Oparin's Reactions Revisited: Enzymatic Synthesis of Poly(adenylic acid) in Micelles and Self-Reproducing Vesicles

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Abstract: The enzymatic polymerization of ADP to poly(A), catalyzed by polynucleotide phosphorylase (PNPase) from *Micrococcus luteus*, has been studied in two supramolecular systems: (a) in reverse micelles formed by sodium bis-(2-ethylhexyl) sulfosuccinate in isooctane and (b) in oleic acid/oleate vesicles at pH 9. In the case of reverse micelles, the reaction proceeded with high yields and with a precipitation of poly(A) out of the micelles. In the case of vesicles, the poly(A) synthesis also proceeded and poly(A) remained entrapped inside the vesicles. The reaction has also in this case been studied under conditions of vesicle autopoietic self-reproduction, namely under conditions in which the vesicles are able to increase their concentrations due to an autocatalytic process which takes place within their boundaries. For this, PNPase was first entrapped inside the vesicles, followed by external addition of ADP and oleic anhydride. ADP permeated across the vesicle bilayer into the interior where PNPase catalyzed the formation of poly(A). In parallel to this endovesicular enzymatic poly(A) synthesis, oleic anhydride was hydrolyzed to oleic acid within the boundaries of the vesicles, which lead to an increase in size and number of vesicles. In this way, we realized a system in which self-reproduction was accompanied by a simultaneous growth of RNA inside the vesicles. This can be seen as a primitive model of a minimal cell.

Introduction

As a support to his theory on the origin of life, Alexander Ivanovic Oparin has carried out a series of significant experiments with his so-called "coacervates". Coacervates are spherical droplets obtained by suspending certain macromolecular compounds in aqueous solution, for example, gum arabic and histone. Oparin developed these coacervates in the pursue of constructing primitive forms of precellular structures.[1-4] These droplets are very heterogeneous and polydisperse (with a diameter between 1 and 500 μm) but have the important properties of hosting enzymes and being permeable to certain low molecular weight substrates. Oparin and co-workers were able to use such enzyme-containing coacervates as microreactors for the enzymatic synthesis of poly(A) starting from ADP,[1-3] or of starch starting from glucose 1-phosphate,[5] and even some oxidoreduction reactions were carried out using NAD-dependent enzymes.[4] Work on coacervates in relation to evolution has been pursuit up to the present time by the Russian school.[6]

Oparin's reactions with coacervates represent an important reference in the field of the chemistry of life, but somehow their importance seems to be more historical than scientific. This is due to the fact that coacervates are generally regarded as dubious structures; in fact, they are devoid of thermodynamic stability (they generally precipitate in a short time and build a water-free layer[6,7]) and as such they are difficult to characterize in terms of structure or of physicochemical properties.

It would then be desirable to check the validity of Oparin's reactions using supramolecular structures which are more defined from the thermodynamic and physical point of view, such as micelles and vesicles; this is actually the main idea underlying the present paper.[8] In the work described here, we will deal with the reaction catalyzed by polynucleotide phosphorylase (PNPase, polyribonucleotide:orthophosphate nucleotidyltransferase, EC 2.7.7.8),[9-12] namely, the synthesis of poly(A) starting from ADP, which Oparin and co-workers first described occurring in coacervates.[1-3]

With this work, we do not want simply to check Oparin's reaction, but we intend to study the chemistry of formation of nucleic acids inside micelles or vesicles and in addition set a more specific and perhaps more interesting goal, stemming from our work on self-reproduction of micelles and vesicles:[13] whether namely a system can be realized in which the formation of poly-(adenylic acid) within the bounded structures proceeds in parallel with the self-reproduction of such structures.

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(8) The necessity of this kind of work had been already expressed by A. Lazcano who—among others—believes that "a decisive step towards the emergence of the first living systems was the appearance of *membrane*-enclosed systems." Today the most significant models for precellular systems are lipid vesicles (liposomes): (a) Lazcano, A.; Fox, G. E.; Oró, J. F. In *The Evolution of Metabolic Function*; Mortlock, R. P., Ed.; CRC Press Inc.: Boca Raton, FL, 1992; pp 237-295. (b) Deamer, D. W. *Origins Life Evol. Biosphere* **1986**, *17*, 3-25.

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Our work with the PNPase reaction which Oparin studied will be reported in two different systems. The first one is the reverse micelle system formed by sodium bis(2-ethylhexyl) sulfosuccinate (AOT) in isooctane, a well-known system for enzymatic reactions;[14] the second one is a vesicle system formed by oleic acid/oleate, a system described by Gebicki and Hicks,[15] Deamer and co-workers,[16] and Li and Haines,[17] as well as by Small et al.[18] In this second case, a very primitive model for a biological cell will be described: the bounded structures (the vesicles) are self-reproducing while ribonucleic acid is produced inside of it.

Materials and Methods

Reagents. The potassium salt of poly(adenylic acid) (Poly(A), prepared from ADP with polynucleotide phosphorylase), oleic anhydride (~99%), bicinchoninic acid solution, and sodium bis(2-ethylhexyl) sulfosuccinate (AOT) were from Sigma. AOT was used as obtained.[19] The commercial AOT sample contained about 0.3 molecules of water/AOT molecule, as determined by Fourier transformed infrared (FTIR) spectroscopy.[20]

Polynucleotide phosphorylase (PNPase) from *Micrococcus luteus* was either from Boehringer Mannheim, Germany, or from Sigma. A change in enzyme supplier was necessary since PNPase from Boehringer was sold out during the course of the work.

