[CONTRIBUTION FROM THE CONVERSE MEMORIAL LABORATORY OF HARVARD UNIVERSITY]

Kinetics of Peptide Bond Formation[1]

BY D. E. KOSHLAND, JR.

Acetyl phosphate has been shown to react at a measurable rate with some amines and amino acids in aqueous solution at 39°. The reaction is first order in acetyl phosphate, first order in amino compound and involves a nucleophilic attack by the nitrogen of the amino compound on the carbonyl carbon of the acyl phosphate. The relationship of the observed rates to the mechanism of peptide bond formation in biological systems is discussed. A new synthesis of acetyl phosphate is described which utilizes the acid-catalyzed reaction of isopropenyl acetate with phosphoric acid in normal butyl ether.

A fundamental aspect of the problem of protein synthesis is the mechanism by which the individual amino acids are linked together to form the polypeptide chain. Although the theories postulated so far have certain attractive features, they are based largely on mechanical analogies, *e.g.*, the "template hypothesis," and it is not readily apparent how they can be modified to be consistent with what is known of the mechanisms of organic and enzymatic reactions. To clarify this part of the problem, a kinetic study of peptide bond formation *in vitro* has been initiated using compounds which are believed to be close analogs of the actual intermediates in the biological system.

Of the many compounds which have been postulated as intermediates in protein synthesis, the amino acid acyl phosphates, originally suggested by Lipmann,[2] seem most in accord with the currently available evidence (see below). It was decided, therefore, to study the kinetics of the simplest acyl phosphate, acetyl phosphate, with various amines and amino acids in aqueous solution in the absence of any enzymes

$$CH_3\overset{O}{\overset{\|}{C}}OPO_3H_2 + H_2NR \longrightarrow CH_3\overset{O}{\overset{\|}{C}}NHR + H_3PO_4 \quad (1)$$

Experimental

Synthesis of Acetyl Phosphate from Isopropenyl Acetate.—Fourteen milliliters of isopropenyl acetate[3] (12.8×10^{-2} mole) dissolved in 15 ml. of dry *n*-butyl ether was added gradually with cooling to 6 ml. of 90% phosphoric acid (9.6×10^{-2} mole) and 2 drops of concentrated sulfuric acid in 15 ml. of the butyl ether. The solution was shaken for 15 minutes at room temperature and then warmed gently to 30–35°, where it was kept under reduced pressure (approximately 20 mm.) for one-half hour. At higher temperatures polymerization was observed to occur. The solution was then poured into a small volume of ice-water containing solid lithium carbonate and neutralized by addition of more solid lithium carbonate to a *p*H of 3–4. The ice-cold solution was extracted twice with approximately two volumes of ether and then neutralized with lithium carbonate and lithium hydroxide to a *p*H of 9. After filtering to remove the precipitate of lithium phosphate, cold absolute alcohol was added gradually in large excess (9 volumes to 1). The milky slurry was allowed to stand for one-half hour at 0° before filtering on a Buchner funnel. The precipitate, which was washed with absolute alcohol and ether and dried in a vacuum desiccator, contained 5.1×10^{-3} mole of lithium acetyl phosphate of 97% purity. The compound was purified further by recrystallization from aqueous alcohol and by conversion to the silver salt.

Anal. Calcd. for $CH_3COOPO_3Li_2$: acetate, 39.5; phosphate, 20.4. Found: acetate, 39.9; phosphate, 20.0.

Materials.—The amines and amino acids were commercial samples, recrystallized before use in the kinetic runs. Glycylglycine, glycine, proline and alanine were recrystallized from aqueous alcohol. Sarcosine and glycine ethyl ester hydrochloride were recrystallized from absolute alcohol. Aniline hydrochloride and benzylamine hydrochloride were crystallized from water.

Kinetic Runs.—The solution containing the appropriate buffer, the amine and enough sodium perchlorate to bring the ionic strength to 0.6 was added to a glass-stoppered volumetric flask and allowed to stand in a 39.00 ± 0.02° thermostat until temperature equilibrium was attained. A few milligrams of the powdered lithium acetyl phosphate was added and the decrease in lithium acetyl phosphate followed as a function of time by removing 2-ml. aliquots at regular intervals.

All experiments were run to greater than 75% reaction of acetyl phosphate, a few to greater than 97%. Since the initial amine concentration was usually greater than 0.1 *M* and the initial lithium acetyl phosphate concentration 0.002–0.004 *M*, no significant decrease in the amine concentration occurred during the course of an experiment and no drift in the values was observed. In the few cases in which this situation did not hold, an approximate correction for the later points using the initial slope gave good agreement.

