# CONDENSATION OF OLIGOGLYCINES WITH TRIMETA- AND TETRAMETAPHOSPHATE IN AQUEOUS SOLUTIONS

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**Abstract.** The dehydration condensation of glycine with trimetaphosphate in aqueous solution has been reinvestigated. Although it has been reported that the condensation of glycine under the alkaline conditions was brought about through the formation of cyclic acylphosphoramidate and hence the condensation of polyglycines could not occur, we found that the condensation of oligoglycines with trimeta- and tetrametaphosphate in aqueous solution are possible through the formation of their acylphosphates under the neutral or weak acidic conditions.

Aqueous solutions of 1.0 M glycylglycine and 1.0 M trimetaphosphate in the various pH from 4.0 to 9.0 were incubated at 38 °C. The solutions were analyzed by HPLC with ninhydrin reaction system. Tetraglycine and hexaglycine were detected and their maximum yields were given in the reaction carried out around pH 7. They are approximately 15% and 4% after 30 days, respectively. Analogous experiments were performed with tetrametaphosphate. The results showed a similar pH dependence for the condensation, but the yields were about one-tenth of those of corresponding experiments with trimetaphosphate.

Relative rates of dimerization of glycine, diglycine and triglycine in the equimolar concentration were also investigated at pH 6.0 at 38 °C. The rates for digylcine and triglycine were approximately twice and four times as large as that for glycine.

Relevance of the experiments to chemical evolution is discussed.

## 1. Introduction

A number of experiments have been performed concerning how peptides were formed during the course of chemical evolution (Miller and Orgel, 1974; Hulshof and Ponnamperuma, 1976). Heating of certain mixture of amino acids, for example, has been reported to yield peptide-like polymers under anhydrous (Harada and Fox, 1958; Fox and Harada, 1960) or aqueous (Yanagawa and Kojima, 1985) conditions. Several authors have reported the condensation of amino acids with various prebiotic condensing agents, such as cyanamide and polyphosphates in aqueous or dried state (Steinman *et al.*, 1964, 1966; Nooner *et al.*, 1977; Hawker and Oro, 1981; Rabinowitz *et al.*, 1969; Sawai and Orgel, 1975; Rabinowitz and Hampai, 1978). Formations of peptides have also been brought about with amino acid derivatives or active amino acids in aqueous solutions (Oro and Guidry, 1960, 1961; Paecht-Horowitz and Katchalsky, 1967; Lewinsohn *et al.*, 1967; Weber and Orgel, 1978). Furthermore, the 'fractuation' method was recently reported (Yanagawa *et al.*, 1984).

Although peptide-like polymers having high molecular weight are synthesized by heating amino acids or their derivatives, suitable environments for the reaction seem to have been limited to some special areas on the primitive earth. Some experiments

using amino acid precursors or active amino acids are of interest in relation to biosynthesis of protein, but there occurs another problem how they were formed and accumulated. It seems more likely to us that peptides were formed with the aid of certain prebiotic condensing agents in the primitive ocean as the most universal place under mild conditions.

A possibility of the formation of tetrametaphosphate (TetMP) via a $P_4O_{10}$ molecule on the primitive earth through volcanic activity was proposed by Griffith (1977) and Griffith *et al.* (1977), and recently Yamagata *et al.* (1982) demonstrated that TetMP was an effective reagent for the phosphorylation of adenosine. Trimetaphosphate (TriMP), having a cyclic structure similar to TetMP, was also likely to have been supplied on the primitive earth (Osterberg and Orgel, 1972). These metaphosphates must have acted as effective prebiotic phosphorylating and condensing agents. Furthermore, Yamagata *et al.* (1982) have discussed a possible phosphate cycle on the primitive earth. The recycling might have made it possible to supply TetMP and TriMP constantly to the primitive ocean.

Condensation of glycine (gly) with TriMP was first reported by Rabinowitz *et al.* (1969). They described that the formation of glycylglycine [(gly)$_2$] in an aqueous solution of TriMP proceeded effectively under alkaline conditions, whereas the yield of (gly)$_2$ was extremely low under neutral and acidic conditions. Afterwards, Chung *et al.* (1971) proposed the reaction mechanism shown in Figure 1 for the formation of (gly)$_2$ in an alkaline solution. Amino group of gly first attacks TriMP to form an open chain molecule gly$-$N$-$triphosphate. Subsequent intramolecular condensation
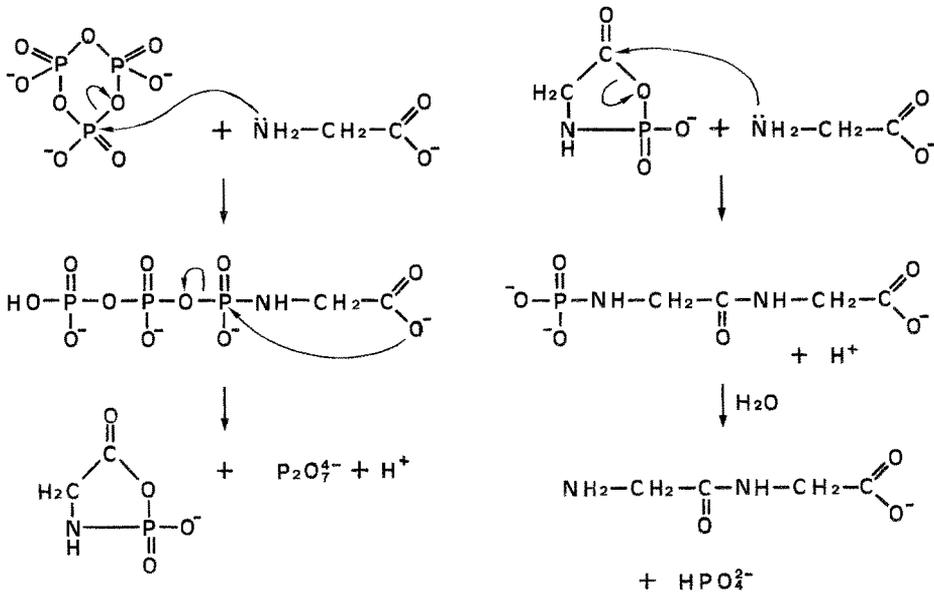


