The Reaction of *cyclo*-Triphosphate with L-Valine

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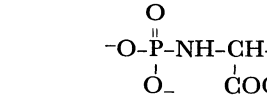
The reaction of *cyclo*-triphosphate(P_{3m}) with L-valine in an aqueous solution was studied by means of anion-exchange chromatography, paper chromatography, chemical analysis for phosphorus and valine, IR spectroscopy, and ninhydrin reaction. 1) P_{3m} reacted with L-valine at pH 10—12 to form an unknown compound, the amount of which reached about 22% at pH 12 and about 9% at pH 10. This compound decomposed into orthophosphate(P_1) and valine at a lower pH. 2) The unknown compound was proved to be a P_1-derivative of valine,  , which has a P–N bond in its molecule. 3) In the reaction of P_{3m} with L-valine at pH 10, in addition to the unknown compound, small amounts of tetra(P_4) and pentaphosphate(P_5) were produced. 4) The disappearance rate of P_{3m} in this reaction was first-order, and its rate constants were 0.317 and 0.138 d^{-1} at pH 12 and 10 respectively at room temperature. 5) Short-chain phosphates, such as P_1, pyro (P_2), and triphosphate(P_3), did not react with L-valine at all. 6) The mechanism of the reaction of P_{3m} with L-valine was discussed.

It was first reported in 1958 by Quimby and Flautt[1] that P_{3m} easily underwent ring cleavage by ammonia to give amidotriphosphate. After that, the reactions of various alkylamines with P_{3m} and *cyclo*-tetraphosphate(P_{4m}) have been studied by Feldmann.[2-5] Furthermore, the application of condensed phosphates as inhibitors to the formation of *N*-nitrosodimethylamine, which is a well known carcinogenic substance and which is easily formed by the reaction of dimethylamine with nitrous acid or nitrite, has recently been reported.[6] That is, when a condensed phosphate is added to an aqueous solution of dimethylamine, they react with each other and the reaction of dimethylamine with nitrous acid or nitrite is inhibited. On the other hand, Rabinowitz,[7-9] applying various phosphates as condensing agents for the polymerization of glycine or alanine, has obtained the corresponding dimers (glycylglycine and alanylalanine) and trimers (glycylgylcylglycine and alanylalanylalanine).

As has been described above, the reactions of cyclic phosphates with ammonia and its derivatives have attracted notice from various points of view. However, up to the present, little attention has been paid to the phosphorus-containing products in the reaction between cyclic phosphates and amino acids or to the mechanisms of the formation of these products. The present investigation was undertaken to see what products, especially phosphorus-containing products, are formed in the reaction of P_{3m} with L-valine, an α-amino acid. The reaction products were examined by means of anion-exchange chromatography, paper chromatography, chemical analysis for phosphorus and valine, IR spectroscopy, and ninhydrin reaction. For the sake of comparison, the reactions of L-valine with P_1, P_2, and P_3 were also tested. From the data obtained, the mechanism of the reaction of P_{3m} with L-valine was discussed.

Experimental

Chemicals. Sodium *cyclo*-triphosphate hexahydrate,[10] $Na_3P_3O_9 \cdot 6H_2O$, was prepared by heating sodium dihydrogenorthophosphate dihydrate at 530 °C for 5 h, cooling it slowly to room temperature, and then crystallizing from an aqueous solution. The $Na_3P_3O_9 \cdot 6H_2O$ thus obtained was identified by paper chromatography and X-ray diffractometry. Reagent-grade L-valine was used without purification. Unless otherwise stated, guaranteed reagents from Wako Chemical Industries, Ltd., were used without further purification.

Reaction between cyclo-*Triphosphate and* L-*Valine.* Two aqueous solutions, 0.4 mol dm^{-3} sodium *cyclo*-triphosphate hexahydrate and 0.4 mol dm^{-3} L-valine, were mixed in a volume ratio of 1:1. The pH of the mixture was about 5.8. The solution was then adjusted with a 6 mol dm^{-3} sodium hydroxide solution to the prescribed pH (10 and 12) and allowed to stand at room temperature. With the progress of the reaction, the pH of the solution was gradually lowered, and then the sodium hydroxide solution was added to maintain the prescribed pH. It was also observed that, at pH values below 9, the solution became moldly.

