The Formation of Amino Acids and Related Oligomers from Formaldehyde and Hydroxylamine in Modified Sea Mediums Related to Prebiotic Conditions*

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Amino acids and related oligomers were produced from formaldehyde and hydroxylamine in modified sea mediums at pH 5.5 and 105 °C. The modified sea mediums are characterized by a lower concentration of sodium chloride and a higher concentration of essential transition metal** ions (Zn^{2+}, Mo^{6+} as MoO_4^{2-}, Fe^{3+}, Cu^{2+}, Co^{2+}, and Mn^{2+}) than in sea water; generally small amounts of clays (kaolin and montmorillonite) were added. About 40 species of amino acids were detected in the automatic amino acid analyzer, and 20 of them were tentatively assigned. Glycine, alanine, and serine among the protein amino acids and β-alanine were further confirmed by thin-layer chromatography. Oligomers with molecular weights of about 200—1000 were produced which gave rise to an amino acid mixture by acid hydrolysis. From the ratio (maximum, about 5) of the content of the amino groups after to before hydrolysis and the molecular size, the oligomers were tentatively regarded as oligopeptides of an unknown nature, resistant to pronase digestion. The large-molecular fraction (M. W. about 700) had a phosphatase-like activity able to hydrolyze *p*-nitrophenyl phosphate.

Since the first experimental demonstration of the synthesis of amino acids under possible primeval conditions published by Miller,[1] a substantial number of investigations have been carried out in this field of research, most of which have been collected in two volumes.[2,3] For the further chemical evolution, that is, for the formation of primary organisms, the existence of an aqueous medium is absolutely required.[4] The dissolved substances in earth's primeval hydrosphere are regarded as the initial materials for the development of life.

Egami, one of the present authors,[5] has reported that a good correlation was found between the biological behaviour of minor elements, such as molybdenum and iron, and their concentrations in the present sea water. A hypothesis has been presented that the composition of the present sea water reflects that of the primeval sea water at the time of early evolution. In accordance with the above hypothesis, iron, molybdenum, and zinc, which are the most abundant transition elements in sea water, are found to be essential components of the enzymes in microorganisms, including *Clostridium*, which is regarded as the most primitive existing organism.[6] These transition elements presumably complexed with compounds accumulated in the primeval sea in the course of chemical evolution, thus forming compounds which subsequently evolved to form protoenzymes with a low activity and a broad specificity.

Taking into consideration the fact that essential transition metal ions had to contribute to chemical evolution in primeval sea water, and anticipating the concerted catalytic action of clays and metal ions, we have been investigating the formation of biomolecules in modified sea mediums, in which the concentration of sodium chloride is lower, and that of transition metal ions in higher, than in sea water. We suppose that the modified sea mediums are essentially similar to primeval sea conditions and are more effective for the formation of organic substances from simple materials.

The present paper will deal with the formation of amino acids and related oligomers from formaldehyde (as the C_1 compound) and hydroxylamine (as the N_1-compound) in the modified sea mediums, with special reference to the effects on their formation of essential transition elements, *i. e.*, iron, molybdenum, zinc, copper, cobalt and manganese, and of such clays as kaolin and montmorillonite. The starting materials, formaldehyde and hydroxylamine, were selected on the basis of the experiments of Oró *et al.*[7] Preliminary experiments under similar conditions by Ventilla and Egami[8] were reported elsewhere.

Materials and Methods

Materials. The formaldehyde was obtained from Wako Chemicals, and the hydroxylamine hydrochloride, from Nakarai Chemicals. They were of a reagent grade. All of the six transition elements, 0.1 M solutions in the chemical forms of $Fe(NO_3)_3$, Na_2MoO_4, $ZnCl_2$, $Cu(NO_3)_2$, $CoCl_2$, and $MnCl_2$, were kind gifts of Miss Kayoko Nakamura (Laboratory of Social Life Science, in our Institute). The kaolin was purchased from Kukita Chemicals and the montmorillonite, from Hohjun Yoko Co. All the other compounds used as the components in the modified sea mediums were of a reagent grade.

The Nuclepore (pore size of 0.4 μ, 47 mm disc) was obtained from the Nomura Micro Science Co. The Dowex 1 (2X, 100—200 mesh, OH form) and Dowex 50 (8X, 100—200 mesh, H^+ form)were purchased from the Muromachi Kagaku Co. The biogel P-2 (100—200 mesh) and P-10 (100—200 mesh) were obtained from Bio-Rad Chemicals. The fluorescamine (Fluram<Roche>) and disodium *p*-nitrophenyl phosphate were purchased from the Japan Roche Co. and Sigma Chemicals respectively. Six M HCl for acid hydrolysis was purified by distillation at a constant boiling temperature. The dioxane was used after distillation over solid NaOH. The other chemicals were reagent-grade.

Preparation of the Modified Sea Mediums for Large-scale Experiments. The original solution (250 ml), with a concentration of 0.6 M of formaldehyde and 0.1 M of hydroxylamine hydrochloride and of containing 0.02 M of magne-

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** The term "transition element" is used here in its broader sense to include zinc and similar elements.

TABLE 1. COMPOSITIONS OF A MODIFIED SEA MEDIUM (500 ml)

Compositions	Concentration
HCHO	0.3 M
NH_2OH	0.05 M
HPO_4^{2-}, SO_4^{2-}, Mg^{2+}, Ca^{2+}	0.01 M each
Na^+	0.015 M
K^+	0.045 M
Cl^-	0.07 M
NO_3^-	0.0005 M
Zn^{2+}, Mo^{6+} as MoO_4^{2-}, Fe^{3+}, Cu^{2+}, Co^{2+}, Mn^{2+}	0.0001 M each
Kaolin, Montmorillonite	2% (wt/vol) each

sium sulfate, 0.02 M of calcium chloride, 0.02 M potassium phosphate (dibasic), and 0.0002 M each of the six transition elements, was adjusted to pH 5.5 by titration with an alkaline solution containing 0.7 M KOH and 0.3 M NaOH. The volume of the solution was diluted to 500 ml, and then 10 g each of kaolin and montmorillonite were added to the solution. The original pH value, 5.5, of the solution was not changed by the addition of clays. The final composition of the reaction mixture, in a total volume of 500 ml, is listed in Table 1. This reaction mixture (namely the modified sea medium) was glass-sealed *in vacuo* under a nitrogen atmospher and kept at 105 °C±5 °C for 35 days in a Dry-Block DB-3H (M ε S Instruments Co.). As the reaction proceeded, the reaction mixture turned yellow after about 2 weeks and reddish brown after about 3 weeks.

