The Reaction of *cyclo*-Triphosphate with L-α- or β-Alanine

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The reaction of sodium *cyclo*-triphosphate(P_{3m}) with L-α- or β-alanine in an aqueous solution was investigated using anion-exchange chromatography, ^{31}P-NMR spectroscopy, chemical analysis for phosphorus and alanine, IR spectroscopy, and thin-layer chromatography(TLC). 1) P_{3m} readily reacted with L-α-alanine at pH 10 and 12 to form two orthophosphate derivatives of α-alanine: *N*-(1-carboxyethyl)phosphoramidate(P_1-(*N*)ala) and 1,3,2-oxazaphospholidin-2-olate derivative(P_1-(*N,O*)ala) of five-membered ring structure. 2) Only triphosphate derivative, *N*-(2-carboxyethyl)triphosphoramidate(P_3-(*N*)ala), was obtained in the reaction of P_{3m} and β-alanine. 3) The total yield of P_1-(*N*)ala and P_1-(*N,O*)ala was about 75% when P_{3m} was allowed to react with α-alanine at a molar ratio of 1:3 and pH 12, while the yield of P_3-(*N*)ala was about 80% in the reaction of P_{3m} with β-alanine at a molar ratio of 1:1 and pH 12. 4) Oligopeptides such as L-alanylalanine(dimer) and L-alanylalanylalanine(trimer) were formed in the reaction of P_{3m} with α-alanine. 5) Under acidic conditions, P_3-(*N*)ala recyclized to the starting material P_{3m} and β-alanine. 6) The mechanism of the reaction of P_{3m} with α- or β-alanine is discussed.

Phosphorus pentaoxide, polyphosphoric acid, phosphoryl chloride,[1–3] and various organic phosphorus compounds[4–6] have been used as phosphorylating agents for organic and bioorganic compounds. Phosphorylation with these compounds, however, requires special and cumbersome techniques and is usually accompanied by the formation of considerable amounts of by-products.

Feldmann reported in 1965—1967 that phosphorylation of alkylamines,[7] phenols,[8] and alcohols[9] can be effected by the use of *cyclo*-triphosphate(P_{3m}). Following this, Rabinowitz[10–12] reported in 1969—1971 that amino acids (glycine and alanine) condense to form oligopeptides in the presence of polyphosphate or P_{3m}. These facts lead one to imagine that inorganic condensed phosphates such as polyphosphate and P_{3m} might have played an important role in the creation of proteins from amino acids in the prebiotic process which preceded the emergence of life on the earth.[11,13,14] However, little is known about the reaction products and mechanism of P_{3m} with amino acids. As a part of our developmental work on the application of P_{3m} as a phosphorylating agent, the present authors investigated the reaction of P_{3m} with L-α- or β-alanine under various conditions (molar ratio of alanine to P_{3m}, pH, and reaction time) in order to characterize the structure and yields of the reaction products as well as the reaction mechanism.

P_{3m} has many advantages over any other phosphorylating agents; it is a component of metaphosphate, which has been approved for safety as a food additive.[15] Furthermore it is very easy to handle and requires no special technique for phosphorylation. The present authors have already reported that P_{3m} is of great value as a phosphorylating agent for nucleosides.[16]

Experimental

Chemicals. Sodium *cyclo*-triphosphate hexahydrate, $Na_3P_3O_9 \cdot 6H_2O$, was prepared by the procedure described in the previous paper[17] and recrystallized three times from aqueous solution. Reagent-grade L-α- and β-alanine, and potassium acetyl phosphate (Rasa Industries Ltd.) were used without further purification. Unless otherwise stated, guaranteed grade reagents from Wako Chemical Industries Ltd., were used.

