Chem. Pharm. Bull.
30(11)3882—3889(1982)

Reaction of Trimetaphosphate with Glycine

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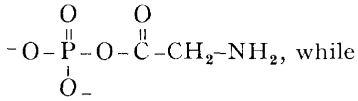
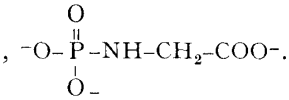
(Received April 15, 1982)

The reaction of trimetaphosphate (P_{3m}) with glycine in an aqueous solution was studied.

1) P_{3m} reacted with glycine at pH 10 and above to form two unknown compounds, A and B. 2) The amounts of these compounds were strongly affected by reaction conditions, such as the mixing ratio of P_{3m} and glycine and the pH, and the yields of compounds A and B were around 26 and 24%, respectively. 3) Compound A was proved to be an orthophosphate derivative of glycine (P_1-derivative of glycine), aminoacetyl phosphate,  $^{-}O-\overset{\overset{O}{\|}}{\underset{O^{-}}{P}}-O-\overset{\overset{O}{\|}}{C}-CH_2-NH_2$, while compound B was found to be a P_1-derivative of glycine, *N*-(carboxymethyl)phosphoramidate,  $^{-}O-\overset{\overset{O}{\|}}{\underset{O^{-}}{P}}-NH-CH_2-COO^{-}$. 4) It was found that glycylglycine (dimer), glycylglycylglycine (trimer), and higher oligopeptides of glycine were formed in the reaction of P_{3m} and glycine. Further, in the reaction at pH 10—14, tetra-(P_4) and pentaphosphate (P_5) were also formed besides compounds A and B, though in small quantities. 5) Short-chain phosphates, such as ortho-(P_1), pyro-(P_2) and triphosphate (P_3), did not react with glycine at all. 6) The mechanism of the reaction of P_{3m} with glycine is discussed.

Keywords——trimetaphosphate; glycine; aminoacetylphosphate; *N*-(carboxymethyl)phosphoramidate; phosphorylation

Among condensed phosphates, linear polyphosphates have found wide practical applications in foodstuffs, pharmaceuticals, detergents and many other industrial fields. However, owing to its weak complex-forming capacity, trimetaphosphate (P_{3m}), which is a six-membered cyclic phosphate, has not found practical applications. It was first reported by Quimby and Flauutt[1] in 1958 that P_{3m} could react with ammonia to form amidotriphosphate. After that, Feldmann studied the reactions of P_{3m} with sodium fluoride,[2] alcohols,[3] alkylamines,[4] and phenols,[5] and succeeded in phosphorylating these compounds. However, few studies on the reactions of P_{3m} with amino acids have been reported; only the reaction of glycine or alanine with P_{3m} was reported by Feldmann[6] and by Rabinowitz[7-9] in 1969. In both of these studies, the formation of phosphate derivatives of glycine by the reaction of P_{3m} with glycine was found, but the molecular structures and the yields of the products and the reaction mechanism were not clarified. Thus, in the present study the reaction of P_{3m} with glycine was carried out under various conditions, and the reaction products as well as their yields were studied by anion-exchange chromatography, paper chromatography, thin-layer chromatography (TLC), chemical analysis for phosphorus and glycine, infrared (IR) spectroscopy, and ninhydrin reaction. The mechanism of the reaction of P_{3m} with glycine is discussed. The present authors have already studied the reaction of P_{3m} or tetrametaphosphate (P_{4m}) with L-valine, an α-amino acid, and reported the formation of an orthophosphate derivative of valine, *N*-(1-carboxy-2-methylpropyl)phosphoramidate.[10,11] Accordingly, in the present paper the difference between the reactivities of glycine and L-valine to P_{3m} is discussed.

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Results and Discussion

Reaction of Trimetaphosphate with Glycine

Aqueous solutions of P_{3m} and glycine were mixed, and the mixture was adjusted to a desired pH (14, 12, or 10) with sodium hydroxide solution, then allowed to stand at room temperature. Reaction products obtained by the reaction of P_{3m} with glycine were separated and determined by anion-exchange chromatography.

As examples, the elution patterns of the reaction mixtures after reaction for 1 and 34 d (P_{3m}: glycine$=1:1$ and pH 12) are shown in Fig. 1. As can be seen, the formation of P_1 and P_2 were already apparent on the 1st day of the reaction. Further, between the elution peaks of P_1 and P_2, elution corresponding to two unknown compounds (hereafter referred to as compounds A and B) appeared. The formation of tetraphosphate (P_4), though in samll quantities, could be observed after 2 d. Crystals of tetrasodium pyrophosphate decahydrate, $Na_4P_2O_7\cdot10H_2O$, were also formed after 2 d.