The buffer used for the runs in neutral solution was a mixture of the mono- and dibasic sodium phosphates made up so that the total phosphate concentration was 0.05 *M*. For the experiments at *p*H 10, the amino compound itself served as buffer when it was present and a 0.05 *M* carbonate buffer was used for the hydrolysis experiments.

The quantitative determination of the acetyl phosphate concentration was performed by a modification of the method of Lipmann and Tuttle[4] in which acetyl phosphate is converted to acetylhydroxamic acid. The 2-ml. aliquot is added to 4 ml. of freshly prepared neutral hydroxylamine solution contained in a 10-ml. volumetric flask. The latter solution, which is 1 *M* in hydroxylamine and 0.05 *M* in acetate buffer, can be conveniently prepared from stock solutions of 4 *M* hydroxylamine hydrochloride, 3.5 *M* sodium hydroxide and acetate buffer as described by Lipmann and Tuttle. After ten minutes standing, 2 ml. of 4 *M* hydrochloric acid and 2 ml. of 0.18 *M* ferric chloride are added, and the solution is again allowed to stand for ten minutes before measurement of the color intensity in a Coleman Junior Spectrophotometer at 540 mμ.

Some fading of the ferric acetylhydroxamic acid color with standing was observed, but by rigorous standardization of the conditions reproducible results (standard error for an individual determination of about 2%) were obtained. It was also noted that some of the ions, *e.g.*, phosphate ion, had a marked effect on the intensity of the color. Since, however, in any one run all aliquots contained essentially identical reagent concentrations, uniform values were observed. The color intensity was shown to obey Beer's law over the entire range of concentrations measured.

Product Yield Runs with Benzylamine.—(a) Ten milliliters of a solution which initially contained 0.28 *M* benzylamine and 0.16 *M* lithium acetyl phosphate was kept in the 39° thermostat. From time to time, drops of a 3.5 *M* sodium hydroxide solution were added to keep the *p*H between 8.5 and 9.0. After 4 hours, the solution was acidified with 4 *M* hydrochloric acid and extracted twice with benzene. The benzene was evaporated off in a stream of dry air and the residue crystallized from ligroin; m.p. observed 59–60°; m.p. reported for $CH_3CONHCH_2C_6H_5$, 60°[5]; amide weight, 0.177 g.; final acetyl phosphate concentration, 0.001 *M*.

(b) In general, the same procedure as in part (a) was

(1) Presented at the American Chemical Society Meeting, Chicago, Ill., September, 1950.

(2) Lipmann, *Adv. in Enzym.*, **1**, 99 (1941).

(3) The isopropenyl acetate was kindly supplied by the Tennessee Eastman Co.

(4) Lipmann and Tuttle, *J. Biol. Chem.*, **159**, 21 (1945).

(5) Amsel and Hofman, *Ber.*, **19**, 1286 (1886).