Adenosine 5′-monophosphate (AMP) disodium salt, adenosine 5′-diphosphate (ADP) monopotassium salt, adenosine 5′-triphosphate (ATP) disodium salt, sodium oleate (>99%), sodium cholate (~95%), isooctane (for UV spectroscopy), and methanol (puriss. p.a.) were from Fluka, Switzerland. Perchloric acid was from Merck, Germany, and urea (puriss.) from Riedel-de Haën, Germany. All other chemicals were of the highest purity available from Fluka or Merck.

Instruments. UV/vis absorption spectra were recorded at 25 °C on a Uvikon 810 spectrophotometer from Kontron, Switzerland, using quartz cells. CD measurements were carried out on a JASCO J-600 spectropolarimeter by using quartz cells of 0.1-cm path length. Molar ellipticities are normalized per nucleotide residue. Fourier transform infrared (FTIR) spectra were recorded on a Nicolet 5SXC FTIR spectrophotometer, using a CaF_2 cell with a fixed path length of 0.02 cm. All centrifugations were carried out at room temperature on a Eppendorf centrifuge or on a microcentrifugette from ALC, Italy. A Bandelin sonorex RK100H instrument was used at room temperature for bath sonications.

HPLC. HPLC measurements were carried out on a Perkin Elmer Series 4 liquid chromatograph with a Nucleogen-DEAE 4000-7 anion exchange column (6 × 125 mm; particle size, 7 μm; pore size, 400 nm) from Machery-Nagel, Switzerland. The UV detector was set to 260 nm. Buffer A was 50 mM potassium phosphate (pH 7.0) containing 4 M urea; buffer B was 50 mM potassium phosphate (pH 7.0) containing 4 M urea and 1.5 M KCl. The flow rate was 1.5 mL/min, and the elution was carried out with a gradient from 10% buffer B to 100% buffer B in 30 min.

Electron Microscopy. The electron microscopic analysis of the vesicles was carried out by the freeze-fracturing method.[21]

Poly(A) Synthesis in Aqueous Solution. General procedure: 0.2 mL of an enzyme-containing stock solution (4.77 mg of PNPase/mL of 50 mM tris-HCl buffer, 1.56 mM $MgCl_2$, pH 9.5) was added to 1.0 mL of an ADP stock solution (1.91 mM ADP in 50 mM tris-HCl buffer, 1.56 mM $MgCl_2$, pH 9.5), and this reaction mixture was incubated at 25 °C. The initial ADP concentration in the reaction mixture was 1.59 mM and the enzyme concentration 0.80 mg/mL.

From time to time, 150 μL of the reaction mixture was withdrawn and mixed with 50 μL of 1 M $HClO_4$ in order to precipitate poly(A) which was formed during the reaction. The solution was centrifuged for 2 min, 50 μL of the supernatant solution was mixed with 1 mL of 50 mM tris-HCl buffer (pH 9.5) containing 1.56 mM $MgCl_2$, and the optical density was measured at 260 nm. The absorbance at 260 nm was taken as a measure for the ADP concentration which remained unpolymerized. The ratio {OD (at time t)}/{OD (at time 0)} corresponds to the fraction of unpolymerized ADP at time t.

As a modification to this general procedure, the initial concentrations of enzyme and ADP were varied. In addition, the polymerization of ADP to poly(A) has further been confirmed by analyzing the reaction mixture by DEAE ion exchange HPLC chromatography (see above).

Preparation of Reverse Micelles. "Dry" AOT/isooctane reverse micelles were prepared by dissolving an appropriate amount of AOT in isooctane to yield a desired concentration of 0.05, 0.1, or 0.3 M. AOT completely dissolves in isooctane, and a transparent solution containing almost "dry" reverse micelles is obtained. To this solution was added a certain amount of buffer solution to give reverse micelles of a desired water content. Generally, complete solubilization of the added buffer was achieved within 1 min. The relative amount of water is expressed as the ratio w_0, which is the ratio between the molar concentration of added water and the molar concentration of AOT ($w_0 = [H_2O]/[AOT]$).

AOT reverse micelles containing guest molecules, e.g. ADP or PNPase, were prepared in the same way as just described, with the exception that the added buffer solution contained the corresponding guest molecule. In order to avoid undesired pH effects,[22] it was necessary to control and eventually adjust all buffer solutions containing guest molecules before solubilization. This is of particular importance in the present work in the case of ADP, since concentrated aqueous solutions of ADP are acidic. In all cases in this study, complete solubilization was achieved in less than 1 min.

Poly(A) Synthesis in Reverse Micelles. The PNPase-catalyzed synthesis of poly(A) in reverse micelles was carried out by applying the "micelle-mix" technique.[23] For this, two reverse micellar solutions having the same AOT concentration and the same w_0 value were first prepared, one containing ADP and the other containing the enzyme. The reaction was then started by mixing the two reverse micellar solutions. Typical conditions were as follows: 2 mL of 0.3 M AOT/isooctane, $w_0 = 20$ (50 mM tris-HCl, 10 mM $MgCl_2$, pH 9.5), containing overall 1.7 mM ADP was mixed with 0.4 mL of 0.3 M AOT/isooctane, $w_0 = 20$ (50 mM tris-HCl, 10 mM $MgCl_2$, pH 9.5), containing overall 0.48 mg of enzyme/mL. The initial ADP concentration were 1.42 mM (overall, $[ADP]_{ov,0}$) or 14.3 mM (with respect to the water pool, $[ADP]_{wp,0}$) and 0.08 mg of enzyme/mL (= $[PNPase]_{ov}$) or 0.817 mg of enzyme/mL (= $[PNPase]_{wp}$). The reaction mixture was incubated at 25 °C in glass test tubes. From time to time, 100 μL of the reaction mixture was withdrawn and added to 1 mL of 1 N $HClO_4$ in a Eppendorf tube. The solution was vortexed for 30 s, sonified for 3 min, and then centrifuged for 2 min. After centrifugation, the isooctane phase was first removed and 0.5 mL from the remaining supernatant solution was withdrawn, mixed with 0.5 mL of methanol, and vortexed. The absorbance of this methanolic solution was then measured at 260 nm against a 1:1 (v/v) mixture of 1 N $HClO_4$/methanol. The amount of poly(A) being produced and adsorbed to the glass wall during the PNPase-catalyzed synthesis in reverse micelles was directly proven in the following way. Firstly, the reverse micellar solution (6 mL) was removed from the test tube with a Pasteur pipette. Then 0.3 mL of the buffer solution (50 mM tris/HCl, 1.56 mM $MgCl_2$, pH 9.5) was added to the tube and sonified for 20 s. After addition of 0.3 mL of chloroform—to remove remaining AOT—the mixture was sonified again for 20 s and then centrifuged to obtain two clear phases. Poly(A)