The changes in the amounts of P_2, P_3, P_{3m}, and the compounds A and B formed by the reaction of P_{3m} and glycine at pH 14 are shown in Fig. 2. At pH 14, the reaction was so rapid that the starting material, P_{3m}, had almost disappeared within 3 d, and the yield of compound A reached about 20% at 4 d, then decreased very slowly. On the other hand, the formation of compound B in a small amount could be observed until 3 d, but after 4 d, compound B was no longer detectable. Crystals of $Na_4P_2O_7\cdot10H_2O$ began to precipitate at 4 d and the amount increased up to 10 d, then remained almost constant. Further, at pH 14 the formation of P_1 could hardly be observed. P_3 was formed immediately after the initiation of the reaction in considerable quantity; this may be caused by hydrolysis of P_{3m}.

Amounts of P_1, P_2, P_3, P_{3m}, and compounds A and B at pH 12 are plotted as a function of reaction time in Fig. 3. Compounds A and B were formed from the beginning of the reaction, and their yields reached about 10.7 and 5.3%, respectively at 7 d, then remained almost cons-

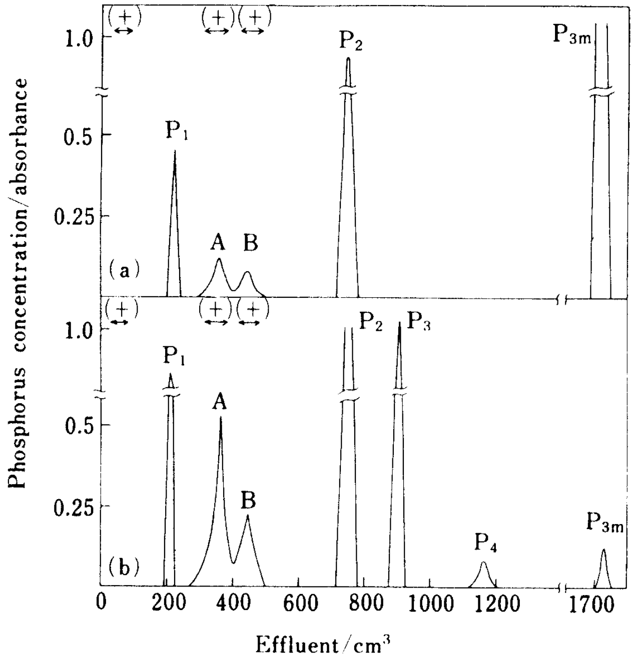

Fig. 1. Elution Patterns of the Reaction Products of P_{3m} with Glycine at pH 12

(a): after 1 d.
(b): after 34 d.
(+): nihydrin reaction positive.

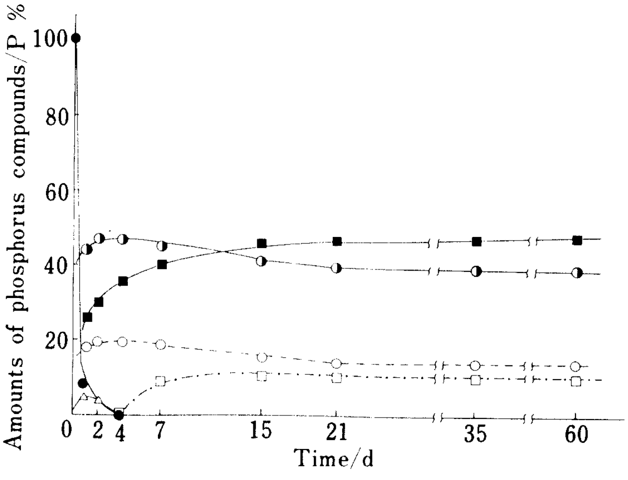

Fig. 2. Changes of the Amounts of Phosphorus Compounds in the Reaction of P_{3m} with Glycine at pH 14

P_{3m}: Gly.$=1:1$.
– –○– –: compound A; ——△——: compound B;
——■——: P_2 (total); —·—□—·—: P_2(precipitated);
——◑——: P_3. ——●——: P_{3m}.

