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Membrane Self-Assembly Processes: Steps Toward the First Cellular Life

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ABSTRACT

This review addresses the question of the origin of life, with emphasis on plausible boundary structures that may have initially provided cellular compartmentation. Some form of compartmentation is a necessary prerequisite for maintaining the integrity of interdependent molecular systems that are associated with metabolism, and for permitting variations required for speciation. The fact that lipid-bilayer membranes define boundaries of all contemporary living cells suggests that protocellular compartments were likely to have required similar, self-assembled boundaries. Amphiphiles such as short-chain fatty acids, which were presumably available on the early Earth, can self-assemble into stable vesicles that encapsulate hydrophilic solutes with catalytic activity. Their suspensions in aqueous media have therefore been used to investigate nutrient uptake across simple membranes and encapsulated catalyzed reactions, both of which would be essential processes in protocellular life forms. Anat Rec 268:196–207, 2002.

Key words: cellular life; membrane; boundary structures; amphiphile vesicles

The emergence of life on the early Earth required the presence of at least three different substances and related physical properties: liquid water, a source of free energy, and organic compounds capable of self-assembly. Liquid water is essential for all life today, and it is highly implausible that life can exist in its absence. Possible energy sources include sunlight (if life began on the Earth's surface) and energy arising from chemical disequilibria in submarine or subterranean sites. Self-assembling compounds must have been available to provide building blocks for polymer synthesis and formation of boundary structures.

The first forms of life were represented by self-assembled molecular systems with specific sets of chemical and physical properties; these are listed here to provide a foundation for later discussion. First, the system must have defined boundaries that separate it from its environment (Fleischaker, 1990; Deamer et al., 1994; Lazcano, 1994a, b; Deamer, 1997; Tawfik and Griffiths, 1998; Luisi et al., 1999; Szostak et al., 2001). A specific set of catalyzed metabolic and polymerization reactions must occur in the encapsulated volume, which implies effective exchange of nutrients and energy from the environment. Perhaps most central to the definition of life is that the entire molecular system must be able to reproduce itself using self-assembly of components and genetically coded polymerization reactions. This capacity implies information transfer between molecules within a cell, then from one generation to the next as the cell reproduces (Varela et al., 1974; Luisi and Varela, 1989). Finally, it must be possible for small changes to be introduced into the general components that direct polymerization. In a population of reproducing systems that compete for energy and nutrients, the changes produce variations in individual molecular systems that affect the efficiency of growth and reproduction. A population of bounded molecular systems can then undergo a variety of selective processes required for Darwinian evolution.

Our research has focused on the nature of boundary structures that defined the first cellular life. Such structures are required for speciation, energy capture and transduction, and development of the complex network of

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catalytic reactions associated with metabolism (Deamer and Oro, 1980; Deamer, 1997; Szostak et al., 2001). In contemporary cells, a fundamental role of membrane boundaries is to provide a selective permeability barrier that is necessary for separating the cytoplasm from the external environment. The transmembrane transport of nutrients and ionic solutes is mediated by a variety of membrane-associated proteins that act as channels, carriers, and active transporters (pumps). Membrane receptors provide a sensor mechanism that permits communication between the intracellular milieu and the outside world. Membranes also capture light energy and redox energy by using pigment systems and electron transport to generate electrochemical proton gradients as a source of free energy.

All of these functions require membrane-associated proteins, which were presumably absent in the first forms of cellular life. It therefore seems likely that the membrane boundaries of the earliest cells simply provided a selective permeability barrier that permitted the permeation of essential nutrients but retained polymeric products of primitive biosynthesis. This concept has guided our research over the past decade, and is the main theme of the current discussion.

MODELS OF PROTOCELLULAR COMPARTMENTS

The concept that life began on the early Earth as self-assembled structures of organic material was first proposed by Oparin (1924). Laboratory investigations of such structures began in the 1950s when Oparin (1957) proposed the concept of "chemical evolution as a transition to life." At that time the role of membranes as boundary structures had not yet been established. Instead it was believed that a living cell could be understood as a collection of aggregated colloidal particles. Therefore, Oparin and his coworkers prepared heterogeneous, spherical aggregates from macromolecular components, such as gum Arabic, gelatin, and histone, which could provide localized sites for enzymatic reactions (Oparin et al., 1976). These aggregates, called coacervates, were not intrinsically stable, and their molecular compositions were highly variable. Furthermore, coacervates have no permeability barrier, so they lacked the capacity for encapsulated metabolism and accumulation of biosynthetic products.

The first suggestion that membranes played a role in the origin of life was put forward by Haldane (1929), who wrote that "[t]he cell consists of numerous half-living chemical molecules suspended in water and enclosed in an oily film. When the whole sea was a vast chemical laboratory the conditions for the formation of such films must have been relatively favourable.. . ." Goldacre (1958) proposed that the first membranes could have been produced by wave action disturbing films of lipid-like surfactants.

As we learned more about the role of membranes in defining cell structures, it became clear that all membranes incorporated lipid bilayers as the primary permeability barrier, and that phospholipid is a nearly ubiquitous amphiphilic component of the bilayer. Bangham and coworkers (1965) first demonstrated that phospholipids spontaneously form bilayer vesicles with dimensions in the range of bacterial cells. Lipid bilayer vesicles are commonly referred to as liposomes, and such self-assembled membrane structures can be used as models of the earliest cell membranes. The first question we address concerns the nature of the lipid-like compounds available on the early Earth. One possibility is that phospholipids were synthesized during prebiotic chemical evolution. In fact, several early papers demonstrated that phospholipids could be synthesized under simulated prebiotic conditions from mixtures of fatty acids, glycerol, and phosphate (Hargreaves et al., 1977; Oro et al., 1978). However, the simultaneous presence of all three components on the early Earth is highly speculative, and we have therefore turned our attention to simpler membranogenic amphiphiles.

Stability and Permeability of Amphiphile Vesicles

Although the ability of phospholipids to self-assemble into membranous vesicles is common knowledge, it is less well known that a variety of membranous structures can also be prepared from single-chain amphiphiles such as fatty acids (see Fig. 1), fatty alcohols, and monoglycerides. We will argue that such vesicles are plausible models for the formation of early cellular compartments.

An important aspect of this argument is that the prebiotic availability of such amphiphiles has been established. Carbonaceous meteorites contain a rich mixture of organic compounds that were synthesized abiotically in the early solar system, and this mixture can be used as a guide to the kinds of organics that likely were available on the early Earth, either delivered during late accretion or synthesized at the Earth's surface. For example, Miller (1953) first demonstrated that amino acids are synthesized in mixtures of reduced gases that are chemically activated by impinging sources of free energy such as electrical discharge. The conjecture that similar reactions could occur in the early solar system was confirmed by the discovery of a variety of amino acids in the Murchison meteorite (Kvenvolden et al., 1970).

A similar argument can be made for monocarboxylic acids. These have been synthesized under a variety of simulated prebiotic conditions (Deamer and Oro, 1980; McCollom et al., 1999; Rushdi and Simoneit, 2001). Furthermore, monocarboxylic acids ranging from 2 to 12 carbons in length are abundant components of the organic mixture present in the Murchison meteorite (Lawless and Yuen, 1979; Komiya et al., 1993; Mautner et al., 1995). It has also been established that certain components of the Murchison organics are amphiphiles and have the capacity to assemble into membranous vesicles (Deamer, 1985; Deamer and Pasley, 1989). Figure 1 shows several examples of such vesicles by light microscopy, and it is clear that certain organic components have the capacity to assemble into recognizable membranes. The presence of a permeability barrier is confirmed by the fact that such vesicles can capture and maintain concentration gradients of pyranine, an anionic fluorescent dye marker (Apel et al., 2002).

