THE PHOSPHORYLATION OF CARBOXYLIC ACIDS AND AMINO ACIDS BY DISERYL PYROPHOSPHATES

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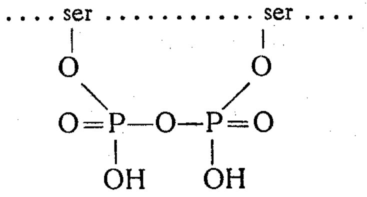
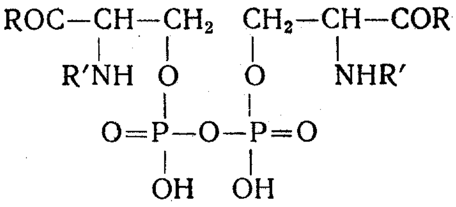
Phosphoproteins containing more than one atom of phosphorus per molecule of protein are characterized by interesting properties. The phosphoserine residues in such proteins are assembled in the peptide chain in the form of blocks of the type (Ser-O-$PO_3H_2)_3$ and (Ser-O-$PO_3H_2)_6$ [1]. This fact has not been explained so far. It is possible that the appearance of such blocks is connected with the functional properties of proteins. It is highly likely that the appearance of the secondary and tertiary structures of these phosphoproteins may lead to the interaction of spatially adjacent phosphoryl groups with the formation of pyrophosphate bonds



(1)

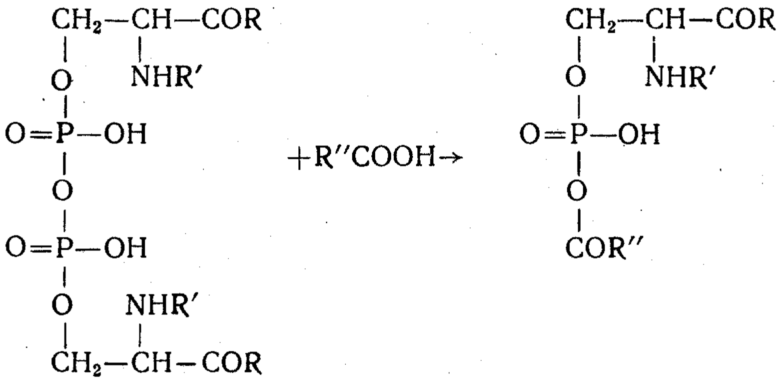
To elucidate the reactivity of such a type of grouping, we have synthesized a number of compounds containing diseryl pyrophosphate groups



(2)

and have begun a study of their properties [2-4].

The present paper gives the results of an investigation of the transphosphorylation reaction taking place when diseryl pyrophosphates react with carboxylic acids. When the pyrophosphate bond is sufficiently reactive, the formation of acyl phosphate derivatives is possible:



(3)

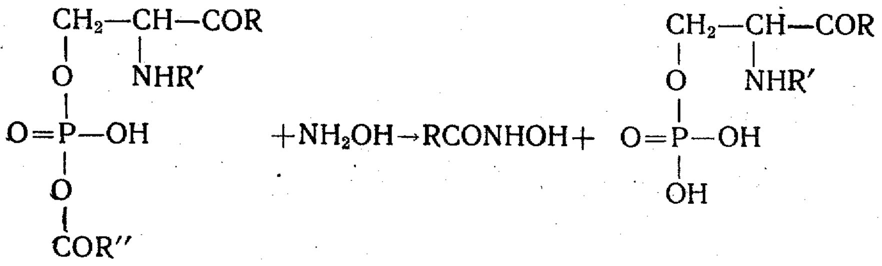
I $R=NHCH_3$
$R'=COC_6H_5$

I

The acyl phosphates belong to the biologically important class of mixed anhydrides which are intermediates in the synthesis of peptide bonds.

The formation of acyl phosphates from a natural compound with a high-energy bond, adenosine triphosphate, and carboxylic acids without the participation of an enzyme has recently been studied in detail by Lowenstein [5-7]. In spite of the fact that such a reaction takes place to a very slight degree, it is considered as the prototype of more important enzymatic processes.

We have studied the reaction of the pyrophosphate of the methylamide of N-benzoyl-DL-serine (I) with acetic anhydride and with glycine and have shown that the reaction takes place by route (3). The amount of acyl phosphate formed was determined by its reaction with hydroxylamine to form the corresponding hydroxamate which gives a colored complex with the iron ion:



$$\mathrm{R''CO{-}O{-}P({=}O)(OH){-}O{-}CH_2{-}CH(NHR'){-}COR} + \mathrm{NH_2OH} \rightarrow \mathrm{RCONHOH} + \mathrm{(HO)P({=}O)(OH){-}O{-}CH_2{-}CH(NHR'){-}COR} \tag{4}$$

The results of the reaction of the pyrophosphate (I) with acetic acid at various pH values for 3 hr at a temperature of 37° C are given in the table.

pH	Acetohydroxamate formed*, %	
	without metal ion	in the presence of Be^{2+} (30 μmole)
2.0	0.2	0.22
3.0	0.24	0.64
4.0	0.76	1.40
5.2	1.18	1.64
6.0	2.2	—
7.0	1.4	—

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*50 μmole of substance (I) was added.

