compound. The yield of the monohydrate was 15.5 g., $[\alpha]^{24}D$ −5.9° (in water). Chromatographic analysis, as described for the D,L-form above, revealed only a single spot.

Anal. Calcd. for $C_{14}H_{27}O_5N_5 \cdot H_2O$: C, 46.3; H, 8.1; N, 19.3. Found: C, 45.8; H, 8.2; N, 18.8.

In order to ascertain that the above compound was, in fact, an epimeric mixture, a sample of 7.27 g. was refluxed with 6 *N* hydrochloric acid for 24 hr. Paper chromatograms, as above, showed only a single spot corresponding to octopine (or isoöctopine). After removal of chloride ions with silver carbonate, the filtrate yielded 2.12 g. of D-octopine and 0.42 g. of L-alloöctopine, isolated *via* their respective picrate and flavianate derivatives. Although such data indeed indicated the presence of an epimeric mixture, the ratio of "D-octopinyl"- to "L-alloöctopinyl"-L-valine could not properly be determined from the final yields of D-octopine and L-alloöctopine subsequently secured, since large losses occur during the isolation of the flavianate of this latter compound.

III. **Rotatory Dispersion Measurements.**—The rotatory dispersion of (+)-octopine and (+)-isoöctopine was determined at 589, 578, 546, 435, 405 and 365 mμ, as 1% solutions in water and 5 *N* HCl, with a Rudolph photoelectric polarimeter. The procedure employed was similar to that described earlier.[19] Replicate readings were reproducible to ±0.003° (Table I).

IV. **Kinetic Measurements.**—Separate solutions of L-α-bromopropionic acid (632.4 mg.) in water (4.14 ml.) and of L-arginine·HCl (435.6 mg.) in 2 *N* sodium hydroxide (4.14 ml.) were allowed to equilibrate for 1 hr. in a water-bath at 39°. The solutions were then mixed and two 0.2-ml. samples removed immediately for Volhard titration and for Van Slyke ninhydrin-CO_2 determination. Similar sampling was effected at succeeding 30-minute intervals for 5.5 hr., by which time about 55% of the arginine had disappeared. Results were as shown in Table III.

BETHESDA 14, MARYLAND

[CONTRIBUTION FROM THE DIVISION OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

An Alanine-dependent, Ribonuclease-inhibited Conversion of AMP to ATP, and its Possible Relationship to Protein Synthesis

BY ROBERT W. HOLLEY[1]

RECEIVED AUGUST 3, 1956

An enzyme preparation from the soluble fraction of rat liver homogenate brings about conversion of radioactive AMP (adenosine 5′-monophosphate) into ATP (adenosine 5′-triphosphate) in the presence of L-alanine. Other amino acids, potassium acetate and sodium pyruvate have no effect. The L-alanine-dependent conversion of AMP to ATP is inhibited by ribonuclease.

There is considerable evidence that the first step in the synthesis of proteins from amino acids is an activation of the amino acid carboxyl groups by ATP.[2] The work of Hoagland[3] and Hoagland, Keller and Zamecnik[4] has suggested a detailed mechanism for this activation (eq. 1). These workers found that an enzyme preparation from the soluble fraction of rat liver homogenate brings about a rapid exchange between pyrophosphate and the terminal phosphates in ATP if amino acids are present. The amino acid activating enzymes could also be studied by demonstrating the formation of amino acid hydroxamic acids when the reaction was run in the presence of a high concentration of hydroxylamine. The activated amino acids have been formulated as enzyme-bound amino acyl–AMP derivatives[3,4]

$$\text{Enzyme} + \text{Amino Acid} + \text{ATP} \rightleftharpoons \text{Enzyme–Amino Acyl–AMP} + \text{Pyrophosphate} \quad (1)$$

The assumption that this type of enzymatic activation of amino acids is the first step in protein synthesis rests primarily on the discovery of the enzymes in a fraction which is required for the *in vitro* incorporation of radioactive amino acids by rat liver microsomes.[3,4]

Strong support for the above formulation of the activation of amino acids has been furnished by DeMoss, Genuth and Novelli[5] who synthesized the L-leucyl derivative of AMP (thought to be the mixed anhydride of L-leucine and AMP) and showed that in the presence of amino acid activating enzyme obtained from *E. coli* this compound reacts with pyrophosphate to form ATP. Similar results were obtained by Berg and Newton[6] using a methionine activating enzyme obtained from yeast.