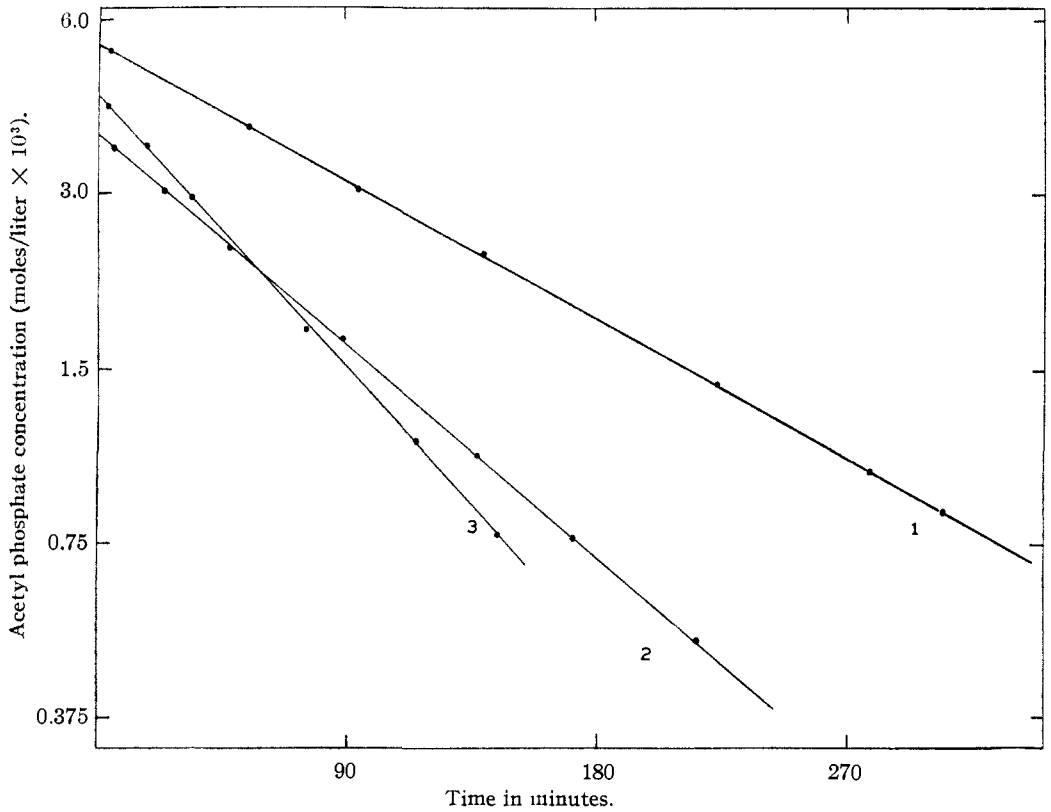


Fig. 1.—Rate of disappearance of acetyl phosphate in aqueous solutions at 39.00°: (1) 0.05 *M* phosphate buffer, *p*H 6.9; (2) 0.200 *M* glycine, 0.05 *M* phosphate, *p*H 6.9; (3) 0.106 *M* glycylglycine, 0.05 *M* phosphate buffer, *p*H 7.3.

followed. The initial concentrations of benzylamine and lithium acetyl phosphate were 0.42 and 0.20 *M*, respectively, and the time of reaction was 5 hours. The *p*H was kept at approximately 7. The final concentration of acetyl phosphate was 0.008 *M* and the amide weight was 0.13 g.

Product Yield Run with Glycine.—Ten milliliters of a solution which was 0.18 *M* in lithium acetyl phosphate and 0.095 *M* in glycine was kept at 39° for seven hours. From time to time, 3.5 *M* sodium hydroxide solution was added to keep the *p*H between 8.5 and 9.0. After removal of phosphate as the barium salt, the solution was neutralized precisely to *p*H 7.0 and the glycine present determined by formol titration. The value observed was 0.044 *M*. The final lithium acetyl phosphate concentration as determined by the hydroxamic acid test was 0.018 *M*.

Recovery of Lithium Acetyl Phosphate from Glycine Solution.—Solid lithium acetyl phosphate (3.2×10^{-3} mole) was added to approximately 10 ml. of a 0.2 *M* glycine solution (*p*H 6.5) which was maintained at 39°. After twelve minutes, the solution was chilled, made basic to phenolphthalein indicator with 4 *M* lithium hydroxide and treated with five portions of ice-cold absolute alcohol. The precipitate was filtered, washed with absolute alcohol and ether and analyzed by the hydroxamic acid method. It contained 2.4×10^{-3} mole of lithium acetyl phosphate which was 75% of the amount initially added. In a similar experiment in which no glycine was present, 79% of the acetyl phosphate initially added was recovered.

Results

The rate of disappearance of acetyl phosphate was first order in acetyl phosphate both in the presence and absence of the amino compounds. Some sample curves are shown in Fig. 1. The observed rate constants showed no appreciable change on varying the initial concentration of lithium acetyl phosphate from 0.002 to 0.008 *M*. Acid is liberated during the course of the reactions at *p*H 7 and 10 (*cf.* equations (2) and (3)) causing the final *p*H to differ in some cases by as much as 0.2 *p*H unit from the initial *p*H. The changes in the rate constants

$$CH_3\overset{O}{\overset{\|}{C}}OPO_3^{--} + H_2O \longrightarrow CH_3COO^- + HOPO_3^{--} + H^+ \quad (2)$$

$$CH_3\overset{O}{\overset{\|}{C}}OPO_3^{--} + {}^+H_3NCH_2COO^- \longrightarrow CH_3CONHCH_2COO^- + H^+ + HOPO_3^{--} \quad (3)$$

with this change in *p*H are negligible at *p*H 7 and small enough to be corrected by linear extrapolation at *p*H 10.

The order of the reaction with respect to glycine was obtained by varying the concentration of the glycine which was always present in large excess. A bimolecular constant could then be calculated in the usual manner from the observed pseudo-unimolecular constant and the independently determined hydrolysis constant. The constancy of the bimolecular constants so calculated, as shown in Table I, demonstrate that the reaction is also first order with respect to the amino compound.

TABLE I

RATE OF ACETYLATION OF GLYCINE BY ACETYL PHOSPHATE AT *p*H 6.9

Glycine concn., mole/liter	Pseudo first order rate constant × 10^3, min.$^{-1}$	First order hydrolysis constant × 10^3, min.$^{-1}$	Second order constant × 10^3, l. mole^{-1} min.$^{-1}$
0.100	4.96	3.90	10.6
.200	6.02	3.90	10.6
.300	7.17	3.90	10.9
.400	8.20	3.90	10.8

The bimolecular constants for a number of amines and amino acids, determined in a similar manner, are summarized in Table II.

