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Synthesis of Phosphatidylethanolamine under Possible Primitive Earth Conditions

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Summary. The synthesis of phosphatidylethanolamine was accomplished when a mixture of phosphatidic acid, ethanolamine, and cyanamide at pH 7.3 was taken to dryness and heated at temperatures ranging from 25 to 60°C for 6 h. Chromatographic, enzymatic, and chemical techniques were used to identify and confirm that phosphatidylethanolamine had been formed. This work indicates that the synthesis of this compound can occur starting with precursors and conditions that are presumed to have existed on the primitive Earth.

Key words: Chemical evolution — Prebiotic synthesis — Phosphatidylethanolamine — Cyanamide — Phospholipids — Protomembranes

Introduction

Phospholipids are a distinct class of biomolecules present in all types of cells, where they constitute the major component of membrane structures. As a result of their amphiphilic molecular structure, they are ideally suited to provide the necessary interphase between the external and internal hydrophilic cell media, bridging both environments with efficient transport systems for different types of molecules and ions.

Because of their structural characteristics, phospholipids readily self-assemble to form liposomes, which are well-known models for primordial cell membranes (Deamer and Oró 1980; Deamer and Barchfeld 1982; Oró and Lazcano 1984). Consequently, considerable attention has been focused on the investigation of phospholipid surface films at interfaces and on the interactions of phospholipid monolayers with biologically active substances. In contrast, not much work has been done until the last few years on the elucidation of the prebiological origin of these compounds (Hargreaves et al. 1977; Hargreaves and Deamer 1978; Oró et al. 1978). The specific work done in our laboratory on the formation of phospholipids and their precursors includes the synthesis of straight-chain fatty alcohols and fatty acids (up to C_{21}) from carbon monoxide and hydrogen (e.g., Leach et al. 1978; Nooner and Oró 1979), glycerol and glycerol phosphates (Epps et al. 1979), acylglycerols (Eichberg et al. 1977), phosphatidic acids (Epps et al. 1978), and other compounds (Sherwood et al. 1978). Furthermore, the abiotic synthesis of phosphatidylcholine has also been reported from our laboratory (Rao et al. 1982). For this study, only reactants considered prebiotic compounds were used. Along these lines, this paper reports the synthesis of phosphatidylethanolamine from simple precursors.

Chemical synthesis of phosphatidylethanolamine was attempted in 1944 (Eibl 1980), and was in part achieved using protecting groups for the amino or the phosphate function or both. In some cases, the diacylglycerol was phosphorylated and reacted with N-blocked ethanolamine or the bromoethylester of 1,2-diacylglycerol-3-phosphate was converted to phosphatidylethanolamine by direct amination with ammonia. Other synthetic schemes have used phosphatidic acid as the starting material. Condensation of phosphatidic acid with suitably protected etha-

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nolamine gives phosphatidylethanolamine. However, most of these laboratory reactions cannot be considered to be likely events in the primordial Earth environment. Consequently, the aim of this study was to show that phosphatidylethanolamine can be synthesized from simple precursors in the absence of any protecting groups. Such a reaction system would constitute a further example illustrating how fairly complex molecules could have been synthesized in the prebiotic era.

Experimental

Materials

Ethanolamine and bee venom phospholipase A_2 were purchased from Sigma Chemical Company (St. Louis, MO). Cadmium acetate and methylamine were obtained from Fisher Scientific Company (Pittsburgh, PA). Phosphatidylethanolamine and lysophosphatidylethanolamine were purchased from Serdary Research Laboratories (London, Ontario, Canada). Phosphatidic acid as the disodium salt [1-(palmitoyl, stearoyl)-2-(oleoyl, linoleoyl) *sn*-glycero-3-phosphate, disodium salt] was obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). POPOP [1,4-bis-2-(5-phenyloxazolyl)-benzene] (scintillation grade) was bought from Packard Instrument Company, Inc. (Downers Grove, IL). PPO (2-5-diphenyloxazole) was obtained through Research Products International Corporation (Elk Grove Village, IL). Chloroform was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI) and methanol and acetone from MBC Manufacturing Chemists, Inc. (Cincinnati, OH). [2-^{14}C]ethanolamine hydrochloride (44 mCi/mmol) was purchased through Amersham (Arlington Heights, IL). All solvents were spectroscopic grade.

