trate) indicated that after 1000 hr at 5°C the intrinsic viscosity was about 6 dl/g in fair agreement with a recently reported value of 8 dl/g for chilled ichthyocol gelatin.[10]

[20] In contrast to reconstitution of native type fibrils, SLS precipitates are more structurally uniform and nearly quantitative. SLS aggregates were formed in excess of 98% yield from the untreated TC preparation used in the present experiments. Modification of ϵ-lysine groups (e.g., by complete acetylation) greatly reduces amounts of SLS formed. Some unusual forms of SLS have been obtained from sonically ruptured tropocollagen (see Hodge, A. J., and F. O. Schmitt, these PROCEEDINGS, **44**, 418 [1958]).

[21] Ewald, A., *Z. Physiol. Chem.*, **105**, 115 (1919).

[22] Wright, B. A. and N. M. Wiederhorn, *J. Polymer Sci.*, **7**, 105 (1951).

[23] However, at least one other laboratory has demonstrated the return of striations by low angle X-ray diffraction. A. Diokio obtained maxima at 495 A, 251 A, and 165 A for re-elongated tanned RTT. (Personal communication from L. Mandelkern, U.S. Bureau of Standards.)

[24] We have obtained electron micrographs of RNA (from TMV and yeast) sprayed in neutral volatile buffers before heating, while hot, and after cooling. Before heating, strands up to 10,000 A long are present; solutions sprayed in an oven show only globules; after cooling strands up to several thousand Angstroms long are found in large amounts and strands 10,000 A long are also present. These observations are obviously similar to the collagen experiments. They confirm the conclusions of Doty *et al.* (these PROCEEDINGS, **45**, 482 [1959]) concerning RNA helix-coil transitions except for present indications of relatively long helical regions (1000 to 10,000 A).

[25] Veis, A., and J. Cohen, *Nature*, **186**, 720 (1960).

[26] Marmur, J., and D. Lane, these PROCEEDINGS, **46**, 453 (1960).

[27] Doty, P., J. Marmur, J. Eigner, and C. Schildkraut, these PROCEEDINGS, **46**, 461 (1960).

---

*CHEMICAL AND ENZYMATIC SYNTHESIS OF CARBAMYL PHOSPHATE**

BY MARY ELLEN JONES AND FRITZ LIPMANN

GRADUATE DEPARTMENT OF BIOCHEMISTRY, BRANDEIS UNIVERSITY, AND THE ROCKEFELLER INSTITUTE

Communicated July 25, 1960

The mechanisms of chemical as well as enzymatic synthesis of carbamyl phosphate (CAP) pose a number of problems which invite closer exploration. This paper is more in the manner of an exposition of these problems and a statement of their scope and implication than an attempt to solve them definitely. The first part will deal with the chemical synthesis of CAP from cyanate and phosphate, and its decomposition which recently we recognized to be largely due to elimination of phosphate rather than to hydrolysis. The second part will deal with biochemical synthesis which, in the relatively simple bacterial system discussed here, goes by way of a carbamate phosphokinase transferring the terminal phosphate of ATP to carbamate.

The animal tissue process of CAP synthesis is a more complex reaction involving additional energy input and cofactors, and although we will not deal here with this process experimentally, we will discuss some of its facets in comparison with the simpler process in bacteria. The chemical synthesis of CAP has been discussed in the context of a pre-biological synthesis of an energy-rich phosphate carrier, and some consideration will be given to this proposition.

Methods and Preparations.—CAP was synthesized and isolated as the lithium

salt as previously described.[1] Ammonium carbamate was prepared by mixing liquid ammonia and dry ice; the compound generally contained approximately 10 per cent of ammonium carbonate.[2] CAP was determined by the differential phosphate determination described by Spector *et al.*[1] As will be shown, strong alkali decomposes CAP to phosphate and cyanate, while enzymatic reactions generally yield carbamate, i.e., ammonia.[3] Ammonia was determined by distillation in Conway vessels under the conditions described by Johnston *et al.*[4]

Free carbamic acid decomposes instantly to NH_3 and CO_2. Ammonium carbamate when dissolved in water, however, decomposes at a slower rate, depending on the temperature and the pH of the solution, until an equilibrium is reached between ammonium carbonate and ammonium carbamate.[5] Carbamate was determined by the method of Faurholt[5] which makes use of the solubility of barium carbamate in contrast to $BaCO_3$. The difference between $BaCO_3$, precipitated immediately at 0°, and the total $BaCO_3$, obtained after heating at 100° for 90 min in an ammonium, barium hydroxide solution, is used as the measure for carbamate.

Cyanate was measured semiquantitatively by a blue color produced in alcoholic solutions of cobaltous ions described as a qualitative test by Schneider.[6] Phosphate was found to interfere with the color development. It was removed as lithium phosphate by addition of $LiCl_2$; the samples were adjusted to pH 8 and sufficient ethanol added to give a final concentration of 70 per cent. After chilling, the Li_3PO_4 was centrifuged off and an equal volume of 95 per cent ethanol was added to an aliquot of the supernatant solution, followed by the addition of sufficient volume of 2 *M* $CoCl_2$ to give a 0.04 *M* final concentration.

