DIKETOPIPERAZINE-MEDIATED PEPTIDE FORMATION IN AQUEOUS SOLUTION
II. CATALYTIC EFFECT OF PHOSPHATE

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Abstract. The previous paper (I) reported that DKP (glycine anhydride) spontaneously reacts with glycine (Gly) or oligoglycines (Gly_n) to produce longer oligoglycines (Gly_{n+2}). This paper presents that phosphate catalyzes the condensation reaction quite effectively.

Formation of Gly_4 from DKP (0.1 M) and Gly_2 (0.1 M) in phosphate solution of various concentrations was investigated at a neutral pH at 41 °C. The yields of Gly_4 increased almost linearly with the concentration of phosphate from 0.06 M to 0.24 M. The yield in 0.24 M phosphate solution was approximately one hundred times as high as that in the absence of the phosphate, whereas in the case of Gly_3 formation from DKP and Gly the effect of the phosphate was of ten times lower than in the former case. Orthophosphate was the most effective catalyst among the various kind of chemicals tried in the present investigation including polyphosphates.

1. Introduction

How prebiotic peptides were formed in the primitive ocean is one of the most interesting subjects in the study of chemical evolution. In the course of the study of prebiotic peptide formation by the dehydration condensation of amino acids with the aid of condensing agents, it has been one of the major problems that dipeptides tend to afford the cyclyzed product, diketopiperazines (DKP), which is believed to interrupt the subsequent elongation reaction. However, we have recently found that DKP (glycine anhydride) is a high energy dipeptide and reacts spontaneously with amino acids or peptides to give the corresponding elongated peptides by ring-opening (Nagayama *et al.*, 1990; this paper is referred to I). Thus, DKP has been proved to be an active intermediate to the peptide formation.

During the continuation of the study, it was found that phosphate accelerates greatly the DKP-mediated peptide formation reaction. This fact allowed us to investigate the reaction at a relatively low temperature where the hydrolysis of the reactants and the products is extremely suppressed. We report here the remarkable catalytic effect of orthophosphate (simply described as phosphate below) on the reaction and compare it with the other various chemical substances.

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2. Material and Method

Glycine (Gly), dipotassium hydrogen phosphate (K_2HPO_4), potassium pyrophosphate (PPi), boric acid, sodium acetate and sodium chloride were purchased from Wako Pure Chemical Co. (Japan), glycine anhydride (DKP), Gly_2, Gly_3, and Gly_4 from Tokyo Kasei Kogyo Co. (Japan), Gly_5, Gly_6, pentasodium tripolyphosphate (PPPi), trisodium trimetaphosphate (TMP) and 5'-AMP from Sigma Chemical Co., potassium dihydrogen phosphate (KH_2PO_4) from Kanto Chemical Co. (Japan) and 2'(3')-AMP and 5'-GMP from Yamasa Biochemicals (Japan).

Oligoglycines were analyzed by a computer controlled amino acid analyzer with a ninhydrin reaction system (Hitachi L-8500, Column: Hitachi #2622SC (cation exchange resin, 3 μm) 4.6 × 60 mm, Elution: successive elution with four different buffer solutions (MCI Buffer L-8500-PH-KIT), Temperature of ninhydrin reaction bath: 130 °C, detection at 570 nm). A column temperature of 57 °C or 70 °C was used and two different modes of the buffer change were used to attain the better separations between oligoglycines.

Oligoglycines were identified and quantified by comparing the elution time and the peak area with those of authentic sample of each of oligoglycines. Moreover, the relative sensitivity was confirmed by the following method: An arbitrary amount of an authentic Gly_n was subjected to the amino acid analyzer and the fraction corresponding to Gly_n was separated without ninhydrin reaction. A portion of the fraction was again applied to the analyzer and the area of the peak of Gly_n was determined after ninhydrin reaction. Another portion of the same volume of the fraction was hydrolized (1 N HCl, 100 °C, 36 hr; Gly is stable under such conditions). The hydrolysate was analyzed on the analyzer to obtain the area of the Gly peak which gives approximately n times larger area than that of the non-hydrolysis sample. The relative sensitivity of Gly_n to Gly is given by a ratio; the area of the Gly_4 (non-hydrolysis)/the area of the Gly (hydrolized) divided by n. The results were Gly (1.0), Gly_2 (1.0), Gly_3 (1.0), Gly_4 (0.93), Gly_5 (0.90), and Gly_6 (0.85). The values were consistent with those by the method described before (Nagayama et al., 1990).

