CATALYTIC CLEAVAGE OF THIOETHERS[1]

Sir:

It has been observed that lanthionine and cystathionine (but not cysteine, homocysteine or methionine) are cleaved in a catalytic manner in neutral or slightly alkaline media in the presence of chelating agents, metal ions and pyridoxal or pyridoxal phosphate. These results are to be contrasted with those of Snell and co-workers[2] in that the cleavages occur at physiological temperatures and at rates comparable with that of the purified enzyme.[3]

L(or meso)-Lanthionine was cleaved with the formation of cysteine, pyruvate and ammonia; hydrogen sulfide was not formed. The products from L(or L-allo)-cystathionine were homocysteine, pyruvate and ammonia; this cleavage was analogous to that of bacterial enzymes[4] but opposite to that of mammalian enzymes which produce cysteine from L-cystathionine. The cleavages were accelerated by chelating agents (Table I) and were metal specific.

TABLE I

CLEAVAGE OF THIOETHERS

0.005 *M* substrate incubated at 37°, *p*H 8.5–9.0, with 0.05 *M* chelating agent and 1×10^{-4} *M* metal ion and pyridoxal. Zinc and cupric ions were used with lanthionine and cystathionine, respectively. There was no cleavage in the absence of pyridoxal or metal ion.

Chelating agent	Per cent. cleavage (1 hr.) L-lanthionine[a]	L-cystathionine[b]
Tris	18	0
Oxalate	45	72
Citrate	57	42
Pyrophosphate	57	56
Glycine	45	40
EDTA	12	2

[a] Ref. 5. [b] Ref. 6, modified (buffer *p*H 7.2, zinc and ammonium salts omitted and 1% EDTA added.)

The effectiveness of chelating agents was related to the stability constant of the chelate complex. Optimal activity for the cleavage of cystathionine (cupric ion) and lanthionine (zinc ion) was observed at or near *p*H 9 and with chelates with stability constants near 10^{-12} and 10^{-9}, respectively. Only cupric ions were found to catalyze the cleavage of cystathionine. Zinc and cupric ions catalyzed the cleavage of lanthionine; zinc ion was more effective with pyridoxal and cupric ion was more effective with pyridoxal phosphate. Other ions tested (including aluminum) were without effect; thus, the metal chelates demonstrated considerable structural specificity. The rate and order of reaction was dependent upon concentration of substrate; first order reaction rates were observed at lower concentrations of substrate (0.005 *M*) and zero order reaction rates were observed at higher concentrations (0.025 *M*) with the system of Table I. A turnover number of 10,000 (maximal) was observed for the cupric ion in the cleavage of cystathionine in oxalate solutions with 10^{-5} *M* cupric ion. Thus, the simple chelate systems were found to have many properties comparable to those of enzymes.

The enzyme responsible for the cleavage of L-cystathionine to cysteine does not conform to the chelate models. First, no chelate model has been found to have the specificity of the mammalian enzyme and, second, the enzyme is not influenced in its activity by a wide variety of chelating agents. Cupric ion was found to activate the enzymatic cleavage but toward the formation of homocysteine; EDTA eliminated this activation without influence on the cleavage to cysteine. Thus, it would be necessary to assume that protein is substituting for metal ion if one is to regard the models as pertinent. The chelate systems may be related to the bacterial enzymes since the specificity of cleavage of cystathionine is identical and there is some evidence that a metal ion may be concerned.[7]

(1) Aided by grants from the Nutrition Foundation and from the U. S. Public Health Service. The following abbreviations are used: Tris, tris-(hydroxymethyl)-aminomethane; and EDTA, ethylenediaminetetraacetic acid.

(2) D. E. Metzler, M. Ikawa and E. E. Snell, THIS JOURNAL, **76**, 648 (1954).

(3) F. Binkley and D. Okeson, *J. Biol. Chem.*, **182**, 273 (1950).

(4) F. Binkley and A. Hudgins, *Federation Proc.*, **12**, 178 (1953).

(5) M. X. Sullivan and W. C. Hess, *J. Biol. Chem.*, **116**, 221 (1936).

(6) E. Brand, G. F. Cahill and B. Kassell, *ibid.*, **133**, 431 (1940).

(7) S. Wijesundera and D. D. Woods, *J. Gen. Microbiol. (Proc.)*, **9**, 3 (1953).