Anion-exchange Chromatography.[11,12] A chromatographic column of 64×1.5 cm I. D. packed with an anion-exchange resin, Bio-Rad AG 1-X8 (chloride form) of 100—200 mesh, was used. The gradient-elution technique was employed. Sodium chloride solutions of 0.32 mol dm^{-3} and 0.12 mol dm^{-3} were stored in a reservoir (3000 cm^3) and a mixing bottle (750 cm^3) respectively. 150 and 40 cm^3 of a mixture (2:1) of the 0.1 mol dm^{-3} tetrasodium ethylenediaminetetraacetate (EDTA-4Na) and 0.1 mol dm^{-3} disodium ethylenediaminetetraacetate (EDTA-2Na) solutions were added to the sodium chloride solutions in the reservoir and the mixing bottle. The pH of the eluent was about 10. The sample solution was diluted 20 times, and 1 cm^3 of it was injected into a column. The effluent was collected in 10-g fractions with a fraction collector of the weight type.

Determination of Phosphorus.[11,13] The phosphorus in the effluent was determined by the orthophosphoric heteropoly-blue method. To 10-g of the fractionated effluent, 2 cm^3 of the molybdenum(V)–molybdenum(VI) reagent[14] and about 5 cm^3 of distilled water were added. The mixture was heated in a water bath at 95 °C for 1 h. After cooling, the solution was made up to 25 cm^3 with distilled water. The absorbance was measured at 830 nm by means of a Hitachi Spectrophotometer, 100-20 type.

Determination of Valine. A Hitachi Amino-acid An-

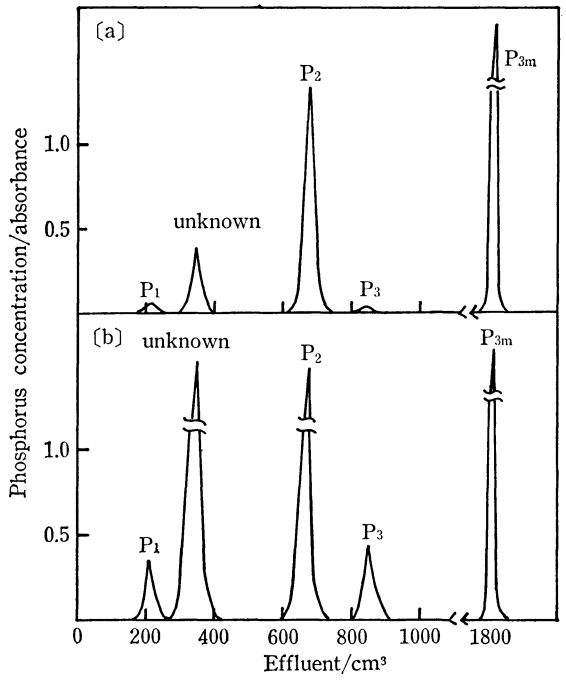


Fig. 1. Elution patterns for the reaction products of P_{3m} with L-valine at pH 12.
(a): After 1 d, (b): after 14 d.

alyzer, KLA-5 type, was used for the determination of valine in the reaction products.

Paper Chromatography. Using Toyo Roshi No. 51A filter paper and acidic and basic solvents,[15-17] the phosphate species in the reaction products were paper-chromatographed by an ascending technique at 5 °C and at room temperature for about 20 h.

Ninhydrin Reaction. To 5 cm³ of the fractionated effluent we added a few drops of a 5% ninhydrin solution, after which the mixture was boiled and then cooled. The reddish purple coloration indicates the presence of valine.