It can be seen from the table that in the relationship between the rate of the reaction and the pH there is a strict correspondence between the percentage of acetohydroxamate formed and the concentration of the acetate anion, which is a function of the pH. The yield of acetohydroxamate reaches a maximum when the concentration of the nucleophilic agent, the acetate anion, is almost a maximum (pK_a CH_3COOH 4.75). The decrease in the yield of acetohydroxamic acid with a further increase in the pH apparently is caused primarily by a decrease in the stability of the acyl phosphate formed.

Under the conditions mentioned (37° C, 3 hr, pH 5.2) adenosine triphosphate (ATP) scarcely reacts with acetic acid [6].

When the experiments were carried out with the pyrophosphate and hydroxylamine alone, the formation of reaction products giving a colored complex with the iron ion was found. (The intensity of the coloration was approximately 1/8 of the intensity of the coloration in experiments with the whole of the reaction mixture.) This is probably due to the direct reaction of hydroxylamine with diseryl pyrophosphate [8]; the possibility of the formation of phosphorylhydroxylamine has been reported by Jencks [9].

It is known that many processes involving the transfer of a phosphate residue are catalyzed by metal ions [10]. In view of this, we have studied the reaction of diseryl pyrophosphate (I) with acetic acid in the presence of a number of metal ions: Be^{2+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, La^{3+}, Ce^{3+}. Only the beryllium ion proved to be active. The pH of the medium was found to have a considerable influence on the rate of the reaction (table). The optimum pH is 5.2; a higher pH values the precipitation of beryllium hydroxide interferes with the quantitative evaluation of the reaction.

The rate of the reaction also depends on the molar ratio of beryllium ions and pyrophosphate. The maximum yield of acetohydroxamate (4%) is found at a ratio of beryllium to pyrophosphate of 2:1. If no metal ions are present, only 1.2% of acetohydroxamate is formed at pH 5.2; thus, the beryllium ion accelerates the reaction of the pyrophosphate (I) with acetic acid by a factor of more than 3.

We give data on the influence of the molar ratio of Be^{2+} (I) on the reaction of diseryl pyrophosphate (I) with acetic acid (37° C, 3 hr, pH 5.2):

Ratio Be^{2+}/(I)	0.6	1.2	2.0	2.4
Formation of acetohydroxamate,* %	1.62	1.94	4.04	2.50

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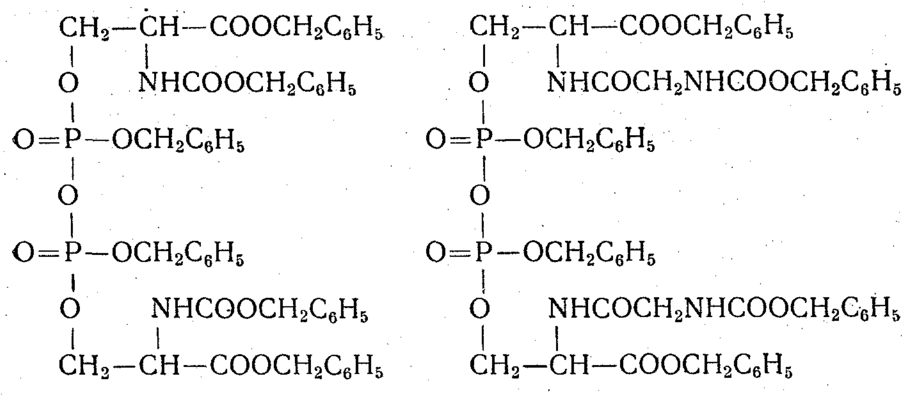
* 50 μmole of (I) was used for the experiment.

Under optimum conditions (37° C, 3 hr, pH 5.2, Be^{2+}) ATP undergoes 2.88% reaction with acetic acid.

The specific interaction of beryllium ions and the pyrophosphate (1) apparently leads to a decrease in the $P_{\pi} - d_{\pi}$ conjugation of the P_{π} electrons of the oxygen with the 3d orbitals of the phosphorus, which facilitates the nucleophilic attack of the phosphorus atom by the acetate ion. In view of this, still greater reactivity in the reaction with acetic acid may be expected for tetraalkyl pyrophosphates having no negatively charged oxygen atoms in the phosphoryl groups. We have studied the reactivity of two such pyrophosphates: P^1, P^2-di (N-benzyloxycarbonylseryl) P^1, P^2-dibenzyl pyrophosphate (II), and P^1, P^2-di (N-benzyloxycarbonylaminoacetylseryl) P^1, P^2-dibenzyl pyrophosphate (III).



