TABLE I

KETONIC HYDRAZONES OF TRIAMINOGUANIDINE

Ketone	Yield,[a] %	M.p., °C.	Formula	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
2-Acetylthiophene	98	217	$C_{19}H_{20}S_3N_6$[b]	53.27	53.14	4.67	4.75[c]
3-Acetylthionaphthalene	93	283	$C_{31}H_{26}S_3N_6$[b]	64.35	64.49	4.50	4.53[d]
Alloxan	94	>300	$C_{13}H_{14}N_{12}O_{12}$[e,f]	29.43	29.3	2.64	2.58
Benzylideneacetone	72	125	$C_{31}H_{35}N_7O_4$[g,h]	65.3	65.85	6.16	6.44
Benzoin	97	190	$C_{43}H_{43}N_7O_8$[g,i]	65.73	65.52	5.47	5.0
Chalcone	75	132	$C_{46}H_{39}N_7O_3$[g]	74.9	74.76	5.29	5.39
Cyclohexanone	56.5	135	$C_{19}H_{27}N_9O_7$[g,k,l]	45.41	45.44	5.57	5.62
Dibenzylacetone	95	227	$C_{52}H_{44}N_6$[g]	83.0	83.0	5.85	5.87
m-Cyanoacetophenone	95	237	$C_{25}H_{23}N_9$[b]	69.28	69.47	4.74	5.0

[a] All yields of crude material. [b] Recrystallized from pyridine and therefore tabulated data are those for the hydrazone free base. [c] Calcd. S, 22.43; found S, 22.49. [d] Calcd. S, 16.61; found S, 16.55. [e] Not recrystallized because of insolubility. [f] This formula is for a trihydrate. [g] Recrystallized from 95% alcohol. [h] This formula is for a monohydrate. [i] This formula is for a dihydrate. [k] Dihydrazone isolated as a picrate. [l] Yellow plates analyzing for 0.5 mole of water of crystallization.

crystals of the dipyrazolemonohydrazone, named above, were almost insoluble in ether, soluble in water and in alcohol. The nitron[4] test proved the absence of nitrate. *Anal.* Calcd. for $C_{16}H_{22}N_6O$: C, 61.1; H, 7.0; N, 26.75. Found: C, 60.8; H, 7.1; N, 26.7.

When the experiment was repeated, under reflux, for half an hour and the product precipitated by pouring into water, a 48% yield of a yellow amorphous powder, which after recrystallization from aqueous ethanol melted at 148° was obtained. Its nature is unknown. Its analysis suggested it to be of empirical formula $C_3H_4N_2$ and it did not form a picrate. *Anal.* Calcd. for $C_3H_4N_2$: C, 52.9; H, 5.8; N, 41.2. Found: C, 52.8; H, 5.7; N, 42.2.

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(4) *Cf.* J. E. Heck, H. Hunt and M. G. Mellon, *Analyst*, **59**, 18 (1934).

COMMUNICATIONS TO THE EDITOR

ENZYMATIC DEGRADATION OF THE CHOLESTEROL SIDE CHAIN IN CELL-FREE PREPARATIONS

Sir:

It has been demonstrated by Bloch, *et al.*,[1] that cholesterol can be converted to cholic acid in intact animals. These earlier studies have recently been extended by Chaikoff and his co-workers[2,3,4] who showed, in both animals and in surviving tissue slices, that the oxidation of the side chain proceeded at a relatively rapid rate while the cyclopentenophenanthrene nucleus was essentially inert. To facilitate a systematic study of the enzyme-catalyzed steps in this oxidation, we have obtained an active cell-free system from mouse liver tissue and report some of its properties below.

Mouse liver mitochondria were prepared by the method of Kielley and Kielley.[5] These preparations were unable to oxidize the C^{26}-carbon atom of 26-C^{14} labeled cholesterol[6] (added as a serum albumin stabilized emulsion) to CO_2, either alone or when supplemented with DPN, nicotinamide, adenylic acid, cytochrome C, magnesium ions and malate. The original sucrose homogenate, on the other hand, oxidized as much as 1.0% of the labeled carbon atom under the incubation conditions described in Table I. Microsome free supernatants, prepared

TABLE I

CHARACTERISTICS OF THE CHOLESTEROL OXIDASE SYSTEM

Individual flasks incubated in air at 37° contained one or more of the following components as indicated: 1.6 cc.[a] washed mitochondria in 0.25 *M* sucrose, M_w; 2.0 cc. homogenate-free of cell-debris and nuclei; 2.0 cc. microsome and mitochondria-free supernatant, S. Other additions were made to each flask unless otherwise indicated, at the following final concentrations: DPN, 0.0005 *M*; AMP, 0.002 *M*; nicotinamide, 0.02 *M*. The final volumes were made to 6.0 cc. with 1:1 0.9% KCl–0.2 *M* potassium phosphate buffer, *p*H 7.4; CO_2 was trapped in the center well in KOH and counted[7] as $BaCO_3$; 0.9 cc. 26-C^{14}-cholesterol was added as a 1% serum albumin stabilized emulsion containing 0.4 mg. cholesterol (42,000 c.p.m.[7])/cc. of saline-phosphate.

Expt.	Time of incubation, min.	Addition	Total c.p.m. in $BaCO_3$
I-1	120	Homogenate	206
2	33	M_w + S	8
3	63	M_w + S	43
4	120	M_w + S	197
5	120	M_w + dialyzed S	157
6	120	M_w + boiled, 3×concentrated S	0
7	120	M_w + S (no AMP)	124
8	120	M_w + S (0.22 cc. cholesterol emulsion)	96
II-1	120	M_w + S (stored at 3° for 24 hr.)	54
2	120	M_w + S (no DPN)	7
3	120	M_w + S (no DPN; TPN 0.0005 *M*)	2

[a] Equivalent to homogenate volume in flask 1.

(1) K. Bloch, B. N. Berg and D. Rittenberg, *J. Biol Chem.*, **149**, 511 (1943).

(2) I. L. Chaikoff, *et al.*, *ibid.*, **194**, 413 (1952).

(3) M. D. Siperstein and I. L. Chaikoff, *ibid.*, **198**, 93 (1952).

(4) J. R. Meier, M. D. Siperstein and I. L. Chaikoff, *ibid.*, **198**, 105 (1952).

(5) W. W. Kielley and R. K. Kielley, *ibid.*, **191**, 485 (1951).

(6) A. I. Ryer, W. H. Gebert and N. M. Murrill, THIS JOURNAL, **72**, 4247 (1950).

(7) C. V. Robinson, *Science*, **112**, 198 (1950).

by centrifugation of homogenates at 120,000 × *g.* for 30 minutes, when added to washed mitochondria, restored the activity to essentially the level originally obtained in the crude system.