IR Spectroscopy. After the fractionated effluent had been dehydrated under reduced pressure, it was submitted to IR spectroscopy. Since, in the sample, sodium chloride was present in a large amount, a direct measurement was possible without any dilution with potassium bromide. A Hitachi Infrared Spectrophotometer, EPI-S2 type, was used.

Results and Discussion

Reaction of cyclo-*Triphosphate with* L-*Valine.* The aqueous solutions of P_{3m} and L-valine were mixed at a molar ratio of 1:1 and adjusted to pH 10 and 12 with a sodium hydroxide solution. When the mixture was allowed to stand at room temperature, the pH of this solution gradually lowered. This may suggest the formation of chain phosphates and/or their derivatives by the reaction of P_{3m} with water and/or L-valine. During the progress of the reaction, the sodium hydroxide solution was added to maintain the prescribed pH. The reaction products of P_{3m} with L-valine were separated by anion-exchange chromatography. As examples, the elution patterns for the reaction mixture at pH 12 after 1 d and after 14 d are shown in Fig. 1. Between the elution peaks of P_1 and P_2, a peak of an unknown compound appeared. P_2 began

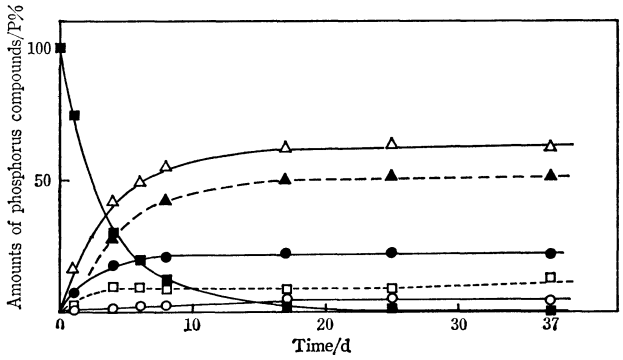


Fig. 2. Change of the amounts of phosphorus compounds in the reaction of P_{3m} with L-valine at pH 12.
—●—: Unknown compound, —○—: P_1, —△—: P_2 (total), --▲--: P_2 (precipitated), --□--: P_3, —■—: P_{3m}.

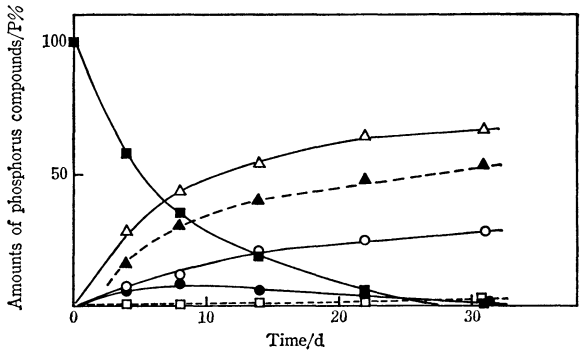


Fig. 3. Change of the amounts of phosphorus compounds in the reaction of P_{3m} with L-valine at pH 10.
—●—: Unknown compound, —○—: P_1, —△—: P_2 (total), --▲--: P_2 (precipitated), —□—: P_3, —■—: P_{3m}.

to precipitate as crystals of tetrasodium pyrophosphate decahydrate on the 2nd day. Figure 2 shows the changes in the amounts of P_1, P_2, P_3, P_{3m}, and the unknown compound during the progress of the reaction. As can be seen from Fig. 2, the amount of the starting material, P_{3m}, rapidly decreased with time and became nearly zero within four weeks. On the contrary, the amounts of P_2 and the unknown compound gradually increased with time, reached about 62 and 22% respectively after 2 weeks, and after that were almost constant. Although the amounts of P_1 and P_3 were less than those of P_2 and the unknown compound, they gradually increased with time.