If it is assumed that amino acid activation of this type is the first step in protein synthesis, the question immediately arises as to the nature of the next step in protein synthesis. Presumably, the activated amino acid must react with something, or be transferred to something. As a working hypothesis, it was assumed that the activated amino acid reacts with some unknown material with liberation of AMP

$$\text{Enzyme–Amino Acyl–AMP} + \text{X} \rightleftharpoons \text{Enzyme} + \text{Amino Acyl–X} + \text{AMP} \quad (2)$$

Such a reaction might be reversible and, if combined with the pyrophosphate exchange reaction (eq. 1), it might be detected by assaying for exchange of AMP into ATP.

A procedure for assay of AMP exchange was therefore devised. Crude enzyme preparations were incubated with radioactive AMP under conditions in which pyrophosphate was known to exchange with ATP. At the end of the incubation period, the ATP was precipitated as the barium salt and its radioactivity was determined.

The assay was first applied to extracts of spinach acetone powder, hog pancreas acetone powder and lyophilized *Lactobacillus plantarum* cells, extracts

(1) New York State Agricultural Experiment Station, Cornell University, Geneva, N. Y. John Simon Guggenheim Memorial Foundation Fellow, 1955–1956.

(2) H. Borsook, *J. Cellular Comp. Physiol.*, **47**, Suppl. 1, 35 (1946).

(3) M. B. Hoagland, *Biochim. Biophys. Acta*, **16**, 288 (1955).

(4) M. B. Hoagland, E. B. Keller and P. C. Zamecnik, *J. Biol. Chem.*, **218**, 345 (1956).

(5) J. A. DeMoss, S. M. Genuth and G. D. Novelli, *Proc. Natl. Acad. Sci.*, **42**, 325 (1956).

(6) P. Berg and C. Newton, *Federation Proc.*, **15**, 219 (1956).

known to contain amino acid activating enzymes.[7] In the presence of these extracts, radioactive AMP is converted rapidly to ATP in the absence of added amino acids. This conversion may be due to the presence of adenylic kinase, which would bring about exchange between AMP and ATP: AMP + ATP $\rightleftarrows$ 2ADP. Whatever the mechanism, the amino acid-independent conversion in these systems is very rapid and obscures any amino acid-dependent exchange which may be present.

These results suggested that it might be difficult to find a crude preparation of amino acid activating enzymes which was sufficiently free of "adenylic kinase" to be used in the AMP exchange assay procedure. Since Hoagland, Keller and Zamecnik[4] reported that their amino acid activating enzyme preparation from rat liver failed to exchange AMP with ATP, the AMP exchange assay procedure was tried with their enzyme preparation. Using material prepared according to their directions for the "*p*H 5 enzyme,"[4] it was found that approximately 10% of the added radioactive AMP was converted to ATP in 5 min. at 37° in the absence of added amino acids, indicating the probable presence of adenylic kinase. However, even in the presence of this high amino acid-independent incorporation, there was a slight increase in the radioactivity of the ATP when amino acids were added to the incubation mixture. Assays on the various fractions from the preparation of the "*p*H 5 enzyme" showed that the supernatant from the precipitation of the "*p*H 5 enzyme" contained a high concentration of "adenylic kinase," and suggested that a second precipitation at *p*H 5 might reduce the "adenylic kinase" activity. This was found to be the case. Using an enyme preparation which had been precipitated twice at *p*H 5.1–5.2, the addition of amino acids to the incubation mixture increased the conversion of radioactive AMP into ATP approximately 100%. With fresh enzyme preparations this amino acid-dependent conversion ("exchange"[8]) of AMP into ATP consistently represented 2–3% of the added AMP (see Fig. 1 and Table I).

Evidence that the radioactive material formed in the presence of added amino acids is ATP is as follows. The radioactivity precipitates with the barium salt of ATP. It chromatographs on paper as ATP. Conversion of the barium salt of ATP to the triacridine salt of ATP, followed by recrystallization of the triacridine salt, causes no significant change in specific radioactivity.