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tant. The yield of compound A at pH 12 was less than that at pH 14. On the other hand, no great difference between the yield of compound B at pH 12 and that at pH 14 could be observed, but it is characteristic that compound B remains stable for long period at pH 12. Further, P_2 was formed from the beginning of the reaction and its amount increased rapidly with time; the amount at pH 12 was somewhat larger than that at pH 14. The formation of P_3 increased gradually for about 20 d, then remained almost constant at about 15%. The yield of P_1 was as little as about 7% even at 49 d, but increased with time, though very slightly. Further, in the reaction of P_{3m} with glycine at pH 12, P_4 and pentaphosphate (P_5) were formed in small quantities, namely about 3.1 and 1.5% respectively, but they are omitted in Fig. 3.

Figure 4 shows the changes in the amounts of P_1, P_2, P_3, P_4, P_5, P_{3m}, and compounds A and B at pH 10. As can be seen from Fig. 4, the yields of compounds A and B at pH 10 were smaller than those at pH 14 and 12. The yields increased with time, reached a maximum at about 14 d (for compound A about 3.3%; for B, about 2.0%), and then decreased. The amount of P_2 increased rapidly until about 14 d, while $Na_4P_2O_7 \cdot 10H_2O$ began to precipitate from 5 d. The formation of P_1 gradually increased with time and was greater than those at

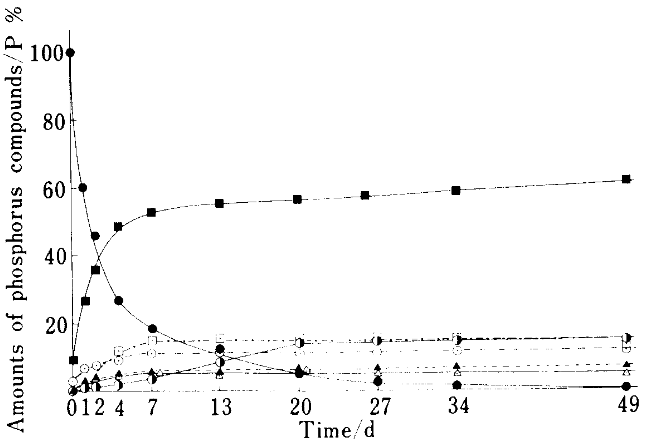


Fig. 3. Changes of the Amounts of Phosphorus Compounds in the Reaction of P_{3m} with Glycine at pH 12

--⊙--: compound A, —△—: compound B, ---▲---: P_1,
—■—: P_2(total), -·-⊡-·-: P_2 (precipitated), —◑—: P_3,
—●—: P_{3m}

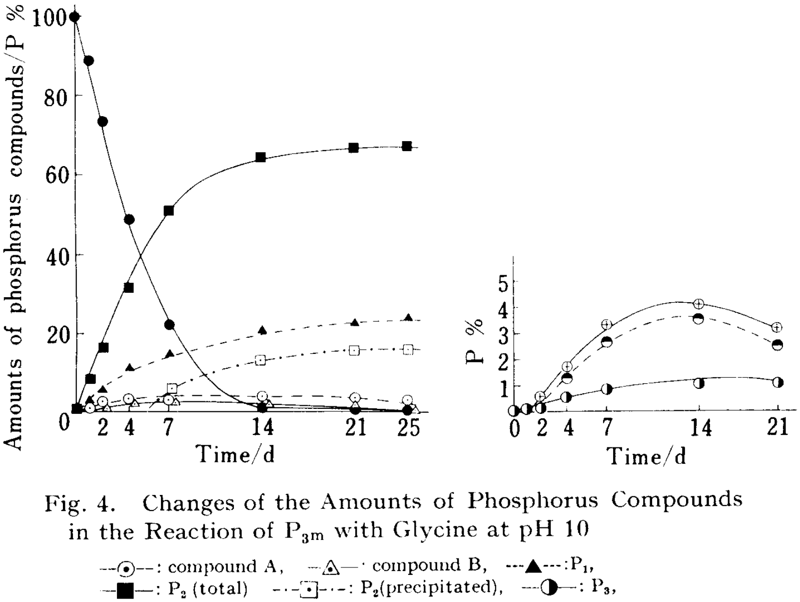


Fig. 4. Changes of the Amounts of Phosphorus Compounds in the Reaction of P_{3m} with Glycine at pH 10

--⊙--: compound A, —△— compound B, ---▲---:P_1,
—■—: P_2 (total) -·-⊡-·-: P_2(precipitated), —◑—: P_3,
—⊕—: P_1, --◓--: P_5, —●—: P_{3m}.