Preparation of carbamate phosphokinase: The enzyme preparation used was a fraction derived from extracts of *Streptococcus faecalis* R. The culture of this organism was obtained from Dr. D. J. O'Kane. Subcultures were maintained as stock agar stabs.[7]

Cells were grown on a medium consisting of: 0.9% glucose, 1% K_2HPO_4, 1% yeast extract, 0.5% tryptone, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% $MnSO_4 \cdot 4H_2O$, and 0.9% arginine monohydrochloride. The cells were transferred from a solid agar stab to 10 ml of the medium and grown for 24 hr at 37°. A drop of this culture was used to inoculate 25 ml of medium and growth was allowed to proceed for 8 hr. The entire 25 ml was used to inoculate 2 liters of medium in a 4-liter Erlenmeyer flask which was incubated overnight at 37°. The cells from several 2-liter batches were collected by centrifuging in a refrigerated Sharples centrifuge. The cells were then washed by suspending in a volume of cold water equal to the volume of the culture medium, and were collected as above. This crude paste can be stored indefinitely at −20° before extraction.

Crude extracts were obtained by mixing 10 gm of cell paste with 50 ml of 0.9% KCl and subjecting this suspension for 40 min to sonic oscillation in a Raytheon 10 kilocycle magnetostrictor oscillator at 1°. The cell debris was removed by centrifuging at 15,000 × *g* in a refrigerated centrifuge for 15 min. The extract is stable indefinitely if held at −20°, and contains approximately 1,500 units (μM ATP synthesized from CAP + ADP per ml enzyme in 10 min at 30°), assayed as described previously,[8] and 18 mg protein per ml;[9] the specific activity is therefore 85 units per mg protein.

This crude extract was fractionated by addition of solid versene-recrystal-

lized ammonium sulfate.[10] The fraction between 65–85% saturation was collected, the precipitate dissolved in 0.9% KCl, and dialyzed thoroughly against the same solvent before use. This gave a preparation with a specific activity of 430. It was stable for several months at −20°, dialysis having caused no loss in activity or stability.

THE CHEMICAL SYNTHESIS AND DECOMPOSITION OF CAP

CAP is unstable in solution. At moderate temperatures it decomposes more or less rapidly, depending on pH. At alkaline reaction there is an almost instantaneous liberation of inorganic phosphate, while at neutral or acid reaction decomposition is slower. At elevated temperature, however, at acid as well as intermediate pH's, decomposition also becomes quite rapid, and at 100° it is completed in a few minutes.

In a previous paper,[8] the decomposition-pH profile was charted. At that time, however, we were not too familiar with the behavior of the breakdown products. Looking at CAP primarily as if it were merely another acid anhydride of the carboxyl phosphate type, we were misled to believe that decomposition was synonymous with hydrolysis of the phosphoanhydride bond, commonly rather rapid with this type of compound. The interpretation of our data, therefore, was made on the assumption that liberation of inorganic phosphate could be equated with hydrolysis to carbamate and phosphate. At this juncture, a number of observations helped us to realize that CAP was not so much hydrolyzed but, rather, was largely reversed to cyanate and phosphate. On heating CAP solutions with ammonium salts or amino acids, urea and ureido acids were found to form. This looked, at first, as if CAP could also act nonenzymatically as a carbamyl donor. CAP determinations, however, showed that urea and ureido acid formation continued after CAP had disappeared from solution. It was found then that if equivalent concentrations of cyanate were used instead of CAP the results obtained were quite analogous. Similar results have recently been reported by Reuter.[11] This was one of the observations that forced us to conclude that CAP yields cyanate which then becomes a carbamyl donor in this system.

Furthermore, our earlier observation that the amount of ammonia liberated at the neutral region was surprisingly low made us begin to doubt that carbamate could be the product of the reaction, because we learned that 80–90 per cent of carbamate at this pH dissociates to $NH_3 + CO_2$. On the other hand, from our experiments on the synthesis of CAP from cyanate and phosphate, this appeared to be due, most likely, to an equilibrium reaction. At that time we were fortunate to find a rather specific color test for cyanate which had been developed in 1895 by Schneider[6] for the determination of small amounts of cyanate present in cyanide. By applying this test to CAP solutions that had been decomposing at alkaline or neutral reaction, it was easy to show that the blue color of the Co^{++}-cyanate complex of Schneider appeared increasingly on standing. Although we found it difficult to standardize the conditions for this test for the quantitation of cyanate, it was invaluable as a qualitative and semiquantitative assay. It was argued, furthermore, that if cyanate was the main product, electrotitration of the decomposition mixture should show the appearance of a fairly strong acid. Cyanic acid, according to Amell,[12] has a pK short of 4. Therefore, an electrotitrometric com-

parison of a CAP solution before and after decomposition would give a good estimate of the amount of cyanate formed.

The electrotitration curves for inorganic phosphate, CAP, and cyanate are shown in Figure 1. The curve for cyanate indicates a dissociation constant

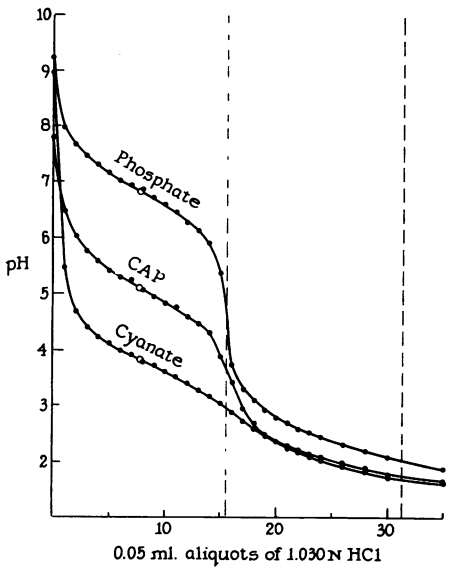

FIG. 1.—Titration curves for potassium phosphate, lithium CAP, and potassium cyanate. The upper curve represents the titration of 4.9 ml of 0.162 *M* K_2HPO_4, the middle curve of 15 ml of 0.053 *M* dilithium CAP, which contained 7.7% orthophosphate when the titration was started and 13.6% orthophosphate at the end, and the lower curve 5.3 ml of 0.15 *M* KNCO, all made up to 15 ml with distilled water before titration. The temperature was 22.5°, and the standard acid used was 1.030 *N* HCl. Additions were made with a calibrated syringe burette and mechanical stirring with vessels placed in a large volume of water.