Although the composition of the membrane-forming amphiphiles present in the Murchison organic mixture has not yet been established in detail, it is clear that substantial amounts of monocarboxylic acids are present (Mautner et al., 1995). For this reason we have begun to investigate the physical properties of self-assembled structures produced by monocarboxylic acids of various chain lengths, and of mixtures with other simple amphiphilic compounds. Gebicki and Hicks (1973, 1976) first established that oleic acid, a fatty acid, forms vesicular structures. Since this discovery, the bilayer-forming potential

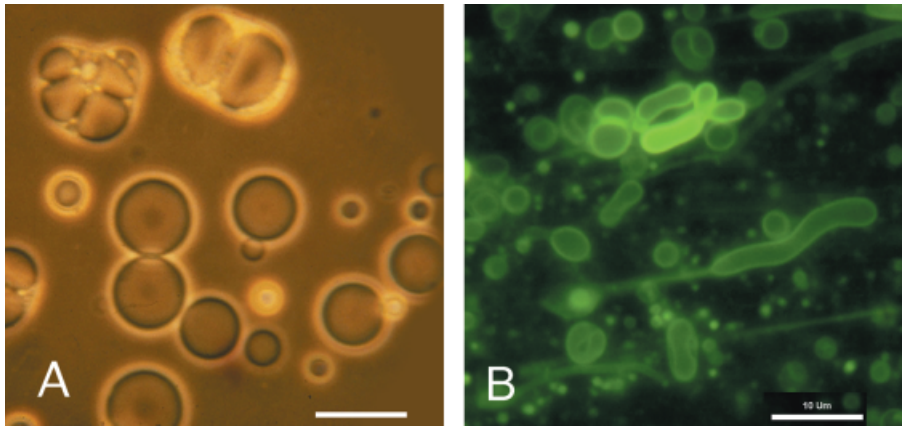

Fig. 1. Primitive membrane structures visualized by light microscopy. **A:** Amphiphilic compounds extracted from the Murchison meteorite form membranous vesicles when exposed to dilute aqueous salt solutions at pH > 7.0. The probable components of the vesicles are monocarboxylic acids ranging from 8 to 11 carbons in length together with admixtures of polycyclic aromatic hydrocarbon derivatives. **B:** Monocarboxylic acids in pure form also self-assemble into membranous vesicles, as shown here for DA:decanol (37 mM:3mM, C10, pH 7.4) stained with rhodamine 6G and observed by epifluorescence microscopy. This dye inserts in the bilayer membranes, which appear green. Bar = 10 μm.

of fatty acids with shorter hydrocarbon chains (C8-C11) has been investigated (Hargreaves and Deamer, 1978; Apel et al., 2002; Monnard et al., 2002). The length and the degree of unsaturation of the hydrocarbon chains play an important role in determining the bilayer-membrane properties, such as permeability and stability, which would have been essential for the primitive life forms.

In suspensions of fatty acids at concentrations above the critical bilayer concentration (CBC) (by analogy to the critical micelle concentration (CMC)), fatty acid bilayer membranes (see part 1 in Fig. 2) are stabilized by van der Waal interactions between their hydrocarbon chains and by hydrogen bonds formed between deprotonated and protonated acid molecules (Rosano et al., 1969; Haines, 1983). For this reason the formation of bilayer vesicles is highly sensitive to pH (see Fig. 3). Below the apparent pK_a of the acid inserted in a structure (this pK_a is higher than that of a single acid molecule), the fatty acid droplets will replace the bilayer structures, and above it micelles will form. In addition, CBC increases with decreasing length and degree of unsaturation of hydrocarbon chains. For instance, octanoic acid (C8:0, the shortest vesicle-forming carboxylic acid), decanoic acid (DA) (C10:0), and oleic acid (C18:1) form bilayers at amphiphile concentrations higher than 130, 43, and 0.85 mM, respectively. This tendency becomes significant when the prebiotic availability of these amphiphiles is considered. The analysis of the fatty acid content of the Murchison meteorites shows that fatty acids with hydrocarbon chain length from C8-C12 are present. Reaction products of Fischer-Tropsch-type synthesis produce mixtures of alkanoic acids with hydrocarbon chains as long as 22 carbons, yet the main products were again heptanoic (C7:0), octanoic (C8:0), and nonanoic acids (C9:0) (Rushdi and Simoneit, 2001). If pure fatty acids form the protocellular bilayers, the relative distribution of fatty acids would result in membranes with short-chain amphiphiles that have a very high CBC, implying that high concentrations of short fatty acids would be required to trigger vesicle formation. This raises the issue of availability in the early Earth environment, in which short-chain fatty acids likely were present in low concentrations, and hence an efficient concentrating mechanism was needed to reach their CBC. One can reasonably speculate that vesicles of short-chain fatty acids could have existed in pools where water evaporation helped to concentrate the amphiphiles, together with other solutes. It follows that this concentrating mechanism would have not only aided formation of the first membranes, but would have also increased the probability for a newly formed vesicle to encapsulate catalytic species that perhaps were already present in the environment, along with an initial substrate supply.

Permeability of primitive membranes is also a significant factor to consider. Recent works on cellular lipid membranes have clearly underscored the role of the lipid moiety as the primary permeability barrier to free diffusion of polar and ionic solutes. Further investigations of vesicle bilayers have shown that their permeability can be modulated by varying bilayer composition. In general, the permeability of bilayers to ionic solutes is inversely proportional to the length and degree of unsaturation of the hydrocarbon chains.

It may be therefore have been advantageous for primitive life forms to have membrane boundaries composed of short-chain amphiphiles. Such bilayers would have significantly facilitated exchange of solutes between the protocell and its environment, allowing for a steady supply of nutrients.

Apel et al. (2002) investigated the permeability of bilayers formed from short-chain monocarboxylic acids (8-11 carbons), and established that these vesicles could efficiently retain polymeric material such as nucleic acids (see Fig. 4) or proteins. Other large ionic molecules, such as ADP, can also slowly diffuse across oleic acid bilayers (C18:1 Δ9), and serve as both as an energy source and substrate for RNA polymerization by polynucleotide phosphorylase (PNPase) (Walde et al., 1994a).