Reaction between cyclo-*Triphosphate with* L-α- *or* β-*Alanine.* In the reaction of P_{3m} with L-α-alanine, 50 cm^3 of 0.5 mol dm^{-3} aqueous sodium *cyclo*-triphosphate(P_{3m}) solution was mixed with 50 cm^3 of 0.5—1.5 mol dm^{-3} aqueous L-α-alanine solutions. The pH values of the mixed solutions were about 5.2—5.3. In the reaction of P_{3m} with β-alanine, on the other hand, 50 cm^3 of 0.5 mol dm^{-3} aqueous P_{3m} solution was mixed with 50 cm^3 of 0.5 mol dm^{-3} aqueous β-alanine solution, and the pH value of the mixed solution was about 6.1. These mixed solutions were then adjusted to the predetermined pH value(pH 12, 10, 7, or 4) by use of a sodium hydroxide solution or hydrochloric acid, and the reaction was allowed to stand at room temperatue. Since the pH of each mixed solution decreased as the reaction progressed, the solution was constantly adjusted to the predetermined pH by adding a sodium hydroxide solution.

^{31}P-NMR Measurement. A JEOL FT-NMR Spectrometer (Model JNN-FX 100) operating at 40.25 MHz for ^{31}P was used. 85% orthophosphoric acid was used as a reference.

TLC Analysis. For analysis of L-α-alanine, L-alanylalanine(dimer), L-alanylalanylalanine(trimer), and β-alanine, TLC was used. A mixed solvent consisting of $CHCl_3$, CH_3OH, and 17% aqueous ammonia (2:2:1) was used, with silica gel plates (made by E. Merck Co. and Wako Chemical Industries Ltd.).

Anion-exchange chromatography, IR spectroscopy, and the determination of phosphorus and α-alanine were carried out by the methods described in the previous paper.[18]

Results and Discussion

Reaction of cyclo-*Triphosphate with* L-*α- or β-Alanine.* After aqueous solutions of P_{3m} and L-α- or β-alanine were mixed, the mixtures were adjusted to a desired pH(12, 10, 7, or 4) with sodium hydroxide or hydrochloric acid, and then allowed to stand at room temperature. Reaction products obtained by the reaction of P_{3m} with α- or β-alanine were separated and determined by anion-exchange chromatography. Figures 1-a and b show the anion-exchange chromatograms of the reaction products of P_{3m} with α- or β-alanine at a molar ratio of 1:1, pH 12, and at room temperature. In the reaction of P_{3m} with α-alanine, two elution peaks of unknown compounds (designated as Compounds I and II) were observed between those of ortho-(P_1) and pyrophosphate(P_2). In the reaction between P_{3m} and β-alanine, on the other hand, a large peak of an unknown compound (Compound III) appeared at 1400 cm^3 of the effluent, other than the peaks of ortho-, pyro-, tri-(P_3), and tetraphosphate(P_4). As can be seen from Fig. 1-a and b, the formation of Compound I and P_2 was predominant in the P_{3m}–α-alanine system, whereas the predominant product in the P_{3m}–β-*alanine system was Compound III, with* P_1, P_2, P_3, and P_4 being formed only in very small quantities.

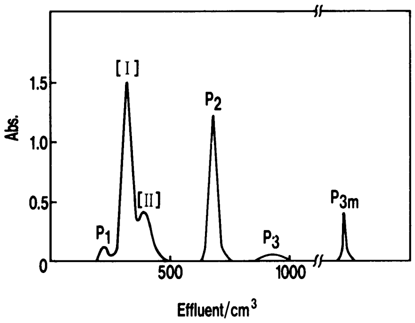


Fig. 1-a. Elution pattern of the reaction products of P_{3m} with L-α-alanine at pH 12.
P_{3m}: $CH_3CH(NH_2)COOH$=0.5 mol dm^{-3}: 1.5 mol dm^{-3}, room temp, after 3 d.

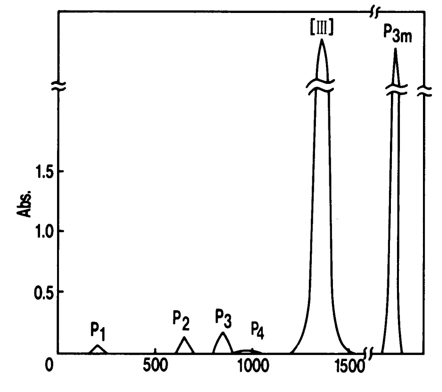


Fig. 1-b. Elution pattern of the reaction products of P_{3m} with β-alanine at pH 12
P_{3m}: $NH_2CH_2CH_2COOH$=0.5 mol dm^{-3}: 0.5 mol dm^{-3}, room temp, after 14 d.