Both the crude and partially resolved systems require the addition of diphosphopyridine nucleotide (DPN) and nicotinamide. Triphosphopyridine nucleotide (TPN) does not replace the DPN requirement. The over-all oxidation process shows a partial dependence on the presence of adenylic acid (AMP) although this phenomenon may be related to the stabilizing effect of adenylic acid and adenosinediphosphate on the general metabolic integrity of mitochondria.[5]

As shown in Table I, dialysis of the soluble fraction of the system does not lead to a significant loss in activity, nor can this fraction be replaced by a concentrated, boiled extract of whole liver or liver fractions. The inactivity of either the mitochondrial or the soluble fractions alone, and the demonstration of a considerable lag phase in the appearance of $C^{14}O_2$ suggests, as one possibility, the accumulation of an intermediary compound derived from the side chain carbon atoms which is subsequently oxidized by the terminal oxidizing systems of the mitochondrial elements.

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RECEIVED JANUARY 23, 1953

ENZYMATIC CLEAVAGE OF THE CITROVORUM FACTOR

Sir:

In our studies with soluble enzyme preparations which liberate bound forms of folic acid from liver, we observed that citrovorum factor (CF) added to such preparations disappeared at a rapid rate. We now have obtained from horse liver a protein fraction which, in the presence of *l*-glutamic acid, effectively destroys CF.

The protein fraction was obtained as the 30% saturated (0°) ammonium sulfate precipitate from a cold water extract (0°) of horse liver.

The influence of *l*-glutamic acid on the rate of inactivation of CF by the liver fraction is shown by the data in Table I. The loss of CF activity as measured by both *Leuconostoc citrovorum* and *Streptococcus faecalis* R, is paralleled by a rise in arylamines indicating a cleavage of the pteridine moiety from the *p*-aminobenzoylglutamic acid residue.

TABLE I

RECOVERY OF CF AFTER INCUBATION WITH LIVER PROTEIN FRACTION

Incubated at 37° for 2 hr. in 0.08 *M* Na_2HPO_4; volume, 7 ml.; initial CF = 56 γ.

L-Glutamic acid, *M*	None	0.001	0.002	0.004	0.0075	0.01
CF, γ	51.0	36.8	31.2	26.6	24.2	23.0

The role of *l*-glutamic acid appears to be specific. Other amino acids including *l*-glutamine and also known metabolic products of *l*-glutamic acid do not replace *l*-glutamic acid in this system. It is significant that *p*-aminobenzoic acid inhibits the reaction (31% inhibition with 0.01 *M* concentration of *l*-glutamic and *p*-aminobenzoic acids). While CF is attacked readily by the protein fraction in the presence of *l*-glutamic acid, neither pteroylglutamic acid nor its N-10-formyl derivative is affected.

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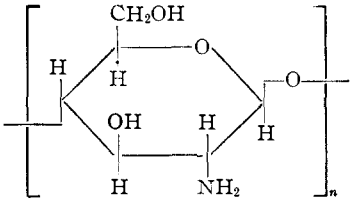
RECEIVED FEBRUARY 20, 1953

DIRECT EVIDENCE OF THE INFLUENCE OF SULFAMIC ACID LINKAGES ON THE ACTIVITY OF HEPARIN-LIKE ANTICOAGULANTS

Sir:

Heparin, the naturally occurring glucosamine-glucuronic acid polysaccharide polysulfate, is characterized by its high anticoagulant activity (U.S.P. Heparin is defined as having not less than 100 International Units per mg.) and by its essentially nontoxic nature (mouse intravenous LD_{50} = 1500–2000 mg./kg.[1]). Extensive work by numerous investigators has indicated that the activity of heparin is dependent, among other things, upon the degree of sulfation of the molecule and recently both Jorpes[2] and Meyer[3] concluded, as earlier considered a possibility[4] and more recently affirmed[5] by Wolfrom, that the amino groups in the molecule are sulfated and demonstrated that hydrolysis of the protected amino linkages resulted in essentially complete inactivation of the material. In order to test the validity of the postulate that the presence of sulfamic acid groups is a major factor essential for the high activity and presumably for the low toxicity of heparin, and also because the stated conclusions were based largely upon indirect evidence, we undertook to obtain direct evidence of the contribution of sulfamic acid linkages to the anticoagulant activity of polysaccharide polysulfate esters of the heparin type.

In this work the polyglucosamine, chitosan



was used as a model substance in sulfation experiments designed for the preparation of products in which the amino and hydroxyl groups were sulfated to varying degrees. Some of the data obtained on some of these products are given in Table I.

Thus, for the first time, there are data which indicate a correlation in agreement with the hypothesis

(1) J. Seifter and A. J. Begany, *Am. J. Med. Sci.*, **216**, 234 (1948).
(2) J. E. Jorpes, H. Boström and V. Mutt, *J. Biol. Chem.*, **183**, 607 (1950).
(3) K. H. Meyer and D. E. Schwartz, *Helv. Chim. Acta*, **33**, 1651 (1950).
(4) M. L. Wolfrom and W. H. McNeely, THIS JOURNAL, **67**, 748 (1945).
(5) M. L. Wolfrom, R. Montgomery, J. V. Karabinos and P. Rathgeb, *ibid.*, **72**, 5796 (1950).

TABLE I

Sample	A	B	C	D	E
η_{sp}/c, c = 1.00% in 0.50 N NaCl, 30°	0.328	0.358	0.370	0.200	0.380
S, %	16.17	16.74	14.28	14.27	15.99
N (Kjeldahl), %	3.75	3.44	3.70	3.12	3.15
N (Van Slyke), %	1.02	0.25	1.09	0.19	0.17
Moles of $-SO_3Na$ group per repeating unit					
Total	1.68	1.80	1.32	1.32	1.64
on OH	0.95	0.87	0.61	0.38	0.69
on NH_2	0.73	0.93	0.71	0.94	0.95
Activity, I.U./mg.	13	59	11	57	57

that, other conditions being equal, the contribution of sulfamic acid groups (above a certain limit) to the anticoagulant activity of polysaccharide polysulfate esters is far greater than that of sulfate ester groups.

That there is no simple relationship, *per se*, between anticoagulant activity and acute toxicity is amply demonstrated by other of our sulfated chitosan products, shown in Table II.

TABLE II

Sample	F	G	H	I
Activity, I.U./mg.	20	57	63	74
LD_{50}, i.v. in mice, mg. of kg.	3250 ±250	1500 ±500	1700 ±300	1250 ±250

In consideration of reports that the U.S.P. method does not give a reliable measure of *in vivo* activity with polysaccharide polysulfate synthetic anticoagulants,[6,7,8] our products which have been assayed by the U.S.P. method are currently being evaluated for activity by *in vivo* methods.

(6) E. G. Snyder (to Wyeth Inc.), U. S. Patent 2,508,433, May 23, 1950.

(7) C. N. Mangieri, R. Engelberg and L. O. Randall, *J. Pharmacol. Exptl. Therap.*, **102**, 156 (1951).

(8) H. E. Stavely, P. J. Baker, Jr., and H. G. Payne, *Federation Proc.*, **11**, 488 (1952).

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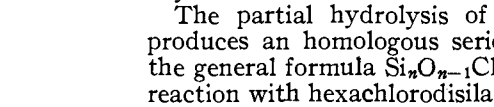
RECEIVED JANUARY 29, 1953

THE PARTIAL HYDROLYSIS OF HEXACHLORODISILANE

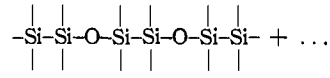
Sir:

Part of the current research program of these laboratories involves a comparative study of the partial hydrolysis, ammonolysis and thiohydrolysis of silicon halides. Using the method of Schumb and Stevens,[1] we have succeeded in partially hydrolyzing hexachlorodisilane, obtaining the first member of what is believed may be a new series of silicon oxychlorides.