We must also take into account the effects on bilayer stability of environmental conditions such as salinity, ionic strength, pH, and divalent cations. For instance, if the emergence of life occurred in a marine environment, small sodium chloride concentration gradients would be expected to have developed osmotic pressure across membranes (Wilson and Maloney, 1976). We have investigated

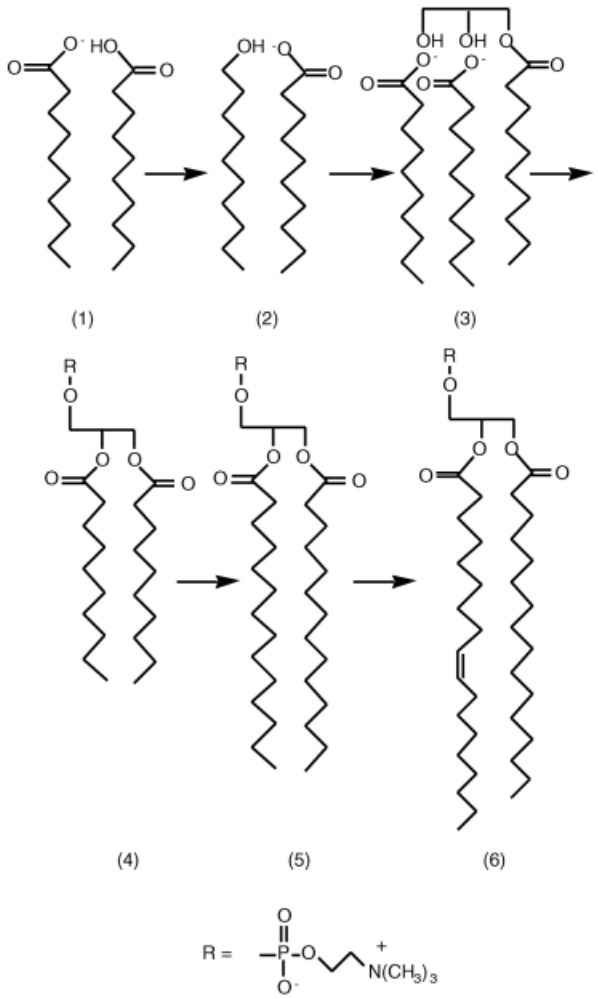

Fig. 2. Chemical structure of amphiphiles and amphiphile mixtures forming bilayer vesicles: 1) pure fatty acid vesicles (DA), 2) DA/decyl alcohol mixtures, 3) DA/glycerol decanoate mixtures, 4) didecyl-sn-glycerol-3-phosphocholine (DCPC), 5) dimysristoyl-sn-glycerol-3-phosphocholine (DMPC), and 6) 1-palmitoyl-2-oleyl-sn-glycerol-3-phosphocholine (POPC).

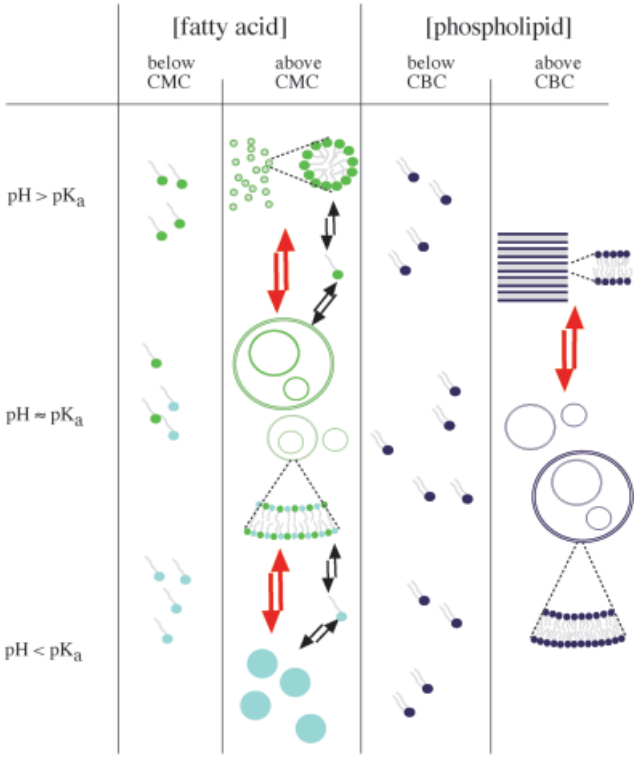

Fig. 3. Structures formed by amphiphilic molecules suspended in aqueous solutions. Fatty acids and phospholipids assemble into a variety of structures when suspended in aqueous media. The self-assembly process depends on the amphiphile concentration, the pH, and the ionic content. Pure fatty acids are deprotonated (green headgroup) at pH values higher than the apparent pK_a, and form micellar aggregates at concentrations higher than the CMC. When the pH of a micellar suspension of fatty acids is slowly lowered to their apparent pK_a ($\pm$0.5–1 unit), vesicles become the predominant structure as the number of protonated molecules increases. Upon further acidification, fatty acid molecules become entirely protonated (turquoise headgroup), and droplets of free acid form. At all pH ranges, fatty acid aggregates are always in dynamic equilibrium with single molecules. Pure phospholipids below their CBC are present as single molecules. As their concentration increases above CBC, two structures are possible: at low water content, PCs form stacked planar bilayer sheets, which then transform into vesicular structures at high water content.

this effect and established that fatty acid vesicles are unstable in sodium chloride at concentrations near that of sea water (Monnard et al., 2002). The self-assembly of DA into vesicles is markedly inhibited at molar ratios of lipid to NaCl exceeding 3:1 (see Fig. 5). Furthermore, millimolar concentrations of divalent cations in marine salts cause fatty acid vesicles to precipitate (Szostak et al., 2001; Monnard et al., 2002). The presence of divalent cations in early seawater would presumably inhibit the self-assembly of fatty acid membranes. These observations suggest an important constraint on aqueous sites related to the origin of cellular life. Unless future research demonstrates a plausible mixture of amphiphiles that can produce stable bilayer membranes in the presence of typical marine salts, it is more likely that cellular life was first established in a fresh-water environment. This implies that land masses were required for the origin of life, so that fresh-water could accumulate in the form of ponds, rivers, and shallow inland seas.

Phosphatidylcholine bilayers. Phosphatidylcholine (PC) is a highly evolved lipid species that is synthesized by a series of enzyme-catalyzed, energy-dependent reactions. As such, PC provides a marked contrast with fatty acid as a membrane component. PC vesicles, also called liposomes, spontaneously form at CBC as low as 10^{-9}–10^{-12} M. These structures are very stable to pH and divalent cations, and can withstand wide temperature variations (Oberholzer et al., 1995b). Although stable vesicles are formed by PC molecules with relatively long hydrocarbon chains (liposomes with hydrocarbon chains shorter than 10 carbons are unstable), the synthesis of PC under prebiotic conditions seems implausible because of its molecular complexity, so these vesicles are unlikely candidates for self-assembly of early membranous boundary structures. Nonetheless, their relative stability also makes PC vesicles a useful model system in the laboratory.

The permeability of PC bilayers to large ionic solutes is generally lower than that of fatty acid bilayers. The per-

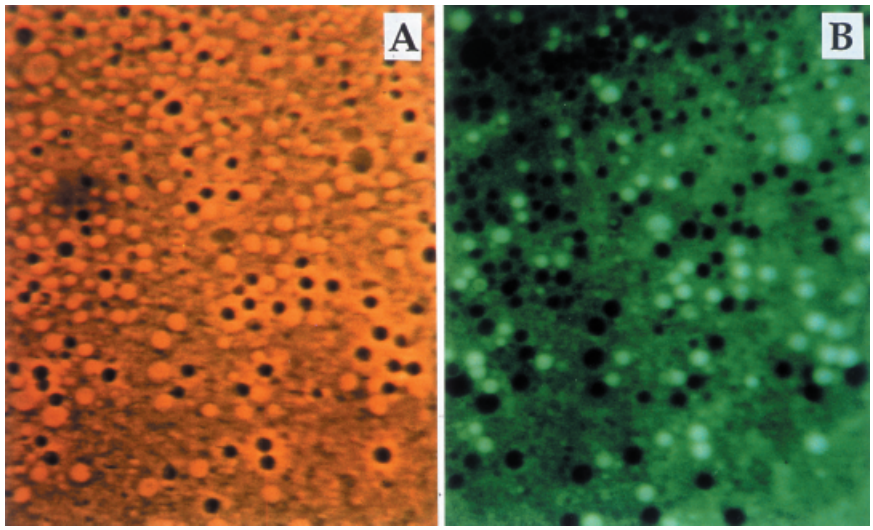

Figure 4.