DEPARTMENT OF BIOCHEMISTRY
DIVISION OF BASIC SCIENCES FRANCIS BINKLEY
EMORY UNIVERSITY, GEORGIA

RECEIVED DECEMBER 18, 1954

INVOLVEMENT OF ATP, 5-PHOSPHORIBOSYL-PYROPHOSPHATE AND L-AZASERINE IN THE ENZYMATIC FORMATION OF GLYCINAMIDE RIBOTIDE INTERMEDIATES IN INOSINIC ACID BIOSYNTHESIS

Sir:

The tentative identification of two aliphatic ribotide derivatives of glycinamide which are intermediates in the *de novo* synthesis of inosinic acid in enzyme systems from pigeon liver has recently been reported.[1] In agreement with these findings, we have isolated and characterized by chemical analysis two metabolic products of glycine, herein designated as glycinamide ribotide (GAR) and (α-N-formyl)-glycinamide ribotide (FGAR), both of which accumulate in relatively large amounts when *de novo* synthesis of purines in soluble enzyme fractions of pigeon liver is carried out in the presence of L-azaserine (O-diazoacetyl-L-serine). Both ribotides can be labeled by 1-C^{14}-glycine, while C^{14}-formate is found only in FGAR. Radioactive bicarbonate labels neither of these two compounds, although bicarbonate ions greatly enhance their formation.

GAR and FGAR behave as typical mononucleotides on ion exchange resins, in that they are not held by Dowex-50 cation exchanger (ammonium cycle), but are retained and can be eluted readily by dilute buffers on Dowex-1 anion exchanger (acetate cycle). A preparation of the barium salt of FGAR, which was obtained enzymatically from 1-C^{14}-glycine, purified by use of these ion exchange techniques and recrystallized several times from water-ethanol, has yielded the analyses reported in Table I.

(1) D. A. Goldthwait, R. A. Peabody and G. R. Greenberg, THIS JOURNAL, **76**, 5258 (1954).

TABLE I

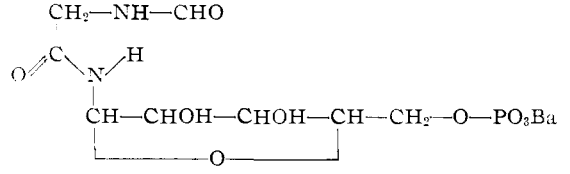
ANALYSIS OF (α-N-FORMYL) GLYCINAMIDE RIBOTIDE

	Molar Ratio (glycine = 1.00)
Glycine[a]	1.00
Formic acid[b]	1.11
Acid-labile N[c]	1.01
Total N[d]	1.90
Pentose[e]	0.96
Organic P[f]	1.07
C^{14}	0.97

[a] Determined by the method of B. Alexander, G. Landwehr and A. M. Seligman, *J. Biol. Chem.*, **160**, 51 (1945), following hydrolysis in 2.5*N* H_2SO_4, 1.5 hours, 150°. [b] Determined by the method of W. M. Grant, *Anal. Chem.*, **20**, 267 (1948), following hydrolysis in 1*N* NaOH, 50 minutes, 100°. [c] Determined by nesslerization, following hydrolysis in 2*N* H_2SO_4, 1.3 hours, 150°. [d] Determined by semi-micro Kjeldahl method. [e] Determined by the orcinol method using adenosine-5-phosphate standard. Hydrolyzed in 1.3 *N* HCl, 40 minutes, 100°, prior to color development. [f] Determined by the method of C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, 66, 375 (1925), following digestion in 10*N* H_2SO_4, 1.5 hours, 150°. Inorganic P was absent.

FGAR possesses only weak end-absorption (below 240 mμ) in the ultraviolet, and does not react in the Pauly diazo test for imidazoles.[2] The stability of the phosphate group of this compound toward hydrolysis in acidic solution is identical to that of adenosine-5′-phosphate. The location of the formyl residue on the α-amino nitrogen atom of the glycinamide moiety is indicated by the following evidence: (a) Absence of the ninhydrin reaction.[3,4] (b) Studies with paper electrophoresis (0.05 *M* ammonium acetate, *p*H 7.0, 90 minutes, 16 volts/cm.) demonstrate that FGAR has a mobility approximately twice that of GAR. This finding suggests that the α-amino N atom in the former compound is substituted and hence uncharged at this *p*H. (c) Absence of a titratable group in the region of the pK_2 of glycine or glycinamide.

An electrometric titration of the barium salt of this compound revealed the presence of only one titratable group (*i.e.*, the secondary phosphate dissociation, *pK* 6.40) between *p*H 3.5 and *p*H 10.5. Based upon this group, an equivalent weight of 450 ± 15 for the barium salt is obtained, a value which is in excellent agreement with the molecular weight (450) of the compound with the formula proposed



(α-N-Formyl)-glycinamide ribotide, barium salt

Both GAR and FGAR are highly efficient precursors of inosinic acid in the enzyme systems of pigeon liver which synthesize purines,[5] when supplemented with bicarbonate, formate, glutamine, aspartic acid and 3-phosphoglyceric acid. Large banks of unlabeled glycine or formate have no effect on the incorporation of isotope from the corresponding C^{14}-labeled acyclic ribotides into the completed purine structure. With C^{14} in the formyl group of FGAR, the inosinic acid formed biosynthetically may be degraded to reveal the location of C^{14} solely in carbon atom 8 of the purine base.