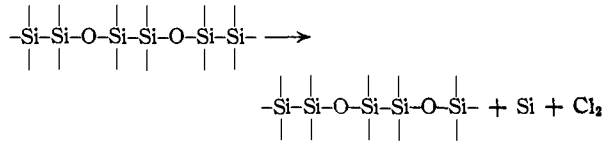
The partial hydrolysis of silicon tetrachloride produces an homologous series of oxychlorides of the general formula $Si_nO_{n-1}Cl_{2n+2}$. The analogous reaction with hexachlorodisilane would be expected similarly to give a series of the type $Si_{2n+2}O_nCl_{4n+6}$, as indicated by the schematic arrangement



(1) W. C. Schumb and A. J. Stevens, THIS JOURNAL, **72**, 3178 (1950).



The first member of this series, Si_4OCl_{10}, has been isolated and identified. Evidence also has been obtained for the existence of higher members, which, however, appear to undergo thermal decomposition during fractional distillation. This decomposition is probably the result of the thermal instability of long chains containing Si–Si linkages and has prevented the isolation of higher members. Analyses and estimated molecular weights of higher boiling fractions, however, are in the expected region. One of these fractions appears to be a decomposition product of the second member of the series referred to above, by such a process as the following



The hexachlorodisilane was added to dry ether in a three-necked round-bottom flask fitted with a slip-seal stirrer. The solution was cooled to −78° in a solid carbon dioxide–trichloroethylene bath and, with constant stirring, a measured amount of water was added dropwise from a buret. The mixture remained in the cold-bath for two hours and was then allowed to come to room temperature. The ether and unreacted hexachlorodisilane were removed by fractional distillation. The partial hydrolysis was then repeated with the recovered hexachlorodisilane. In a series of six such reactions, a total of 230 g. of Si_2Cl_6 was partially hydrolyzed.

The higher boiling residues remaining after the removal of the ether and unreacted hexachlorodisilane were combined and fractionated under reduced pressure. During the fractionation the contents of the distillation pot darkened gradually and a considerable quantity of black residue remained after removal of all liquid material. All fractions were clear liquids, increasing in viscosity with increasing temperature. While the first two fractions exhibited relatively narrow boiling point ranges, no well defined holds were observed at higher temperatures.

The first fraction, b.p. 120–123° (13 mm.), weighed about 12 g. *Anal.* Calcd. for Si_4OCl_{10}: Si, 23.3; Cl, 73.4; Si–Si bonds, 2/mole; mol. wt., 483. Found: Si, 23.2; Cl, 73.4; Si–Si bonds, 1.96/mole; mol. wt., 475. The second fraction, b.p. 140–143° (14 mm.), weighed about 5 g. *Anal.* Calcd. for $Si_5O_2Cl_{12}$: Si, 23.5; Cl, 71.2; Si–Si bonds, 2/mole; mol. wt., 598. Found: Si, 23.4, Cl, 71.0, Si–Si bonds, 1.93/mole; mol. wt., 595. The number of Si–Si linkages was determined by measuring the volume of hydrogen resulting from decomposition of the sample with dilute alkali while molecular weights were obtained from the freezing point depression of *p*-dioxane.

DEPARTMENT OF CHEMISTRY
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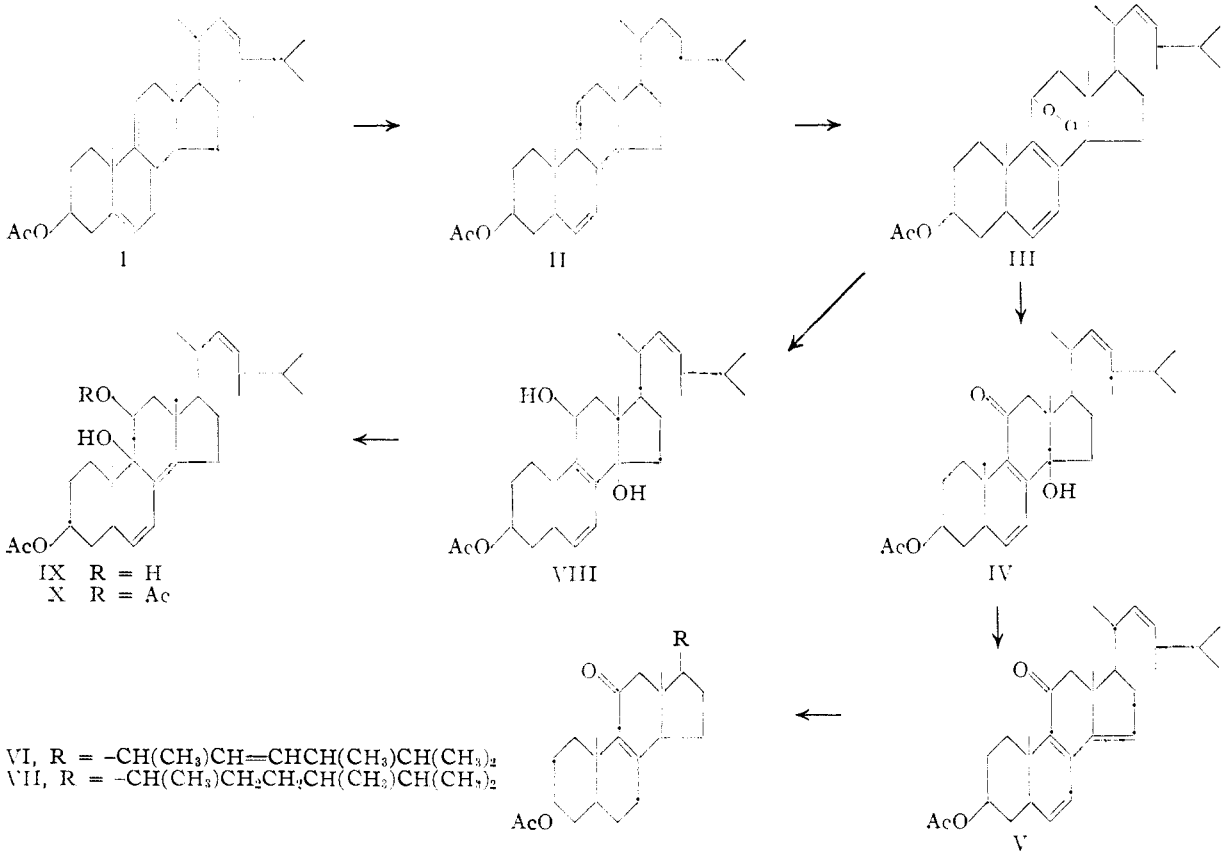
RECEIVED MARCH 4, 1953

CORTICOSTEROID INTERMEDIATES. II. A NEW ROUTE TO 11-OXYGENATED STEROIDS

Sir:

A new synthetic route has been devised for the conversion of C-ring unsubstituted steroids to cortisone. In contrast to recently published methods, which involve epoxidation of steroid 7,9(11)-dienes,[1-6] our synthesis employs photochemical peroxidation of homoannular C-ring dienes to introduce 11-oxygen as an 11,14-peroxide bridge. The C-ring endoperoxide system undergoes facile rearrangement to form directly 11-keto steroids suitable for conversion to cortisone. The preparation of the C-ring dienes required in this synthesis is accomplished by a heretofore unreported isomerization of nuclear trienes of the dehydroergosterol type. This communication reports the application of the new synthesis to ergosterol.