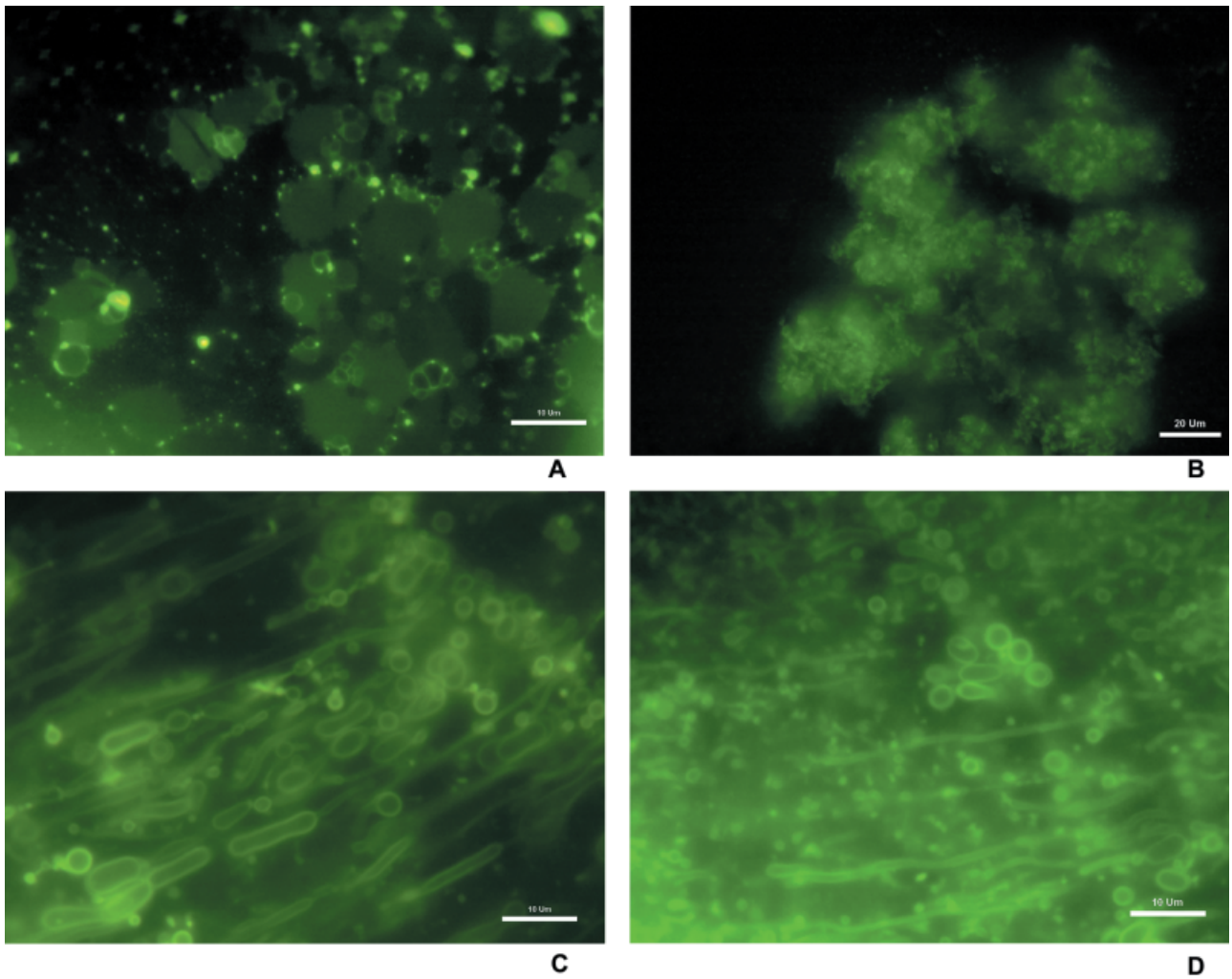

Figure 5.

meability properties of PC membranes have been extensively investigated for small ionic solutes (Kanehisa and Tsong, 1978; Rosenquist et al., 1981; Deamer and Bramhall, 1986; Chakrabarti et al., 1994; Paula et al., 1996) and small solutes (Langner and Hui, 1993), in part because these lipids represent a significant portion of the amphiphiles that form biological membranes. The diffusion of charged solutes is clearly dependent on the gel-fluid phase transition (Mouritsen et al., 1995), and the length of the hydrocarbon chains. Chakrabarti et al. (1994) have further established that bilayer permeability to larger ionic molecules, such as ADP, has three distinct regimes. If the lipid chains are shorter than 12 carbons in length, no selective permeability is observed. Bilayers composed of 16- or 18-carbon chains are relatively impermeable, and can maintain concentration gradients of ADP for hours or days. On the other hand, dimysristoyl-*sn*-glycerol-3-phosphocholine bilayers (DMPC; C14:0) are sufficiently permeable so that diffusion of solutes the size of ADP can provide substrates for entrapped enzymes. For this reason DMPC is often chosen for the experimental model systems described below.

Mixed amphiphile systems. So far we have considered membranes composed of a single amphiphilic species, and these systems are very interesting as experimental models. However, membranous boundaries composed of a pure amphiphile are implausible under prebiotic conditions, so it is reasonable to propose that primitive cellular life forms relied on mixed bilayers from abiotic sources of amphiphiles. In fact, all organisms today use lipid mixtures to construct cell membranes, and some also require self-assembling amphiphiles from the environment. In the case of human beings, examples include tocopherols and carotenoids (vitamins E and A) and essential fatty acids such as linoleic and arachidonic acid. Admixture of cholesterol is also used by eukaryotic cells to modulate properties of bilayers, such as permeability and robustness.

Studies of mixed bilayers have demonstrated that similar modulating effects can be achieved by mixing prebiotically plausible amphiphiles. For instance, adding small amounts of a second amphiphile, such as a fatty alcohol, to pure fatty acid bilayers can substantially lower their CBC and mitigate disruptive effects of inorganic solutes on fatty acid bilayers (see Fig. 5). Such amphiphiles are considerably less complex than cholesterol, and can be synthesized in Fischer-Tropsch-type reactions that are plausible for early Earth organic synthesis.

As an example of such effects in mixed bilayers, we have prepared vesicles composed of mixed fatty acids (C8-C11) with relative concentrations corresponding to their distribution in the Murchison meteorite. The concentration of each amphiphile was substantially below its CBC, and yet the resulting mixture produced stable vesicular structures (unpublished data).