Fig. 1.    Reaction mechanism for the dimerization of gly with TriMP under the alkaline conditions.

gives an active intermediate, cyclic acylphosphoramidate (CAPA). CAPA is then attacked by another gly to form $(gly)_2-N-$phosphate which is readily hydrolyzed to $(gly)_2$.

When $(gly)_2$ was used instead of gly, $(gly)_2$-N-triphosphate was similarly formed under alkaline conditions, however, no active cyclic intermediate was given. Therefore, they concluded that the condensation of $(gly)_2$ could not take place. It means that a peptide can merely grow by means of the attack of the amino group of the peptide to CAPA, and the degree of polymerization increases only one by one.

If the condensation could occur between two peptides, it would enable more effective synthesis of large peptides. We have found that the condensation of oligoglycines with TriMP and TetMP took place under the conditions around neutral through a different mechanism from the case of condensation of gly under alkaline conditions.

## 2. Material and Methods

Gly and TriMP trisodium salt were purchased from Wako Pure Chemical Co. (Tokyo, Japan) and Sigma Chemical Co., respectively, and were used without further purifications. $(Gly)_2$ and $(Gly)_3$ were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Analysis with HPLC showed that they did not contain any impurity besides quite small amount of gly.

An aqueous solution of TetMP was prepared by adding phosphorus pentoxide little by little into cold water cooled on an ice bath with stirring. The solution was analyzed with $^{31}$P-NMR (JEOL ltd., JNM GX-400), and it was confirmed that almost all of the material was converted to TetMP.

Each 5.0 ml of aqueous solutions of gly, $(gly)_2$, or $(gly)_3$ and TriMP or TetMP sodium salt at several pH values between 4.0 and 10.0 was allowed to stand at 38 °C to observe the pH dependence for the condensation of gly. The pH's were initially adjusted with concentrated HCl or NaOH (solid or 10 N aqueous solution). During the course of the reactions, the pH's decreased owing to the decomposition of TriMP or TetMP, and they were occasionally adjusted back to the initial values with NaOH. A small amount of toluene was also added as an antiseptic.

The analyses of oligoglycines were carried out with HPLC system which is similar to amino acid analyzing system by ninhydrin reaction [Hitachi Model 638−50; Column: Hitachi gel No. 2619 (cation exchange porous polymer, 5.25 $\mu$m) 4 × 150 mm: column temperature 70 °C; flow rate 0.4 ml/min; elution: computer controlled gradient elution of aqueous solution of sodium citrate, sodium chloride, ethanol and thiodiglycol]. The elution gradient is shown in Figure 5 with an example of HPLC analyses. It was preliminarily confirmed with authentic samples that oligoglycines could be separated up to at least $(gly)_6$ by the present analytical system. Identifications of the products were achieved by comparing their retention time on HPLC with those of authentic samples, and in some cases, the products were additionally confirmed by analyzing their DNP-derivatives with another HPLC system.
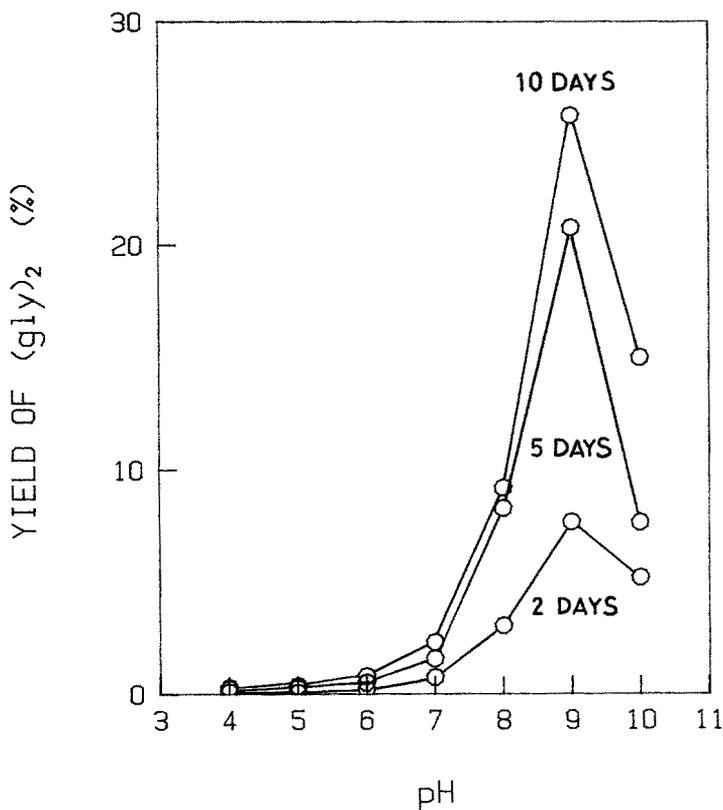
Fig. 2. pH dependence of the yield of (gly)$_2$ in the solution of 0.5 M gly and 0.5 M TriMP trisodium salt at 38 °C. The yield means the percentage of the starting material converted to the product.