Structures of Compounds I, II, and III. The molecular structures of Compounds I, II, and III were determined by means of chemical analysis, IR spectroscopy, and ^{31}P-NMR spectroscopy. In addition, the reaction of Compounds I, II, and III in an acidic solution was investigated in order to determine the structure.

Compound II was fractionated by anion-exchange chromatography according to the method described in the previous paper.[17] The amounts of phosphorus and α-alanine in Compound II were determined by chemical analysis and with an amino acid analyzer.[18] It was found that the ratio of phosphorus to α-alanine in Compound II is 1:0.96, showing that Compound II may be an orthophosphate derivative of α-alanine containing P_1 and α-alanine in the molar ratio of 1:1.

The IR spectrum of compound II is shown in Fig. 2. Characteristic absorptions that can be assigned to α-alanine were noticed at about 2850 ($\nu_{s\,CH_2}$), 1595 ($\nu_{C=O}$), 1450 (ν_{CH_2}), 1412 ($\nu_{C=O}$), and 1358 cm^{-1} (ν_{C-H}).[19] In addition, characteristic absorptions due to the P-O-C(↑) and P-N bonds(⇡) were observed at the vicinity of 1150 and 900 cm^{-1},[20,21] and 710 cm^{-1},[22-24] respectively. These results demonstrate that Compound II is a phosphate derivative of α-alanine having both P-O-C and P-N bonds in its molecule. For reference, the IR spectrum of the raw material, L-α-alanine, is also shown in Fig. 2-(1).

Figure 3 shows the ^{31}P-NMR spectra of the reaction products between P_{3m} and α-alanine. As can be seen in the ^{1}H-decoupling spectrum, singlet peaks corre-

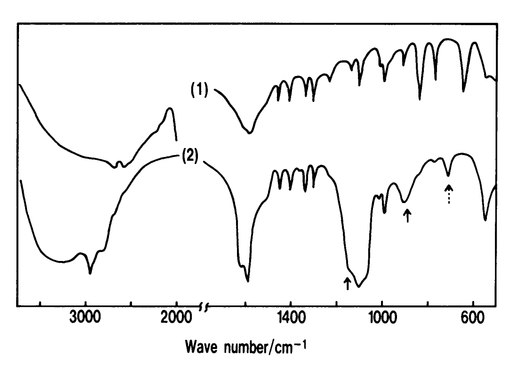


Fig. 2. IR spectra of L-α-alanine and product (II)
(1): L-α-alanine, (2): product (II)
↑ : P-O-C bond, ⇡ : P-N bond.

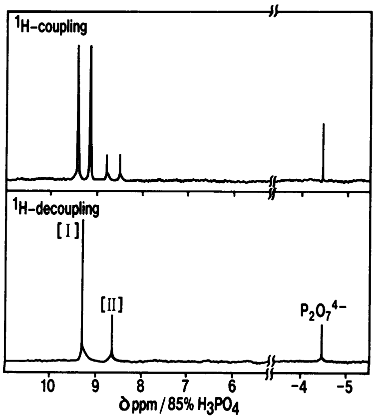


Fig. 3. ^{31}P-NMR spectra of the reaction products in the L-α-alanine—P_{3m} system
P_{3m}: L-α-alanine=0.5 mol dm^{-3}: 1.5 mol dm^{-3}
pH 12, room temp.

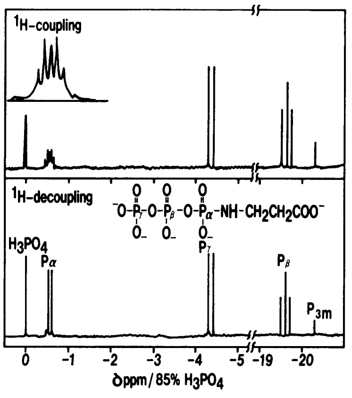
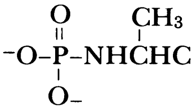
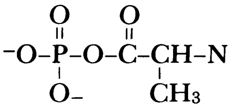
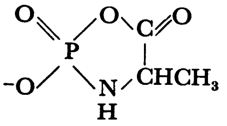
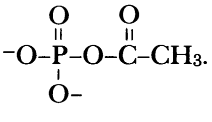