pH 14 and 12. On the other hand, the yield of P_3 reached about 1.2% in the range of 14—21 d, and then gradually decreased. The amount of P_3 at pH 10 was quite small compared with those at pH 14 and 12. Further, the yields of P_4 and P_5 at pH 10 reached about 4.0 and 3.5%, respectively, at 14 d; these values are larger than those at pH 12. Thus, it was proved that the lower the pH, the more P_4 and P_5 was formed. On the other hand, in the reaction of P_{3m} with L-valine, the formation of P_4 and P_5 was also observed.[10]

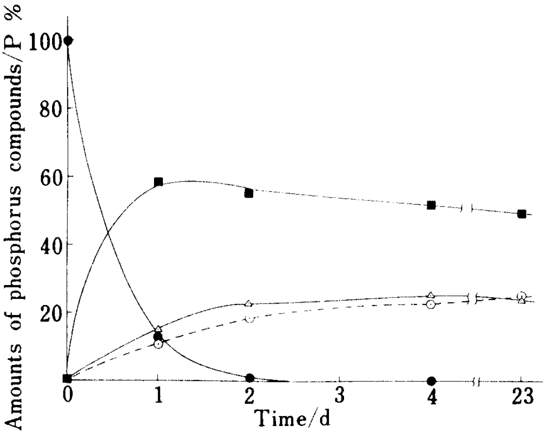


Fig. 5. Changes of the Amounts of Phosphorus Compounds in the Reaction of P_{3m} with Glycine at pH 12

P_{3m}: Gly=1: 5.
---⊙---: compound A, —△—: compound B;
—■—: P_2, —●—: P_{3m}.

The formation of compounds A and B was also investigated by varying the mixing ratio of P_{3m} and glycine. As an example, the results at pH 12, using a mixture containing P_{3m} and glycine (1: 5), are shown in Fig. 5. As can be seen, when glycine is used in large excess over P_{3m}, the reaction was so rapid that it was almost completed in 2 d. In this case, only P_2 and compounds A and B were formed, while the formation of P_3 and P_1 was hardly observed. The formation of compounds A and B was especially remarkable and their yields were fairly high (about 25.3 and 22.7%, respectively after 4 d).

At pH 12 and with P_{3m}: glycine=5: 1, the yield of compound A was about 4%, but the formation of compound B could hardly be observed, while the formation of P_3 increased gradually with time. This may be caused by hydrolysis of excess P_{3m}. It was found that unreacted P_{3m} was present in fairly large quantities in the solution and it amounted to about 52% even after 50 d.

In neutral or acidic solution, P_{3m} did not react with glycine at all. The reason why compounds A and B are not obtained in neutral and acidic solutions is because of the formation of the unreactive H_3N^+–CH_2–COO^- zwitterion or H_3N^+–CH_2–COOH at lower pH.

Molecular Structures of Compounds A and B

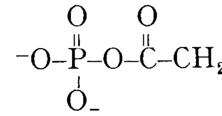
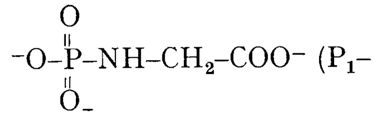
The molecular structures of compounds A and B were determined by means of ninhydrin reaction, chemical analysis, paper chromatography, and IR spectroscopy. Also, the structural change of compounds A and B in an acidic solvent was investigated. The results of ninhydrin reaction for each fraction obtained by ion-exchange chromatography are shown in Fig. 1. The components in fractions of 50—100, 310—380, and 410—470 ml were positive to the ninhydrin reaction. The positive ninhydrin-reaction of 50—100 ml may be attributed to unreacted glycine. On the other hand, as the positive reactions of fractions of 310—380 and 410—470 ml were caused by compounds A and B, it was suggested that both compounds A and B may be phosphate derivatives of glycine.

The amounts of phosphorus and glycine in compounds A and B fractionated by ion-exchange chromatography were determined by chemical analysis and with an amino acid analyzer. It was found that the ratios of phosphorus to glycine in compounds A and B are unity in both, showing that these compounds are P_1-derivatives of glycine containing P_1 and glycine in the molar ratio of 1: 1.

Compounds A and B were analyzed by paper chromatography with an acidic solvent. Compounds A and B both gave a strong color-developing spot at the position corresponding to the standard P_1 and a weak color-developing spot at a position between those of P_1 and P_2. The latter may be attributed to compounds A and B themselves, and the former to P_1, formed by hydrolysis of compounds A and B during the development process. Thus, it may be considered that compounds A and B are both P_1-derivatives of glycine. However, when compound B was developed with an acidic solvent, the formation of P_2 (though only a small quantity) was observed. This finding will be discussed later in detail. Further, no great difference between the *Rf*-values of compounds A and B was observed with the present solvent.