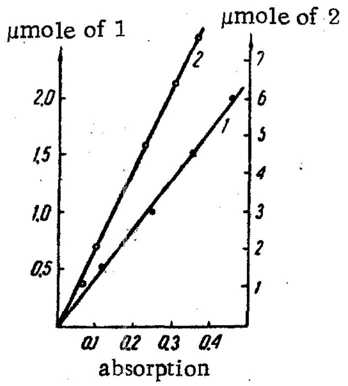
II III

(5)

The results obtained in the incubation of the diseryl phosphates (II) and (III) with acetic acid (37° C, 2 hr, pH 5.2) are given below:

Diseryl pyrophosphate, μmole	(II) 40	(III) 16	(I) 41
Formation of acetohydroxamate, %	5.3	13.8	0.81

The substituted pyrophosphates (II) and (III) are considerably more reactive than the pyrophosphate (I). However, the activities of substances (II) and (III) differ considerably from one another, and the same feature is observed as in the hydrolysis of these compounds [4]: the pyrophosphate (III) is more reactive than the pyrophosphate (II).



Calibration curves for acetohydroxamic acid (1) and glycinedroxamic acid (2).

Still more interesting results were obtained in the reaction of the diseryl pyrophosphate (I) with glycine which took place by the route given above (3). After 13 hr (37° C, pH 5.2), 8.5% of glycinehydroxamate was formed (beryllium ions accelerate this reaction), and after the same period at a ratio of beryllium ion to pyrophosphate of 1:1 13.3% of glycinehydroxamate was formed. ATP reacts with glycine only in the presence of a metal ion (Mn^{2+}) and only to the extent of 0.86% [7].

The rate of the reaction of the pyrophosphate (I) with glycine also depends on the pH of the medium (37° C, 26 hr):

pH	2.15	3.0	3.8	4.3	5.0
Formation of glycinehydroxamate, *	3.4	6.0	14.0	22.3	19.0

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*34 μmole of substance (I) was used.

The maximum formation of glycinehydroxamate (22%) took place at pH 4.5. In this case the glycine was almost completely dissociated (pK of glycine 2.34). This correspondence between the degree of dissociation of the carboxy group and the optimum pH of the reaction of diseryl pyrophosphate with carboxylic acids may serve as yet another confirmation of the mechanism of the reaction as a nucleophilic substitution at the phosphorus atom.

Experimental

Procedure. 1. 1 ml of a reaction mixture containing the amounts given above of a 0.5 N solution of pyrophosphat (I), (II), or (III), 800 μmole of buffer solution, and 400 μmole of 1 N hydroxylamine hydrochloride was incubated at 37° C. Then 0.5 ml of water and 1.5 ml of a 3% solution of $FeCl_3 \cdot 6H_2O$ in 1.2 N hydrochloric acid were added to the hydrochloric acid were added to the reaction mixture. The amount of acetohydroxamic or glycinehydroxamic acid was determined spectrophotometrically in a SF-4 spectrophotometer at 540 mμ from a previously constructed calibration curve (figure). In control experiments in the absence of buffer solution or pyrophosphate, the absorption was zero.

2. The buffer solutions used in the reactions of the pyrophosphates with acetic acid (pH 2, 2 M CH_3COOH; pH 3.3, and pH 4.3) were obtained by adding to 2 M acetic acid the required amount of 4 M tris solution and pH 5.6 by mixing equal volumes of 4 M acetic acid and 4 M tris solution.

The addition of 400 μmole of hydroxylamine hydrochloride to 800 μmole of the solutions mentioned gave the pH values shown above.

3. The following buffer solutions were used in the reaction of pyrophosphate with glycine: pH 2.1, a mixture of 3 ml of 2 M glycine and 3.5 ml of 10% HCl; pH 3.0, 4 ml of 2 M glycine and 3 ml of 10% HCl; pH 4.0, 5 ml of 2 M glycine and 0.2 ml of 10% HCl; pH 5.0, 5 ml of 2 M glycine and 0.05 ml of 10% HCl; pH 6.0 2 M glycine. Mixing equal volumes of the buffer solution and a 1 M solution of hydroxylamine hydrochloride gave the pH values mentioned above.

4. The P^1, P^2 pyrophosphate of the methylamide of N-benzoylserine (I) was obtained as described by Avaeva et al., [3].

5. P^1, P^2-di (N-benzyloxycarbonylseryl)-P^1, P^2-dibenzyl pyrophosphate (II) was obtained by the addition to an acetone solution of 1 mole of the benzyl ester of N-carbobenzoxy-O-benzylphosphorylserine [2] and 0.25 mole of dicyclohexylcarbodiimide. After 2 hr, the dicyclohexylurea was filtered off, the solution was evaporated, and the residue was dissolved in peroxide-free dioxane and treated as in paragraph 1. In this case, the calibration curve for the acetylhydroxamic acid was also constructed in the presence of dioxane.

6. P^1, P^2-di (N-benzyloxycarbonylaminoacetylseryl)-P^1, P^2-dibenzylpyrophosphate (III) was obtained in a similar manner to compound (II).

7. The acetohydroxamic acid was synthesized by the method of Eichberg and Dauson [11].

8. The glycinehydroxamic acid was synthesized by the method of Safir and Williams [12].

Summary

The transphosphorylation reaction taking place in the interaction of diseryl pyrophosphates with acetic acid and glycine has been studied. It has been found that the rate of this reaction depends on the pH of the medium and on the presence of metal ions. A high reactivity of the pyrophosphate bond in the transphorylation reaction has been found which exceeds the reactivity of a natural compound with a high-energy bond, ATP.

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