CONSTRUCTION OF PROTOCELLULAR STRUCTURES UNDER SIMULATED PRIMITIVE EARTH CONDITIONS

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Abstract. We have developed experimental approaches for the construction of protocellular structures under simulated primitive earth conditions and studied their formation and characteristics. Three types of envelopes; protein envelopes, lipid envelopes, and lipid-protein envelopes are considered as candidates for protocellular structures. Simple protein envelopes and lipid envelopes are presumed to have originated at an early stage of chemical evolution, interaction mutually and then evolved into more complex envelopes composed of both lipids and proteins.

Three kinds of protein envelopes were constructed *in situ* from amino acids under simulated primitive earth conditions such as a fresh water tide pool, a warm sea, and a submarine hydrothermal vent. One protein envelope was formed from a mixture of amino acid amides at 80 °C using multiple hydration-dehydration cycles. Marigranules, protein envelope structures, were produced from mixtures of glycine and acidic, basic and aromatic amino acids at 105 °C in a modified sea medium enriched with essential transition elements. Thermostable microspheres were also formed from a mixture of glycine, alanine, valine, and aspartic acid at 250 °C and above. The microspheres did not form at lower temperatures and consist of silicates and peptide-like polymers containing imide bonds and amino acid residues enriched in valine. Amphiphilic proteins with molecular weights of 2000 were necessary for the formation of the protein envelopes.

Stable lipid envelopes were formed from different dialkyl phospholipids and fatty acids.

Large, stable, lipid-protein envelopes were formed from egg lecithin and the solubilized marigranules. Polycations such as polylysine and polyhistidine, or basic proteins such as lysozyme and cytochrome *c* also stabilized lipid-protein envelopes.

1. Introduction

What is life on earth? Life primarily consists of three elements. First, life has a phase boundary or an envelope within which is isolated a portion of the environment. The smallest unit of life is the cell. Second, life is self-replicating. RNA, proteins and the substances of living cells are synthesized on the basis of information stored in DNA sequences. Third, life requires metabolism conducted by enzymes. Life, in other words, is a molecular machine, driven and grown by molecular interactions of living matter synthesized on the basis of genetic information of DNA in a cell.

Biomembranes are composed of lipids and proteins in fluid sheets only two molecules thick [1]. The thickness of bilayer of lipid is 8–10 nm. The lipids, containing both hydrophobic and hydrophilic domains, form fluid bilayers in water that are selective barriers to polar molecules. Proteins serve distinct functions in membranes

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such as enzymes, receptors, pump, energy transducer, or as gates allowing ions or molecules to pass through the membrane.

An envelope which isolates a portion of environment is essential for the genesis of life. In aqueous systems such an envelope may be hydrophobic. Oparin's coacervate [2] and Fox's proteinoid microsphere [3] demonstrated that envelopes may self-assemble in an aqueous system from partly hydrophobic materials.

To date, several models for protocellular structures have been proposed [4–8]. It is not known how protocellular structures might have originated on the primitive earth. We suggest that simple protein envelopes and simple lipid envelopes first evolved at an early stage of chemical evolution, and then interacted to develop into more complex envelopes composed of lipids and proteins: lipid envelopes embedded with proteins (I), protein envelopes coated with lipid envelopes (II), or lipid envelopes coated with protein envelopes (III) (Figure 1).

We have attempted to build models for protocellular structures composed of lipids, proteins, or both under simulated primitive earth conditions. This paper presents experimental approaches for constructing such protocellular structures.

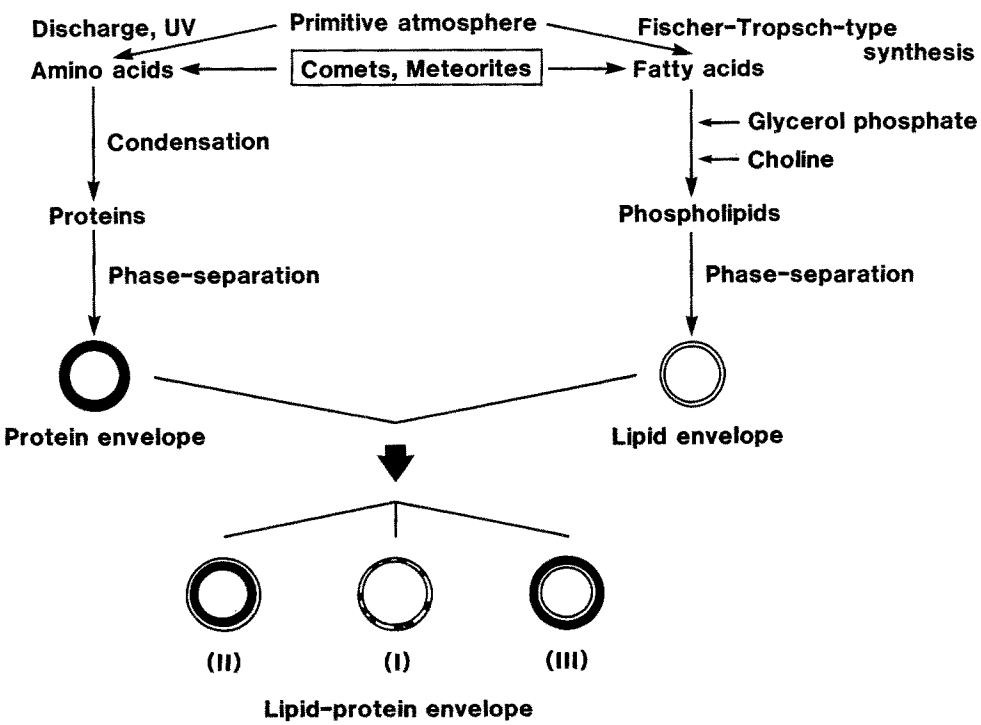

Fig. 1. The origin and organization of possible protocellular structures. Three types of envelopes; protein envelopes, lipid envelopes, and lipid-protein envelopes are considered as candidates for protocellular structures. Three kinds of lipid-protein envelope are also possible; lipid envelopes embedded with proteins (I), protein envelopes coated with lipid envelopes (II), and lipid envelopes coated with protein envelopes (III).

2. Materials and Methods

2.1. Materials

Glycinamide hydrochloride, *L*-alaninamide hydrobromide, *L*-valinamide hydrochloride, triglycine, hexaglycine, polyaspartic acid (M.W. 14000), polyglutamic acid (M.W. 14000), polylysine (M.W. 14000), polyarginine (M.W. 100000), polyhistidine (M.W. 9300), cytochrome *c*, RNase A, egg lecithin, dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylcholine (DOPC), n-octyl glucoside, trypsin, hog pancreatic elastase, and fluorescamine were obtained from Sigma Chemical Company (St. Louis, MO). Sodium dodecyl sulfate (SDS), lithium bromide, and oleic acid were from Wako Pure Chemical Industries (Osaka, Japan); lysozyme from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan); Bio-Gels P-2 and P-4 (200–400 mesh) from Bio Rad Laboratories (Richmond, CA); Blue dextran 2000 from Pharmacia Fine Chemicals (Uppsala, Sweden)); proteinase K from Merck (Rahway, NJ); and Pronase (a protease from *Streptomyces griseus*) from Kaken Chemicals, Ltd. (Tokyo, Japan); amino acids from Takara Kosan Co., Ltd. (Tokyo, Japan). *L*-Aspartic acid α-amide hydrochloride was prepared from N-carbobenzoxy-*L*-aspartic acid-β-benzylester [9]. A synthetic polypeptide (YRKLRKRLLRD) was kindly provided by Dr. K. Sato of this Institute. All chemicals were analytical grade.

2.2. Synthesis of polypeptides during repeated hydration - dehydration cycles

A reaction mixture (10 ml) containing 0.03–0.1 M amino acid amides and 0.14 M $KHCO_3$–NaH_2PO_4 buffer (pH 7.2) was placed in a beaker (100 ml, Ø60 mm × 70 mm). The solution was completely evaporated to dryness by heating at 80 °C for an hour and then heated for another hour at 80 °C. Fresh reaction mixture (10 ml) was added to the residue. The pH was adjusted to 7.2 with 1 N HCl and the suspension heated at 80 °C for another hour. This series of operations was repeated 20 times. The residue was then extracted with water and divided into water-soluble and water-insoluble fractions. Both fractions were lyophilized and stored at − 20 °C.

2.3. Synthesis of marigranules in a modified sea medium

Marigranules were synthesized from *L*-tryptophan or a mixture of glycine and acidic, basic and aromatic amino acids in a modified sea medium or in water at 80 °C. The modified sea medium contained 0.01 M (each) $MgSO_4$, $CaCl_2$, and K_2HPO_4, and 0.1 mM (each) of six transition elements, Na_2MoO_4, $Fe(NO_3)_3$, $ZnCl_2$, $Cu(NO_3)_2$, $MnCl_2$, and $CoCl_2$. *L*-Tryptophan (0.05 M) or a mixture of glycine (0.05 M) and *L*-glutamic acid, *L*-aspartic acid, *L*-lysine, *L*-arginine, *L*-histidine, *L*-phenylalanine, *L*-tryptophan and *L*-tyrosine (each 5 mM) were added to the medium. The pH was adjusted to 6.0 with 0.1 N KOH and 2.5 liters of the mixture was placed in a 3 liters Erlenmeyer flask and heated at 80 °C. After 3 weeks highly organized particles were produced in the reaction mixture. The particles were filtered on a Nuclepore membrane filter of 0.2 μm pore size. The precipitates were collected and suspended in a

small amount of the filtrate. We have designated the organized particles produced in the modified sea medium 'marigranules' because they are granules produced under a condition similar to marine environments [6].

2.4. Synthesis of thermostable microspheres in superheated hydrothermal environments

Thermostable microspheres were synthesized from a mixture of glycine, alanine, valine, and aspartic acid in aqueous solutions at 200–350 °C. A reaction mixture (15 ml) containing 0.3 M glycine, 0.1 M *L*-alanine, 0.3 M *L*-valine, 0.1 M *L*-aspartic acid, and 0.1 M $KHCO_3$–NaH_2PO_4 buffer (pH 7.2) was put into a Pyrex tube (Ø20 × 105 mm), which was capped and placed in a stainless steel autoclave (70 ml) which was encased in an aluminum heating block. The autoclave was heated at 200 °C, 250 °C, 300 °C, or 350 °C for 6 hr. The resulting solution was centrifuged and the precipitate was collected.

2.5. Preparation of reconstituted protein envelopes (marisomes) from solubilized marigranules by dialysis

Marigranules (100 mg) synthesized from *L*-tryptophan, were added to methanol (50 ml) and stirred at room temperature overnight. The mixture was centrifuged at 3500 rpm for 30 min. The supernatant was evaporated under reduced pressure to give solubilized marigranules (70 mg). Solubilized marigranules (1.4 mg) were added to a 2% aqueous *n*-octyl glucoside solution (3 ml), heated at 80 °C for 15 min, and dialyzed against distilled water at room temperature for 20 hr.