## 3. Condensation of Gly, (Gly)$_2$ and (Gly)$_3$ with TriMp

### 3.1. CONDENSATION OF Gly

As described above, condensation of gly with TriMP has been already reported by Rabinowitz *et al*. The following experiments were performed in order to investigate the dependence of the yield of (gly)$_2$ upon pH in detail. It was found that the optimum pH of the reaction is around 9.0 as shown in Figure 2. The formation of (gly)$_3$ was also observed in the solutions at pH 8, 9 and 10 after 15 days. The yield of (gly)$_3$ did not exceed 0.1% in any case. No (gly)$_4$ was detected at any pH. These results were almost consistent with those reported by Rabinowitz *et al*. The decrease of the yield of (gly)$_2$ at pH 10 seems to be due to the lack of the gly having free amino group, because the amino group undergoes phosphorylation more effectively at higher pH.
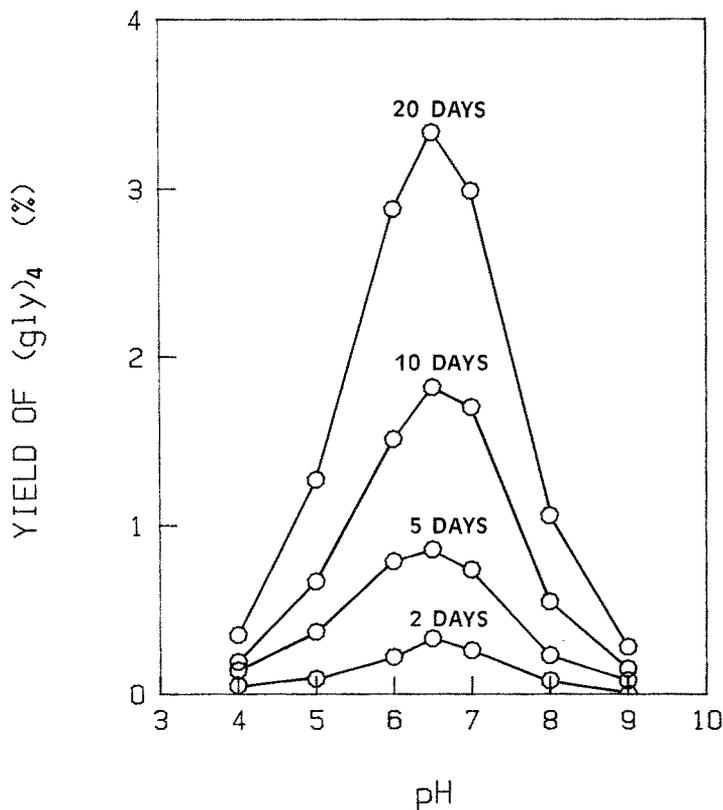
Fig. 3.   pH dependence of the yield of (gly)$_4$ in the solution of 0.5 M (gly)$_2$ and 0.5 M TriMP trisodium salt at 38 °C

## 3.2. CONDENSATION OF (Gly)$_2$

The following aqueous solutions were allowed to stand at 38 °C at severl pH's (4−9).

Exp I: 0.5 M (gly)$_2$, 0.5 M TriMP tridosium salt,

Exp II: 1.0 M (gly)$_2$, 1.0 M TriMP trisodium salt.

There were small amounts of insoluble materials in the case of Exp II.

