTON

Department of Biology, Josiah Willard Gibbs Research Laboratories, Yale University, New Haven, Connecticut, USA

Incubation of the plant growth hormone indole-3-acetic acid (IAA) with peroxidase yields a substance of unknown structure (IAA′) which forms a complex with purified RNA from pea shoots, at pH values of 4.8 or below. Once formed at low pH values, the complex is stable at higher pH values. The complex is not formed if IAA itself is added to RNA, or if the RNA is first treated with ribonuclease.

The plant growth hormone, auxin, has a basic role in the regulation of many processes in plant growth and development. Indole-3-acetic acid (IAA), which is probably the major native auxin, is effective at the relatively low concentrations of 10^{-8} to 10^{-5} M. Thus, for some time, some basis for amplification has been sought as part of the mechanism of action of this auxin. An effect of IAA upon or through a catalytic macromolecule would provide one obvious means of amplification. Siegel and Galston[1] found evidence for the attachment of IAA to a macromolecule in pea tissues. This phenomenon was later resolved by Meudt and Galston[2] into two component reactions. These were (a) the conversion of IAA to a derivative of unknown structure (to be referred to as IAA′) by a protein fraction of pea homogenate and (b) the attachment of IAA′ to another macromolecular fraction which resembled ribonucleic acid (RNA). These findings suggested that in plant tissues, IAA may be activated, then attached to RNA. This could be part of the system of amplification possibly required for the growth regulating functions of IAA. In this paper the nature of this interaction between RNA and IAA′ is studied.

METHODS

Plant material. Pea (*Pisum sativum* cv. Alaska) seedlings grown in darkness at 26°C for seven days were used.

Isolation of RNA. A modification of the method of Gierer and Schramm[3] was used. Acetone powder of 50 g of frozen pea shoots was stirred with 500 ml of 0.01 M pH 8 Tris buffer at

4°C for 30 min and the mixture emulsified with 500 ml of phenol. The emulsion was stirred at 20°C for one hour and was then broken by centrifugation in the cold. The supernatant fraction was removed and emulsified with a further 50 ml of phenol. After centrifugation the supernatant fraction (now 600 ml) was removed. To this fraction was added 60 ml of 20 % potassium acetate solution (pH 5), then 1800 ml of cold ethanol. The precipitate, collected by centrifugation, was freed of phenol by two further precipitations with ethanol after redissolving in 200 ml of Tris buffer. For use, the precipitate of RNA was dissolved in 0.1 M phosphate buffer pH 7. The concentration of RNA is given as the equivalent number of grams of pea acetone powder per 1 ml of reaction mixture. In one experiment the RNA was pretreated with ribonuclease (Sigma Chemical Co., bovine pancreas, 5 × crystallized) at pH 7 for 30 min at 25°C.

Formation of IAA′ and its reaction with RNA. Meudt and Galston[2] found that IAA′ could be formed from the following defined mixture: 10^{-6} M crystalline horseradish peroxidase (Worthington Biochemical Corp.), 10^{-4} M 2,4-dichlorophenol, 10^{-4} M manganous chloride and 10^{-4} M or 2×10^{-4} M IAA in 0.01 M phosphate buffer pH 7. The mixture was incubated at 25°C for 20 min. For convenience in the text, the concentration of IAA′ is given as the concentration of the IAA used in its preparation. During the last 10 min of the above incubation, RNA was added, then the mixture was cooled to 4°C.

Precipitation of RNA and detection of indolic compounds. Aliquots of 2 ml of reaction mixtures were made 0.2 M with perchloric acid, yielding a precipitate. The precipitate was centrifuged, washed twice with 0.2 M perchloric acid, dissolved in 2 ml of 0.1 M phosphate buffer pH 7 and treated with 2 ml of Salkowski reagent (0.01 M ferric chloride in 14 N sulphuric acid). After 70 min the optical density of the pink color produced by IAA or IAA′ was measured in a Klett colorimeter using a green filter (Corning 54).

Dialysis of RNA-IAA′ mixtures. Dialysis was used to detect the formation of macromolecular complexes of IAA′. RNA-IAA′ mixture was added to an equal volume of cold 0.1 M phosphate-citrate buffer of known pH value. Aliquots of 4 ml were then dialyzed in cellophane dialysis tubing at 4°C for 24 h against 4 × 50 ml of 0.01 M buffer of the same or different pH. After dialysis, 2 ml of the bag contents were made 0.2 M with perchloric acid. The precipitated material was centrifuged, washed twice with 2 ml of 0.2 M perchloric acid, dissolved in 2 ml of 0.1 M phosphate buffer and treated with Salkowski reagent.

Gel filtration of RNA-IAA′ mixture or a pea acetone powder extract-IAA′ mixture. Twin columns, 2 × 30 cm, of the dextran gel Sephadex G-50, medium mesh (Pharmacia, Sweden),

Table 1. Interaction of IAA′ and RNA and of IAA and RNA shown by precipitation of RNA with perchloric acid.

Reaction mixture			
Ribonucleic acid g equiv./ml	IAA M × 10^4	IAA′ M × 10^4	Salkowski reaction Klett scale divisions
–	–	–	0.0
0.1	–	–	2.0
–	1.0	–	1.0
–	2.0	–	1.0
–	–	1.0	1.5
–	–	2.0	13.0
0.1	1.0	–	2.0
0.1	2.0	–	4.0
0.1	–	1.0	24.0
0.1	–	2.0	47.0

Table 2. Effect of preincubation with ribonuclease of the interaction of RNA and IAA'.

Reaction mixture			Salkowski reaction Klett scale divisions
Ribonucleic acid g equiv./ml	IAA' M × 10^4	Ribonuclease μg/ml	
–	–	–	0.0
0.1	–	–	0.4
–	1.0	–	4.0
0.1	1.0	–	33.0
–	–	0.5	0.1
0.1	–	0.5	4.0
–	1.0	0.5	5.5
0.1	1.0	0.5	11.5

were prepared in phosphate buffer pH 7. To one was added 18 ml of a mixture of 0.1 g/ml RNA and 10^{-4} M IAA', to the other was added 18 ml of 10^{-4} M IAA'. The columns were developed with 0.01 M phosphate buffer at 4°C. Approximately 5 ml fractions were collected and their ultraviolet absorption spectra and Salkowski reactions, without prior perchloric acid treatment, were recorded. In another experiment the same columns were used. To one column, 5 ml of a mixture of phosphate buffer extract of 3.0 g of pea acetone powder and 10^{-4} M IAA' was added and to the other 5 ml 10^{-4} M IAA'. The columns were developed and the fractions assayed as above.

RESULTS

When RNA from peas was incubated with IAA', then precipitated with perchloric acid (Table 1) the precipitated material gave a positive reaction with the Salkowski reagent. Thus, an indolic compound was precipitated together with the RNA. In the same experiment, no Salkowski color was obtained if RNA was incubated with unchanged IAA, then precipitated with perchloric acid.

Table 3. Salkowski reaction of RNA precipitated by perchloric acid or alcohol following reaction with IAA'.

Reaction mixture		Salkowski reaction Klett colorimeter units	
RNA g equiv./ml	IAA' M × 10^4	Perchloric acid precipitation	Alcohol precipitation
–	–	0.0	0.0
0.1	–	1.5	2.5
–	1.0	2.0	4.5
–	2.0	5.5	1.5
0.1	1.0	19.5	4.5
0.1	2.0	36.5	4.5

Table 2 shows that the incubation of RNA with ribonuclease reduced the Salkowski color to a figure expected from the controls. Ribonuclease treatment of the RNA also reduced the amount of material precipitated by perchloric acid and increased the absorption at 260 mμ of the supernatant fraction.

If, after incubation with IAA′, RNA was precipitated with 70 % ethanol containing 0.05 M potassium acetate, no Salkowski reaction was obtained from the precipitated material (Table 3). The contrasting effects of perchloric acid and alcohol as precipitants suggested that the attachment of IAA′ to RNA might be effected by the hydrogen ion concentration. A study of the effect of pH upon the binding of IAA′ to RNA was made by incubating these substances together or separately at pH 7, adding 2 ml aliquots to 2 ml of phosphate-citrate buffers of pH range 2.2 to 6.8, and then dialyzing these mixtures against 0.01 M buffer of the same pH as that which had been added. After dialysis, the Salkowski reactions of the material in the dialysis bags precipitated by perchloric acid were recorded. The data in Fig. 1 show the binding of IAA′ to RNA, as indicated by the Salkowski positive reaction of the non-dialysable material, occurred only at pH values below 4.8.

The effect of pH upon RNA-IAA′ complex, already formed at pH 2.2, has also been studied. RNA and IAA′ were incubated together at pH 7 and added to an equal volume of 0.1 M phosphate-citrate buffer, pH 2.2. Aliquots of 4 ml were then dialyzed against 0.1 M buffers over the pH range 2.2 to 6.8. The amount of nondialyzable, indolic material, as indicated by the Salkowski reaction, was unchanged by dialysis against any of the buffers up to at least pH 6.8.

Gel filtration has been used, in addition to dialysis, for the separation of large and small molecules. The behavior of a mixture of RNA and IAA′ and IAA′ alone on Sephadex columns, developed with phosphate buffer at pH 7, is shown in Fig. 2, A and B. The RNA, as indicated by absorption at 260 mμ, passed

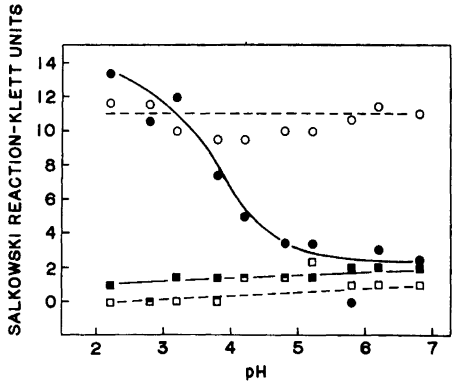


Fig. 1. Effect of pH upon the formation (full lines and symbols) and the stability (broken lines and open symbols) of the RNA-IAA′ complex. The optical density of the Salkowski reaction upon RNA plus IAA′ (circles) or IAA′ alone (squares) after dialysis against buffers of various pH values. In treatments represented by open symbols and broken lines, the RNA plus IAA′ or IAA′ alone were treated with buffer pH 2.2 prior to dialysis.

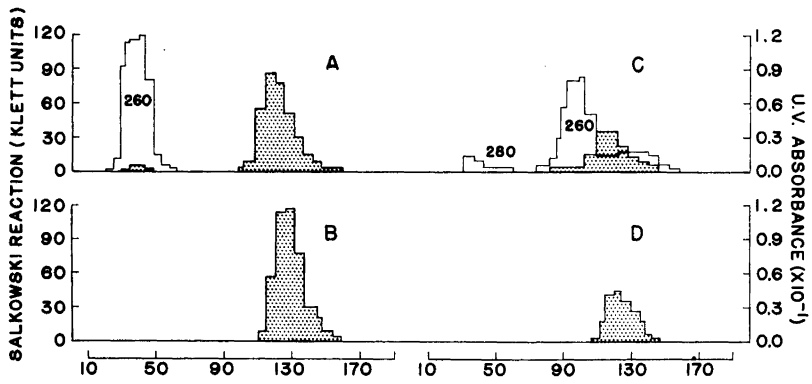


Fig. 2. The absorption at 260 or 280 mμ and the Salkowski reaction (stippled) of fractions from gel filtrations using buffer pH 7 as eluant. A and B represent twin columns to which were applied RNA plus IAA′ (A) or IAA′ alone (B), C and D represent subsequent runs on the same columns, this time a buffer extract of pea shoot acetone powder plus IAA′ (C) or IAA′ alone (D) were applied.

through the column near the solvent front and was clearly separated from the bulk of the Salkowski reacting material. The latter was eluted from the column at the same rate in the absence and presence of RNA except for a small, possibly insignificant quantity which travelled with the RNA peak.

The behavior of a buffer extract of pea shoot acetone powder incubated with IAA′, and of IAA′ alone, on the columns used in the previous experiment, is shown in Fig. 2, C and D. In this case, the first material eluted from the column holding extract of pea acetone powder had maximum absorption at 280 mμ and was presumed to be protein. This was followed by RNA-like material with a maximum absorption at 260 mμ. The Salkowski reacting material overlapped with the 260 mμ absorbing material and was eluted by the same volume in the absence and presence of acetone powder extract. Thus, these gel filtration experiments provide no evidence for the formation of the RNA-IAA′ complex at the higher pH values.

DISCUSSION

Purified RNA from peas has been shown to complex with a derivative of IAA, but not with IAA itself. The ability of the RNA preparation to complex with IAA′ was destroyed by treatment with ribonuclease, confirming that the complexing macromolecule is RNA. Both dialysis and gel filtration show that the complex between RNA and IAA′ is not formed at pH 7. It is, however, formed at pH values less than 4.8 and is subsequently stable to higher pH values. The formation of the complex only at low pH values makes its role in physiological processes questionable.