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product was identified in the supernatant aqueous phase by HPLC and CD spectroscopy.

Preparation of Vesicles. (a) Spontaneous Formation of Vesicles. A 0.08-mmol sample of sodium oleate was dissolved in 1 mL of 50 mM tris-HCl buffer (pH 9). The pH of the resulting solution was adjusted to 9 with 1 N HCl. During this pH adjustment, the solution got turbid as a result of the spontaneous transformation of oleate micelles into oleic acid/oleate vesicles.

(b) Extrusion of Spontaneously Formed Vesicles. The spontaneously formed vesicles were sized down at room temperature by repeated extrusions through polycarbonate filters by the use of "The Extruder" from Lipex Biomembranes Inc., Vancouver, Canada.[24] Repeated extrusions (10 times) were first made by using two stacked filters with pores of 200-nm size, followed by 10 extrusions through two stacked 100-nm filters.

Vesicles Containing Entrapped PNPase. Vesicles containing entrapped PNPase were prepared in the same way as described above, with the exception that the tris-HCl buffer contained PNPase (4.3 mg of PNPase product/mL). For the separation of non-entrapped PNPase from vesicles containing entrapped enzyme, a sepharose 4B column (diameter, 1.6 cm; length, 30 cm) was used and samples of 1 mL were applied (flow rate, 0.5 mL/min; 2 mL/fraction). The amount of entrapped enzyme was determined with the bicinchoninic acid protein assay[25] after concentrating with centricon-30 concentrator (from Amicon). The calibration was made with PNPase samples.

Determination of the Concentration of Oleic Acid/Oleate. The concentration of oleic acid/oleate in aqueous vesicle samples was determined by FTIR spectroscopy. For this, 0.5 mL of the vesicle sample was first acidified by adding 0.5 mL of 1 N HCl to protonate all the oleate molecules. After addition of 1.5 mL of isooctane, the mixture was vortexed for 2 min and then equilibrated for phase separation during 2 h at room temperature. The upper isooctane phase was then removed and analyzed by FTIR spectroscopy, measuring the intensity of the carbonyl stretching frequency of the carboxylic acid group at 1715 cm^{-1} using an appropriate calibration with known amounts of oleic acid (molar extinction coefficient, 855 M^{-1} cm^{-1}).

Uptake of AMP, ADP, and ATP by Oleic Acid Vesicles. A 0.3-mL portion of concentrated nucleotide solution (100 mM AMP, 100 mM ADP, or 100 mM ATP in 50 mM Tris-HCl (pH 9) readjusted to pH 9 with 1 N HCl) was added to 3 mL of a suspension containing oleic acid vesicles ([oleic acid/oleate] = 80 mM in 50 mM Tris-HCl (pH 9) extruded 10 times through 100-nm polycarbonate filters). The suspension was chromatographed on a sepharose 4B column (diameter, 1.6 cm; length, 45 cm) in order to separate free nucleotides from nucleotides which eluted together with the vesicles. The concentration of the nucleotides in the vesicle fractions was determined spectrophotometrically at 260 nm after solubilization of the vesicles by adding sodium cholate (final concentration, 16 mM). The molar extinction coefficient of the nucleotides at 260 nm in the presence of 16 mM cholate was 15 000 ± 400 M^{-1} cm^{-1} (pH 9).

PNPase-Catalyzed Synthesis of Poly(A) in Vesicles. Extruded oleic acid/oleate vesicles containing entrapped PNPase were first prepared as described above. After gel filtration on sepharose 4B, the turbid fractions containing vesicles were pooled (10 mL of 8 mM oleic acid/oleate). A 2-mL portion of these vesicles was mixed with 0.2 mL of 100 mM ADP (in 50 mM tris-HCl (pH 9)), and the mixture was incubated at 25 °C. From time to time, 0.1-mL samples were withdrawn, stored at −20 °C, and later analyzed for poly(A) formation by HPLC. In order to prove that the synthesis of poly(A) occurred within the vesicles and that the synthesized poly(A) remained within the vesicles, the reaction mixture was chromatographed on sepharose 4B (diameter, 1.6 cm; length, 30 cm). Control measurements have shown that externally added poly(A) elutes later than the vesicles.

PNPase-Catalyzed Synthesis of Poly(A) in Vesicles with Simultaneous Hydrolysis of Oleic Anhydride. A 0.024-mL (0.039-mmol) portion of oleic anhydride was added to 6.6 mL of a reaction mixture containing oleic acid/oleate vesicles (7.5 mM) with entrapped PNPase and externally added ADP (9.9 mM) at 25 °C and pH 9 (50 mM tris-HCl). The rate of anhydride hydrolysis was followed with FTIR spectroscopy (increase in oleic acid concentration) as described above by analyzing samples of 0.1 mL.