Preparation of Lipid Vesicles

Lipid films were prepared by rotatory evaporation of a chloroform solution (100 mg/ml of chloroform, 0.15 ml) under vacuum. The resulting thin films were swollen in a mixture of distilled water (1.8 ml), 0.2 M Tris-HCl buffer, pH 7.5 (0.3 ml) and 1 M KCl (0.3 ml). To the suspension, 10% aqueous *n*-octyl glucoside solution (0.6 ml) was added. The mixture was put in cellulose tubing and dialyzed against 0.1 M KCl–0.02 M Tris–HCl buffer (pH 7.5). The dialysis solution was changed six times at 1-hr intervals, then left overnight for a total period of 20 hr. The dialysis was performed above the phase transition temperature (Tc) of the lipid; egg lecithin, DOPC and oleic acid at room temperature, DPPC at 50 °C. The vesicles from oleic acid were prepared according to the method of Haines [10].

2.6. Preparation of lipid-protein envelopes

The lipid films were prepared by evaporating a chloroform solution of lipid (100 mg/ml, 0.35 ml) under vacuum and swollen in a mixture of distilled water (4.2 ml), 0.2 M Tris–HCl buffer, pH 7.5 (0.7 ml) and 1 M KCl (0.7 ml). To the suspension, 10% aqueous *n*-octyl glucoside solution (1.4 ml) was added. Methanol-solubilized marigranules (0.7 mg) were dissolved in 10% aqueous *n*-octyl glucoside solution (0.6 ml) by heating at 80 °C for 10 min. To the solution of the solubilized

marigranules, a suspension of lipid was added and the mixture was put in cellulose tubing and dialyzed against 0.1 M KCl–0.02 M Tris-HCl buffer (pH 7.5). The dialysis was performed as above. Proteins (cytochrome *c*, lysozyme, RNase A and bovine serum albumin) and polyamino acids (polyaspartic acid, polyglutamic acid, polylysine, polyarginine and polyhistidine) were brought into contact with the lipids using the same procedure.

2.7. GEL CHROMATOGRAPHY

The molecular weights of the resulting polypeptides were estimated by gel chromatography on a Bio-Gel P-2 or P-4 (200–400 mesh) column (Ø1.0 cm × 28.3 cm, 22.2 ml). Waterinsoluble polypeptides (2 mg) were dissolved in 200 μl of 10 M LiBr solution, heated at 40 °C and then diluted with three 10 μl portions of 10 mM H_3PO_4-Tris buffer (pH 6.8) – 1% SDS – 8 M urea solution. After centrifugation at 3500 rpm for 15 min, 110 μl of the supernatant was applied to the column equilibrated with 2.5 mM H_3PO_4-Tris buffer (pH 6.8) – 0.25% SDS – 2 M urea.

Polypeptides were eluted with the same buffer solution at room temperature using 3 ml/hr flow rate. Fractions of 0.5 ml were collected and analyzed for polypeptides with a Gilford 2400-2 spectrophotometer at 230 nm. Blue dextran was used to determine the void volume. Triglycine (M.W. 189), hexaglycine (M.W. 360), and a synthetic polypeptide (M.W. 1500) were used to calibrate the column.

2.8. AMINO ACID ANALYSIS

Polypeptides (1 mg) were hydrolyzed with 6 N HCl in an evacuated and sealed glass tube at 110 °C for 72 hr. After hydrolysis, hydrochloric acid was removed by rotary evaporation and the residues were dissolved in 0.2 M sodium citrate buffer (pH 2.20). Polypeptides containing valine residues were also hydrolyzed with trifluoroacetic acid-12N HCl (1 : 1) at 175 °C for 24 hr [11]. Amino acid analysis was performed with a Hitachi KLA-5 amino acid analyzer.

2.9. LIGHT AND FLUORESCENCE MICROSCOPIES

Envelopes were routinely examined with a Nikon phase-contrast microscope and a Zeiss-WL fluorescence microscope. Fluorescence was monitored at 485 nm after by excitation at 419–443 nm.

2.10. ELECTRON MICROSCOPY

For scanning electron microscopic (SEM) observation of envelopes, the aqueous suspension of envelopes was placed on a clean glass coverslips and gently dried at room temperature. The dried specimen was coated with platinum : palladium (80 : 20) in an Eiko IB-3 ion coater. Each specimen was examined under a JEM 100CX-ASID operated at 40 kV. For negatively stained electron microscopic observation of envelopes, a drop of the suspension of envelopes was placed on a carbon-coated collodion grid and after excess samples had been removed with filter paper 1% phosphotungustate or 2% uranyl acetate was applied to the grid. An excess of staining solution was re-

moved with filter paper after 10 s and the grid was immediatelly examined under a JEM 100B operated at 60–80 kV. In order to observe the interior structure of envelopes transmission electron microscopy (TEM) was used. The thin sections were cut with glass knives of a LKB Cryo Nova ultramicrotome operated at −120 to −150 °C, mounted on a carbon-coated collodion grid, dried, stained in vapor of 3% aqueous ruthenium tetroxide solution, and photographed with a JEM 1200 EX operated at 120 kV. Marigranules were also embedded in Epon 812. The thin sections (800 Å) were cut and photographed with a JEM 100B operated at 80 kV.

2.11. Determination of size distribution of lipid vesicles and lipid-protein envelopes

Lipid vesicles and lipid-protein envelopes were prepared as described above. Light and fluorescence micrographs of lipid vesicles and lipid-protein envelopes taken at magnification × 400 were used for their size determination. The photographs were printed at 6 times the negative enlargements. Negative stain electron micrographs of lipid-protein envelopes were prepared as described above. The photographs were printed at 1.7 times the negative enlargements. The diameters of individual vesicles were measured with a Kontron MOP-AMO2 semi-automatic image analyzer. Two hundred vesicles were measured for each sample.

2.12. Determination of phospholipids and marigranules

Phospholipids in lipid or lipid-protein envelopes were estimated by phosphate assay [12]. Marigranules were measured with a Shimadzu RF-502 fluorescence spectrophotometer. The samples were excited at 360 nm and emitted at 431 nm.

2.13. Differential scanning calorimetry

The phase-transition temperature (Tc) of lipid dispersions were determined with a differential scanning calorimeter (Daini Seikosha SSC-560) [13]. The lipid suspension (1 mg of lipid/60 μl of 0.1 M KCl – 0.02 M Tris-HCl buffer, pH 7.5) was encapsulated in a calorimeter aluminum pan. The heating rate was 0.5 °C/min. Transition enthalpies (ΔH) were determined after measuring the area under the excess specific heat curve by paper weighing.

2.14. Polyacrylamide gel electrophoresis

Microspheres (0.2 mg) or peptide-like polymers (0.2 mg) were treated with 100 μl of 10 mM H_3PO_4 –Tris (pH 6.8) – 1% SDS – 8 M urea solution at 100 °C for 10 min and then centrifuged at 3500 rpm for 20 min. The supernatant (30 μl) was applied to electrophoresis. The electrophoresis was carried out in a 12.5% polyacrylamide slab gel (1 × 140 × 115 mm, acrylamide:N,N′-methylenebisacrylamide = 10:1) at room temperature in 0.1 M H_3PO_4–Tris (pH 6.8) – 0.1% SDS – 8 M urea solution at 28 mA for 4 hr [14]. Bromophenol blue was the tracking dye. Myoglobin fragments (SDS-PAGE Marker III, MW 16950, 14400, 8160, 6210, 2510, Fulka AG) were used for a standard. The gel was stained for peptide-like polymers with Coomassie Brilliant Blue and silver [15].

2.15. Other analytical methods

The infrared spectrum was recorded on a Hitachi 260–50 infrared spectrophotometer. Ultraviolet absorption was measured with a Gilford 2400–2 spectrophotometer.

3. Results

3.1. Formation of protein envelope under three simulated primitive earth conditions

We have developed experimental approaches for the formation of polypeptides and their envelopes from amino acids under three simulated early earth conditions. The first consists of heating at 80 °C in an aqueous and then solid phase with repetition. This system simulates a hydration-dehydration cycle of a fresh water tide pool. The second is a system heating at 50–105 °C in a sea medium enriched with transition metal ions. This system simulates warm or hot sea. The third is at high temperatures (200–350 °C) and under high pressure (120–160 atm) in an aqueous solution. Here we have simulated a submarine hydrothermal vent in the deep sea.

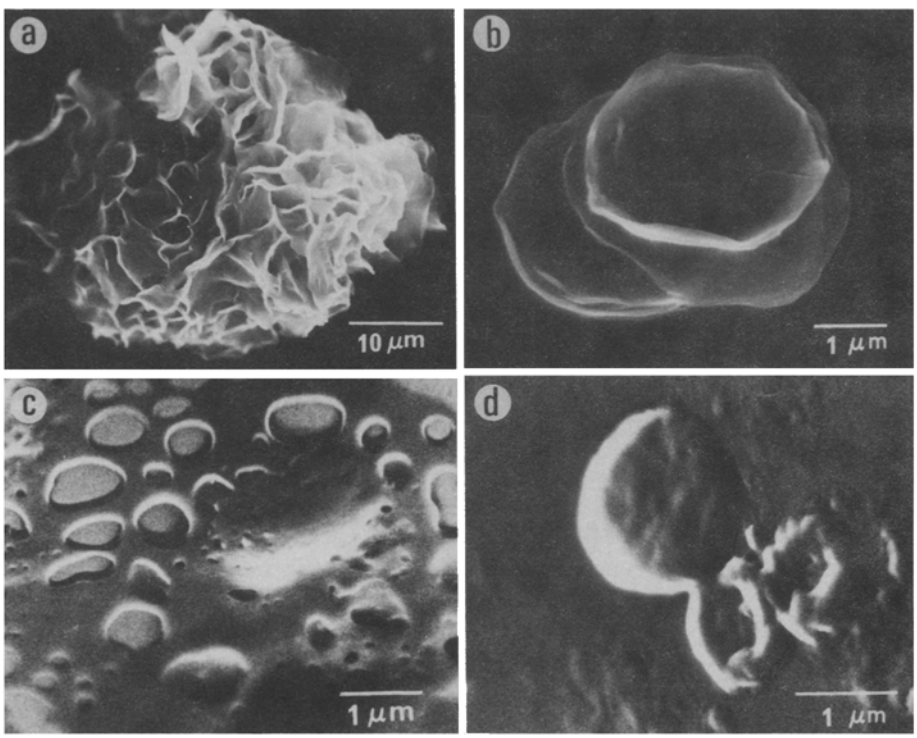

Fig. 2. Morphology of the polypeptides synthesized from 0.1 M glycinamide (a), 0.1 M glycinamide – 0.1 M alaninamide (b), 0.1 M glycinamide – 0.03 M alaninamide – 0.1 M valinamide (c), and 0.1 M glycinamide – 0.03 M alaninamide – 0.1 M valinamide – 0.03 M aspartic acid α-amide (d) during 20 hydration-dehydration cycles at 80 °C.

3.1.1. *Formation of Polypeptides and their Envelopes in a System Repeating a Hydration-Dehydration Cycle at 80 °C*

Glycine, alanine, valine and aspartic acid were more abundantly formed in the studies on the prebiotic synthesis of amino acids [16] and were found in the Murchison meteorite [17]. Thus glycine, alanine, valine and aspartic acid could have been formed in abundance at an early stage of chemical evolution [18–20]. Amino acid amides were formed from aldehyde, hydrogen cyanide and ammonia by the Strecker synthesis that is a prebiotically possible route to the formation of amino acid [21, 22]. We chose these four amino acid amides as starting materials for our studies.