Both (gly)$_4$ and (gly)$_6$ were found to be formed in both Exp I and Exp II. Figure 3 shows pH dependence of the yield of (gly)$_4$ in Exp I. The yield of (gly)$_6$ at pH 6.5 after 20 days was approximately 0.5%. The pH dependence of yields of (gly)$_4$ and (gly)$_6$ in Exp II are shown in Figures 4a and 4b, respectively. The optimum pH for the condensation of (gly)$_2$ was in the range of 6.0 to 7.0 being different from 9.0 in the case of gly.
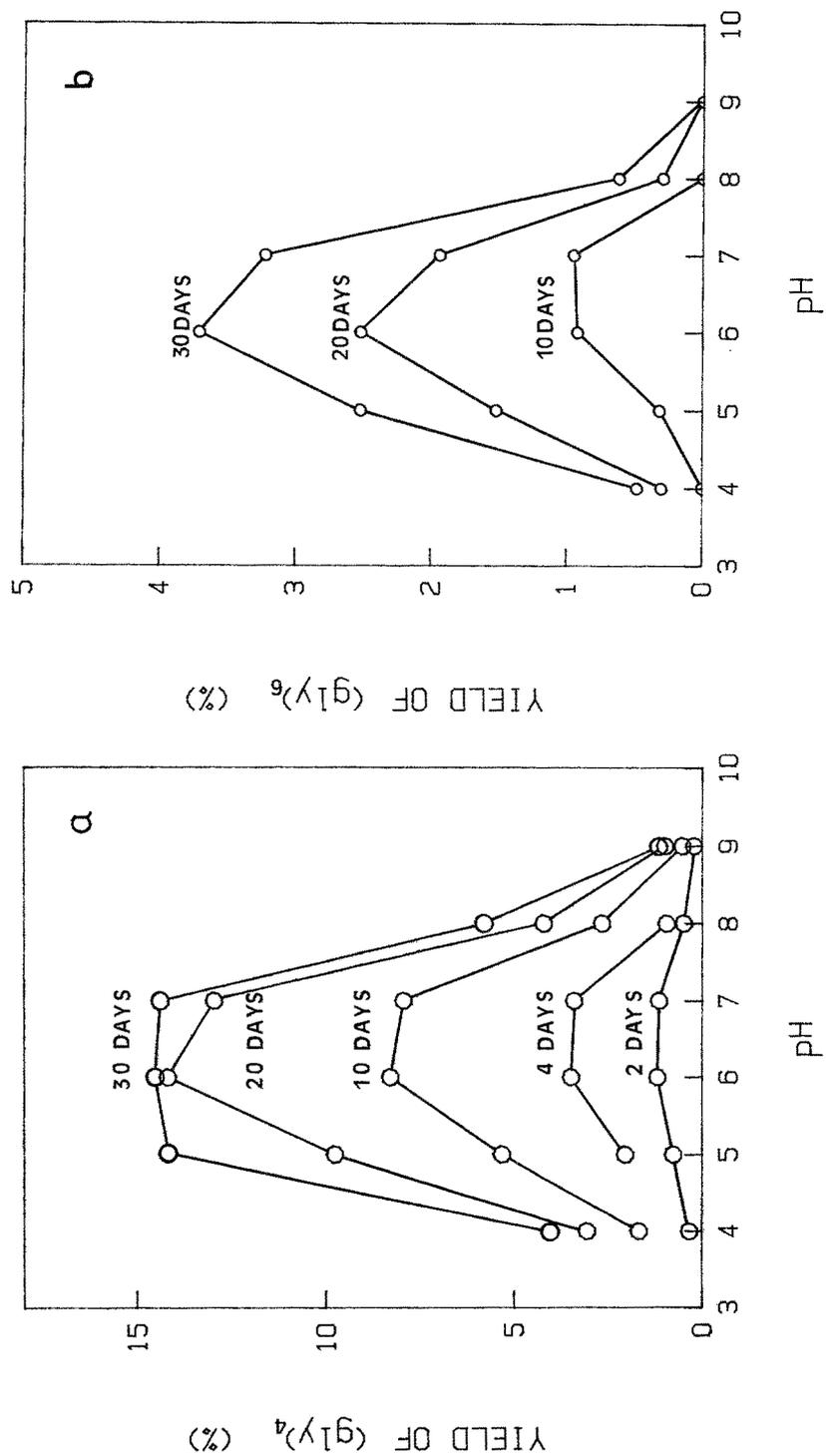
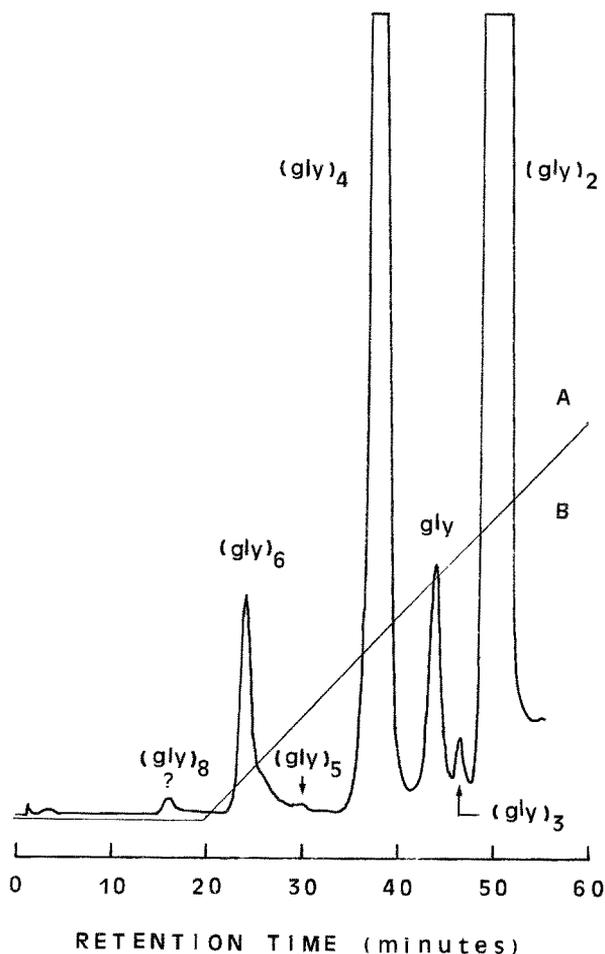Fig. 4a–b.   pH dependence of the yield of (gly)$_4$ and (gly)$_6$ in the solution of 1.0 M (gly)$_2$ and 1.0 M TriMP trisodium salt at 38 °C.