A crude preparation of phospholipase D was obtained by homogenizing 200 g of fresh cabbage leaves in 170 ml of ice cold distilled water in a prechilled Waring blender for 3 min in a cold room. The mixture was filtered through a plastic funnel and centrifuged at 1800 × g for 3 min to remove whole cells and debris. The supernatant was then centrifuged at 15,000 × g for 25 min to sediment the chloroplast fraction. The green pellet was washed by suspension in 80 ml of water and recentrifuged at 15,000 × g.

Scintillation bags were bought from Nalge Company (Rochester, NY). Silica gel 60 high performance thin-layer chromatography (HPTLC) plates without fluorescent indicator (manufactured by E. Merck) were obtained through Scientific Products (Houston, TX). Cellulose MN-300 250-μm prescored thin-layer plates were purchased from Analtech (Blue Hen Industrial Park, Newark, DE).

Synthetic Procedure

The standard reaction mixture that gave optimal yields of phosphatidylethanolamine was composed of a mixture of 2.6 μmol phosphatidic acid, disodium salt, 47 μmol cyanamide, 4.2 μmol ethanolamine, and 0.4 μCi [^{14}C]ethanolamine hydrochloride dissolved in 100 μl chloroform in a scintillation vial. A small amount of HCl (0.25 μmol) was added to bring the pH of this standard mixture to 7.3, which is well within the range of possible pHs on the primitive Earth. The mixture was vortexed for 30 sec and evaporated to dryness in a stream of nitrogen. The vial was capped and heated for 4–6 h at 37°C.

Recovery of Reaction Product

The reaction was terminated either by placing the vials in a freezer or by extracting them immediately. Each vial was rinsed with 1 ml 0.9% NaCl and 5 ml chloroform : methanol (2:1, v/v). The solution was vortexed and allowed to stand until the biphasic mixture separated. The upper phase was discarded and the lower phase was washed with ⅔ its volume of theoretical upper phase, chloroform : methanol : 0.9% NaCl (3:48:47, v/v). The upper phase was again discarded and the lower phase was dried under nitrogen. The lipids in the residue were dissolved in chloroform : methanol : water (75:25:2, v/v) in preparation for thin-layer chromatography (TLC).

Chromatography

The following solvent systems were employed for TLC of lipids on HPTLC silica gel 60 plates: for separations in one dimension: *Solvent System 1:* chloroform : methanol : ammonia (70:30:5, v/v), *Solvent System 2:* chloroform : methanol : acetone : acetic acid : water (6:2:8:2:1, v/v); for separations in two dimensions: *Solvent System 3:* chloroform : methanol : acetic acid : water (52:20:7:3, v/v) followed by *Solvent System 4:* chloroform : methanol : 20% methylamine : water (65:31:5:5, v/v). After being run in the first dimension, the plate was washed thoroughly with acetone in the direction of Solvent System 3 and turned 90° for development in the second dimension.

The water-soluble products from mild methanolic alkaline hydrolysis of phospholipids were chromatographed on plates coated with MN-300 cellulose in *Solvent System 5:* isopropanol : 15 N NH_4OH : water (7:2:1, v/v).

Radioassay

For radioactivity measurements, the TLC plates were scanned using a Berthold LB 2860 Radiochromatogram scanner. The radioactive bands were scraped off and the silica gel was suspended in 10 ml of toluene scintillation fluid and counted in a Packard Model 3380 Liquid Scintillation Spectrometer. The toluene scintillation fluid was prepared by dissolving 16 g of PPO and 0.2 g of POPOP in 4 l of toluene.

Elution and Characterization of Synthesized Phospholipids

When desired, the radioactive band that migrated like authentic phosphatidylethanolamine was scraped from a thin-layer plate developed in Solvent System 1 and eluted with chloroform : methanol : water : glacial acetic acid (50:39:1:10, v/v) according to Arvidson (1968). The eluate was dried under nitrogen and the residue was characterized.

Phospholipase A Degradation. The putative phosphatidylethanolamine was mixed with a sample of authentic phosphatidylethanolamine and hydrolyzed with phospholipase A_2 as described in our previous paper (Rao et al. 1982). The enzyme hydrolyzed the synthetic phosphatidylethanolamine to lysophosphatidylethanolamine.