Glycine polymers were synthesized from glycinamide during 20 hydration-dehydration cycles at 80 °C [23]. The polymers were found to have aggregated leaflet-like structures (Figure 2a). Polypeptides consisting of glycine and alanine were ob-

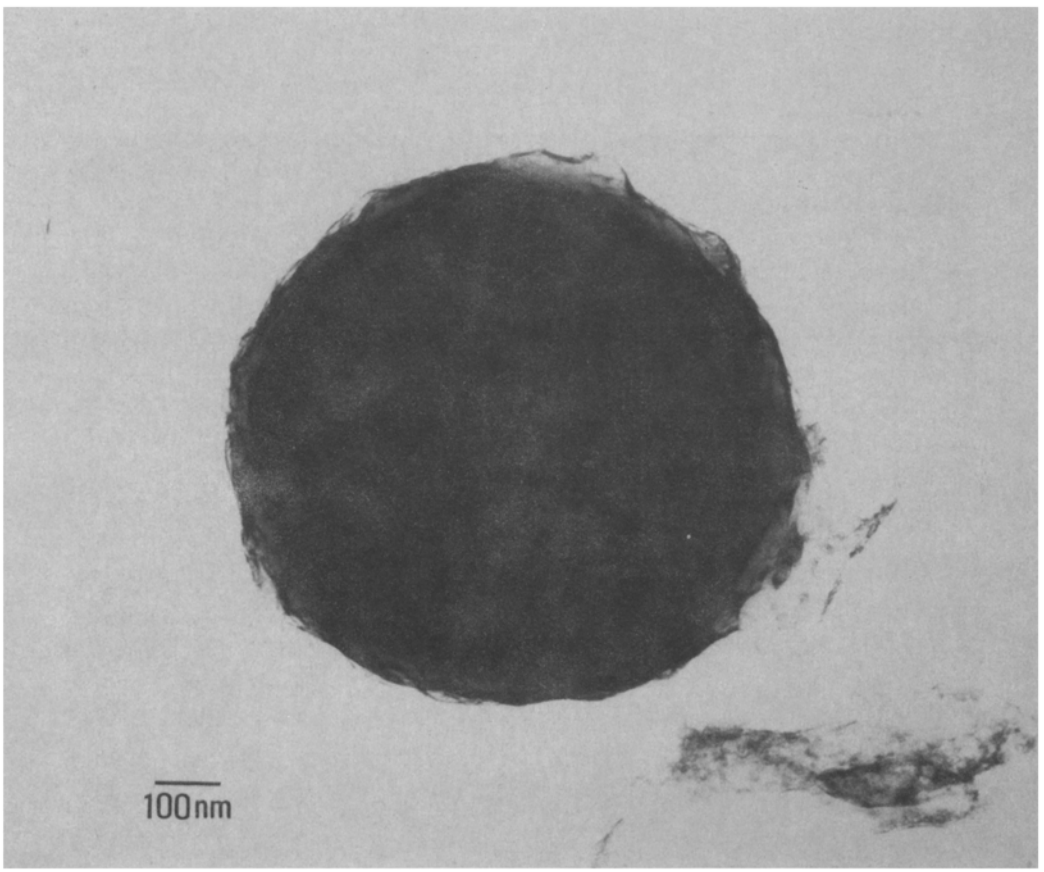

Fig. 3. An electron micrograph of cryogenic thin section of the polypeptides synthesized from a mixture of glycinamide, alaninamide, valinamide, and aspartic acid α-amide. The thin sections of microspheres were cut with glass knives of a LKB Cryo Nova ultramicrotome operated at − 120 to − 150 °C. The details are given in the Methods

TABLE I

Molecular weights, amino acid composition, and polarity index of water-insoluble fractions of resulting polypeptides

Reaction system[a]	Molecular weights[b]	Amino acid composition (%)[c]				Polarity index (%)[d]
		Gly	Ala	Val	Asp	
Gly	200–600	100				50.0
Gly-Ala	200–600	66.8	33.2			33.4
Gly-Ala-Val	200–1300	65.7	8.7	25.6		32.9
Gly-Ala-Val-Asp	500–2000	47.0	12.9	30.3	9.7	33.2

[a] Amino acid amide concentrations of reaction mixtures are given in the legend of Figure 2.
[b] Molecular weights of resulting polypeptides were determined by gel chromatography as described in the Methods.
[c] Amino acids were measured after hydrolysis of polypeptides at 110 °C for 72 hr.
[d] Polarity index of resulting polypeptides was calculated according to the method of Capaldi [24].

tained from a mixture of glycinamide and alaninamide under the same conditions (Figure 2b). These polypeptides assumed a structure whose center was concave. This structure was different from that of glycine polymers. Addition of alanine was directed toward the formation of a spherical structure. Polypeptides synthesized from a mixture of glycinamide, alaninamide and valinamide under the same conditions formed spherical structures (Figure 2c). These structures appear to have been crushed by the drying process. Fixation of the polypeptides with glutaraldehyde was unsuccessful, presumably because they had no amino group except for the terminal amino. Addition of valine clearly caused a significant change in the morphology of the polypeptides. Polypeptides synthesized from a mixture of glycinamide, alaninamide, valinamide and aspartic acid α-amide are shown in Figure 2d. These polypeptides had relatively stable spherical envelopes. The spherical envelopes appear to have a membrane-like structure. The presence of the membrane-like structure was supported by electron microscopic observation of cryogenic thin sections (Figure 3).

Table I shows the molecular weights, amino acid composition, and polarity index of the water-insoluble fraction of the resulting polypeptides. Polypeptides, synthesized from only glycinamide or from a mixture of glycinamide and alaninamide showed molecular weights of 200–600. Addition of valinamide to the mixture increased the molecular weight of the resulting polypeptide to 1300. Polypeptides with molecular weights of up to 2000 were obtained by the addition of aspartic acid α-amide. This result suggests that polypeptides with molecular weights of at least 2000 may be necessary for the formation of definite spherical structures. Alternatively, sphere formation may be enhanced by either branching or the addition of negative charges to the polypeptide as it is found.

The content of glycine was markedly high in all of the polypeptides formed. However, alanine, valine, and aspartic acid were incorporated into the corresponding polypeptides in proportion to their ratio in the starting materials. This finding suggests that the reactivity of glycine is much higher than that of alanine, valine, or aspartic acid.

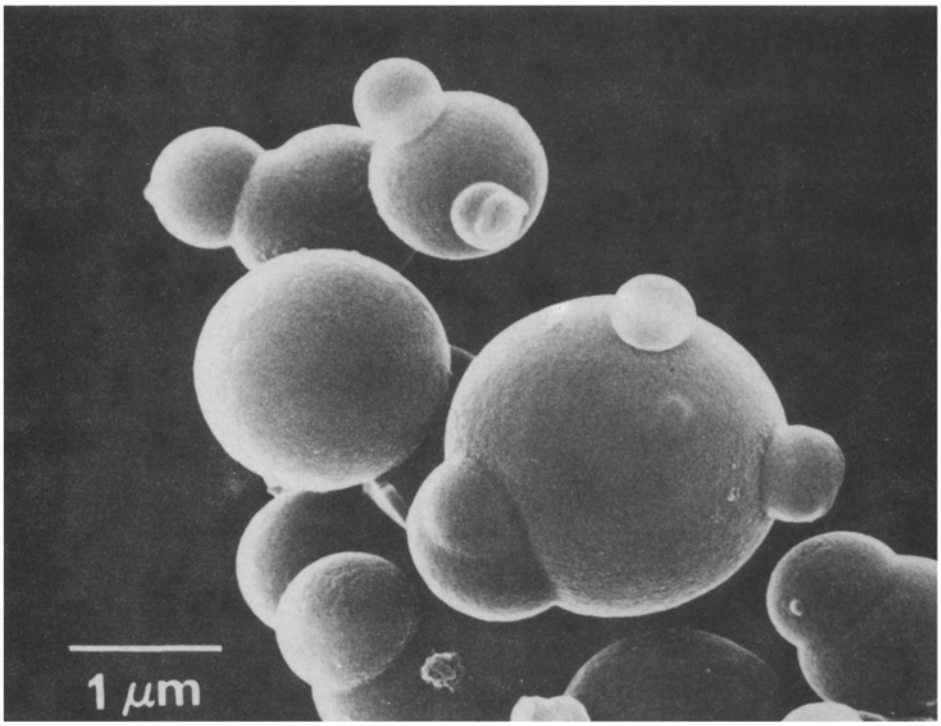

Fig. 4. A scanning electron micrograph of marigranules produced from glycine and acidic, basic and aromatic amino acids in a modified sea medium.

The polarity index of the polypeptides was calculated according to the method of Capaldi [24]. Polypeptides with a polarity index of below 40% gave definite spherical structures. This evidence was further supported by other different resulting polypeptides containing glycine, alanine, valine, and aspartic acid in appropriate ratios, except for high glycine polypeptides (data not shown).

3.1.2. *Formation of Marigranules in a Modified Sea Medium*

Egami has recently found that a close correlation between the concentration of transition elements in sea water and their biological behavior and he has suggested that the transition elements relatively rich in contemporary sea water such as molybdenum, iron, and zinc must have played important roles in the course of chemical evolution in the primitive sea [25]. Based upon this idea, we have used a modified sea medium to simulate chemical conditions in the primitive sea. Such transition element enrichment would be expected to accelerate reactions in a laboratory. The enriched sea water contains 1000 to 100000 times higher concentration of the six transition elements, iron, zinc, molybdenum, copper, manganese, and cobalt, than does contemporary sea water.

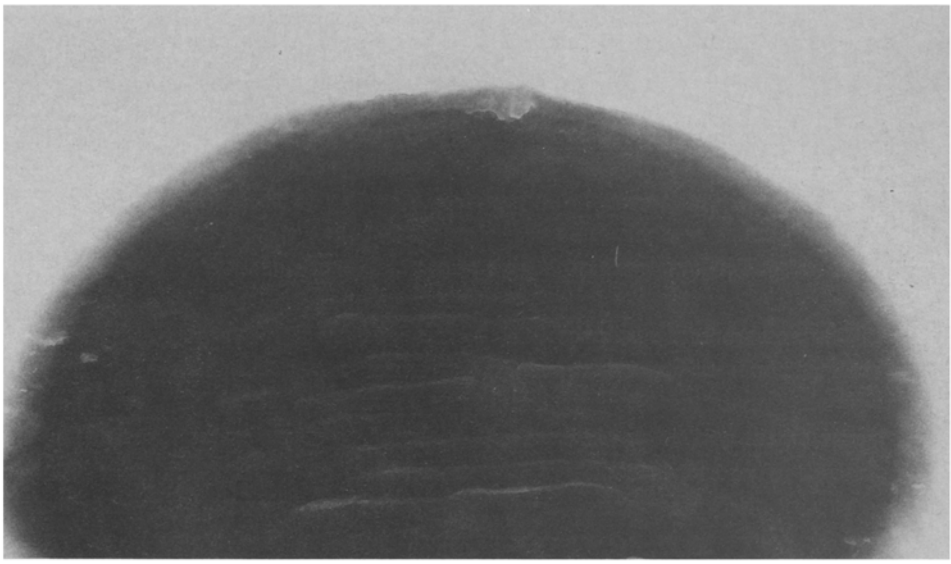

Fig. 5. An electron micrograph of a thin section of marigranules. Marigranules were embedded in Epon 812. The thin section (800 Å) were cut and photographed.