These results bear on earlier investigations of the interactions between IAA′ and macromolecules. The association between RNA-like material and IAA′, observed by Meudt and Galston[2] during the gel filtration of mixtures of IAA′ and

extract of pea acetone powder, were probably due to a coincidence of elution volumes. The difference found in the present experiments between the elution volume for purified RNA and the 260 mμ absorbing material from incubated acetone powder extract suggests some differences between the purified RNA and the RNA-like material of acetone powder extract.

The failure of Andreae and van Ysselstein[4] to detect a Salkowski positive macromolecular fraction in IAA treated pea tissue, as had been done by Siegel and Galston[1], may be explained by the respective use of alcohol and trichloroacetic acid as precipitant for the macromolecules.

This work was supported in part by the *National Science Foundation* and by the *Herman Frasch Foundation*. We thank George Brawerman and Werner J. Meudt for advice. N. P. Kefford is on leave from the Division of Plant Industry, C. S. I. R. O., Canberra, Australia.

REFERENCES

1. Siegel, S. M. and Galston, A. W. *Proc. Natl. Acad. Sci. U. S.* **39** (1953) 1111.
2. Meudt, W. J. and Galston, A. W. *Plant Physiol. Suppl.* **37** (1962) xiv. (Full paper submitted for publication, March, 1963).
3. Gierer, A. and Schramm, G. Z. *Naturforsch.* **11B** (1956) 138.
4. Andreae, W. A. and van Ysselstein, M. W. H. *Plant Physiol.* **35** (1960) 225.

Received April 1, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 319–S 324

Studies on the Phosphate Metabolism in Multiple Sclerosis

I. HUSZÁK and F. SZÉCHENYI

Institute for Brain Research, University of Szeged, Hungary

In order to elucidate the pathological processes of MS on the basis of the anatomo-pathological events it was attempted to gain insight into the patho-physiological processes of the organism in the various phases of the disease.

To elucidate the metabolic changes of the organism of MS patients different loadings were used (ACTH, Corticoids, exptl. fever, etc.).

The results concerning the change of energy rich phosphate esters in the erythrocyte with different loadings are presented. In the case of experimental febrile loads at 38°C the inorganic phosphate content shows an increasing tendency in the blood of most of the MS patients, whilst in that of the controls it decreases.

The change of the energy rich phosphate ester content of the cells is the opposite to the change of the inorganic P of the blood inasmuch as with febrile loadings the energy rich phosphate ester content of the erythrocytes of MS patients decreases, as compared to that of the controls which rather show an increasing tendency.

The amount of other organic phosphate esters does not vary significantly in the case of the MS patients as compared to that of the controls.

Following ACTH administration the same significant differences were obtained as when applying febrile loads.

The conclusion can be drawn that the erythrocytes of the MS organism cannot produce the energy rich phosphate esters corresponding to the energy requirements of the experimentally enhanced metabolism. The significance of these metabolic disturbances is discussed.

The pathological process in multiple sclerosis (MS) with its focal and essentially selective attack on the myelin sheaths, seems to be peculiar to the disease. On the basis of the pathomorphological picture these types of reaction are usually termed demyelinisation reactions or leukoencephalitides. In general they can be considered as a disease of the white matter. Among the different demyelinating diseases multiple sclerosis is a special disease of humans, it cannot be transferred to animals. Thus, the pathochemical processes of the damaged nervous tissue cannot be studied directly. We are completely dependent upon clinical human material, blood, cerebrospinal fluid, urine, etc. This fact naturally limits the possibilities to elucidate in details the pathological processes of the organism.

At the elaboration of our research plan relating to the pathology of MS the following aspects were taken into consideration:

1) In view of the fact that this illness is essentially the disease of the central white matter of the brain it seemed an important task to obtain as many data as possible relating to the normal biological and biochemical processes of the white matter[1–5].

2) On the basis of the anatomo-pathological events we attempted to gain insight into the patho-physiological processes of the organism in the various phases of the disease: acute, reparative and stationary[9–14].

3) We attempted to elucidate the metabolic changes of the organism according to an integrated view of the fundamental correlation of the structure, function and metabolism. Only later, after recognising many details of the pathological chain reactions taking place in the organism, we shall be able to gain information about the causative factors of this disease[15].

The organisation of the resting and functional structures of the cells are controlled by the energy producing oxidation processes of the cells. Any disturbance in the function of the oxido-reduction system may induce a structural damage of the cells. Thus the impairment of the myelin sheath with its very complex components and strictly organised layers may be produced by disturbances in the nutrient[8] or oxygen[6,7] supply respectively, as well as by the disturbance of the function of any catalyst of the oxido-reduction system. In every cell the decomposition and synthetic processes must be in equilibrium; if the decomposition processes increase at the expense of the synthetic processes structural damage ensues.

Glucose is the main energy source of the grey and white matter of the central nervous system[1]. Acetylcholine, cholesterols and the different long chain fatty acids of the nervous tissue are built up by the intermediary products of the glucose metabolism. Thus our attention was focussed on the carbohydrate metabolism in patients suffering from multiple sclerosis.

It is most important to bring the evaluation of laboratory results into correlation with the different phases of the illness. As the active, reparative and stationary periods of the disease cannot always be well differentiated clinically, the correct evaluation of the laboratory data obtained is also difficult. The many discrepancies contained in the literature may partly be due to this fact. Taking these considerations into account it seems preferable to examine the responses of the organism to various well-defined loads. If loads are applied to pathologically impaired or less efficient enzyme systems the functional or metabolic disturbances ensue earlier in the region of the pathological enzyme system. Owing to the above considerations therefore, instead of concentrating our attention on the various chemical changes occurring in the blood in the different phases of the disease we have rather observed the various alterations resulting from varying loads. Patients suffering from different phases of this disease were submitted to various loads: ACTH, corticoids and fever induced by milk. These proved to be the most suitable means. On examining the different metabolites, electrolytes and enzyme activities at a temperature of 38°C (the blood samples were taken at this temperature) it could be established that the organism of MS patients reacts not only quantitatively but also qualitatively differently as compared to the controls[13,14].

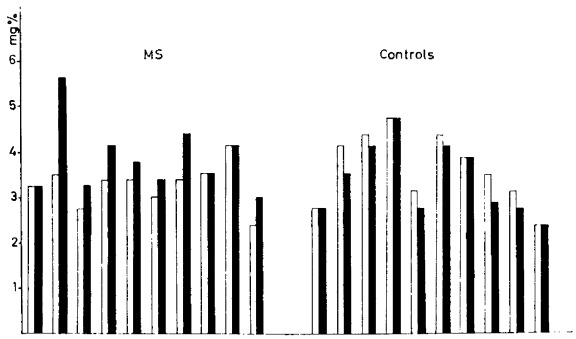


Fig. 1. Changes in the inorganic phosphate level of the blood induced by fever loads. MS: $0.02<P<0.05$; controls: $P>0.05$. □: before fever ■: at a temperature of 38°C.

Concerning the keto body formation we could establish that whilst the keto body content of the blood of MS patients in a resting state does not show a significant difference as compared to the controls, in the case of a febrile load (at 38°C) far more keto bodies and lactate form in the MS patients than in the controls. The keto body formation runs parallel with the activity of the disease. In a febrile state the metabolic processes are enhanced. The sugar utilization also increases[15].

The enhanced keto body and lactate production of MS patients in a febrile state points to the fact that there is some disturbance in their carbohydrate metabolism. And if this is the case some disturbance may be assumed in the related phosphate metabolism too. Owing to these considerations the next task was to examine the phosphate metabolism in different loading states.

METHOD

After three to six days of rest fasting blood samples were taken and then 10 ml sterile milk was administered intramuscularly. The patients were not allowed to move during the examination. Normal subjects, acting as controls, were submitted to the same procedure. In order to perform hormonal loadings 20 units of ACTH was administered intramuscularly, or 25 mg Di-Adreson-F aquosum (Organon) intravenously. The blood samples were taken at a temperature of 38°C; after the ACTH injections twice in intervals of 2 hours and following Di-Adreson-F aquosum administration 4 times every hour. For the inorganic phosphate determinations the method of Lohmann and Jendrassik[16] was applied. The different acid soluble phosphate esters were estimated according to Lohmann[17].

RESULTS

Fever loads: In the case of febrile loads at 38°C the inorganic phosphate content shows an increasing tendency in the blood of most of the MS patients, whilst in that of the controls it decreases (Fig. 1).

The change of the energy rich phosphate ester (acid soluble phosphate esters hydrolysable in 7 min) content of the blood cells is the opposite to that of the blood inorganic P content, inasmuch as with febrile loadings the energy rich

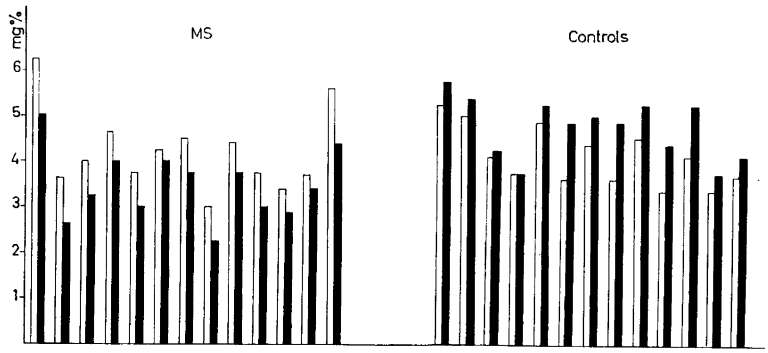


Fig. 2. Changes in the energy-rich phosphate ester content of the blood after fever loads. MS and controls: $0.02<P<0.05$. □: before fever ■: at a temperature of 38°C.

phosphate content of the cellular elements of MS patients decreases, while that of the controls rather shows an increasing tendency (Fig. 2).

The amount of other acid soluble, hydrolysable and nonhydrolysable phosphate esters does not show any significant difference in the case of the MS patients as compared to that of the controls.

Hormonal loads: Every load – also a febrile one – increases the activity of the hypophyseo-adrenal system. The ACTH production is enhanced. Hence we examined the effect of the experimentally administered ACTH and corticoids too.

Following ACTH administration the same significant differences could be observed in the energy rich phosphate ester content of the blood as on applying fever loads. The significant changes could be observed 2 hours after the administration of the ACTH (Fig. 3).

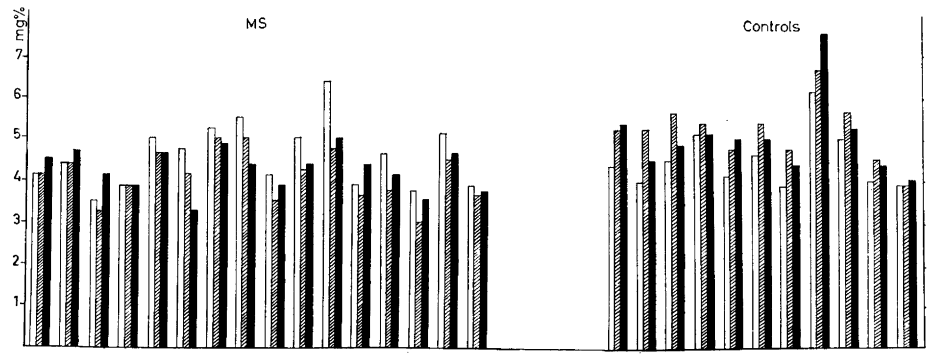


Fig. 3. Changes in the energy-rich phosphate ester content of the blood after administration of ACTH. MS (16 cases) and controls (11 cases): 2nd h: $0.02 < P < 0.05$; 4th h: $P > 0.05$. □: before ACTH ▨: 2nd h after ACTH ■: 4th h after ACTH.

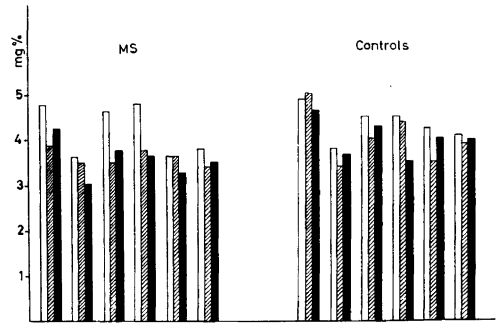


Fig. 4. Changes in the energy-rich phosphate ester content of the blood after administration of Di-Adreson-F aquosum. MS: 1st h: $0.02<P<0.05$; 2nd h: $P>0.05$; controls: 1st and 2nd h: $P>0.05$. □ : before Di-Adreson-F aquosum ▨ : 1st h after injection ■ : 2nd h after injection.

On administering a glucocorticoid (Di-Adreson-F aquosum), (Organon) the energy rich phosphate ester content of the blood decreased in the first two hours in both the MS patients and the controls, but the change was only significant in the case of MS patients (Fig. 4).

DISCUSSION

The conclusion may be drawn that the erythrocytes of normal individuals can produce the energy rich phosphate esters corresponding to the energy requirements of the enhanced febrile metabolism, whereas the cellular elements of the blood of MS patients fail to do this. The cause of the decreased energy-rich phosphates in the blood of MS patients in a febrile state as compared to the controls might be attributed partly to the weakness of the processes generating energy rich phosphate esters and partly to the increased ATP-ase activity in such conditions.