Extraction Procedure of the Products from Modified Sea Mediums after the Reaction. After a 35-day reaction at 105 °C, the sealed ampoule was cut off in an ice bath. After centrifugation at 10000 rpm for 30 min, the sediment was at once washed by 100 ml of distilled water. The resulting supernatants (590 ml) were combined and filtered off on a 0.4 μ pore size Nuclepore membrane. The washed sediment was then resuspended in 400 ml of 2 M aqueous ammonia, and the suspension was vigorously shaken at 37 °C for 1 day. After the centrifugation of the suspension, the supernatant (390 ml) was filtered off. Further extraction from the resulting sediment with 200 ml of 2 M aqueous ammonia was repeated twice. After the final extraction at 37 °C for 2 days, the resulting sediment was washed twice with 150 ml of distilled water.

All of the extracts (total volume of 1670 ml) were lyophilized by means of a Virtis-model 10—145 MR-BA freeze-mobile. The lyophilized dark brown powder was redissolved in distilled water and filtered off on a Nuclepore membrane. The color of the resulting extract (19 ml) was a deep reddish brown. It was designated "Golden Primordial Broth" from the color tone and the richness of the products.

Biogel P-2-column Chromatographic Separation of Products. Biogel P-2 column chromatography was used for the initial separation of oligomeric products with different molecular weights. An aliquot (15 ml) of concentrated "Golden Primordial Broth" was placed in the column beds under an overlying buffer layer. Biogel P-2 (100—200 mesh) was equilibrated with 0.1 M ammonium hydrogencarbonate (approximate pH value of 9.0) and then packed in a large column (2.65×130 cm=700 ml). The fraction (5.0 ml each) was eluted at 13 ml/h with a constant pressure head of 15 cm by means of 0.1 M ammonium hydrogencarbonate.

For the purpose of the further characterization of each fraction, for example, the determination of the primary amino groups before and after acid hydrolysis, and the measurement of the alkaline phosphatase activity, each fraction was lyophilized in order to remove the ammonium hydrogencarbonate and then, with distilled water, brought back to the original volume.

The molecular weight of each fraction was calibrated on the basis of the interpolation of semi-logarithmic plots (elution volume *versus* molecular weight) obtained from separate experiments with the following standard compounds; horse heart cytochrome *c*, oxidized and reduced glutathione, adenosine triphosphate, and sodium chloride.

Small-scale Experiments. In order to examine the reaction requirements of catalysts such as transition elements and clays, small-scale (total volume of 10 ml) experiments were carried out under essentially the same reaction conditions and using the same extraction procedures as those of the above large-scale experiments except for their experimental scale. After the reaction an aliquot of each extract was placed in a small Biogel P-2 column (1.25×49 cm=60 ml) equilibrated with 0.1 M ammonium hydrogencarbonate. Each fraction (5 ml) was lyophilized and then brought back to the original volume with distilled water.

The same small-scale experiments used to discover the time course of the formation of amino acids and related oligomers were carried out in the same way.

Determination of the Content of Primary Amino Groups. The fluorescamine reagent has been employed for the fluorometric determination of the content of primary amino groups. The advantages of the use of this reagent instead of ninhydrin are the high sensitivity of amino acid, the non-sensitivity of hydroxylamine, and the low sensitivity of ammonia. The manual determination procedure was undertaken according to Böhlen *et al.*[9] An aliquot of the sample (10—250 μl, 1—25 nmol of primary amino groups) was transferred into a 18×105 mm test tube, and then the volume was brought to 2.5 ml with a 0.05 M sodium phosphate buffer (pH 8.0). While the test tube was being vigorously shaken on a vortex-type mixer [a Thermomixer (Termonics Co.)], 0.5 ml of a fluorescamine solution in dioxane (30 mg/100 ml) was rapidly added to the buffer solution by means of a Finnpipette (Kemistien Oy). A reagent blank and a standard leucine solution in two different concentrations (5 and 10 nmol) were run routinely. The fluorescence was measured on a Shimadzu model RF-502 recording spectrofluorometer with the excitation wavelength at 390 nm and the emission at 470 nm with a slit width of 5 μm in each. Measurement was made with a regular quartz cuvette (1 cm light path).

As a more sensitive method, we also employed a one-fifth-scal-down procedure. Its sensitivity range for leucine was 0.1—10 nmol. The final volume was 0.6 ml. The quartz cuvette used had a 0.25 cm light path.

Acid Hydrolysis. An aliquot of the sample was transferred to a 15×105 mm test tube (made of Pyrex glass) and lyophilized to a powder; the volume was then brought up to about 1 ml of 6M HCl. This solution was glass-sealed *in vacuo* and kept at 110 °C for 20 h in a hydrolysis furnace (Mitamura Riken Co.). After hydrolysis, the HCl was removed *in vacuo* at 40 °C.

Amino Acid Analysis. Amino acid analyses were conducted using a JEOL model JLC-6AH automatic amino acid analyzer. Before the amino acid analysis, the sample was placed in a small Dowex 1 (OH^- form) anion-exchange column (1.2×5 cm=5.7 ml). The pass-through fraction (neutral) was discarded. After washing with distilled water, the amino acid fraction (acidic) was eluted with about 10 ml of 4 M acetic acid. The acetic acid solution was evaporated to dryness *in vacuo* at 40 °C. Then the sample was dissolved

in a 0.2 M sodium citrate–citric acid buffer (pH 2.2) and eluted from a long column (0.8×50 cm) with a pH gradient of 3.30 to 4.25 by a step-by-step procedure. The sample solution was eluted from a short column (0.8×15 cm) with a pH of 5.29. JEOL custom spherical resin LCR-2 (strong-acidic cation exchange resin) was used to pack the column.