When a reaction mixture containing glycine, and acidic, basic and aromatic amino acids was heated in this enriched sea medium at 105 °C for 3 weeks, marigranules were formed in the reaction mixture [6]. The marigranules were examined by SEM. They had the appearance of fused sphere of 0.3–2.5 μm in diameter (Figure 4). Many of the marigranules had junctions.

Figure 5 shows the electron micrograph of a thin section of the marigranules. Marigranules appear to have a defined boundary layer. The interior appeared to be packed with polymers. The interior structure of marigranules was solubilized by treatment of KOH solution. The KOH-solubilized component was characterized by polyacrylamide gel electrophoresis. The component consisted of polymers with molecular weights of about 2000.

We examined environmental conditions required for the formation of the marigranules. Molecular oxygen and aromatic amino acids such as tryptophan, tyrosine and phenylalanine were essential for the formation of the marigranules (data not shown). Of the three aromatic amino acids, tryptophan gave the best yield of marigranules. The presence of divalent metal ions such as calcium ions and magnesium ions resulted in the formation of larger particles (2.5 μm in diameter) with junctions. The absence of metal ions resulted in the formation of smaller particles (0.2–0.6 μm in diameter) without junctions.

Elemental analysis of the marigranules formed from a mixture of glycine, acidic, basic and aromatic amino acids gave a percentage composition of carbon 58.22%, hydrogen 3.76%, nitrogen 14.23%, ash 7.62%, and others *e.g.* oxygen 16.17%. This

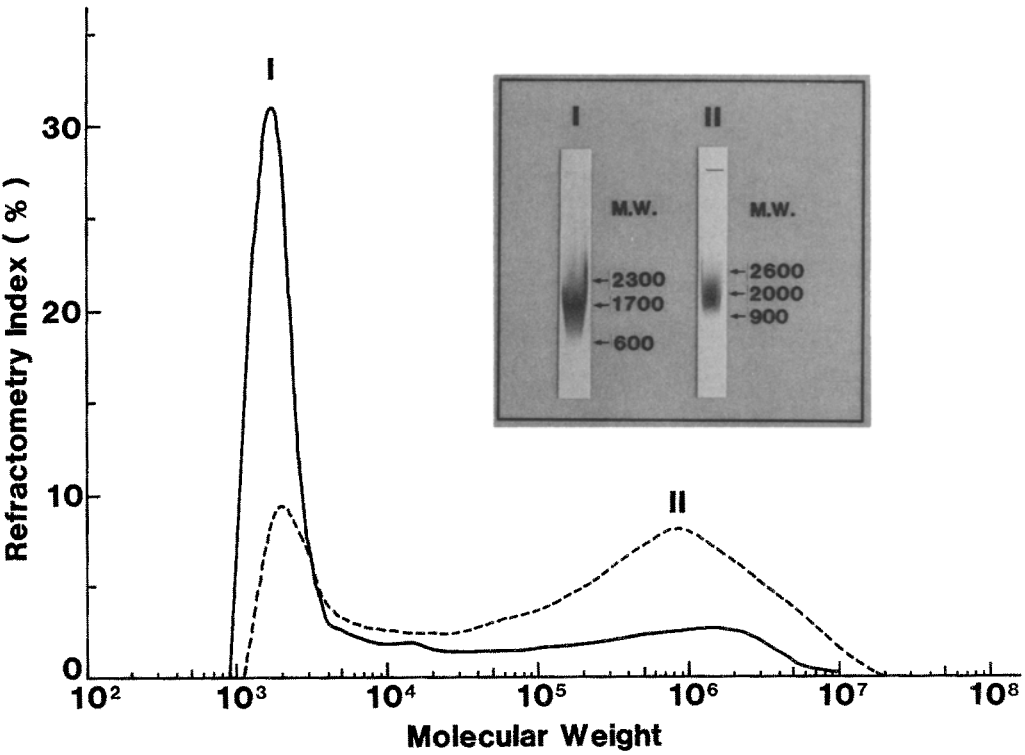

Fig. 6. Molecular weights of methanol-soluble fraction (–) and dimethyl sulfoxide-soluble fraction (...) of marigranules. The molecular weights of each fraction were determined by high-performance liquid chromatography (HPLC) using a silica gel column (3/8 inch × 25 cm, Du Pont PSM60S and 1000S) with dimethyl formamide as a mobile phase at 100 °C. The flow rate was 1 ml/min and the column effluent was monitored with a refractometer. The column was calibrated by measuring the elution volume of polystyrene's markers. Polyacrylamide gel electrophoresis of peak I and peak II was carried out as in the Methods.

result indicated that the marigranules are mostly made up of organic substances and the composition is somewhat similar to that of proteins. The infrared spectrum of the marigranules showed strong absorptions at 3560–2000, 1710, 1680–1610, 1550, 1510, 1450, 1380, 1260, 1160, 1130, 850, and 750 cm^{-1}. In the spectrum, two bands at 1680–1610 cm^{-1} and 1550 cm^{-1} strongly suggested the presence of peptide bonds. Eight amino acids were detected after acid hydrolysis of the marigranules. However, the recovery of total amino acids was very low (1.6 wt %). It seems likely that a part of the marigranules was not susceptible to acid hydrolysis. These results suggest that the marigranules contain bonds which are resistant to acid hydrolysis, besides amide bonds susceptible to it.

We confirmed the presence of peptide bonds in solubilized marigranules. The most useful reagent to confirm the presence of peptide in a protein is a proteolytic enzyme. We determined from solubilized marigranules on treatment with proteolytic enzymes [6]. The KOH-solubilized components were resistant to treatment with trypsin and

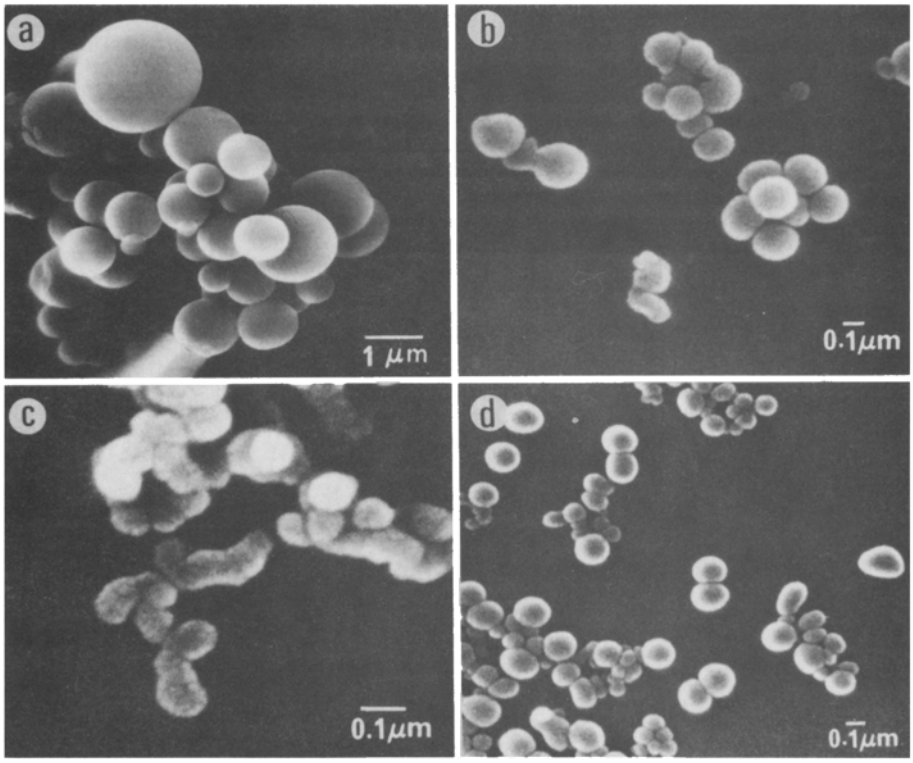

Fig. 7. Scanning electron micrographs of marisomes produced by different procedures. a, Sonication; b, dialysis, c, dilution; d, evaporation.

proteinase K, but susceptible to treatment with Pronase and elastase and 35% of the total nitrogen was revealed as primary amino groups after treatment with elastase. Thus, this result indicated that at least about one third of the total residues of KOH-solubilized components of marigranules have peptide bonds.

Marigranules were suspended in methanol and also in dimethyl sulfoxide. The methanol-soluble fraction consisted of polymers with molecular weights of 2000 which were determined by gel filtration and gel electrophoresis (Figure 6). The dimethyl sulfoxide-soluble fraction consisted of polymers with much larger molecular weights (about 10^6) as determined by gel filtration. However, the fraction gave much smaller molecular weights (2000) by gel electrophoresis. The discrepancy is due to the aggregation on the marigranules. The sonication, dialysis, dilution and evaporation of the methanol-soluble fraction of marigranules gave envelopes (Figure 7), which were termed marisomes. The marisomes had a definite boundary layers (Figure 8). Their interiors were ununiformly packed with polymers.

3.1.3. *Formation of Thermostable Microspheres in Superheated Hydrothermal Environments*

There are many submarine hydrothermal vents along tectonic rifts and ridges of the

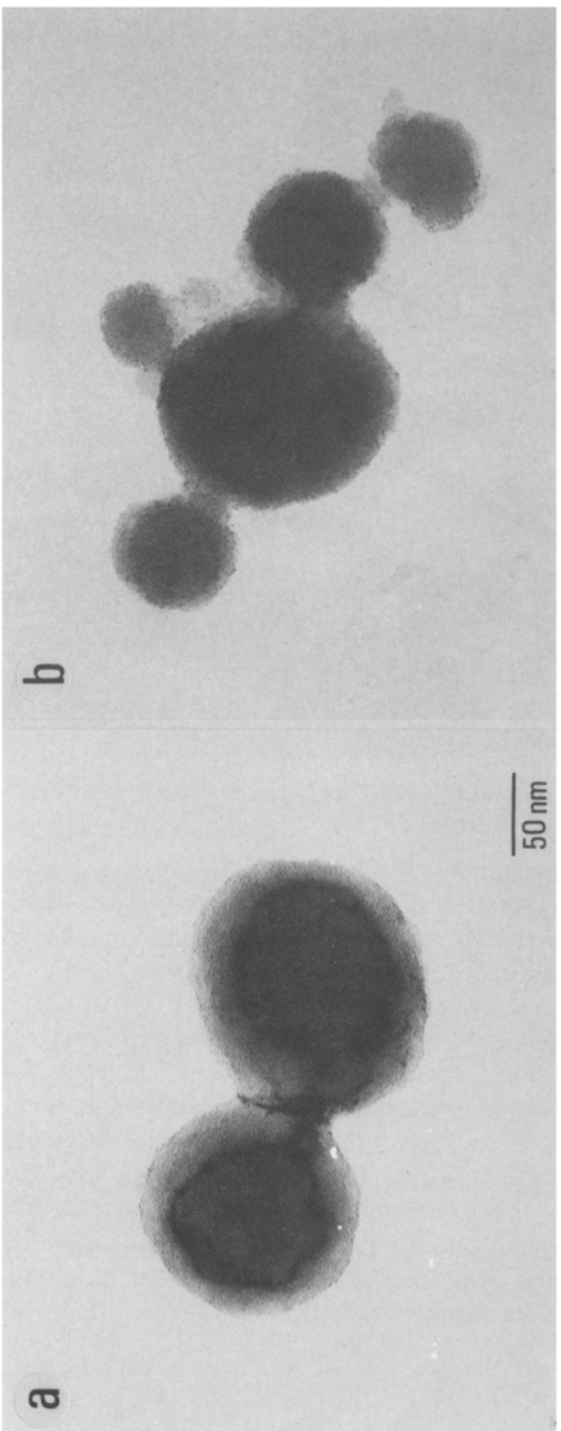

Fig. 8. Electron micrographs of marisomes. a, A drop of the suspension of marisomes was placed on a carbon-coated collodion grid, stained with 1% phosphotungstate, and examined under an electron microscope (EM) operated at 80 kV. b, The thin sections of marisomes were cut with glass knives of a LKB Cryo Nova ultramicrotome operated at -120 to -150 °C. The details are described in the Methods.