Examinations to establish whether the cause of the metabolic disturbances of the erythrocytes of MS patients mentioned above should be sought for in the enzyme system of the erythrocytes themselves, or whether the composition of the surrounding plasma exerts a damaging influence on the metabolism of the otherwise normal erythrocytes are in progress.

The question seems justified how the metabolic changes described above can be brought into correlation with the etiologic factors of MS. We assume that as a causative factor is not involved, but that the above metabolic loads indicate a disorder of the general adaptation mechanism in MS. They can, on the other hand, explain the well known clinicopathologic facts that the state of every MS patient deteritorates as a result of physical or metabolic loads. Namely, as has already been stated in the case of experimental metabolic loads (fever, ACTH) one of the tissue elements of the MS organism: the red blood cell is not able to produce the energy rich phosphate esters in amounts corresponding to the enhanced metabolic requirements. This can of course have an unfavourable effect on the restitutional processes.

REFERENCES

1. Huszák, I. *Biochem. Z.* **313** (1942) 315.
2. Huszák, I. *Acta Chem. Scand.* **1** (1947) 813.
3. Bennetts, H. W. and Chapman, F. E. *Australian Vet. J.* **13** (1937) 138.
4. Porter, H. and Folch-Pi, J. *Progr. Neurobiol.* **1** (1956) 40.
5. Huszák, I. and Domonkos J. *Aktuelle Probleme der Neurologie. Sammlung von Abhandlungen, dargebracht zum 60. Geburtstag Akademiker Kamil Henner, Praga 1955. Ideggyógyászati Szemle, Hungary Suppl. 178* (1954) 178.
6. Meyer, A. Z. *Neurol. Psychiat.* **112** (1928) 187.
   Putnam, T. J., JcKenna, J. B. and Morrison, L. R. *J. Am. Med. Assoc.* **97** (1931) 1591.
7. Hurst, E. W. *Australian J. Exptl. Biol. Med. Sci.* **20** (1942) 297.
   Lumsden, C. E. *J. Neurol. Neurosurg. Psychiat.* **13** (1951) 1.
8. Hicks, S. P. *Arch. Pathol.* **49** (1950) 111.
9. Huszák, I. *Psychiat. Neurol.* **136** (1958) 215.
10. Huszák, I. and Szák, J. *Acta Med. Scand.* **138** (1950) 48 and 57.
11. Szák, J., Könyves-Kolonics, L. and Huszák, I. *Wien. Z. Nervenheilk.* **4** (1951) 265.
12. Könyves-Kolonics, L., Szák, J. and Huszák, I. *Wien. Z. Nervenheilk.* **6** (1952) 60.
13. Huszák, I., Könyves-Kolonics, L., Domonkos, J. and Tass, G. *Acta Physiol. Acad. Sci. Hung.* **6** (1954) 1.
14. Könyves-Kolonics, L., Tass, G., Domonkos, J. and Huszák, I. *Psychiat. Neurol.* **135** (1958) 190.
15. Huszák, I. and Széchenyi, F. *Brain* **82** (1959) 427.
16. Lohmann, K. and Jendrassik, Z. *Biochem. Z.* **178** (1926) 419.
17. Lohmann, K. *Biochem. Z.* **202** (1928) 466.

Received April 2, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 325–S 331

Effects of Isonicotinic Acid Hydrazide (INH) Upon Plant Growth and Auxin (Indoleacetic Acid) Synthesis*

GEORGE D. LUDWIG** and JOHN M. RUSK

Departments of Medicine and Johnson Foundation of Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania, USA

Isonicotinic acid hydrazide (INH) is shown to inhibit growth of both tomato and pea seedlings as well as the conversion of exogenous tryptophan to indoleacetic acid by lyophilized tomato leaf preparations. The inhibitory effect is at least partially prevented by pyridoxine or pyridoxal phosphate. The inhibition is ascribed to formation of isonicotinyl-hydrazone between INH and pyridoxal, resulting ultimately in a depletion of pyridoxal phosphate, the coenzyme required for an essential transaminase involved in the conversion of tryptophan to indoleacetic acid. Evidence is presented which adds support to the hypothesis that the main biosynthetic pathway between tryptophan and indoleacetic acid involves indolepyruvic acid rather than tryptamine or indoleacetonitrile.

The studies described here*** were undertaken as a result of developments in two independent and unrelated fields; one in clinical medicine and pharmacology, the other in plant physiology and biochemistry. The first consisted of reports appearing soon after isonicotinic acid hydrazide (INH) was introduced for the treatment of tuberculosis, that a significant proportion of patients developed a peripheral sensory neuropathy, especially when given the drug in large dosage[1–4]. Because of the close chemical similarity between INH and nicotinamide with the resultant possibility of anti-metabolite action, it was at first suspected that the neuropathy resulted from niacin deficiency[1]. However, nicotinamide was ineffective in preventing or ameliorating the neuritis. Biehl and

* This work was supported in part by funds from USPHS Grant CA-05394-03 (Studies of Indole Metabolism), Institute for Arthritis and Metabolic Disease, National Institutes of Health, Bethesda, Maryland, USA.

** Address reprint requests to George D. Ludwig, Assoc. Prof. of Medicine, Hospital University of Pennsylvania, 3600 Spruce St. Philadelphia, Penn., USA.

*** The first part of the work reported here was performed by one of us (G. D. L.) during 1957 in Professor Hugo Theorell's laboratory, Biochemical Department, Nobel Medical Institute, Stockholm, and a preliminary report has been made (Ludwig, G. *Federation Proc.* **17** (1958) 524, no. 2049). The work was completed in the Johnson Foundation of Biophysis (Professor Britton Chance) and Departments of Medicine (Professor Francis C. Wood) and Botany (Professor David Goddard) of the University of Pennsylvania. The authors are indebted to the above named for use of facilities and for advice rendered during the course of this work.

Vilter, recognizing the similarity of the neuritis to that produced by desoxypyridoxine[5] showed that the administration of INH led to an increased urinary excretion of pyridoxine (B_6); that the patients with the neuritis displayed other biochemical evidence of pyridoxine deficiency; and that the neuritis could be prevented by administration of pyridoxine along with the drug[6,7]. The evidence suggested that the increased urinary excretion of pyridoxine consisted of a bound form of pyridoxal, *i. e.*, the isonicotinyl hydrazone, which has been shown to occur readily *in vitro*[8].

The second set of observations that prompted this study concerned the formation of the plant growth hormone or auxin, indoleacetic acid. From the time that Thimann[9] first isolated indoleacetic acid (IAA) from a mold and suggested that it might be the main auxin of higher plants, it was suspected that it was derived from tryptophan. Abundant evidence has accumulated to support both hypotheses[10–22]. However, especially with the isolation of other indolic compounds from plants, the precise biosynthetic pathway by which tryptophan is converted to indoleacetic acid by plants has remained controversial[18–22]. The present work was based on the assumption that the conversion by plants of tryptophan to indoleacetic acid, by whatever route, would require both transamination and decarboxylation, reactions known to require pyridoxal phosphate as a coenzyme[23–27]. Moreover, pyridoxine has been shown to be required for growth of higher plants[28], but its exact role has not been further defined. Since INH is capable of binding pyridoxal phosphate, it was postulated that INH should inhibit the formation of indoleacetic acid from tryptophan, and thus, plant growth. To test this hypothesis the experiments reported here were designed.

MATERIALS AND METHODS

Tomato seeds (garden variety) were germinated in sand, and the seedlings transferred to small pots containing soil, one seedling to a pot for the initial series of experiments. All experiments were carried out in a greenhouse with controlled heat and humidity, but with natural variation of light and dark. The control plants were watered daily with de-ionized distilled water, the experimental plants with various concentrations of INH. Some plants were watered with pyridoxine, alone or in addition to the INH. In subsequent series of experiments, to exclude any effect of soil, the seedlings were transferred to 125 ml Erlenmeyer flasks containing Hoagland's nutrient solution[29], three seedlings to a flask. Various concentrations of INH varying between 10^{-6} and 10^{-2} M were added to the experimental flasks. All flasks were buffered to pH 5.1 with 0.01 M phosphate buffer. Pyridoxine (10^{-2} to 10^{-4} M) was added to some flasks with and without INH. All the flasks were continuously aerated by bubbling air through glass capillary tubes connected to the air supply through a manifold.

Measurements of stem length were made daily, and the plants were photographed at four day intervals. At the completion of each experiment, the plants in each flask were weighed after blotting and allowing to dry 8 h at room temperature. When the inhibition of growth of tomato plants by INH had been demonstrated, similar experiments were conducted using garden peas (var. "Gnesta favorit") in order to show that the inhibitory effect applied to other higher plants.

When the anticipated inhibition of plant growth by INH was observed, experiments were then performed in an attempt to show that the conversion of tryptophan to indoleacetic acid by plant tissue is inhibited by INH. Plant tissue containing the enzymes necessary for the above conversion were prepared according to the procedure described by Wildman and Muir[17] and incubated with L-tryptophan in the manner described by Henderson and Bonner[30]. Leaves from greenhouse-grown 14-day-old tomato plants were minced by sharp scissors directly into liquid nitrogen, lyophilized, pulverized, and stored in a dessicator over P_2O_5 at 0°C. Soon after preparation, aliquots of the pulverized leaf preparation (50 mg) were incubated in 100 ml Erlenmeyer flasks, with and without 50 mg L-tryptophan buffered to pH 7.0 in 0.01 M phos-

phate buffer. Various concentrations of INH between 10^{-5} and 10^{-2} M or INH plus pyridoxal phosphate were added to the experimental flasks. The flasks were incubated in the dark for 5, 10, and 15 h at 25°C. The residual plant tissue was then removed by filtration and washed with peroxide-free ether. To extract the indoleacetic acid produced, the filtrate was adjusted to pH 2.8 with 0.1 N HCl and extracted twice with 10 ml portions of peroxide-free ether, which was combined with the ether used to wash the plant particles; and the combined fractions then shaken vigourously with 2 ml of 2 % $NaHCO_3$. The bicarbonate solution was readjusted to pH 2.8 with HCl, extracted with fresh peroxide-free ether (10 ml). The ether was removed without heat (vacuum) and the residue again taken up in 2 ml of 2 % $NaHCO_3$. The IAA in the bicarbonate solutions was then assayed by two methods. A 1 ml aliquot was concentrated *in vacuo* and chromatographed in two dimensions on Whatman No. 1 filter paper using isopropanol : ammonia : water (200 : 10 : 20 v/v) for the first solvent and butanol : acetic acid : water (120 : 30 : 50 v/v) for the second solvent, according to the procedure of Jepson[31]. The papers were sprayed with Ehrlich's reagent (1 % in 1 N HCl). Indoleacetic acid was clearly identified as a red-purple spot with R_F values 0.37 and 0.91 in the two solvent systems, respectively. It is possible to detect as little as 1 μg of IAA by this method. Another 1 ml aliquot of the bicarbonate solution was treated with an equal volume of Gordon and Weber's modification[32] of Salkowski reagent. Indoleacetic acid yielded a red color, with an absorption maximum at 525 mμ. Absorbancy readings were made in a Beckman spectrophotometer at 15, 30, and 60 min, and the amount of indoleacetic acid present quantitated by comparison with a standard curve prepared by adding authentic IAA to Salkowski reagent in a manner similar to that used for the experimental solutions.

RESULTS

Tomato plants in soil; watered daily with INH: The appearance of tomato plants at the end of 19 days is shown in Fig. 1. The control plant received only distilled water, the experimental plants 10^{-5}, 10^{-4}, 10^{-2} M INH, respectively. The inhibitory effect of INH became apparent within 5–7 days, especially with 10^{-2} M INH, which completely inhibited growth; the latter concentration caused the plant to be stunted; the leaves wrinkled, dry, and shrivelled. The inhibition of growth varied in an approximately linear manner with the log of the concentration of INH, although in some experiments little difference in inhibitory effect was seen between 10^{-3} and 10^{-4} M INH.

Tomato and pea seedlings in Hoagland's nutrient solution: The results obtained with tomato plants in nutrient solution, to which were added various concentra-

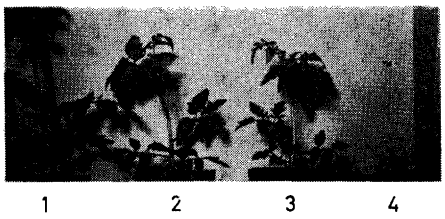


Fig. 1. Appearance of tomato plants grown in soil for 19 days; the control plant was watered with deionized distilled water, and the experimental plants with various concentrations of isonicotinic acid hydrazide (INH). 1) Control; 2) 10^{-5}, 3) 10^{-4} and 4) 10^{-2} M INH.

