Polymerization of Aminoacetonitrile

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If the protein is absolutely essential for all living things, the non-enzymic formation of protein must have been prerequisite for the origination of life.

In 1936 Oparin[1] postulated that α-amino acids could have been formed non-biologically from hydrocarbons, ammonia and hydrogen cyanide at the age of the earth when atmosphere contained these substances in high concentrations. Bernal[2] emphasized the role played by ultraviolet light in the formation of organic compounds at a certain stage of the evolution of the earth. He also suggested that life

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1) A. I. Oparin, "The Origin of Life", Macmillan Co. N. Y. (1936).

2) J. D. Bernal, "Physical Basis of Life", Routledge and Kegan Paul Ltd., London (1951).

might have originated on the surface of clay which accumulated large amounts of organic substances. Oparin's hypothesis has received strong experimental support from the recent work of Miller[3].

It has generally been accepted that the first proteins or fore-proteins were non-biologically formed by the polycondensation of preformed free amino acids. This belief is solely based on the fact that proteins in the present-day organisms are synthesized via free amino acids. Such non-biological formation of polypeptides and proteins, however, seems to be very difficult owing to the requirement of free energy, though Bresler[4] reported the reconversion of tryptic hydrolysates to the original proteins under an extremely high pressure.

In 1955 Akabori proposed[5] a hypothesis concerning the origin of the fore-protein and speculated that it must have been produced through reactions consisting of the following three steps.

I) $CH_2O+NH_3+HCN \rightarrow NH_2-CH_2-CN+H_2O$

II) $n(NH_2-CH_2-CN) \rightarrow (-NH-CH_2-C(=NH)-)_n$

$\xrightarrow{+H_2O} (-NH-CH_2-CO-)_n+NH_3$

III) $(-NH-CH_2-CO-)_n \rightarrow (-NH-CH(R)-CO-)_n$

The first step is the formation of aminoacetonitrile from formaldehyde, ammonia and hydrogen cyanide. The second is the polymerization of aminoacetonitrile on a solid surface, probably in the state adsorbed on clay, followed by the hydrolysis of the polymer to polyglycine and ammonia. The third step is the introduction of side chains into polyglycine by the reaction with aldehyde or with unsaturated hydrocarbons. The reaction in the first step is well known as Strecker's reaction. Akabori and co-workers[6] reported some experimental results on the possibility of the third step.

The present paper deals with experiments concerning the second step in the formation of the fore-protein, that is the polymerization of aminoacetonitrile. Although acetamidine is formed from ammonia and acetonitrile in methanol solution, the condensation of primary amine with nitrile has been known very little. In the synthesis of purine derivatives, Traube[7] synthesized diamino thiopyrimidine by the condensation of thiourea and malonitrile. In this reaction sodium ethoxide was used as catalyst, and the anhydrous condition had been kept in the course of reaction.

Aminoacetonitrile prepared by the usual method was heated at 120~140°C for 3~5 hr. in the presence of clay, which was used as adsorbent of the nitrile and seemed to play a role as catalyst in this reaction. The product was extracted with water, dilute hydrochloric acid and sodium hydroxide solution. The presence of glycyl-glycine and glycyl-diglycine in extract and partial hydrolysate of extraction residue was confirmed by paper and column chromatography.

Experimental

Materials.—*Aminoacetonitrile sulfate;* According to Anslow and King's method[8] aminoacetonitrile sulfate was obtained, m. p. 164°C(decomp.). *Kaolin;* Commercial "Kaolin" was refluxed with hot water. After 2 hr., it was centrifuged, washed with distilled water and dried in an air bath. Kaolin treated with concentrated hydrochloric acid was used in some cases. *Other clays;* Japanese acid clay and bentonite were treated in the same way as kaolin. *Glycyl peptides;* Gly-Gly** and Gly-Gly-Gly** were synthesized in our laboratory and the purity was confirmed on paper chromatogram.

Procedures.—Several procedures were tried for polymerization of aminoacetonitrile. A typical method is the following.

One gram of amino acetonitrile sulfate was mixed with 20 times of kaolin and heated at 130~135°C for five hours. To heat uniformly, the mixture was taken in a wide bottomed flask and kept in an air bath at 130°C. As the liberation of ammonia was detected in the course of reaction, the top of flask was connected with a small tube of Dowex-50 (H-form). The reaction mixture, being of violet-grey color, was extracted with each 30 ml. of water, N/10 hydrochloric acid and N/10 sodium hydroxide successively in the cold (see Fig. 1). The pH of the water extract was about 5.5, and each of extracts showed a strong positive biuret reaction. A part of the residue was hydrolyzed with N/10 hydrochloric acid at 100°C for 1 hr. (KB) and another part was

3) S. L. Miller, *Science*, **177**, 528 (1953); *J. Am. Chem. Soc.*, **77**, 2351 (1955); *Biochim. et Biophys. Acta*, **23**, 480 (1957).