Based on the above-mentioned results of chemical analysis and paper chromatography, it is suggested that compounds A and B are P_1-derivatives of glycine, in which phosphorus is combined with glycine in 1: 1 ratio.

The IR spectra of compounds A and B, fractionated by ion-exchange chromatography, are shown in Fig. 6. In the spectra of both compounds A and B, characteristic absorptions attributed to glycine were observed at about 1600 ($\nu_{C=O}$), 1413 ($\nu_{C=O}$), 1334 (ν_{C-N}), 900, and 500 cm^{-1}.[12] In addition, the spectrum of compound A showed characteristic absorptions at about 1150, 930, and 760 cm^{-1} due to the P–O–C bond.[13,14] Further, absorptions due to PO_3 were observed at about 1080 and 980 cm^{-1}.[15,16] On the other hand, the spectrum of compound B showed, in addition to the above-mentioned absorptions of glycine, characteristic absorptions due to the P–N bond at about 1260, 800, and 720 cm^{-1}.[17–19] As in compound A, absorptions due to PO_3 were observed at about 1080 and 980 cm^{-1}. These results demonstrate that compound A is a P_1-derivative of glycine having a P–O–C bond in its molecule, while compound B is a P_1-derivative having a P–N bond in its structure.

Based on all the experimental results, compound A was concluded to be aminoacetyl phosphate  $^{-}\text{O}-\underset{\text{O}^{-}}{\overset{\text{O}}{\overset{\|}{\text{P}}}}-\text{O}-\overset{\text{O}}{\overset{\|}{\text{C}}}-\text{CH}_2-\text{NH}_2$ (P_1–(O)Gly.), while compound B was concluded to be *N*-(carboxymethyl)phosphoramidate  $^{-}\text{O}-\underset{\text{O}^{-}}{\overset{\text{O}}{\overset{\|}{\text{P}}}}-\text{NH}-\text{CH}_2-\text{COO}^{-}$ (P_1–(*N*)Gly.).

Formation of Oligopeptides

It has already been reported by Feldmann[6] and Rabinowitz *et al.*[7–9] that in the reaction of P_{3m} and glycine, glycylglycine (dimer) and glycylglycylglycine (trimer) were formed. Therefore we investigated whether or not the condensation of glycine would also occur under the conditions of the present study.

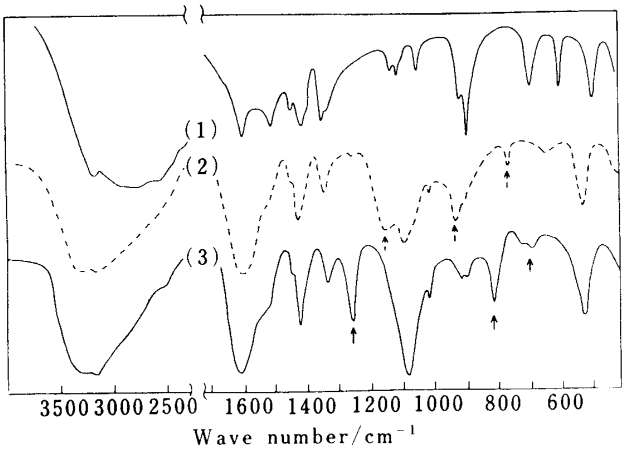


Fig. 6. IR Spectra of Glycine and Compounds A and B

(1): glycine, (2): compound A, (3): compound B.
↑ : P-O-C bond; ↑ : P-N bond.

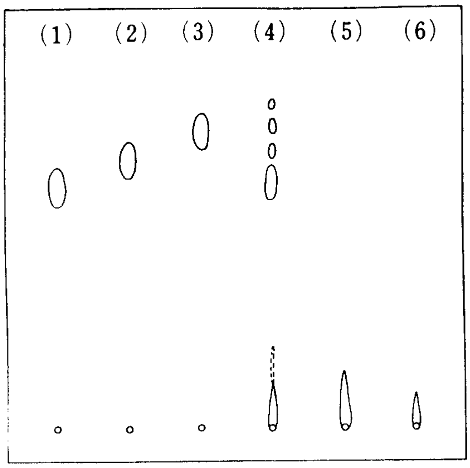


Fig. 7. Thin Layer Chromatogram of the Reaction Products of P_{3m} with Glycine

(1): glycine, (2): glycylglycine, (3): glycylglycylglycine, (4): 20 d after the reaction of P_{3m} with glycine, (5) compound A, (6): compound B.