In another example, adding alcohols with the same hydrocarbon chain length to fatty acid vesicles (Apel et al., 2002) not only reduced the CBC of the fatty acid, but also dramatically increased the stability of vesicles in alkaline conditions (see part 2 in Fig. 2). When a micellar solution of nonanoic acid (at pH 11) was mixed with 1-nonanol, which by itself does not form vesicles, vesiculation was immediately induced upon vortexing. An aqueous solution of nonanoic acid required a concentration at least 85 mM to form vesicles, but only 20 mM acid when mixed with 2 mM nonanol. Longer-chain alcohols exhibit the same property when mixed with the corresponding fatty acids. When these vesicles containing encapsulated nucleic acid, or proteins are exposed to digestive enzymes (Apel et al., 2002), the encapsulated species remain functional, which establishes the relative stability of the mixed structures.

The mechanism underlying increased vesicle stability upon addition of an alcohol presumably involves hydrogen-bonding alcohol headgroups that are not pH-dependent, which enhances the stability of the structures. This interaction does not inhibit the electrostatic interaction between carboxylic headgroups and divalent cations; thus, fatty acid/fatty alcohol vesicles are not significantly more stable in the presence of divalent cations than those composed of pure fatty acids. On the other hand, polyols (e.g., monoglycerides (Hargreaves et al., 1977)) are able to mitigate this disruptive interaction and allow the formation of more robust membranes (see part 3 in Fig. 2). Mixed vesicles composed of glycerol monodecanoate (GMD)/DA at a molar ratio of GMD/DA near 1:2 were exposed to increasing concentrations of NaCl, and divalent cations (Monnard et al., 2002).We found that these vesicles could cope with ionic inorganic solutions that were 10 times more concentrated than the pure fatty acid counterparts.

We also note that the addition of fatty acids or fatty alcohols to phospholipid vesicles markedly increases their permeability to large ionic solutes, in marked contrast to the stabilizing alcohol effects on fatty acid bilayers. For example, addition of myristic acid (MA; C14:0) to DMPC (C14:0, C14:0) increases the permeability of the bilayers toward large charged molecules, such as NTPs, by one order of magnitude (Monnard and Deamer, 2001). Similarly, the addition of amphiphilic detergents to phospholipid membranes at sublytic concentrations permits the passive diffusion of molecules as large as proteins across phosphatidylcholine bilayers that are otherwise totally impermeable (Oberholzer et al., 1999).

Prebiotic Plausibility of Various Membrane Models

To summarize, it is likely that the components of early membranes have undergone a considerable evolution as the first forms of life evolved and slowly acquired new

Fig. 4. Encapsulation of macromolecules in fatty acid bilayers visualized by (**A**) epifluorescence microscopy phase and (**B**) epifluorescence micrographs of vesicles produced from n-dodecanoic acid, n-dodecanol (5:1 molar ratio) at pH 8. The mixed vesicles formed by pH vesiculation were mixed in a 2:1 mass ratio with sonicated salmon testis DNA (approximately 600 bp in length), and then dried. A dilute solution of acridine orange dye was added, and the newly formed vesicles were photographed by phase and fluorescence. Bar = 20 μm. Vesicles with entrapped DNA that are dark in part A contain DNA that becomes fluorescent under UV illumination in part B. B: Phase-light vesicles that do not contain DNA do not take up dye, and therefore do not fluoresce. The dark phase contrast is produced by the refractive index difference in those vesicles containing DNA.

Fig. 5. Stability of fatty acid vesicles compared to fatty acid/glycerol alkanoate mixed vesicles in the presence of ionic solutes. **A:** DA (64 mM) in the presence of sodium chloride (636 mM) at a molar ratio DA to NaCl of 1:10. **B:** DA (64 mM) in the presence of calcium chloride at a molar ratio DA to divalent cation of 2:1. **C:** A mixture of GMD/DA (7.5 mM/15 mM) in the presence of sodium chloride (447 mM) at a molar ratio DA to NaCl of 1:33. **D:** A mixture of GMD/DA (7.5 mM/15 mM) in the presence of calcium a molar ratio DA to divalent cation of 2:1. DA vesicle suspensions (see Fig. 1B) when exposed to either NaCl or $CaCl_2$, precipitate, whereas GMD/DA mixed vesicles remain intact.

TABLE 1. Summary of the properties of various compartments models

Compartment type	Amphiphile	Properties	
		Limitations	Strengths
Coacervates		Aggregates of proteins, gelatine Low stability No selective permeability	Localized sites for enzymatic reactions
Vesicle	Fatty acid	High CBC Sensivity to salts	Prebiotic synthesis Entrapment efficiency Selective permeability membrane growth
	Phosphatidylcholine	Complex molecule Low permeability	Stability (temperature, salts) Encapsulation efficiency
	Mixed amphiphiles	Sensivity to salts	Prebiotic synthesis Encapsulation efficiency Selective permeability
	Isoprenoids	Synthesis/availability	Encapsulation efficiency Selective permeability
Mineral		No enclosed volume	Availability Selectivity by absorption

catalytic capacities. Figure 2 summarizes the chemical structures of six different amphiphile systems, all of which are capable of forming vesicles, and the increase in molecular complexity is readily apparent. Understanding the emergence of life and its early evolution is closely related to comprehending the origin and evolution of cellular boundaries. At first, the requirements imposed on early membranes by the lack of protein transport assemblies, and of stabilizing structures, such as cytosomal support proteins or cell walls suggests relatively permeable membrane boundaries which are best modeled by fatty-acid vesicles (see Table 1). As the early cells began to synthesize polymeric molecules (perhaps RNA fragments (Khvorova et al., 1999; Vlassov et al., 2001), small polypeptides (Ghadiri et al., 1994; Oliver and Deamer, 1994; Clark et al., 1998; Kim et al., 1998), or other molecules that could mediate simple transport processes), more robust mixed amphiphile membranes that are modeled by short-chain phospholipid/fatty acids mixtures (such as DMPC/MA) would have replaced them. Finally, as populations of microbial organisms increasingly were able to produce their own specific amphiphiles, membranes of more homogenous composition would have appeared.

We note that amphiphile candidates other than fatty acids have been proposed, such as polyprenyl derivatives (Nomura et al., 2001). These molecules also form vesicles that can capture and retain macromolecules, such as DNA, but are limited by the lack of a prebiotically plausible synthetic pathway (Ourisson and Nakatani, 1999).

Non-amphiphile compartments. Bernal (1951), Wächtershäuser (1988), and Cody et al. (2000) have proposed a different approach to the self-assembly of organic compounds, in which mineral surfaces act as templates for assembling organic solutes into ordered structures. Indeed, a variety of polar substances can adsorb with considerable specificity on clays, followed by polymerization reactions (Ferris, 1994, 1999). The adsorption on clays, although effective, does not have clear continuity with an evolutionary process leading to cellular life. Assuming that newly produced polymers can be released from binding sites on mineral surfaces, the lack of true cellular compartmentation would prevent their accumulation and significantly reduce further reactions that are essential for the evolution of increasingly complex catalytic reaction networks. Although it is highly probable that mineral surfaces played a role in the synthesis of complex organic molecules, they do not intrinsically possess the properties of compartmentation provided by amphiphile vesicles.