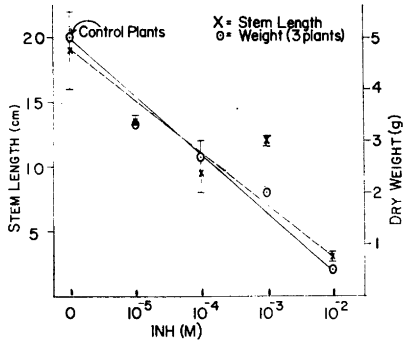


Fig. 2. Stem length and dry weight of tomato plants expressed as a function of INH concentration to which they were exposed for 21 days while growing in nutrient solution.

Fig. 3. Pea plants at the end of 10 days in nutrient solution containing various concentrations of INH. The control flasks contained no INH. 1) and 6) controls; 2) 10^{-2}, 3) 10^{-3}, 4) 10^{-4} and 5) 10^{-5} M INH.

Fig. 4. Effect of pyridoxine HCl (10^{-3} M) in preventing inhibition by INH (10^{-3} M) (compare flasks 2 and 3). High concentrations of pyridoxine HCl alone (10^{-2} M) appear to be slightly inhibitory (Flask 4). 1) Control; 2) 10^{-3} M INH; 3) 10^{-3} M INH + 10^{-3} M pyridoxine; 4) 10^{-2} M pyridoxine.

tions of INH, were similar to those obtained when the seedlings were grown in soil. The measurements of stem length and dry weight at the end of 21 days, as a function of INH concentration, are given in Fig. 2. Similar results were obtained when pea seedlings were grown under these conditions, as shown in Fig. 3. Definite evidence of at least partial protection against inhibition by INH was obtained when pyridoxine HCl ($10^{-3} - 10^{-4}$ M) was added along with INH to either tomatoes or peas. However, the reversal of inhibition was never complete as indicated by the results obtained with tomato plants shown in Fig. 4. Evidence was obtained that suggested that pyridoxine HCl alone may be slightly inhibitory to growth under these conditions, if it is applied at very high concentrations (Fig. 4, flask 4).

Conversion of tryptophan to indoleacetic acid by tomato leaf preparations: The amounts of IAA produced at 5, 10, and 15 h as a result of incubating quick-frozen, lyophilized, minced tomato leaf preparations with L-tryptophan with and without INH of various concentrations are shown graphically in Fig. 5. The highest values were obtained at 5 h incubation. The lower values at longer times may reflect destruction by an oxidase system of some of the IAA formed. It is apparent that 10^{-2} M INH almost completely inhibited IAA formation. When no tryptophan was added to the flask containing the plant material, no IAA was formed as measured by spectrophotometric assay after extraction or by paper chromatography. In this series of experiments 10^{-4} pyridoxal phosphate appeared to almost completely reverse the inhibition of color formation (*i. e.*, IAA formation) obtained with 10^{-2} M INH.

DISCUSSION

It is noteworthy that growth of both tomatoes and peas, as well as the conversion of exogenous L-trypthophan to indoleacetic acid by plant tissue, was practically completely inhibited by 10^{-2} M INH. This same concentration of INH has been shown to inhibit completely the growth of certain strains of *Lactobacilli* and *Saccharomyces*[33], and decarboxylation reactions[34], and tryptophan to indole conversion[35] by *E. coli*. However, the latter inhibitory effects were attributed to competitive metabolic antagonism between INH and pyridoxal phosphate for a site on the enzyme protein. It appears more likely that

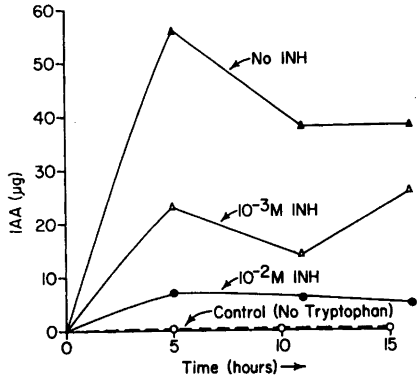


Fig. 5. Indoleacetic acid formed from L-tryptophan by quick-frozen, lyophilized tomato leaf preparations in 5, 10, and 15 h incubations at 25°C.

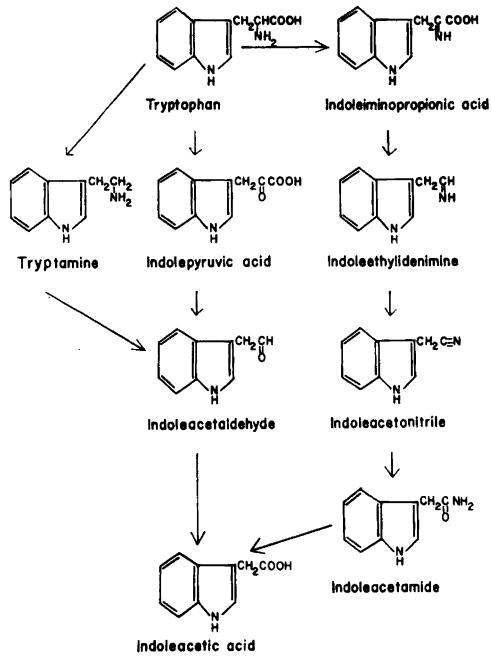


Fig. 6. Possible biosynthetic pathways for conversion by higher plants of tryptophan to indoleacetic acid that have been postulated and for which some experimental evidence exists.

the latter results as well as those reported here are due to formation of the pyridoxal-isonicotinyl hydrazone as suggested by the urinary excretion studies in the human neuropathy induced by INH[6] and by effects on certain other bacterial and mammalian enzyme systems[36,37]. In the course of these studies we have confirmed the observations of Sah[8] that the isonicotinyl-hydrazone forms easily between INH and pyridoxal phosphate, *in vitro*.

Wort[38], who tested the effect of INH on plants on an empirical basis, also has observed inhibition of growth. However, he attributed it to a decrease in catalase, to a depletion of available photosynthate for cell material synthesis and also of available energy, and in part to an inhibition of respiration.

The possible biosynthetic pathways for conversion of tryptophan to indoleacetic acid by higher plants that have been postulated and for which some experimental support exists are depicted in Fig. 6. For years, the main pathway was considered to involve either indolepyruvic acid (IPyA)[10,44] or tryptamine[18,19,39]. The demonstration of the activity and presence of indoleacetaldehyde (IAc) and its conversion to IAA in plants[22] placed it as a logical intermediate since it could arise from either IPyA or tryptamine. With the isolation of indoleacetonitrile (IAN) from plants[40,41] and the demonstration of its convertibility to IAA[10,42], it was proposed that IAN and possibly indoleacetamide (IAM) might be on the direct pathway between tryptophan and IAA[40]. However, later work showed that IAM arises from exogenous IAA added to respiring plants[43], and it has been speculated further that IAN might arise as a hydrated condensation product of IAc and hydroxylamine[21].

The demonstration that INH inhibits both plant growth and the conversion

of tryptophan to IAA, especially when considered in conjunction with certain previously reported findings of others, permits some conclusions to be drawn regarding the principle biosynthetic pathway involved in higher plants. Gordon[20] has reported that isopropyl-isonicotinic acid hydrazide (Iproniazid, Marsilid), a potent monoamine oxidase inhibitor, completely blocked conversion of tryptamine to IAA by plant tissue, but had no inhibitory effect upon the conversion of tryptophan to IAA or upon plant growth. This result, by itself, suggests that the pathway is not through tryptamine, leaving as the most likely alternative, the tryptophan → indolepyruvic acid → indoleacetaldehyde → indoleacetic acid pathway, as Thimann's work[10,44] had previously suggested. The results obtained with INH contained in the present report add further support to this hypothesis, since INH, which has little or no effect on monoamine oxidase[45,46], is a potent inhibitor of both transamination[47] and decarboxylation reactions[36,37] by virtue of its ability to form the isonicotinyl-hydrazone, depriving the above enzymes of their requisite coenzymes. Conversely, the isopropyl analogue is a potent monoamine oxidase inhibitor[45,46]; but cannot bind with pyridoxal to form a hydrazone and consequently does not inhibit transaminases or decarboxylases[36].

Therefore, we conclude that INH probably inhibits plant growth by blocking the conversion of tryptophan to indoleacetic acid. The probable mechanism is inhibition of an essential transaminase involved in converting tryptophan to indolepyruvic acid due to formation of the isonicotinyl-hydrazone with pyridoxal, ultimately depriving the enzyme of its requisite co-factor. McCormick and Snell[48] have shown that pyridoxal-isonicotinyl hydrazone is a potent inhibitor of pyridoxal-phosphate kinase of brain tissue. They suggest that this mechanism is at least partly responsible for the depletion of pyridoxal phosphate in a tissue, and thus for the physiological effects of INH.

The next step in the sequence, the decarboxylation of IPyA, would be expected to resemble pyruvate oxidation which requires co-factors other than pyridoxal phosphate and, therefore, would not be likely to be inhibited by INH. The decarboxylation of tryptophan to tryptamine could conceivably be inhibited by INH, but as mentioned above, the previously reported results obtained with iproniazid[20] provide evidence against tryptamine being on the main biosynthetic pathway. The pathway proposed by which IAA would be formed from tryptophan through intermediate participation of indoleacetonitrile[40] would require an oxidation followed by decarboxylation, or an oxidative decarboxylation followed by subsequent oxidation and hydrolysis (Fig. 6). None of these reactions would be expected to require pyridoxal phosphate as a co-factor, hence, inhibition by INH cannot be ascribed to this pathway.

Although these results point toward the tryptophan → IPyA → IAc → IAA pathway being the major one under normal conditions, they do not exclude the possibility of tryptamine or indoleacetonitrile being intermediates in the alternate routes that become active under certain circumstances[18–20]. Indeed, the conversion by plant tissue of radioactively labelled tryptophan to both tryptamine and indoleacetonitrile has been demonstrated[21]; but even in those experiments the main pathway appeared to be through IPyA and IAc.

It is also suggested by these studies that at least one of the specific functions of pyridoxine, which has been shown to be a requirement for growth of tomatoes

and other higher plants[28], is to provide coenzyme (pyridoxal phosphate) needed for a transaminase which is, in turn, required for the synthesis of the main plant growth hormone, indoleacetic acid.

REFERENCES

1. Jones, W. A. and Jones, G. P. *Lancet* **1** (1953) 1073.
2. Gammon, G. D., Burge, F. W. and King, G. *Arch. Neurol. Psychiat.* **70** (1953) 64.
3. Biehl, J. P. and Skavlem, J. H. *Am. Rev. Tuberc.* **68** (1953) 296.
4. Loring, H. N. *Am. Rev. Tuberc.* **68** (1953) 458.
5. Vilter, R. W., Mueller, J. F., Glazer, H. S., Jarrold, T., Abraham, J. P., Thompson, C. and Hawkins, V. R. *J. Lab. Clin. Med.* **42** (1953) 335.
6. Biehl, J. P. and Vilter, R. W. *Proc. Soc. Exptl. Biol. Med.* **85** (1954) 389.
7. Biehl, J. P. and Vilter, R. W. *J. Am. Med. Assoc.* **156** (1954) 1549.
8. Sah, P. P. T. *J. Am. Chem. Soc.* **76** (1954) 300.
9. Thimann, K. V. *J. Biol. Chem.* **109** (1935) 279.
10. Stowe, B. and Thimann, K. V. *Arch. Biochem. Biophys.* **51** (1954) 499.
11. Berger, J. and Avery, G. S. *Am. J. Botany.* **31** (1944) 199.
12. Haagen-Smit, A., Leech, W. D. and Bergren, W. R. *Am. J. Botany.* **29** (1942) 500.
13. Haagen-Smit, A., Dandliker, W., Wittwer, S. and Murneck, A. *Am. J. Botany.* **33** (1946) 118.
14. Wildman, S. G., Ferri, M. G. and Bonner, J. *Arch. Biochem.* **13** (1947) 131.
15. Wildman, S. G. and Bonner, J. *Am. J. Botany.* **35** (1948) 740.
16. Tsui, C. *Am. J. Botany.* **35** (1948) 172.
17. Wildman, S. G. and Muir, R. M. *Plant Physiol.* **24** (1949) 84.
18. Gordon, S. A. and Nieva, F. S. *Arch. Biochem.* **20** (1949) 356.
19. Gordon, S. A. and Nieva, F. S. *Arch. Biochem.* **20** (1949) 367.
20. Gordon, S. A. In Wain, R. L. and Wightman, F. (Ed) *The Chemistry and Mode of Action of Plant Growth Substances,* Academic Press, New York, 1952, p. 65.
21. Dannenberg, W. N. and Liverman, J. L. *Plant Physiol.* **32** (1957) 263.
22. Larsen, P. *Ann. Rev. Plant Physiol.* **2** (1951) 169.
23. Snell, E. E. *J. Am. Chem. Soc.* **67** (1945) 194.
24. Schlenk, F. and Snell, E. E. *J. Biol. Chem.* **157** (1945) 425.
25. Meister, A. *Science* **120** (1954) 43.
26. Umbreit, W. W. and Gunsalus, I. C. *J. Biol. Chem.* **159** (1945) 333.
27. Lichstein, H. C., Gunsalus, I. C. and Umbreit, W. W. *J. Biol. Chem.* **161** (1945) 311.
28. Bonner, J. and Bonner, J. *Vitamins Hormones* **6** (1948) 225.
29. Hoagland, M. B. *Arch. Biochem. Biophys.* **35** (1952) 249.
30. Henderson, J. H. M. and Bonner, J. *Am. J. Botany.* **39** (1952) 444.
31. Jepson, J. B. *Lancet* **2** (1955) 1009.
32. Gordon, S. A. and Weber, R. P. *Plant Physiol.* **26** (1951) 192.
33. Boone, I. U. and Woodward, K. T. *Proc. Soc. Exptl. Biol. Med.* **84** (1953) 292.
34. Yoneda, M., Kato, N. and Okajima, M. *Nature* **170** (1952) 803.
35. Yoneda, M. and Asano, N. *Science* **117** (1953) 277.
36. Davison, A. N. *Biochim. Biophys. Acta* **19** (1956) 131.
37. Hoare, D. S. *Biochim. Biophys. Acta* **19** (1956) 141.
38. Wort, D. J. *Science* **120** (1954) 72.
39. Skoog, F. *J. Gen. Physiol.* **20** (1937) 311.
40. Jones, E. R. H., Henbest, H. B., Smith, G. F. and Bentley, J. A. *Nature* **169** (1952) 485.
41. Henbest, H. B., Jones, E. R. H. and Smith, G. F. *J. Chem. Soc.* **1953** 3796.
42. Thimann, K. V. *Arch. Biochem. Biophys.* **44** (1953) 242.
43. Good, N. E., Andreae, W. A. and Van Ysselstein, M. W. H. *Plant Physiol.* **31** (1956) 231.
44. Stowe, B. B. and Thimann, K. V. *Nature* **172** (1953) 764.
45. Zeller, E. A. and Barsky, J. *Proc. Soc. Exptl. Biol. Med.* **81** (1952) 459.
46. Zeller, E. A., Barsky, J., Fonts, J. R., Kirchheimer, W. F. and Van Orden, L. S. *Experientia* **8** (1952) 349.
47. Meister, A. and Downey, P. F. *Proc. Soc. Exptl. Biol. Med.* **91** (1956) 49.
48. McCormick, D. B. and Snell, E. E. *Proc. Natl. Acad. Sci. U. S.* **45** (1959) 1371.