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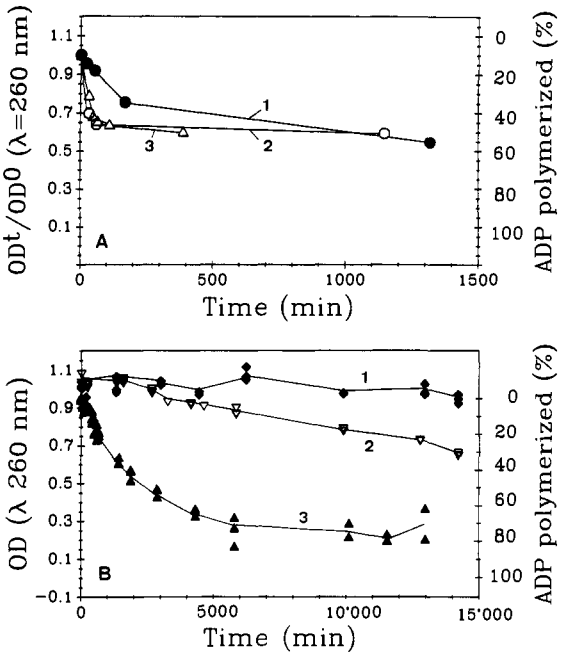

Figure 1. (A) PNPase-catalyzed synthesis of poly(A) in aqueous solution (50 mM tris-HCl, 1.56 mM $MgCl_2$, pH 9.5): (1, ●) [PNPase] = 0.40 mg/mL, $[ADP]_0$ = 2.0 mM. (2, ○) [PNPase] = 0.87 mg/mL, $[ADP]_0$ = 2.0 mM. (3, △) [PNPase] = 0.80 mg/mL, $[ADP]_0$ = 1.66 mM. (B) PNPase-catalyzed synthesis of poly(A) in AOT/isooctane reverse micelles at 25 °C. Influence of the $MgCl_2$ concentration on poly(A) formation: 0.3 M AOT/isooctane, w_0 = 20 (50 mM tris-HCl, pH 9.5), $[PNPase]_{wp}$ = 0.82–0.89 mg/mL, $[ADP]_{wp,0}$ = 14.3–14.8 mM. $[MgCl_2]_{wp}$ = 1.56 mM (◆, 1), 5 mM (▽, 2), or 10 mM (▲, 3).

Results and Discussion

The Micellar System. The AOT–isooctane–water system is one of the most used in the field of reverse micellar enzymology.[14] Generally, enzymes maintain their activity and display a behavior similar to that in aqueous solutions. To the best of our knowledge, PNPase has not been studied in reverse micelles.

PNPase can easily be incorporated in AOT reverse micelles by the injection technique, i.e. by adding to the AOT isooctane solution a small amount of water containing the buffered enzyme solution. The added water forms the water pool of the reverse micelles and defines the w_0 value of the system, i.e. the molar water to surfactant concentration, which is one of the most important parameters for characterizing a reverse micellar system. As it is well-known, w_0 is directly proportional to the radius of the water pool.[14]

For the synthesis of poly(A) in reverse micelles, two reverse micellar solutions at the same w_0 have first been prepared, one containing PNPase and the other ADP, which were then mixed. Since ADP is not soluble in isooctane, the reaction occurs obviously in the water pool of the micelles, where the enzyme is hosted. The ADP water pool concentration corresponds to the concentration of the aqueous stock solution. Since reverse micelles form a highly dynamic system with rapid exchanges of water among all micelles, it can be safely assumed—as commonly accepted in the field—that all micellar enzymes and ADP molecules coexist in the same water microphase.[14]

From this reaction mixture, samples were withdrawn at given time intervals and the concentration of unreacted ADP was determined. Typical results are shown in Figure 1: after an incubation of ca. 7 days at 25 °C, up to about 80% of the initial ADP concentration is used up. Figure 1 also reports the time progress of the reaction in water under the conditions used by Oparin and co-workers: in this case, only 40% of ADP is used when the equilibrium is reached. Another interesting difference with respect to the water solution lies in the fact that, with a

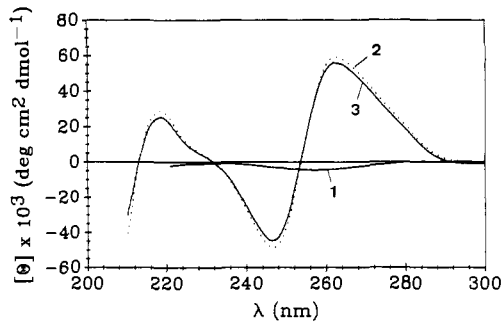

Figure 2. Circular dichroism spectrum of ADP and poly(A): (solid line, 1) ADP in 50 mM tris-HCl, 10 mM $MgCl_2$, pH 9.5; (dotted line, 2) poly(A) from Sigma in 50 mM tris-HCl, 10 mM $MgCl_2$, pH 9.5; (solid line, 3) poly(A) synthesized in AOT reverse micelles and then measured in 50 mM tris-HCl, 1.56 mM $MgCl_2$, pH 9.5.