Fig. 5. HPLC chromatogram illustrating the formations of $(gly)_4$ and $(gly)_6$ in the solution of Exp II (pH 6, 35 days: sample; 2 $\mu$l). One liter of eluent A contains sodium citrate dihydrate (2.80 g), citric acid monohydrate (20.0 g), sodium chloride (7.07 g), ethanol (100 ml) and thiodiglycol (5.0 ml), and that of eluent B contains sodium citrate dihydrate (27.67 g), citric acid monohydrate (6.10 g) and sodium chloride (54.35 g).

In Figure 5 a typical chromatogram of HPLC analyses of the reaction mixture of Exp II (pH 6, 35 days) is illustrated. A small peak having the retention time of 16 min appeared after about 20 days and then it continued to grow. It seemed to be due to $(gly)_8$.

### 3.3. CONDENSATION OF $(Gly)_3$

Each 5.0 ml of aqueous solutions of 0.3 M $(gly)_3$ and 0.3 M TriMP trisodium salt at pH between 4.0 and 9.0 was maintained at 38 °C. The reaction mixtures were
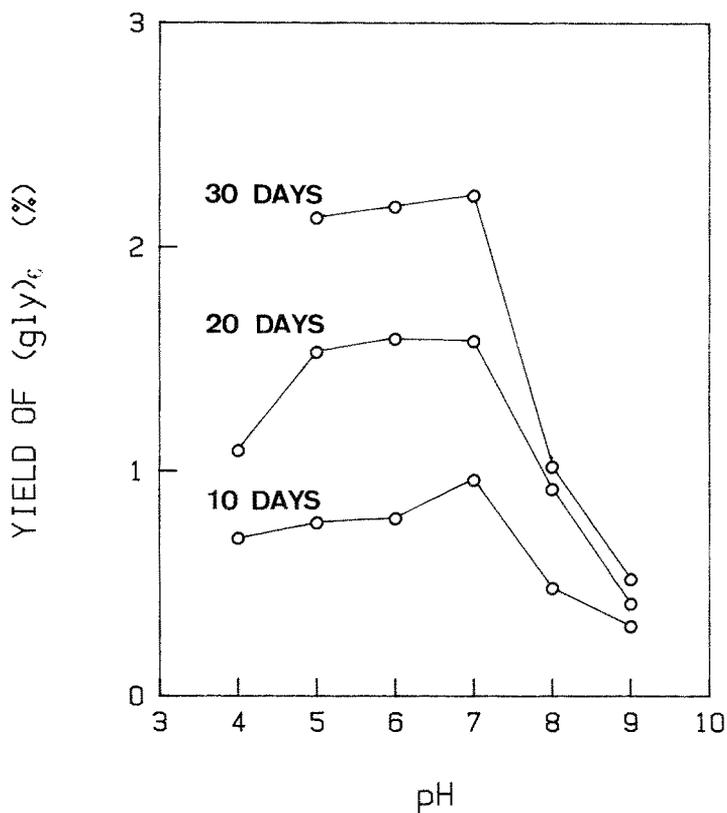
Fig. 6.    pH dependence of the yield of (gly)$_6$ in the solution of 0.3 M (gly)$_3$ and 0.3 M TriMP trisodium
salt at 38 °C.

analyzed with HPLC and the formation of (gly)$_6$ was confirmed. The optimum yield
of (gly)$_6$ was obtained around pH 7.0 as shown in Figure 6, similarly to the case of
the condensation of (gly)$_2$.