Fig. 4. ^{31}P-NMR spectra of the reaction products in the β-alanine—P_{3m} system
P_{3m}: β-alanine=0.5 mol dm^{-3}: 0.5 mol dm^{-3}
pH 12, room temp.

sponding to the two unknown compounds were observed at δ (chemical shift) 9.3 and 8.7 ppm. From the peak heights (amounts) of Compounds I and II on anion-exchange chromatogram (Fig. 1), peaks at δ 9.3 and 8.7 ppm were attributable to Compounds I and II, respectively. It was found from the ^{1}H-decoupling spectrum that these compounds are both orthophosphate derivatives of α-alanine. The fact that each singlet peak split into doublets due to ^{1}H-coupling suggests that Compounds I and II might be two of the following three compounds: *N*-(1-carboxyethyl)phosphoramidate(P_1-(*N*)ala) containing P–N bond in the molecule,  $^-O-P(=O)(O^-)-NHCH(CH_3)COO^-$, 2-aminopropionyl phosphate having P–O–C bond,  $^-O-P(=O)(O^-)-O-C(=O)-CH(CH_3)-NH_2$, and 4-methyl-5-oxo-1,3,2-oxazaphospholidin-2-olate 2-oxide (P_1-(*N,O*)ala) of five-membered ring structure, 

Since the proton on a nitrogen atom undergoes rapid exchange, the spin–spin coupling to phosphorus atom(P–N–H) has not been observed. Of these, the possibility of the formation of 2-aminopropionyl phosphate was excluded because no ^{1}H-coupling of phosphorus atom was observed in ^{31}P-NMR spectrum of authentic acetyl phosphate,  $^-O-P(=O)(O^-)-O-C(=O)-CH_3$. Therefore, the two unknown compounds could be P_1-(*N*)ala and P_1-(*N,O*)ala. It may thus be deduced that Compound II(δ 8.7 ppm) would be P_1-(*N,O*)ala, because its IR spectrum (Fig. 2) showed the presence of both P–O–C and P–N bonds in the molecule. On the other hand, when an aqueous solution of Compound II was acidified to pH 4, hydrolysis took place to give P_1 and α-alanine. Based on all the experimental results, Compound II was identified as an orthophosphate derivative of α-alanine, 1,3,2-oxazaphospholidin-2-olate derivative(P_1-(*N,O*)ala).

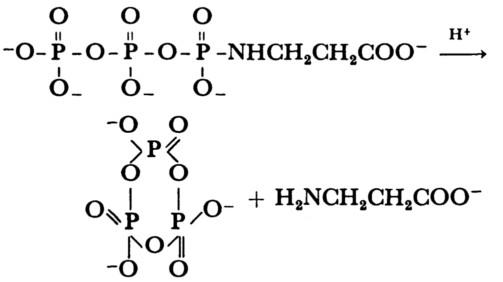
It was difficult to investigate the structure of Compound I by use of IR spectroscopy and chemical analysis, because this compound could not be isolated in a pure form by the chromatographic separation due to its unstability at neutral conditions. However, the result of ^{31}P-NMR spectrum (^{1}H-decoupling) shows that Compound I is an orthophosphate derivative of α-alanine. Since this readily undergoes hydrolysis under neutral and acidic conditions to form P_1 and α-alanine, it is expected that this compound contains P–N bond in its molecule. Based on these facts, we deduced that Compound I may probably be *N*-(1-carboxyethyl)-phosphoramidate(P_1-(*N*)ala).