By studying the effect of individual amino acids it was found that L-alanine accounted for all of the activity of the mixture of 15 amino acids which had been used in the initial experiments. (D-Alanine is inactive.) Other natural amino acids, tested individually, did not cause significant conversion of AMP to ATP. This finding suggested that perhaps the conversion of AMP to ATP resulted from conversion of L-alanine to pyruvate,

(7) Unpublished work (a) J. M. Clark, Jr., with spinach; (b) R. S. Schweet with hog pancreas; (c) R. W. Holley, with *L. plantarum*.

(8) Presumably the radioactivity of the ATP results from a two-way exchange process between AMP and ATP, but since this has not been proved the process is termed simply a *conversion* of AMP into ATP.

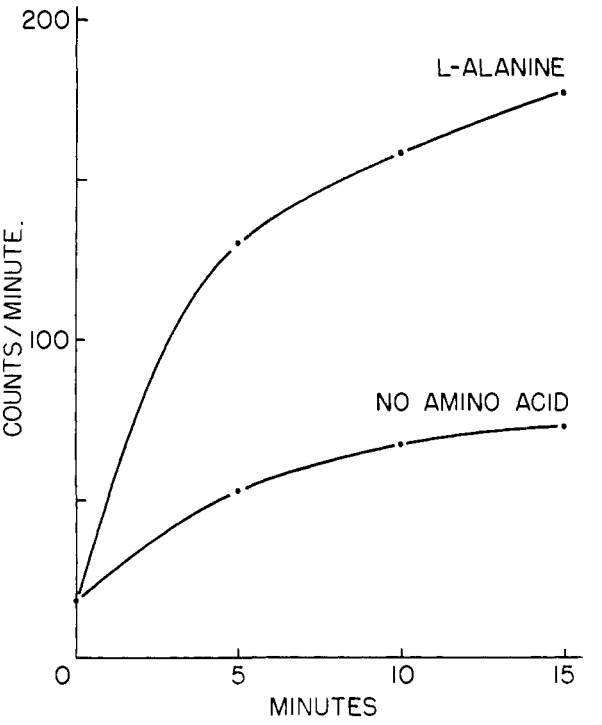


Fig. 1.—Effect of L-alanine on the conversion of radioactive AMP into ATP at 37°. The ATP was counted as the barium salt.

with subsequent formation of acetyl coenzyme A and involvement of the acetate activation enzyme system.[6,9] Such a possibility was excluded by the finding that pyruvate, acetate and coenzyme A have no effect.

Of particular interest is the finding that the L-alanine-dependent conversion[8] of AMP to ATP is sensitive to ribonuclease (see Table I). The addition of 0.1 μg. of ribonuclease at the start of the 10 min. incubation significantly reduces the L-alanine-dependent incorporation of radioactivity. The addition of 1 μg. suppresses the L-alanine-dependent incorporation completely. Ribonuclease has no effect on pyrophosphate exchange (eq. 1). As controls, to determine if the action of ribonuclease might be attributed to its basicity,[10] the basic proteins histone[11] and protamine sulfate[11] were added at 1 and 10 μg. levels and were found to have no effect. Thus far, attempts to increase the conversion of AMP to ATP by the addition of ribonucleic acid preparations or preparations of microsomes have been unsuccessful. These experiments are summarized in the Experimental Part.

The extent of incorporation of radioactive AMP into ATP was compared with the incorporation of radioactive pyrophosphate into ATP. Using identical reaction mixtures, except for the use of radioactive AMP in one instance and radioactive pyrophosphate in the other, the incorporation of AMP is approximately 9% of the incorporation of pyrophosphate. If pyrophosphate exchange (eq. 1) is an intermediate step in the incorporation of

(9) P. Berg, THIS JOURNAL, **77**, 3163 (1955).

(10) J. Brachet, *Biochim. Biophys. Acta*, **19**, 583 (1956).

(11) Purchased from Nutritional Biochemicals Corp.