### 3.4. COMPARISON AMONG DIMERIZATION RATES OF Gly, (Gly)$_2$ AND (Gly)$_3$.

In order to compare the dimerization rate of gly, (gly)$_2$ and (gly)$_3$ under the same
conditions, each aqueous solutions of equal molar concentration (0.3 M) of them
containing TriMP trisodium salt (0.3 M) were allowed to stand at 38 °C adjusting to
pH 6.0. The yields of their dimers, namely, (gly)$_2$, (gly)$_4$ and (gly)$_6$ were 0.19, 0.37
and 0.83% after 10 days and 0.36, 0.76 and 1.47% after 20 days, respectively. It
indicates that under these conditions the dimerization rate of (gly)$_n$ increases with
the increase of the number of $n$. This tendency seems to depend on the strength of
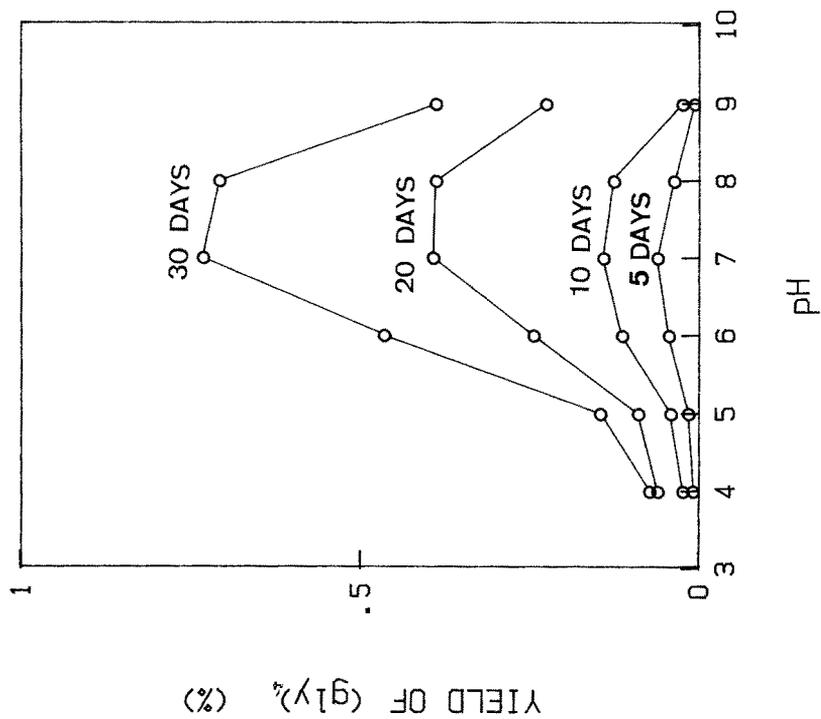the interaction between oligoglycines.

Fig. 7b. pH dependence of the yield of $(gly)_4$ in the solution of 0.5 M $(gly)_2$ and 0.5 M TetMP at 38°C.
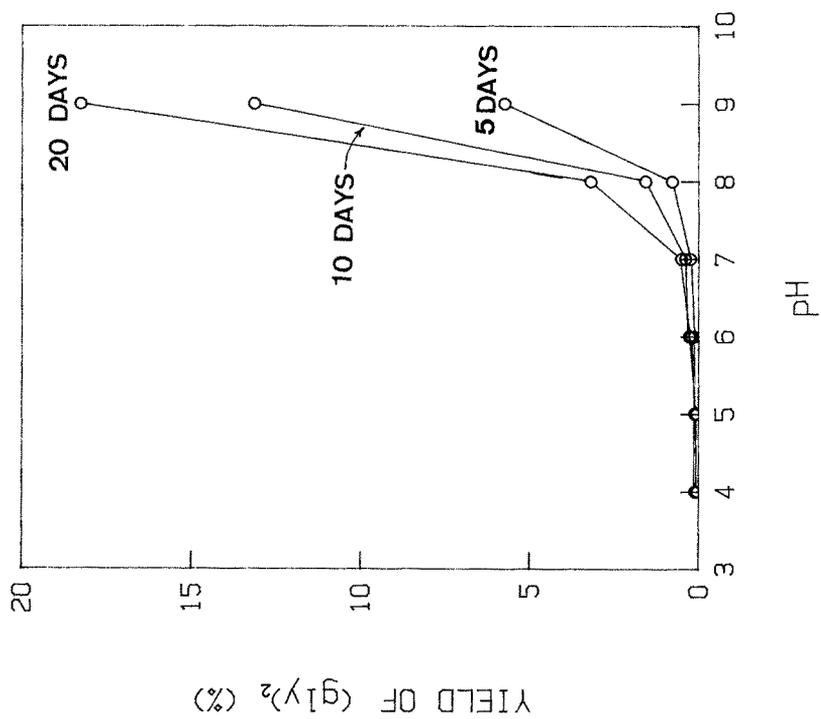
Fig. 7a. pH dependence of the yield of $(gly)_2$ in the solution of 0.5 M gly and 0.5 M TetMP at 38°C.
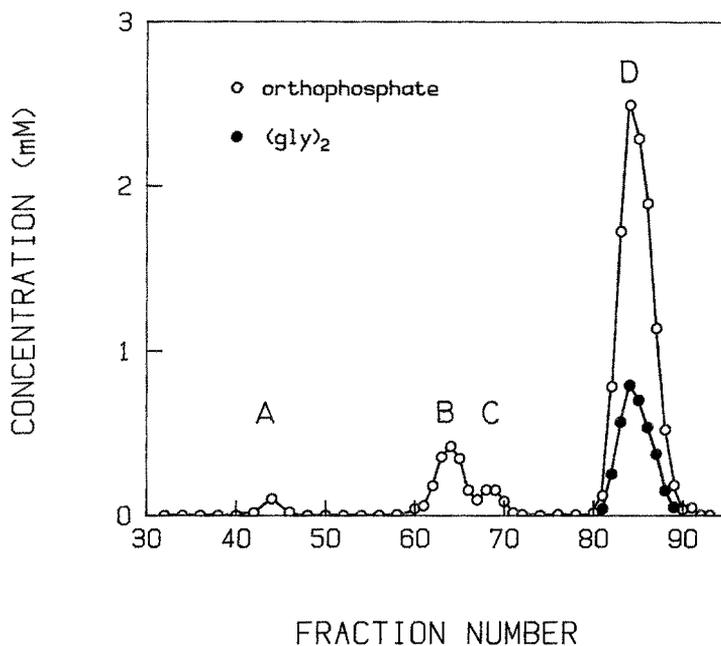
Fig. 8.   Chromatogram showing the formation of (gly)$_2$-N-triphosphate in aqueous solution of 0.5 M (gly)$_2$ and 0.5 M TriMP at pH 9.0 (38 °C, 2 days). Peaks A, B and C correspond to ortho-, pyro- and triphosphate, respectively.