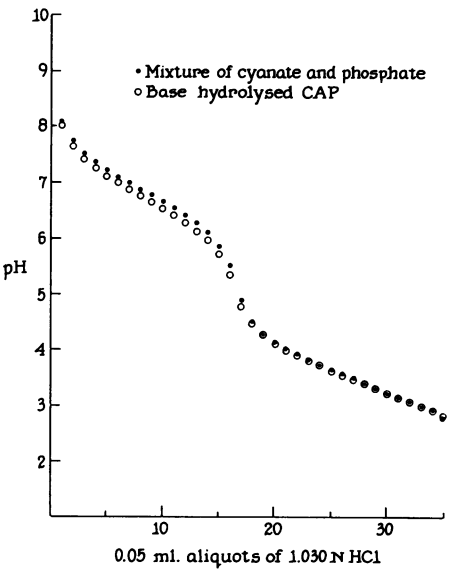

FIG. 2.—Comparison of electrotitration curves of alkali-decomposed CAP and a mixture of cyanate and phosphate. Alkali decomposition was produced by the addition of 0.1 ml of 10 *N* KOH to 15 ml of 0.053 *M* CAP solution; the vessel was allowed to stand at room temperature (23°) for 15 min, at which time all of the phosphate was present as orthophosphate. Open circles represent the curve for the decomposed CAP, the solid circles represent the curve for a solution containing a mixture of 0.053 *M* phosphate and 0.053 *M* cyanate. Small aliquots of this vessel were tested at various time intervals during the titration, and the CAP content was always less than 10% (cf. Fig. 5).

of 3.8 at 23°, which checks well with the reported value.[12, 13] The second CAP dissociation constant of 5.3 at 20° is rather similar to the second dissociation constant of 4.7 for acetyl phosphate,[14] both being nearly 2 pK units lower than for inorganic phosphate.

A comparison of the titration curve of alkali-decomposed CAP with a mixture of inorganic phosphate and cyanate is presented in Figure 2. The curves overlap rather convincingly. Solutions of partially decomposed CAP at intermediate pH's under conditions analogous to those described in Figure 1 of the paper by Jones *et al.*[8] were also examined by electrotitration. In these more complex data the liberation of cyanate could be recognized, and approximate estimates confirm that between pH 6 and 9 cyanate and phosphate are the main products of CAP decomposition. A re-evaluation of the pH-decomposition profile[8] is presented in Figure 3.

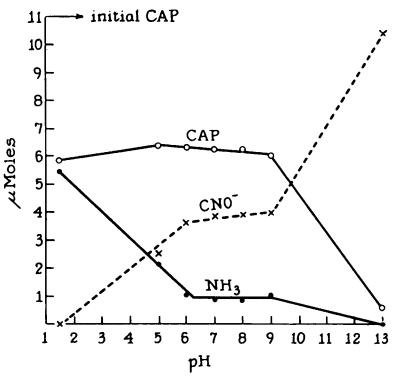

FIG. 3.—The pH-dependence of CAP decomposition. All vessels were incubated at 37° for 45 min. For vessels at pH 5–9 each ml of solution contained: 11 μmoles of dilithium CAP, and 100 μmoles each of acetate, imidazole, Tris, and glycine adjusted to the indicated pH. For pH 1.5 and 13, the solutions were 0.05 *M* HCl and KOH respectively, containing the same amount of CAP per ml. The curve of the open circles represents CAP present, while the solid circle represents ammonia, or ammonium bicarbonate, present. The broken line is the difference between these two curves and represents the cyanate present.

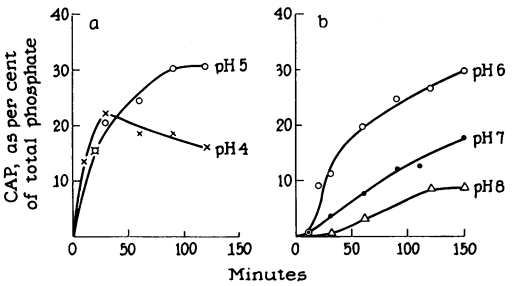

FIG. 4.—Course of CAP synthesis at various pH's. Each beaker contained at zero time, 3 ml of 1.5 *M* KH_2PO_4 and 3 ml of 1.5 *M* KCNO, as well as distilled water and appropriate amounts of HCl or KOH to obtain the desired pH made up to 15 ml and a final concentration of 0.3 *M*. The vessel was placed in a bath of water at room temperature, and supplied with a mechanical stirrer, the electrodes of the pH-stat, and the microtip of the syringe burette containing 2.98 *N* HCl added automatically to keep the indicated pH. At the intervals noted, samples were analyzed for orthophosphate and orthophosphate + CAP.

From these observations we conclude as follows: at strongly alkaline reaction, CAP decomposes, or one might say reverses, to cyanate by phosphate elimination with practically no hydrolysis; at intermediate pH's, down to 6, elimination prevails over hydrolysis. At strongly acid reaction, the final result is hydrolysis to phosphate, CO_2, and ammonia; however, if free cyanic acid were formed primarily by elimination it would hydrolyze rather rapidly to ammonium carbonate. But an indication that, at the acid situation, hydrolysis is largely direct was obtained by decomposition in H_2O^{18}. In unpublished experiments, Allison and Jones[15] found at pH 1.0 that about 70 per cent of the O^{18} expected for exclusive hydrolysis was found in phosphate. At intermediate pH's, less O^{18} transfers from H_2O^{18} to phosphate; at strongly alkaline reaction very little, if any, such O^{18} transfer is observed. It appears, then, that decomposition by hydrolysis and elimination occur side by side, elimination becoming exclusive with increasing alkalinity and hydrolysis becoming the most important path at strongly acid reaction.