Figure 4 shows the ^{31}P-NMR spectra of the reaction products of P_{3m} and β-alanine at a molar ratio of 1 : 1 and pH 12. By comparing with the ^{31}P-NMR spectra of triphosphoramidate in the literature,[25] Compound III was identified as a triphosphate derivative of β-alanine, *N*-(2-carboxyethyl)triphosphoramidate(P_3-(*N*)ala). In the ^{1}H-decoupling spectrum of Fig. 4, the doublets at δ −0.6 and −4.3 ppm were assigned to the end phosphorus atoms, P_α and P_γ, respectively, and the triplet at −19.6 ppm corresponded to the middle phosphorus atom, P_β. Each of the doublets of P_α should in principle split into triplets in ^{1}H-coupling by the influence of the two hydrogen atoms of $-NHCH_2COO^-$, but actually quintet was observed as a result of overlapping.

TABLE 1. CHANGE OF THE AMOUNTS OF PHOSPHORUS COMPOUNDS DUE TO pH

Compound / pH	P_1 (%)	P_2 (%)	P_3 (%)	[III] (%)	P_{3m} (%)
pH 12	0.8	0.6	0.6	69.3	28.6
pH 4	0.8	0.6	0.8	0.0	97.9
pH4 - pH12	0.0	0.0	+0.2	−69.3	+69.3

The reaction solution containing a large amount of Compound III was divided into two portions, one being set at pH 12 and the other being controlled to pH 4 by adding dilute hydrochloric acid, and both samples were analyzed by anion-exchange chromatography. The results are shown in Table 1; the amounts of phosphorus compounds at pH 12 in top row, those at pH 4 in middle row, and the difference between the two pH levels in bottom row. Compound III formed in 69.3% yield at pH 12 disappeared completely at pH 4, regenerating the starting material P_{3m}. This result indicates that Compound III recylized to P_{3m} as shown in formula(1) under acidic conditions.



(1)

Quimby[25] and Feldmann[7] have reported in the study of the reaction of P_{3m} with ammonia or various alkylamines that amidotriphosphate or *N*-alkyltriphosphoramidate formed were readily recyclized to produce P_{3m} under acidic conditions.

Amounts of Compounds I, II, and III. Figure 5 shows the changes in the amounts of P_1, P_2, P_3, P_1-(*N*)ala, and P_1-(*N*,*O*)ala formed by the reaction of P_{3m} with α-alanine at a molar ratio of 1 : 1, pH 12 and at room temperature. As can be seen from Fig. 1-a, elution peaks of P_1-(*N*)ala and P_1-(*N*,*O*)ala overlapped with each other, so the amounts of these compounds are shown as total in Fig. 5. The total amount of P_1-(*N*)ala and P_1-(*N*,*O*)ala gradually increased with the passage of reaction time, reaching about 33.5% after 22 d. P_2 was formed in a considerable quantity at the initial stage of the reaction, while the amounts of P_1 and P_3 gradually increased as the reaction progressed.

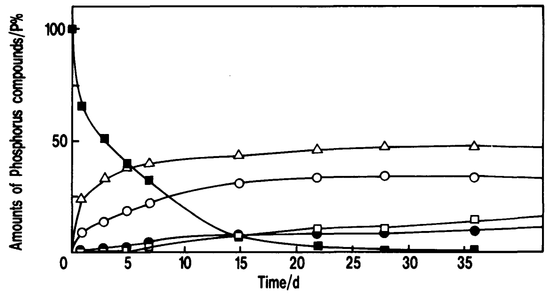


Fig. 5. Change of the amounts of phosphorus compounds in the reaction of P_{3m} with L-α-alanine at pH 12.

P_{3m} : L-α-alanine = 0.5 mol dm^{-3} : 0.5 mol dm^{-3}
—○—: P_1-(N)ala + P_1-(N,O)ala, —●—: P_1, —△—: P_2, —□—: P_3, —■—: P_{3m}

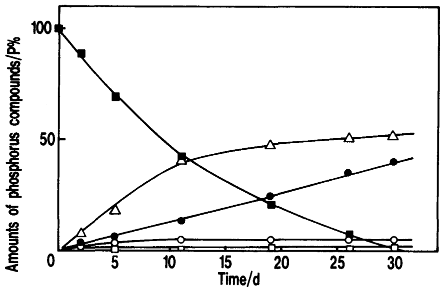


Fig. 6. Change of the amounts of phosphorus compounds in the reaction of P_{3m} with L-α-alanine at pH 10

P_{3m} : L-α-alanine = 0.5 mol dm^{-3} : 0.5 mol dm^{-3}
—○—: P_1-(*N*)ala + P_1-(*N*,*O*)ala, —●—: P_1, —△—: P_2, —□—: P_3, —■—: P_{3m}.