TABLE II

RATE OF ACETYLATION OF AMINO COMPOUNDS BY ACETYL PHOSPHATE

Amino compound	pK_a	pH	$k_2 \times 10^3$, l. mole^{-1} min.$^{-1}$
Glycine	9.8	7.3	10.7
		10.0	110
Alanine	9.9	7.3	1.3
Phenylalanine	9.1	7.3	0.5
Proline	10.6	7.3	0.9
Glycylglycine	8.2	7.3	36
Glycine ethyl ester	7.7	7.0	55
Aniline	4.7	7.3	2.9
Ammonia	9.3	7.2	2.1
Benzylamine	9.3	7.3	17
		8.6	34

To establish that the increased rate of disappearance of acetyl phosphate in the presence of the amino compound was caused by acetylation of the amine and not by catalysis of the hydrolytic reaction, the yield of acetylated product was determined in several experiments in which the acetyl phosphate concentration was made comparable to the concentration of the amino compound. The final product was isolated in the case of benzylamine and determined by formol titration in the case of glycine.

The yields to be expected in such experiments can be calculated from the independently determined rate constants in the following manner. If x represents the instantaneous acetyl phosphate concentration and y the concentration of amino compound, then

$$-dx/dt = k_1x + k_2xy \quad (4)$$

and

$$-dy/dt = k_2xy \quad (5)$$

Dividing (4) by (5) and integrating, we have

$$x_0 - x_f = y_0 - y_f - k_1/k_2 \ln (y_f/y_0) \quad (6)$$

where the subscripts o and f refer to the initial and final concentrations of the respective reagents. Due to the nature of the experiments which are described in the experimental part, the pH was not precisely constant and the volume increased by about 10% during the run. Nevertheless, the agreement, shown in Table III, between the observed yields and those calculated using equation (6) is sufficient to demonstrate that the acetylation accounts for the increased rate of reaction.

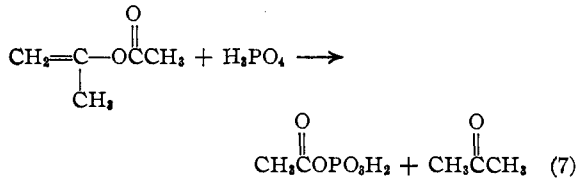
TABLE III

OBSERVED AND CALCULATED PRODUCT YIELDS FOR THE ACETYLATION OF GLYCINE AND BENZYLAMINE

Amine	pH	Yield of acetylated product in mole/liter Calcd.	Obsd.
Benzylamine	7	0.11	0.08
Benzylamine	8.5–9	.12	.12
Glycine	8.5–9	.040	.044

Discussion

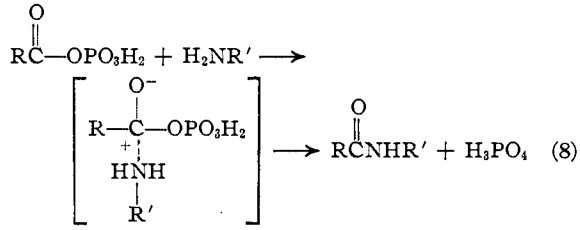
Synthesis of Acetyl Phosphate.—Acetyl phosphate has been prepared previously by the reactions of acetyl chloride with silver dihydrogen phosphate[6] and ketene with phosphoric acid.[7] Each procedure has inconveniences and the reported ready acetylation of inorganic acids by isopropenyl acetate[8] indicated that a simpler and less expensive procedure might be developed in which the isopropenyl acetate was allowed to react directly with phosphoric acid using a trace of sulfuric acid catalyst. This was found to be the case and, in addition, the over-all yield was higher than those previously reported.



Normal butyl ether was used as an inert solvent since the acetone produced in the reaction could be removed at reduced pressure without appreciable loss of solvent. The temperature was kept below 35° to prevent polymerization, which caused the solution to acquire a deep orange-red coloration at 50°. In the procedure finally adopted, the solution was colorless throughout. The isolation of the dilithium salt followed the procedure of Lipmann and Tuttle with the modification that solid lithium carbonate was used as a neutralizing agent below pH 7. This has the advantage of preventing local excesses of base and of keeping the volume of solution small. The over-all yield was 53%.

The synthesis of acetyl phosphate by means of isopropenyl acetate has been independently developed by Stadtman and Lipmann, who used excess isopropenyl acetate as solvent, instead of the inert ether.[9] Their initial crude product contained a slightly higher yield of acetyl phosphate (60–70%), but the product was less pure (80%).