TABLE I

CONVERSION OF RADIOACTIVE AMP INTO ATP

		Counts/min. in Ba_2ATP	Conversion of AMP into ATP, %
(a)	Complete system[a]	152 ± 27	5
(b)	L-Alanine omitted	74 ± 13	2.5
(c)	Control[b]	20 ± 1	0.7
(d)	As (a), plus 1 μg. ribonuclease[c]	75 ± 18	2.5
(e)	As (b), plus 1 μg. ribonuclease	74 ± 20	2.5
(f)	As (a), plus 0.1 μg ribonuclease	115 ± 30	4

[a] The complete system contained 100 μmoles of tris buffer, *p*H 7.5, 10 μmoles of potassium ATP, *p*H 7.0, 5 μmoles of potassium pyrophosphate, *p*H 7.5, 5 μmoles of magnesium chloride, 10 μmoles of potassium fluoride, 0.5 μmole of AMP-8-C^{14} containing 3000 counts/min., 2 μmoles of L-alanine, and approximately 2 mg. of protein, in 1 ml. The results given are for 10 min. incubation at 37°, and are averages of several experiments, with standard deviations. In over 20 experiments, the smallest increase in radioactivity of the ATP brought about by the addition of L-alanine (increase of (a) over (b)) was 60%. [b] Trichloroacetic acid was added immediately after addition of the enzyme solution. [c] Crystalline, salt-free ribonuclease (Worthington Biochemical Corp.).

AMP into ATP (eq. 1 and 2 combined), it is clear that AMP incorporation could not exceed pyrophosphate incorporation.

The results described in this paper are compatible with a combination of eq. 1 and a more detailed formulation of eq. 2 as

$$\text{Enzyme} + \text{L-Alanine} + \text{ATP} \rightleftharpoons \text{Enzyme-L-Alanyl-AMP} + \text{Pyrophosphate} \quad (1')$$

$$\text{Enzyme-L-Alanyl-AMP} + X \xrightarrow[\text{Including ribonuclease-sensitive material}]{\text{Cofactors? Enzymes?}} \text{Enzyme} + \text{L-Alanyl-X} + \text{AMP} \quad (2')$$

Whether this formulation is correct, or not, only further work will tell.

As has been indicated, it is suggested, as a working hypothesis, that eq. 2 (or 2′) describes the second step in the synthesis of proteins from amino acids. If this postulate is correct, amino acids other than L-alanine should undergo a similar type of reaction. It is known that enzymes active with several amino acids in pyrophosphate exchange (eq. 1) are present in the rat liver preparation.[4] Therefore, it might have been anticipated that AMP exchange, if it took place with this enzyme preparation, would not be limited to one amino acid. One possible explanation for the limitation of activity to L-alanine in the AMP exchange assay would be that essential factors ("X," etc. in eq. 2′) for the other amino acids are lost or inactivated during preparation of this enzyme system. Since it is thought that an amino acid must find its specific place on the "coding system" or "template"[2] before it is incorporated into a protein, it would not be surprising to find that individual factors are involved in the pathways of individual amino acids.

Whatever interpretation of the results is considered most plausible, it is certain that the enzyme system described in this paper catalyzes reactions which directly involve an amino acid, ATP and ribonuclease-sensitive material. Because of the evidence that ribonucleic acid is involved in some way in protein synthesis,[2] studies of the nature of the ribonuclease-sensitive material and elucidation of its role in the present enzyme system will be of special interest.

Experimental Part

AMP Exchange Assay Procedure.—Assays were normally run using a volume of 1.0 ml. in 13 × 100 mm. test-tubes. In each tube was placed 0.10 ml. of 0.05 *M* $MgCl_2$ in 0.5 *M* tris (tris-(hydroxymethyl)-aminomethane) buffer, *p*H 7.5, 0.10 ml. of 0.1 *M* dipotassium ATP (crystalline, Pabst) neutralized to *p*H 7.0–7.2 with either sodium hydroxide or potassium hydroxide, 0.10 ml. of 0.1 *M* potassium fluoride in 0.05 *M* potassium pyrophosphate solution neutralized to *p*H 7.5, and 0.05 ml. of 0.010 *M* AMP-8-C^{14} containing approximately 3000 counts/min.[12] Amino acids or other compounds were added at the concentrations indicated elsewhere, and the volume was made up to 0.50 ml. by the addition of water. To this solution was added 0.50 ml. of cold enzyme solution, and the mixture was incubated, with shaking, in a water-bath at 37°. At the end of the incubation period, the tubes were cooled in ice and 1.0 ml. of 12% trichloroacetic acid was added immediately to each tube. (If necessary, the tubes can be stored briefly in the cold at this stage.)