Phospholipase D Degradation. To a suspension containing labeled reaction products (2000–4000 cpm) and 3 μmol of unlabeled phosphatidylethanolamine in two drops of chloroform : methanol (2:1, v/v) were added 0.3 ml of 0.2 M acetate buffer, 0.5 ml of 1 M $CaCl_2$, 0.2 ml of enzyme solution (20 mg protein/ml), and 0.2 ml of diethyl ether. The mixture was vortexed and

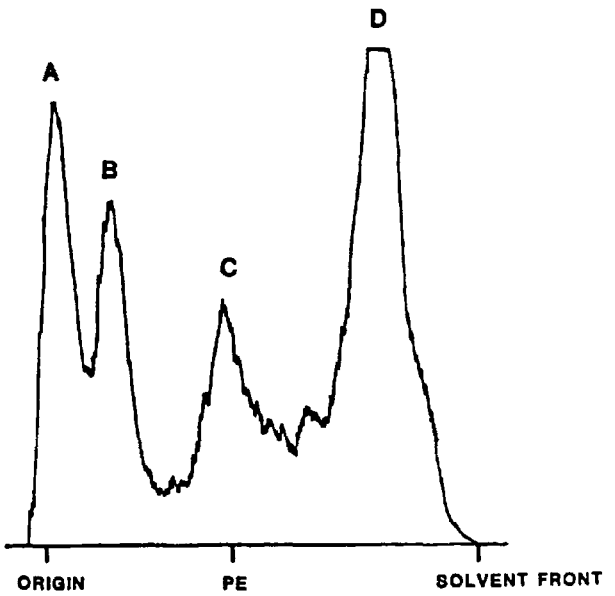

Fig. 1. Scan of ^{14}C radioactive products separated by TLC in Solvent System 1 following the reaction of the standard reaction mixture for 4 h at 37°C. Peak C cochromatographed with authentic phosphatidylethanolamine standard

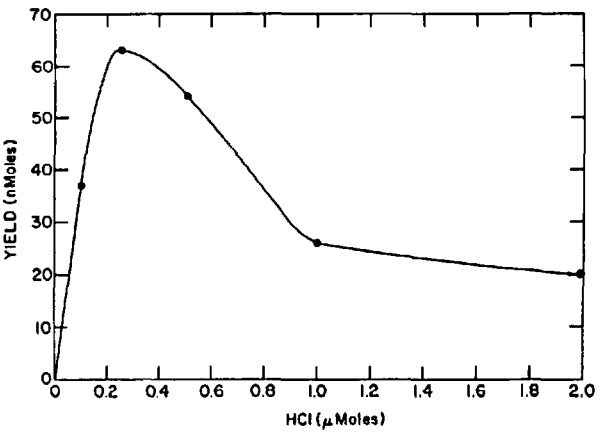

Fig. 2. Effect of different amounts of 0.1 N HCl included in the reaction mixture on the yield of phosphatidylethanolamine. A mixture consisting of 2.6 μmol phosphatidic acid, disodium salt, 47 μmol cyanamide, 4.2 μmol ethanolamine, and varying amounts of HCl (0–2 μmol) was incubated for 6 h at 37°C. The yield of phosphatidylethanolamine is plotted against the μmoles of HCl added. Each value is a mean of two independent determinations. An optimal yield was obtained with the addition of 0.25 μmol of HCl. The initial pH of this mixture before evaporation was 7.3

incubated overnight at 37°C. At the end of the incubation period, the mixture was evaporated to dryness in a stream of nitrogen and partitioned using 2.5 ml chloroform, 2.5 ml methanol, and 1.25 ml water. Aliquots from both the upper and lower phases were removed for radioassay and the water-soluble products in the upper aqueous phase were chromatographed in Solvent System 1 to identify the free base.

Chemical Degradation. Mild alkaline methanolysis of the presumed [^{14}C]phosphatidylethanolamine and separation of water-soluble products carried out in Solvent System 5 were performed according to the procedures outlined in our previous paper (Rao et al. 1982).