Figure 6 shows the changes in the amounts of phosphates formed by the reaction of P_{3m} and α-alanine at a mixing ratio of 1 : 1 and pH 10. In this case, the total amount of P_1-(*N*)ala and P_1-(*N*,*O*)ala was about 6%, which was much lower than that at pH 12. The amount of P_1, on the other hand, increased linearly with the reaction time, amounting to about 39% after 30 d. The formation of P_1 was notable compared with the case of pH 12. No reaction took place between P_{3m} and α-alanine under neutral and acidic conditions. It is clear from these results that P_{3m} readily reacts with α-alanine under alkaline conditions and that the reactivity lowers with decreasing pH, until eventually no reaction occurs when the pH is lowered to 7.

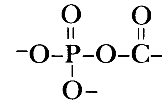
Figure 7 shows the changes in the amounts of various phosphates when the reaction was carried out using 0.5 mol dm^{-3} P_{3m} and 1.5 mol dm^{-3} α-alanine (mixing ratio = 1 : 3) at pH 12. As is apparent from Figs. 5 and 7, the reaction proceeded much faster at a mixing ratio of 1 : 3 than at that of 1 : 1. The total amounts of P_1-(*N*)ala and P_1-(*N*,*O*)ala formed at a reaction ratio of 1 : 3 were much greater than those formed at 1 : 1. P_1-(*N*)ala and P_1-(*N*,*O*)ala began to be formed immediately at the initial stage of the reaction, whose total amount reached about 65% after

3 d. After that, the amount continued to increase slowly until a nearly constant level of about 75% was reached. The slight decrease in the amount of P_2 after 2 d was due to the precipitation of crystals of tetrasodium pyrophosphate decahydrate, $Na_4P_2O_7 \cdot 10H_2O$, which were removed from the solution phase.

Figure 8 shows the changes in the amounts of phosphorus compounds formed by the reaction between P_{3m} and β-alanine (molar ratio=1:1) at pH 12 and room temperature. A rapid formation of P_3-(*N*)ala was observed. This amount reached about 54% in 10 d and increased slowly thereafter. The amount of P_3 increased linearly with the reaction time, which was presumably due to the hydrolysis of P_{3m}. Since P_1, P_2, and P_4 were formed in quite small quantities (about 1.3, 2.5, and 0.4%, respectively), they are not shown in Fig. 8. Such low yields of P_1 and P_2 provided further evidence that Compound III is a triphosphate derivative of β-alanine. The formation of P_4 when the reaction was continued for long periods is considered to be a result of a reaction of free P_1 with P_3-(*N*)ala having a P–N bond.[26]

Formation of Oligopeptides. The reaction products of P_{3m} with α- or β-alanine were analyzed by TLC to investigate the peptide bond formation of alanine in the reaction solutions. As can be seen in Fig. 9, which indicates, other than the spot of monomeric alanine, those of its dimer(L-alanylalanine) and trimer(L-alanylalanylalanine) in the reaction of P_{3m} with α-alanine were formed. On the other hand, no oligopeptide was formed in the reaction of P_{3m} with β-alanine.

Feldmann,[27] Paecht and Katchalsky,[28] and Koshland[29] reported that a compound having a



$$-O-\overset{\overset{O}{\|}}{\underset{\underset{O^-}{|}}{P}}-O-\overset{\overset{O}{\|}}{C}-$$

structure reacts with a free amino acid to form a peptide bond. Based on these results, the authors have postulated a reaction mechanism for the

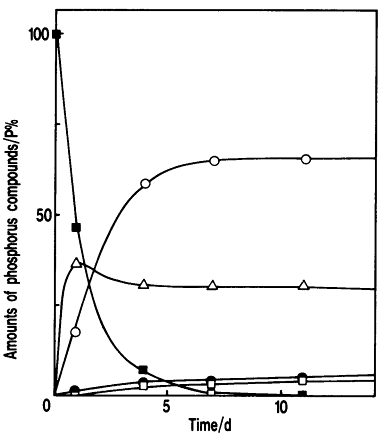


Fig. 7. Change of the amounts of phosphorus compounds in the reaction of P_{3m} with L-α-alanine at pH 12.