The tubes were centrifuged and the supernatant trichloroacetic acid solutions were decanted into centrifuge tubes. The protein precipitates were washed with 1.0 ml. of water, each, and the washes were added to the trichloroacetic acid solutions. Each trichloroacetic acid solution was neutralized to *p*H 7–8 by the addition of a measured volume (approximately 0.75 ml., determined by prior titration of an aliquot of the 12% trichloroacetic acid solution) of 1 *N* sodium hydroxide. The ATP in each tube was precipitated by the addition of 0.50 ml. of 1 *M* barium acetate solution. The tubes were left in a refrigerator 20 min. or longer, and then the precipitates were collected by centrifugation. Each precipitate was washed twice with 1 ml. of water and twice with 0.5 ml. of water, and was finally suspended in 0.15 ml. of water and transferred to a metal planchet, by means of a small pipet. The centrifuge tube and pipet were rinsed with a few drops of water. The suspensions of solid were evaporated to dryness, by means of an infrared lamp. (In order to obtain residues firmly bound to the planchets, to permit easy handling, discs of lens tissue were cemented on the surface of the planchets by means of thinned Tygon paint.) The radioactivities of the dried precipitates were measured using a thin-mica-window Geiger–Müller tube or a flow counter.

Preparation of the Enzyme System from Rat Liver.—(The procedure as far as resuspension of the first *p*H 5 precipitate is essentially that of Hoagland, *et al.*,[4] but is described here because their report that AMP fails to exchange with ATP suggests that the slight modifications may be important.) White rats were stunned and decapitated, and the livers (wt. 4–12 g.) were removed and immediately chilled in an ice-bath. The livers were homogenized individually. Unless otherwise indicated all operations were carried out in a cold room at 2°. In a typical preparation, an 8-g. liver was minced quickly with scissors in 4 ml. of 0.05 *N* potassium chloride solution and the mixture, with 8 ml. of additional potassium chloride solution, was homogenized in a Potter–Elvehjem type homogenizer with a Teflon pestle (A. H. Thomas Cat. No. 4288-B, size C, diameter of pestle 0.4 mm. less than the diameter of the glass tube). The pestle was turned by a motor at approximately 75 r.p.m. Homogenization was continued only long enough for all the material to pass between the pestle and tube. The mixture was diluted with 14 ml. more 0.05 *N* potassium chloride solution, and homogenization was continued very briefly, to mix the material. The homogenate (homogenates of several livers can be combined) was centrifuged for 50 min. at 40,000 r.p.m. (108,000 × *g*, average) in a refrigerated Spinco Model L preparative ultracentrifuge. The red supernatant

(12) Radioactive AMP labelled with C^{14} in the 8-position was purchased from Schwarz Laboratories (0.7 μcuries/mg.) and was diluted approximately 10-fold with non-radioactive AMP purchased from Pabst Laboratories.

was removed with a pipet, avoiding, as much as possible, the scum of fatty material, and leaving approximately 1 ml. of solution in each centrifuge tube. The supernatant solution, in a beaker immersed in an ice-bath, was adjusted to *p*H 5.1–5.2 by the dropwise addition of approximately 1.7 ml. of 0.1 *N* hydrochloric acid. (A Beckman Model G *p*H meter was used in the Laboratory (room temperature) with the temperature adjustment at the lowest possible setting, which by extrapolation was approximately 2°.) The precipitate was collected by centrifugation for 5 min. at 12,000 r.p.m. (10,000 × *g* average) in the refrigerated ultracentrifuge. The supernatant was decanted and the last drops were allowed to drain from the tubes onto absorbent tissue. The tan-colored pellets were resuspended in a total of 14 ml. of cold 0.1 *M* (*p*H 7.5 at room temperature) tris buffer, homogenized briefly, and centrifuged 5 min. at 15,000 r.p.m. (16,000 × *g*, average) in the refrigerated ultracentrifuge. The tan-colored supernatant was frozen at −20°. With present knowledge, storage at −20° is best at this stage, and the second *p*H 5 precipitation is carried out immediately before the enzyme is used. Present data indicate that the solution should be frozen before the second *p*H 5 precipitation is carried out.

