Trimetaphosphate-Induced Addition of Aspartic Acid to Oligo(glutamic acid)s

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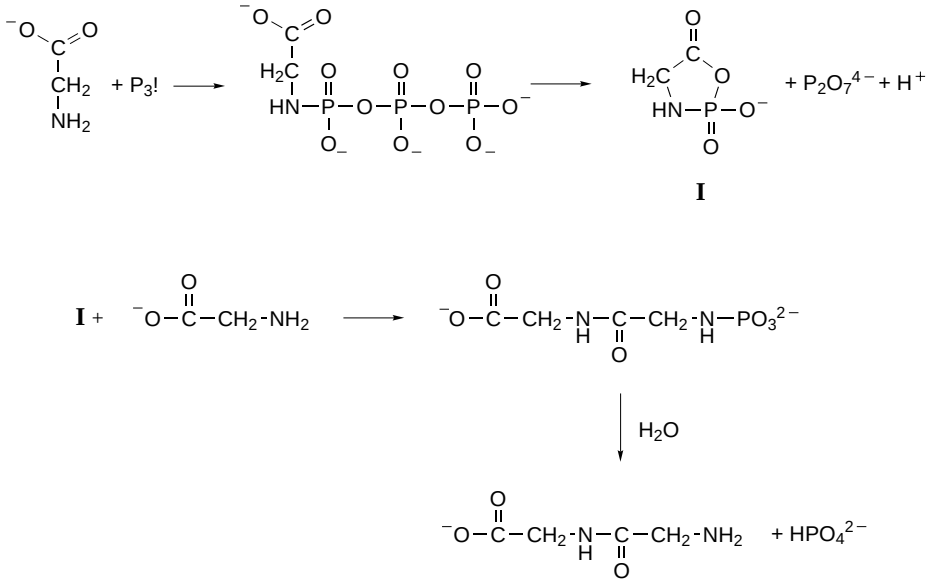
Dedicated to Professor *Dieter Seebach* on the occasion of his 65th birthday

When a solution containing 0.01M decaglutamic acid, 0.1 – 1.0M aspartic acid, 1.0M $MgCl_2$, and 0.5M sodium trimetaphosphate is allowed to stand at temperatures in the range 0 – 50°, addition products containing up to ten aspartic acid residues are formed. Addition occurs to the side-chain carboxy moieties, not to the terminal amine of the decaglutamic acid. A number of other amino acids including glutamic acid, glycine, and histidine fail to react with decaglutamic acid under the same conditions. We believe that the activation of aspartic acid leads to the formation of a cyclic anhydride that is the key intermediate in the reaction.

1. Introduction. – The oligomerization of amino acids in aqueous solution with prebiotically plausible condensing agents has been studied intensively, but with limited success. Cyanamide and its dimer, for example, induce the synthesis of dipeptides from glycine, but only in low yield [1]. There is one partial exception to this generalization. Inorganic trimetaphosphates have been shown, under some conditions, to bring about a fairly efficient oligomerization of glycine or ligation of short oligoglycines.

Rabinowitz et al. first reported that diglycine is formed in good yield, when glycine is maintained in alkaline solution in the presence of a high concentration of sodium trimetaphosphate [2]. The reaction was subsequently shown to proceed *via* diglycine *N*-phosphate (*Scheme 1*). Since phosphoramidates of this kind are very stable under alkaline conditions, only very small amounts of oligomers longer than the dimer are formed [3]. It would, in principle, be possible to produce longer oligomers by this reaction, by cycling between alkaline and acidic conditions, but a different solution was suggested by *Yamagata* and co-workers [4]. They showed that, under mildly acidic conditions, the carboxylate group rather than the amino group of glycine attacks the trimetaphosphate ion. The resulting acyl triphosphate can then react with a second glycine molecule to yield a dipeptide (*Scheme 2*). This reaction, unlike the reaction at alkaline pHs, which is restricted to the monomeric amino acid, is quite general and can be used to oligomerize glycine or to ligate oligoglycines to form products containing more than two of the input molecules. In a subsequent paper, it was shown that the Mg^{2+} ion is an effective catalyst for these reactions. In the presence of 0.5M $MgCl_2$, a solution containing 0.5M gly_2 and 0.5M trimetaphosphate held at 38° for 10 d at pH 5 yielded 12% diglycine and some longer peptides [5]. The Mg^{2+} ion had previously been shown to catalyze the oligomerization of glycine efficiently in the solid state in the presence of the trimetaphosphate ion and 1*H*-imidazole [6].