Equilibrium and mechanism: The expectation of a synthesis of CAP by condensation of cyanate, or isocyanic acid, and phosphate had prompted Spector[1] to try such a procedure analogous to the known synthesis of acetylphosphate from ketene and phosphoric acid,[16] formulating here without regard to state of ionization:

$$H_2C{:}C{:}O + HOPO_3H_2 \rightarrow H_3C{\cdot}COOPO_3H_2$$

$$HN{:}C{:}O + HOPO_3H_2 \rightarrow H_2N{\cdot}COOP_3H_2$$

Accordingly, the rate of reaction should correlate with the availability of the react-

ant, isocyanic acid. When the dependence of initial rate on pH was tested, as shown in Figure 4 (*a* and *b*), initial rate was found to be highest at pH 4 but nearly as fast at pH 5, while at higher pH's the rate decreased strongly. It may be seen in Figure 5*a* that at pH 4, CAP initially forms rapidly but starts to disappear from 30 min onwards. With a pK of 3.8 at pH 4, the ratio of free acid to cyanate is approximately 1:1, the former hydrolyzing very rapidly. This rapid disappearance of the reactant complicates the rate evaluation in strongly acid medium where the initial rate is probably higher than it appears to be in the crude assay. Optimal synthesis obtains between pH 5 and 6, a region which had been chosen empirically for the preparative method. At this pH, the equilibrium is reached fairly rapidly; the decomposition of cyanate by way of hydrolysis of free acid is relatively slow, although not negligible.

Figure 5*b* reveals an interesting feature, that at alkaline or neutral reaction a brief induction period precedes the synthesis. This may indicate that, at this pH range where cyanate is present almost exclusively as the anion, CNO^-, it takes some time to accumulate enough reactive compound for the condensation to get going. Since isocyanic acid seems to be the reactant, the induction period should represent the time allowed for build-up of isocyanic acid by way of $H^+ + CNO^- \rightarrow CNOH \rightarrow HNCO$.

Infrared spectra of the vapor and liquid[17] under these conditions indicate the equilibrium cyanic $\rightleftharpoons$ isocyanic acid to be 99.8 per cent in favor of the latter. It is suggested that in solution[18] an equilibrium exists between the two forms. However that may be, in an over-all manner, the preliminary data indicate the condensation isocyanic acid + phosphate to be the likely mechanism of CAP synthesis.

To determine the equilibrium between the three components, cyanate, phosphate, and CAP, experiments were carried out at pH 6 using a pH-stat to keep the pH constant. In the routine preparation, the mixture of cyanate and dihydrogen phosphate had shown a rather rapid change at 30° from an initial pH of about 5.3 to 6 during the first 5 min, and from then on had changed slowly to a higher pH. The main reason for this rise is the hydrolysis of cyanate to ammonium carbonate (cf. also legend to Table 1). To maintain homogenous conditions, a constant supply

TABLE 1

DETERMINATION OF EQUILIBRIUM BETWEEN CYANATE, PHOSPHATE, AND CAP

Initial, Cyanate *M*	Initial, Phosphate *M*	Final, Cyanate *M*	Final, Phosphate *M*	CAP, *M*	$K = \frac{[CAP]}{[cyanate]\ [phosphate]}$
0.3	0.28	0.18	0.19	0.08	2.4
0.3	0.29	0.16	0.19	0.10	3.5
0.3	0.56	0.12	0.41	0.15	3.1
0.6	0.28	0.34	0.13	0.15	3.4
				Av.	3.1

Cyanate and phosphate were analytical reagents. 3 or 6 ml aliquots of 1.5 *M* KNCO or KH_2PO_4-K_2HPO_4, pH 6.0, were added to sufficient distilled water to give a total volume of 15 ml. All vessels were incubated at 30° in a beaker containing a mechanical stirrer, and equipped with a syringe burette filled with 2.98 N HCl and connected to the pH-stat. HCl was added automatically to the beaker to maintain the pH at 6.

During incubation, the reaction, if not adjusted, shifts towards a higher pH because of a slow, steady hydrolysis of CNO^- to $CO_2 + NH_4^+$ whereby two base equivalents appear; the conversion of $H_2PO_4^-$ to $CNO^- \rightarrow CAP^{2-}$ occurs at pH 6, practically without change of pH because the disappearance of CNO^- is compensated for by the increase in the second dissociation constant in CAP.