Received April 2, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 332 – S 338

Studies on Myeloperoxidase Activity

I. Spectrophotometry of the MPO-H_2O_2 Compound

KJELL AGNER

Department of Clinical Chemistry, S:t Görans Hospital, Stockholm, Sweden

Myeloperoxidase, MPO, forms a dissociable hydrogen peroxide compound with an apparent dissociation constant $K = 1 \times 10^{-4}$ M.

The hydrogen peroxide compound formed at peroxide concentrations with a numerical value below the one found for the dissociation constant contains only one perhydroxyl per molecule. This compound is stable and may be used for the oxidation of suitable substrates.

Each of the two iron atoms in the MPO molecule combines at increased hydrogen peroxide concentrations with separate perhydroxyls. The latter are most probably held by the iron atoms in a favourable position for a mutual exchange of electrons, which process initiates reactions resulting in a disintegration of the peroxide into water and molecular oxygen.

Hydrogen peroxide concentrations above 2×10^{-3} M destroy the iron porfyrin structure and denature the enzyme.

The majority of the white blood cells, *i. e.* the myelocytes or granulocytes, enclose in their granules large quantities of the green coloured protein originally named verdoperoxidase and later renamed myeloperoxidase, MPO[1]. Besides the protein moiety, which has a basic character – I. P. > pH 10 –, the MPO molecule contains two identical iron porfyrin structures[2,3]. MPO was originally termed a peroxidase because of its capacity to form a spectroscopically demonstrable peroxide compound and to use the peroxide in this complex in a subsequent oxidative reaction when suitable substrates were added.

The peroxidative activity of the MPO was early found to differ in many respects from those of peroxidases of other origin, especially peroxidases from the vegetable kingdom. Its activity could not be checked by the usual peroxidase activity tests. MPO was inhibited and even destroyed by the prescribed peroxide concentrations. Some recent investigations have even more strongly emphazised the importance of an adequte control of the hydrogen peroxide concentration when the catalytic activity of MPO is to be tested[4]. It has been found that MPO can utilize hydrogen peroxide for peroxidation most effectively, if the hydrogen peroxide concentration is kept extremely low. Certain reactions require a con-

tinuous addition at a rate even as slow as 5 – 10 moles per mole of MPO per min if they are to be brought into effect at all.

It has for these reasons been found important to make a thorough study of the properties of the MPO-hydrogen peroxide compound at various hydrogen peroxide concentrations. The present investigation has been restricted mainly to spectrophotometry of these compounds.

EXPERIMENTAL

Material. The MPO used in these investigations was prepared according to the procedure published in 1958[2]. The analytical data for this preparation were almost identical with those given in that report: Fe-content 0.074 % and an absorbancy value of 1.2 at 427 mμ for the concentration 1 mg MPO per ml.

Technique. Light absorption measurements have been carried out using a Zeiss PMQ 11 recording spectrophotometer, and 10 mm optical cells. As the light passes the optical cells twice in this instrument the recorded values have been devided by two in order to refer the experimental data to an absorbing path of 10 mm.

Evolution of molecular oxygen from hydrogen peroxide has been measured by the use of Warburg-apparatus "V 85", B. Braun Co. The test solution were prior to the experiments freed from dissolved oxygen and kept under argon. The evolution of oxygen was measured by the usual technique and the total amount calculated as the sum of the gaseous and the dissolved oxygen. The amount of MPO used was calculated to be large enough for complete disintegration of the hydrogen peroxide.

Experiments. Measurements of the absorbancy of the MPO · H_2O_2 compound in equilibrium with the remaining MPO have been carried out by recording:

1. the values within the whole spectral region 700 – 400 mμ, Figs. 1 and 2, or the values at wavelengths for the maximal increase and decrease, 452 and 427 mμ, as soon as possible after the addition of H_2O_2 and the formation of the MPO · H_2O_2 compound, Fig. 6.
2. the differences between the absorbancy values for MPO and the values for the MPO – – MPO · H_2O_2 mixture at specified H_2O_2 concentration, Fig. 3.
3. the absorbancy at 452, 443 and 427 mμ during the thirty min period following the establishment of the equilibrium state between MPO and its H_2O_2 compound, Fig. 4. Fig. 5 reproduces values estimated for the hydrogen peroxide concentration during this 30 min period.

Manometric measurements of the effect of MPO upon hydrogen peroxide, experiment 7, have, when tested by Warburg technique, given the following results:

H_2O_2 added:	O_2 evolved:
3 ml 2×10^{-3} M; 6×10^{-6} moles	3.4×10^{-6} moles
3 ml 1×10^{-3} M; 3×10^{-6} „	1.2×10^{-6} „
3 ml 0.5×10^{-3} M; 1.5×10^{-6} „	0.4×10^{-6} „

RESULTS

The light absorption spectrum for MPO has within the visible part of the spectrum, 400–700 mμ, two well-defined absorption maxima at 565 and 427 mμ, and three minor ones at 675, 620 and 490 mμ.

The addition of hydrogen peroxide results in the formation of an MPO · H_2O_2 compound, which spectrophotometrically is characterized by increased absorbancy in the wave-length regions 675–590, max. 622 mμ and 490–433, max. 452 mμ, Figs. 1 and 2, dotted lines.

Fig. 1 demonstrates the absorbancy for the MPO · H_2O_2 compound at an initial hydrogen peroxide concentration of 2×10^{-4} M and after various intervals of time. The hydrogen peroxide complex formed is fairly stable. The hydrogen peroxide disintegrates slowly. Twenty-four hours after the addition of the peroxide the absorbancy curve has returned to its original appearance. In the experiment demonstrated in Fig. 2 the hydrogen peroxide concentration is initially ten times higher than in the one discussed above. During the first 10 min there

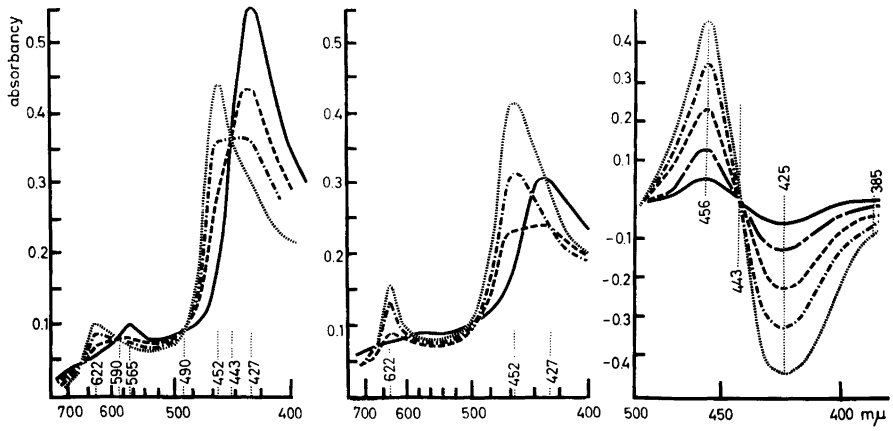


Fig. 1. Spectra of MPO, 0.46 mg/ml (Fe-conc. 6.14×10^{-6} M), after addition of H_2O_2, 2×10^{-4} M:

immediately .....
after 30 min · · · · · · · · ·
after 90 min - - - - - - - - -
after 24 h ————————

Fig. 2. Spectra of MPO, 0.46 mg/ml (Fe-conc. 6.14×10^{-6} M), after addition of H_2O_2, 2×10^{-3} M:

immediately .....
after 5 min · · · · · · · · ·
after 20 min - - - - - - - - -
after 24 h ————————

Fig. 3. Difference spectra between MPO and its H_2O_2-compound. MPO-conc. 0.92 mg/ml (Fe-conc. 12.3×10^{-6} M).

1.96×10^{-4} M H_2O_2 .....
1.28×10^{-4} M H_2O_2 · - · - · -
6.4×10^{-5} M H_2O_2 - - - - - -
3.2×10^{-5} M H_2O_2 —— - - ——
1.6×10^{-5} M H_2O_2 ————

is a continuous disappearance of absorbancy over the whole spectral region, while at the same time the shape of the absorption curve, characteristic for the peroxide complex, is retained throughout. This indicates a saturation of the MPO with peroxide and a destruction of the enzyme during that period of time, when hydrogen peroxide was bound to the iron porfyrin structures. Not until the amount of hydrogen peroxide exceeding the level of about 1×10^{-4} M has become disintegrated the spectrum will adopt the characteristics of the non-complex-bound enzyme.

The absorption spectrum of MPO becomes completely destroeyed and its protein moiety denatured in experiments with an initial hydrogen-peroxide concentration of 2×10^{-3} M and higher.

By the symmetrically shaped curves and the well-defined isosbestic points, 490 and 443 mμ, Fig. 3, the differences in absorbancy for test solutions of which one contains MPO only and the other a mixture of MPO and MPO · H_2O_2 in proportions determined by the concentration of hydrogen peroxide, demonstrates that the two iron atoms in each MPO molecule most probably form identical peroxide compounds, and also that each iron atom forms only one type of hydrogen peroxide complex. This statement is valid for hydrogen peroxide concentrations below the level of 2×10^{-4} M. At concentrations above this value the absorbancy increases at 622 mμ more than expected. At 1×10^{-3} M, for instance, there is a continuous increase at 622 mμ during the initial 30–40 sec, while at 452 mμ the absorbancy value at the same time decreases as a consequence of the destruction of the enzyme. This increase in absorbancy at 622 mμ seems, despite the fact that part of the iron-porphyrin structures is destroyed, to indicate the formation of a reaction product which can provoke an analogous and more pronounced light absorption then the perhydroxyls themselves.