Scheme 1. *Glycylglycine Formation with the Trimetaphosphate Ion as Condensing Agent under Alkaline Conditions*



We have been interested in the oligomerization of the negatively charged amino acids, aspartic acid, and glutamic acid in the context of mineral-assisted polymerizations [7]. We hoped that the presence of charged side chains on an amino acid or peptide might lead to the coordination of Mg^{2+} ions and hence to an augmentation of its catalysis of trimetaphosphate-induced peptide bond formation. To facilitate the analysis of oligomerization products , we decided to study the addition of aspartic acid to the decapeptide of glutamic acid (glu_{10}), since mixtures of multiply charged peptides are readily resolved by chromatography on *RPC-5* [7]. In this paper, we describe in some detail the formation of adducts of glu_{10} from glu_{10} and aspartic acid in the presence of sodium trimetaphosphate and $MgCl_2$.

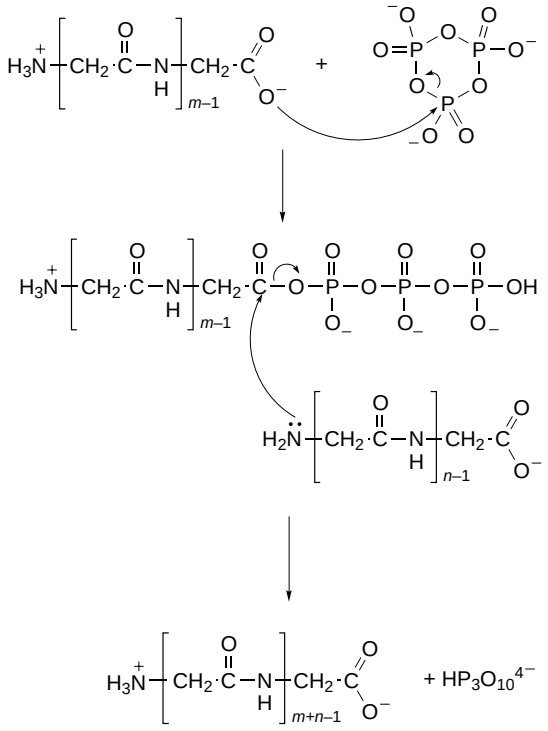
2. Experimental. – Sodium trimetaphosphate was obtained from *Johnson Matthey Electronics*, decaglutamic acid (glu_{10}) was a gift from the *Peptide Biology Laboratory* at the *Salk Institute*, and *N*-acetyldecaglutamic acid (*N*-Ac-glu_{10}) was prepared in the *Molecular Biology and Virology Laboratory* at the *Salk Institute.* All amino acids and other reagents were obtained from standard commercial sources.

We prepared a soln. (100 µl) containing 100 µmol of aspartic acid, 100 µmol of $MgCl_2$, and 0.04 µmol of glu_{10} in a 0.65-ml microfuge tube and then evaporated the soln. to dryness. Next, 100 µl of a freshly prepared 0.5M soln. of sodium trimetaphosphate was added to the tube, and the contents of the tube were dissolved by thorough mixing. The other reaction mixtures used in our experiments were prepared in the same way with appropriate changes in the nature of the amino acid and the peptide involved and in the concentrations of the various reagents. Heavy precipitates, presumably of the Mg^{2+} salt of linear tripolyphosphoric acid, formed after short times (1–2 h) in tubes held at 50° and after much longer times (1–2 d) at 25°.

After appropriate times, an aliquot (usually 20 µl) of the mixture was dissolved in 1 ml of HPLC buffer A. An aliquot of this soln. containing 5 µmol of the amino acid was diluted to 1 ml with 2 mM *Tris* · $HClO_4$ at pH 8 containing 10 µmol of the K salt of EDTA, and the resulting soln. was analysed by HPLC on *RPC-5*.

In a series of experiments designed to determine approx. the pH dependence of the reaction, we prepared a mixture as described above, adjusted the pH to 5, 6, or 7 with NaOH, and then warmed the soln. to 50°. The pH

Scheme 2. *Activation of Peptides by the Trimetaphosphate Ion in Slightly Acidic Solution* (redrawn from [4])



dropped as the reaction proceeded, but was readjusted to the starting pH after each 15-min interval. The products were analysed by HPLC after 2 and 6 h.

Paper chromatography was carried out on *Whatman 3MM* paper by the descending technique. The papers were developed for *ca.* 24 h at r.t. with $PrOH/H_2O/NH_4OH$ 55 : 35 : 10. A 10-µl aliquot of the product mixture (1M asp, 1M $MgCl_2$, 0.5M trimetaphosphate after 50° for 24 h) was added to 10 µmol of the K salt of EDTA in *ca.* 10 µl of H_2O. One 1-µl aliqot of the resulting soln. was chromatographed directly and another was co-chromatographed with 0.1 µmol each of asp_2, asp_4, and asp_6. After developement, the paper was dried and sprayed with ninhydrin.