The products obtained by the reaction of P_{3m} with L-valine at pH 10 were somewhat different from those obtained at pH 12 and consisted of six kinds of phosphates, *i.e.*, the unknown compound, P_1, P_2, P_3, P_4, and P_5. The changes in the amounts of the unknown compound, P_1, P_2, P_3, and P_{3m} with time are shown in Fig. 3. The data for P_4 and P_5 are omitted, because their amounts are very small. Unlike the results in the case of pH 12, the yield of the unknown compound at pH 10 reached a maximum, about 9%, after 7—8 d, and after that gradually decreased. It was only about 2.6% after 31 d. The amounts of P_4 and P_5 formed at pH 10 were about 0.7 and 0.5% respectively after

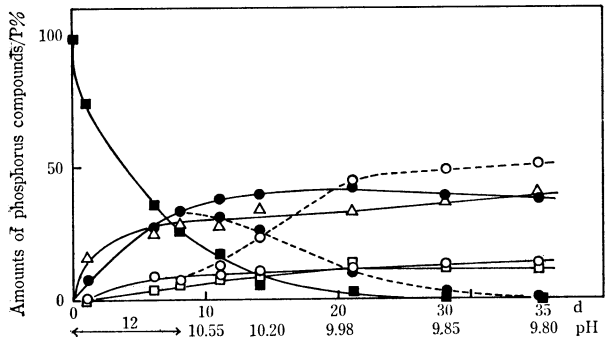


Fig. 4. Change of the amounts of phosphorus compounds in the reaction of P_{3m} with L-valine at pH 12 and when pH was not controlled.
——: pH 12, ……: pH not controlled, —●—: unknown compound, —○—: P_1, —△—: P_2, —□—: P_3, —■—: P_{3m}.

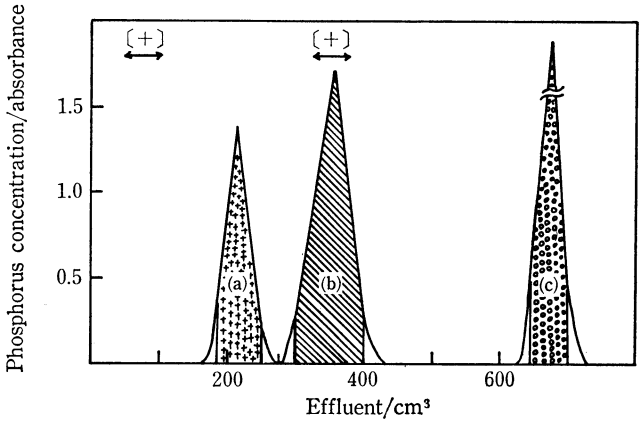


Fig. 5. An elution pattern for the reaction products of P_{3m} with L-valine at pH 12 and the tests by ninhydrin reaction and IR spectroscopy.
Ninhydrin reaction; (+) positive. IRS: (a): orthophosphate, (b): unknown compound, (c): pyrophosphate.

31 d. It is an interesting phenomenon that, at pH 10, P_4 and P_5 are produced. The mechanism of their formation has not yet been definitely proved, but will be discussed later.

The above experimental results indicate that the yield of the unknown compound was higher at pH 12 than that at pH 10. In order to confirm this fact, the reaction of P_{3m} and L-valine was tried at a lower pH. A mixture of P_{3m} and L-valine solutions at a molar ratio of 1:1 was allowed to react at pH 12 for a week. After that, this sample solution was divided into two portions, one being set at pH 12 and the other being allowed to stand without any adjustment of the pH. The yields of the products in these two series of experiments are shown in Fig. 4, in which the amount of precipitated P_2 has been omitted. The variations in the pH are indicated below the abscissa. The amounts of P_2, P_3, and P_{3m}, when the pH was not controlled, are also omitted, because they are almost the same as those when the pH was controlled at 12. When the pH was not controlled, the pH of the solution gradually decreased with the progress of the reaction, and the yield of the unknown compound decreased and became nearly zero at pH 9.8. On the contrary, the yield of P_1 was found to increase sharply. The curve of the formation of the unknown compound has a tendency reverse-symmetric to that of P_1. This suggests that the unknown compound, initially formed in a large quantity at pH 12, decomposed into P_1 as the pH of the solution was lowered. This means, in turn, that the unknown compound may be a P_1-derivative of valine.