Fig. 4 demonstrates absorbancy values at 452 mμ (max. increase for the H_2O_2 compound), 443 mμ (isosbestic point), and 427 mμ (max. decrease) during a thirty min period following the addition of hydrogen peroxide. The initial hydrogen-peroxide concentrations were 2×10^{-4}, 2.5×10^{-4}, 5×10^{-4} and 10×10^{-4} M. The curves for the absorbancy values at 452 and 427

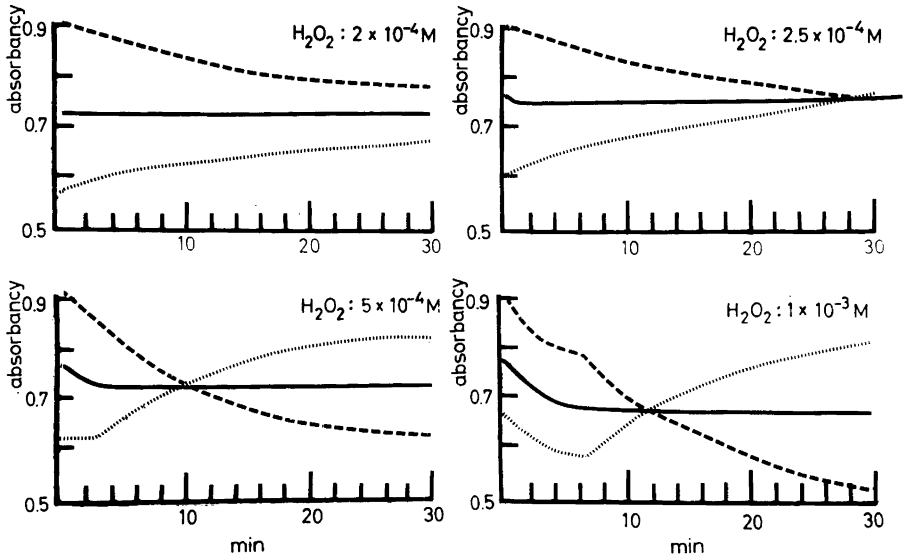


Fig. 4. Absorbancy readings for the MPO-H_2O_2 compound during a 30 min period after admixture of H_2O_2 in amounts giving the concentrations indicated in the figure. MPO-conc. 0.46 mg/ml = 3.07 × 10^{-6} M. (Fe-conc. 6.14 × 10^{-6} M). Absorbancy at 452 mμ - - - - - - - - -; 443 mμ ——————; and 427 mμ ·····. The lines indicating the continuous readings intersect when the H_2O_2-conc. is 0.75 × 10^{-4} M.

mμ are only slightly convergent for the experiment where the initial hydrogen peroxide concentrations is 2 × 10^{-4} M. The absorbancy values after a thirty min period correspond to a concentration of about 1.5 × 10^{-4} M. An increase in the initial H_2O_2 concentration from 2 × 10^{-4} to 2.5 × 10^{-4} M results in a significantly faster hydrogen peroxide disintegration. The concentration decreases during the thirty min period from 2.5 × 10^{-4} to 0.75 × 10^{-4} M. The hydrogen peroxide concentration values decrease in the other two experiments in Fig. 4 with a pronounced increased rate. The initially high values, 5 × 10^{-4} and 10 × 10^{-4} M, reach the 0.75 × 10^{-4} M level after 10 and 12 min respectively.

The absorbancy values as well as the ratio between them in these experiments depend upon the hydrogen peroxide concentrations. Thus, knowledge of the values for the absorbancy at 452 and 427 mμ makes it possible to calculate the hydrogen peroxide concentrations at any time during the experiment. Fig. 5 summarizes calculations made for six different experiments. It is quite evident that the hydrogen peroxide decomposes most rapidly in the experiment with the highest initial hydrogen peroxide concentration. It is also clear that the fast initial disintegration does not decline when the concentration reaches a certain lower level. It continues even after the concentration has decreased below the value reached in experiments with a low initial hydrogen peroxide concentration. A theory to explain these facts will be discussed below. According to this theory it seems most likely that one peroxide molecule attaches to each of the two iron atoms and that these two molecules react with each other. The product formed might thereafter react with excess of hydrogen peroxide and disintegrate into water and molecular oxygen. It seems likely that this intermediate may have a structure able to give the increased absorbancy at 622 mμ, discussed above.

The absorbancy readings at 452 and 427 mμ from twentyfour separate experiments with varying initial hydrogen peroxide concentrations have been plotted versus the negative logarithm for the hydrogen peroxide concentration in Fig. 6. The absorbancy values indicate the ratio of iron atoms forming a peroxide complex to non-complex-bound atoms. It has therefore been possible to use these experimental values for a calculation of this ratio and to plot these

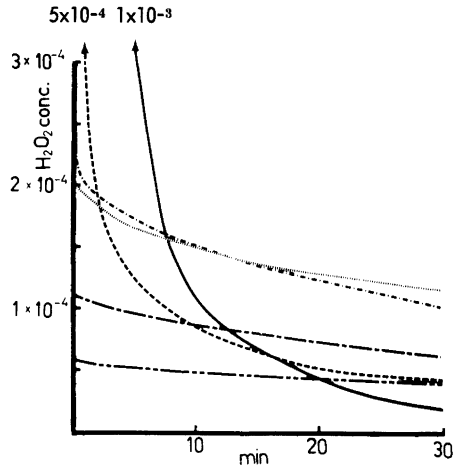


Fig. 5. Decrease of H_2O_2 conc. during the first 30 min period following the addition of H_2O_2 to MPO, calculated from absorbancy values found in experiments with known H_2O_2 concentration. MPO-conc.: 0.46 mg/ml = 3.07×10^{-6} M. Initial conc. of H_2O_2 is indicated by the intercent on the axis of ordinates.

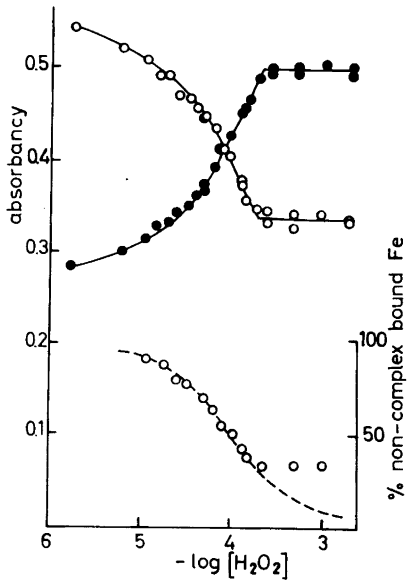


Fig. 6. Upper curves: Absorbancy readings for MPO – MPO · H_2O_2 at 427 ⊙ and 452 mμ ⊗ in solutions with known H_2O_2 concentrations. MPO-conc. 0.46 mg/ml = 3.07×10^{-6} M. (Fe-conc. 6.14×10^{-6} M).
Lower curve: The percentage of iron atoms not combined with H_2O_2 as a function of H_2O_2 conc. ⊙ : Evaluated values from the experimental points in the upper curves.
Dotted line: Theoretically calculated dissociation curve on the assumption of a pK_{app} value of 4.0.

results on a diagram in the same figure. The distribution of the experimental points agree with a theoretical, simple dissociation curve with an apparent dissociation constant of 1×10^{-4} M.

The results from the manometric measurements in experiment 7 show that the disappearence of hydrogen peroxide in the expriments demonstrated by Fig. 4 is followed by a formation of molecular oxygen. Thus, MPO acts in these experiments as a catalase. The ratio between the number of moles of oxygen formed per mole of hydrogen peroxide varies in the different experiments. Therefore, the results obtained can not at present be used for a true estimation of the number of hydrogen peroxide molecules used up in the reactions resulting in its disintegration into oxygen and water.

DISCUSSION

Each MPO molecule contains two identical iron-porphyrin structures, and each of these enters, on the addition of hydrogen peroxide, into a dissociation equilibrium:

$$\begin{bmatrix} Fe^{3+} \\ Fe^{3+} \end{bmatrix} + H_2O_2 \rightleftarrows \begin{bmatrix} Fe \cdot H_2O_2 \\ Fe^{3+} \end{bmatrix} \text{ or } \begin{bmatrix} Fe^{3+} \\ Fe \cdot H_2O_2 \end{bmatrix} \quad (1)$$

where the MPO molecule with its two iron atoms is described by the

symbol $\left[\begin{matrix}Fe^{3+}\\ \\ Fe^{3+}\end{matrix}\right.$

The value for the apparent dissociation constant, 1×10^{-4} M, has been determined.

The complex formed is fairly stable when the hydrogen-peroxide concentration is kept below the value for the dissociation constant, *i. e.* when on an average not more than one of the two iron atoms is occupied by peroxide.

Both iron atoms take up hydrogen peroxide at higher peroxide concentration. Formation of the compound with two perhydroxyls in the same molecule is followed by a decomposition of hydrogen peroxide. If the formation and consumption of intermediates are neglected, the reaction may be written:

$$\left[\begin{matrix}Fe \cdot H_2O_2\\ \\ Fe \cdot H_2O_2\end{matrix}\right. \rightarrow \left[\begin{matrix}Fe^{3+}\\ \\ Fe^{3+}\end{matrix}\right. + 2\ H_2O + O_2 \qquad (2)$$

Provided that its concentration does not exceed a limit of about 2×10^{-3} M, hydrogen peroxide decomposes faster, the higher the initial hydrogen peroxide concentration. At and above this concentration the iron-porfyrin structures are destroyed and the enzyme denatured by intermediates formed and accumulated at the increased decomposition rate.

The results obtained highly emphasizes the close relationship between peroxidative and hydrogen-peroxide decomposing effects for catalysts containing two or more structures able to form weakly dissociated hydrogen peroxide compounds. The compound formed by attaching only one hydrogen peroxide per molecule is stable and may be used for the oxidation of substrates. The formation of a compound with two hydrogen peroxide molecules bound to favourably located active centres with adaptable charge might result in a mutual exchange of electrons and the generation of a highly reactive derivative, presumably an ozonide. This might then decompose rapidly in the presence of an excess of hydrogen peroxide through the over-all reaction:

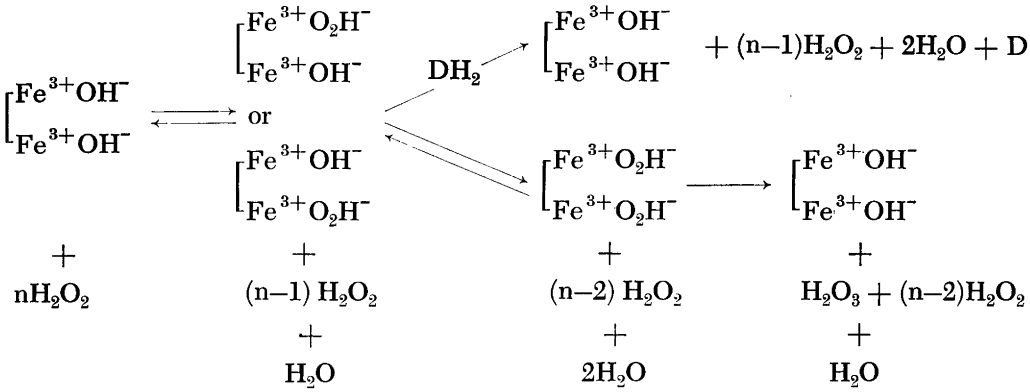
$$H_2O_3 + 2\ H_2O_2 \rightarrow 3\ H_2O + 2\ O_2 \qquad (3)$$

Hydrogen peroxide has complete electron shells and its electrons have a symmetrical distribution. Acceptance of electrons from donors and a mutual exchange between two hydrogen-peroxide molecules within the same MPO structure necessitate a shift of charge, a break in the state of equilibrium and even disruption of the molecule. This is most probably brought into effect by the charge capacity of the tri-valent porphyrin iron.

The results described do not permit a definite decision as to whether hydrogen peroxide forms its complex with the iron atoms in competition with some other group whose concentration may be dependent upon the actual hydrogen ion concentration, *e. g.* hydroxyl. Variations in pH values from 5 to 9 in experiments with an equal hydrogen-peroxide concentration do not have any influence upon the spectral reading indicating the ratio between complex bound to non-complex bound iron. However, as will be described and discussed in a forthcoming report, the formation of a chloride compound is governed not only by the chloride ion,

but also by the hydroxyl ion concentration in conformity with the law of mass action. These differences in the two types of experiment may well be explained by the fact that hydrogen peroxide is a weak acid, $K = 1.5 \times 10^{-12}$. The perhydroxyl ion concentration is therefore dependent upon the actual pH value, while the chloride ion concentration is independent of this. A shift in the hydrogen ion concentration will influence the dissociation of hydrogen peroxide to the same extent but in opposite direction as it influences a tri-valent iron-hydroxyl group of the MPO. The probability of a competitive mechanism operating in the formation of the hydrogen peroxide compound supports the idea that the complex is established through an attraction between the tri-valent iron and the negatively charged perhydroxyl ions, and not as result of juxtaposition with molecular hydrogen peroxide.

Spectrophotometry registers changes in absorbancy only and gives no information concerning the character of the exposed reactions or concerning the physicochemical properties of the reaction products. Complementary investigations with other techniques will be required for a more complete understanding of the reactions discussed. However, even with our present partial knowledge it may be permissible to summarize the experimental results and considerations in the foregoing in a schematic formula indicating the processes initiated by MPO in a milieu containing hydrogen peroxide together with substances acting as hydrogen donors, DH_2, or hydrogen-peroxide only.



$$H_2O_3 + (n-2)H_2O_2 \rightarrow 3H_2O + 2O_2 + (n-4)H_2O_2$$

Acknowledgement. This investigation has been supported by a grant from *Statens Medicinska Forskningsråd.*

REFERENCES

1. Agner, Kj. *Acta Physiol. Scand.* **2** (1941) *Suppl. 8.*
2. Agner, Kj. *Acta Chem. Scand.* **12** (1958) 89.
3. Ehrenberg, A. and Agner, Kj. *Acta Chem. Scand.* **12** (1958) 95.
4. *Unpublished observations.*

Received April 17, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 339–S 342

The Theorell-Chance Mechanism

JOHN S. McKINLEY-McKEE

Medical Research Council and Postgraduate Medical School, Hammersmith Hospital, London, England

The Theorell-Chance compulsory order mechanism applies to liver alcohol dehydrogenase. This appears dependent on the coenzyme first attaching and inducing an appropriate conformational change in the enzyme before the substrate can attach.