HPLC Analysis of samples was performed on an anal. *RPC-5* column with a *Waters* model *680* gradient controller and a *Waters* model *510* solvent-delivery system. Reaction products were eluted with a linear gradient of $NaClO_4$ (0 – 0.04M in 40 min) at pH 8 in the presence of 2 mM *Tris* · $HClO_4$ at a flow rate of 1 ml/min. The eluate was monitored at 220 nm on an *ABI Analytical Kratos Division Spectraflow 757* absorbance detector.

Samples for mass spectrometry were obtained by collecting and combining appropriate eluates from several runs on the *RPC-5* column, with a slower gradient (0.01 – 0.03M in 80 min). We carried out a standard reaction with 1M aspartic acid at 37° for 1 d. Fractions corresponding to three major peaks in the chromatograms were concentrated, dialyzed against 2 l of H_2O for 24 h, and finally evaporated to dryness.

3. Results. – 3.1. *Addition of Aspartic Acid and Other Amino Acids to glu_{10}.* In preliminary experiments, we incubated 0.1M or 1.0M aspartic acid with 0.5M sodium trimetaphosphate and 0.1 mM glu_{10} in the presence of 1.0M $MgCl_2$ at 25°, and analyzed the products after various times. We were surprised to find that a much more rapid reaction than that reported for glycine and glycine peptides took place, and much of the

glu_{10} was converted to higher molecular-weight products overnight (*Fig. 1*). These experiments also established that the reduction of the $MgCl_2$ concentration to 0.1M resulted in a greatly reduced rate of reaction, and that the replacement of aspartic acid by glutamic acid almost abolished the reaction. Consequently, our more detailed work was largely restricted to the reactions of aspartic acid and its peptides in the presence of 1.0M $MgCl_2$.

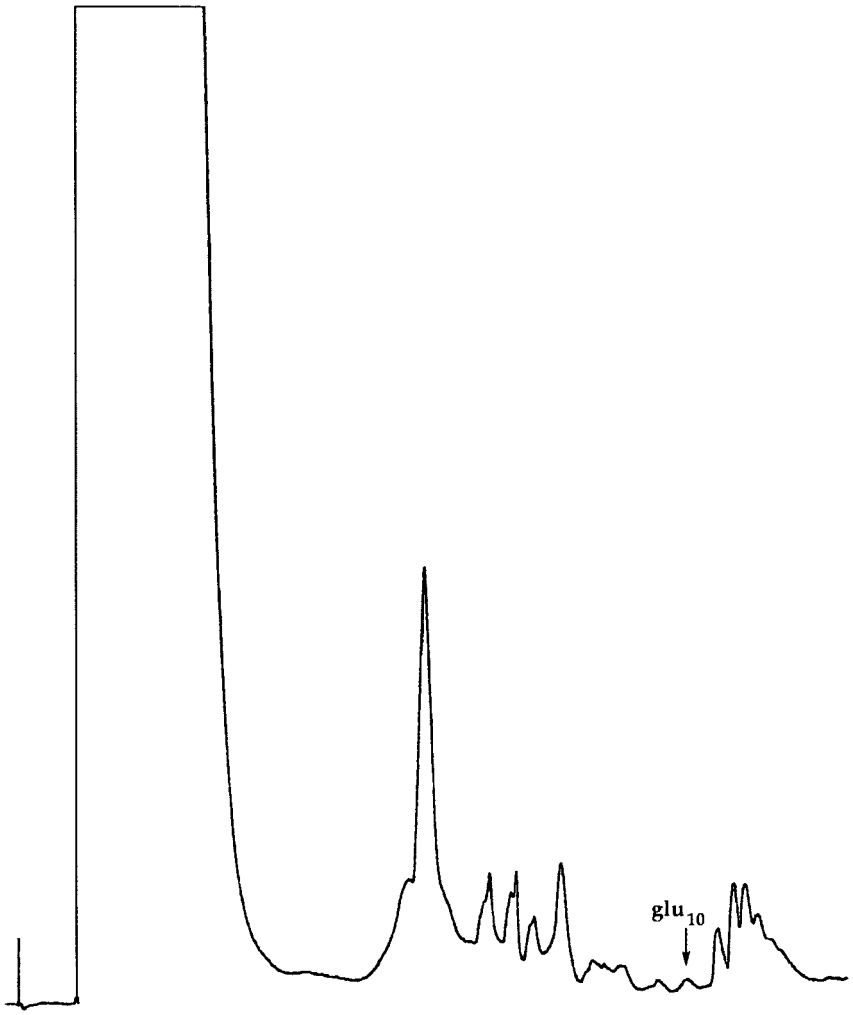


Fig. 1. *HPLC Elution profile of products from the reaction of 0.2 mM glu_{10}, 1M aspartic acid, 0.5M sodium trimetaphosphate, and 1M $MgCl_2$ at 25° for 1 d*

The Mg^{2+}-catalyzed addition of multiple aspartic acid residues to glu_{10} in the presence of the trimetaphosphate ion is efficient over a wide temperature range. In *Fig. 2*, we show representative data for reactions carried out at 25°, 35°, and 50°. As

expected, the reaction proceeds faster at higher temperatures and with higher concentration of aspartic acid, but good yields of products are ultimately obtained in all of these experiments. We also found that glu_{10} was almost completely converted to adducts up to the adduct with seven aspartic acid residues in 2 weeks at 0°. Replacement of aspartic acid by D-aspartic acid or D,L-aspartic acid or replacement of Mg^{2+} by Ca^{2+} had little effect on the pattern of reaction products (data not shown).