Manual Handling of Amino Acid Analyzer for the Preparation of Individual Amino Acids. The acid hydrolysates of an aliquot of the concentrated "Golden Primordial Broth" were subjected to Dowex 1 (OH^- form)-column treatment. The resulting acidic fraction was evaporated and dissolved with a 0.2 M sodium citrate-citric acid buffer (pH 2.2). An aliquot (0.8 ml; 12.9 μmol of primary amino groups) was placed in a moderately long column (0.8×50 cm) of the amino acid analyzer. The elution was carried out by the step-by-step pH gradients of 3.30 and 4.25 at a constant elution speed of 0.84 ml/min. At the end of the buffer (pH 4.25) elution, the residual amino acids retained on the resin were eluted off with 0.2 M NaOH. The fractionation (0.5 ml for each fraction volume) was done by means of a LKB model 7000 Ultrolac fraction collector. An aliquot (10 μl) of each fraction was estimated for the primary amino groups with fluorescamine. The fractions—peak No. 9 shown in Fig. 1 (possibly containing serine), Peak No. 15 (possibly containing glycine), Peak No. 16 (possibly containing alanine), Peak No. 30 (possibly containing β-alanine) and the 0.2 M NaOH eluate (the basic amino acid fractions)—were pooled. The pooled fractions except for the 0.2 M NaOH eluates were then adjusted to pH 1.0 with HCl and placed in a Dowex 50 (H^+ form) resin column (1.2×5 cm=5.7 ml). After the drying of the 2 M aqueous ammonia eluate, they were subsequently placed in a Dowex 1 (OH^- form) resin column (1.2×2 cm=2.3 ml). The 4 M acetic acid eluate was evaporated to dryness and then dissolved with a small volume of distilled water. An aliquot of each desalted sample was placed on a thin-layer chromatogram coated with cellulose on a plastic roll (DC-Plastikrolle Cellulose; 20 cm height; Merick). The solvent system used was butyric acid : acetic acid : water (4 : 1 : 5, by volume).[10] After developing for 8 h, each spot was visualized by means of a spray of 0.2% ninhydrin butyric acid saturated in a water solution and heating at 95 °C for 10 min.

Spectrophotometric Analysis of the Oligomers. The oligomers used for analysis were concentrated pooled fractions (Fractions Number 51—58 in the Biogel P-2 column shown in Fig. 2) which contained 4.25 μmol of primary amino groups per ml. The ultraviolet spectrum was measured in a diluted aqueous solution (17.9 nmol of primary amino groups per ml) of the oligomer, using a Hitachi model 323 spectrophotometer. The infrared spectrum was recorded as potassium bromide disc on a JASCO model IRA-1 spectrophotometer.

Attempted Pronase-catalyzed Hydrolysis of the Oligomers. In order to remove the small-molecular weight peptides contaminating the commercially available pronase preparation (Pronase E from the Kaken Kagaku Co.), a 30-mg portion of Pronase E was placed in a small column (1.25×47 cm =58 ml) packed with Biogel P-10 equilibrated with a 0.02 M potassium phosphate buffer (pH 7.5). The main peak just behind the void peak of Biogel P-10 chromatography was collected and then used immediately for the following hydrolysis experiment. The oligomers used were the same preparation as the one above used for the spectrophotometric analysis. The reaction mixture for pronase-catalyzed hydrolysis, in a total volume of 0.5 ml, contained 0.904 μmol of the oligomers, as calculated from the content of the primary amino groups, 0.63 mg of Biogel P-10-treated pronase, and 20 μmol of a potassium phosphate buffer (pH 7.5). After incubation at 37 °C for 17 h, the mixture was placed in a Biogel P-10 column (1.25×47 cm=58 ml) equilibrated with a 0.02 M potassium phosphate buffer (pH 7.5). The fractions (1 ml each) were then collected by eluting at 2.5 ml/h with a pressure head of 15 cm. The effluents were monitored in terms of their absorbance at 280 nm, using a LKB Uvicord II absorption spectrophotometer, and by then measuring their content of primary amino groups by the use of fluorescamine.

Measurement of Alkaline Phosphatase Activity. The reaction rate of alkaline phosphatase activity in the oligomer fractions formed was determined by measuring the *p*-nitrophenol released from *p*-nitrophenyl phosphate according to the method of Oshima,[11] with slight modifications. The assay mixture, in a total volume of 0.9 ml, contained 90 μmol of the Tris-acetic acid buffer (pH 7.5), 9 μmol of $MgCl_2$, 0.9 μmol of $ZnCl_2$, 9 μmol of disodium *p*-nitrophenyl phosphate, 80 μl of the oligomer fraction, and 5 μl of toluene. The reaction was carried out at 37 °C in a 13×100 mm test tube (Corning Co.) with a tight cap, and the reaction rate was directly measured at 400 nm, using a Zeiss PMQ II spectrophotometer, at suitable time intervals (1—5 days).

Results and Discussion

Extraction of "Golden Primordial Broth" after the Reaction in Modified Sea Mediums Containing Formaldehyde and Hydroxylamine. After 35 days at 105±5 °C, the following four-step extractions from a sealed ampoule containing 500 ml of modified sea mediums were done:

Table 2. Extraction of the products from a modified sea medium (500 ml)

Steps of extraction	Volume	Content of primary amino groups		Ratio of (B) to (A)
		Before acid hydrolysis (A)	After acid hydrolysis (B)	
	ml	μmol		
H_2O extract	590	154	1214	7.88
1st 2 M NH_4OH extract	390	178	423	2.38
2nd 2 M NH_4OH extract	190	33.3	100	3.00
3rd 2 M NH_4OH extract	500	34.6	107	3.09
Summation	1670	400	1844	4.61
Concentrated "Golden Primordial Broth"	19	404	1519	3.76

The extraction procedures are described in the text.

extraction with distilled water, and 1st, 2nd, and 3rd extractions with 2 M aqueous ammonia. The extraction volumes and their contents of primary amino groups before and after the acid hydrolysis are listed in Table 2. As is shown in Table 2, the compounds with a high ratio of the content of the primary amino groups after acid hydrolysis to that before the procedure were extracted with distilled water. The extraction percent reached 65% of the total extraction. However, it appeared that even the third extraction with 2 M aqueous ammonia did not accomplish the complete extraction of the products. Although tightly bound compounds seem to be present in the clays, most of the products with primary amino groups might be recovered in the concentrated "Golden Primordial Broth." In order to eliminate the clays finely pulverized in 2 M aqueous ammonia, the concentrated solution was filtered through a Nuclepore membrane (pore size 0.4 μ). As can be seen in Table 2, though, this operation did not reduce the recovery of primary amino groups.