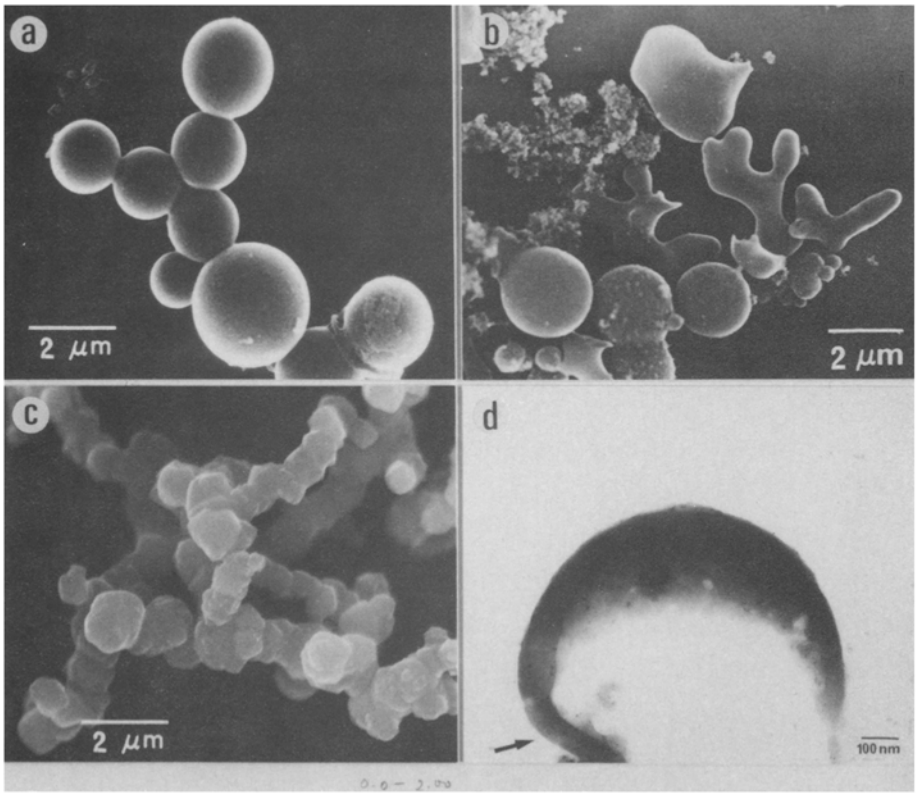

Fig. 9. Formation of microspheres on a reaction of a mixture containing glycine, alanine, valine, and aspartic acid at 250–350 °C under a hydrostatic pressure of 130 atm. The reaction procedures are described in the Methods. a, Heated in a glass tube at 250 °C for 6 hr; b, heated in a glass tube at 350 °C for 6 hr; c, heated in a stainless steel vessel at 250 °C for 6 hr; d, the interior structure of the microspheres produced in a glass tube at 250 °C. The arrow indicates membrane-like structure. The thin sections of microspheres were cut with glass knives of a LKB Cryo Nova ultramicrotome operated at −120 to −150 °C. The details are given in the Methods.

Galapagos Islands and along the East Pacific Rise [26]. Among these submarine hydrothermal vents, black smokers at 21° N along the East Pacific Rise spout superheated water at temperatures exceeding 350 °C.

Baross and Deming recently reported that extremely thermophilic bacteria isolated from one of the black smokers grew at temperatures of up to at least 250 °C [27].

Since such submarine hot springs might serve as a possible model for primitive environments on the earth, we have studied chemical evolution in superheated hydrothermal environments. We describe here the formation of thermostable microspheres and peptide-like polymers in aqueous solutions at 250–350 °C.

When an aqueous solution containing glycine, alanine, valine and aspartic acid was heated in a glass tube at 250 °C and 130 atm for 6 hr, numerous microspheres of 1.5 to 2.5 μm in diameter were formed (Figure 9a) [28]. Microspheres were also obtained in reactions at 300 °C, and 350 °C, however, their spherical structures were

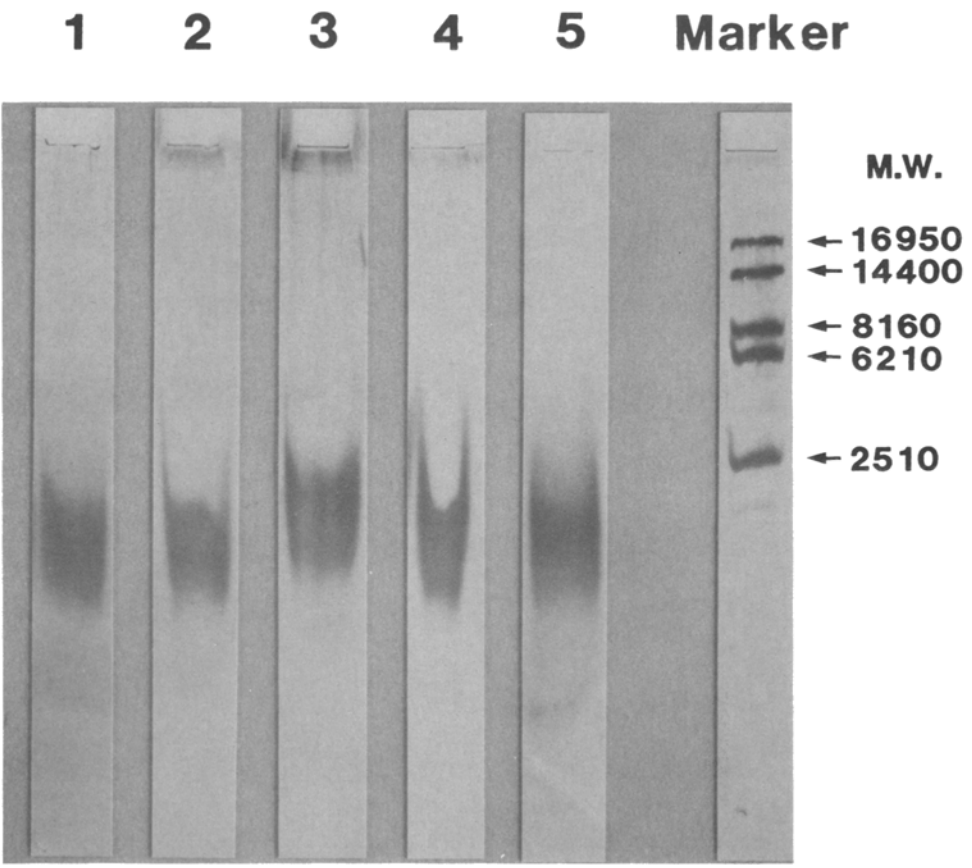

Fig. 10. Polyacrylamide gel electrophoresis of thermostable microspheres, supernatants of reaction mixtures heated at different temperatures, and proteinoid microspheres [3]. 1, 1% SDS-8 M urea-solubilized components of microspheres produced at 250 °C; 2, 3, and 4, supernatants of reaction mixtures heated at 250 °C, 300 °C, and 350 °C, respectively; 5, 1% SDS-8 M urea-solubilized components of proteinoid microspheres. The detailed procedures are given in the Methods.

deformed (Figure 9b). Microspheres were not obtained in the reaction under the same pressure condition at 200 °C.

When the reaction mixture was heated at 250 °C, 130 atm for 6 hr in a stainless steel vessel, fused structures were obtained (Figure 9c). Microspheres were not obtained from mixtures of either only glycine, or of glycine-alanine-valine at 250 °C for 6 hr in a glass vessel. Polar amino acids such as glutamic acid, lysine, arginine, histidine, serine, threonine and 4-hydroxyproline could replace aspartic acid to form microspheres in glass vessels. Basic amino acids resulted in the formation of larger microspheres, 4–8 μm in diameter. These results indicate that polar amino acids, glass vessels and reaction temperatures of 250 °C or above are necessary for the formation of the microspheres.

Microspheres have a definite boundary layer with a thickness of about 70 nm (Figure 9d). The interior is empty.

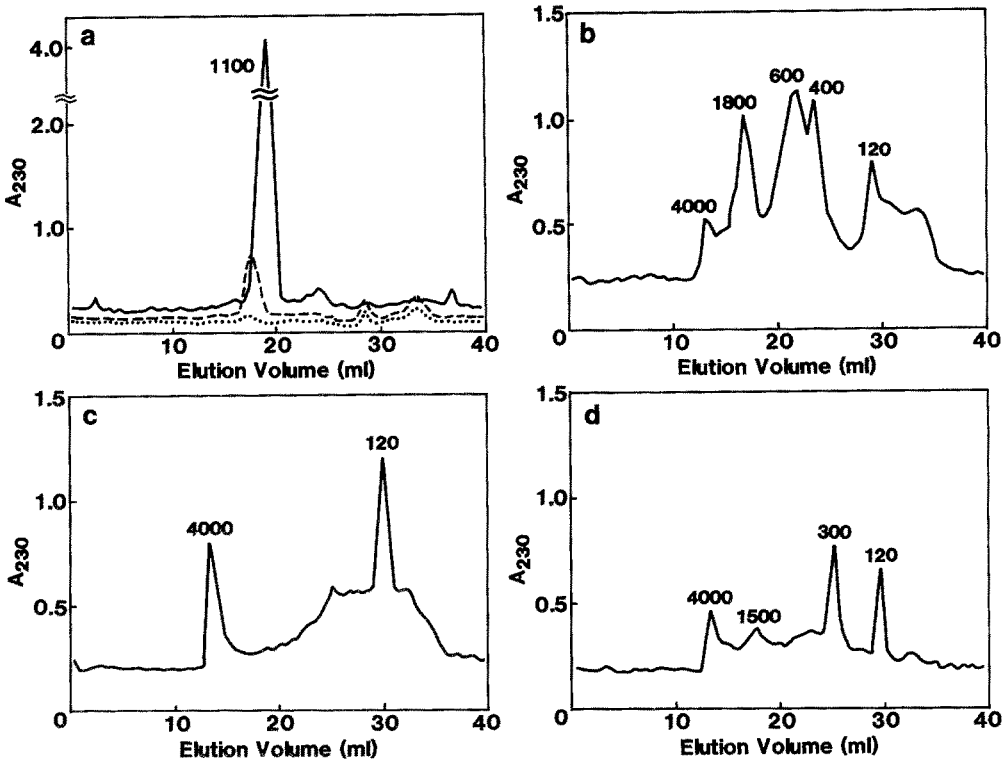

Fig. 11. Bio-Gel P-4 chromatography of supernatants of reaction mixtures heated at different temperatures. a, Supernatants of reaction mixtures heated at 100 °C (...), 150 °C (– – –), and 200 °C (–) for 6 hr; b, c, and d, supernatants of reaction mixtures heated at 250 °C, 300 °C, and 350 °C for 6 hr, respectively. The details are given in the Methods. Numerical values over peaks represent molecular weight of the peaks.