The final cyanate concentration was estimated by subtracting from the initial value the CAP formed and one-half of the HCl consumed. The latter correction amounted to approximately 20% of the initially added cyanate. Phosphate and CAP were determined as described. At intervals, 0.1 ml aliquots were removed for orthophosphate and CAP analysis. Equilibrium was attained between 120 and 180 min.

of acid was needed and was applied automatically through the instrument. An exhaustive study of the equilibrium was not attempted. The effect of change of concentrations of the components was studied largely to find the best conditions for synthesizing radioactive CAP from either radioactive cyanate or radioactive phosphate. It appears from Table 1 that, as expected, the yield increased by increasing the concentration of either the cyanate or the phosphate. The data

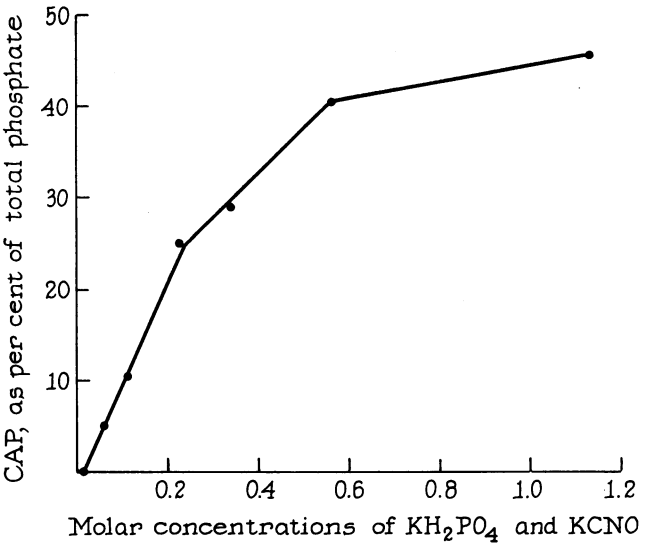

FIG. 5.—Dependence of CAP synthesis on concentration of cyanate and phosphate. Each vessel contained in 10 ml appropriate aliquots of 1.5 *M* KH_2PO_4 and 3 *M* KCNO to give the concentration indicated on the abscissa. To obtain the synthesis for 0.34 *M* concentration, 2.25 ml. of 1.5 *M* KH_2PO_4 and 6.8 ml H_2O were mixed and placed at 30°. At zero time, 1.12 ml of 3 *M* KCNO was added (total volume 10 ml) and thoroughly mixed. At 60 min, aliquots were taken and the orthophosphate and the sums of CAP and orthophosphate were determined. If dilution was required for analysis, an aliquot was added to a suitable volume of ice-cold water.

indicate, as a rough approximation, a value of 3 for the equilibrium constant, corresponding to a change of free energy on synthesis, ΔF (cyanate + phosphate $\rightleftharpoons$ CAP), of -680 cal. As expected for a bimolecular reaction, the yield is quite dependent on concentration, falling sharply toward lower concentrations. The relative yield at different concentration levels is shown in Figure 5.

THE ENZYMATIC SYNTHESIS OF CAP FROM CARBAMATE AND ATP

If, as in Figure 6, the microbial enzyme is incubated with a mixture of carbamate and ATP, the synthesis of CAP is very rapid; in the first 8 minutes three times as much CAP is formed from carbamate as is formed from a freshly prepared equimolar solution of ammonium carbonate. With carbamate as the initial substrate, CAP concentration reaches a maximum at 10 minutes, after which it declines. When about two-fifths of the excess CAP have disappeared, it levels off, having reached the same level to which CAP concentration slowly climbs when freshly dissolved ammonium carbonate is used as the source of carbamate. At that time, the initially

added 0.134 M carbamate has adjusted to an equilibrium mixture of 0.011 M carbamate and 0.123 M ammonium carbonate. It is a decline in carbamate concentration that causes the observed decomposition of CAP and eventually the system adjusts to the same CAP level whether carbamate or ammonium carbonate is added at the beginning. The rapid initial burst of CAP formation from carbamate and ATP, however, nicely illustrates that carbamate is the true substrate of phosphorylation.

A further clarification of the rather involved equilibria between ammonium bicarbonate, CO_2, NH_3, and carbamate was obtaincd by the use of carbonic anhydrase:

reaction 1: $NH_4HCO_3 \xleftarrow{\text{carbonic anhydrase}} H_2O + CO_2 + NH_3$

reaction 2: $NH_3 + CO_2 \xleftarrow{\text{kinase}} H_2NCOOH$

reaction 3: $H_2NCOO^- + ATP^{-4} \longleftrightarrow H_2NCOOPO_3^{-2} + ATP^{-3}$

Roughton[19] had already observed that, under certain conditions, carbonic anhydrase will increase the slow rate of carbamate formation from an ammonium-bicarbonate-carbonate mixture. This is so, because $NH_3 + CO_2$ are the reactants that condense to carbamate. Therefore, when ammonium carbonate-bicarbonate is freshly dissolved without the enzyme, the CO_2 concentration builds up quite slowly and carbamate, and consequently CAP, form sluggishly: this can be seen on the lower curve of Figure 6. Carbonic anhydrase speeds up the dehydration of ammonium bicarbonate to $CO_2 + NH_3$ and thereby carbamate condensation which, in turn, furnishes more substrate to react with ATP in the carbamate phosphokinase reaction. This is illustrated in Figure 7 where CAP formation in the carbamate phosphokinase system with ammonium carbonate as substrate is compared with and without carbonic anhydrase: the figure again demonstrates the sluggishness of the equilibrium between CO_2, NH_3, and carbamate using *freshly* dissolved ammonium carbonate without enzyme. If an *aged* solution of ammonium carbonate is used instead, the effect of carbonic anhydrase disappears. A further illustration of the effect of carbonic anhydrase is given in Figure 8 where the course of CAP formation is charted with and without carbonic anhydrase using carbamate as the sub-