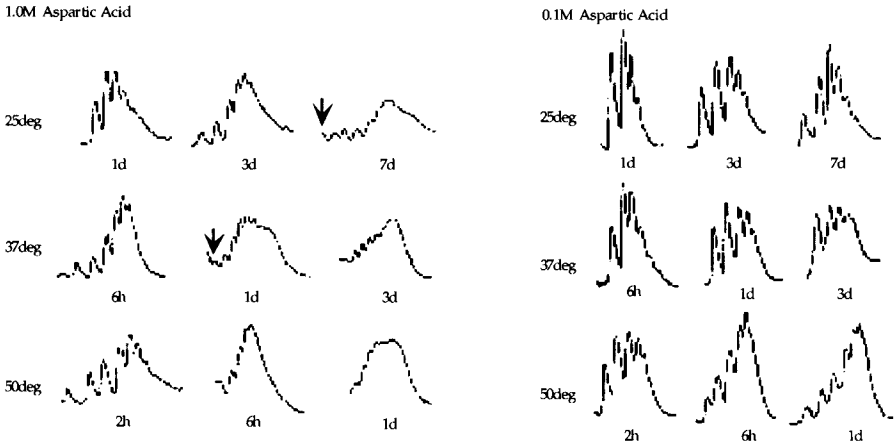


Fig. 2. *Sections of HPLC elution profiles for reactions under various conditions.* The reaction times and temperatures as well as the concentration of aspartic acid are shown. Arrows show the position of glu_{10} when co-injected with the sample.

The composition of the products of the reaction was determined by MALDI and electrospray mass spectrometry. The peaks corresponding to different products resolved when we used a slow gradient (0.01 – 0.03M in 80 min) of eluent (*Fig. 3*). The material corresponding to each of three peaks was collected separately, dialyzed against H_2O to remove salts, dried down, and subjected to mass spectrometry. The MALDI mass spectrum of the material corresponding to peak 4 in the chromatogram included peaks corresponding to the tetra adduct $glu_{10}asp_4$ and its mono-sodium salt. In addition, there was a large peak at a mass corresponding to the loss of one H_2O molecule from the monosodium salt (*Fig. 4*). The material corresponding to the next two series of peaks in the chromatogram had corresponding mass peaks attributable to $glu_{10}asp_5$ and $glu_{10}asp_6$, and their dehydration products. The presence of large amounts of a compound with 18 mass units less than that anticipated for the peptide $glu_{10}asp_4$ was confirmed by electrospray mass spectrometry (data not shown).

Having established that the successive peaks in the elution profile correspond to successive addition products, and that adducts containing at least ten aspartic acid residues are formed, one can roughly estimate the average number of aspartic acid residues that attach to glu_{10} under different conditions. The most extensive reaction occurs at 50° after 1 d when *ca.* 7 residues on average have been added. Even at 25° an average of *ca.* 6 residues are added in 7 d. This is a remarkably efficient addition reaction. Even after 1 d at 37°, a small amount of the deca-addition product is present (*Fig. 3*).