Mechanism of the Reaction.—The reaction has been shown to be first order in amino compound and first order in acyl phosphate, and undoubtedly both molecules are present in the transition state (*cf.* Equation 8). The only mechanisms in which this would not be true and which seem at all reasonable involve either a preliminary disproportionation



or an ionization to acylium ion. Disproportionation of unsymmetrical anhydrides to the corresponding symmetrical compounds has been observed in non-aqueous solution by Sheehan and Frank[10] for the acyl phosphates and by Emery and Gold[11] for the carboxylic anhydrides, but such a reaction in

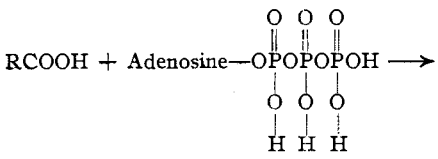
(6) Lipmann and Tuttle, *Arch. Biochem.*, **13**, 373 (1947).
(7) Bentley, THIS JOURNAL, **70**, 2183 (1948).
(8) Hagemeyer and Hull, *Ind. Eng. Chem.*, **41**, 2920 (1949).
(9) Stadtman and Lipmann, *J. Biol. Chem.*, **185**, 549 (1950).
(10) Sheehan and Frank, THIS JOURNAL, **72**, 1312 (1950).
(11) Emery and Gold, *J. Chem. Soc.*, 1443 (1950).

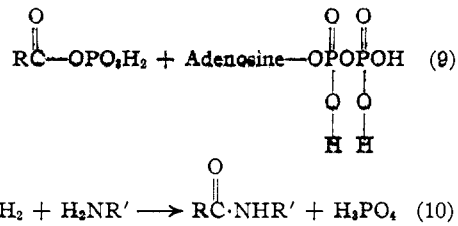
the present case is not consistent either with the product yields or with the recovery of lithium acetyl phosphate from glycine solution (*cf.* Experimental section). Dissociation to acylium ion, $(CH_3CO)^+$, has been proposed in some reactions of acetic anhydride,[12] but the high reactivity of this type of cation[13] would require that the reaction rate be zero order in amino compound instead of first order as is the case.

The nucleophilic attack of amino compounds on the carbonyl carbon atom has been established for ketones in such reactions as semicarbazone, phenylhydrazone and oxime formation[14] and in the formation of amides from acyl halides.[15] In the present case, it has been established that the carbonyl and amino compounds are both present in the activated complex and an analogous electronic mechanism would be expected.

The formulas in the equation for the mechanism are designated as the neutral acids for convenience with no intended implication that this corresponds to the actual proton distribution in the activated complex; this problem will be treated in a forthcoming article. It is seen, however, that the reaction is base-catalyzed, the rate of acetylation at *p*H 10 where a considerable fraction of the amino acid exists in solution as the free base being about an order of magnitude greater than the rate of *p*H 7 where the amino group is mainly present as the onium ion.

Mechanism of Peptide Bond Formation *in Vivo*. —The degree of pertinence of this study to the enzymatic formation of peptide or amide bonds depends largely on the evidence that the acyl phosphates are intermediates in the reaction. The formation of the –CO–NH– bond in such compounds as acetyl sulfanilimide,[16] hippuric acid[17] and glutamine[18] has been shown to require a high energy phosphate donor. In the case of glutamine, a stoichiometric relationship of one mole of phosphate donor hydrolyzed per mole of amide formed was demonstrated. Since the reactions of a phosphate donor with a carboxylic acid to form an acyl phosphate[19] and of an acyl phosphate with an amine to form an amide[20] have been independently demonstrated, it is highly probable that these reactions occur in at least two phases of the type illustrated in equations (9) and (10).





(9)

(10)

These steps may be complex. A coenzyme activator may be required, and the acyl phosphate derivative may never have an existence separate from the enzyme surface. Nevertheless, from the evidence cited above and the known acylating power of acid anhydrides, it is difficult to devise a reasonable mechanism which does not include an acyl phosphate intermediate.

The evidence that the acyl phosphates are intermediates in protein synthesis is more indirect, yet the accumulating data support the hypothesis of Lipmann. Since the standard free energy of formation of a peptide bond from the free amino acids is positive by approximately 3 kcal.[21] and the equilibrium in protein hydrolysis lies well on the side of hydrolysis, it is clear that energy must be supplied to obtain a reasonable yield of peptide. The observations that in the absence (or inhibition) of energy-yielding reactions such as glycolysis or respiration, protein synthesis does not occur (or is reduced in rate) indicate that the above thermodynamic considerations are not obviated *in vivo* by some special insolubility or complex formation. Energy is supplied in such synthetic reactions by means of phosphorylated compounds and the recent evidence[22] that dinitrophenol inhibits protein synthesis without inhibiting respiration supports the assumption that these compounds are involved.