Elemental analysis of the microspheres showed a percentage composition of C, 41.7; H, 4.3; N, 5.0; Si, 12.0%. The IR spectrum of the microspheres showed strong absorptions at 3400, 2970, 2940, 2870, 1670–1630, 1090, and 470 cm^{-1}. When the microspheres were treated with hydrofluoric acid, two clear bands appeared at 1670 and 1630 cm^{-1} that can be attributed to an imide bond and an amino group (NH_3^+ deformation vibration of an amino acid). This suggests the possibility that the amino group may be linked with silicates. Two bands at 1090 and 470 cm^{-1} could be attributed to the Si–O–Si bond.

The microspheres were dissolved with 1% SDS-8 M urea or 0.5% hydrofluoric acid. They completely disappeared on treatment with 1% SDS-8 M urea at 100 °C for 10 min or 0.5% hydrofluoric acid at room temperature for 10 min.

Polyacrylamide gel electrophoresis of the 1% SDS-8 M urea-solubilized component of the microspheres gave a broad band with a molecular weight of 1000–2000 daltons (Figure 10).

Microspheres were partically hydrolyzed with 6 N HCl at 110 °C for 72 hr. The

amino acid composition was glycine 45.7%, alanine 9.5%, and valine 44.8%. Aspartic acid was not detected.

These results suggest that the microspheres are made of peptide-like polymers that, are resistant to acid hydrolysis, contain silicates, and have molecular weights of about 1000–2000.

Peptide-like polymers were also present in the supernatant of the reaction mixture. Figure 11 shows gel chromatography of the products of several reaction mixtures heated at different temperatures. The reaction mixture at 200 °C gave a single peak with molecular weights of 1100 daltons. It can hardly be detected at 100 °C, increases at 150 °C and is the sole product at 200 °C (Figure 11a). At 250 °C several peaks with molecular weights up to 4000 appear in the chromatogram (Figure 11b). A gel chromatogram of the product of the reaction mixture heated at 250 °C in a stainless steel vessel was much the same as that of the reaction mixture heated at 250 °C in a glass tube. At 300 °C these peaks disappeared and a peak with a large molecular weight and a peak with a small molecular weight survived (Figure 11c). Judging from the fact that polyacrylamide gel electrophoresis of this peak gave a broad band with a molecular weight of 1000–2000 daltons (Figure 10), this peak seems to be an aggregated form of peptide-like polymers. Namely, at 250 °C and 300 °C aggregation and degradation of the peptide-like polymers seems to occur simultaneously. At 350 °C these peaks decreased considerably (Figure 11d). The NMR spectrum of the peptide-like polymers showed high content of valine.

The resulting peptide-like polymers were resistant to acid hydrolysis. The fraction of the polymers susceptible to hydrolysis decreased with a rise in the temperature of hydrolysis.

Based upon the physical data described above, a possible chemical structure of the peptide-like polymers of microspheres is considered. They consist of imide bonds and amino acid residues having an abundance of valine. The presence of imide bonds results in the branched structure of the polymers. Some amino groups near the branching point are linked by an aminosilyl bond to a silicon of silicates. The silicates would presumably contribute to the hydrophilicity of a moiety of the polymers.

Concerning the stability of the aminosilyl bond, there must be some protective mechanism. A peptide bond between two valine residues is very resistant to acid hydrolysis [29]. For example, only 5% of the total peptide bonds of polyvaline is hydrolyzed with 6 N HCl 110 °C for 72 hr. Polyvaline is completely hydrolyzed with trifluoroacetic acid-12N HCl (1 : 1) at 175 °C for 24 hr. Therefore, the aminosilyl bond may be protected by the hydrophobic moiety of valine residues from attack by water.

It may be that at lower temperatures of the reaction, polypeptides are produced that are rich in glycine, alanine, valine, and aspartic acid. Peptide bonds composed of glycine, alanine, and aspartic acid, which may be preferentially cleaved because of their relative instability at 250 °C as compared to peptide bonds between valine residues which withstand hydrolysis at high temperature. Thus, the content of valine increases and peptide-like polymers having a preponderance of valine survives.

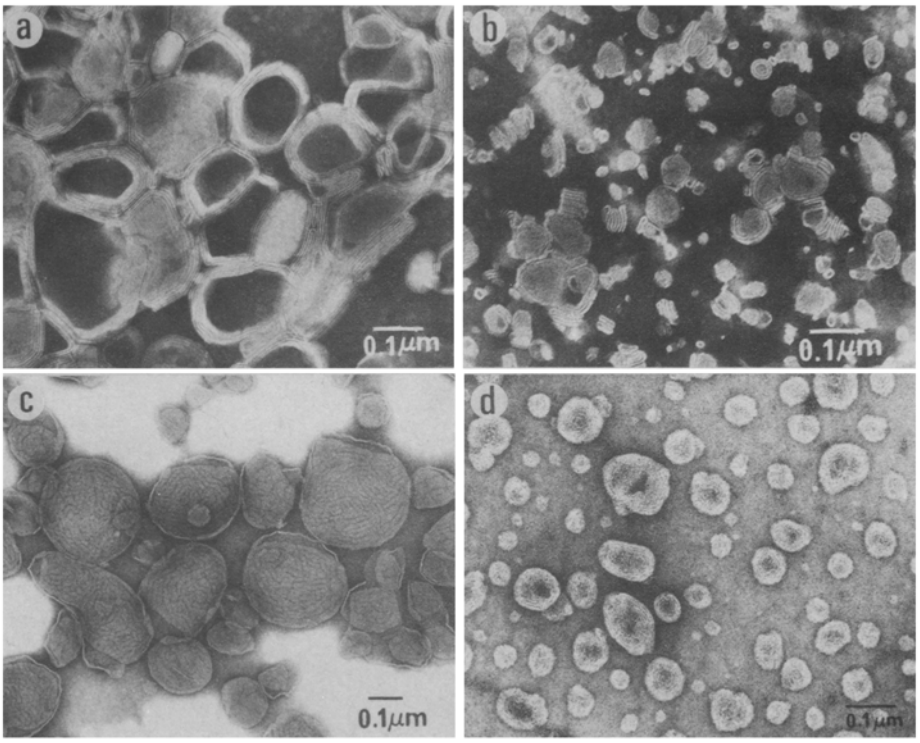

Fig. 12. Electron micrographs of different lipid vesicles. a, Egg lecithin; b, DOPC; c, DPPC; d, oleic acid. The preparation and electron microscopic observation of lipid vesicles are described in the Methods.

3.2. Formation of Lipid Vesicles

Lipid vesicles were prepared by the dialysis method to examine their interaction with proteins. When lipids were solubilized with a detergent such as *n*-octyl glucoside [30] and dialyzed against water, numerous small lipid vesicles form. Figure 12 shows negatively stained electron micrographs of lipid vesicles prepared from four lipids. The vesicles derived from egg lecithin and DOPC clearly have multilamellar membrance structures (Figures 12a and 12b). It is difficult to tell if the vesicles formed from DPPC and oleic acid had unilamellar of oligolamellar membrane structures (Figures 12c and 12d).

3.3. Formation of Lipid-Protein Envelopes

Methanol-solubilized marigranules were mixed with egg lecithin in the presence of n-octyl glucoside and dialyzed against water. Numerous large envelopes were formed (Figure 13). Figure 13a shows an optical micrographs of reconstituted lecithin-marigranule envelopes. The envelopes were relatively uniform and their size was 1-3 μm in diameter. Figure 13b shows a fluorescence micrograph of the same vesicles. This indicates that marigranule components were strongly bound to the lipids.

Figure 14 illustrates the size distribution of lecithin-marigranule envelopes pre-

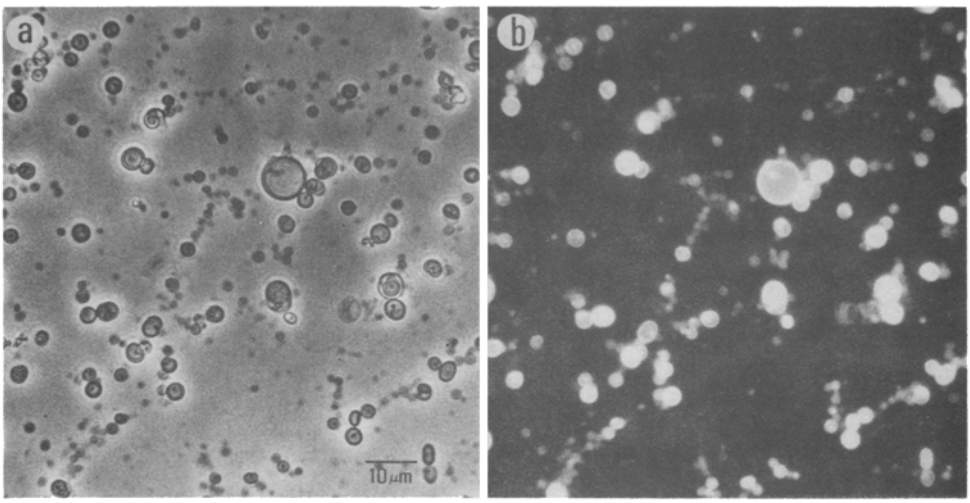

Fig. 13. Phase-contrast (a) and fluorescence (b) micrographs of lecithin-marigranule envelopes. The preparation of lecithin-marigranule envelopes are given in the Methods.

pared at different lecithin-to-solubilized marigranules ratios. Addition of solubilized marigranules to lecithin increased both the mean size and the heterogeneity of the preparation (Figure 14b). The maximal mean size was obtained at a lecithin to solubilized marigranules ratio of 10:1.1 (Figure 14c). Further addition of solubilized marigranules, however, reduced both the mean size and the size heterogeneity of the preparations (Figures 14d and 14e).

Figure 15 illustrates the size distribution of DPPC-marigranule envelopes prepared at DPPC to solubilized marigranules ratios of 10:0.3–4.0. The size distribution of the envelopes is similar to that of the lecithin-marigranule envelopes. The hybrid envelopes from DPPC and the solubilized marigranules were markedly stable and we could not observe any aggregation of the DPPC-marigranule envelopes during standing of a few weeks at room temperature.

Addition of solubilized marigranules to DPPC decreased in the Tc of the lipid (Figure 16). The Tc of the main peak became broader at high solubilized marigranule-to-lipid ratios, and the pre-transition temperature was eliminated by solubilized marigranules. The ΔH for the transition of the envelopes decreased with each increase in the solubilized marigranule content.