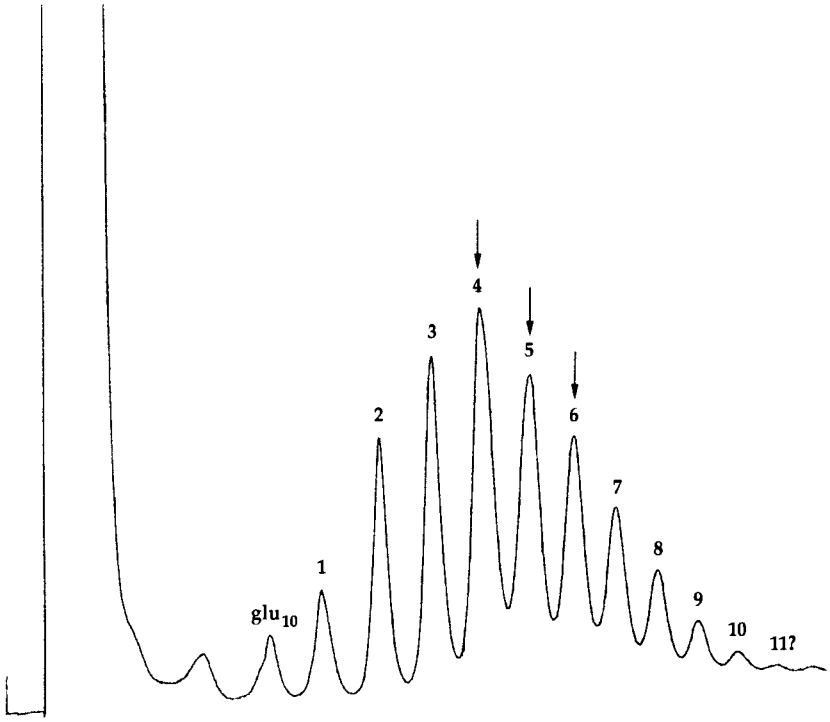


Fig. 3. *HPLC Elution profile, with a slow gradient, of products from a reaction with* 1M *aspartic acid, 2 mM* glu_{10}, *0.5M trimetaphosphate,* 1M $MgCl_2$ *at 37° for 1 d*. The numbers above the peaks indicate the oligomer lengths. The arrows mark the material that was collected for analysis by mass spectrometry.

We carried out a few experiments to determine very approximately the pH dependence of the efficiency of the reaction. When the pH is maintained in the range 6.6–7.5 at 50° for 6 h, most of the glu_{10} is unchanged, and only mono- and di-addition products are detected. When the pH is maintained between 5.6 and 6.5, the reaction is much more efficient and adducts with up to at least 7 aspartic acid residues are detected after 6 h. The reaction is most efficient in the pH range 4.8–5.2, and the products formed in that pH range mirror those obtained in our most successful reactions. Our experiments do not establish the source of the pH dependence of the overall reaction because each of the many sub-reactions involved might be affected by variations in pH (see below).

To differentiate between the addition of activated aspartic acid to the N-terminal amine of glu_{10} and the reaction of aspartic acid with activated side-chain carboxy moieties of glu_{10}, we replaced glu_{10} in the above reaction by *N*-Ac-glu_{10}. We found that the reaction proceeded in essentially the same way with the *N*-Ac derivative as with the unmodified peptide. This establishes that the side-chain carboxy moieties are the major sites of amide-bond formation.

We attempted to add a number of other amino acids to glu_{10} under the experimental conditions described above. We could detect little or no reaction with glycine, serine, valine, histidine, arginine, or glutamic acid.