COMPARTMENTALIZATION OF CATALYTIC SPECIES

Even though the membranous boundaries of early forms of cellular life were more permeable than those of contemporary cells, they would have been sufficiently impermeable to polymeric materials so that complex networks of catalyzed reactions could develop. It follows that encapsulation of primitive catalytic molecular assemblies assumed to be present in the environment would have required a reversible process by which the bilayers were first disrupted, allowing the entry of these assemblies, and then resealed. The encapsulation of single molecules may have been fairly common on the early Earth in vesicles with an internal volume ranging from 5×10^{-13} L to 5×10^{-18} L for vesicular structures with a 10-μm to 200-nm diameter. Encapsulation of specific multiple-component catalytic systems would have been increasingly difficult as the number of components increased. This suggests that the metabolism of early cells might have been necessarily simple until protein-mediated transport systems evolved.

Under prebiotic conditions, three plausible entrapment processes can be envisioned: 1) simultaneous dehydration/rehydration of the vesicles and solutes (Deamer and Barchfeld, 1982; Shew and Deamer, 1985; Monnard et al., 1997); 2) production of amphiphile molecules, from non-bilayer-forming precursors, in an environment containing solutes followed by amphiphile self-assembly (Walde et al., 1994b); and 3) aerosol-based vesicle formation (Dobson et al., 2000).

Two of these processes have been extensively used in experimental studies of catalytic assemblies entrapped in amphiphile vesicles. The dehydration/rehydration process is based on the simultaneous drying of vesicles and macromolecules, which results in the formation of multilayered structures of lipid bilayers with intercalated solutes. Solute molecules are captured upon rehydration when the

lipid bilayers reseal into vesicles, and it seems likely that multiple cycles of dehydration/rehydration could have occurred in intertidal zones on the early Earth. The second process was delineated by Luisi and coworkers (Bachmann et al., 1992; Walde et al., 1994b), who proposed that membranous structures were produced at the expense of chemical energy stored in water-insoluble amphiphile precursors, or delivered to them from external sources. As the concentration of amphiphiles increased to values above the CBC, vesicles would have formed, entrapping macromolecules.

Once the catalytic species has been encapsulated in vesicles, access to nutrients and energy sources becomes crucial. As stated above, early life forms presumably lacked specialized membrane transport systems, so simple uptake mechanisms, such as passive diffusion, would play an essential role in the nutrient transport across boundary membranes. Could transmembrane diffusion be fast enough to keep up with the demands of a primitive metabolism? The metabolic rates of early cellular life are difficult to estimate, but it is likely that nutrient requirements may have been less than those of contemporary cells. For instance, if in fact an RNA world was a stepping stone toward contemporary life, it is clear that reactions catalyzed by ribozymes proceed much more slowly than those catalyzed by similar enzymes (Cech and Golden, 1999; McKay and Wedekind, 1999).

As soon as a recognizable metabolism is established within cellular boundaries, an energy supply becomes paramount. Several aspects of primitive metabolism have been studied, including energy uptake, compartmentalization of catalytic species pertaining to genetic-code information transfers (see Fig. 6), and general issues related to metabolism. (See Walde and Ichikawa (2001) for a review of catalytic species entrapped in vesicular structures.) Three sources of energy could be harvested by primitive cells: chemical energy in the form of chemical bonds or oxidation-reduction reactions, and light energy (Deamer, 1997). Abundant sources of chemical energy are highly plausible components of the prebiotic environment (Morowitz, 1992). However, as the membranous boundaries evolved and became less permeable to free diffusion of solutes, the uptake of chemical energy would have decreased due to slower permeation rates (Deamer, 1991).

At some point, light energy became the most abundant source of energy, as it is today, but how could it have been harvested by primitive cellular life? To capture light energy, photons must first be absorbed by a pigment system and then transduced into usable forms of chemical energy. It is reasonable to assume that photosynthetic assemblies comparable to those of contemporary life were absent, so that pigment systems such polycyclic aromatic hydrocarbons (PAHs), organic iron complexes, porphyrins, and proteinoids could have been incorporated into the structure of bilayer membranes (Deamer, 1991, 1997). The presence of PAH as an abundant organic component of carbonaceous meteorites materials has been established (Cronin et al., 1988), and it seems likely that PAHs and their derivatives would have been among the most common organic compounds in the early Earth environment. A few preliminary studies of PAHs as pigment systems have been reported. For instance, upon illumination of amphiphile membranes containing small amounts of pyrene carboxylaldehyde or other PAH derivatives, substantial pH gradients can be established, the vesicular internal volume becoming acidic.

Encapsulated "genetic" information transfer. Even an early form of life must have possessed some sort of genetic apparatus that could direct the synthesis of polymers and pass genetic information from one generation of cells to the next. Although it is unlikely that nucleic acids and proteins as such were components of the apparatus, analogous polymers, which were capable of the linked interactions leading to evolutionary selection, must have been synthesized by an as yet unknown pathway. For this reason, we have been exploring simple "genetic" information transfer within vesicles.

A minimal transcription system should be composed of a molecule with the dual functions of catalysis and information storage (Joyce, 1998; Rogers and Joyce, 1999). RNA seems to be a plausible candidate, as first proposed by Gilbert (1986) in the "RNA world" conjecture. Even though the search for an RNA fragment with polymerase activity has progressed rapidly (Johnston et al., 2001), an efficient RNA-dependent RNA polymerase remains elusive, and no working experimental system is yet available. Therefore, most of the research on genetic information transfer within amphiphile vesicles has been conducted using RNA/DNA polymerase enzymes, PNPase (Chakrabarti et al., 1994; Walde et al., 1994a), *Taq* polymerase (Oberholzer et al., 1995b), Qβ replicase (Oberholzer et al., 1995a), and T7 RNA polymerase (Monnard and Deamer, unpublished results). These systems can help us determine which mechanisms might have been involved in the early information transfer.

In his work with coacervates, Oparin (1976) studied RNA polymerization mediated by polynucleotide phosphorylase (PNPase). The PNPase in living cells normally functions to hydrolyze RNA to monomeric species, but if NDPs are in excess it will produce random RNA polymers thousands of nucleotides in length. To demonstrate that polynucleotides can be synthesized by encapsulated PNPase using transmembrane transport of substrates, two groups independently encapsulated PNPase within different vesicular systems: DMPC (C14:0) (Chakrabarti et al., 1994), a phospholipid; and oleic acid (C18:1 Δ9) (Walde et al., 1994a), an unsaturated fatty acid. In both experiments, ADP was the substrate and RNA in the form of poly(A) was produced after 1–5 days incubation, and remained within the amphiphile-bound compartment (Fig. 6A). The reaction rate with the DMPC-encapsulated enzyme was determined to be approximately 20% of that with PNPase in an aqueous buffer, showing that the bilayer was a substantial barrier to substrate permeation. These systems demonstrated that ADP could permeate across lipid bilayers at rates sufficient to support polymer synthesis by PNPase.