The new antibiotic L-azaserine, which exerts an inhibitory effect on the growth of Crocker mouse sarcoma 180[6] and significantly reduces the incorporation of C^{14}-formate into tumor nucleic acids[7] has been used in relatively low concentrations in our *in vitro* experiments to effect a considerable increase in the accumulation of GAR and FGAR.[8] *De novo* synthesis of inosinic acid is markedly inhibited by this compound and the inhibition can be overcome with higher levels of L-glutamine. Azaserine also blocks the further conversion of the acyclic ribotide intermediates to inosinic acid. On this basis it is concluded that L-azaserine exerts an inhibitory action on the *de novo* synthesis of purines at a metabolic site subsequent to the formation of FGAR.

L-Glutamine has been found to be the specific source of the amide nitrogen atom of GAR and FGAR. Since evidence from isotopic tracer studies[9] has shown that N^{15}-(amide labeled)-L-glutamine is the precursor of nitrogen atom 9 of the purine ring of hypoxanthine, it can be concluded that the amide N of glutamine is the source of the amide N of these acyclic ribotides.

The donor of the ribose-5-phosphate moiety of GAR was investigated in ethanol-precipitated fractions of pigeon liver extract. The enzyme fraction precipitating between 15 and 45% ethanol was dialyzed against distilled water, thereby completely removing the enzyme synthesizing 5′-phosphoribosylpyrophosphate (PRPP)[10,11] from ribose-5-phosphate and ATP, which is present predominantly in the 0 to 15% ethanol fraction. Treatment of the enzyme with Norite removed residual nucleotide coenzymes and transformylating cofactors.[1] The reaction mixture described in Table II was deproteinized in boiling water for one minute, acidified, and placed on a Dowex-50 column in the ammonium cycle. The initial eluate was combined with the washings obtained by passing 0.05*M* ammonium formate buffer, *p*H 3.35, through the column. These combined fractions contained the acyclic ribotides while C^{14}-glycine remained on the resin. The amount of acyclic ribotide formed was determined by direct plating of this fraction. Further chromatography of this fraction on Dowex-1 in the acetate cycle showed that all the radioactivity could be accounted for as GAR.

(2) K. K. Koessler and M. T. Hanke, *J. Biol. Chem.*, **39**, 497 (1919).

(3) S. Moore and W. H. Stein, *ibid.*, **176**, 367 (1948).

(4) In a personal communication, Dr. James Baddiley has informed us that synthetic glycinamide glucoside is ninhydrin-positive, while (α-N-formyl)-glycinamide glucoside is ninhydrin-negative.

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

(6) C. C. Stock, H. C. Reilly, S. M. Buckley, D. A. Clarke and C. P. Rhoads, *Nature*, **173**, 71 (1954).

(7) H. E. Skipper, L. L. Bennett, Jr., and F. M. Schabel, Jr., *Federation Proc.*, **13**, 298 (1954).

(8) We are indebted to Dr. Alexander Moore for the generous samples of L-azaserine used in this study.

(9) J. C. Sonne, I. Lin and J. M. Buchanan, THIS JOURNAL, **75**, 1516 (1953).

(10) A. Kornberg, I. Lieberman and E. S. Simms, *ibid.*, **76**, 2027 (1954).

(11) PRPP was prepared by the unpublished method of C. N. Remy, W. T. Remy and J. M. Buchanan.

TABLE II

INVOLVEMENT OF PRPP AND ATP IN THE SYNTHESIS OF GAR

Basic system: 5 μM C^{14}-glycine, 5 μM L-glutamine, 7 μM $NaHCO_3$, 2 μM L-azaserine, 0.3 ml. 0.03 *M* phosphate buffer, *p*H 7.4, containing 0.13 *M* KCl and 0.01 *M* $MgCl_2$, and 10 mg. of dialyzed, Norite-treated 15–45% ethanol fraction of pigeon liver extract; incubated 45 minutes at 38°.

Addition to basic system	μM. GAR synthesized
None	0.00
Ribose-5-phosphate, 2 μM.	0.02
ATP, 2 μM.	0.04
Ribose-5-phosphate + ATP, 2 μM. each	0.07
PRPP, 2 μM.	0.13
PRPP + ATP, 2 μM. each	0.43

It is seen in Table II that PRPP is the active ribose phosphate moiety in this ribotidation reaction but that maximal synthesis is realized only when PRPP and ATP are present together. These experiments demonstrate the requirement for ATP in a step in the formation of GAR other than that concerned with pyrophosphorylation of ribose-5-phosphate.