The yield of primary amino groups in the concentrated "Golden Primordial Broth" was calculated to be 6.06% from the amount of hydroxylamine used as the starting N_1 compound. The fraction eluted from a Dowex 1 (OH^- form) anion-exchange resin column by 4 M acetic acid contained 1311 μmol of primary amino groups (5.24% yield calculated from the hydroxylamine). That is, most (86.3%) of the compounds with primary amino groups in the hydrolyzate of the "Golden Primordial Broth" were acidic ones (probably with a carboxyl or sulfonate group). This amphoteric character strongly suggested that most of the hydrolyzates were amino acids.

Amino Acid Analysis of the Hydrolyzate Formed from "Golden Primordial Broth". After the treatment of the Dowex 1 (OH^- form), an aliquot of the hydrolyzate formed from the "Golden Primordial Broth" was examined by means of the automatic amino acid analyzer using cation-exchange resin. The resulting chart is shown in Fig. 1. As is shown in Fig. 1, the peaks of the ninhydrin-positive materials consisted of 40 species. The retention times of natural protein amino acids are presented in the same chart (shown in Fig. 1). In the separate experiments, the retention times of 16 non-protein amino acids (β-alanine, α-aminobutyric, α-aminoisobutyric, β-aminobutyric, β-aminoisobutyric, γ-aminobutyric and α,γ-diaminobutyric acids, homoserine, phosphoserine, taurine, norvaline, norleucine, alloisoleucine, sarcosine, citrulline, and ornithine) were also measured. The substances with the same retention times as these authentic amino acids are listed in Table 3. The identities with respect to the retention times on the amino acid analyzer were further confirmed by a mixed experiment using the samples and authentic amino acids. The individual peak area calculated on the basis of the response factor to the peak area of a known amount of glycine gave the content of the products as is shown in Table 3. The recovery (46.9%) introduced by the summation of the peak area was rather wrong. This might be due to the difference in sensitivity between the fluorescamine and ninhydrin detections. Another possibility was that the sample might contain high-basic compounds yet retained on the resin. To confirm the former possibility, the determination of the amino groups in the hydrolyzate by the ninhydrin method is required, but this is difficult because of the unavoidable contamination of the ammonia used in a large amount in the extraction procedure. However, the lesser sensitivity of β-amino groups compared with that of α-amino groups is well-known. It seems to explain the incomplete recovery in the amino acid analyzer with ninhydrin detection.

In a separate experiment, several individual peaks were obtained by means of the manual handling of the amino acid analyzer. The total recovery was about 70% by the use of fluorescamine. Especially, the amount of Peak No. 30 was 5.22 mol%. The difference between the yields determined by ninhydrin (2.40 mol%) and by fluorescamine seems to show, together with the fact of the same retention time, that this peak (Peak No. 30) was β-alanine. Furthermore, the fractions corresponding to Peaks No. 9, 15, 16, and 30, shown in Fig. 1, were analyzed by thin-layer chromatography coated with cellulose powder. The R_f values

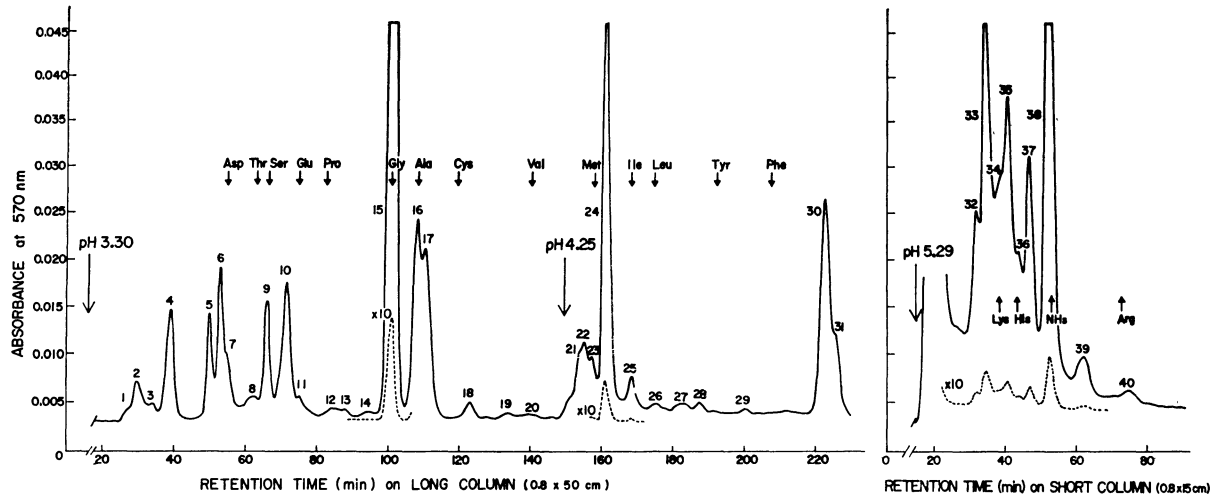

Fig. 1. Amino acid analysis of the acid hydrolyzate from the concentrated "Golden Primordial Broth." The experimental conditions were given in the text.

Table 3. Amino acid analysis of the hydrolysate of "golden primordial broth"

Peak No.	t_R	Amounts of amino acid residues		Authentic amino acids with the same t_R value
	min	μ mol	mol %	
1.	27.6	3.20	0.244	Phosphoserine
2.	29.8	6.08	0.466	
3.	34.1	2.06	0.157	Taurine
4.	39.1	13.55	1.03	
5.	49.9	9.36	0.714	
6.	52.9	15.44	1.18	
7.	55.0	3.43	0.262	Asp
8.	62.0	6.24	0.476	Thr
9.	66.0	11.68	0.891	Ser
10.	71.5	18.37	1.40	Homoserine
11.	75.0	4.72	0.360	Glu
12.	84.4	2.50	0.191	
13.	87.8	1.52	0.116	
14.	94.6	1.82	0.139	
15.	100.9	114.20	8.71	Gly
16.	108.0	25.95	1.98	Ala
17.	110.2	28.51	2.17	
18.	123.0	2.32	0.177	α-Aminobutyric acid
19.	133.6	1.14	0.087	
20.	140.0	0.95	0.072	Val
21.	153.4	7.40	0.564	
22.	154.8	8.55	0.652	
23.	157.0	4.98	0.380	Met
24.	161.0	42.59	3.25	Norvaline
25.	168.2	4.71	0.359	Ile
26.	175.0	1.71	0.130	Leu
27.	182.5	1.71	0.130	Norleucine
28.	187.4	1.46	0.112	
29.	200.0	0.40	0.031	
30.	222.6	31.46	2.40	β-Alanine
31.	225.2	12.52	0.955	
32.	32.0	33.85	2.58	
33.	34.8	72.02	5.49	
34.	39.0	3.53	0.269	Lys and γ-Aminobutyric acid
35.	41.0	59.87	4.57	
36.	44.0	1.37	0.104	His
37.	47.3	34.47	2.63	
38.	52.9			Ammonia
39.	62.6	12.08	0.921	
40.	75.0	6.75	0.515	Arg