4) E. S. Bresler et al., *Izvest. Akad. Nauk, SSSR, Ser. Biol.*, **13**, 392 (1949).

5) S. Akabori, *Kagaku* (*Science in Japan*), **25**, 54 (1955).

6) S. Akabori, K. Okawa and M. Sato, This Bulletin, **29**, 608 (1956).

7) W. Traube, *Ann. Chem.*, **331**, 64 (1904).

** Glycine, glycyl-glycine and glycyl-diglycine are abbreviated as Gly, Gly-Gly, Gly-Gly-Gly, respectively.

8) W. K. Anslow and H. King, *J. Chem. Soc.*, **1929** 2463.

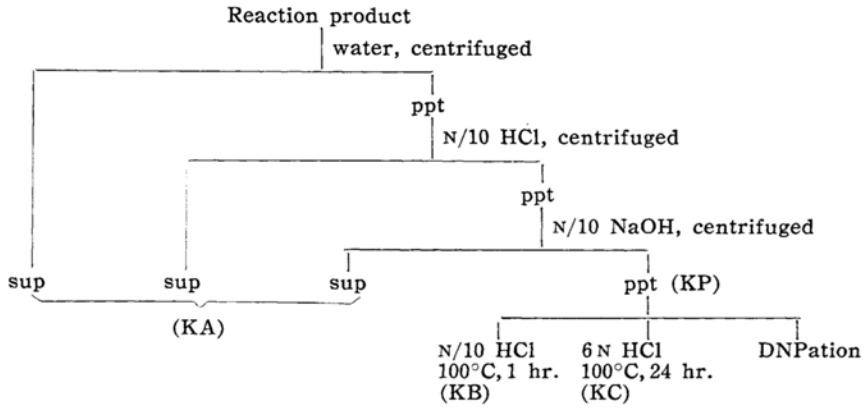

Fig. 1. The Scheme of extraction of reaction product

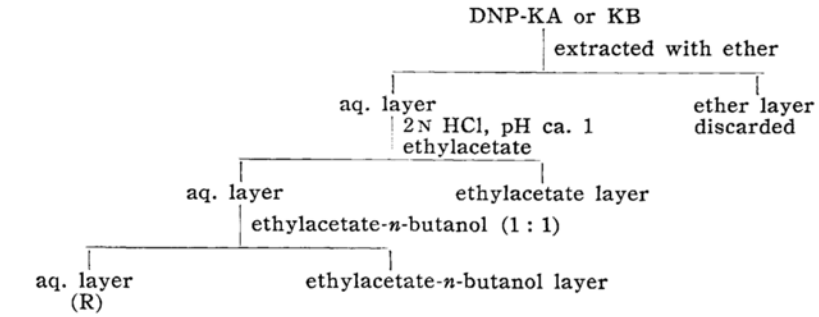

Fig. 2. Procedure for the extraction of DNP-peptides.

hydrolyzed with 6N hydrochloric acid at 100°C for 24 hr. (KC). For the purpose of the hydrolysis of imino group of polymerized product, combined extract (KA) was refluxed at pH 1 for 1 hr. After neutralization the component of each fraction was examined by paper chromatography and by paper and column chromatography after dinitrophenylation.