The above, "*p*H 5," enzyme preparation is thawed and the cold solution is adjusted to *p*H 5.1–5.2 by the addition of cold 0.1 *N* hydrochloric acid. The precipitate is collected by centrifugation for 5 min. at 12,000 r.p.m., and the precipitate is resuspended in approximately eight-tenths of the original volume of 0.1 *M* tris buffer at *p*H 7.5. After centrifugation for 5 min. at 15,000 r.p.m., the very-light-colored supernatant is ready for use in the AMP exchange assay. The solution contains 3–4 mg. of protein/ml., and its absorption maximum at 260 mμ corresponds to the presence of 0.3–0.4 mg. of nucleic acid/ml.

Identification of the Radioactive Product as ATP.—To establish that the radioactive material formed in the presence of added amino acid in the AMP exchange assay is actually ATP, samples of the barium salt were converted to the potassium salt and these were chromatographed on Whatman No. 1 paper using the procedure of Cohn and Carter.[13] After chromatography, the ratios of radioactivity to optical density (260 mμ) of the eluted ATP samples were determined. The results were the same as had been obtained by determining the radioactivity of the barium salts.

As a further check on the identity of the radioactive material, samples of the barium salt of ATP from assays with and without added amino acids were treated with an equivalent of dilute sulfuric acid and, after removal of the barium sulfate and addition of carrier ATP, ATP was precipitated as the triacridine salt.[14] The samples of triacridine salt were washed with ethanol and ether and dried. Aliquots were counted, and the remainder of each sample was recrystallized from water and ethanol. The specific radioactivities of the samples of ATP remained constant, within experimental error. The radioactivities of the samples of recrystallized triacridine salt showed the same increase due to the addition of L-alanine as had been found with the samples of barium salt.

Pyrophosphate Exchange.—In order to compare AMP incorporation with pyrophosphate incorporation, two sets of reaction mixtures were prepared according to the AMP exchange assay procedure (above), but with one set containing radioactive AMP (3000 counts/min. in the 0.5 μmole present) and the other set containing radioactive pyrophosphate[15] (115,000 counts/min. in the 5 μmole present). At the end of the incubation, the tubes containing radioactive pyrophosphate were worked up according to the method of Crane and Lipmann,[16] which involves adsorption of the ATP on charcoal. In the presence of 2 μmoles of L-alanine, the ATP contained 158 counts/min. from radioactive AMP and 4420 counts/min. from radioactive pyrophosphate. In the absence of added amino acid the values were 67 and 700 counts/min., respectively. Thus, the L-alanine-dependent radioactivity was 90 counts/min. from AMP and 3700 counts/min. from pyrophosphate. In terms of μmoles of radioactive compound converted to ATP, these values are 0.015 μmole from AMP and 0.16 μmole from pyrophosphate. Therefore, under these conditions, incorporation of radioactive AMP is approximately 9% of the incorporation of pyrophosphate.

The addition of 1 μgram of ribonuclease did not inhibit the L-alanine-dependent incorporation of pyrophosphate into ATP.

Addition of Various Preparations Containing Ribonucleic Acid.—Addition of the following materials did not increase the amino acid-dependent conversion of AMP to ATP in the standard AMP exchange assay. (A mixture of 2 μmoles of each of the following amino acids was used in this set of experiments: L-alanine, L-arginine, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine and L-valine.)

(1) Commercial yeast ribonucleic acid, 0.3 mg. added/assay tube.

(2) Freshly prepared beef liver ribonucleic acid,[17] 0.4 mg. or 0.04 mg. added/assay tube.

(3) Freshly prepared rat liver microsomes[18]: the pellet obtained in one centrifuge tube was homogenized with 0.5 ml. of 0.1 *M* tris buffer, and 0.05 ml. of the mixture was added to the assay mixture. (The addition of this rat liver microsome preparation greatly increased the amino acid-independent conversion of AMP to ATP. Abood and Romanchek[19] have reported the presence of adenylic kinase in rat liver microsomes.)

(4) Rat liver microsomes treated with sodium desoxycholate[18]: the same fraction of a microsome pellet was used as in (3).

(5) Rat liver microsomes treated with sodium lauryl sulfate[20]: in the cold room, a microsome pellet was homogenized with 5 ml. of 0.1 *M* *p*H 7.5 tris buffer and 0.5 ml. of a 5% solution of sodium lauryl sulfate in 45% ethanol was added. After 2 hr. at 2°, 320 mg. of sodium chloride was added, and the mixture was centrifuged 20 min. at 25,000 *g* average. To 4 ml. of the supernatant was added 8 ml. of cold 95% ethanol and the precipitate was collected by centrifugation in a small centrifuge in the cold room. The precipitate was washed three times with cold acetone, and was allowed to dry briefly in air. It was mixed with 0.5 ml. of 0.1 *M* *p*H 7.5 tris buffer, and 0.050 ml. was added to the assay mixture.