DIVISION OF BIOCHEMISTRY
DEPARTMENT OF BIOLOGY
MASSACHUSETTS INSTITUTE OF TECHNOLOGY
CAMBRIDGE 39, MASSACHUSETTS

STANDISH C. HARTMAN
BRUCE LEVENBERG[12]
JOHN M. BUCHANAN

RECEIVED DECEMBER 2, 1954

(12) Public Health Service Research Fellow of National Institute of Neurological Diseases and Blindness. Work supported by Grants from the Damon Runyon Memorial Fund for Cancer Research and the National Cancer Institute, National Institutes of Health, United States Public Health Service.

A NEW CRITICAL VOLUME EFFECT IN HIGH POLYMER SOLUTIONS

Sir:

Staudinger[1] and several other authors[2] have predicted from the length of the macromolecules that these would be separated from each other in very dilute solution, but on the contrary would form a continuous network in concentrated solution. The transition from a discontinuous to a continuous solution would be expected to take place at a critical value of the concentration.

Streeter and Boyer[3] by viscosity measurements of very dilute solutions of polystyrene in toluene, have observed a phenomenon which they have attributed to this critical concentration. Daoust and Rinfret,[4] studying the heats of mixing of polyvinyl acetate in S-dichloroethane and S-tetrachloroethane, discovered at high dilution the existence of a concentration at which the graph of heat of mixing *vs.* volume fraction shows an inflection point. Parent and Rinfret[5] have subsequently shown that the value of these critical concentrations varied inversely as the molecular weight of the P.V.A.

We have thought here that it was possible to determine those critical concentrations by very precise measurements of the specific partial volume at high dilution. Using a magnetically controlled float similar to that of Lamb and Lee,[6] we have attained a precision of $\pm 1 \times 10^{-6}$ g. ml.$^{-1}$. Five series of density measurements of carefully fractionated P.V.A. in dilute chlorobenzene solutions were made; Fig. 1 shows that at concentrations lower than one gram per hundred grams of solution, each molecular weight gives rise to a different inflection point in the graph of density *vs.* concentration.

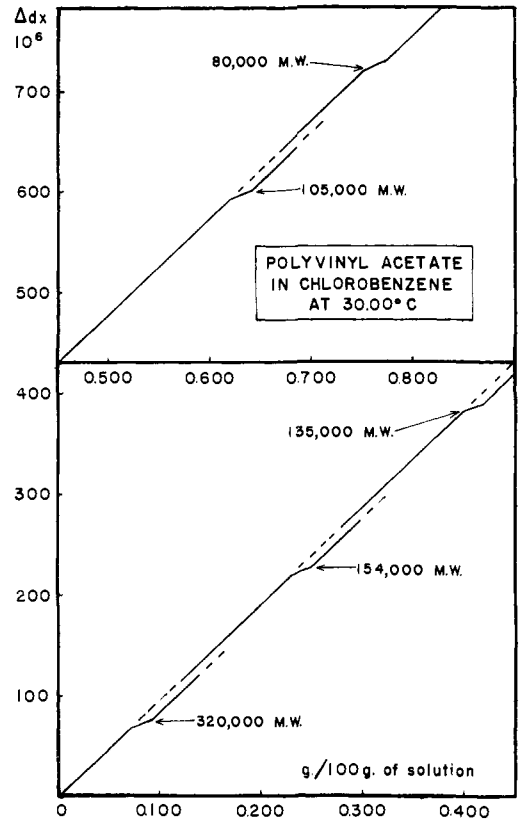


Fig. 1.

As the position of these inflection points shifts downward with increasing molecular weight, it would seem that the phenomenon observed confirms the prediction of Staudinger, *et al.*, about the change of structure of the polymer in solution with concentration.

DÉPARTMENT DE CHIMIE
UNIVERSITÉ DE MONTRÉAL
MONTREAL, CANADA

ALCIDE HORTH
MARCEL RINFRET

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PHOSPHORYLATION COUPLED WITH ELECTRON TRANSPORT TO CYTOCHROME C BY SOLUBLE ANIMAL ENZYMES

Sir:

Intramitochondrial diaphorase is inhibited by hydroxylamine. Soluble diaphorase is not inhibited. A factor necessary for this inhibition was isolated from guinea pig liver.[1] We observed that

(1) H. Staudinger, "Die hochmolekularen organischen Verbindungen," Springer, Berlin, 1932, p. 128.
(2) R. F. Boyer and R. S. Spencer, *J. Polymer Sci.*, **5**, 375 (1950).
(3) D. J. Streeter and R. F. Boyer, *ibid.*, **14**, 5 (1954).
(4) H. Daoust and M. Rinfret, *Can. J. Chem.*, **32**, 492 (1954).
(5) M. Parent, Ph.D. Thesis, Université de Montréal, 1954.
(6) A. B. Lamb and R. E. Lee, THIS JOURNAL, **35**, 1668 (1913).

(1) I. Raw, *Science*, **118**, 159 (1953).