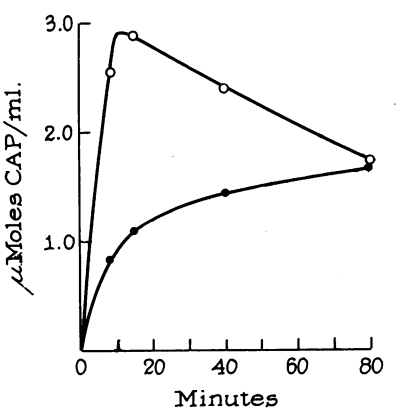

FIG. 6.—Comparison of CAP synthesis from carbamate and ammonium carbonate + ATP. Each vessel contained per ml: a mixed buffer composed of 100 μM Tris and Bis(2-amino-2-methyl-1,3-propanediol) pH 9.5, 8.8 μM ATP, 7 μM $MgCl_2$, and 6 mg of carbamate phosphokinase (2600 units). The open circles represent the vessel to which solid ammonium carbamate (133 μmoles per ml of reaction medium) was added at zero time. The solid circles represent the vessel to which solutions of potassium carbonate (134 μmoles per ml of reaction medium) and ammonium chloride (268 μmoles per ml of reaction medium were added). The incubation was at 10°. The total volume was 1.5 ml, of which samples were taken at the interval noted for P_i and CAP determinations.

strate instead of ammonium carbonate. In this case, the addition of carbonic anhydrase causes a decrease rather than an increase in the CAP that forms initially: the anhydrase effects a rapid disappearance of carbamate for the same reason that it speeds carbamate formation from ammonium bicarbonate. In the presence of

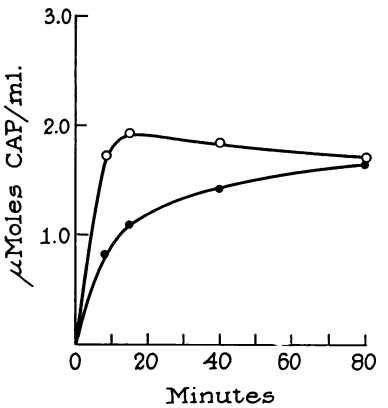

FIG. 7.—Effect of carbonic anhydrase on CAP synthesis from ammonium carbonate + ATP. Conditions were the same as for Fig. 6. The vessel represented by solid circles contained ammonium carbonate alone, while the open circles represent the vessel that contained ammonium carbonate and 0.1 mg of highly purified carbonic anhydrase (courtesy of Mr. Arlan Roberts, Rohrig and Co., Chicago, Ill.).

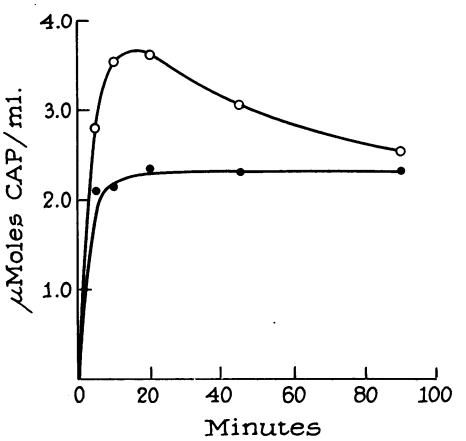

FIG. 8.—The effect of carbonic anhydrase on CAP synthesis from carbamate + ATP. Each vessel contained in 1 ml: 66 μmoles of Tris buffer pH 9.4, 13 μmoles of ATP, 13 μmoles of $MgCl_2$, and 6 mg of a purified carbamate phosphokinase containing 430 units per mg protein. Tube 1 contained 120 μmoles of ammonium carbamate per ml. Tube 2 contained 0.14 mg of carbonic anhydrase per ml in addition to 120 μmoles of ammonium carbamate. The total volume was 3 ml. and the tubes were incubated at 10°. At interval noted, 0.5 ml was added to 1 ml ice-cold TCA, and the protein was removed by centrifugation at 0°. The supernatant fraction was analyzed for orthophosphate and for orthophosphate + CAP using base hydrolysis to obtain total phosphate.

anhydrase, CAP formation becomes nearly identical whether one starts with carbamate or ammonium carbonate. Comparison of the curves in Figures 6, 7, and 8 confirms the assumption that carbamate is the true substrate of the reaction catalyzed in microbial CAP synthesis.

Table 2 gives data on over-all equilibrium determination, and an equilibrium

TABLE 2

EQUILIBRIUM CONSTANT FOR THE REACTION: $ATP + NH_2COO^- \rightleftharpoons ADP + NH_2COOPO_3^=$

Experiment of figure	Time, min	Substrate concentration ATP, μM/ml	CA, μM/ml	ADP, μM/ml	CAP, μM/ml	$K = \frac{[CAP][ADP]}{[CA][ATP]}$
6 and 7	0	8.80	11.3	0	0	..
	80	7.07	11.3	1.73	1.73	0.037
8	0	13.0	10.2	0	0	..
	90	10.7	10.2	2.3	2.3	0.048

The final concentration of CAP and the initial ATP concentration were determined; the final ATP concentration was estimated by difference and the final ADP was assumed to be equal to that of CAP. With the purified preparation there is essentially no phosphatase activity toward the three substrates, and at 10° the chemical half life of CAP is greater than 12 hr. The carbamate concentration was estimated from a separate experiment in which ammonium carbamate and ammonium carbonate were allowed to come to equilibrium and the carbamate was estimated by the method of Faurholt.[5] Under the conditions of these experiments at equilibrium, 8.5% of the initial ammonium carbonate was present as carbamate.

constant of 4×10^{-2} for carbamate + ATP = CAP + ATP is obtained. Roughly, it had already been observed[3] that equilibrium favors a formation of ATP from ADP and CAP, and for this reason the enzyme is generally more easily assayed in the direction of ATP synthesis. The equilibrium constant now obtained shows that the phosphoryl potential of CAP at pH 9.5 is approximately −1.8 kcals. higher than that of the terminal phosphate of ATP, and CAP formation is slightly uphill. To appreciate the over-all energy required for $NH_3 + CO_2 + ATP \rightleftharpoons CAP$, the ratio of carbamate to ammonium carbonate needs to be considered, which is approximately 0.05. Therefore, the over-all reaction is still more uphill by about 0.8 kcal. On the other hand, CAP + ADP is a good feeder system for ATP.