The amounts of amino acid residues corresponding to the respective peaks shown in Fig. 1 were calculated on the basis of the response factor to the peak area of the known amount of glycine obtained from a separate experiment under the same operation conditions of the automatic amino acid analyzer.

in the butyric acid : acetic acid : water (4 : 1 : 5, by volume) solvent system were 0.13, 0.15, 0.22, and 0.23 respectively. These values agreed with those obtained with the authentic amino acids, serine, glycine, alanine, and β-alanine respectively. The purple colorations after the spray of ninhydrin except in the case of β-alanine were practically the same (the coloration for β-alanine was blue-violet). Since these compounds had the same retention times on the automatic amino acid analyzer as those of the respective authentic amino acids, these results indicate that the compounds eluted at the positions of Peaks No. 9, 15, 16, and 30, shown in Fig. 1, correspond to serine, glycine, alanine, and β-alanine respectively. The other compounds eluted on the automatic amino acid analyzer could not be analyzed by means of the thin-layer chromatography because of their small amounts.

Gel-filtration of Concentrated "Golden Primordial Broth" using Biogel P-2. Most (15 ml) of the concentrated "Golden Primordial Broth" was placed in a column packed with Biogel P-2 (fractionation range; MW 100—1800) equilibrated with 0.1 M ammonium hydrogencarbonate. The chromatographic patterns of the products containing, as expected, the high-molecular-weight

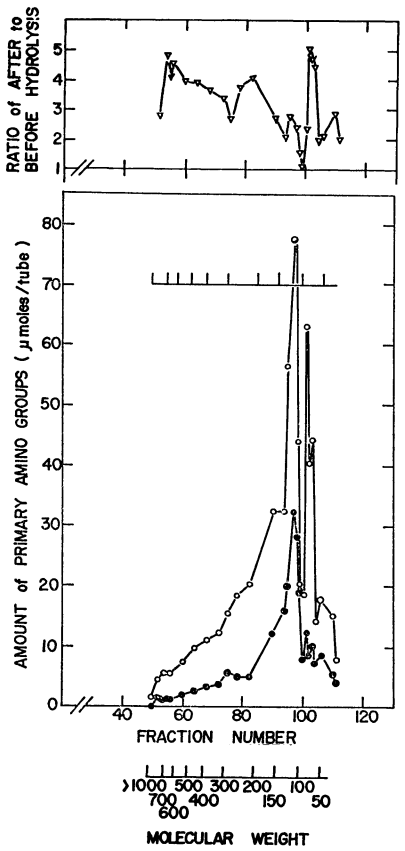

Fig. 2. Elution profiles of Biogel P-2 column chromatography of the concentrated "Golden Primordial Broth." The procedure of the column chromatography was given in the text. Closed and open circles represent the amount of primary amino groups determined by the usage of fluorescamine before and after acid hydrolysis, respectively. Reversed open triangles in the upper figure represent the ratio of the amount of primary amino groups after to before acid hydrolysis. The given scale of molecular weight was obtained from the separate experiment using the several authentic compounds listed in the text.

compounds are shown in Fig. 2. After the lyophilization of each fraction in order to remove the excess of ammonium hydrogencarbonate, the resulting powder was brought back to the original volume with distilled water. The amount of primary amino groups in each fraction before and after acid hydrolysis was measured by using fluorescamine, with leucine as the standard. As can be seen in Fig. 2, a considerable amount of the compounds with primary amino groups was found in the high-molecular-weight fractions. In addition, the ratio of the amount of primary amino groups after to before acid hydrolysis in the column effluents increased by about 5-fold, together with the molecular size. These results mean that the compounds present in the high-molecular-weight fractions possessed latent primary amino groups which appeared as primary amino groups upon 6 M HCl hydrolysis, and suggest that they were oligomers consisting of several amino acid residues.

The sharp peak having a rather high ratio of the amount of primary amino groups after to before acid hydrolysis but eluted in the small-molecular-weight region (corresponding to a molecular weight of about 100) might contain a cyclic dipeptide of glycine, 2,5-piperadinedione.

Three column effluents — *i. e.*, Fraction Number 55 (at the position with 700 daltons as the average molecular weight), Fraction Number 75 (at 300 daltons), and Fraction Number 95 (at 130 daltons) — were analyzed for amino acid composition using the automatic amino acid analyzer. The amount of 40 species of ninhydrin positive materials is shown in Table 4 in mol%. The difference between the composition in the high- and low-molecular-weight fractions was tended to be small under the present experimental conditions.

Some Characteristics of the Oligomer Fractions Eluted from the Biogel P-2 Column. The collected fractions from Fraction Numbers 51 to 58 (oligomer fractions) were subjected to the following spectrophotometric measurements. Figure 3 shows the ultraviolet and infrared absorption spectra of the oligomer fraction. A shoulder peak in the neighborhood at 280 nm was observed in the ultraviolet spectrum. A rather broad, low-resolution band in the region at 1500—1600 cm^{-1} was also detected in the infrared spectrum. These results were not in contradiction with the existence of oligopeptides in the oligomer fraction. However, this fraction certainly contains various compounds other than oligopeptides. In fact, a considerble amount of sugar derivatives was detected in a separate experiment. Thus, the above findings can not be regarded as evidence for the presence of oligopeptides or peptide bonds.