The fact that the phosphate transfer system supplies the energy does not, of course, establish that the acyl phosphates are intermediates. The energy might be used, for example, in some undisclosed manner to achieve exceptionally high local concentrations of amino acids. The simplest pathway, however, is one which is essentially similar to that outlined for the formation of the amides in the isolated enzyme systems (*cf.* equations (13) and (14)). Isotopic and nutrition studies show that the amino acids themselves, and not smaller or deaminated fragments, are the compounds which are activated in some way to form the polypeptide chain. Thus, the thermodynamic, chemical and biological evidence are consistent with an amino acid acyl phosphate intermediate.

The fact that the acyl phosphates acylate amines in aqueous solution adds further support to the hypothesis. It has been shown that acetyl phosphate acetylates aniline in aqueous solution[23] and that the substituted acyl phosphates react at an appreciable rate with amino acids in the absence of enzymes.[24,25] The reaction of the unsubstituted

(12) MacKenzie and Winter, *Trans. Faraday Soc.*, **44**, 159 (1948); Benton and Praill, *J. Chem. Soc.*, 1203 (1950).

(13) Hammett and Treffers, THIS JOURNAL, **59**, 1708 (1937); Newman, *ibid.*, **63**, 2431 (1941).

(14) Conant and Bartlett, *ibid.*, **54**, 2881 (1932); Westheimer, *ibid.*, **56**, 1962 (1934); Stempel and Schaffel, *ibid.*, **66**, 1158 (1944).

(15) Williams and Hinshelwood, *J. Chem. Soc.*, 1079 (1934).

(16) Lipmann, *J. Biol. Chem.*, **160**, 173 (1945).

(17) Cohen and McGilvery, *ibid.*, **166**, 26 (1946).

(18) Speck, *ibid.*, **179**, 1405 (1949).

(19) Bucher, *Biochem. et Biophys. Acta*, **1**, 292 (1947); Lipmann, *J. Biol. Chem.*, **155**, 55 (1944).

(20) Chou, Novelli, Stadtman and Lipmann, *Fed. Proc.*, **9**, 160 (1950).

(21) Borsook and Dubnoff, *ibid.*, **132**, 307 (1940); Borsook and Huffman in Schmidt, "Chemistry of the Amino Acids and Proteins," C. C. Thomas, Springfield, Illinois, 1945.

(22) Frantz, Zamecnik, Rees and Stephenson, *J. Biol. Chem.*, **174**, 773 (1948); Melchior and Klotz, Abstracts of Am. Chem. Soc., Sept., 1950, p. 17c.

(23) Lipmann, *J. Biol. Chem.*, **160**, 173 (1945).

(24) Chantrenne, *Nature*, **160**, 603 (1949).

(25) Sheehan and Frank, THIS JOURNAL, **72**, 1312 (1950).

compounds with amino acids, however, was not detectable.[26,27] Thus, dibenzoyl phosphate or benzoyl phenyl phosphate acetylated glycine whereas no reaction with benzoyl phosphate was observed.[26] Similarly, diacetylphosphate was reported to react under conditions which gave no reaction with acetyl phosphate.[27] These results led to the suggestion[26] that the diacyl phosphates or acyl ester phosphates might be the active agents in the biological acylation reactions rather than the monosubstituted compounds. Since the unsubstituted compounds exist as the dinegatively charged ion in neutral solutions, it might be expected from electrostatic considerations that their rate of acylation would be lower than the substituted phosphates. In this work it is observed that the reactions are very slow in some cases. In other cases, however, and in basic solution the rate is easily measurable and a high yield of acetylated product may be obtained. This indicates that no qualitative change occurs on substitution and that the primary contribution of the second covalently bonded group is to decrease the electrostatic repulsion toward the electron-sharing amino group. Positively charged groups on the enzyme surface could cause activation similar to that contributed by the covalent bonding. This is of particular interest since the unsubstituted acyl phosphate would be expected to be formed in the enzyme-catalyzed reaction between adenosine triphosphate and a carboxylic acid.

It is further noted that the rate of acylation is moderate, a k_2 of 0.01 liter moles^{-1} minutes^{-1} corresponding to a half-life of approximately 11 hours for acetyl phosphate in 0.1 M glycine solution. Thus, although the acyl phosphates are "high energy compounds" in terms of equilibrium free energy, they are not rapidly reacting compounds as, for example, are the aliphatic free radicals. This is a desirable property for a biological intermediate since it allows a moderately active enzyme to direct the reaction into the desired channel without significant loss from the competing non-enzymatic reaction.