The next experimental step involved encapsulation of a more complex enzyme system capable of catalyzing replication or transcription. Two such reactions include the amplification of RNA template by Qβ replicase entrapped in oleic acid vesicles (Oberholzer et al., 1995a) and the amplification DNA by PCR within phospholipid vesicles (Oberholzer et al., 1995b) (Fig. 6B). Both reactions are template-dependent and require metal ions as cofactors, as well as primers for the PCR experiment. All components of the reaction (three for the Qβ replicase, and five for the PCR) must be captured simultaneously in a single vesicle with their respective substrates because of the low permeability of the bilayer membranes to NTPs and dNTPs. In both cases, the expected products were formed

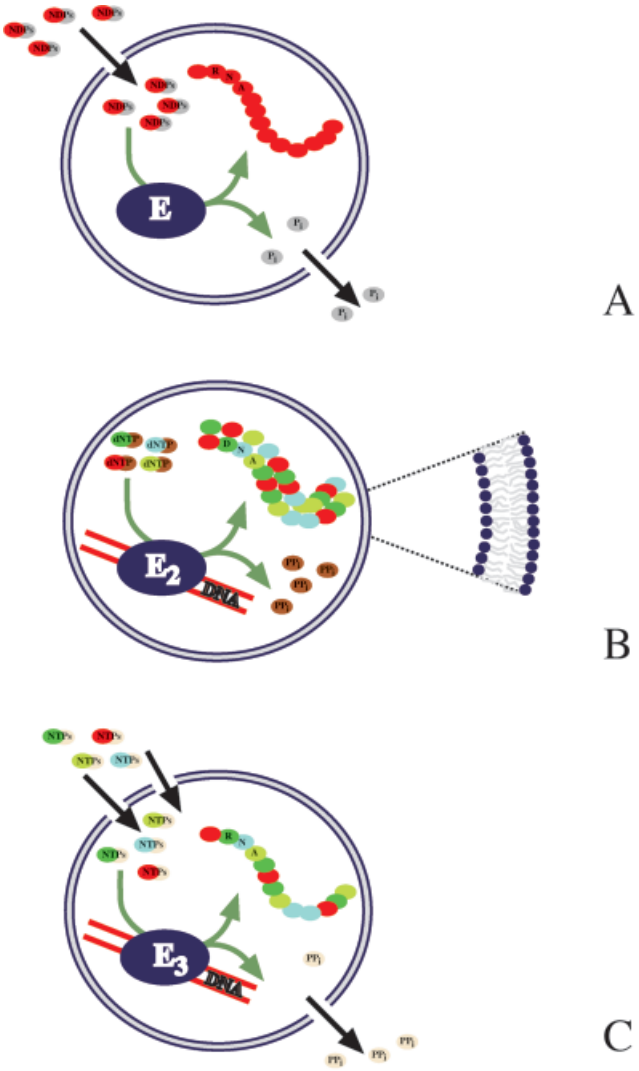

Fig. 6. Schematic representation of enzymatic reactions in vesicles. RNA polymerization mediated by polynucleotide phosphorylase. **A:** E represents the PNPase enzyme encapsulated within liposomes. ADP is added in the external medium, and must passively diffuse across the amphiphile bilayers to be processed by the enzyme. **B:** PCR in POPC liposomes. The DNA polymerase enzyme (E_2) with its template (the primers are omitted), and its substrates were encapsulated simultaneously. **C:** RNA transcription mediated by T7 RNA polymerase. The T7 RNA polymerase enzyme (E_3) and its template are encapsulated. NTPs are added externally as an energy source and substrates for the enzyme.

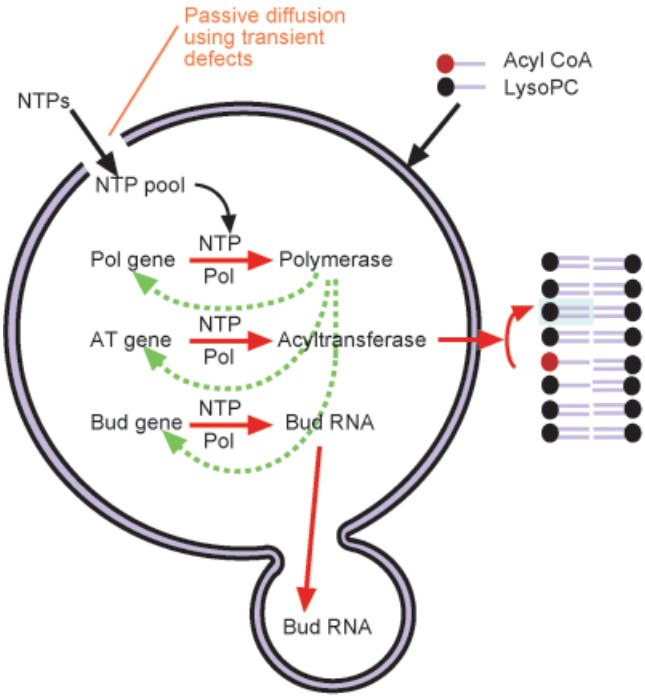

Fig. 7. A minimal cell based on catalytic/structural RNA. A laboratory version of a minimal cell depicted here would possess a rudimentary genetic library coding for three catalytic/structural RNA required for its growth and self-reproduction: Pol gene (polymerase for replication and transcription), AT gene (acyltransferase for lipid synthesis), and bud RNA. Substrates in the form of NTPs passively diffuse across the bilayer membranes using transient defects, while precursors of bilayer compounds (Acyl CoA and lysophosphatidylcholine (LysoPC)) partition into the existing bilayers (black arrows) where the AT RNA synthesizes phospholipids. The red arrows represent the actual reaction network of the minimal cells: RNA polymerization, lipid formation, and structural promotion of membrane budding. The green dashed arrows represent the secondary activity of the polymerase ribozyme that produces copies of the genes. Note that the polymerase acyltransferase and three genes would also be present in the aqueous volume of the budding membrane.

in low yields. Oberholzer et al. (1995b) calculated that the average aqueous vesicular volume was 3.3×10^{-18} L in the PCR experiments, which represents approximately 8,000 dNTP molecules per reaction volume, enough to produce approximately 10 double-stranded DNA products.

In the Oberholzer et al. (1995a, b) studies, polymerization was limited by the low permeability of PC bilayers to substrate molecules. However, the PNPase experiments using DMPC vesicles established that passive diffusion of substrate under certain conditions may be fast enough to allow template-directed enzymatic amplification of encapsulated genetic material. We have attempted to develop this approach further by designing a system capable of relatively high rates of substrate permeation. Because of their stability and permeability properties, DMPC liposomes were chosen to encapsulate a template-directed T7 RNA polymerase (Monnard and Deamer, unpublished results). Even though DMPC liposomes are more permeable to NTPs than the POPC vesicles used for the PCR reactions, their permeability coefficient is in the range of 10^{-11} cm s^{-1} at 37 °C (Monnard and Deamer, 2001), a rate that permits only a few NTPs per second to enter a given vesicle. To increase permeability, the reactions were cycled between 23.3°C and 37°C. Under these conditions the vesicles undergo a fluid- to gel-state phase transition at 23.3°C, at which temperature they are orders of magnitude more permeable than in the fluid phase. As a result, substrate uptake by a vesicle increases to approximately 1,500 molecules during a 5-min incubation at 23.3°C, followed by a 1-min incubation at 37°C to induce optimal T7 RNA polymease activity. RNA product was detected within the liposomes, and could be reverse-transcribed.

These results were enlightening in several ways. First, they demonstrate that the bilayer stability allows the encapsulation of a complex enzymatic assembly in an aqueous volume as small as 3.3×10^{-18} L. Second, passive

diffusion of substrate as large as nucleotides provided enough substrate for gene amplification to take place. Third, the localization of the products inside the vesicles, where they remain effectively protected from degradation by proteases and nucleases, underlines the importance of the compartment in the early evolution of cellular life. Membrane-bound compartments, such as the amphiphile vesicles described herein, allow the accumulation of polymeric products, which in turn can further interact and produce more complex systems.