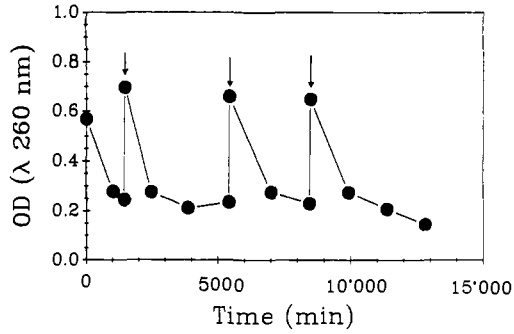

Figure 3. PNPase-catalyzed synthesis of poly(A) in 0.3 M AOT/isooctane reverse micelles at 25 °C, w_0 = 20 (50 mM tris-HCl, 10 mM $MgCl_2$, pH 9.5), $[ADP]_{wp,0}$ = 29.7 mM, and $[PNPase]_{wp}$ = 0.83 mg/mL. The supernatant reverse micellar solution was replaced by a fresh reverse micellar solution containing $[ADP]_{wp}$ = 35.6 mM at the time indicated by the arrows.

$MgCl_2$ concentration of 1.56 mM (the value used by Oparin in water), no reaction takes place in reverse micelles. A considerably larger Mg^{2+} concentration is needed in reverse micelles, and the reaction rate is proportional to the $MgCl_2$ concentration (see Figure 1). Measurements at two different AOT concentrations (50 and 100 mM), the water pool concentration of ADP and PNPase being constant, gave the same results (data not shown). Note finally from Figure 1 that the reaction proceeds without any lag phase. The relevance of this observation will be apparent later on, when discussing the reaction in vesicles.

The formed poly(A) can be easily detected on the basis of the CD spectrum, which is significantly different from that of ADP.[26] Figure 2 shows the CD spectrum of the so-obtained poly(A), which is identical to the CD spectrum of an authentic, commercial sample. Note the strong positive bands at ca. 260 and 220 nm and the negative peak at around 246 nm, which arise mostly from the base stacking in the polymer.[26]

Interestingly, however, no poly(A) was spectroscopically detected in the micellar solution. It was easy to check that this was due to the fact that poly(A) precipitates out of the reverse micelle solution, and in fact, poly(A) is found at the bottom of the reaction vial during and at the end of the reaction, as detected by HPLC and circular dichroism measurements. A similar polymer precipitation occurs in the case of peroxidase-catalyzed synthesis of poly(ethylphenol) in AOT reverse micelles.[27] In view of the fact that PNPase forms a tight complex with poly(A),[28] we asked whether the enzyme precipitates out together with the polymeric product. This was indeed shown to be the case with the following experiment: a micellar reaction mixture was allowed to react for 24 h, so that a considerable amount of ADP was polymerized. The micellar solution was then removed from the test tube and replaced by a fresh reverse micellar solution containing ADP at the same initial concentration as before. As shown in Figure 3, this resulted in a new start of the reaction with the same kinetics, until a new equilibrium was reached. The procedure (see Figure 3) could be repeated several times, indicating that the catalytically active PNPase precipitates together with poly(A).

However, it is also clear from Figure 1B that the enzyme remains active in the micellar system for a long time (days). In other words, whereas poly(A) is removed immediately after its synthesis from the micelle water pool, this is not so for the enzyme, which precipitates only in the last phase of the reaction. This is to be understood on the basis of the competition between ADP and poly(A) for the enzyme. While ADP is in large excess, the enzyme remains preferentially bound to it, and only when the concentration of the mononucleotide tends to vanish can the poly(A)–PNPase complex be formed in a significant amount to induce an enzyme precipitation. The dissociation constant of the PNPase–poly(A) complex is in the range 10^{-8}–10^{-9} M.[10]

At this point, we can draw a general conclusion regarding the reverse micellar system hosting the PNPase reaction. Comparison with water shows that the reverse micellar environment operates favorably on the reaction yield: the elimination of the polymer from the micelles brings about a shift of the equilibrium toward the product. However, the precipitation of poly(A) and eventually of the enzyme–product complex at the end of the reaction, although interesting from the point of view of reactor chemistry, makes the reverse micellar system not suitable as a minimal cell model for the poly(A) synthesis reaction studied by Oparin.

The Vesicle System. In fact, in order to realize a better model for a minimal cell, it would be necessary that the nucleic acid material—after its synthesis—remains inside the structure. Vesicles should be in this sense a better system than micelles: it is known in fact that vesicles are much less dynamic structures than micelles,[29] and, in particular, that the transport of molecules across the interface is much restricted. This is particularly so for biopolymers, whereas the permeability of low molecular weight substances might be somewhat higher, depending on the chemical structure, charge, and other factors.[30] In our case, ideally, one can then work with vesicles containing the entrapped PNPase in a stable form, such that ADP can permeate inside whereas poly(A) does not leak outside.

Preparation and Properties of Oleic Acid/Oleate Vesicles. Let us begin by describing the preparation and characterization of oleate vesicles. Since the work of Gebicki and Hicks[15] and Hargreaves and Deamer[16] it has been known that vesicles can be prepared from fatty acids under selected conditions. In the case of oleic acid, the pH region for vesicle formation lies between 7.5 and 9.3,[18] namely, at a pH close to the pK of the acid in the bilayer.[17] This is about 3–4 pH units higher than the pK of monomeric alkanoic acids.[18] Under those conditions the protonated and the dissociated forms of the acid form a hydrogen-bonded dimeric structure which favors formation of vesicles.[17] The spontaneously formed vesicles are rather polydisperse, as apparent from Figure 4A, and actually, also giant vesicles with diameters of several micrometers can be directly seen by light microscopy (data not shown). These vesicles can be sized down with a repeated filtration through polycarbonate filters of defined pore size, and Figure 4B shows the micrographs of vesicles extruded first through 200-nm, then 100-nm, filters. It is perhaps interesting to mention that in the case of 100-nm vesicles the