From the relatively low values of these rate constants, it is possible to exclude a non-enzymatic acylation by the free acyl phosphate as a step in peptide formation. In the formation of glutamine[18] the reaction is not second order, but the data at 30° in a solution containing 0.05 M glutamate and 0.01 M ammonium chloride show that the bimolecular constant for a non-enzymatic reaction would have to be greater than 0.69 liter moles^{-1} minutes^{-1}. The reactivity of γ-glutamyl phosphate will certainly not be greatly different from that of acetyl phosphate since the charged groups are several carbon atoms removed from the site of the reaction. When one considers that the bimolecular constant for the reaction of acetyl phosphate with ammonium chloride is only 0.002 at a higher temperature (39°), a non-enzymatic reaction cannot account for the rate of product formation. In the amino acid phosphates the charged group is in the α-position where its effect will be much larger, but even in this case the low amino acid levels[28] in the tissues seem to preclude a significant contribution from a non-enzymatic pathway.

A non-enzymatic reaction can also be excluded on the basis of the relative rates of hydrolysis and amine acylation. In glutamine formation, assuming that the ratio of rates is the same for γ-glutamyl phosphate as for acetyl phosphate, only 0.5% of the glutamyl phosphate formed (as measured by adenosine triphosphate hydrolysis) would appear finally as the amide. Instead, one mole of glutamine is formed for each mole of adenosine triphosphate converted to the diphosphate. In the case of the unsubstituted amino acid acyl phosphates, the assumption that the ratio of hydrolysis and amine acylation rates remains constant may be in greater error, but in view of the low amino acid concentrations, it seems safe to conclude that a non-enzymatic acylation is too inefficient to be a step in the mechanism of protein synthesis.

Since a direct acylation by an unsubstituted acyl phosphate must take place on an enzyme surface if it is to contribute to peptide bond formation, it is worthwhile considering what the kinetics of the non-enzymatic reaction indicate about the active portion of the enzyme surface. In general, the kinetic and thermodynamic behavior of substances in the absence of enzymes may clarify (a) the nature of the groups which attract the substrate to the enzyme surface, and (b) the nature and mode of action of the groups which catalyze the reaction by affecting the electron distribution in the reacting molecules. One group may, of course, have both of these functions. In acetylcholinesterase, for example, enzyme kinetics and action of inhibitors have been interpreted[29] by postulating two active sites, a negative site which attracts the positively charged quaternary nitrogen and an esteratic site, which acts in the region of the carboxyl group. From the known effects of substituents on the velocity of ester hydrolysis[30] the negative site would be expected to have, if anything, a decelerating effect on the rate of reaction at the carbonyl group. Similarly the acidic and basic groups which are postulated to be present in the esteratic site would be expected to accelerate the reaction from the observed acid and base catalysis of ester hydrolysis.

From these observations an active surface for acylation by an acyl phosphate might have the features shown schematically in Fig. 2. The two positively charged groups attract the corresponding negatively charged groups in the acyl phosphate and amino acid. A basic group at B would tend to attract a proton from the quaternary nitrogen, thus liberating the free amine form of the amino acid and enhancing its nucleophilic activity.

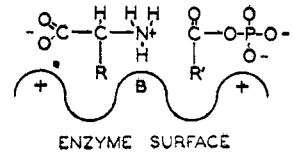


Fig. 2.—Schematic representation of the postulated enzyme surface.

(26) Chantrenne, *Compt. rend. trav. Lab. Carlsberg*, **26**, 10, 297 (1948); *Biochem. Biophys. Acta*, **2**, 286 (1948).

(27) Lipmann and Kaplan, *Ann. Rev. Biochem.*, **18**, 267 (1949).

(28) Christensen, *et al.*, *J. Biol. Chem.*, **163**, 741 (1946); **166**, 87 (1946); **172**, 515 (1948).

(29) Wilson and Bergman, *ibid.*, **185**, 479 (1950).

(30) Hammett, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1940.

The positive group adjacent to the phosphate would tend to decrease the electron releasing effect of the dinegatively charged acyl phosphate. It should, therefore, serve to catalyze the reaction by causing a favorable shift of electrons as well as by attracting the substrate to the enzyme surface. The effect of the positively charged group near the carboxyl ion portion of the amino acid is more complicated. In neutral solution, glycine ethyl ester reacts more rapidly than glycine, but a comparison on the basis of both reactants being present in the form of the free amino compound indicates that the glycine reacts more rapidly, presumably because of its greater electron releasing tendency. A positive site should, therefore, attract the substrate to the enzyme surface, but cause an unfavorable shift of electrons at the amino nitrogen. In future publications, the nature and mode of action of the catalytic groups will be discussed in more detail.

Acknowledgment.—The author wishes to express his appreciation to Dr. Paul D. Bartlett for his stimulating discussions during the course of this work. He also wishes to thank the Atomic Energy Commission for the fellowship grant.