$\Delta^{5,7,9(11),22}$-Ergostatetraen-3β-ol acetate (dehydroergosterol acetate) (I),[7] prepared by mercuric acetate dehydrogenation of ergosterol acetate, was catalytically isomerized with liquid sulfur dioxide in over 80% yield to the C-ring diene $\Delta^{6,8(14),9(11),22}$-ergostatetraen-3$\beta$-ol acetate (II), m.p. 149.0–151.0°; $[\alpha]_D$ −94° ($CHCl_3$); $\lambda_{max.}$ 287.5 mu (log ϵ = 3.82), $\lambda_{max.}$ 232.5 mu (log ϵ = 4.25) (ether); found: C, 82.39; H, 10.26.[8] Photoperoxidation[9] of II afforded $\Delta^{6,8,22}$-ergostatrien-3β-ol acetate 11,14-peroxide (III), m.p. 164.6–166.4°; $[\alpha]_D$ −19° ($CHCl_3$); $\lambda_{max.}$ 272 mu (log ϵ = 3.61) (ether); found: C, 77.03; H, 9.50. Experimental evidence in support of the structure III was obtained by selective hydrogenation over a lead-palladium catalyst to form a glycol, $\Delta^{6,8,22}$-ergostatrien-3β,11,14-triol 3-acetate (VIII), m.p. 160.8–163.4°; $[\alpha]_D$ −34° ($CHCl_3$); $\lambda_{max.}$ 274 mu (log ϵ = 3.63) (ether); found: C, 76.61; H, 9.75, which underwent acid-catalyzed anionotropic rearrangement to a readily acylated isomer, $\Delta^{6,8(14),22}$-ergostatrien-3β,9,11-triol 3-acetate (IX), m.p. 203.0–206.0°; $\lambda_{max.}$ 248 mu (log ϵ = 4.42) (ether); found: C, 76.68; H, 9.80; active hydrogen, 1.87 moles per mole. 11-Acetate (X), m.p. 169.2–170.6°; $[\alpha]_D$ −47° ($CHCl_3$); $\lambda_{max.}$ 247 mu (log ϵ = 4.43) (ether); found: C, 75.05; H, 9.60. The ultraviolet spectra of III, VIII and IX and the acetylation of IX to X are consistent with the structures shown.

Mild base-catalyzed rearrangement of the per-

(1) L. F. Fieser, J. E. Herz and W. Huang, THIS JOURNAL, **73**, 2397 (1951); L. F. Fieser, J. C. Babcock, J. E. Herz, W. Huang and W. P. Schneider, *ibid.*, **74**, 4054 (1951).

(2) (a) E. M. Chamberlin, W. V. Ruyle, A. E. Erickson, J. M. Chemerda, L. M. Aliminosa, R. L. Erickson, G. E. Sita and M. Tishler, *ibid.*, **73**, 2396 (1951); (b) E. Schoenewaldt, L. Turnbull, E. M. Chamberlin, D. Reinhold, A. E. Erickson, W. V. Ruyle, J. M. Chemerda and M. Tishler, *ibid.*, **74**, 2696 (1952).

(3) (a) G. Stork, J. Romo, G. Rosenkranz and C. Djerassi, *ibid.*, **73**, 3546 (1951); (b) F. Sondheimer, R. Yashin, G. Rosenkranz and C. Djerassi, *ibid.*, **74**, 2697 (1952).

(4) H. Heusser, K. Eichenberger, P. Kurath, H. Dallenbach and O. Jeger, *Helv. Chim. Acta*, **34**, 2106 (1951).

(5) R. C. Anderson, R. Budziarek, G. T. Newbold, R. Stevenson and F. S. Spring, *Chem. and Ind.*, 1035 (1951).

(6) P. Bladon, R. B. Clayton, C. W. Greenhalgh, H. R. Henbest, E. R. H. Jones, B. J. Lovell, G. Silverstone, G. W. Wood and G. F. Woods, *J. Chem. Soc.*, 4883 (1952).

(7) A. Windaus and O. Linsert, *Ann.*, **465**, 148 (1928).

(8) Studies in this Laboratory indicate that this isomerization reaction is general for steroid 5,7-dienes.

(9) W. Bergmann and M. J. McLean, *Chem. Rev.*, **28**, 367 (1941).

oxide[10,11] III yielded $\Delta^{6,8,22}$-ergostatrien-3β,14-diol-11-one 3-acetate (IV), m.p. 188.8–192.4°; $[\alpha]_D$ +34° ($CHCl_3$); $\lambda_{max.}$ 308 mu (log ϵ = 3.84) (ether); found: C, 76.72; H, 9.65. Acid-catalyzed dehydration, followed by reacetylation led to $\Delta^{6,8,14,22}$-ergostatetraen-3β-ol-11-one acetate (V), m.p. 145.0–146.8°; $[\alpha]_D$ −82° ($CHCl_3$); $\lambda_{max.}$ 326 mu (log ϵ = 3.95), $\lambda_{max.}$ 233 mu (log ϵ = 4.18) (ether); found: C, 79.72; H, 9.54. The position of the carbonyl group at C-11 in V was established by hydrogenation over palladium-charcoal or W-7 Raney nickel[12] to form Δ^{8}-ergosten-3β-ol-11-one acetate (VII), m.p. 137.8–138.6°; $[\alpha]_D$ +125° ($CHCl_3$); $\lambda_{max.}$ 248 mu (log ϵ = 3.90) (ether); found: C, 78.91; H, 10.74; melting point undepressed on admixture with an authentic sample prepared by hydrogenation of VI obtained by an independent route.[2b,4]

Intermediates retaining the unsaturated side chain and suitable for conversion to cortisone were prepared by selective hydrogenation of V over W-2 nickel[13] to form $\Delta^{8,14,22}$-ergostatrien-3β-ol-11-one acetate, m.p. 127.0–128.2°; $[\alpha]_D$ +20° ($CHCl_3$); $\lambda_{max.}$ 291 mu (log ϵ = 4.06) (ether); found: C, 79.51; H, 9.92; or over W-7 nickel to form the known cortisone intermediate, $\Delta^{8,22}$-ergostadien-3β-ol-11-one acetate (VI), m.p. 131.4–131.8°; $[\alpha]_D$ +110° ($CHCl_3$); $\lambda_{max.}$ 248 mu (log ϵ = 3.95) (ether); melting point undepressed on admixture with an authentic sample.[2b,4]

Details of this work and alternate conversions of III and IV will be the subject of later communications from this Laboratory.

(10) W. Bergmann, F. Hirschmann and E. L. Skau, *J. Org. Chem.*, **4**, 29 (1939).