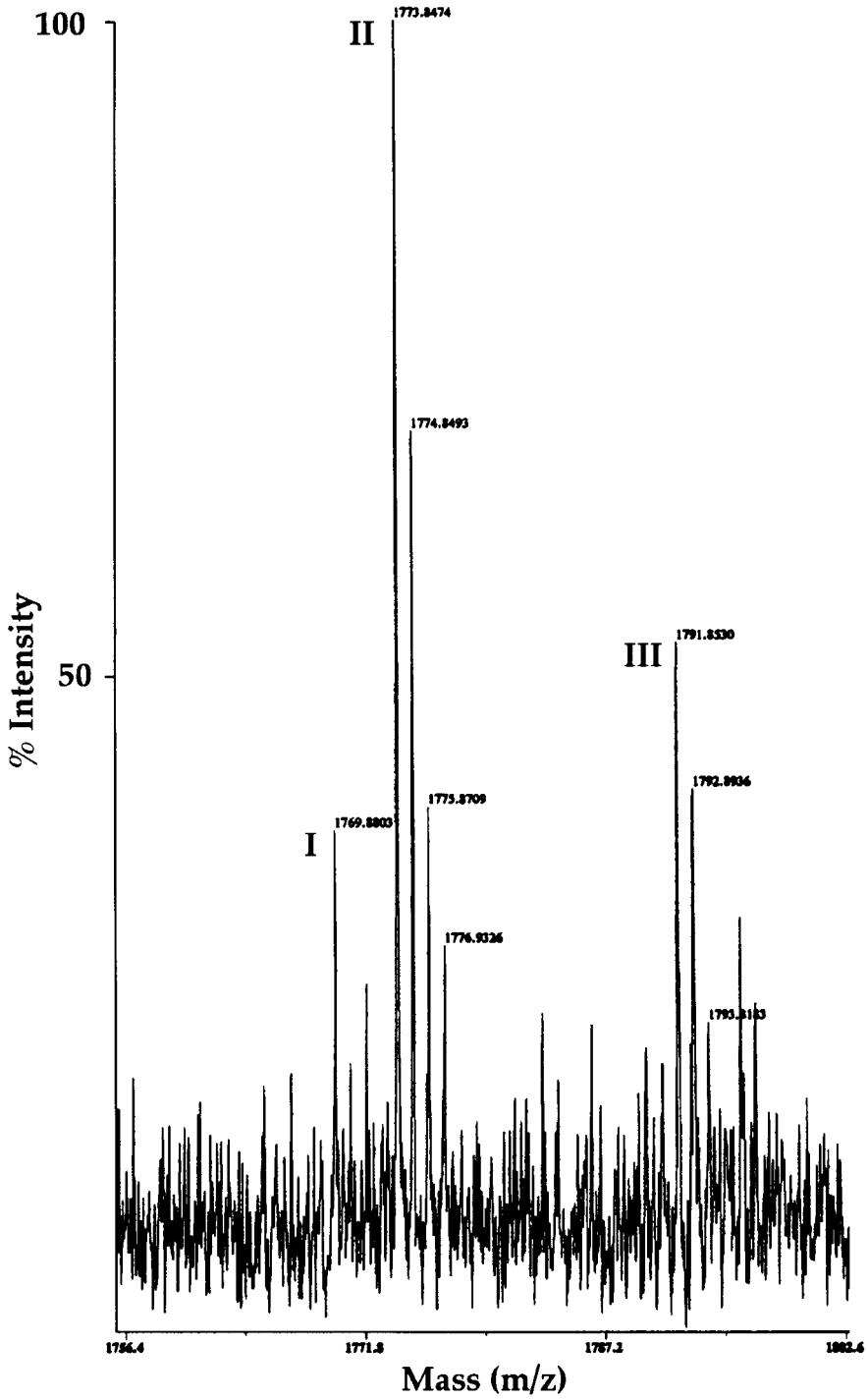


Fig. 4. *MALDI Spectrogram of material in peak* 4 *in* Fig. 3. I is $glu_{10}asp_4H^+$; II is $glu_{10}asp_4Na^+ - H_2O$; III is $glu_{10}asp_4Na^+$.

3.2. *Addition of Aspartic Acid to asp$_6$.* When asp$_6$ was incubated with 1.0M aspartic acid at 25° under the conditions used in our experiments with glu$_{10}$, a substantial yield of a mono-addition product was obtained and a small yield of a di-addition product. However, the reaction was much less efficient than that with glu$_{10}$, so we did not investigate it further.

3.3. *Oligomerization of Aspartic Acid.* The HPLC profiles of the region in which oligomers of aspartic acid elute was always complicated and rarely reproducible. This is readily understood. The hydrolysis of the trimetaphosphate ion leads to the formation of the linear triphosphate anion, which adsorbs much more strongly than the trimetaphosphate ion to anion exchangers. Pyrophosphate is known to be an excellent eluent for displacing strongly bound polyanions, and the linear triphosphate anion would, no doubt, be an even better eluent. In the course of our reactions, the extent of hydrolysis of the trimetaphosphate ion depends on the temperature, the time of reaction, the pH *etc.*, so varying amounts of the linear triphosphate are applied to the column along with the polypeptide products. This leads to a wide variation in the retention time of the shorter poly(aspartic acid)s. The effect of the triphosphate on the elution of more tightly bound oligomers is smaller, so that glu$_{10}$ and its adducts are much less affected. However, even for glu$_{10}$, we noticed more variation in the retention time in these experiments than we are accustomed to.

The above difficulty prevented our making a detailed analysis of the products of the direct oligomerization of aspartic acid. We did, however, obtain a qualitative understanding of the nature of the reaction products by co-chromatographing the reaction mixture with an authentic sample of asp$_4$. Comparison of numerous elution profiles make it clear that the longest detectable oligomers are *ca.* 5–6 residues long. It is also clear from the elution profiles that many isomers of oligomers of each length are present in the reaction mixture. Paper chromatography shows that asp$_3$ and asp$_4$ are formed in small amounts in these experiments, but longer oligomers are not detectable by this technique.

3.4. *Ligation of Short Oligo(Aspartic Acid)s.* We attempted to ligate asp$_4$ with sodium trimetaphosphate as condensing agent under the same conditions as described above for aspartic acid. However, the lower solubility of the oligomers forced us to lower the concentration of substrate to 0.45M. The results of a typical experiment are presented in *Fig. 5*. Clearly substantial ligation occurs and oligomers up to the 16- or 20-mer are formed, but the efficiency is much lower than the efficiency of addition of 0.1M aspartic acid to glu$_{10}$.

4. Discussion. – Our results are best explained in terms of the general conclusion reached by *Yamagata* and *Inomata*, namely that the primary function of the trimetaphosphate ion under the conditions of our reactions is the Mg^{2+}-catalyzed activation of carboxylate groups [5]. The rate and efficiency of the addition reactions, however, are much greater than those observed for comparable reactions of glycine carried out under similar conditions. The details of the reaction mechanism are not immediately obvious, since the site of activation in the reaction of aspartic acid with glu$_{10}$ could be a carboxylate group of aspartic acid, a carboxylate group of glu$_{10}$, or both.