Results

Identification of Synthesized Phosphatidylethanolamine

Figure 1 shows a typical scan of a thin-layer chromatogram developed in Solvent System 1 to separate the radioactive products formed when phosphatidic acid, [^{14}C]ethanolamine, cyanamide, and hydrochloric acid were heated for 4 h at 37°C. Four radioactive peaks (A, B, C, and D) were observed. The radioactivity in Peak C cochromatographed with authentic phosphatidylethanolamine, as did a radioactive area when reaction products were separated by TLC using Solvent System 2. Other reactions under identical conditions were performed using [^{32}P]phosphatidic acid and unlabeled ethanolamine. When the reaction products were examined after one-dimensional separation in Solvent System 1, as well as in a two-dimensional separation in Solvent Systems 3 and 4, radioactivity was found in a position identical to that occupied by phosphatidylethanolamine.

Treatment of the putative phosphatidylethanolamine after elution using phospholipase A_2 resulted in the formation of a product that cochromatographed with lysophosphatidylethanolamine in Solvent System 1. Hydrolysis with phospholipase D resulted in the breakdown of the synthetic product to phosphatidic acid and ethanolamine. Mild alkaline methanolysis of the product resulted in the formation of a water-soluble compound, which, when chromatographed in Solvent System 5, moved to a position identical to that of glycerophosphorylethanolamine generated from authentic [^{32}P]phosphatidylethanolamine prepared as described by Hauser and Eichberg (1975). All three analytical results provide evidence for synthesis of phosphatidylethanolamine under the above conditions.

Synthesis of Phosphatidylethanolamine under Different Reaction Conditions

In these experiments, the composition of the reaction mixture was varied systematically to define the conditions for optimum yield of phosphatidylethanolamine. Each reaction was performed in a separate vial and analyzed individually.

Effect of Acid

The inclusion of small amounts of acid was found to be essential for the synthesis of phosphatidylethanolamine under the conditions studied. The reaction mixtures studied were composed of 2.6 μmol of phosphatidic acid, disodium salt, 47 μmol of cyanamide, 4.2 μmol of ethanolamine, and varying volumes of 0.1 N HCl. Figure 2 shows that the maximum yield of phosphatidylethanolamine was obtained with 0.25 μmol of HCl and that the yield declined as the amount of acid added increased. The pH of this specific mixture (containing 0.25 μmol of HCl) before evaporation was 7.3.

Effect of Temperature

Mixtures of 2.6 μmol of phosphatidic acid, disodium salt, 47 μmol of cyanamide, 4.2 μmol of ethanolamine, and 0.25 μmol of HCl were incubated at room temperature and 37, 60, 80, and 100°C for 6 h to determine the effect of temperature on the yield of phosphatidylethanolamine. Figure 3 indicates that the reaction was most efficient at relatively low temperatures, with maximum yield in the range of 37–60°C.

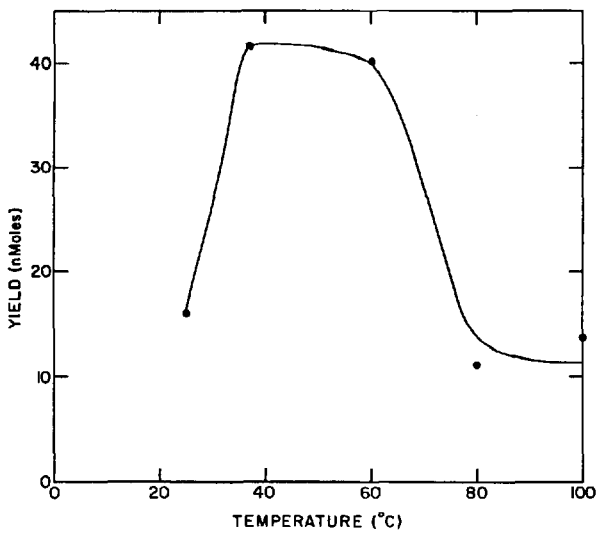

Fig. 3. The effect of temperature on the synthesis of phosphatidylethanolamine. The standard reaction mixture was incubated for 6 h at temperatures ranging from 25 to 100°C. The yield of phosphatidylethanolamine is plotted vs the temperature. Each value is an average of two independent experiments

Time Course of the Reaction

The standard incubation mixture was heated at 37°C for varying lengths of time ranging from 1 to 10 h. The yield of phosphatidylethanolamine increased gradually, with little change between 3 and 6 h of incubation, after which there was a decline with incubation time (Fig. 4). The decline probably resulted from the cessation of new phosphatidylethanolamine formation and the gradual hydrolysis of previously existing phosphatidylethanolamine to lysophosphatidylethanolamine or other breakdown products.