## 4. Condensation of Gly and (Gly)$_2$ with TetMP

The effect of TetMP as a condensing agent was also examined. Aqueous solutions of 0.5 M gly and 0.5 M TetMP, and those of 0.5 M (gly)$_2$ and 0.5 M TetMP were allowed to stand at 38 °C at several pH between 4.0 and 9.0. By means of HPLC as described in Section 3.1, the formation of (gly)$_2$ from gly and (gly)$_4$ from (gly)$_2$ were observed. Their yields are shown in Figures 7a and 7b, respectively. The dependence of the yields on pH was almost similar to the case with TriMP, however, the yields of (gly)$_4$ were only about one-tenth comparing with those using TriMP.

## 5. Analysis of (Gly)$_2$-N-Triphosphate

As described in Section 1, Chung et al. reported that (gly)$_2$ was phosphorylated by the reaction with TriMP in an alkaline aqueous solution giving inactive (gly)$_2$-N-triphosphate. Although they detected it with paper chromatography, we tried to separate it with an anion exchange column chromatography to confirm the structure and to examine the pH dependence of the yield.

    An aqueous solution of 0.5 M (gly)$_2$ and 0.5 M TriMP trisodium salt was allowed to stand for 2 days at 38 °C at pH 9.0. 100 $\mu$l aliquot of the reaction mixture was
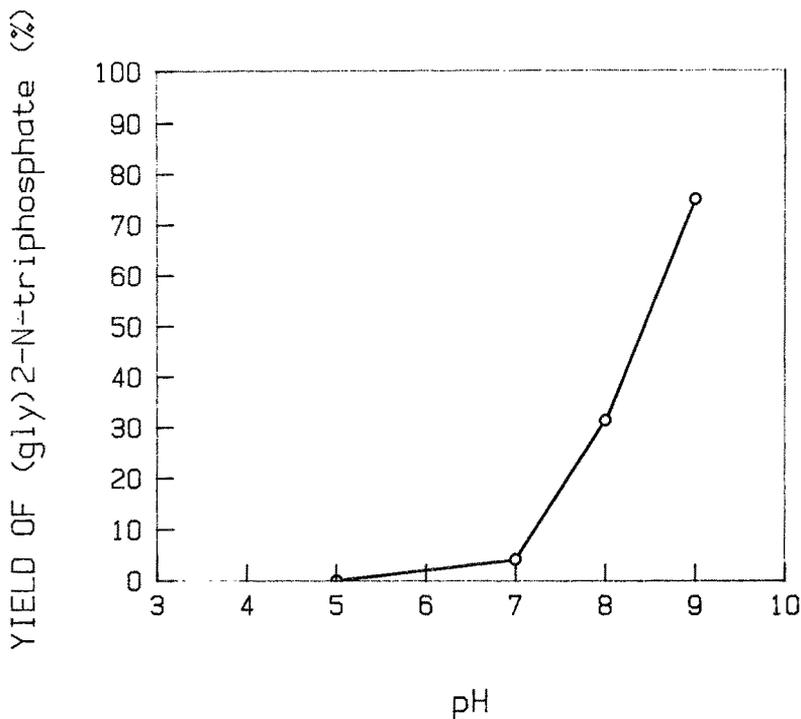
Fig. 9. pH dependence of the yield of (gly)$_2$-N-triphosphate in aqueous solution of 0.5 M (gly)$_2$ and 0.5 M TriMP.

subjected to DEAE-Sephadex A-25 column (1.5 × 37 cm), and was eluted with a linear gradient of aqueous solution of ammonium hydrogen carbonate [0.02 M (500 ml)−0.4 M (500 ml)]. Then 100 $\mu$l aliquots of each fraction were hydrolyzed in $^2/_3$ N H$_2$SO$_4$ for 1 hr on a boiling water bath, and then the resulting orthophosphate was detected according to the procedure of Chen et al. (1956). The chromatographic pattern is shown in Figure 8. Comparing with those of authentic samples, the peaks, A, B, and C are considered to be ortho-, pyro-, and triphosphate, respectively.

The peak D was identified to be (gly)$_2$-N-triphosphate from the following analyses. 100 $\mu$l aliquots of the fractions belong to the peak D (No. 81−No. 89) were hydrolyzed with 100 $\mu$l of 1 N HCl in sealed tubes at 100 °C for 15 min, and were analyzed for gly and oligoglycine with HPLC-ninhydrin reaction system. (gly)$_2$ was only detected except a small amount of gly probably formed by the hydrolysis of (gly)$_2$. The molar ratio of orthophosphate to (gly)$_2$ was 3 : 1 within an experimental error. This analysis cannot be disturbed with free (gly)$_2$ and TriMP, because TriMP is eluted in the fractions over No. 100 and (gly)$_2$ in the fractions from No. 30 to No. 40.