The aspartic acid residues are clearly attached to glu$_{10}$ *via* the carboxylate groups of the peptide, since replacement of glu$_{10}$ by *N*-Ac-glu$_{10}$ has little effect on the pattern of

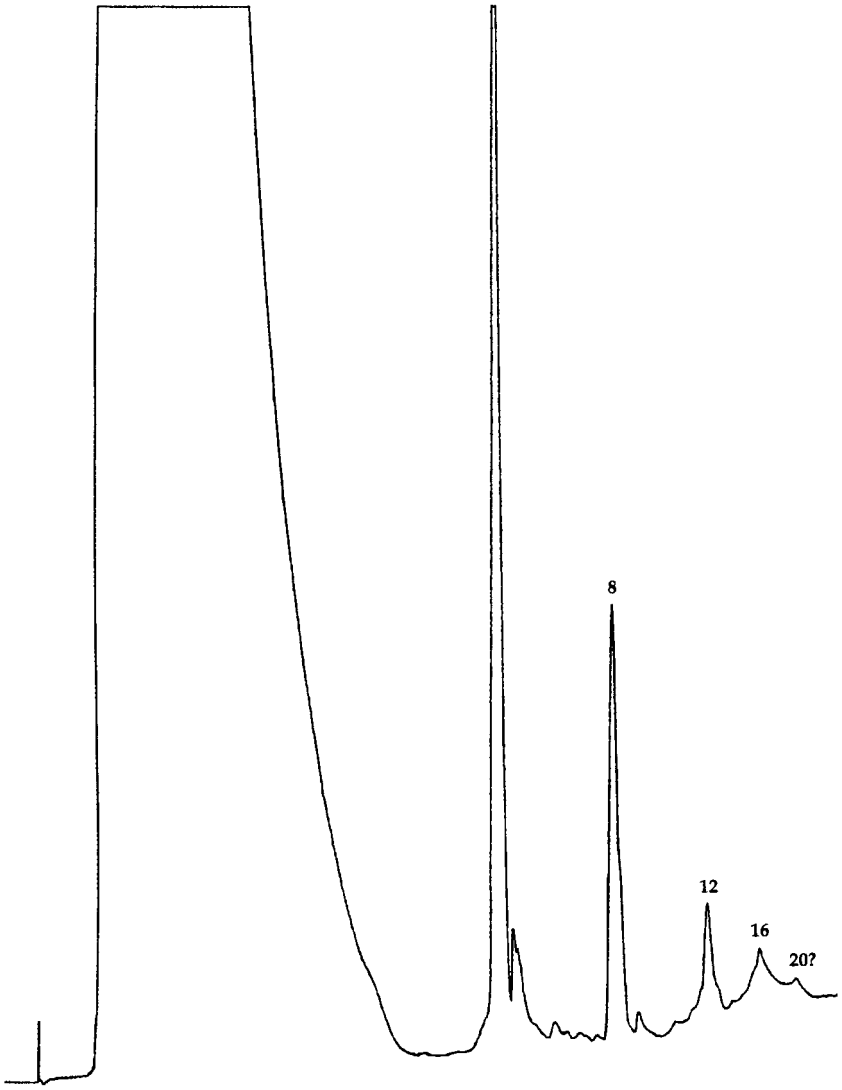


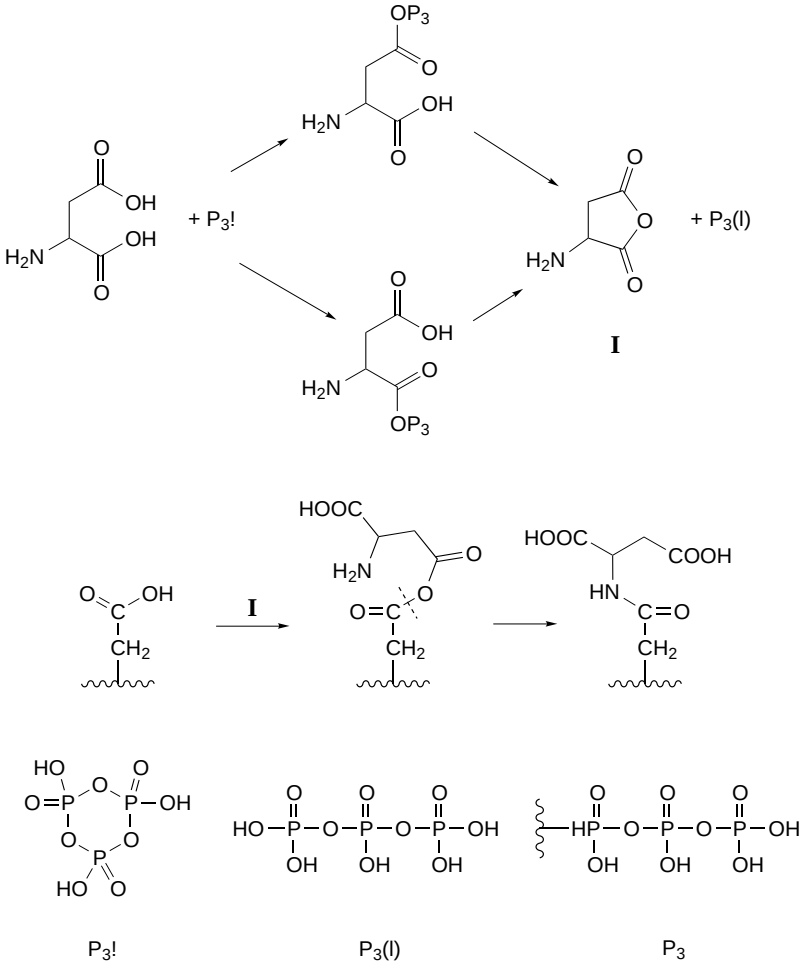
Fig. 5. *Elution profile of products from a reaction of 0.45*M *of* asp_4*, 0.5*M *trimetaphosphate, and I*M $MgCl_2$ *at 25° for 3 d*. The numbers above the peaks are the assumed asp content of the products.

products. At first sight, this would suggest that the side-chain carboxylate groups are the primary site of activation. However, this is hard to reconcile with the failure of glutamic acid, glycine, serine, histidine, arginine, or valine to undergo significant reaction with glu_{10} under our standard conditions. This strongly suggests that reaction is not the straightforward aminolysis of activated carboxylate side chains of the peptide, but depends on some particular aspect of the structure of aspartic acid.

We suggest that aspartic acid reacts with trimetaphosphate to yield an anhydride. This anhydride is able to undergo exchange with a side-chain carboxylate of glu_{10} to

give a product that readily rearranges to the final amide-linked product (*Scheme 3*). This mechanism is not available to the other amino acids, although glutamic acid could, in principle, form a six-membered cyclic anhydride. Our hypothesis is based on the expectation that an acyl triphosphate would be so reactive that it would not discriminate between nucleophiles and would hydrolyse immediately in H_2O. The anhydride would be much more stable and so would survive long enough to undergo exchange with a carboxylate group of glu_{10}.

Scheme 3. *Proposed Steps in the Addition of Aspartic Acid to the Side Chains of glu_{10}*



The results from mass spectrometry identify the tetra-, penta-, and hexa-adducts of aspartic acid to glu_{10}. From the HPLC elution profile in *Fig. 3*, it is clear that adducts at least up to the deca-adduct are present in small amounts in the product mixture. The shape of the elution profile of products carried out for longer times at 50° (*Fig. 2*)