(11) N. Kornblum and H. E. DeLaMare, THIS JOURNAL, **73**, 880 (1951).

(12) H. Adkins and H. R. Billica, *ibid.*, **70**, 695 (1948).

(13) R. Mozingo, *Org. Syn.*, **21**, 15 (1941).

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RECEIVED JANUARY 28, 1953

THE ENZYMATIC OXIDATION OF *d*- AND *l*-β-HYDROXYBUTYRATE[1]

Sirs:

Although the *l*-isomer of β-hydroxybutyric acid is often regarded as the "naturally occurring" isomer[2] the *d*-isomer is also known to undergo biological oxidation.[3,4,5]

We have found that the mechanisms of enzymatic oxidation of the two pure isomers[6] in clear, dialyzed extracts of acetone-dried rat liver mitochondria are quite different. Such extracts contain the already known[2] diphosphopyridine nucleotide (DPN)-linked, *l*-specific β-hydroxybutyric dehydrogenase catalyzing the following reaction

$$\text{1-}\beta\text{-Hydroxybutyrate} + \text{DPN} \rightleftharpoons \text{acetoacetate} + \text{DPNH} \quad (1)$$

This reaction requires no components beyond extract, DPN and *l*-β-hydroxybutyrate. Under these circumstances *d*-β-hydroxybutyrate is not oxidized.

However such extracts will cause the reduction of DPN by the *d*-isomer if they are supplemented with adenosine triphosphate (ATP), Coenzyme A, and Mg^{++}. Such additions have no stimulatory effect on the reduction of DPN by *l*-β-hydroxybutyrate. Furthermore, the *l*-specific β-hydroxybutyric dehydrogenase is not involved in the oxidation of the d-isomer in the presence of these additional cofactors, since fractionation of the extracts yielded preparations with high activity toward *d*-β-hydroxybutyrate and little or none toward the *l*-isomer (Table I). These findings therefore suggested

TABLE I

ENZYMATIC OXIDATION OF *d*-β-HYDROXYBUTYRATE

The test system contained 0.10 ml. of dialyzed fraction of acetone-dried rat liver mitochondria, 10 μM. cysteine, 2.5 μM. ATP, 5 μM. $MgCl_2$, 50 μM. tris-(hydroxymethyl)-aminomethane buffer *p*H 8.0, 0.5 μM. CoA, 100 μM. KCl, 1.0 μM. DPN, 25 μM. *d*- or *l*-β-hydroxybutyrate and H_2O to make 1.00 ml.; temp. 20°; time, 20 min. Appearance of DPNH measured spectrophotometrically at 340 mμ.

System	Substrate	$\Delta \log I_0/I$
Complete	None	0.015
Complete	*d*-Isomer	.670
ATP omitted	*d*-Isomer	.005
CoA omitted	*d*-Isomer	.062
Mg^{++} omitted	*d*-Isomer	.149
Complete	*l*-Isomer	.040
ATP + CoA + Mg^{++} omitted	*l*-Isomer	.052

that the reduction of DPN by the *d*-isomer proceeds as follows

$$d\text{-}\beta\text{-Hydroxybutyrate} + \text{CoA} \xrightarrow{\text{ATP}} d\text{-}\beta\text{-hydroxybutyryl-CoA} \quad (2)$$

$$d\text{-}\beta\text{-Hydroxybutyryl-CoA} + \text{DPN} \rightleftharpoons \text{acetoacetyl-CoA} + \text{DPNH} \quad (3)$$

Further evidence for this formulation follows: In the absence of DPN but with hydroxylamine present as a "trapping" agent the *d*-isomer forms a hydroxamic acid derivative, detected by colorimetry and paper chromatography.[7] This reaction requires the presence of ATP, Mg^{++} and CoA. The formation of acetoacetyl-CoA as the end-product of the over-all reaction was established by the finding that citrate was formed as product of oxidation of *d*-β-hydroxybutyrate when oxalacetate, excess CoA, and "condensing enzyme" were present, *via* the following known reactions[8]

$$\text{Acetoacetyl-CoA} + \text{CoA} \rightleftharpoons 2\ \text{acetyl-CoA} \quad (4)$$

$$\text{Acetyl-CoA} + \text{oxalacetate} \rightleftharpoons \text{citrate} + \text{CoA} \quad (5)$$

Free acetoacetate formed no citrate under these conditions.

(1) This work was supported in part by grants from the Nutrition Foundation, Inc., the U. S. Public Health Service, and the John Simon Guggenheim Foundation.

(2) A. Magnus-Levy, *Arch. exptl. Path. Pharmakol.*, **45**, 389 (1901); D. E. Green, J. G. Dewan and L. F. Leloir, *Biochem. J.*, **31**, 934 (1937).

(3) A. McKenzie, *J. Chem. Soc.*, **81**, 1402 (1902).

(4) H. D. Dakin, *J. Biol. Chem.*, **8**, 97 (1910).

(5) A. L. Grafflin and D. E. Green, *ibid.*, **176**, 95 (1948).

(6) H. T. Clarke, unpublished improvement of method of McKenzie.[3]

(7) E. R. Stadtman and H. A. Barker, *J. Biol. Chem.*, **184**, 769 (1950).

(8) J. R. Stern, M. J. Coon and A. del Campillo, *Nature*, **171**, 28 (1953).

Since *l*-β-hydroxybutyrate also forms a hydroxamic acid in the presence of ATP, CoA, Mg^{++} and hydroxylamine (*cf.* equation (2)) but causes little if any reduction of DPN (Table I) it would appear that reaction (3) is responsible for the optical specificity of the over-all scheme.

The dehydrogenase catalyzing reaction (3), which has been found to be reversible, may be identical with that recently described by Lynen, *et al.*[9] In view of the present work it would appear probable that the β-hydroxybutyryl-CoA participating in this reaction contains the *d*-isomer. Since reactions (3) and (4) are reversible, *d*-β-hydroxybutyrate may be regarded as a "naturally occurring" metabolite in the form of its CoA derivative and may possibly be an intermediate in the enzymatic oxidation and synthesis of butyric acid.

(9) F. Lynen, L. Wessely, O. Wieland and L. Rueff, *Angew. Chem.*, **64**, 687 (1952).