It was further confirmed by $^{31}$P-NMR analysis of one of the fractions of the peak D. Three peaks corresponding to the three different phosphorus in the molecule were

Fig. 10. Reaction mechanism for the condensation of oligoglycines with trimetaphosphate.

observed in the spectrum. On the other hand $(gly)_2$-N-triphosphate is known to be readily decomposed to give $(gly)_2$ and TriMP under acidic conditions (Feldman and Thilo, 1964; Chung *et al.*, 1971). Therefore, this sample was allowed to stand for 1 min at 60 °C at pH 1.3, and was again applied to NMR. Only one peak is observed and its chemical shift was in agreement with that of TriMP. $(gly)_2$ was also detected in this sample by HPLC. These facts prove that the peak D is attributed to $(gly)_2$-N-triphosphate.

Furthermore, pH dependence of the yield of $(gly)_2$-N-triphosphate was investigated. Aqueous solutions of 0.5 M $(gly)_2$ and 0.5 M TriMP trisodium salt at pH 5.0, 7.0 and 8.0 were maintained at 38 °C for 2 days similarly to the above case at pH 9.0, and analyzed with DEAE-Sephadex A-25 column in a similar way to the case described above. The results are shown in Figure 9. The yield of $(gly)_2$-N-triphosphate was extremely high under alkaline conditions and decreased suddenly at lower pH. Namely, under alkaline conditions the most part of $(gly)_2$ molecule was converted to this inactive compound, whereas almost all of $(gly)_2$ was left free under neutral or acidic conditions.

## 6. Discussion

We found that condensation of oligoglycines with TriMP and TetMP took place most efficiently under the neutral or weakly acidic conditions differently from the

case of condensation of gly. It was also revealed that $(gly)_2$-N-triphosphate was scarcely formed under neutral or acidic conditions.

Condensation of oligoglycines cannot be explained by a mechanism similar to that for the condensation of gly under alkaline conditions (Figure 1). The reaction seems to proceed through the formation of an active intermediate, oligoglycyl-O-triphosphate (Figure 10), though an attempt to detect this molecule was unsuccessful because of its presumed instability. This reaction mechanism was proposed first by Rabinowitz (1969, 1970) for the condensation of gly under alkaline conditions, and afterwards denied by Chung *et al.* as described above.

As the amino group of gly or oligoglycine is free under alkaline conditions, TriMP may be attacked more easily by the amino group rather than the carboxylate anion to give their N-triphosphates. This was supported by the pH dependence of the yield of $(gly)_2$-N-triphosphate as shown in Figure 9. From a considerably large difference between pK values 9.60 and 8.13 for the amino group of gly and $(gly)_2$ respectively (Cohn and Edsall, 1943), we can conclude that the formation of $(gly)_2$-N-triphosphate from $(gly)_2$ and TriMP is much easier than that of gly-N-triphosphate from gly and TriMP in weak alkaline solution.

Those facts explain why the polymerization can hardly proceed to form $(gly)_3$ via the formation of $(gly)_2$ from gly under alkaline conditions (Section 3.1), that is, the formed $(gly)_2$ is quickly converted to $(gly)_2$-N-triphosphate which cannot attack cyclic acylphosphoramidate to produce $(gly)_3$.

On the other hand, when the amino group is converted to an ammonium salt, it cannot attack the active intermediate, acyltriphosphate. Therefore the condensation is difficult to proceed under strong acidic conditions, too. Hence the optimum pH for the condensation of oligogycines is around neutral.

Our findings concerning the condensation of peptides will have an essential meaning for the chemical evolution. This type of the condensation make the elongation of peptides possible in an exponential way, whereas the growth of peptides via cyclic acylphosphoramidate under the alkaline condition is limited to a linear manner. Besides, the optimum pH around neutral for the condensation of peptides presents the most favorable conditions for the survival of the produced peptides. It is not so difficult to imagine the weak acidic or neutral primitive ocean, if we consider a constant supply of acidic volcanic gas on the primitive earth followed by its moderate neutralization with the primitive rocks and/or if we suppose an equilibrium the ocean and the atmosphere containing carbon dioxide. We also found that the rate of dimerization of $(gly)_2$ and $(gly)_3$ amounts to approximately twice and four times as large as that of gly, respectively. Those facts suggest that the larger peptides have the greater dimerization rates. If there exists such a general tendency for the condensation reactions, it would further contribute to the acceleration of the growth of peptides in the chemical evolution.

Recently an evidence to support our idea regarding the phosphate cycle on the earth through the volcanic activity was found in our laboratory, namely pyrophosphate and triphosphate which are considered to be the molecules

degradated from $P_4O_{10}$ were detected in the condensed water of volcanic gas. Although the pH value of the primitive ocean is not evident, if it was in the range around neutral and if $P_4O_{10}$ was sufficiently supplied, it seems to have been possible that the condensation of peptides took place and large peptides were formed effectively in the primitive ocean.

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