Samples containing orthophosphate were prepared by mixing both aqueous solutions of K_2HPO_4 and KH_2PO_4 in suitable proportions to obtain the required concentration and pH, and then the pH was adjusted at the set values with 10 N NaOH and 5 N HCl. The pH of all of the samples was maintained at the set values within ±0.1 during the incubation by adding 10 N NaOH or 5 N HCl at intervals.

In long term experiments, a small amount of toluene was added to the sample as an antiseptic.

3. Results and Discussions

Aqueous mixed solutions (pH 7.0) containing DKP (0.1 M), Gly (0.1 M) or Gly_2

(0.1 M) and phosphate in different concentration were incubated at 41 °C. The analysis of the solutions showed the formation of Gly_3 from Gly, Gly_4 and Gly_6 from Gly_2, respectively, depending on the phosphate concentration. The phosphate catalyst was much more effective for Gly_2 than for Gly. The results are given in Figures 1a, b (as the dependence of the yield of Gly_3 and Gly_4 on the phosphate concentration, respectively) and in Figures 2a, b (as the time course of the yield of Gly_4 and Gly_6). From the figures, it is found that the yields of Gly_3, Gly_4 and Gly_6 increase with the phosphate concentration and with the reaction time. The formation of Gly_6 was accelerated with the reaction time, probably due to the accumulation of Gly_4 which reacts with DKP to form Gly_6.

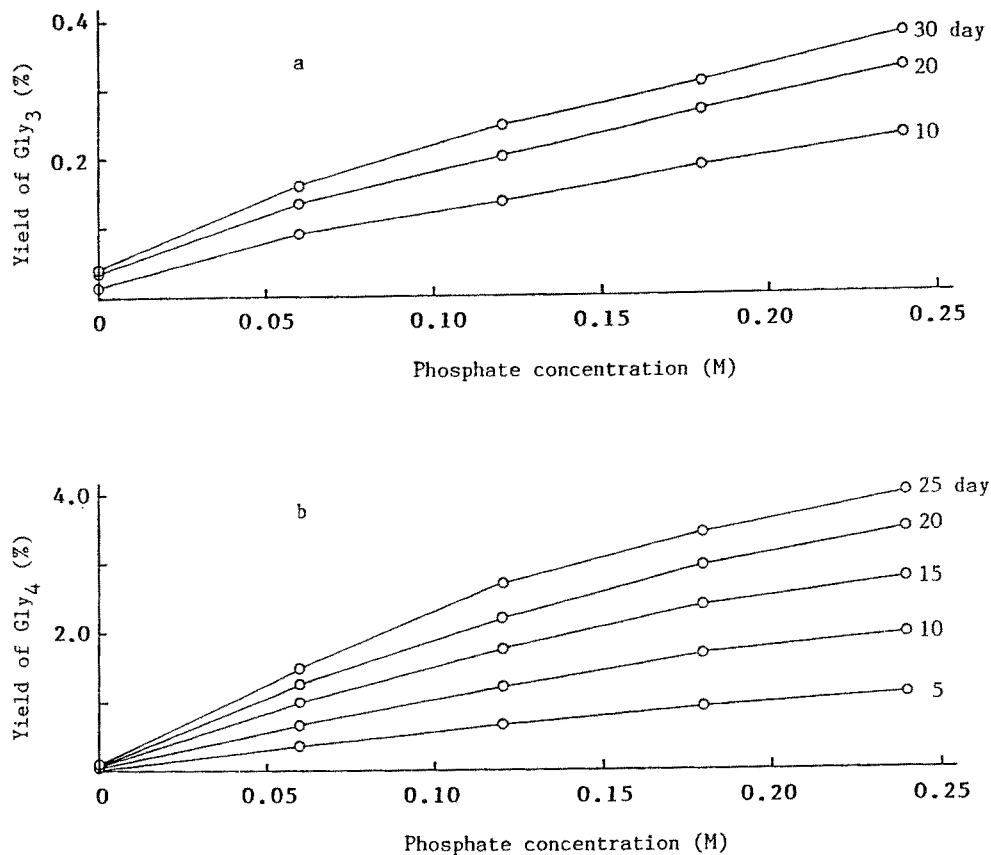In order to examine the pH dependence of the yield of Gly_4 from DKP and

Fig. 1. (a) Effect of phosphate on the reaction, DKP (0.1 M) + Gly (0.1 M) ⇌ Gly_3 at 41 °C at pH 7.0 in the presence of toluene. (b) Effect of phosphate on the reaction, DKP (0.1 M) + Gly_2 (0.1 M) ⟶ Gly_4 at 41 °C at pH 7.0 in the presence of toluene.
    Yield means percent of the concentration of the product to that of the starting Gly_2.
    The similar experiment without toluene corresponding to Figure 1a afforded almost the same result. However, the yields of the one corresponding to Figure 1b were higher by a factor of 1.3–1.35 throughout the experiment than those with toluene.