Comments.—Comparison of CAP synthesis in mammalian tissue and bacteria: In view of the interest in urea synthesis as an important phase in animal metabolism, citrulline synthesis, particularly in mammalian liver where urea synthesis occurs, has been more intensively studied in animal tissue than in the just discussed microbial system. The work of Grisolia and Cohen[20] provided the earliest indication of energy derivation from ATP. Later, a dependence of this reaction on the presence of N-acylated glutamate, such as acetyl or carbamyl glutamate, was discovered in Cohen's laboratory.[21] It has been pointed out in earlier communications[3, 22] that acetyl glutamate is not a component of our bacterial system. Purified preparations of the enzyme synthesizing CAP from ATP and carbamate did not show any requirement for this cofactor. Nevertheless, it soon appeared that in animal tissue CAP is also the precursor of the carbamyl group in citrulline, and that carbamyl *transfer* from CAP to ornithine is acetyl glutamate-independent.[8] The additional catalyst, acetyl glutamate, only functions in the animal system of CAP *synthesis*. This is thus a more complex reaction which, according to recent work,[23] involves the participation of two ATP's, in contrast to the straight formation of CAP from carbamate and one ATP in the microbial enzyme system, at least in that of *S. faecalis*. In *E. coli*, Schwartz in Werner Maas' laboratory,[24] observed that several mutants requiring both uridine and arginine, implying a deficiency of the common precursor CAP, still contain carbamate kinase although in reduced amounts. This made them wonder if there might be an additional enzyme implicated in CAP synthesis in *E. coli*. No effect, however, of acetyl glutamate has been found so far, neither has there been direct confirmation of such a possibility.

The equilibrium data indicate that a formation of CAP from ammonium carbonate + ATP is an uphill reaction. In the case of the animal, this reaction is responsible for a fast removal of ammonia which, even at rather low concentrations, is a powerful poison. Therefore, apparently an additional activation is needed to form CAP easily from the relatively low concentration level of ammonia that is permissible to keep the animal out of trouble. The activation, through a second phosphoryl expenditure, would effect the equivalent of a boost in concentration of one of the reactants. It now seems that it is the CO_2 that is activated by the acetyl glutamate-linked ATP. The over-all effect of such a CO_2 activation will be to attract the ammonia to enter into the link with CO_2 from low concentration level. But obviously the close coordination of the formation of carbamate and of its phosphorylation to CAP is not clear, nor is the implication of acetyl glutamate in this sequence clarified. From early work by Lardy and Peanasky[25] there are indications that citrulline formation in the animal has a relation to a biotin-catalyzed reaction. In this connection, after rather extensive purification,

Peck[26] tested the carbamate phosphokinase in *S. faecalis* for the presence of biotin. No accumulation of biotin was observed in the course of purification of this enzyme; although the final preparation was not completely free of biotin, the ratio of activity to biotin appeared so low that its presence in microbial enzymes seems to be excluded (cf. also Ravel *et al.*[27]).

To summarize, there now appears to be general agreement that CAP is the intermediary in the carbamylization of ornithine and aspartic acid in animal tissue as well as in the microbial system. The earlier much discussed "active carbamate," once called compound X, is now considered to be identical with CAP.[28] The effect of acetyl glutamate, in which the animal system differs from the microbial system, is localized exclusively in the formation of CAP and not in the transfer of carbamyl from CAP.

Biochemical evolution of urea synthesis: Reflecting on the enzymatic carbamyl transfer found presently in biological systems and in particular in urea synthesis, we often wondered during this work why, in urea synthesis, CAP did not react directly with ammonia to form urea rather than with ornithine to form δ-ureido-α-amino pentanoic acid (citrulline) followed by a laborious energy-consuming cycle.[29, 30] It may be that ammonia concentration, which has been discussed, is an issue here. The generally low concentrations of free ammonia may make it necessary to employ the cyclic pathway. There seems, however, to be another possibility. In biochemical evolution, the arginine synthesis pathway must have developed very early since every organism needs arginine as part of its protein complement. One might therefore consider that this pathway, once developed, presented a smooth procedure and had to attach only one further step, the arginase-catalyzed hydrolysis of arginine, to yield urea. In view of the availability of arginine synthesis as a universal reaction, it offered itself for adoption to urea synthesis, the possibility of a less complex chemical path notwithstanding. Still, urea appears in large quantities in some plants, and one wonders if it could serve as a source of metabolic energy since, in analogy to citrulline phosphorylysis, urea could yield CAP by phosphorylysis.

Prebiological synthesis of CAP: The spontaneous condensation of cyanate and phosphate to CAP seems to be a uniquely easy synthesis of a carrier of energy-rich phosphate. In recent discussions on the origin of life[31] the need of a spontaneous formation of an energy-carrying phosphoryl compound was considered. Given the existence of cyanate in the preorganismic period, there should have been phosphate available to form CAP, although its concentration would be expected to be rather low. Its use as a source of energy in some microorganisms makes such a speculation more attractive. As mentioned, in *S. faecalis*, the phosphorylysis of citrulline may be adaptively developed into a major pathway of energy derivation:[22, 32–34]

$$\text{Citrulline} + \text{phosphate} \rightarrow \text{CAP} + \text{ornithine}$$
$$\text{CAP} + \text{ADP} \rightarrow \text{ATP} + CO_2 + NH_3.$$

This citrulline fermentation seems to support life and growth in such adapted organisms. They live on CAP as the sole source of energy.