Effect of Cyanamide Concentration

The effect of varying the concentration of cyanamide while all other factors are held constant is shown in Fig. 5. Concentration of the condensing agent influenced the yield of phosphatidylethanolamine. A broad peak was seen, with little change in yield as cyanamide concentration increased from 25 to 70 μmol. A gradual decline was observed thereafter. No phosphatidylethanolamine was synthesized in the absence of cyanamide. In summary, the standard reaction mixture (2.6 μmol of phosphatidic acid, disodium salt, 47 μmol of cyanamide, 4.2 μmol of ethanolamine, and 0.25 μmol of HCl dissolved in 100 μl of chloroform) evaporated to dryness and heated 6 h at 37°C gave a yield of 1.5% of phosphatidylethanolamine on the basis of the ethanolamine used (Fig. 2).

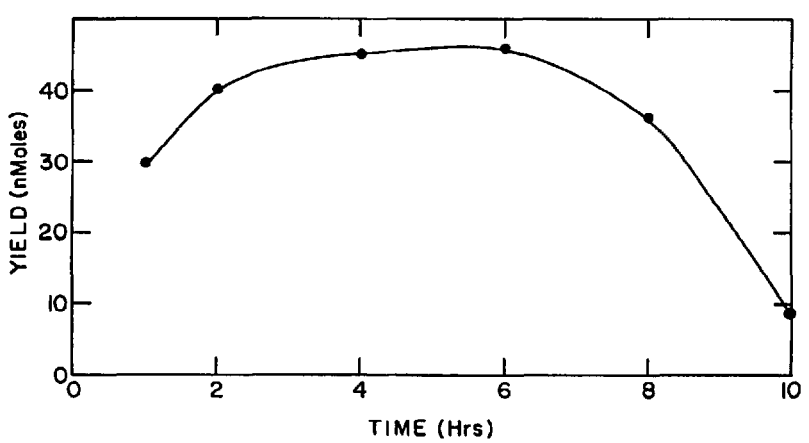

Fig. 4. Time course of formation of phosphatidylethanolamine. The standard reaction mixture was incubated at 37°C for varying lengths of time. The yield of phosphatidylethanolamine is plotted against the time of incubation. Each value is a mean of two independent determinations

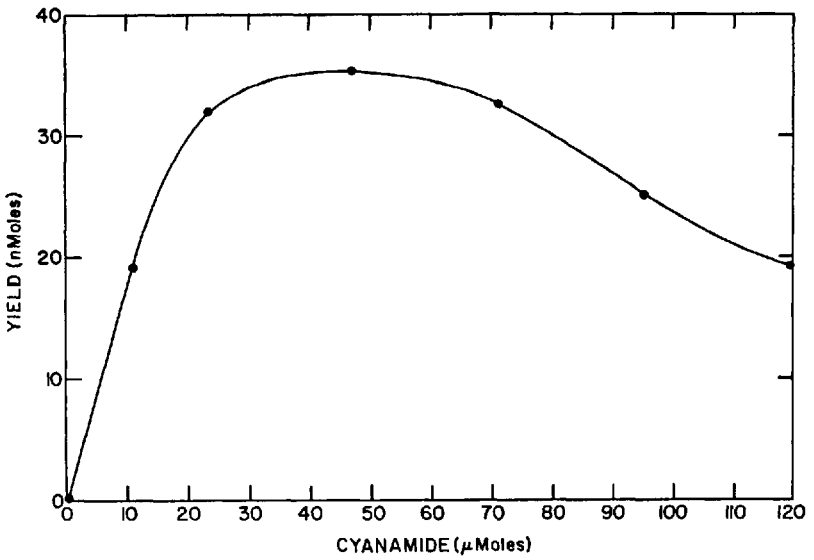

Fig. 5. Effect of cyanamide concentration on the yield of phosphatidylethanolamine. A mixture consisting of 2.6 μmol of phosphatidic acid, disodium salt, 4.2 μmol of ethanolamine, 0.25 μmol of HCl, and varying amounts of cyanamide was dried and incubated at 37°C for 4 h. The yield of radioactive phosphatidylethanolamine is plotted against cyanamide concentration. Each value is an average of two individual determinations