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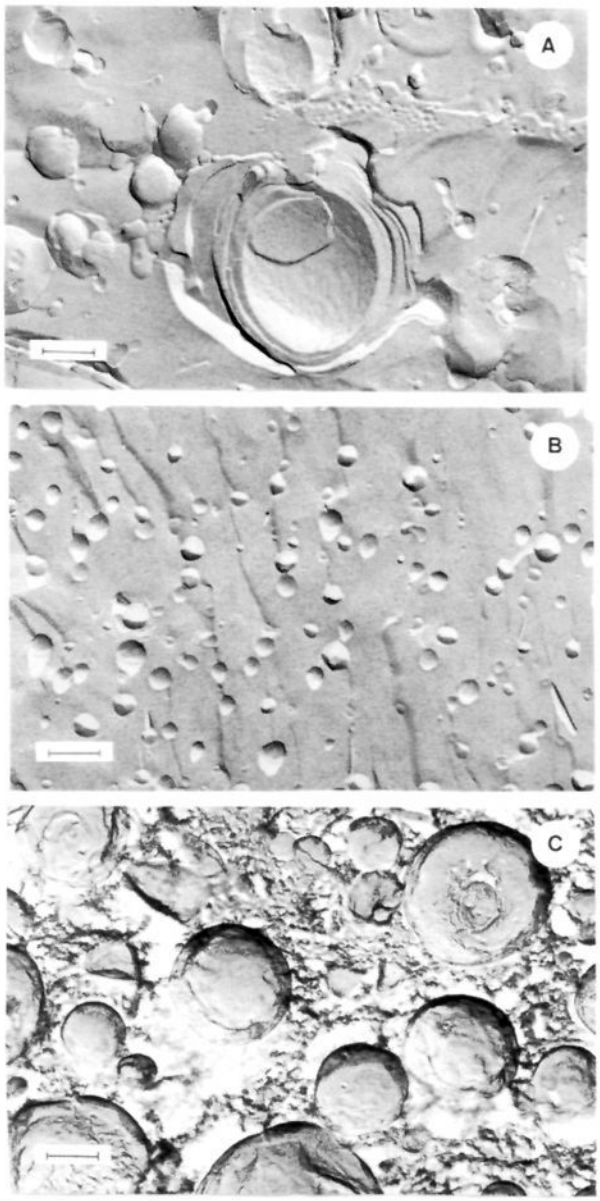

Figure 4. Freeze fracture electron micrographs of 80 mM oleic acid/oleate vesicles at pH 9 (50 mM tris-HCl). The spontaneously formed vesicles (A) have been extruded at room temperature through polycarbonate filters with final pore sizes of 100 nm (B). (C) Spontaneously formed vesicles (20 mM) which were reacted with 25 mM oleic anhydride. The length of the bar corresponds to 200 nm.

internal volume is about 1.4 μL/μmol of lipid,[31] which under our present conditions (80 mM oleic acid/oleate) corresponds to a total internal volume of 10%.

We report elsewhere the detailed physicochemical properties of oleic acid/oleate and caprylic acid/caprylate vesicles.[32] Here we limit ourselves to describe two features which are relevant for the present PNPase reaction. The first one is the autopoietic self-reproduction of the oleate vesicles, the second one is the uptake of ADP.

Autopoietic Self-Reproduction. By "autopoietic self-reproduction" we mean[33] an increase of the number of vesicles due to a reaction which takes place within the spherical boundary of the vesicular structure itself. For that, an aqueous suspension of the oleic acid/oleate vesicles is allowed to react with a supernatant amount of oleic anhydride: the water insoluble anhydride binds to the oleic acid/oleate vesicles and is rapidly hydrolyzed into the acid, which brings about an increase of the number of oleic acid/oleate vesicles.[32] Figure 4C shows the results in electron microscopy after one such self-reproduction experiment: starting from spontaneously formed vesicles (20 mM), we obtain that the average number of particles, as well as their average size, has increased as a consequence of a gross increase of the oleate concentration by a factor of 3.5.

Table 1. Uptake of AMP, ADP, and ATP by "Extruded" Oleic Acid Vesicles at 25 °C[a]

nucleotide	incubation time	uptake (%)
AMP	5 h 45 min	3.4
	29 h 45 min	3.2
	114 h 30 min	4.0
ADP	5 h	3.3
	29 h 45 min	4.0
	146 h	2.8
ATP	4 h 30 min	2.9
	24 h 30 min	2.7
	48 h 30 min	2.8

[a] 100-nm vesicles (72.7 mM oleic acid/oleate) were incubated in the presence of 9.1 mM nucleotide at pH 9 (50 mM Tris/HCl).

Uptake of AMP, ADP, and ATP. The question of liposome permeability to small molecules added in the bulk medium is of central importance, particularly when the aggregates are used as microreactors. In the case of phospholipids, the transport across the bilayer has been studied intensively, and it is usually accepted that the permeability for charged and in particular large molecules is rather low.[23] We decided to study here the permeability of oleic acid vesicles to negatively charged molecules AMP, ADP, and ATP, having two, three, and four, respectively, negative charges at pH 8–9. After incubating preformed 100-nm vesicles of oleic acid (72.7 mM at pH 9) with externally added nucleotides (9.1 mM), it is found that in all cases about 3% of the nucleotides coelute with the vesicles, independent of whether AMP, ADP, or ATP is used (see Table 1). Control experiments showed that in the case of POPC liposomes, as expected from the literature,[34] the uptake under the same conditions—in the absence of divalent cations—is negligible. The difference can be ascribed to the fact that oleic acid vesicles are equilibrium systems in which the rapid dynamics favor the permeability of molecules present in the milieu.[32]

The local ADP concentration reached when ADP is furnished externally to the oleic acid/oleate vesicles is in any case sufficient to start the enzymatic reaction.[35]

Entrapment of PNPase in Vesicles. Finally, for starting the reaction, it is necessary to entrap PNPase inside the oleate vesicles. Entrapment in this kind of vesicles of water soluble low molecular weight compounds[15,16] as well as enzymes[36] has been shown already. Here, PNPase has been entrapped according to the classic procedure (see Materials and Methods), whereby the non-entrapped enzyme has been separated from the enzyme-containing vesicles by gel permeation chromatography on sepharose 4B (Figure 5B). The vesicle fractions containing PNPase were pooled as indicated in Figure 5B and analyzed for protein concentration. This resulted in 15% of the initial protein concentration. In turn, this means that we have on average ca. 7–8 enzyme molecules/vesicle.[37]

(31) Taking into account a bilayer thickness of 4 nm and a head group area of 0.32 nm^2.