Attractive as such a scheme might look it remains to be considered that CAP, metabolically speaking, is only a phosphoryl feeder to the nucleotide polyphos-

phate system and may not really present a good primary energy source. The inorganic polyphosphates which are found in a great variety of organisms seem more attractive as primary donors. Although now they give the impression of being metabolically fairly inert, they may represent an early means of energy distribution which was abandoned later on. Their availability from inorganic sources seems to be rather likely.

* This work was aided in part by a grant from the National Cancer Institute, National Institutes of Health, U.S. Public Health Service. Most of the work was carried out in the Biochemical Research Laboratory of the Massachusetts General Hospital.

† The abbreviations used in most cases are those accepted by the *Journal of Biological Chemistry*. Exceptions are: CA, carbamate; CAP, carbamyl phosphate; TCA, trichloroacetic acid.

[1] Spector, L., M. E. Jones, and F. Lipmann, in *Methods of Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, 1957), vol. **3**, p. 653.

[2] Brooks, L. A., and L. F. Audrieth, *Inorganic Synthesis* (New York: McGraw-Hill, 1946), vol. **2**, p. 85.

[3] Jones, M. E., L. Spector, and F. Lipmann, *J. Am. Chem. Soc.*, **77**, 819 (1955).

[4] Johnston, R. B., M. J. Mycek, and J. S. Fruton, *J. Biol. Chem.*, **185**, 629 (1950).

[5] Faurholt, C., *Det. Kgl. Danske Videnshaberne Selskab. Mathematisk-fysiske Meddelelsen III*, 20 (1921).

[6] Schneider, E. A., *Ber. Deut. Chem. Ges.*, 1540 (1895).

[7] O'Kane, D. J., and I. C. Gunsalus, *J. Bact.* **56**, 499 (1948).

[8] Jones, M. E., L. Spector, and F. Lipmann, in *Proc. 3rd International Congress of Biochemistry*, Brussels, 1955, ed. C. Liebecq (New York: Academic Press, 1956), p. 270.

[9] Stadtman, E. R., G. D. Novelli, and F. Lipmann, *J. Biol. Chem.*, **191**, 365 (1951).

[10] Beisenherz, G., H. J. Boltze, Th. Bücher, R. Czok, K. H. Garbade, E. Meyer-Arendt, and G. Pfleiderer, *Z. Naturforsch.*, **8b**, 555 (1953).

[11] Reuter, G., *Z. Naturforsch.*, **14b**, 475 (1959).

[12] Amell, A. R., *J. Am. Chem. Soc.*, **78**, 6234 (1956).

[13] Taufel, K., C. Wagner, and H. Dunwald, *Z. Electrochem.*, **34**, 115 (1928).

[14] Lipmann, F., and L. C. Tuttle, *J. Biol. Chem.*, **153**, 571 (1944).

[15] Allison, W., and M. E. Jones, unpublished data.

[16] Bentley, R., *J. Am. Chem. Soc.*, **70**, 2183 (1948).

[17] Eyster, E., R. Gillette, and L. Brockway, *J. Am. Chem. Soc.*, **62**, 2326 (1940).

[18] Werner, E. A., and W. R. Fearon, *J. Chem. Soc.*, **117**, 1356 (1920).

[19] Roughton, F. J. W., and A. M. Clark, in *The Enzymes*, ed. J. B. Sumner and K. Myrbäck (New York: Academic Press, 1951), vol. **1**, p. 1250.

[20] Grisolia, S., and P. P. Cohen, *J. Biol. Chem.*, **191**, 189 (1951).

[21] *Ibid.*, *J. Biol. Chem.*, **204**, 753 (1953).

[22] Korzenovsky, M., in *A Symposium on Amino Acid Metabolism*, ed. W. D. McElroy and B. Glass (Baltimore: The Johns Hopkins Press, 1955), p. 309.

[23] Metzenberg, R., L. M. Hall, M. Marshall, and P. P. Cohen, *J. Biol. Chem.*, **229**, 1019 (1957).

[24] Schwartz, J. H., Dissertation in partial fulfillment of the requirements for the degree of doctor of medicine with honors in microbiology, New York University College of Medicine, 1959.

[25] Lardy, H. A., and R. Peanasky, *Physiol. Rev.*, **33**, 560 (1953).

[26] Peck, H., unpublished data.

[27] Ravel, J. M., M. L. Grona, J. S. Humphreys, and W. Shive, *J. Biol. Chem.*, **234**, 1452 (1959).

[28] Marshall, R. O., L. M. Hall, and P. P. Cohen, *Biochim. et Biophys. Acta*, **17**, 279 (1955).

[29] Krebs, H. A., and K. Henseleit, *Z. Physiol. Chem.*, **210**, 33 (1932).

[30] Ratner, S., *Adv. in Enzymol.*, **15**, 319 (1954).

[31] Bernal, J. D., in Proc. of 1st International Symposium on *The Origin of Life on the Earth*, ed. A. I. Oparin *et al.* (London: Pergamon Press, 1959), p. 38.

[32] Knivett, V. A., *Biochem. J.*, **56**, 602 (1954).

[33] Oginsky, E. L., in *A Symposium on Amino Acid Metabolism*, ed. W. D. McElroy and B. Glass (Baltimore: The Johns Hopkins Press, 1955), p. 300.

[34] Slade, H. D., in *A Symposium on Amino Acid Metabolism*, ed. W. D. McElroy and B. Glass (Baltimore: The Johns Hopkins Press, 1955), p. 321.