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Reaction products of P_{3m} with glycine were subjected to TLC ; an example of the results is shown in Fig. 7. As can be seen from Fig. 7, dimer, trimer, and higher oligopeptides of glycine were formed. For reference, the results for compounds A and B are also shown; these compounds were hardly developed by the present solvent.

Reaction of Short-Chain Phosphates with Glycine

A solution of trisodium orthophosphate (P_1), tetrasodium pyrophosphate (P_2), or pentasodium triphosphate (P_3) was mixed with glycine solution in a molar ratio of 1 : 1, and then the mixed solution was adjusted to pH 12 with sodium hydroxide solution and allowed to react at room temperature. No reaction products at all were observed in the reactions of glycine with P_1, P_2, and P_3. Therefore, it may be concluded that no phosphate derivatives of glycine can be formed from short-chain phosphates, such as P_1, P_2 ,and P_3.

Mechanism of the Reaction of Trimetaphosphate with Glycine

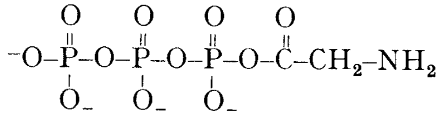
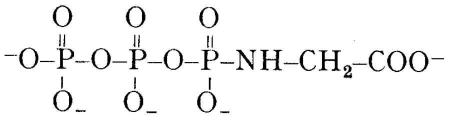



Fig. 8 shows the mechanism of the reaction of P_{3m} and glycine at pH 10—14. By the attack of carboxyl and amino groups of glycine, the ring of P_{3m} is easily opened and P_3-derivatives of glycine, ${}^{-}O-\underset{O_-}{\overset{\overset{O}{\|}}{P}}-O-\underset{O_-}{\overset{\overset{O}{\|}}{P}}-O-\underset{O_-}{\overset{\overset{O}{\|}}{P}}-O-\overset{\overset{O}{\|}}{C}-CH_2-NH_2$ (P_3-(O)Gly.) and ${}^{-}O-\underset{O_-}{\overset{\overset{O}{\|}}{P}}-O-\underset{O_-}{\overset{\overset{O}{\|}}{P}}-O-\underset{O_-}{\overset{\overset{O}{\|}}{P}}-NH-CH_2-COO^-$ (P_3-(N)Gly.) are formed. However, P_3-(O)Gly and P_3-(N)Gly could not be detected either by paper chromatography or anion-exchange chromatography. This may be explained by assuming that these compounds are unstable and easily hydrolyzed not only to P_3 and glycine but also to P_2 and P_1-derivatives of glycine, P_1-(O)Gly and P_1-(N)Gly.