Different polyamino acids and proteins were mixed with egg lecithin and their effects on the morphology of the resulting envelopes were examined by electron microscopy. Polycations such as polylysine and polyhistidine produced envelopes of mean size 0.084 μm and 0.074 μm in diameter with stable unilamellar membrane structures, respectively (Figures 17a and 17b). However, there was no interaction between lipids and polyanions such as polyglutamic acid and polyaspartic acid. Basic proteins such as lysozyme and cytochrome *c* produced very small envelopes of mean

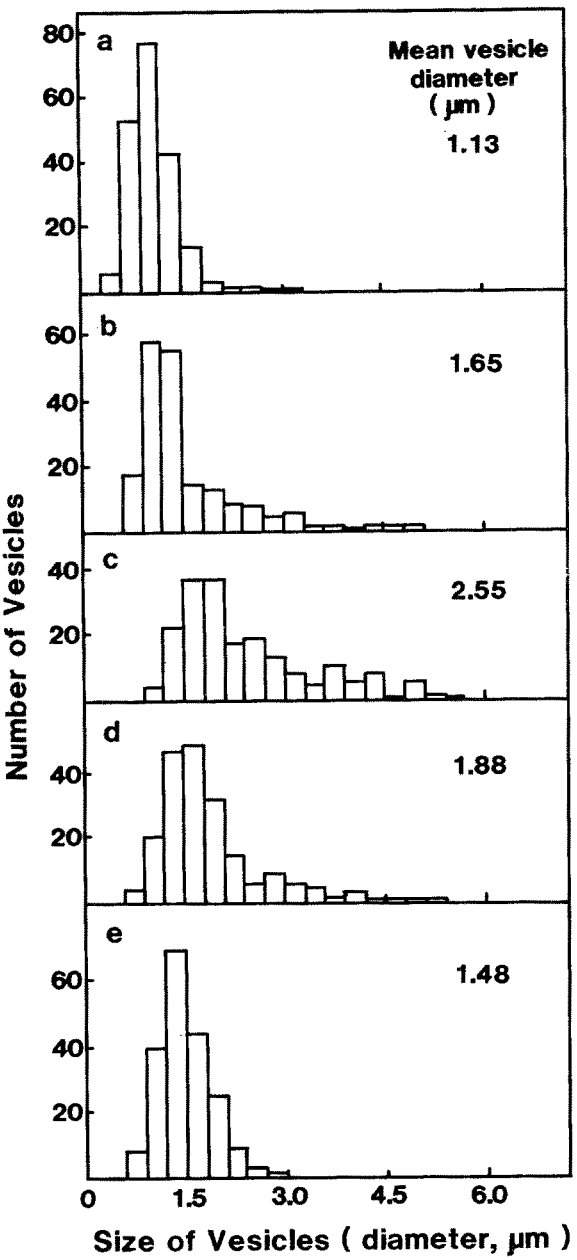

Fig. 14. Size distribution of lecithin-marigranule envelopes. Lecithin to methanol-solubilized marigranules ratios are 10:0 (a), 10:0.6 (b), 10:1.1 (c), 10:2.1 (d), and 10:4.2 (e). The preparation and determination of size distribution of lecithin-marigranule envelopes are described in the Methods.

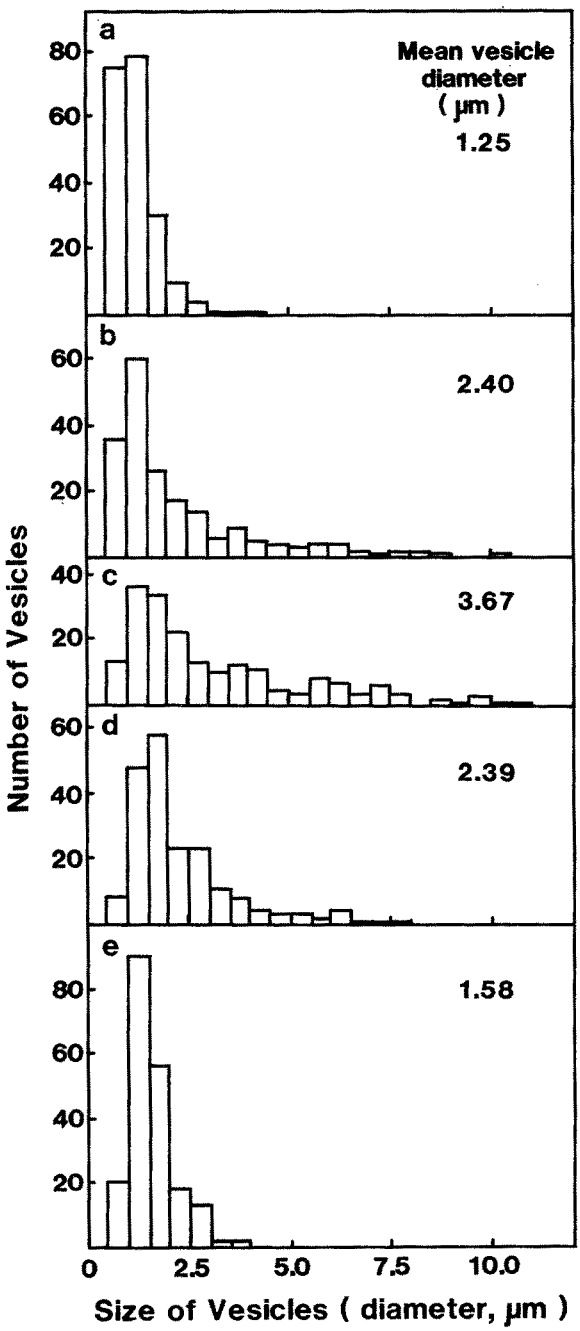

Fig. 15. Size distribution of DPPC-marigranule envelopes. DPPC to methanol-solubilized marigranules ratios are 10:0 (a), 10:0.3 (b), 10:0.5 (c), 10:1.0 (d), and 10:4.0 (e). The preparation and determination of size distribution of DPPC-marigranule envelopes are given in the Methods.

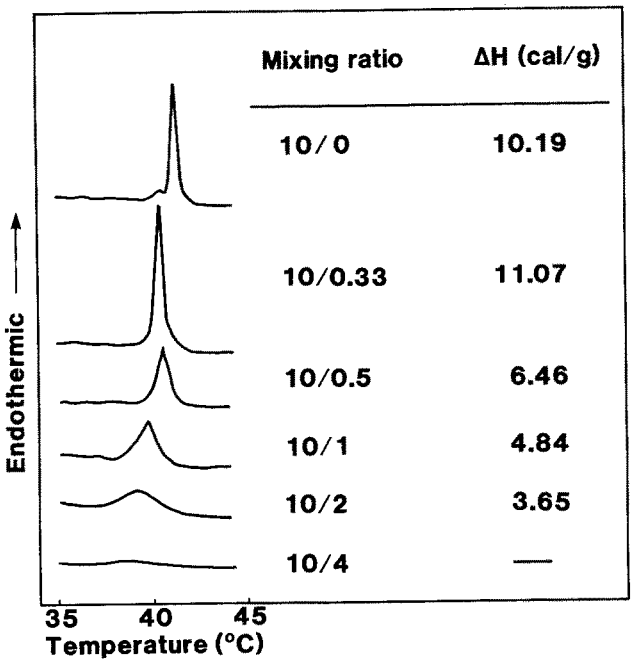

Fig. 16. Differential scanning calorimetry of DPPC and DPPC-marigranule envelopes. The determination of differential scanning calorimetry and transition enthalpies are given in the Methods. Mixing ratio is a DPPC-to-solubilized marigranules molar ratio.

size 0.027 μm and 0.017 μm in diameter with stable unilamellar membrane structures, respectively (Figures 17c and 17d). The lipid in the absence of polypeptides gave small envelopes of 0.02–0.4 μm in diameter with unstable multilamellar membrane structures. These results indicate that polypeptides affect the size, membrane structure and stability of lipid envelopes.

We examined incorporation of marigranule components synthesized from *L*-tryptophan into lipid envelopes *in situ* as well as their contribution to the stability of lipid envelopes. Lipid vesicles derived from egg lecithin were heated in the presence or absence of *L*-tryptophan at 80 °C for two weeks. The vesicles were completely degraded in the absence of *L*-tryptophan by 6 days, whereas they were protected by *L*-tryptophan during 14 days of the same treatment (Figure 18). Fluorescence microscopic observation of the envelopes proved that considerable amounts of the resulting marigranule components are incorporated into the lipid membranes. It is clear that the incorporation of the marigranule components contributes to the stability of lipid envelopes.

In addition, we tried to construct models for protein envelopes coated with lipid envelopes (II in Figure 1) and lipid envelopes coated with protein envelopes (III in Figure 1). As shown in Figure 19, protein envelopes coated with lipid envelopes were formed from a mixture of marisome and egg lecithin. Lipid envelopes coated with

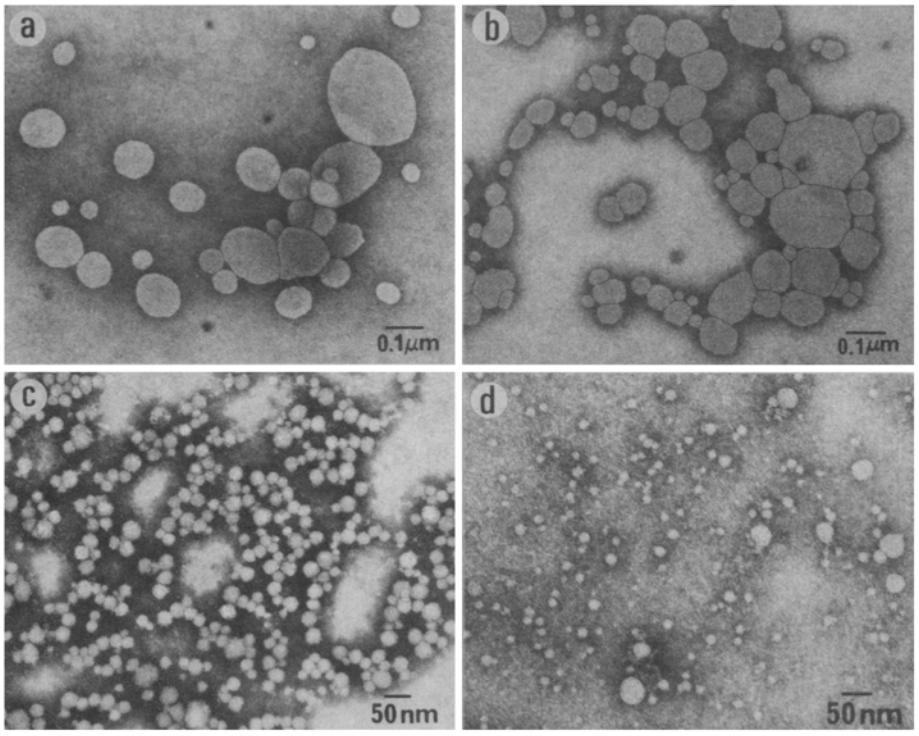

Fig. 17. Electron micrographs of different lipid-protein envelopes produced from lecithin-polylysine (a), lecithin-polyhistidine (b), lecithin-lysozyme (c), and lecithin-cytochrome *c* (d). The preparation of lipid-protein envelopes are described in the Methods.

protein envelopes, however, were not obtained from a mixture of lipid envelopes and protein envelopes.