In a separate experiment, an enzymatic degradation of the oligomers was attempted using pronase, a bac-

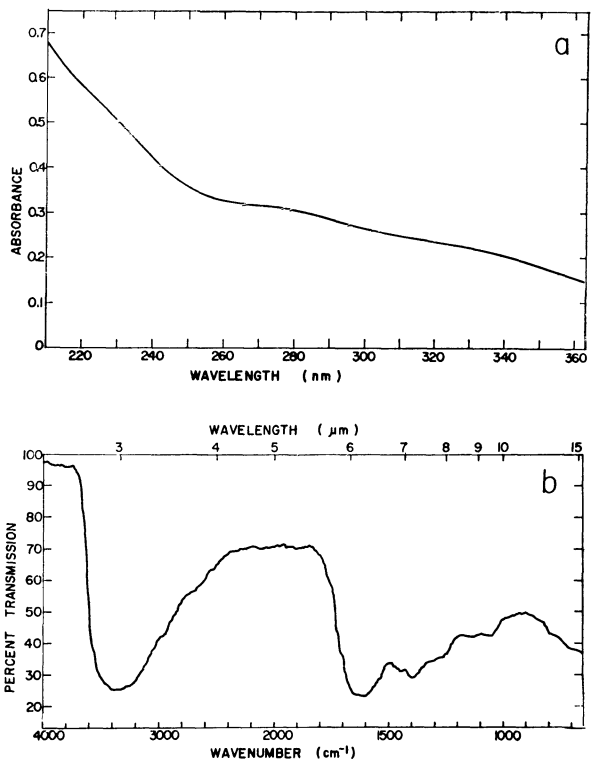

Fig. 3. Ultraviolet (a) and infrared (b) spectra of the oligomers. Experimental conditions were given in the text.

Table 4. Amino acid analysis of the hydrolysate of biogel *P*-2-column effluents

Peak No.	t_R	Amounts of amino acid residues in these fractions			Authentic amino acids with the same t_R value
		No. 55	No. 75	No. 95	
	min		mol %		
1.	27.6	0.090	0.0832	0.141	Phosphoserine
2.	29.8	0.489	0.670	0.441	
3.	34.1	0.206	0.254	0.220	Taurine
4.	39.1	1.23	1.66	0.679	
5.	49.9	0.504	0.832	1.21	
6.	52.9	0.0326	0.208	1.55	
7.	55.0	0.662	1.04	0.397	Asp
8.	62.0	0.348	0.208	0.727	Thr
9.	66.0	0.557	1.02	1.27	Ser
10.	71.5	0.472	0.924	2.22	Homoserine
11.	75.0	0.574	0.333	0.304	Glu
12.	84.4	0.0869	0.259	0.0529	
13.	87.8	0.0326	0.0555	0.264	
14.	94.6	0.104	0.222	0.0353	
15.	100.9	9.81	8.65	13.74	Gly
16.	108.0	1.33	1.47	3.31	Ala
17.	110.2	0.424	0.970	2.01	
18.	123.0	0.335	0.162	0.370	α-Aminobutyric acid
19.	133.6	0.209	0.0693	0.0441	
20.	140.0	0.313	0.0104	0.0441	Val
21.	153.4	0.804	0.243	0.645	
22.	154.8	0.804	1.22	0.705	
23.	157.0	1.09	0.635	0.487	Met
24.	161.0	1.46	2.51	2.38	Norvaline
25.	168.2	0.417	0.403	0.317	Ile
26.	175.0	0.209	0.0924	0.0661	Leu
27.	182.5	0.109	0.0693	0.0661	Norleucine
28.	187.4	0.304	0.243	0.0551	
29.	200.0	0.0326	0.0139	0.0220	
30.	222.6	1.44	4.46	1.78	
31.	225.2	0.526	0.776	0.446	β-Alanine
32.	32.0	0.522	1.39	4.17	
33.	34.8	4.90	9.98	7.29	
34.	39.0	0.261	3.70	3.53	Lys and γ-Aminobutyric acid
35.	41.0	4.07	3.70	6.08	
36.	44.0	3.00	0.693	4.26	His
37.	47.3	2.29	6.70	1.98	
38.	52.9				Ammonia
39.	62.6	1.28	0.834	1.96	
40.	75.0	0.176	0.231	0.848	Arg

terial non-specific protease. Contray to expectations, no amino acids and no newly-formed primary amino groups were detected — only the autolysis of pronase itself. A similar observation was reported by Ferris *et al.*,[12] who used the oligomers derived from HCN. They reported that no amino acids were released from the oligomers by pronase. Based on their results, they concluded that the oligomers did not contain peptide bonds.

In our case, however, taking into consideration the abundant formation of amino acids in the monomer fractions and the reasonable ratio of the primary amino group content after acid hydrolysis to before the procedure for the molecular size, the apparent non-susceptibility to pronase may be attributed, not to the absence of peptide bonds, but to the complex nature of the products with the predominant amount of unnatural peptide bonds due to D-amino acids, other nonprotein amino acids such as β-alanine, and those due to diamino or dicarboxylic amino acids.

Phosphate Ester Hydrolysis by the Oligomer Fractions Formed from Formaldehyde and Hydroxylamine. The oligomers formed in the modified sea mediums containing several transition elements were tested for the hydrolysis of the phosphate ester bond of *p*-nitrophenyl phosphate, as is shown in Fig. 4. Four column effluents, *i.e.*,

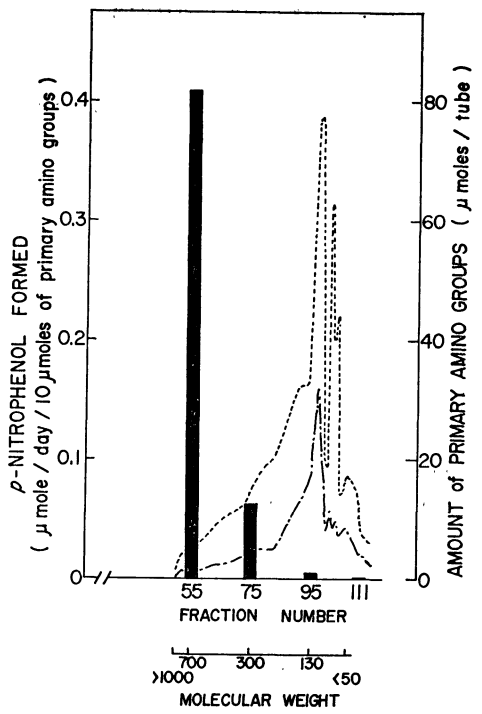

Fig. 4. Phosphate ester hydrolysis by the oligomer fractions. Closed bar represent the specific activity of alkaline phosphatase in the fractions obtained from the Biogel P-2 column chromatography shown in Fig. 2. Dotted and broken lines were the trace lines of the amount of primary amino groups before and after acid hydrolysis, respectively, obtained from the experiment shown in Fig. 2.