(6) Rat liver microsomes treated with phenol[21]: in the cold room, a microsome pellet was homogenized with 0.5 ml. of 0.1 *M* *p*H 7.5 tris buffer and the mixture was extracted four times with 0.5–1.0 ml. of phenol saturated with water. The aqueous solution was extracted repeatedly with ether, the remaining ether was allowed to evaporate into the air in the cold room, and 0.050 ml. of the solution was added to the assay mixture.

(7) Freshly prepared pea microsomes[22]: 0.7 mg. or 0.07 mg. added/assay tube.

Addition of Other Materials.—The following compounds were tested in the absence of added L-alanine in the standard AMP exchange assay procedure at the concentrations indicated (number of μmoles added/assay tube is given in parentheses) and had no significant effect on the conversion of radioactive AMP to ATP, *i.e.*, the radioactivity of the ATP at the end of the incubation was the same as if nothing had been added: amino acids (2 each), D-alanine, L-arginine, L-aspartic acid, L-asparagine, L-cysteine, L-glutamic acid, L-glutamine, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine and L-valine; coenzyme A (1); glucose (30); potassium acetate (2); sodium propionate (2); sodium pyruvate (2); cytidine 5′-triphos-

(13) W. E. Cohn and C. E. Carter, THIS JOURNAL, **72**, 4273 (1950).

(14) A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 2487 (1949).

(15) Kindly furnished by J. M. Clark, Jr. Prepared by pyrolysis of the dipotassium salt of P^{32}-labeled orthophosphate obtained on allocation from the U. S. Atomic Energy Commission.

(16) R. K. Crane and F. Lipmann, *J. Biol. Chem.*, **201**, 235 (1953).

(17) Kindly furnished by V. D. Burrows. Prepared according to S. E. Kerr and K. Seraidarian, *ibid.*, **180**, 1203 (1949).

(18) J. W. Littlefield, E. B. Keller, J. Gross and P. C. Zamecnik, *ibid.*, **217**, 111 (1955), but homogenization was in 0.05 *N* potassium chloride.

(19) L. G. Abood and L. Romanchek, *Exptl. Cell Research*, **8**, 459 (1955).

(20) Procedure patterned after that of E. R. M. Kay and A. L. Dounce, THIS JOURNAL, **75**, 4041 (1953).

(21) Procedure patterned after that of A. Gierer and G. Schramm, *Nature*, **177**, 702 (1956).

(22) Kindly furnished by P. O. P. Ts'o. Prepared according to P. O. P. Ts'o, J. Bonner and J. Vinograd, *J. Biophysic. Biochem. Cytol.*, in press.

phate (1); guanosine 5'-triphosphate (1); uridine 5'-triphosphate (1); and yeast extract (0.5 mg.). Coenzyme A, the triphosphates, and yeast extract were also tested in the presence of added L-alanine and had no significant effect.

Acknowledgment.—The author wishes to thank Professor James Bonner for the opportunity of working in his laboratories, and he wishes to thank Professor Bonner, Mr. John M. Clark, Jr., Dr. Peter H. Lowy and Dr. Richard S. Schweet for many stimulating discussions.

PASADENA, CALIFORNIA

[CONTRIBUTION FROM THE GEORGE HERBERT JONES LABORATORY, UNIVERSITY OF CHICAGO]

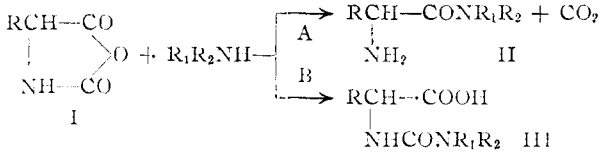
The Reaction of Amines with Amino Acid N-Carboxy Anhydrides

BY KENNETH D. KOPPLE

RECEIVED SEPTEMBER 11, 1956

The reaction of amines with N-carboxyanhydrides of α-amino acids proceeds to yield either a ureido acid (attack at the 2-carbonyl of the oxazolidinedione ring) or an amino amide (attack at the 5-carbonyl). The actual course of the reaction is dependent on the steric requirements of the amine, its concentration and its basicity. A number of new ureido acids are reported.