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THE ROLE OF N^{15} GLYCINE, GLUTAMINE, ASPARTATE AND GLUTAMATE IN HYPOXANTHINE SYNTHESIS[1]

Sir:

Previous investigations *in vivo*[2,3,4] have shown that carbon dioxide, formate and glycine are precursors of the carbon atoms of the purine ring, and that glycine also supplies one of the four nitrogen atoms of the ring, nitrogen atom 7. It has also been shown that in an extract of pigeon liver where many of the side reactions complicating quantitative work *in vivo* are negligible, carbon dioxide, formate and glycine combine in the definite molecular proportion of 1:2:1 in the synthesis of hypoxanthine.[5] With the use of this technique of comparing the number of molecules of two different substrates utilized in the synthesis of hypoxanthine by pigeon liver extract, a search has been made for the nitrogen precursors of the three nitrogen atoms of the purine ring not supplied by glycine.[5,6] The N^{15}-labeled substrates were individually incubated with pigeon liver extract synthesizing hypoxanthine from glycine-1-C^{14}. At the termination of the incubation the hypoxanthine formed was converted to uric acid by the action of xanthine oxidase. After the addition of carrier, the uric acid was isolated, purified and analyzed for C^{14} and N^{15} content. The C^{14} of the radioactive glycine and the N^{15} of the other labeled substrates were determined and found to have remained sufficiently constant during the 30-minute incubation. From these analytical values it was possible to calculate the number of atoms of N^{15} which each of the nitrogenous compounds tested contributed to the synthesis of each molecule of hypoxanthine formed *de novo* from radioactive glycine. These ratios are reported in the accompanying table. It may be seen that, although ammonia is readily incorporated into purines *in vivo*, its low incorporation here indicates that it is not an immediate precursor of any one of the hypoxanthine nitrogen atoms. The other nitrogenous substrates, however, contributed significantly to hypoxanthine synthesis. The amide group of glutamine supplied approximately two atoms of nitrogen, and the α-amino group of the other substrates, N^{15} labeled glycine, aspartic and glutamic acids supplied one atom each. These integer ratios suggested that these substrates were contributing to specific nitrogen atoms of the purine ring. Degradation of the uric acid samples was carried out into fractions containing nitrogen atoms 1 and 3 combined, 7 and 9 combined and 7 alone. The analyses from the several experiments revealed considerable variation in the pattern of distribution of the N^{15} in the purine molecule and supported the belief that the substrates were specific donors. In the glycine N^{15} experiment the isotope was primarily in position 7; in the glutamine experiment the N^{15} donated by the amide group was found to be about half in the 9 atom and half in the 1 + 3 fraction. In the aspartic and glutamic acid experiments, almost all of the N^{15} was found in the 1 + 3 fraction, with a small amount in the 7 + 9 fraction. In the 7 + 9 fraction, therefore, the 7 atom is supplied by the known precursor glycine, and the 9 atom by the amide group of one of the two molecules of glutamine used in hypoxanthine synthesis. The 1 + 3 fraction has not been further separated, but the biological similarity of the transaminating dicarboxylic acids favors the probability that their α-amino groups both supply the same one atom of this pair, and that the other nitrogen atom of the pair is donated by the amide group of

TABLE I

LOCATION OF N^{15} IN THE PURINE RING

Expt.	N^{15} labeled substrate	Moles of N^{15} utilized for hypoxanthine synthesis per mole of C^{14} labeled glycine	N^{15} in various N atoms[a] of purine molecule (atom per cent. excess N^{15}) 1 + 3	7 + 9	7	9
1	NH_4Cl	0.27	...	...	...	...
2	Aspartic	1.20	0.091	0.009	...	...
3	Glutamic	1.20	.185	.025	...	...
4	Glutamine (amide N^{15})	1.90	.186	.176	.018	0.334[b]
5	Glycine	1.00	.058	.378	.750	.058

[a] The distribution of N^{15} in the purine ring in individual experiments was determined after further dilution of the original sample with varying amounts of uric acid. Therefore, only the N^{15} values of the nitrogen fraction of uric acid within each experiment are comparable. [b] Estimated from the N^{15} determination of nitrogen atom 7 and nitrogen fraction 7 + 9.

(1) Supported by grants from National Cancer Institute, National Institutes of Health, United States Pulic Health Service and the Damon Runyon Memorial Fund for Cancer Research, Inc. One investigator (J. C. S.) was a fellow of the Jane Coffin Childs Memorial Fund for Medical Research 1950–1952.

(2) J. C. Sonne, J. M. Buchanan and A. M. Delluva, *J. Biol. Chem.*, **166**, 395 (1946).

(3) J. M. Buchanan and J. C. Sonne, *ibid.*, **166**, 781 (1946).

(4) D. Shemin and D. Rittenberg, *ibid.*, **167**, 875 (1947).

(5) M. P. Schulman, J. C. Sonne and J. M. Buchanan, *ibid.*, **196**, 499 (1952).

(6) J. C. Sonne and I. Lin, *Federation Proc.*, **11**, 290 (1952).

the second molecule of glutamine used in hypoxanthine synthesis.

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ACETOACETYL COENZYME A AS INTERMEDIATE IN THE ENZYMATIC BREAKDOWN AND SYNTHESIS OF ACETOACETATE[1]

Sir:

In a recent note[2] evidence was presented that enzyme preparations from pig heart catalyze the following reversible reactions[3]

(1) Succinyl-S-CoA + acetoacetate $\rightleftharpoons$ succinate + acetoacetyl-S-CoA

(2) Acetoacetyl-S-CoA + CoA-SH $\rightleftharpoons$ 2 acetyl-S-CoA

(3) 2 Acetyl-S-CoA + 2 oxalacetate $\rightleftharpoons$ 2 citrate + 2 CoA-SH

The CoA transferase catalyzing Reaction 1 is analogous to the acetyl-propionyl CoA transphorase discovered by Stadtman[4] in *C. kluyveri*. Reaction 3 is catalyzed by the citrate condensing enzyme.[5] By various methods, including low temperature ethanol fractionation in the presence of zinc ions, the enzymes catalyzing Reactions 1 and 2 have now been separated and the intermediate acetoacetyl CoA has been isolated.

When succinyl CoA[6] and acetoacetate are incubated with the heart CoA transferase, an acid-stable intermediate accumulates which can be precipitated as a crude, alcohol insoluble barium salt. The intermediate yields no citrate (*i.e.*, no acetyl CoA) in the presence of CoA-SH, oxalacetate and citrate condensing enzyme, unless the acetoacetate condensing enzyme (Reaction 2) from either heart or liver is present. As shown in Table I one molecule of CoA-SH (determined as sulfhydryl) is released for each two molecules of citrate formed (*cf.* Reactions 2 and 3).

Acetoacetyl CoA can be further purified by paper chromatography. In ethanol–acetate[7] its R_F is 0.52 at 24° (R_F of acetoacetate, 0.75). Like acetyl CoA[8] it gives a nitroprusside reaction only after treatment with alkali. The absorption spectrum of acetoacetyl CoA eluates is similar to that of Lynen's analog S-acetoacetyl-N-acetyl thioethanolamine.[9] At *p*H 8.1 the compound shows a strong absorption band in the range 290–320 mμ with a peak at 305 mμ. The absorption is markedly increased by magnesium ions. The formation and disappearance of acetoacetyl CoA, through progress of Reaction 1 or 2 in either direction, can thus be readily followed spectrophotometrically (Fig. 1). By means of the optical method it has been possible to obtain an approximate estimate of the equilibrium constant (*p*H 8.1) of Reaction 2 (K^2_{eq}: (Acetyl-S-CoA)^2/(Acetoacetyl-S-CoA) (CoA-SH) $\simeq 5 \times 10^4$) which greatly favors acetoacetyl CoA cleavage. The equilibrium constant of Reaction 1 (K^1_{eq}: (Succinate) (Acetoacetyl-S-CoA)/(Succinyl-S-CoA) (Acetoacetate)) is about 10^{-2}.