strongly suggest that, under such conditions, most of the side chains of glu_{10} has been converted to adducts with aspartic acid.

Mass spectrometry showed that the peptide products of the reaction are accompanied by molecules related by the loss of a H_2O molecule. This is not unexpected, since activation of a peptide with an N-terminal glutamic acid leads to the formation of the cyclic amino acid, pyroglutamic acid. The formation of these products establishes that the direct activation of side-chain carboxylate groups, as well as the activation of aspartic acid occurs under the conditions that we use.

The MALDI mass spectra display major peaks due to the protonated forms of the parent peptides, accompanied by small peaks, if any, due to the protonated forms of the dehydration products. The situation is reversed for the peaks attributed to the metal salts, for which the peaks due to the dehydration products are larger than those due to the parent peptides (*Fig. 4*). This is probably explained by the presence of an ionized carboxylic acid side chain in the zwitterionic peptide, which is eliminated in the dehydration product, because the basic N-terminal amino group is no longer available to accept a proton from a carboxylic acid side chain.

While the addition of aspartic acid to glu_{10} is a very efficient reaction, the addition of aspartic acid to $(asp)_6$ yields only mono- and possibly di-adducts. The direct oligomerization of aspartic acid is also relatively inefficient, yielding detectable oligomers only up to a mixture of isomeric pentamers or hexamers. It is not clear why the side chains of oligo(aspartic acid)s are so much less reactive than those of oligo(glutamic acid)s.

The reactions described in this paper do not lead to the synthesis of linear peptides as we had hoped. They emphasize the difficulty of selectively elongating peptides containing unprotected side-chain carboxylate groups. In the context of prebiotic chemistry, they re-emphasize the generality of the activation of carboxylic acids by the trimetaphosphate ion [4–6], a reaction, which is in some way the 'poor man's' equivalent of enzymatic or ribozymic activation by ATP.

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