The Theorell-Chance mechanism[1] was put forward for the liver alcohol dehydrogenase-coenzyme system* in 1951. It is to the credit of the authors that our thinking about the mechanism of action of the dehydrogenases is to-day dominated by this mechanism. It has been considered to also apply to yeast alcohol dehydrogenase[2], muscle lactic dehydrogenase[3], malic dehydrogenase[4], and possibly ribitol dehydrogenase[5]. The mechanism implies that only the binary coenzyme complexes are rate limiting.

$$E + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ER$$

$$ER + ald \overset{k_3}{\rightleftharpoons} (ERald \rightleftharpoons EOalc) \underset{k_3'}{\rightleftharpoons} alc + EO$$

$$EO \underset{k_1'}{\overset{k_2'}{\rightleftharpoons}} E + O$$

For LADH after first receiving support[6], the mechanism was questioned on the basis that the ternary complexes were also rate limiting[7–9]. This was later shown not to be the case, as sensitive kinetic tests for the mechanism[10], were fulfilled[11]. The accuracy of these experiments is exemplified by the fact that $^{Km}(DPNH) \times {}^{Km}(DPN)/K_{E,R} \times K_{E,O}$ was 0.8. Theoretically it should be $k_2'/k_1 \times k_1/k_2 \times k_2/k_1' \times k_1'/k_2'$ or unity[11]. Experiments with inhibitors[12] also showed

* Abbreviations: LADH: Liver alcohol dehydrogenase; E: μN LADH, since each enzyme molecule has two independent binding sites; R and O: Reduced and oxidized diphosphopyridine nucleotide, DPNH and DPN.

Theorell-Chance kinetics. However, they did not rule out the possibility that the general two substrate mechanism applied with certain restrictions and that the binary enzyme-substrate complexes Eald and Ealc with dissociation constants of 10 μM and 6100 μM might also exist[11]. The existence of these complexes with stabilities as indicated has now been tested.

EXPERIMENTAL AND RESULTS

(1) At pH 7 and 9, alcohol or aldehyde concentrations of up to 0.2 M do not protect LADH against inactivation by iodoacetamide while small coenzyme concentrations do[13].

(2) Experiments using a recording spectrophotofluorometer. When (a) 1 μM enzyme was excited at 280 mμ and the protein fluorescence at 350 mμ measured and (b) 1 μM LADH-DPNH complex was excited at 280 mμ and 330 mμ and the resultant protein fluorescence at 350 mμ and coenzyme fluorescence at 460 mμ examined, in no case was the fluorescence appreciably affected by alcohol concentrations of up to 0.61 M. While the protein fluorescence might not be expected to reflect $E \rightleftharpoons Ealc$ or $ER \rightleftharpoons E \rightleftharpoons Ealc$ competition, the coenzyme fluorescence certainly would. Acetaldehyde has an absorption maximum at 277 mμ, and experiments which had to use the protein fluorescence were therefore more difficult to carry out. However, there was no evidence for an enzyme acetaldehyde complex with a dissociation constant of 10 μM.

(3) In optical rotatory dispersion experiments the Cotton effect at 325 mμ due to the LADH-DPNH complex[14] was also not affected by high alcohol concentrations. A Bellingham and Stanley automatic spectropolarimeter[15] was used with enzyme, coenzyme and alcohol concentrations of 20 μN, 40 μM and 140 mM, respectively, in 0.2 μ phosphate, pH 7.1.

DISCUSSION

The experiments indicate that binary enzyme-substrate complexes with stabilities as indicated from inhibition experiments[12] do not exist. The competitive behaviour from which they were adduced must be considered to involve ternary complexes. Lack of agreement in the enzyme-alcohol dissociation constants obtained from the inhibition experiments with imidazole and caprate, had already caused their existence to be in doubt. Thus, there is now no longer any need to consider non coenzyme containing ternary complexes or whether the substrates affect the "on" velocity of the enzyme with the coenzyme[11]. The Theorell-Chance mechanism with rate limiting binary complexes and non rate limiting ternary complexes must be considered to apply in its entirety. The ternary complexes were of course manifest in the experiments with imidazole and inhibitors[12]. They can also be demonstrated by product inhibition experiments[16,17].

Recently on the basis of isotope experiments the Theorell-Chance mechanism has been questioned for the alcohol dehydrogenases [18,19] and for lactic dehydrogenase[20]. With A-DPND or CH_3CD_2OH, it is a consequence of the mechanism that k_2' $(1/\Phi_0)$ or k_1' $(1/\Phi_1')$ should show no isotope effects. For rabbit muscle lactic dehydrogenase Φ_0^H/Φ_0^D was 0.71[20]. However, this may be due to the fact that the reaction from the reduced coenzyme side is particularly susceptible to

inhibitors[21], and DPND preparations have been repeatedly found to be more impure than commercial DPNH. Impurities would result in k_2' for DPND being too low, Φ_0^D too high, and the ratio less than unity. This appears to also apply to the deuterium work with the alcohol dehydrogenases. With LADH although the results are somewhat invalidated by the type of buffer used, there was as would be expected no isotope effect on k_1', but Φ_0^H/Φ_0^D was 0.63[18]. For the yeast enzyme, where binary substrate complexes have been assumed, the latter value was 0.57[19].

The problem of purity may also arise with the substrates. On the basis of the Theorell-Chance mechanism different alcohols and aldehydes should not affect k_2' $(1/\Phi_0)$ or k_2 $(1/\Phi_0')$. With LADH for some twentyseven different alcohols and aldehydes, they have been stated to do so[22], although the three carbon homologues behave as expected, showing no such influence[21]. However, unless the substrates are carefully purified spurious effects could be caused by inhibitors present. These problems are currently being examined using 3,3-dimethylallyl alcohol and 1,1-dideutero-3,3-dimethylallyl alcohol purified by gas chromatography.

It is interesting to enquire why no binary substrate complexes are formed and why the maximum rate is the rate of breakdown of the binary coenzyme complexes. Experiments with the thiol reagent iodoacetamide designed to test the theory of conjugate catalysis[13,23] bear on this. Iodoacetamide reacts much more slowly than mercurials with thiol groups and so protection effects can be studied with greater precision. Protection against iodoacetamide by DPNH and DPNH + + isobutyramide was found to depend on the amount of ER formed. Isobutyramide appeared to exert its effect not by tightening the complex further but by increasing the amount of coenzyme complexed[24]. The experiments indicated that protection was by the coenzyme alone. A conformational change with a resultant change in the distribution of the polar-non polar groups in the enzyme must be involved when the coenzyme attaches[24], and the rate limiting step is the conformation change involved when ER or EO break down. It seems likely that the octahedral zinc in the coenzyme complexes, with three bonds to the protein, two to the coenzyme-adenine and one to water or the substrate[12,25], reverts to the tetrahedral configuration in the free enzyme.

It may be general for pyridine nucleotide coenzyme reactions that as for LADH a compulsory order mechanism is involved due to the coenzyme inducing a conformational change which is necessary before the substrate can attach. Also the stability of ternary substrate complexes may be determined by non aqueous properties of the enzyme, such that they dissociate as well as interconvert so rapidly that they do not affect the rate and give added significance to the Theorell-Chance mecha[illegible].

REFERENCES

1. Theorell, H. and Chance, B. *Acta Chem. Scand.* **5** (1951) 1127.
2. Vallee, B. L. *The Enzymes*, Academic Press, New York and London, 1960, Vol. III, p. 247.
3. Zewe, V. and Fromm, H. J. *J. Biol. Chem.* **237** (1962) 1668.
4. Ravel, D. N. and Wolfe, R. G. *Biochemistry* **1** (1962) 263.
5. Fromm, H. J. and Nelson, D. R. *J. Biol. Chem.* **237** (1962) 215.

6. Theorell, H., Nygaard, A. P. and Bonnichsen, R. *Acta Chem. Scand.* **9** (1955) 1148.
7. Dalziel, K. and Theorell, H. *Biochem. J.* **66** (1957) 34 P.
8. Theorell, H. *Advan. Enzymol.* **20** (1958) **471**.
9. Theorell, H. and Winer, A. D. *Arch. Biochem. Biophys.* **83** (1959) 291.
10. Dalziel, K. *Acta Chem. Scand.* **11** (1957) 1706.
11. Theorell, H. and McKinley-McKee, J. S. *Acta Chem. Scand.* **15** (1961) 1797.
12. Theorell, H. and McKinley-McKee, J. S. *Acta Chem. Scand.* **15** (1961) 1834.
13. McKinley-McKee, J. S. *Biochem. J. In press.*
14. Ulmer, D. D., Li, T and Vallee, B. L. *Natl. Acad. Sci.* **47** (1961) 1155.
15. Jennings, J. P. *Biochem. J.* **86** (1963) 16 P.
16. Alberty, R. A. *J. Am. Chem. Soc.* **80** (1958) 1777.
17. Dalziel, K. *Trans. Faraday Soc.* **54** (1958) 1247.
18. Baker, R. H. *Biochemistry* **1** (1962) 41.
19. Shiner, V. J., Mahler, H. R., Baker, R. H. and Hiatt, R. R. *Ann. N. Y. Acad. Sci.* **84** (1960) 583.
20. Thompson, J. F. and Darling, J. J. *Biochem. Biophys. Res. Commun.* **9** (1962) 334.
21. Dalziel, K. *Biochem. J.* **84** (1962) 240, 244.
22. Baker, R. H. *Private communication.*
23. Rabin, B. R. and Whithead, E. P. *Nature* **196** (1962) 658.
24. McKinley-McKee, J. S. *Biochem. J. In press.*
25. McKinley-McKee, J. S. *Biochem. J.* **84** (1962) 70 P; **85** (1962) 24 P.

Received April 8, 1963.

ACTA CHEMICA SCANDINAVICA 17 (1963) S 343–S 347

Crystalline Leghemoglobin

VII. Magnetic and Spectrophotometric Properties of Leghemoglobin and its Derivatives

ANDERS EHRENBERG and NILS ELLFOLK

Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm, Sweden

The paramagnetic susceptibilities of soya bean leghemoglobin (Lhb) and some of its derivatives have been determined. Acidic and alkaline ferriLhb have molar paramagnetic susceptibilities of 9 500 and 5 200$\times 10^{-6}$ cgs emu at 20°C, respectively. The susceptibilities of the complexes of ferriLhb formed upon addition of cyanide, fluoride and acetate and of ferroLhb show no large deviations from values for other hemoglobins. Difference optical absorption spectra at 8 and 38°C show for both acidic and alkaline ferriLhb characteristic maxima and minima. This indicates that both forms of ferriLhb are temperature dependent equilibria between high- and low-spin states. No such temperature dependent difference spectrum was obtained for the acetate complex of ferriLhb, showing that this complex is a pure high spin form, which is also inferred from its magnetic moment. The electron spin resonance at low temperatures verify that acidic ferriLhb is a mixed spin compound.

Leghemoglobin (Lhb), the hemoglobin(Hb) of the root nodules of leguminous plants, has been found to differ from ordinary Hb in several respects. The molecular weight of Lhb is similar to that of myoglobin (Mb), *i. e.* only one fourth of that of Hb[1]. The affinity for oxygen of ferroLhb is even higher than that of Mb. In fact it is the highest measured for any hemoprotein[2]. This might be due to a partially exposed heme group on the surface of the globin molecule of Lhb[3], whereas the heme groups of Hb and Mb are more shielded by the globin helices. It has been shown that a positive correlation exists between the amount of nitrogen fixed and the concentration of Lhb in the nodules[4,5]. More recently it has been demonstrated by gas chromatography that one molecule of Lhb is able to fix one molecule of nitrogen[6]. This interesting observation might help to explain the biological function of Lhb.

In order to understand the reactions of Lhb it seemed desirable to obtain further information about the mode of binding of the heme iron of Lhb to the globin and to other coordinated ligands. In this paper the magnetic properties of Lhb and some of its derivatives are reported and are correlated to spectroscopic data.

EXPERIMENTAL

Leghemoglobin. The *fast* and *slow* components of crystallized Lhb were prepared chromatographically as described previously[7]. The preparations were checked by electrophoresis as well as by light absorption spectroscopy. The concentration of a preparation was evaluated on the basis of determination of the pyridine hemochrome[8]. For the magnetic measurements it was desirable to use concentrated solutions. However, with the technique applied, dialysis of ammonium sulphate precipitate, it was not possible to obtain higher concentrations than about 1 mM.