The second point also illustrates the required properties of semipermeable bilayer membranes in primitive cellular systems. Stable bilayer membranes could only have appeared after the first transmembrane protein carriers became available, because the permeation rates of substrates across bilayer membranes of contemporary phospholipids are too low to support an extensive metabolism that depends on external nutrient solutes. It seems reasonable that mixed membranes composed of short-chain amphiphiles available in the environment were best suited for early cell membranes, while a coevolution of catalysts, metabolism, and membrane-forming compounds would occur later in the evolutionary process.

SELF-REPRODUCING COMPARTMENTS

As discussed above, even the earliest cellular forms of life must have had membranous boundaries, a specific set of catalyzed metabolic and polymerization reactions, and the capacity for self-reproduction. Self-reproduction entails not only the replication of a genetic material and catalysts, but also the production of additional membrane surface to accommodate growth and to permit the budding of daughter cells. Figure 7 illustrates a hypotheticical minimal cell of an RNA world. The genetic material must code for three essential components of the cell: 1) A ribozymal polymerase activity is required for transcription of the gene into other active ribozymes, including replication of the genetic RNA itself. 2) A ribozymal acyltransferase activity synthesizes new membrane molecules from their precursors. Acyl CoA and lysophophatidylcholine are shown as examples of such precursors, and in fact have been demonstrated to generate new membranes from soluble precursors (Gavino and Deamer, 1982). 3) An RNA fragment, "bud RNA," is required to trigger the budding of the compartment boundaries once these exceed a threshold surface area.

Luisi and coworkers (Walde et al., 1994a, b; Oberholzer et al., 1995a; Morigaki et al., 1997) at the ETH in Zürich have approached the question of self-reproducing compartments by using vesicles formed from fatty acids that are plausible amphiphile candidates for self-assembling boundary structures of early cellular life. Starting with preformed oleic acid vesicles, a source of amphiphile precursors was added in the form of a water-immiscible anhydride of oleic acid. The anhydride does not form vesicles, but instead is present as fluid droplets. As the anhydride hydrolyzes, it provides additional membrane building blocks, and the number and size of the vesicles in the aqueous medium increase. Significantly, when the hydrolysis is carried out without vesicles in the aqueous phase, two distinct kinetic regimes are observed: an initial slow phase before vesicles are present, and an autocatalytic fast phase as the number of vesicles in the suspension increases.

Two additional series of experiments using oleic acid vesicles with entrapped PNPase and Qβ replicase demonstrated vesicle self-reproduction and simultaneous polymerization of RNA from substrates added to the external medium. These represent the first model systems to incorporate a catalyzed membrane growth in concert with a catalyzed synthesis of a nucleic acid (Walde et al., 1994a; Oberholzer et al., 1995a).

It is interesting to consider this experiment in terms of the prebiotic environment. The anhydride droplets are models for an external source of membrane precursors, and the hydrolysis of the anhydride linkage proceeds at the expense of chemical energy stored in the anhydride bond. Furthermore, the fatty acid bilayers themselves catalyze the reaction by which their components are produced. A system similar to the self-reproducing oleic acid vesicles might have preceded a lipid synthesis coded in the genetic material of early cells, even though such systems lack feedback regulation and could result in the production of empty vesicles. Indeed, if the rate of amphiphile formation substantially exceeds the reproduction rates of the metabolic network elements (genetic codes and catalytic species), these species will be rapidly diluted in the expanding internal aqueous compartment, and the newly formed vesicles will lose the characteristics of the parent cell. For this reason we believe it would be worthwhile to investigate how regulatory feedback is incorporated into early cellular systems.

CONCLUSIONS AND OUTLOOK

The study of carbonaceous meteoritic material and laboratory models of plausible Fischer-Tropsch-type reactions show that vesicle-forming amphiphiles likely were present on the early Earth, and therefore could have participated in the formation of boundary membranes required by early cellular life. Moreover, short-chain amphiphile-based vesicles have properties similar to those of liposomes formed from phospholipids that are primary components of contemporary cellular membranes. They tend to be less stable and more permeable to ionic solutes, but as discussed herein, higher permeability can be an advantage in the absence of specialized transport proteins.

Recent investigations have established that encapsulation procedures such as dehydration/rehydration and pH vesiculation permit simultaneous trapping of all components of complex catalytic systems, which remain active in the vesicular compartment. Furthermore, the membrane boundaries of such vesicles can protect the encapsulated catalysts from agents such as proteases and nucleases. On the early Earth, it seems likely that a membrane-protected microenvironment would be conducive to survival of spontaneous molecular systems on the evolutionary path to the first forms of life.

Another interesting aspect of amphiphile vesicles is their selective permeability, which permits the passive diffusion of substrates and prevents the release of metabolic products, thereby leading to accumulation of polymeric products. This observation demonstrates that an early form of life could have relied on a similar mechanism to take up nutrients and energy from the environment. The fact that enzymes with high turnover rates can be supplied with substrate by this simple mechanism supports the idea that a rather complex, perhaps ribozyme-based metabolism could be sustained in a closed compartment without need for transmembrane protein carrier systems. We also note that accumulation of polymeric products would permit and promote additional reactions

that lead to increased metabolic complexity in cellular compartments.

Experiments have also demonstrated that simple vesicles composed of fatty acids could have undergone membrane growth using external amphiphile precursors, thereby undergoing a kind of self-reproduction, in the sense that new vesicles are produced by growth and dispersion of preformed vesicles. This concept has been discussed in detail as a "lipid world" scenario (Segre et al., 2001).

However, the vesicle-based model systems described here also demonstrate the limitations of membrane-encapsulated reactions that must be overcome before we can design a model system endowed with the properties of a minimal cell (see Fig. 7). Even though single-component vesicles are useful laboratory models, they may not accurately reflect the compartment boundaries of early protocells in terms of mixed lipid components, permeability, and substrate transport. When we attempt to model a protocell using highly evolved polymerase enzymes with high turnover rates, the low permeability of model bilayer membranes imposes significant limitations on successful outcomes. Recent studies tend to emphasize one aspect of a protocell, such as RNA polymerization, and lack the interacting metabolic pathways characteristic of living systems. Amphiphile vesicular systems with entrapped catalytic species possess no feedback regulation, which is a hallmark of life. Even though early cells may have incorporated simplified metabolic pathways, it would still be necessary to control their metabolism in some still unknown way to survive and undergo further evolutionary development.

In terms of future research directions, it is necessary to develop new membrane compositions to enhance interactions between the compartment and its environment. A steady supply of nutrients/energy relying on transport mechanisms, such as passive diffusion, will be a milestone in designing a plausible model for the minimal cell. Furthermore, as our knowledge of ribozyme chemistry increases, it may be possible to bypass the requirement of protein enzymes as catalysts. This will close an enormous gap in our understanding of the origin of life, because ribosomes are necessary for protein synthesis. It seems unlikely that we will reach a point at which it would be possible to include a full complement of DNA, RNA, ribosomes, and protein synthesis in a defined minimal cell system. Finally, by investigating both the synthesis of encapsulated polymeric molecules and the growth of membrane boundaries, a mechanism may be discovered that provides essential insights into the origin of feedback regulation.

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