P_{3m}: L-α-alanine=0.5 mol dm^{-3}: 1.5 mol dm^{-3}

—○—: P_1-(*N*)ala+P_1-(*N,O*)ala, —●—: P_1, —△—: P_2, —□—: P_3, —■—: P_{3m}.

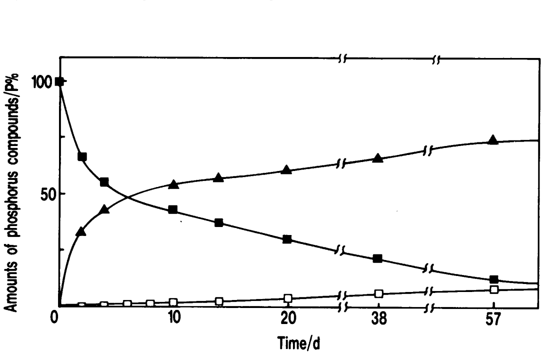


Fig. 8. Change of the amounts of phosphorus compounds in the reaction of P_{3m} with β-alanine at pH 12.

P_{3m}: β-alanine=0.5 mol dm^{-3}: 0.5 mol dm^{-3}

—▲—: P_3-(*N*)ala, —□—: P_3, —■—: P_{3m}.

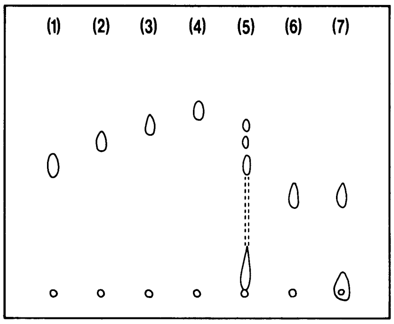


Fig. 9. Thin-layer chromatogram for the reaction products of P_{3m} with L-α-alanine or β-alanine.

(1): L-alanine (monomer), (2) L-alanine (dimer), (3): L-alanine (trimer), (4): L-alanine (tetramer), (5): 24 d after the reaction of P_{3m} with L-α-alanine, (6): β-alanine (monomer), (7): 14 d after the reaction of P_{3m} with β-alanine.

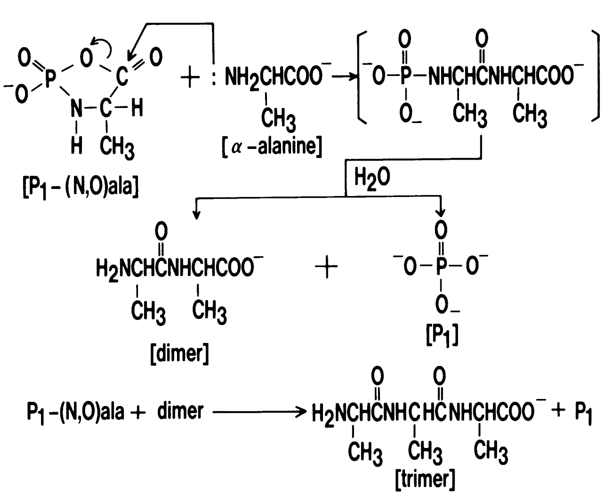


Fig. 10. Mechanism of formation of oligopeptides in the reaction of P_{3m} with L-α-alanine.