CAMBRIDGE 38, MASS. RECEIVED JANUARY 29, 1951

[CONTRIBUTION NO. 90 FROM THE INSTITUTE OF ATOMIC RESEARCH AND THE DEPARTMENT OF CHEMISTRY, IOWA STATE COLLEGE]

Composition and Stability of the Beryllium-sulfosalicylate Complex Ion[1]

BY H. V. MEEK[2] AND CHARLES V. BANKS

The composition and stability of the "colorless" beryllium-sulfosalicylate complex ion was studied by means of its ultraviolet absorption spectra. The Vosburgh and Cooper extension of Job's method of continuous variations was used to show that in the optimum *p*H range (9 to 11) only one complex ion is present in appreciable quantity and that the mole ratio of sulfosalicylate to beryllium in this complex ion is 2 to 1. Evidence is presented that the phenolic hydrogen of the participating sulfosalicylate ions is removed in the process of forming the beryllium-sulfosalicylate complex ion. The true equilibrium constant for the dissociation of $Be(O_3SC_6H_3(O)COO)_2^{-4}$ into $O_3SC_6H_3(OH)COO^{-2}$ and an unknown beryllium species, Be_x, was found to be approximately 2.1×10^{-9} at 25 ± 1°.

Introduction

Recently a new spectrophotometric method for determining beryllium was reported by Meek and Banks[3] which is based on the fact that the absorption maximum characteristic of $O_3SC_6H_3(OH)COO^{-2}$ is displaced appreciably toward the longer wave lengths by the presence of beryllium (Fig. 1). This displacement is not sufficient to give a "color" but is sufficient to make it possible to establish the composition of the beryllium-sulfosalicylate complex ion and to estimate its instability constant.

Experimental

Materials.—Only reagent-grade materials or materials carefully purified or synthesized by the authors were used in this study. Extreme care was taken to select only those lots of materials in which the iron content was negligible since its presence would be particularly undesirable.[4]

The source and purification of the beryllium basic acetate and the N,N,N',N'-tetrakis-(carboxymethyl)-ethylenediamine are described elsewhere.[3]

Sulfosalicylic acid from two commercial sources was recrystallized several times from water and then compared with 5-sulfosalicylic acid, prepared according to the method of Meldrum and Shah,[5] by potentiometric titration and by checking the ultraviolet absorption spectra of equal molar solutions of these acids at the same *p*H with and without beryllium being present (Fig. 1). All of these experiments indicated that the behavior of the commercial acids is, at least for the purpose of this study, identical with 5-sulfosalicylic acid. Stock solutions of sulfosalicylic acid were standardized by titration with standard alkali.

Apparatus.—Silica cells (1.000 cm.) and a Cary recording spectrophotometer (Model 12) were employed in the spectrophotometric studies. Beckman glass electrode *p*H meters (Models H-2, M and G) were used for the *p*H measurements. Appropriate sodium ion corrections were made where accurate knowledge was considered necessary.

Results

The Effect of *p*H.—The wave length of 317.0 mμ was chosen for the examination of the beryllium-sulfosalicylate complex ion because at this wave length the maximum change in absorbancy due to the presence of beryllium is observed. Solutions 6.00×10^{-4} *M* in sulfosalicylate, 0.1 *M* in sodium perchlorate (to maintain constant ionic strength), and having various *p*H values were scanned with the Cary instrument using distilled water as the reference solution. The absorbancies of these solutions are plotted in curve 2, Fig. 2. Solutions containing appreciably higher or lower sulfosalicylate concentrations show, respectively, shorter or longer *p*H intervals of constant absorbancy due to mass action.

Curve 1, Fig. 2, is a plot of similarly observed absorbancies for solutions 6.00×10^{-4} *M* in sulfosalicylate, 0.10 *M* in sodium perchlorate, 3.00×10^{-4} *M* in beryllium sulfate, and having various *p*H values. Again, the limits of the *p*H interval, through which the absorbancy remains constant, vary slightly with the concentration of the beryllium-sulfosalicylate complex ion.

Method of Continuous Variations.—The Vosburgh and Cooper[6] extension of Job's[7] method of continuous variations was used to study the number and composition of beryllium-sulfosalicylate complex ions present in the optimum *p*H range.

(1) This document is based on work performed in the Ames Laboratory of the Atomic Energy Commission, Ames, Iowa.

(2) Abstracted from a dissertation submitted by Homer V. Meek to the Graduate Faculty of Iowa State College in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1950. Procter and Gamble Company, Ivorydale, Cincinnati 17, Ohio.

(3) H. V. Meek and C. V. Banks, *Anal. Chem.*, **22**, 1512 (1950).

(4) R. T. Foley and R. C. Anderson, THIS JOURNAL, **70**, 1195 (1948).

(5) A. N. Meldrum and M. S. Shah, *J. Chem. Soc.*, **123**, 1986 (1923).

(6) W. C. Vosburgh and G. R. Cooper, THIS JOURNAL, **63**, 437 (1941).

(7) P. Job, *Ann. chim.*, [10] **9**, 113 (1928).