Gly_2, aqueous mixed solutions containing DKP (0.1 M), Gly_2 (0.1 M) and phosphate (0.3 M) in different pH values were incubated at a lower temperature of 19 °C to reduce the ring-opening of DKP in alkaline solutions. The results are given in Figure 3 together with the case of the absence of the phosphate.

Effect of various substances was investigated for the reaction, DKP (0.1 M) + Gly_n (0.1 M) $\longrightarrow Gly_{n+2}$ in aqueous solution at pH 7.0 at 41 °C. The concentration of each catalyst was 0.24 M for all cases. The results are shown in Table I.

The present experiments demonstrated that orthophosphate was the most effective catalyst for the reaction, DKP + $Gly_n \longrightarrow Gly_{n+2}$ among the investigated substances. Especially in the case of Gly_2 as nucleophilic reagent, the reaction rate in 0.24 M phosphate solution comes up to approximately 80 times (more than 100 times at the lower temperature as can be seen in Figure 3) as high as that in the absence of phosphate (Table I).

However, there still remains a troublesome problem for our present experiments using ortho- and pyrophosphate as a simulation of primitive earth conditions, since such molecules could not exist in the soluble forms in the primitive ocean as have been discussed for a long time (Gulick, 1957; Miller and Urey, 1959; Miller and Parris, 1964; Schwartz, 1971; Yamagata *et al.*, 1982). Some probable conditions to make apatite soluble have been discussed by Schwartz (1971). If such conditions were actually allowed in the primitive ocean, the DKP-mediated peptide formation might have been effectively catalyzed by phosphate in primitive ocean.

Although we can not discuss the mechanism of the catalytic reaction with

TABLE I

Reaction rate [(yield/day)×100] at pH 7.0 at 41 °C

Catalyst (0.24M)	DKP + Gly $\longrightarrow Gly_3$ (0.1M)(0.1M)	DKP + $Gly_2 \longrightarrow Gly_4$ (0.1M)(0.1M)	DKP + $Gly_3 \longrightarrow Gly_5$ (0.1M)(0.1M)
Control	0.24	0.36	0.37
Phosphate	2.3	30.3	23.1
PPi	1.6	13.9	13.6
PPPi	0.96	7.0	6.8
TMP	–	3.3	–
5′-AMP	–	5.4	–
2′(3′)-AMP	–	3.6	–
5′-GMP	–	5.0	–
$Ca_3(PO_4)_2$*	–	0.38	–
Borate	–	0.43	–
Acetate	–	1.7	–
Na_2SO_4	–	0.35	–
NaCl	–	0.42	–
$NaHCO_3$	–	4.6	–

Toluene was not added.
pH was adjusted by 10N NaOH and 5N HCl.
* Suspension.
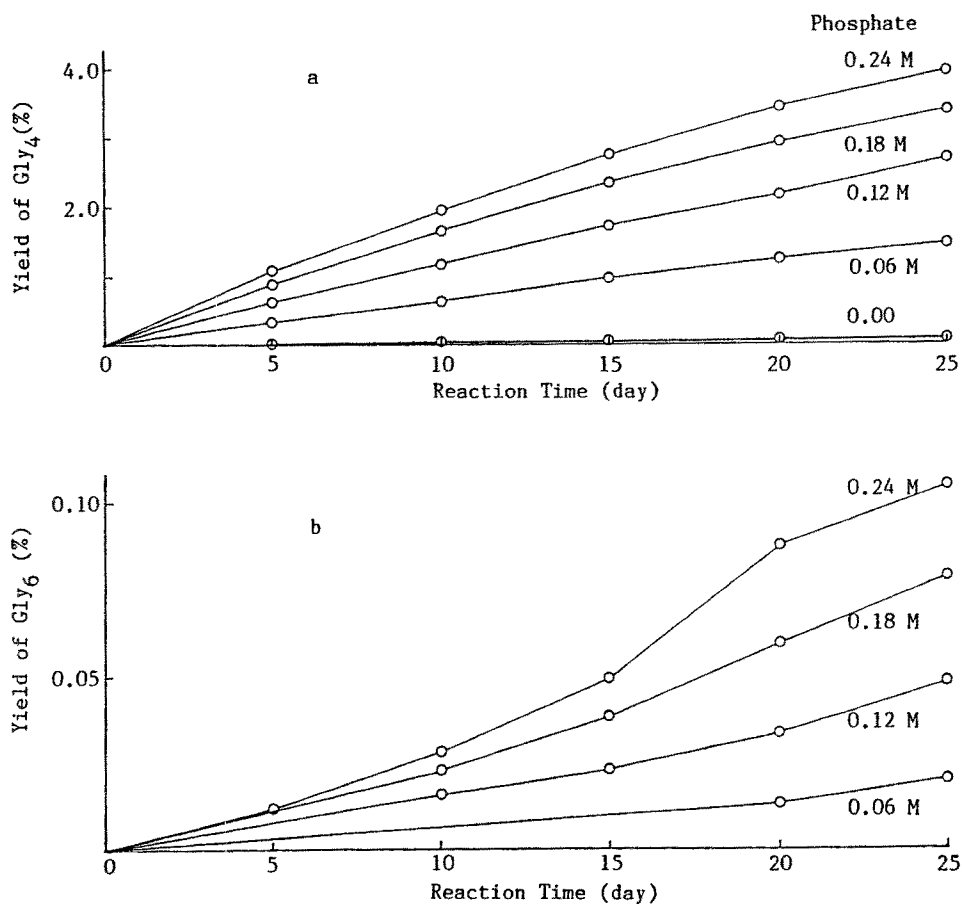
Fig. 2. Time course of the yield of Gly_4 and Gly_6 formed in the reaction, DKP (0.1 M) + Gly_2 (0.1 M) $\longrightarrow$ Gly_4 + Gly_6 at 41 °C at pH 7.0. (a) Yield of Gly_4 (replotted from Figure 1b). (b) Yield of Gly_6.