(32) Walde, P.; Wick, R.; Fresta, M.; Mangone, A.; Luisi, P. L. Submitted for publication.

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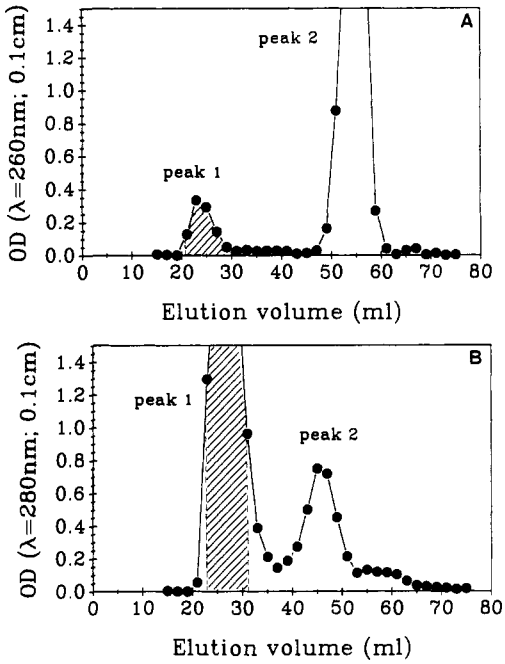

Figure 5. (A) ADP uptake by oleic acid/oleate vesicles. Extruded 100-nm vesicles (72 mM oleic acid/oleate) were incubated at room temperature and pH 9 (50 mM tris-HCl) for 3 days in the presence of externally added ADP (9.9 mM) and then chromatographed on Sepharose 4B. Peak 1 corresponds to the vesicles, and peak 2 corresponds to the free ADP. (B) Entrapment of PNPase inside 80 mM oleic acid/oleate vesicles (50 mM tris-HCl, pH 9). Separation on Sepharose 4B of non-entrapped enzymes (peak 2) from vesicles containing entrapped PNPase (peak 1).

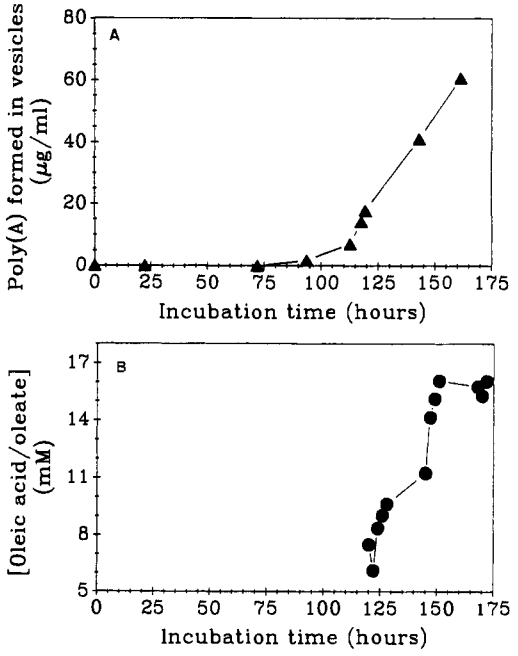

Figure 6. (A) PNPase-catalyzed synthesis of poly(A) in vesicles; poly-(A) yield as a function of incubation time. Oleic acid/oleate vesicles (6.3 mM) containing 32 μg of PNPase/mL were incubated in the presence of 9.9 mM ADP at 25 °C and pH 9 (50 mM tris-HCl). (B) Hydrolysis of oleic acid anhydride during the PNPase-catalyzed synthesis of poly-(A) in vesicles. Oleic anhydride was added to the vesicle suspension as soon as a measurable amount of poly(A) was synthesized in the vesicles.

Poly(A) Synthesis in Vesicles. Having established that oleic acid/oleate vesicles can host the enzyme in a stable form, and that ADP can permeate inside, we can study the chemical transformation of ADP catalyzed by the internally entrapped enzyme when ADP is added in the external aqueous medium. Results are shown in Figure 6. Poly(A) is formed by a slow reaction. In contrast to the case of reverse micelles, no poly(A) is found outside during the course of the reaction or at the end of it as shown by gel permeation chromatography.

Note the lag phase in Figure 6A. This feature is well-known in the PNPase-catalyzed reactions in aqueous solutions. It has actually been shown that the lag phase may depend on several factors, for example, on the enzyme preparation (whether the enzyme has been treated or not with a protease) and on the presence of primer polynucleotides (the lag phase can be eliminated when primers like $(pA)_4$ are added at the beginning of the reaction), and it has been observed when working at suboptimal Mg^{2+} concentrations.[9,11] Particularly interesting is the observation that after the lag phase the process of polymerization may proceed autocatalytically.[9]

In vesicles, contrary to the case of reverse micelles, we have been working without further addition of divalent cations (which would interact with the negatively charged carboxyl groups of the surfactant) and without addition of primers—so that actually a lag phase is to be expected.