The Component of Product. — After concentration, an aliquot of each fraction was chromatographed on filter paper using 80% phenol as solvent. From this paper chromatogram, it was found that each fraction contained glycine, and KA and KC contained also glycyl peptide, probably Gly-Gly and Gly-Gly-Gly. These findings were confirmed by dinitrophenylation technique. This method is excellent for the reason that non-peptide basic substance which is ninhydrin positive can be separated from dinitrophenylated (DNP-) glycyl peptide. After each fraction was dinitrophenylated by the usual method, DNP-peptides were extracted with ethylacetate and *n*-butanol as shown in Fig. 2. Ethylacetate extract and ethylacetate-*n*-butanol extract were combined and dried in vacuo. After removing dinitrophenol by sublimation using cold-finger condenser[9], a part of this DNP-peptide fraction was subjected to one-dimensional chromatography on filter paper using *n*-butanol saturated with 1% ammonia as solvent. Several spots of R_f values 0.30, 0.17, 0.11 and lower ones were obtained; the first three spots were identified as DNP-derivatives of Gly, Gly-Gly and Gly-Gly-Gly in comparison with standard samples. Another part of the DNP-peptide fraction was chromatographed on a buffered celite column by a partly modified Perrone's method[10]. The solvent system used was ethylacetate-0.5M phosphate buffer (1:1). After 10 parts of celite were mixed with 6.6 parts of the lower layer of the solvent mixture, celite was poured into the column of 20 cm. high and 2 cm. in diameter with the upper layer of solvent and packed with glass rod compactly. The upper half of the column was bufferized to pH 5.5 and the lower half to pH 6.4. Samples were charged on the column and developed with the upper layer of the solvent mixture of pH 5.5. DNP-Gly, DNP-Gly-Gly and DNP-Gly-Gly-Gly were separated each other forming sharp bands. The bands corresponding to DNP-Gly-Gly and DNP-Gly-Gly-Gly were cut off and eluted with ethanol acidified by small quantity of hydrochloric acid. The eluates were dried up and dissolved in 1% sodium bicarbonate. The optical densities were measured photometrically. After measurement, such DNP-peptides were hydrolyzed with 6N hydrochloric acid for 5 hr. and DNP-derivative was removed by ethylacetate extraction. Aqueous layer was redinitrophenylated, extracted again with ethylacetate, separated by column chromatography and the quantity of DNP-Gly was measured. If the molecular extinction coefficients of dinitrophenyl

9) G. L. Mills, *Biochem. J.*, **50**, 707 (1952).

10) J. L. Perrone, *Nature*, **167**, 513 (1951).

amino acid and peptide are the same, the ratio of optical density of DNP-derivative before and after acid hydrolysis must indicate the length of the peptide. These ratios were 1:1.07 and 1:2.23 respectively for the two DNP-peptides, and were agreed with the expected value for DNP-Gly-Gly and DNP-Gly-Gly-Gly.

Residual aqueous layer (R) in Fig. 2, the yellow color of which shows the presence of unextracted DNP-derivatives, was hydrolyzed with 1 N hydrochloric acid at 100°C for 2 hr. By the same procedure for DNP-peptides, newly formed extractable DNP-derivatives were taken into ethylacetate-*n*-butanol layer. From this fraction, also, DNP-Gly-Gly and DNP-Gly-Gly-Gly were obtained. This fact shows that the extracts (KA and KB) might contain the longer glycyl peptide. From the procedures described above it was found that the reaction products contain many non-peptide compouds, for example, on the celite column chromatography there appeared DNP-derivatives which have an absorption maximum at 365~380 mμ and do not yield DNP-Gly by hydrolysis. Further, the first extracted solution of reaction product (KA) produces a black colloidal substance on standing at room temperature for 24 hr., and this substance precipitates on acidification.

In order to study the possibility that the polymerized substance remains in the residue as an adsorbed form, determination of nitrogen content of the residue and estimation of mean chain length of peptides were tried using micro-Kjeldahl method and dinitrophenylation technique. Nitrogen distribution in the process of reaction and extraction was shown in Table I. From these data obtained it seems reasonable to assume that not only the expected reaction, i. e., polymerization of aminoacetonitrile, but also the

Table I. Nitrogen distribution in the process of reaction and extraction

	mg. N	%
Starting material	540	100
After reaction period	376	70
Extract (KA)	149	28
Residue (KP)	29	5.3

reaction liberating nitrogen as ammonia or volatile form may take place. Nitrogen content of the residue was lower than expected, but this value varied from one reaction to another between 4~20 per cent. In order to determine the mean chain length of adsorbed polymer, the same procedure as used in the confirmation of two bands on the celite chromatogram was applied to the residue (KP) after refluxing at pH 1 for one hour.

The result of this experiment shows that the mean chain length of adsorbed substance is 1.8. However, it is hard to say that the reaction product involves long-chain, high molecular polyglycine although the residue might adsorb still low molecular peptide and contain the not-straight chain compound as diketopiperazine which produces DNP-Gly by hydrolysis and dinitrophenylation.

The presence of diketopiperazine in the reaction product was supposed by the qualitative test using picrate[11] and the different content of diglycine of the solution with and without alkaline treatment of the hot water extract of reaction product.