Fraction Numbers 55, 75, 95, and 111, were tested; only the high-molecular-weight fractions were found to show any hydrolytic activity for *p*-nitrophenyl phosphate. This catalytic activity was presented as μmol of *p*-nitrophenol formed per day per 10 μmol of primary amino groups in its hydrolyzate. This representation was regarded as μmol/day/mg oligopeptide, by assuming that each amino acid residue has 100 daltons as its molecular weight. Oshima[11] reported that proteinoid (molecular weight, 2500—4000), termally prepared polyamino acids containing 18 common amino acids, promotes the hydrolysis of the ester bond of *p*-nitrophenyl phosphate; its specific activity, 3 μmol/day/mg proteinoid at 30 °C. As can be seen in Fig. 4, the oligomers in Fraction Number 55 (average molecular weight, 700) had 0.409 μmol/day/mg oligopeptide at 37 °C. Its specific activity seems reasonable considering the different molecular weight. The possibility that the activity found was due to contamination by microorganisms or by phosphatases from them can be rejected on the basis of the following observations: toluene was added to hinder the growth of microorganisms; the time course of the reaction was linear, unlike the logarithmic growth curve occurring in the case of bacterial growth; only the high-molecular-weight fractions showed catalytic activities. Of course, another possibility — that this catalytic hydrolysis of *p*-nitrophenyl phosphate might be due to a high-molecular-weight metal complex of different constituents — cannot be excluded. At any rate, it is remarkable that a phosphatase-like activity appeared.

Dependency of the Production of Amino Acids and the Related Oligomers from Formaldehyde and Hydroxylamine on Transition Elements and Clays. The requirements for the production of amino acids and related oligomers from formaldehyde and hydroxylamine were investigated in small-scale (10 ml) experiments, as is shown in Table 5. The production of primary amino groups after acid hydrolysis in the complete system (Exp. a) was calculated as a 5.52% yield from hydroxylamine; this value is almost the same as that in the former large-scale experiment. However, the production of primary amino groups in the hydrolyzates in the minus experiments, such as minus transition elements (Exp. b) and minus clays (Exp. c), was considerably decreased, by 81.5% and 75.7% respectively. The composition of the amino acid residues was examined by using the automatic amino acid analyzer in separate experiments. The composition was almost the same as that in the former large-scale experiments. The no-reaction control (stocked at −20 °C instead of reacted at 105 °C) experiment (Exp. d) gave a small amount of the primary amino groups (0.67% yield). It was found to consist practically exclusively of glycine (41.3 mol%) by a separate experiment using the automatic amino acid

Table 5. Requirements of the production of amino acids and the related oligomers from formaldehyde and hydroxylamine in modified sea mediums

Experiments	Amounts of primary amino groups		Ratio of (B) to (A)
	Before acid hydrolysis (A)	After acid hydrolysis (B)	
	μmol		
a. Complete	9.51	27.6	2.90
b. Minus transition elements (Mo^{6+}, Zn^{2+}, Cu^{2+}, Fe^{3+}, Co^{2+}, Mn^{2+})	7.18	22.5	3.13
c. Minus clays (kaolin, montmorillonite)	7.69	20.9	2.71
d. No-reaction control	1.95	3.34	1.71

The reaction in the complete system (Exp. a) with the reaction mixture listed in Table 1 was carried out in a glass-sealed ampoule at 105±5 °C for 35 days on a small scale (10 ml of total volume). The reaction in the minus system (Exp. b and c) was carried out in the same way except for the omission of transition elements and clays respectively. The no-reaction control (Exp. d) with the same reaction mixture as the complete system was stocked in a glass-sealed ampoul at −20 °C for 35 days. The extraction procedures and the determination of primary amino groups are described in the text.

analyzer. This result shows that most of the compounds with primary amino groups formed in the no-reaction control were not due to the contamination of the impurities in the starting materials such as clays or to the contamination during the experimental handling, and that the formation of glycine in modified sea mediums was so easy that such a detectable amount of glycine could be formed during storage at −20 °C and operation at room temperature. It should be pointed out that clays were not absolutely required for either the formation of amino acids or that of oligomers, as had been observed in preliminary experiments by Ventilla and Egami.[8] It remains unsolved, however, whether the formation of amino acids and oligomers in the case of minus transition elements was realized by the clays themselves or by transition elements contained in the clays.

An aliquot of each product formed from Exps. a, b, c, and d was placed in a small column packed with Biogel P-2 and equilibrated with 0.1 M ammonium hydrogencarbonate. The elution profile obtained by measuring the amount of primary amino groups before and after acid hydrolysis is shown in Fig. 5. All these elution patterns except for that of Exp. d (no-reaction control) were observed to be almost the same. However, the formation of primary amino groups in the high-molecular-weight fraction in Exp. b (minus transition elements) was considerably decreased compared with that in Exp. a (complete). The relatively low production suggests that these transition elements were required for the formation of oligomers in the modified sea mediums. In fact, a separate experiment using a higher concentration (0.001 M) of molybdenum was found to give about twice as much of the primary amino groups.

Effect of the Reaction Time on the Formation of Amino Acids and the Related Oligomers from Formaldehyde and Hydroxylamine in Modified Sea Mediums. The time-dependent formation of primary amino groups before and after acid hydrolysis was measured as is shown in Fig. 6. The formation of primary amino groups before acid hydrolysis was observed to reach its maximum in about 30 days. However, the formation of primary amino groups after acid hydrolysis seemed to progress over a reaction time of about 40 days. As a consequence, the ratio of the content of primary amino groups after to before acid hydrolysis was found to increase linearly for 30 days. This means that the formation of oligomers required rather a long reaction time at 105 °C. The products obtained in different reaction times were measured for their amino acid composition using the automatic amino acid analyzer in separate experiments. No amino acids required a specially long reaction time for the formation, but the formation of alanine, β-alanine, and especially glycine was found to reach the maximum at rather an early stage.