Let us consider now the poly(A) synthesis under conditions of vesicle autopoietic self-reproduction. For that, as already mentioned, we need to carry out the reaction leading to poly(A) in the presence of added oleic anhydride so as to elicit the situation depicted in Figure 4C. In order to synchronize the autopoietic vesicle self-reproduction with the growth of RNA, the anhydride was added to the reacting vesicle at the end of the lag phase, at the point at which poly(A) was beginning to be formed. During the following period of 30 h (see Figure 6), all oleic anhydride was hydrolyzed, leading to a doubling of the initial oleic acid/oleate concentration. The time course of the self-reproduction of vesicles simultaneously to the synthesis of poly(A) is shown in Figure 6B. In this time interval the number of vesicles increases due to an increase in oleic acid/oleate concentration by a factor of ca. 2, whereas an amount of poly(A) corresponding to 60 μg/mL is being formed.

Concluding Remarks

The aim of this work was to utilize micelles and vesicles for the PNAase reaction which Oparin studied in coacervates, and it is clear from our data that these two supramolecular aggregate systems operate with different compartimentation principles. The higher dynamics of the reverse micelle allow an interesting separation procedure between substrate and product but do not permit its consideration as a suitable model for a primitive synthetic chemical cell; conversely, the polymeric product remains entrapped in the vesicles, which are also better models for a cell because of the inner aqueous environment and because of the membrane-like surfactant double layer.

We have accomplished an autopoietic vesicle self-reproduction simultaneously with the synthesis of a RNA molecule. The system is similar to and, in a way, simpler than the one recently described in our laboratory, in which the replication of a MDV-RNA template[38] was accomplished in oleic acid/oleate vesicles containing the enzyme Q_β-replicase, and the synthesis of RNA proceeds with the self-reproduction of oleic acid/oleate vesicles.[38]

In the present case, the overall process can be pictorially represented as shown below (Figure 7), whereby the details of the mechanism are not yet known. In particular we do not know through which intermediates the reproduction of vesicles proceeds,

(37) Although SDS–polyacrylamide gel electrophoresis has shown that the PNPase samples used in the present study were not homogeneous, we assumed for the estimation of the approximate enzyme content per vesicle 100% purity and a relative molecular mass of 230 000.[10]

(38) Oberholzer, T.; Böhler, C.; Wick, R.; Luisi, P. L.; Biebricher, C. K. Submitted for publication.

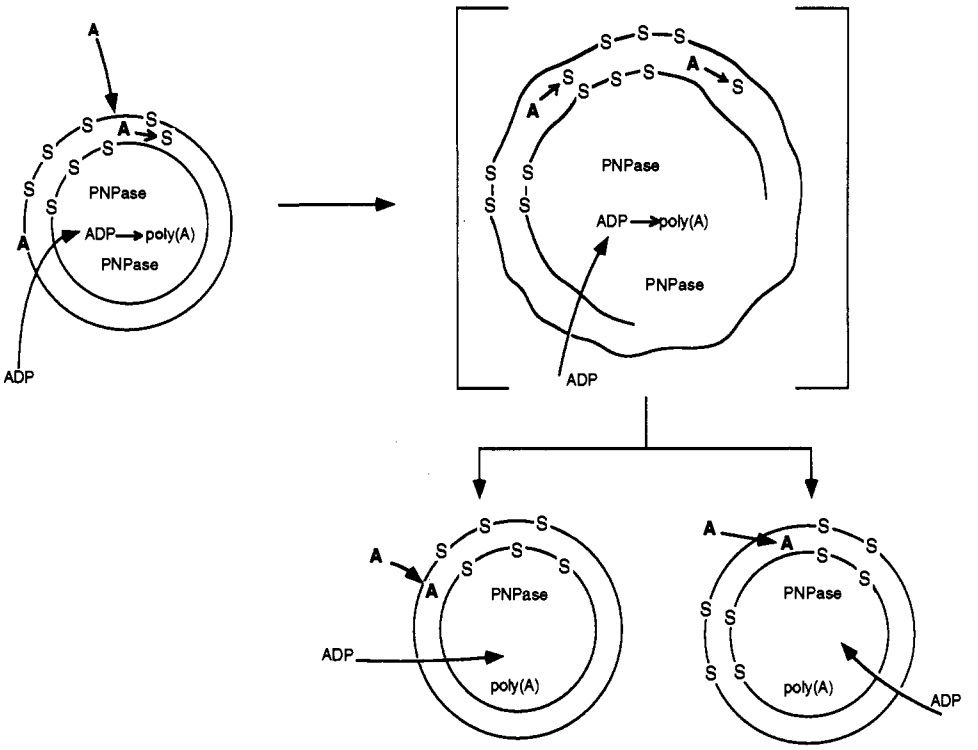

Figure 7. Schematic representation of the PNPase-catalyzed synthesis of poly(A) in oleic acid/oleate vesicles under conditions of autopoietic self-reproduction caused by oleic anhydride hydrolysis at the vesicle boundary: S, oleic acid/oleate; A, oleic anhydride.

and we do not know whether there is a redistribution of PNPase between the original vesicles and the newly born ones following self-reproduction (as depicted in the above cartoon) or whether the enzyme molecules remain confined in the original vesicles (so that the newly born vesicles would be unable to host the polymerization of ADP). Also, although the two synthetic processes (RNA and new vesicles) proceed simultaneously, they are mutually independent, i.e. the self-reproduction of the vesicle wall is not caused by the growth of RNA.

These limits indicate the line of research in our future work, and it can safely be said that, despite these limits, the present system represents a significant improvement toward the construction of a primitive synthetic minimal cell.

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