The disappearance rates of P_{3m} in the reactions with L-valine at pH 12 and 10 at room temperature were found to be first-order. The rate constants were 0.317 and 0.138 d^{-1} at pH 12 and 10 respectively.

The reactions of P_{3m} with L-valine at molar ratios of 0.1:1, 0.5:1, 5:1, and 10:1 were also studied at pH 12, but it was found that the products were completely the same kinds as those at the molar ratio of 1:1. By the reactions of P_{3m} with L-valine in neutral and acidic solutions, no unknown compound was obtained at all.

Molecular Structure of the Unknown Compound. As has been described in the preceding section, it was concluded that the unknown compound may be a P_1-derivative of valine. In order to get more information on the molecular structure of this compound various methods were employed. A solution of the unknown compound, separated by ion-exchange chromatography, was paper-chromatographed with an acidic solvent. The unknown compound gave a spot with a strong coloration at the position corresponding to P_1 and another spot with a weak coloration at a position between those of P_1 and P_2. The latter may be due to the unknown compound. The former may be due to the P_1 produced by the hydrolysis of the unknown compound in the acidic solvent. This fact also supports the idea that the unknown compound is a P_1-derivative of valine.

The amounts of phosphorus and valine in the unknown compound separated by ion-exchange chromatography were determined by chemical analysis. It was found that the ratio of phosphorus to valine in the unknown compound is unity, suggesting that this unknown compound is a P_1-derivative of valine containing P_1 and valine in the molar ratio of 1:1.

Each solution fractionated by ion-exchange chromatography was dehydrated under reduced pressure to obtain the respective components in a solid state and tested by ninhydrin reaction and IR spectroscopy. The results are shown in Fig. 5. In this elution, an eluent containing no EDTA was employed, because EDTA disturbs the ninhydrin reaction. The components in the fractions of 50—100 and 300—400 cm^3 are ninhydrin-reaction positive. The component in the former fractions is attributable to the unreacted valine. The component in the latter fractions is attributable to the unknown compound. These results also support the idea that the unknown compound is a P_1-derivative of valine. Furthermore, from the IR spectra, the components in the fractions of 180—250 and 630—700 cm^3 were proved to be P_1 and P_2 respectively. Figure 6 shows the IR spectrum of the component in the fraction with an elution peak at

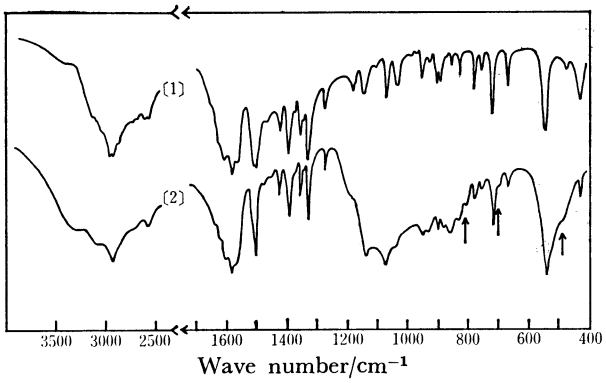
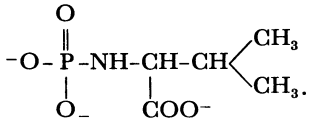


Fig. 6. IR spectra of L-valine and the unknown compound.
Arrow (↑): absorption due to the P-N bond, (1): L-valine, (2): unknown compound.

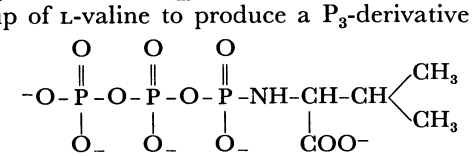
about 350 cm^3 (the unknown compound). The unknown compound indicates absorptions at about 2950, 1610—1560, 1500, 1420, 1390, 1350, 1330, 1270, 780, 720, and 540 cm^{-1} attributed to valine. In addition, at about 1150—1050, 1000—950, and 860 cm^{-1} absorptions attributable to P_1 [18,19] are found. Further, at about 810—800 (ν_{P-N}), 700 (ν_{P-N}), and 490 cm^{-1} (δ_{P-N}) weak absorptions, which may be due to the P–N bond,[20-23] are found. Also, strong absorptions caused by the carboxylato group of valine are observed at about 1610—1550 and 1400 cm^{-1}. Therefore, this unknown compound probably has the P–N bond and the COO^- group in its molecule.