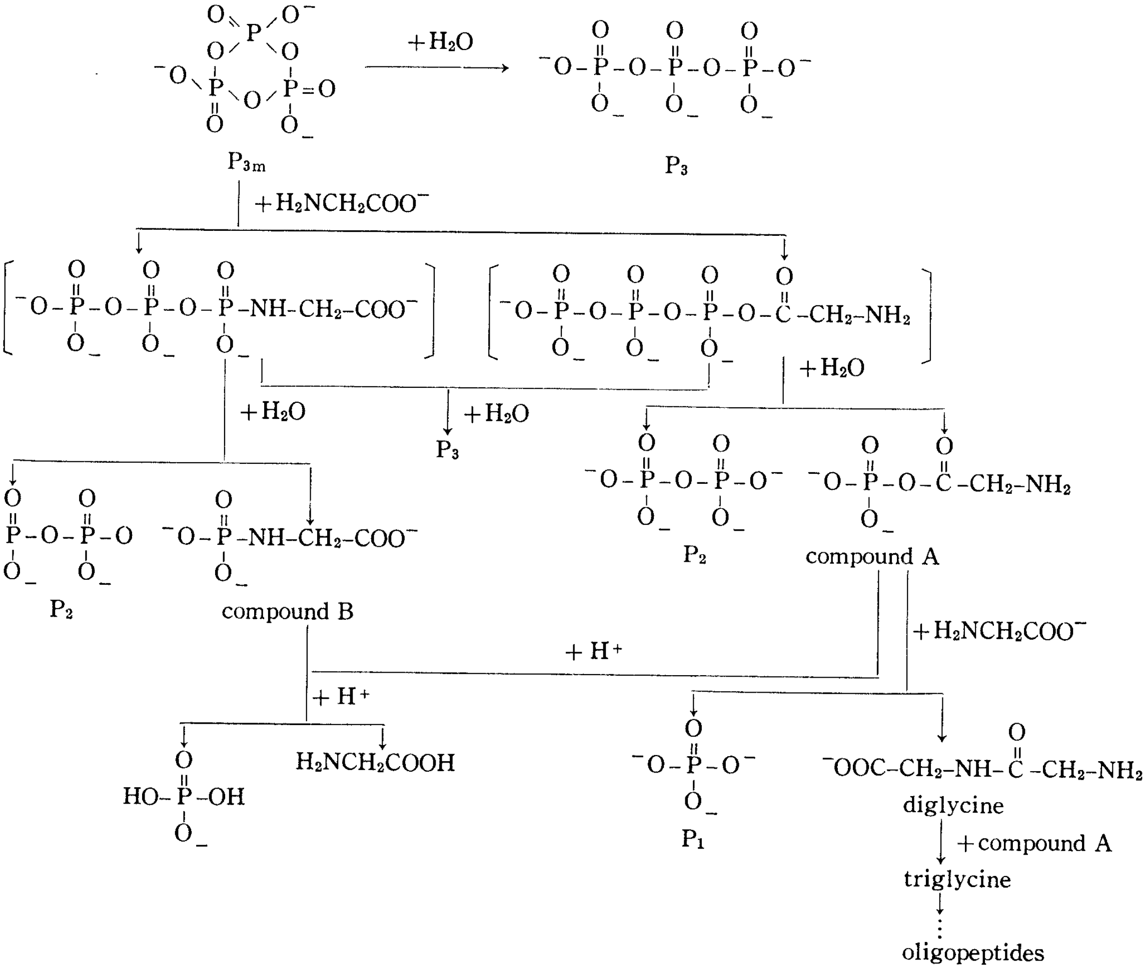
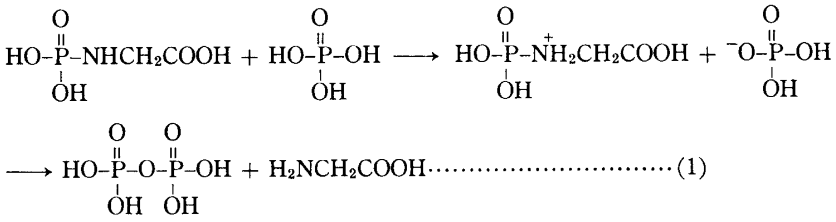


Fig. 8. Mechanism of the Reaction of P_{3m} with Glycine

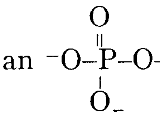
Solutions of compounds A and B were adjusted to pH 3, allowed to stand for 5 h, and then subjected to paper chromatography and anion-exchange chromatography. It was found that compounds A and B were both transformed to P_1 and glycine. Further, compound B gave P_2 (though in small quantity), in addition to P_1, while the formation of P_2 from compound A could not be detected. When a solution of compound B was acidified, P_2 was formed in addition to P_1. This may be interpreted by the following mechanism:

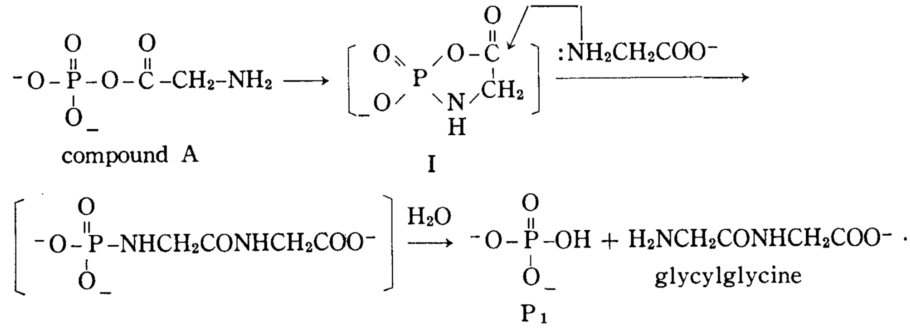


$$\mathrm{HO{-}\underset{OH}{\overset{O}{\overset{\|}{P}}}{-}NHCH_2COOH + HO{-}\underset{OH}{\overset{O}{\overset{\|}{P}}}{-}OH \longrightarrow HO{-}\underset{OH}{\overset{O}{\overset{\|}{P}}}{-}\overset{+}{N}H_2CH_2COOH + {}^{-}O{-}\underset{OH}{\overset{O}{\overset{\|}{P}}}{-}OH}$$

$$\longrightarrow \mathrm{HO{-}\underset{OH}{\overset{O}{\overset{\|}{P}}}{-}O{-}\underset{OH}{\overset{O}{\overset{\|}{P}}}{-}OH + H_2NCH_2COOH} \cdots\cdots (1)$$

That is, compound B reacted with P_1 to give P_2. Quimby,[1,20] Feldmann and Thilo,[21] and Clark *et al.*[22,23] have already reported that when amido and imidophosphates containing the P–N bond are acidified, deamination was accompanied by the condensation of phosphates. This also supports the conclusion that compound B is a P_1-derivative of glycine having a P–N bond in its structure.