4. Discussion

In this study, we have examined the formation of polypeptides from four amino acid amides; glycine, alanine, valine and aspartic acid in a system simulating a fresh water tide pool on the earth. In a study of their morphology it was shown that glycine polymers gave sheet structures, polymers consisting of glycine and alanine produced disc structures and finally polymers synthesized from the four amino acids, glycine, alanine, valine and aspartic acid resulted in the formation of stable spherical structures. On the other hand, we have recently found that polypeptides synthesized from these four amino acid amides and histidine amide under almost the same condition have partly definite secondary structures such as α-helix and β-sheet and exhibit catalytic activities for hydrolysis of *p*-nitrophenyl acetate and dehydrogenation of NADH [31]. In particular, the four amino acids were essential for the formation of the defined secondary structures. Therefore, polymers of these four amino acids were good

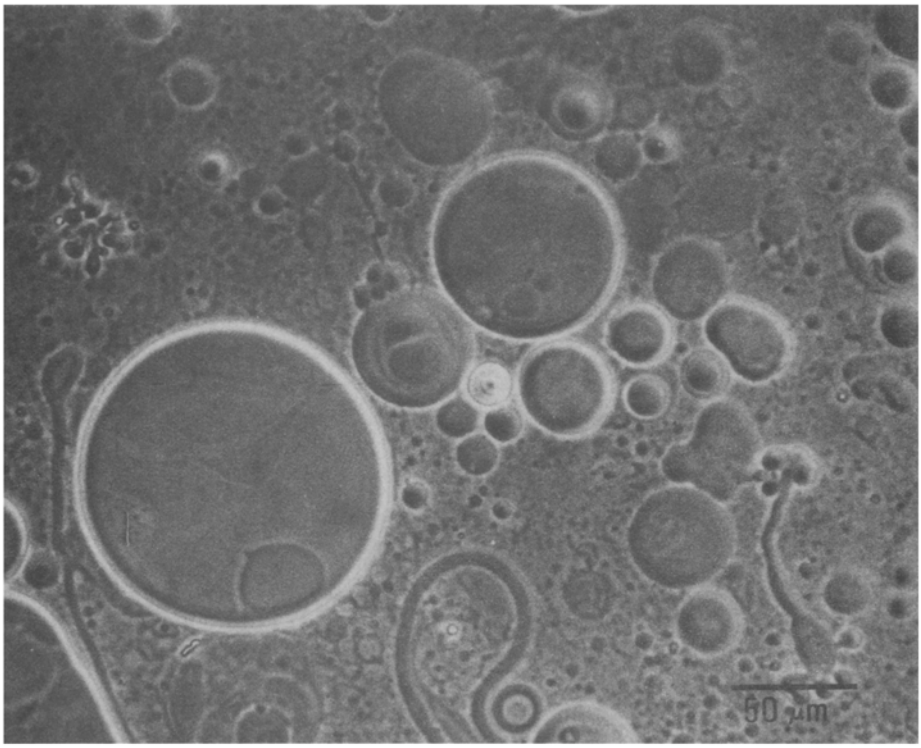

Fig. 18. Formation of lipid-protein envelopes produced *in situ* from egg lecithin and *L*-tryptophan. A reaction mixture (5 ml) containing 140 mg of egg lecithin and 0.25 µmol of *L*-tryptophan was put into a glass tube, capped, placed in a heating block, and heated at 80 °C for 3 days.

candidates for the formation of both protein envelopes and enzymes. The results suggest that primitive protein envelopes and enzymes might have been spontaneously formed from glycine, alanine, valine and aspartic acid under such conditions during the early stages of chemical evolution.

We have also demonstrated that two other types of protein envelopes may have formed *in situ* from amino acids under primitive earth conditions: one a warm or hot sea and the other a submarine hydrothermal vent. A common characteristic of the polypeptides formed under these three primitive earth conditions is that they all have molecular weights of 2000. This was further supported by evidence that the proteinoid microspheres [32] consisted of proteinous polymers with molecular weights of about 2000 (Figure 10). Furthermore, among the characteristics of these polypeptides, the most conspicuous characteristic is that stable envelopes can be constructed readily *in situ* from amino acids under all three primitive earth conditions: a fresh water tide pool, a warm or hot sea and a submarine hydrothermal vent.

Oró [33] and Hargreaves [34] demonstrated that phospholipid vesicles were formed *in situ* from glycerol, fatty acid, phosphate in the presence of a condensing agent under possible prebiotic conditions. Some investigations also demonstrated

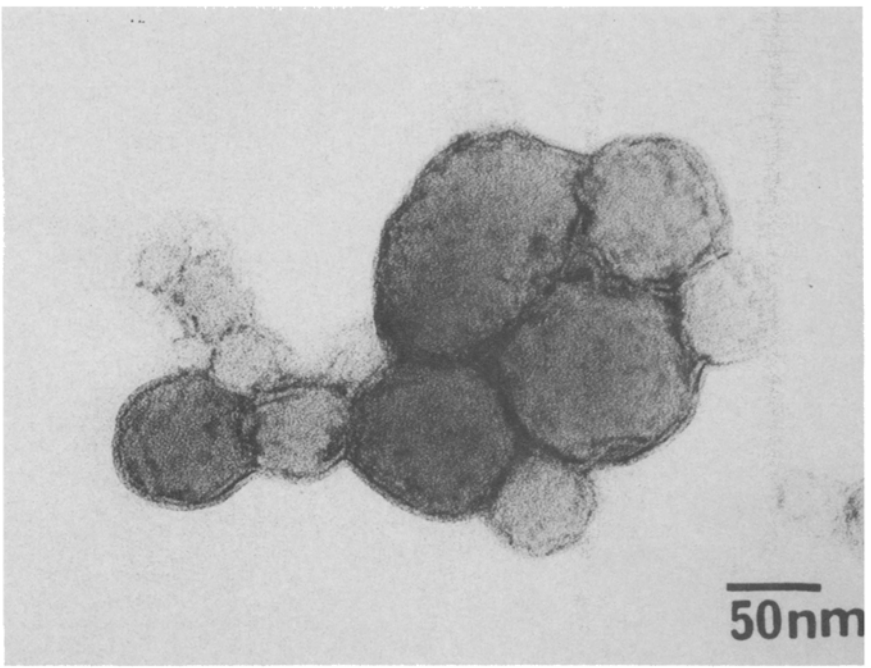

Fig. 19. Formation of protein envelopes coated with lipid envelopes. Lipid films were prepared by evaporating a chloroform solution of egg lecithin (2.8 mg). Marisomes were prepared from methanol-solubilized marigranules as described in the Methods. To the lipid films was added 1.5 ml of the suspension of the marisomes and the mixture shook gently. The resulting envelopes were negatively stained and photographed with a JEM 100B operated at 80 kV.

that even relatively simple lipids such as fatty acids [10,35] and other single-chain amphiphiles [36] are able to assemble into membranes under certain conditions. Deamer found that definite boundary structures are formed from organic components of the Murchison carbonaceous chondrite [37].

We have prepared *in situ* lecithin-marigranule envelopes as a model for lipid-protein envelopes. The hydrophobic polypeptide components interact with the lipids to produce large stable envelopes with unilamellar or multilamellar membrane structures. Generally, polypeptide components affected the stability, membrane structure and size of lipid envelopes. The lipid-protein interactions are grouped into three categories each having different effects on the morphology of the lipid-protein envelopes. Polycations such as polylysine and polyhistidine can interact with phosphate anion groups of phospholipids, therefore, polycations attach the surface of the envelopes by electrostatic interactions. On the other hand, polyanions such as polyglutamic acid and polyaspartic acid cannot interact with the phosphate anion groups. Until now, extensive study has been conducted on the interaction between peptides or proteins and lipids [38]. Positively charged polypeptides such as polylysine and polyhistidine interact with negatively charged liposomes and induce fusion of the liposomes [39, 40]. In addition, positively charged peptides can interact strongly with

TABLE II

Characteristics of protein, lipid, and lipid-protein envelopes

Characters	Protein envelope	Lipid envelope	Lipid-protein envelope
Can it self-assemble?	Yes	Yes	Yes
Has it a phase boundary?	Yes	Yes	Yes
Compartment to keep water	Small	Large	Large
Permeability barrier to small molecules	Low	High	Medium
Permeability barrier to large molecules	Medium	High	High
Fluidity & Elasticity	Low	High	High
Stability	High	Low	Medium
Has it catalytic functions?	Yes	No	Yes

even zwitterionic phospholipids [41]. Negatively charged peptides, however, cannot interact with the lipids. This result supports strongly our result that polycations such as polylysine and polyhistidine interacted with zwitterionic phospholipids and induced the formation of the extremely small vesicles, whereas polyanions such as polyglumatic acid and polyaspartic acid could not interact with the lipids. The basic proteins, lysozyme and cytochrome *c*, which were used in this experiment, have high isoelectric points (11 and 10.1, respectively). Both positively charged amino groups and hydrophobic groups of the proteins can interact with the phosphate anion groups and the alkyl chains of lipids, respectively. Thus, the proteins may penetrate into the lipid membranes. It has already been reported that amphiphilic polypeptides [42] or hydrophobic proteins [43, 44] interact with phospholipids strongly and they are incorporated into the bilayer of the lipids. Hydrophobic marigranule components can presumably interact with the alkyl chains of lipids strongly. In this way the hydrophobic regions of the marigranule polymers can penetrate into the lipid bilayers. This is indirectly elucidated by the decrease in ΔH, which can be interpreted as indicating that fewer phospholipid molecules participate in the cooperative melting of the bulk lipid [38].

To date, different types of plausible models for protocellular structures have been proposed. Among the models, Oparin's coacervates [2] and Fox's 'protenoid microspheres' [3] have been extensively studied. Protocellular structures must satisfy the following requirements: (1) They are formed from simple molecules under simulated primitive earth conditions and self-assembled to thermodynamically into a stable condition; (2) they have a phase boundary which isolates a portion of the environment and retain water; (3) they have selective permeability to water, ions and small organic molecules and permeability barriers to large molecules; (4) they have fluidity and elasticity and are capable of fusion and fission; (5) they have catalytic functions.

Some characteristics of protein envelopes, lipid envelopes and lipid-protein envelopes were summarized in Table II. These three types of the envelopes self-assembled

in aqueous solutions and displayed phase boundaries. Concerning entrapment of water, protein envelopes contained compartments that were too small since most of the interior was packed with proteinous polymers. The lipid envelopes and the lipid-protein envelopes had a large internal compartment. In the permeability barrier to small molecules, the barrier capacity of the protein envelopes was low, the lipid envelopes high, and lipid-protein envelopes medium. For example, glucose freely passed through the protein envelopes [45]. Concerning permeability barrier to large molecules, the barrier capacity of the protein envelopes was medium, and the lipid envelopes and the lipid-protein envelopes high. Furthermore, the fluidity and elasticity of the protein envelopes were low but their stability were very high. On the contrary, the fluidity and elasticity of the lipid envelopes were high but their stability was low [46]. The lipid-protein envelopes had medium stability. The stabilization is due to the addition of proteins. Protein envelopes and lipid-protein envelopes had catalytic functions, whereas lipid envelopes had no catalytic function [46].

Protein envelopes can satisfy the requirements 1, 2, and 5 described above, whereas they cannot satify the requirements 3 and 4. On the other hand, lipid envelopes are able to satisfy the requirements 1, 2, and 4, whereas they are unable to satisfy the requirements 3 and 5. Lipid-protein envelopes can satisfy all the requirements. Though lipid envelopes are inferior to protein envelopes in stability, they are assumed to surpass protein envelopes with regard to their fluidity and elasticity. Thus simple protein envelopes and/or simple lipid envelopes [47, 48] presumably originated at an early stage of chemical evolution. They may then have interacted mutually. Envelopes containing lipids and proteins must have developed structurally and functionally during both prebiotic and biological evolution.

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