From all the results presented above, it was concluded that the unknown compound is a P_1-derivative of valine, an anion of *N*-(1-carboxy-2-methylpropyl)phosphoramidic acid,



Reaction of Short-chain Phosphates with L-*Valine.*
A solution of trisodium orthophosphate, tetrasodium pyrophosphate, or pentasodium triphosphate was mixed with the L-valine solution in a molar ratio of 1:1, and then the mixed solution was adjusted with a sodium hydroxide solution to pH 12 and allowed to react at room temperature. No reaction products were observed at all in the reactions of L-valine with P_1 and P_2. Only in the reaction of P_3 was a slight amount of P_2, produced by the hydrolysis of P_3, observed. Therefore, it may be concluded that, from short-chain phosphates, such as P_1, P_2, and P_3, no phosphate derivatives of valine can be formed.

Mechanism of the Reaction of cyclo-*Triphosphate with* L-*Valine.* The mechanism of the reaction of P_{3m} with L-valine at pH 10—12 is summarized in Fig. 7. At pH 10—12, P_{3m} is easily attacked by an amino group of L-valine to produce a P_3-derivative of valine;



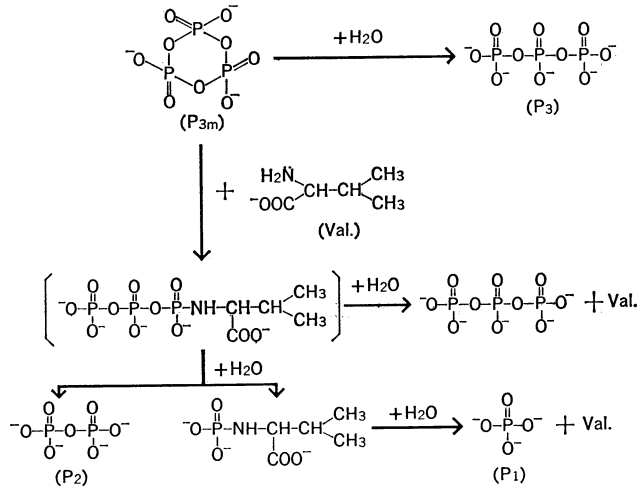


Fig. 7. Mechanism of the reaction of P_{3m} with L-valine.

(P_3-(N)Val). However, since P_3-(N)Val can not be detected at all by means of either ion-exchange chromatography or paper chromatography, this compound may be extremely unstable and immediately hydrolyzed to P_2 and a P_1-derivative of valine, P_1-(N)Val, as well as to P_3 and valine. The latter route of hydrolysis is suggested because the yield of P_3 is much higher than that to be expected from only the hydrolysis of P_{3m}. As has been described before, small amounts of P_4 and P_5 were produced in the reaction of P_{3m} with L-valine at pH 10. This phenomenon may have some connection with Quimby's finding that the amidotriphosphate produced by the reaction of P_{3m} with ammonia in an alkaline solution returns to being P_{3m}, accompanying the abstraction of ammonia, when the solution is acidified. In the present reaction, the following mechanism may be one of the possibilities; P_3-(N)Val reacts with P_1 or P_2 to form P_4 or P_5, accompanying the abstraction of valine. A similar phenomenon has been reported by Feldmann and Thilo,[3] who found that, when a solution of amidotriphosphate is acidified, small amounts of P_4, P_5, and P_6 are produced. On the other hand, in the reaction of P_{3m} with L-valine, divaline and trivaline can not be detected by thin-layer chromatography.

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