The reaction of an α-amino acid N-carboxy anhydride (4-alkyl-2,5-oxazolidinedione, I, abbreviated here to NCA) with an amine may, in principle, take either of two paths, A or B.



Path A, attack at the 5-carbonyl leading to an α-amino amide (*via* an intermediate carbamic acid derivative), is a normal chain propagation step in the polymerization of I to yield poly-α-amino acids.[1] Path B, although occurring much less frequently under normal circumstances, has been reported as an important chain-terminating reaction in such polymerizations.[2] In view of current interest in the polymers prepared from NCA's, some observations of the path of reaction between the NCA's and various amines are here reported.

The use of reaction path A to provide amides of amino acids is well established. Sigmund and Wessely have reported a 90% yield of phenylalanine amide and a 73% yield of the corresponding ethylamide obtained by treatment of phenylalanine NCA with excess amine in ethyl acetate solution.[3] The dimethylamides of glycine, DL-alanine, DL-phenylalanine and sarcosine have been prepared by action of excess dimethylamine on the corresponding N-carboxyanhydride.[4] The reaction of phenylalanine NCA with two equivalents of aniline yields di- and polypeptide anilides along with the amino acid amide.[5] By reaction in the presence of picric acid, however, the same reactants afford only phenylalanine anilide picrate.[5] The reaction of NCA's with amino acid and dipeptide esters has also been reported, the principal products observed being diketopiperazines and di- and tripeptide esters.[6]

A clear-cut case of reaction *via* path B is found in the reaction of phenylalanine NCA with two equivalents of sodium methoxide. A 3% yield of the urethan IV is obtained in addition to the ester V.[7]

$CH_3OCONHCH(CH_2C_6H_5)COONa$

IV

$NaOCONHCH(CH_2C_6H_5)COOCH_3$

V

In the present work, N-carboxyanhydrides were treated with a large excess of each of several amines; the ureido acid III formed by reaction along path B was isolated. In most cases the amino amide II, product of path A, was isolated as the picrate. With DL-phenylalanine NCA and aliphatic amines, the ratio of III to II observed was found to be related to the steric requirements of the attacking amine; with *t*-butyl and diethylamine the yield of ureido acid reached preparative values, but with dimethylamine and ethylamine, the amino amide was the dominant product. Glycine NCA behaved similarly, indicating that the benzyl group of the phenylalanine derivative is not an important factor in directing the course of reaction. Sarcosine NCA reacted only by path A, yielding no ureido acid, whatever the amine. The actual results of these experiments are given in Table I.

The facts just cited, taken alone, would indicate that polymerization of phenylalanine NCA could not proceed very far, for the amino group of a growing peptide chain, which must react by path A to continue the chain, cannot have steric requirements less than those of isopropylamine; isopropylamine, according to Table I, reacts by path B about one-half of the time. However, the amino group at the end of a peptide chain during polymerization is neither so strongly basic (pK_a of the amino group of glycine amide, 7.93[8]; that of isopropylamine, 10.72[9]) nor present in so high a concentration as

(1) D. H. G. Ballard and C. H. Bamford, *Proc. Roy. Soc. (London)*, **223A**, 495 (1954).

(2) M. Sela and A. Berger, THIS JOURNAL, **77**, 1893 (1955); L. A. Arcos, Shyterman and B. Labruyere, *Rec. trav. chim.*, **73**, 347 (1954).

(3) F. Sigmund and F. Wessely, *Z. physiol. Chem.*, **157**, 91 (1926).

(4) W. E. Hanby, S. G. Waley and J. Watson, *J. Chem. Soc.*, 3009 (1950).

(5) F. Wessely and M. John, *Monatsh.*, **48**, 1 (1927).

(6) J. L. Bailey, *J. Chem. Soc.*, 3461 (1950).

(7) A. Berger, M. Sela and E. Katchalski, *Anal. Chem.*, **25**, 1651 (1953).

(8) M. Zief and J. T. Edsall, THIS JOURNAL, **59**, 2245 (1937).

(9) G. Bredig, *Z. physik. Chem.*, **13**, 289 (1894).