phosphate at the present stage, the following experimental facts allow us to speculate somewhat on the mechanism. The first one is that orthophosphate accelerates the ring-opening reaction of DKP only two or three times at most, and the second is that the catalytic effect of phosphate is of one order lower in the reaction, DKP + Gly $\longrightarrow$ Gly_3 than in the reaction, DKP + Gly_2 $\longrightarrow$ Gly_4. These facts would mean that the effect of phosphate is not merely in the acceleration of the ring-opening reaction, but it serves in to bind both reactants so as to increase the probability of the collision between them. Apparently phosphate binds to Gly_2 more strongly than to Gly. It is also conceivable that phosphate enhances the basicity of the amino group of Gly_2 more effectively than that of Gly, thus increasing its nucleophilic potential.
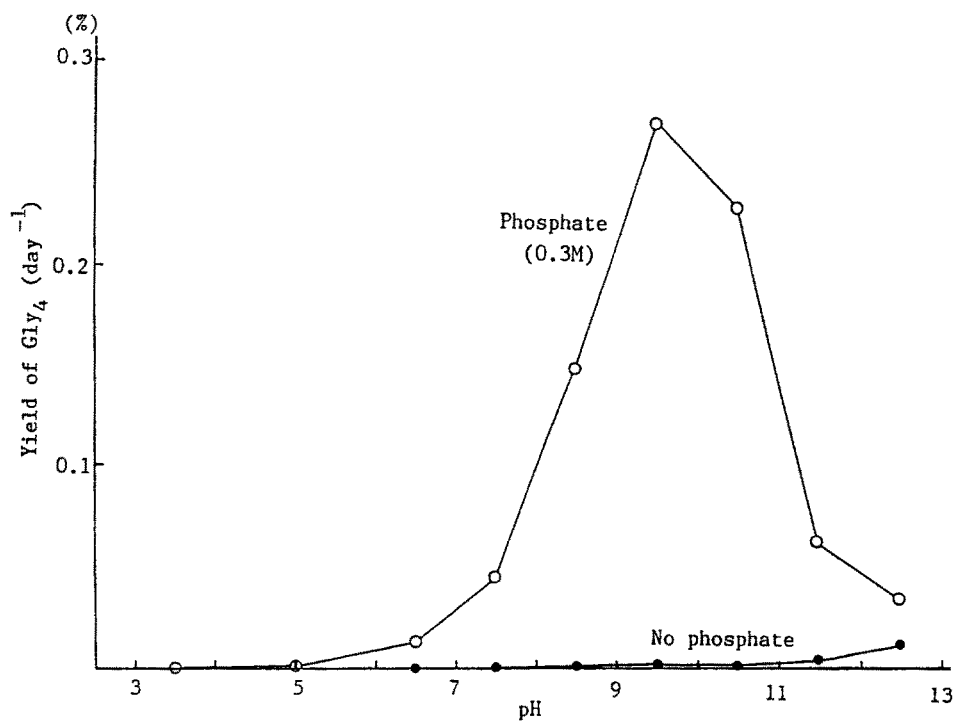
Fig. 3. pH Dependence of the reaction, DKP (0.1 M) + Gly$_2$ (0.1 M) —→ Gly$_4$ in phosphate buffer (0.3 M) and without phosphate at 19 °C.

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