Change of Condition.—If the presence of the clay such as kaolin is advantageous for the proposed reaction, one of the effects must be the adsorption of starting material. The degree of adsorption of aminoacetonitrile on several kinds of clay was determined by the following way. The solution of 200 mg. of aminoacetonitrile sulfate in 4 ml. of water was mixed with 1 g. of clay; after shaking the suspension was allowed to stand overnight at 5°C and filtered. The concentration of free aminoacetonitrile in the filtrate and the control solution (without clay) was measured by ninhydrin reaction[12], which is used generally for the determination of amino acid. By a preliminary test it was confirmed that the aminoacetonitrile was not hydrolyzed in the process of determination and could be determined quantitatively, though the color yield is very low. As shown in Table II, the nitrile was adsorbed most

Table II. The degree of adsorption of aminoacetonitrile on the clay

A. pH 4.5

	Adsorption %
Control	0
Kaolin	3.7
Silicic acid	0
Bentonite	27.0
Silica gel	1.0
Japanese acid clay	37.8

B. Adsorption of Japanese acid clay at different pH

pH	Adsorption %	pH	Adsorption %
4.6	37.8	6.8	54.0
4.6	40.0	7.5	45.0
5.5	51.4	8.7	36.5

on Japanese acid clay and at pH 7. This pH coincides with that of the solution of aminoacetonitrile sulfate neutralized with sodium hydroxide solution.

In an effort to study the effect of adsorption on the reactivity, aminoacetonitrile in an adsorbed state was used as starting material. After adsorption, as described above, Japanese acid clay was filtered, dried and heated at 130°C for 3 hr. in the same way as the typical method. In this case, yield of peptides was slightly high, but black colloidal material was produced abundantly at the same time. It may be concluded from the above fact that the adsorption on the clay

11) M. Frankel and E. Katchalski, *J. Am. Chem. Soc.*, **64**, 2264 (1942).

12) S. Moore and W. H. Stein, *J. Biol. Chem.*, **176**, 367 (1948).

activates the starting material to some extent but the effect is not specific for peptide synthesis.

When bentonite, silicic acid, silica gel and alumina were used as adsorbent, the yields of peptides were poor. Kaolin and Japanese acid clay treated with concentrated hydrochloric acid did not adsorb the aminoacetonitrile.

The effect of temperature was investigated between 100~150°C; below 120°C the reaction did not occur, and above 150°C the decomposition was predominant.

When methylene aminoacetonitrile was used instead of aminoacetonitrile as starting material, many spots appeared on the paper chromatograms of the extract of reaction product, but Gly-Gly-Gly could not be detected. In this case, the extracted solution (corresponding to KA) gave positive biuret reaction, but since several positive compounds besides poly-peptide have been known, biuret test is not suitable to such solutions which involve various unknown substances similarly as the test of diketopiperazine by picrate.

As cited in the introduction of this paper, diaminopyrimidine was synthesized from thiourea and malonitrile in ethanol in the presence of sodium ethoxide by Traube. If aminoacetonitrile reacts analogously, diimino-piperazine may be obtained from two molecules of aminoacetonitrile to yield diketopiperazine and diglycine by hydrolysis.

Four grams of aminoacetonitrile sulfate was suspended in 10 ml. of ethanol. To this suspension, 20 ml. of ethanol dissolving 1.4 g. of sodium was allowed to run from a dropping funnel under stirring. Two aliquots of the mixture were heated to 40°C and to boiling point, respectively, for 3 hr. In each case, the product was neutralized, dried in vacuo, and dissolved in 1N sodium hydroxide. After 1 hr. the product was neutralized again, dried and subjected to paper chromatography. Only a trace of glycyl-glycine was observed, and here again the black colloidal substance appeared in the solution of the product.

Discussion

As aminoacetonitrile has a bifunctional group and an active methylene group, it may be reactive and be able to cause various reactions simultaneously. These characteristics are a strong point as well as a weak point in the case of polymerization which was tried. From the result of investigation, it was obvious that peptides were formed from aminoacetonitrile, but the presence of high polymer which might be firmly adsorbed on the clay was not confirmed. If the conditions may be found, in which polypeptide synthsis is promoted while the side reaction and decomposition are suppressed, poly-glycine should be yielded more easily. Because the heat reaction of aminoacetonitrile without clay was unsuccessful, the solid surface of clay seems to play a certain role in this reaction as in the case of activation of the methylene group of polyglycine[6].

In some cases, the formation of only glycyl-glycine was observed in the typical reaction procedure. Though it is hard to explain, this phenomenon suggests that the reaction proceeds in two directions, the one toward diketopiperazine formation and the other toward polyglycine formation similarly as the polymerization of glycine ethyl ester.

Summary

Aminoacetonitrile sulfate was heated with kaolin and/or Japanese acid clay at 120~140°C for 5 hr. The presence of glycyl-glycine and glycyl-diglycine in the extract and the partial hydrolysate of this reaction product was confirmed by paper and column chromatography.

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