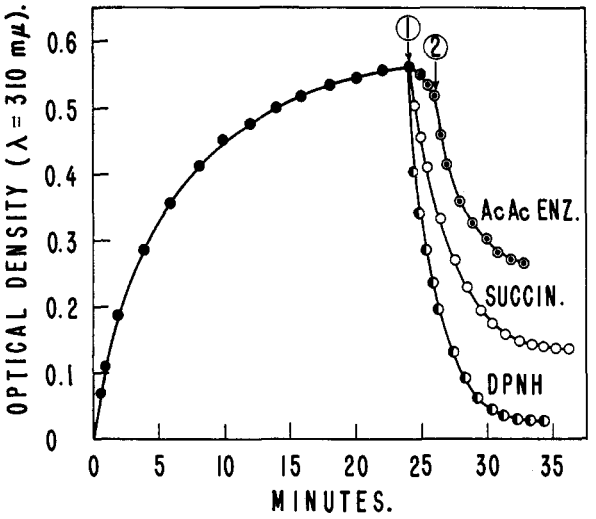

Fig. 1.—Ascending curve represents formation of acetoacetyl CoA on mixing, at *p*H 8.1, 0.4 μM. succinyl CoA and 100 μM. acetoacetate with 0.15 mg. CoA transferase protein (Reaction 1). The descending curves represent three separate experiments: (a) addition of 20 μM. succinate (arrow 1) shifts equilibrium of Reaction 1 to the left. (b) Addition of heart acetoacetate condensing enzyme fraction (0.35 mg. and 1.0 mg. protein at arrows 1 and 2) and CoA–SH causes cleavage of acetoacetyl CoA (Reaction 2). (c) Addition of 0.2 μM. DPNH (arrow 1) causes decrease in extinction through reduction of acetoacetyl CoA to β-hydroxybutyryl CoA.

TABLE I

50 μM. potassium phosphate buffer, *p*H 7.4, 4 μM. $MgCl_2$, 16 μM. potassium oxalacetate, 0.15 μM. CoA–SH (Pabst), potassium salt of intermediate (equivalent to 5.5 mg. of Ba salt), 0.03 mg. crystalline citrate condensing enzyme and, where indicated, ox liver acetoacetate condensing enzyme fraction (free of acetoacetyl CoA deacylase[2]); volume 1.2 ml.; incubation, 30 minutes at 38°.

Acetoacetate enzyme, mg. protein	0	4.3
SH released, μM.	...	0.12[a]
Citrate formed, μM.	0	0.26
SH/Citrate	...	0.46

[a] Corrected for CoA–SH liberated by spontaneous hydrolysis of succinyl CoA present in the acetoacetyl CoA preparation.

The CoA transferase, which has been purified over 100-fold from pig heart extracts, contains as a contaminant the β-hydroxybutyryl CoA dehydrogenase recently described by Lynen, *et al.*[9] DPNH is rapidly oxidized by acetoacetyl CoA (but not by

(1) Supported by grants from The U. S. Public Health Service, The American Cancer Society (recommended by the Committee on Growth, National Research Council), The Williams-Waterman Fund of Research Corporation, and by a contract (N6onr279, T.O. VI) between the Office of Naval Research and New York University College of Medicine.

(2) J. R. Stern, M. J. Coon and A. Del Campillo, *Nature*, **171**, 28 (1953).

(3) Abbreviations: Coenzyme A (reduced), CoA or CoA-SH; acyl coenzyme A derivatives, acyl CoA or acyl-S-CoA; reduced diphosphopyridine nucleotide, DPNH; μM., micromoles.

(4) E. R. Stadtman, *Federation Proc.*, **11**, 291 (1952).

(5) S. Ochoa, J. R. Stern and M. C. Schneider, *J. Biol. Chem.*, **193**, 691 (1951).

(6) Prepared synthetically from succinic anhydride and CoA-SH by an unpublished method of E. Simon and D. Shemin.

(7) E. R. Stadtman, *J. Biol. Chem.*, **196**, 535 (1952).

(8) F. Lynen, E. Reichert and L. Rueff, *Ann.*, **574**, 1 (1951).

(9) F. Lynen, L. Wessely, O. Wieland and L. Rueff, *Angew. Chem.*, **64**, 687 (1952).

acetoacetate) in the presence of transferase preparations; the reaction can be followed by the decrease in optical density at 310 (Fig. 1) or 340 mμ. The product, β-hydroxybutyryl CoA, has been isolated by chromatography as above (R_F, 0.64) and characterized enzymatically.

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COFACTOR REQUIREMENTS FOR THE DECARBOXYLATION OF SUCCINATE

Sir:

The decarboxylation of succinate, considered to be the main pathway of propionate formation in the propionibacteria and certain micrococci, was discovered in experiments with suspensions of whole cells[1,2,3] and consequently the cofactor requirements have not been determined. This communication presents evidence, obtained from experiments with cell-free preparations, that several cofactors are involved in the decarboxylation of succinate.

Micrococcus lactilyticus, strain 221, an anaerobic micrococcus capable of fermenting organic acids[4] and purines,[5] was used. Cells were grown in a medium composed of inorganic salts,[5] 2–3% lactate, 1% Difco yeast extract, 2% peptone and 0.0015% thiamin. Extracts were prepared by grinding the harvested cells with alumina,[6] extracting the paste with 0.01% cysteine solution, and centrifuging at high speed.

The quantitative decomposition of succinate to carbon dioxide and propionate by such extracts is considerable without the addition of any cofactors, although the presence of reducing agents and magnesium chloride increases the rate of carbon dioxide production. It can be demonstrated, however, that coenzyme A (CoA), adenosine-tri-phosphate (ATP), and cocarboxylase take part in succinate decarboxylation. A great decrease in decarboxylase activity is produced by treatment of the extract with Dowex-1 and Norit.[7] It was found that this treatment reduces the CoA content by 98% (determined by the assay method of Kaplan and Lipmann[8]) and the ATP content by 90%. Since the method of treatment does not remove all of the ATP and CoA from the extract, some decarboxylation occurs without the addition of cofactors, and a rather large increase in CO_2 production can be observed with the addition of CoA alone (Table I). Since small amounts of ATP (data not presented here) are sufficient to initiate decarboxylation and are as effective as large amounts, it appears that ATP acts as a "sparker." Decarboxylase activity is almost completely regained by the addition of ATP, CoA and cocarboxylase, as shown in Table I.

TABLE I

EFFECT OF COFACTORS ON SUCCINATE DECARBOXYLATION

Warburg vessels contained: 50 μM. succinate, 50 μM. acetate buffer *p*H 5.5, 100 μM. NaF, 10 μM. glutathione, 10 μM. $MgCl_2$, 1.0 ml. extract containing 20 mg. protein, total volume 2.5 ml. Incubated under pure N_2, at 30° for 2 hours. Pabst ATP and CoA (approximately 200 units/-mg.) used.

Extract	Additions	μl. CO_2[a]
Untreated	None	458
Treated	None	35
Treated	10 μM. ATP	48
Treated	10 units CoA	249
Treated	10 units CoA, 10 μM. ATP	390
Treated	10 units CoA, 10 μM. ATP, 25γ cocarboxylase	416

[a] Values corrected for bound CO_2.