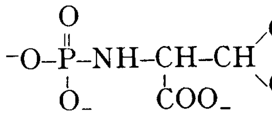
On the other hand, Feldmann,[6] Paecht and Katchalsky,[24] and Koshland[25] have reported that a peptide bond –CONH– could be formed by the reaction of a compound having an  $\mathrm{{}^{-}O{-}\underset{O_-}{\overset{O}{\overset{\|}{P}}}{-}O{-}\overset{O}{\overset{\|}{C}}{-}}$ structure with an amino acid. Thus, it is considered that the mechanism of formation of glycylglycine, glycylglycylglycine, and higher oligopeptides by the reaction of P_{3m} with glycine in the present study may be as follows; the attack of the COO^- group of glycine upon a P atom of P_{3m} causes ring opening of the latter to produce compound A, a part of which forms a five-membered ring intermediate (I), and I may react with unreacted glycine to form glycine dimer. Further, I reacts with the dimer to form a trimer. By a similar mechanism, the tetramer and pentamer of glycine may be successively formed, but the amounts may be extremely small.



$\cdots\cdots (2)$

$\mathrm{I + glycylglycine \longrightarrow H_2NCH_2CONHCH_2CONHCH_2COO^- + P_1} \cdots\cdots (3)$

glycylglycylglycine

On the other hand, as previously reported by the present authors, no dimer and trimer of valine was formed by the reaction of P_{3m} with L-valine.[10] This is consistent with the fact that the reaction of P_{3m} with L-valine afforded only *N*-(1-carboxy-2-methylpropyl)phosphoramidate,  $\mathrm{{}^{-}O{-}\underset{O_-}{\overset{O}{\overset{\|}{P}}}{-}NH{-}\underset{COO_-}{CH}{-}CH\begin{smallmatrix}\nearrow CH_3\\ \searrow CH_3\end{smallmatrix}}$, containing a P–N bond and no phosphate derivative of valine containnig a P–O–C bond was formed. Thus, a great difference is observed between glycine and L-valine in their reactivity to P_{3m} and also in the nature of the reaction products.

Experimental

Chemicals——Sodium trimetaphosphate hexahydrate, $Na_3P_3O_9 \cdot 6H_2O$, was prepared by the procedure described in the previous paper[10] and recrystallized five times from an aqueous solution. Reagent-grade glycine was used without purification. Unless otherwise stated, guaranteed grade reagents from Wako Chemical Industries Ltd., were used without further purification.

Reaction between Trimetaphosphate with Glycine——Aqueous solutions of 0.5 mol/l sodium trimetaphosphate hexahydrate $Na_3P_3O_9 \cdot 6H_2O$ and 0.5 mol/l glycine were mixed in volumetric ratios of 5: 1—1: 5. The pH values of the mixed solutions were in the range of about 5.6—5.9. The solutions were then adjusted to the desired pH (14, 12, or 10) by use of 6 mol/l sodium hydroxide solution, and allowed to stand at room temperature to react. With the progress of the reaction, the pH of the solution gradually fell. Thus, the solution was constantly adjusted to the desired pH by adding the sodium hydroxide solution. For comparison, the reaction of P_{3m} with glycine was also studied in neutral (pH 7) and acidic (pH 3) solutions.

Determination of Glycine——A Nippon Denshi Amino Acid Analyzer, JLC-6AS type, was used for the determination of glycine in the reaction products.

For analysis of glycine, glycylglycine, and glycylglycylglycine, TLC was used. A mixed solvent consisting of $CHCl_3$, CH_3OH, and 17% NH_4OH (2: 2: 1)[8] was used, with silica gel plates (made by Wako Chemical Industries and E. Merck Co.).

Anion-exchange chromatography, paper chromatography, IR spectroscopy, ninhydrin reaction, and the determination of phosphorus were carried out by the methods described in the previous paper.[10]

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