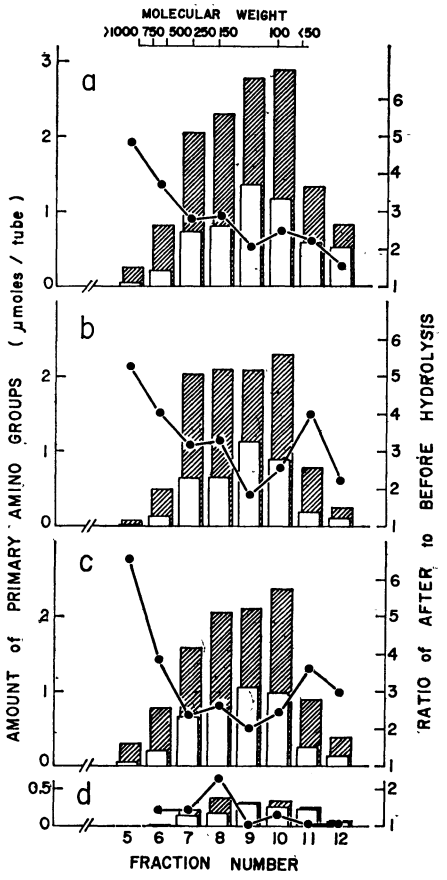

Fig. 5. Elution profiles of small-scale Biogel P-2 column chromatographies of the products obtained from the requirement experiments. Four column profiles; a, b, c, and d, represent that obtained from the Exp. a, b, c, and d, respectively, shown in Table 5. Open and shadowed bars represent the amount of primary amino groups before and after acid hydrolysis, respectively. Closed circles represent the ratio of the amount of primary amino groups after to before acid hydrolysis.

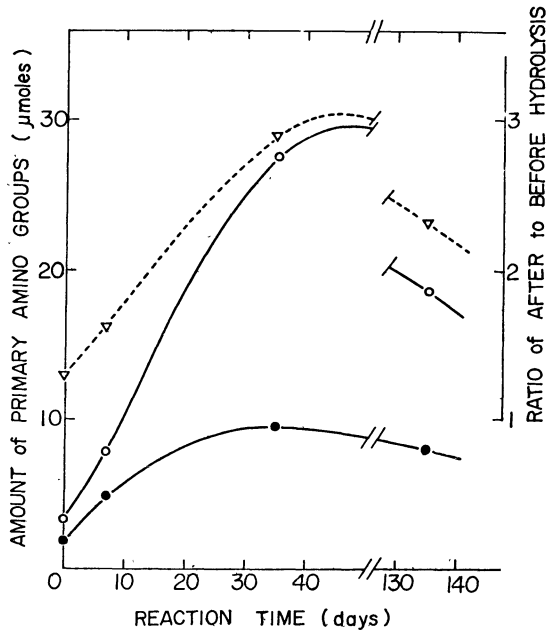

Fig. 6. Time course of the formation of amino acids and related oligomers from formaldehyde and hydroxylamine. Closed and open circles represent the amount of primary amino groups before and after acid hydrolysis, respectively. Reversed open triangles represent the ratio of the amount of primary amino groups after to before acid hydrolysis.

General Discussion. Amino acids were the first organic compounds of biological interest produced under simulated primitive earth conditions.[1] Reactions in one-step processes from simple compounds of carbon with simple nitrogen compounds have been accomplished by several investigators using highly activating forms of energy, such as electric discharges, ultraviolet light, and ionizing radiation [shown in the literature listed in the monograph[13]]. A common feature of all these experiments is the employment of highly activating forms of energy. In these experiments, part of the energy was found to be used to oxidize methane to formaldehyde, and in the reduction of nitrate. Thus, certain C_1 and N_1 compounds, such as formaldehyde and hydroxylamine respectively, of intermediate degrees of oxidation could be expected to produce amino acids even in the absence of electric discharges, ultraviolet, and ionization radiation. Based on the above consideration, Oró *et al.*[7] first reported that it is possible to synthesize appreciable amounts of glycine and certain amino acids by heating aqueous solutions of paraformaldehyde and hydroxylamine hydrochloride at a moderate temperature [Compare 14].

Egami's hypothesis that the composition of the present sea water reflects that of the primeval sea water at the time of early evolution suggests the possibility that the abundant transition elements in sea media, such as iron, molybdenum, and zinc, might play an important role in the prebiotic amino acid synthesis.[5] In addition, these transition elements would be expected to evolve to form protoenzymes.[6] In fact, as is reported in the present paper, the possible direct formation of amino acids and the related oligomers from formaldehyde and hydroxylamine, with six species of essential transition elements and clays, was realized in modified sea mediums. It was of especial interest to ascertain the phosphatase activity in the oligomeric products. The hydrolytic activity of the oligomers (with 700 daltons as the average molecular weight) was found to be 0.409 μmol/day/mg oligopeptide by regarding the oligomers as oligopeptides. This protoenzyme-like activity is in good correlation with the consideration by Egami[6] that the zinc complex in the early stage of evolution may be regarded as a precursor of hydrolytic and transferring enzymes, including enzymes participating in the metabolism of macromolecules and information transfer.

However, attempts to find positive evidence for the existence of peptide bonds in the oligomers formed from formaldehyde and hydroxylamine were unsuccessful. It must be considerably difficult to find the peptide linkage in the crude oligomers mixture, for the oligomers would consist of various compounds of different natures, but all highly microheterogeneous. We hope that it will be elucidated by future experiments.

In the former paper of Oró *et al.*,[7] they reported that the formation of amino acids from paraformaldehyde and hydroxylamine occurred under acidic as well as basic conditions, but no measurable formation of amino acids took place between pH 3 and 6.

Contrary to their finding, in our experiments with modified sea mediums amino acid formation took place at pH 5.5. We chose the pH expecting the accumulation of oligopeptides. As can be seen in Fig. 6, although a rather long time (approximately 40 days) was required for the maximum yield of primary amino groups in the acid hydrolysate, oligomers accumulated as expected. The total yield of primary amino groups was comparable with that reported by Oró *et al.*[7]

In course of the reaction the reaction mixture gradually colored and finally gave rise to the "Golden Primordial Broth." The nature of the colored substance has not been studied, but in view of the nature of the starting materials and intermediates, it might be regarded as a melanoidin-like polymer, the important role of which in chemical evolution has recently been suggested by Nissenbaum *et al.*[15]

It is generally accepted that the origin of life took place in the primeval sea of the earth and that most of the organic substances of general biological interest accumulated in the primeval sea. Our research may be regarded as an attempt to search for the synthesis of organic compounds in modified or improved primeval sea mediums. We hope that it will not only contribute to the elucidation of chemical evolution, but will also open a new way to organic synthesis in general.

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