The effect of cocarboxylase can be demonstrated more clearly by using extracts of cells grown in media with suboptimal concentrations of yeast extract and thiamin, as shown in Table II.

TABLE II

EFFECT OF COCARBOXYLASE ON SUCCINATE DECARBOXYLATION

Experimental conditions as given under Table I. Extract from cells grown under suboptimal conditions

Cocarboxylase added per vessel	μl. CO_2[a]
None	209
0.5γ	214
1.5γ	226
5.0γ	255
15.0γ	270
50.0γ	270

[a] Values corrected for bound CO_2.

An investigation of the possible role of these cofactors has shown that succinate is activated prior to decarboxylation. This conclusion is based on the following evidence: (a) in the presence of hydroxylamine, succinhydroxamic acid, identified by paper chromatography,[9] is formed (Table III), (b) with

TABLE III

THE EFFECT OF ATP AND CoA ON THE FORMATION OF SUCCINHYDROXAMIC ACID

Extract used and experimental conditions as listed under Table I except that 1000 μM. hydroxylamine present as a trapping agent and M TRIS buffer, *p*H 7.0, substituted for acetate buffer.

Extract	Additions	μM. succinhydroxamic acid[a]
Untreated	None	4.54
Treated	None	0.20
Treated	10 μM. ATP	3.02
Treated	10 units CoA	0.22
Treated	10 units CoA, 10 μM. ATP	3.06

[a] Values corrected for endogenous control.

treated extracts, in the presence of added ATP and the absence of added CoA, the same amount of succinhydroxamic acid is formed whether hydroxylamine is added initially as a trapping agent or at the end of the incubation period, (c) the amount of succinhydroxamic acid formed is dependent upon the

(1) A. T. Johns, *Biochem. J.*, **42**, Proc. Biochem. Soc., ii (1948).
(2) E. Delwiche, *J. Bact.*, **56**, 811 (1948).
(3) A. T. Johns, *J. Gen. Microbiol.*, **5**, 326, 336 (1951).
(4) E. L. Foubert and H. C. Douglas, *J. Bact.*, **56**, 35 (1948).
(5) H. R. Whiteley and H. C. Douglas, *ibid.*, **61**, 605 (1951).
(6) H. McIlwain, *J. Gen. Microbiol.*, **2**, 288 (1948).
(7) E. R. Stadtman, *et al.*, *J. Biol. Chem.*, **191**, 365 (1951).
(8) N. O. Kaplan and F. Lipmann, *ibid.*, **174**, 37 (1948).
(9) E. R. Stadtman and H. A. Barker, *ibid.*, **184**, 769 (1950).

amount of ATP and is independent of CoA, thus suggesting that succinyl phosphate may be formed, (d) in experiments with sulfanilamide,[10] a CoA- and ATP-dependent decrease in free amide is observed; this decrease is greater if carbon dioxide production is inhibited, suggesting that succinyl-CoA, as well as succinyl phosphate, may be formed. A detailed treatment of the experimental results and of the postulated mechanism of succinate activation will be presented elsewhere.

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(10) D. R. Sanadi and J. W. Littlefield, *J. Biol. Chem.*, **193**, 683 (1951).

(11) Part of this work was done at the Hopkins Marine Station, Pacific Grove, California, on an Atomic Energy Commission Postdoctoral Fellowship.

SULFATED NITROGENOUS POLYSACCHARIDES AND THEIR ANTICOAGULANT ACTIVITY[1]

Sir:

N-Deacetylated chitin, previously swollen with pyridine, was heterogeneously sulfated at 100° for 1 hour with chlorosulfonic acid and pyridine to yield a product, isolated (inorganic salts were removed by dialysis) as the amorphous, water-soluble sodium salt, containing essentially two N-sulfate and one O-sulfate groups per anhydrodisaccharide unit; $[\alpha]^{25}D$ −23° (*c* 1.5, water). *Anal.* Calcd. for $C_{12}H_{19}O_7(NSO_3Na)_2(OSO_3Na)$: C, 22.93; H, 3.05; N, 4.46; S, 15.31; Na, 10.98. Found: C, 22.68; H, 3.08; N, 4.02; S, 15.6; Na, 11.2; $-NH_2$ (by ninhydrin), absent; NAc, absent. This preparation exhibited the behavior in the Van Slyke amino acid assay characteristic of the acid-labile N-sulfate group present in heparin.[2,3] Its anticoagulant activity was 56 International Units (I. U.)/mg. The animal (mouse intravenous) toxicity was approximately double that of heparin, a finding believed to be due to the unsuitably high molecular size of the substance.

Chondroitinsulfuric acid (from cartilage) was essentially homogeneously N-deacetylated with 45% NaOH (25, 48 hr.) under nitrogen and in the presence of antioxidants (benzyl alcohol and sodium sulfite) and was sulfated as described above (but at 80–90°). The product was isolated as the amorphous sodium salt and is under further analytical characterization; $-NH_2$ (by ninhydrin), absent. The anticoagulant activity was 48 I. U./mg. The same sample of sodium chondroitin sulfate was subjected to the above sulfation procedure without preliminary N-deacetylation and the product, isolated in the same manner, showed an anticoagulant activity of *ca.* 10 I. U./mg. Sodium heparinate was re-sulfated under these conditions with a reduction in its activity from 110 to 55 I. U./mg. and an increase in the sulfur content from 12 (initial) to 14.4%.

Methyl 2-amino-2-deoxy-β-D-glucopyranoside hydrochloride[4] was sulfated as above to produce the amorphous, water-soluble barium salt of the N-sulfate, tri-O-sulfate; $[\alpha]^{25}D$ +4° (*c* 3.4, H_2O). *Anal.* Calcd. for $C_7H_{11}NO_{17}S_4Ba_2 \cdot 2H_2O$: S, 15.63; Ba, 33.48. Found: S, 15.64; Ba, 32.98. A 3×10^{-4} *M* solution of this substance in 0.004 *N* HCl at 95° lost 1.0 mole of sulfate in ≦20 min. with the concomitant release of the free amino group (ninhydrin). The O-sulfate was removed relatively more slowly and only completely so after 12 hr. Previously reported results[2] on the inactivation of heparin by mild acidity were considered to involve a negligible sulfate loss. On the basis of our present knowledge of the heparin molecule,[5] this sulfate loss is about equivalent to the amino group released so that a sulfate group shift[5] is not a required postulation.

The above results show that the sulfamic acid group is a potent contributor to anticoagulant activity. Experiments are now underway to determine the optimum molecular size for these chemically modified polysaccharides.

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(1) Supported by the Bristol Laboratories, Inc., Syracuse, N. Y., (R. F. Project 432).

(2) M. L. Wolfrom and W. H. McNeely, THIS JOURNAL, **67**, 748 (1945).

(3) J. E. Jorpes, H. Boström and V. Mutt, *J. Biol. Chem.*, **183**, 607 (1950).

(4) J. C. Irvine, D. McNicoll and A. Hynd, *J. Chem. Soc.*, **99**, 250 (1911).

(5) M. L. Wolfrom, R. Montgomery, J. V. Karabinos and P. Rathgeb, THIS JOURNAL, **72**, 5796 (1950).