Magnetic susceptibility measurements. The apparatus described by Theorell and Ehrenberg[9,10] was used. The instrument was calibrated with a dilute solution of nickel chloride for which the molar susceptibility was assumed to be $4\,434 \times 10^{-6}$ cgs emu at 20°C. The calibration constant was 2.200×10^{-11} volume susceptibility units per scale division (μ). The samples were made anaerobic by rapid evacuation in Thunberg tubes and twice flushing with nitrogen or argon. The pH of each sample was adjusted by addition of small amounts of 1 N NaOH for the alkaline pH values and 1 N HCl and/or buffer for other values. Reduction was obtained by addition of 20 μl 15 % $Na_2S_2O_4$ per ml of sample. The reaction with CO was performed by flushing Thunberg tubes with this gas.

ESR (electron spin resonance) measurements were performed with a Varian V-4500 X-band spectrometer with 100 kc/s magnetic field modulation. Liquid nitrogen and variable temperature accessories were used.

pH measurements were made at 20°C with a Radiometer PHM3 pH-meter which was standardized against phtalate and borate buffers.

Spectrophotometric measurements. A Beckman spectrophotometer model DK2 was used. A special cell holder was constructed in order to study the effect of temperature on the spectra of the different Lhb derivatives. Each cuvette in the holder was provided with a separate well insulated thermospacer. By circulating thermostated water from two thermostates through the thermospacers each cuvette could separately be kept at a desired temperature. The temperature was checked in the cuvettes before the spectra were recorded by means of a thermistor in a bridge device. Phosphate and borate buffers with known temperature dependence were used. Care was taken to adjust the high temperature buffer to the same pH at the high temperature as the pH of the low temperature buffer at the low temperature.

RESULTS AND DISCUSSION

The CO compound of ferroLhb was found to be diamagnetic. The diamagnetic susceptibility per g of protein was calculated to be $-0.597\ (\pm 0.015) \times 10^{-6}$ cgs emu, when the value 0.740 ml/g was used for the partial specific volume[1]. The paramagnetic susceptibilities of other Lhb derivatives have been referred to the diamagnetic level of the CO compound, taking into due consideration the residual orbital paramagnetism of the iron of a compound of this type[11].

The paramagnetic data of the derivatives investigated are shown in Table 1. No significant difference could be detected between the fast and slow components. When calculating the mean value of susceptibility from several determinations with different samples the values were given weights in proportion to the concentrations, because the relative errors within a series are roughly inversely proportional to the concentrations. The effective magnetic moments, μ_{eff}, were calculated under the assumption that Curie-Weiss constant was zero.

A comparison of the data of Table 1 and the predicted μ_{eff}-values for different spin forms[12] shows the following. The cyanide complex of ferriLhb is a pure low-spin form with 1 unpaired electron. The fluoride and acetate complexes of ferriLhb are, or very nearly are, pure high-spin forms with 5 unpaired electrons. The ferroLhb at pH 10.5 is a pure high-spin form with 4 unpaired electrons. All

Table 1. Paramagnetic data of some derivatives of leghemoglobin (Lhb).

Derivative	Valence of iron	Component	Conc. μM	pH		$\chi_{Fe} \times 10^6$ cgs emu	μ_{eff} Bohr magnetons
Acidic form	Fe^{3+}	fast	265	6.10		8 900	
		„	265	6.10		7 600	
		„	1 000	5.70		10 100	
		slow	710	5.80		9 700	
					mean	9 500	4.74
Alkaline form	Fe^{3+}	fast	251	10.75		4 500	
		„	251	10.80		4 500	
		„	960	10.80		5 200	
		slow	701	10.80		5 800	
		„	612	10.80		5 000	
					mean	5 200	3.50
Cyanide complex	Fe^{3+}	slow	601	10.80		2 500	
		„	601	10.80		2 200	
					mean	2 350	2.36
Fluoride complex	Fe^{3+}	fast	333	–		13 600	5.66
Acetate complex	Fe^{3+}	slow	640	5.5		14 000	5.75
Ferro Lhb	Fe^{2+}	„	395	10.5		12 300	5.39

these data are similar to what has been found for the corresponding derivatives of other hemoglobins.

The magnetic moments 4.17 B. m. (Bohr magnetons) of the acidic form and 3.50 B. m. of the alkaline form, however, are both intermediate between the expected moments of the high- and low-spin forms, 5.92 and 1.73–2.8 B. m., respectively. On several occassions it has been discussed that intermediate magnetic properties of Hb derivatives were due to mixtures of forms with different numbers of unpaired electrons[13–16]. Experimental support for this hypothesis was obtained by George *et al.*[16], who studied the temperature dependence of the light absorption and the paramagnetic susceptibility of alkaline ferriMb, and further evidence was obtained by Ehrenberg[10] by means of the ESR technique. The temperature dependence of the susceptibility of alkaline ferriHb indicates the presence of a thermally balanced mixture of two spin forms in this case also[12]. Experiments of the mentioned types were thus indicated for both alkaline and acidic Lhb.

The temperature dependence of the light absorption was investigated by recording the difference spectra of samples kept at two different temperatures (Fig. 1). Curves A show that alkaline ferriLhb at low temperatures has increased absorption in the regions 405–475 mμ and 515–580 mμ with maxima of the increase at 425, 540 and 565 mμ. At high temperatures the absorption is increased in the complementary regions with maxima of the increase at 385, 495 and 595 mμ. This is essentially the same as observed by George *et al.*[16] for alkaline ferriMb and would suggest an increase in the portion of low-spin form as the temperature is decreased.

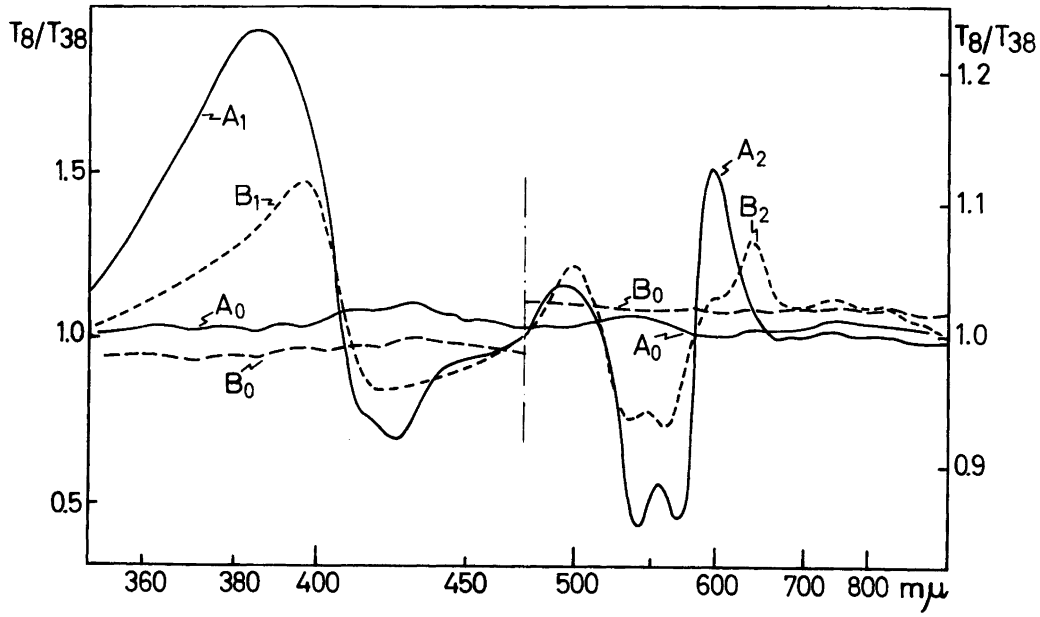


Fig. 1. Difference spectra of ferrileghemoglobin at 8 and 38°C recorded as the transmission ratio T_8/T_{38}. Curve A_1 with ordinate scale to the left, other curves with ordinate scale to the right. ———: glycine buffer pH 10.8, 52.2 μM Lhb, 350–475 mμ (A_1), 475–800 mμ (A_2) and baseline with both samples at room temperature (A_0). – – – –: phosphate buffer pH 5.8, 6.65 μM Lhb at 350—475 mμ (B_1), 66.6 μM Lhb at 475–800 mμ (B_2) and baselines with both samples at room temperature (B_0).

Curves B of Fig. 1 show that the light absorption of acidic Lhb changes in much the same way with temperature as that of alkaline Lhb. The positions of the peaks are, however, slightly shifted. The maximum increase of light absorption at low and high temperatures are at 420, 533 and 558 mμ and at 397, 500 and 635 mμ, respectively. It should be noted that the difference spectra of Fig. 1 are in most respects quite different from those between acidic and alkaline ferriLhb. The size of the changes of the light absorption with temperature suggests that in case of acidic ferriLhb there is a relatively smaller increase in the portion of low-spin form as the temperature is decreased than in case of alkaline ferriLhb.

The light absorption of the acetate complex was investigated in the same way. No temperature effect of the type demonstrated in Fig. 1 was detected. If there was any effect at all it was in the opposite direction. This confirms our conclusion that the acetate complex is a pure high-spin form, which, however, could be dissociated at elevated temperatures.

Frozen solutions of the acetate complex of ferriLhb show the ESR absorption with $g \approx 6$, typical for high-spin heme compounds. This absorption was identical in size and shape to the absorption of acidic ferriMb[10], and both compounds are

classified as essentially pure high-spin forms. Acidic ferriLhb also gives an ESR absorption of the same shape but with reduced intensity. The susceptibility data (Table 1) indicate a high-spin portion of about 60 % for acidic ferriLhb at room temperature. A comparison of the intensities of the ESR absorptions shows that the high-spin portion is only slightly reduced with decreasing temperature and is in the range of 35–50 % at 77°K.

Alkaline ferriLhb has a high-spin portion of about 25 % at room temperature as calculated from the susceptibility (Table 1). The ESR recordings at 77°K revealed only the anisotropic g-values around $g = 2$ typical of low-spin compounds. The concentration of the sample was not high enough to allow a definite conclusion whether any high-spin character remained at 77°K.

The magnetic and spectral data demonstrate unanimously that both acidic and alkaline ferriLhb are thermally balanced mixtures of high- and low-spin forms. The assumption by George *et al.*[16] that alkaline ferriLhb should represent a pure low-spin compound at room temperature has not been confirmed. Acidic ferriLhb has a smaller magnetic moment and more low-spin character than most other "acidic" forms of the hemoproteins investigated so far. In this respect it resembles most closely the intermediate form of the peptic hemopeptide from cytochrome *c*[12,17] and some of the "cytochromoids"[18] which have recently been investigated magnetically[19]. Another common feature of these compounds is that their heme groups are more exposed to the solvent medium than in other related compounds. At the present state of our knowledge it might be permitted to speculate about this feature as a possible common denominator of the increased low-spin character of these compounds.

Acknowledgements: The investigations have been supported by grants from *Statens Medicinska Forskningsråd, Statens Naturvetenskapliga Forskningsråd* and by PHS Research Grant A-5895 from the *National Institute of Arthritis and Metabolic Diseases, Public Health Service.*

REFERENCES

1. Ellfolk, N. *Acta Chem. Scand.* **14** (1960) 1819.
2. Appleby, C. A. *Biochem. Biophys. Acta* **60** (1962) 226.
3. Ellfolk, N. *Acta Chem. Scand.* **15** (1961) 975.
4. Virtanen, A. I., Jorma, J., Linkola, H. and Linnasalmi, A. *Acta Chem. Scand.* **1** (1947) 861.
5. Virtanen, A. J., Erkama, J. and Linkola, H. *Acta Chem. Scand.* **1** (1947) 861.
6. Abel, K., Bauer, N. and Spence, J. T. *Arch. Biochem. Biophys.* **100** (1963) 339.
7. Ellfolk, N. *Acta Chem. Scand.* **14** (1960) 609.
8. Paul, K. G., Theorell, H. and Åkeson, Å. *Acta Chem. Scand.* **7** (1953) 1284.
9. Theorell, H. and Ehrenberg, A. *Arkiv Fysik* **3** (1951) 52.
10. Ehrenberg, A. *Arkiv Fysik* **19** (1962) 119.
11. Griffith, J. S. *Biochim. Biophys. Acta* **28** (1958) 439.
12. Ehrenberg, A. *Svensk Kem. Tidskr.* **74** (1962) 103.
13. Theorell, H. and Ehrenberg, A. *Acta Chem. Scand.* **5** (1961) 823.
14. Scheler, W., Schoffa, G. and Jung, F. *Biochem. Z.* **329** (1957) 232.
15. Havemann, R. and Haberditzl, W. *Z. Phys. Chem.* **209** (1958) 135.
16. George, P., Beetlestone, J. and Griffith, J. S. In Falk, J. E. *et al. Haematin Enzymes,* Pergamon Press, London, 1961, Part 1, p. 105.
17. Paléus, S., Ehrenberg, A. and Tuppy, H. *Acta Chem. Scand.* **9** (1955) 365.
18. Kamen, M. *Acta Chem. Scand.* **17** (1963) Suppl. 1, 41.
19. Ehrenberg, A. and Kamen, M. *In preparation.*

Received April 1, 1963.