Discussion and Implications for Chemical Evolution

The following experimental evidence strongly supports the conclusion that the synthesis of phosphatidylethanolamine occurred under the conditions described: 1) the product obtained by the reaction of prebiotic compounds had the same mobility as authentic phosphatidylethanolamine in three different solvent systems; 2) when subjected to enzymatic hydrolysis with phospholipase D, the product released ethanolamine and when treated with phospholipase A_2, lysophosphatidylethanolamine was formed; and 3) glycerophosphorylethanolamine was liberated upon mild methanolic alkaline hydrolysis. Together these findings confirm that the radioactive material formed was phosphatidylethanolamine. The yield of phosphatidylethanolamine was similar to that achieved for phosphatidylcholine (Rao et al. 1982). This is of interest in view of the fact that the unprotected amino group of ethanolamine could lead to the formation of other products. On the other hand, the conditions for optimum synthesis of phosphatidylethanolamine are not very stringent, as the broad peaks obtained on varying reaction conditions indicate (Figs. 2–5). The synthesis of the phospholipid proceeds over a wide range of temperature, pH, and cyanamide concentration, a situation that would have been advantageous on the primitive Earth because it would have allowed the synthesis and accumulation of these compounds under a wide variety of environmental conditions.

In an attempt to elucidate the nature of the other radioactive products formed as shown by peaks A, B, and D (Fig. 1), palmitic acid was heated with ethanolamine and acid at 37°C for 6 h. Extraction and chromatography revealed the presence of peaks cochromatographing with B and D; one of these is probably *N*-palmitylethanolamine. This compound could be formed from the hydrolysis of newly synthesized phosphatidylethanolamine and the esterification of the released free fatty acid with ethanolamine.

As was discussed in our previous paper (Rao et al. 1982), all of the reactants used can be considered as prebiotic compounds. Thus, phosphatidic acid can be obtained in relatively high yields by heating *sn*-glycero-3-phosphate, ammonium palmitate, and cyanamide together at moderate temperatures (Epps et al. 1978); glycerophosphate can in turn be obtained abiotically by phosphorylation of glycerol (Epps et al. 1979), and palmitic acid by Fischer-Tropsch processes from carbon monoxide and hydrogen (Nooner and Oró 1979). Cyanamide can be confidently considered a prebiotic compound, as it has been shown to be present in interstellar space (Turner et al. 1976), and can be synthesized from sunlight-irradiated ammonium cyanide under possible primitive Earth conditions (Lohrmann 1972). Finally, ethanolamine could arise from decarboxylation of serine (Vallentyne 1964), which is one of the products obtained by different abiotic processes (Miller 1955; Oró et al. 1959; Weber and Miller 1981). In fact, production of ethanolamine would be even simpler than that of choline, which would require methylation of the amine group. Thus, in view of the ready synthesis of phosphatidylcholine under prebiotic conditions (as previously demonstrated by us: Rao et al. 1982), the present synthesis of phosphatidylethanolamine by condensation of phosphatidic acid and ethanolamine in the presence of cyanamide would not be totally unexpected.

The important role of both phosphatidylcholine and phosphatidylethanolamine as likely structural

components of protocellular membranes (Oró and Lazcano 1984) has already been emphasized in the literature (Deamer and Oró 1980; Deamer and Barchfeld 1982).

In the absence of more complete knowledge, we prefer not to speculate on the mechanism by which phosphatidylethanolamine synthesis takes place. It would be of interest first to try to synthesize phosphatidylserine under prebiotic conditions, and then do a comparative study of the pH effects and mechanisms of synthesis of the three key membrane phospholipids (phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine).

In summary, the abiotic synthesis of phosphatidylcholine (Rao et al. 1982) and phosphatidylethanolamine (this work) has been demonstrated using laboratory conditions that can be related to those existing on the primitive Earth. This indicates the likely primordial availability of membrane-forming phospholipids that can readily self-assemble into microvesicular structures or liposomes enclosing the necessary information and catalytic molecules required for the development of primordial living systems.

A recent observation that adds realism to the model studies on the formation of protocellular interphases is the fact that nonpolar organic components extracted from the Murchison carbonaceous chondrite also have physical properties that lead to the formation of boundary structures (Deamer 1985).

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