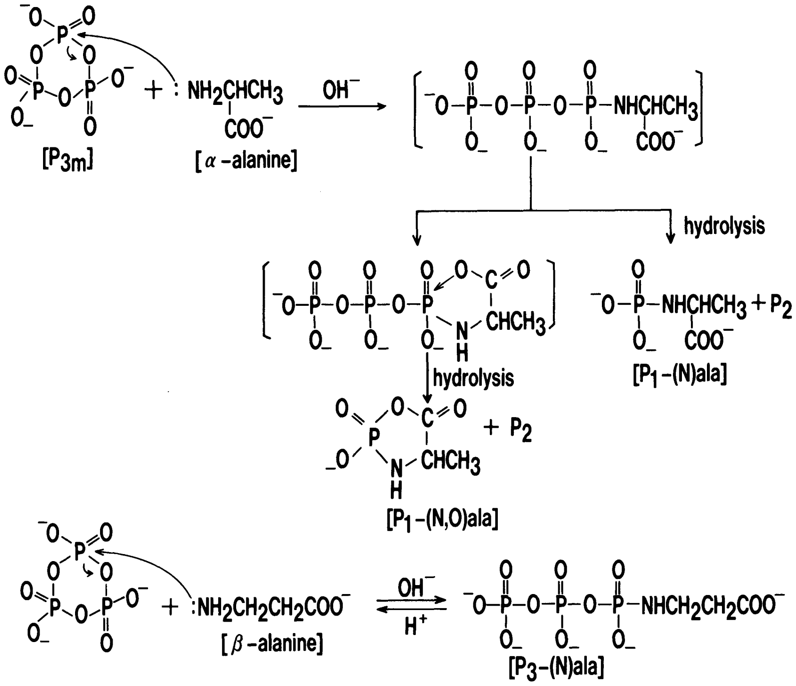


Fig. 11. Mechanism of the reaction of P_{3m} with L-α- alanine or β-alanine.

formation of the dimer and trimer of α-alanine as shown in Fig. 10. A nucleophilic attack by the amino group of free α-alanine on the carboxylate carbon atom of P_1-(*N,O*)ala, gives an orthophosphate derivative of alanylalanine as a reaction intermediate, which is unstable and readily undergoes hydrolysis to form alanylalanine and P_1. The dimer then reacts with P_1-(*N,O*)ala in a similar manner to afford the trimer. Tetramer and higher oligomers should also be formed through the same mechanism, which, however, could not be detected by TLC probably because of the small amounts present. Such oligopeptide formation has already been observed by our previous investigation on the reaction of P_{3m} with glycine.[18]

On the contrary, oligopeptides were not formed at all in the reaction of P_{3m} with β-alanine, showing that P_3-(*N*)ala formed in this reaction did not react with free β-alanine to produce its oligomers. It may be apparent from the foregoing that the five-membered ring compound, P_1-(*N,O*)ala, plays an important role in the formation of oligopeptides in the reaction of P_{3m} with alanine.

Mechanism of the Reaction of cyclo-*Triphosphate with α- or β-Alanine.* The mechanism of the reaction between P_{3m} and α- or β-alanine in alkaline solutions is shown in Fig. 11. In the reaction of P_{3m} with α-alanine, a nucleophilic attack by the amino group of α-alanine upon the phosphorus atom of P_{3m} causes a ring opening of the latter to form a triphosphate derivative of α-alanine having a P–N bond in the molecule as an intermediate. This intermediate, which is very unstable (as evidenced by the fact that it cannot be detected by either HPLC or anion-exchange chromatography), is considered to undergo hydrolysis through the following two routes: (1) an attack by the carboxyl group upon the phosphorus atom of the intermediate to form propionyl triphosphate with a five-membered ring structure, followed by hydrolysis into P_1-(*N,O*)ala and P_2, and (2) a direct hydrolysis of the intermediate to form P_1-(*N*)ala and P_2.

There is no possibility of a direct attack by the carboxyl group of α-alanine upon the phosphorus atom of P_{3m}, because no reaction takes place between CH_3COO^- and P_{3m} under the same conditions. In the reaction between P_{3m} and β-alanine, on the other hand, it is assumed that the ring opening of P_{3m} is caused by a nucleophilic attack of the amino group of β-alanine upon its phosphorus atom, producing P_3-(*N*)ala. This compound is stable in an alkaline solution, but readily recyclizes to P_{3m} under acidic conditions. Thus, the reaction of P_{3m} with β-alanine is reversible, depending on the pH.

The difference in the reaction mechanisms of *cyclo*-triphosphate with α- and β-alanine is outstanding, that is, the formation of a heterocyclic compound takes place with α-alanine but not with β-alanine. A further precise study is necessary in order to clarify any differences in the mechanisms.

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