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Enzymatic reactions in liposomes using the detergent-induced liposome loading method

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Abstract

Microcompartmentalization is a crucial step in the origin of life. More than 30 years ago, Oparin et al. proposed models based on biochemical reactions taking place in so-called coacervates. Their intention was to develop systems with which semipermeable microcompartments could be established. In the present work we follow their intuition, but we use well-characterized bilayer structures instead of the poorly characterized coacervates. Liposomes from phospholipids can be used as microreactors but they exhibit only a modest permeability and, therefore, chemical reactions occurring inside these structures are depleted after a relatively short period. Here it is shown that even highly stable liposomes from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) can be used as semipermeable microreactors when treated with sodium cholate. Using this kind of mixed liposomes, we describe a biochemical reaction occurring inside the liposomes while the same reaction is prevented in the external medium. In addition, we show that this cholate-induced permeability of POPC bilayers can even be used to load macromolecules such as enzymes from the outside.

Keywords: Liposome; Cholate-induced loading; Permeability; Detergent; Enzymatic reaction

1. Introduction

In the middle of this century, following his idea of the chemical evolution as a transition to life, Oparin et al. published a series of papers describing the use of so-called coacervates as prebiotic cell-like structures which were able to take up all the reagents necessary for carrying out enzymatic reactions [1,2]. Coacervates are heterogeneous, spherical aggregates formed by macromolecular components like gum arabic and gelatine or histone [3], and their formation is not always well reproducible. In addition, coacervates are also not stable, and they do not have a well defined chemical composition. For all these reasons, they were never widely used in research. Recent investigations have aimed at clarifying whether the reactions in coacervates described by Oparin et al. would also occur in the interior of well-defined liposomes. For this reason a similar system was selected, namely the polymerization of ADP into poly(A) by polynucleotide phosphorylase (EC 2.7.7.8) [4,5].

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There are actually two lines of interest behind this kind of work. On the one hand, as mentioned above, there is the interest in confirming Oparin et al.'s intuition using a more reproducible and chemically characterized system, which may also contribute to the general notion of the early biochemical cells. Liposomes are these relatively well-characterized systems. Enzymatic reactions in liposomes are of particular interest [6–10] and, therefore, it is important to document in detail the feasibility of enzymatic reactions occurring in this closed bilayer microenvironment.

One of the restrictions concerning long-chain phospholipid vesicles as protocells is their poor permeability. Such liposomes – e.g. 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) liposomes in contrast to vesicles from fatty acids such as oleic acid/oleate vesicles – are almost impermeable to hydrophilic (and charged) molecules. Therefore, an enzymatic reaction taking place within their water pool can only be catalyzed as long as enough substrate molecules are available. Mainly polymerizing reactions tend to run out of substrate molecules when done in liposomes [11] and for this reason occur only for relatively short periods. In this report, we apply a previously described method, namely the detergent-induced loading of liposomes (DILL) [12,13], in order to develop a liposomal system in which a primer-dependent enzymatic reaction (the polymerization of glucose-1-phosphate to glycogen by phosphorylase) can take place in the internal volume of the liposomes, and the substrate molecules are delivered from the external medium. Liposomes, permeable for small molecules but impermeable for the macromolecular components, can be of great interest because they offer new possibilities as simple models for protocells which can take up their nutrients from the bulk environment and harbor their macromolecular components. The advantage of such a system is that the macromolecules are protected by the bilayer. In a second series of experiments we show that, under well defined conditions, even macromolecular components, such as the digestive enzyme DNase I, can permeate across the bilayer retaining their biological activity after reaching the other compartment.

2. Materials and methods

2.1. Reagents

Glycogen (from oyster), glucose-1-phosphate, maltooligosaccharide (from corn syrup), phosphorylase a (EC 2.4.1.1, from rabbit muscle, specific activity of ca. 25 U/mg protein), amyloglucosidase (EC 3.2.1.3, from *Aspergillus niger*, specific activity of 30 U/mg protein), and myoglobin (from horse skeletal muscle) were purchased from Sigma (St. Louis, MO). The DNA polymerase was bought from Finnzymes Oy (Espoo, Finland). The pancreatic DNase I (EC 3.1.21.1, with a specific activity of 2000 U/mg) was purchased from Boehringer (Mannheim, Germany). The oligonucleotides were from Mycrosynth (Balgach, Switzerland). α-D-[U-^{14}C]Glucose-1-phosphate ($>$150 mCi/mmol), [α-^{35}S]ATP (3000 Ci/mmol), and [α-^{35}S]dATP (3000 Ci/mmol) were obtained from Amersham. BioGel A15m was from Bio-Rad, Sephadex G50, ATP and the deoxynucleotides were bought from Pharmacia. POPC was obtained from Avanti Polar Lipids. Sodium cholate, sodium deoxycholate, and tRNA (from wheat germ) were purchased from Fluka (Buchs, Switzerland).

2.2. Enzymatic elongation of glycogen by phosphorylase a in an aqueous solution and detection of the product by column chromatography and β-scintillation counting

The elongation of glycogen in aqueous solution was carried out in principle as described earlier [14]. In brief, the enzymatic reaction was performed in 25 mM Tris (pH 6.8), 2.5 mM EDTA, 1.25 mM DTT, 400 μg/ml glycogen, 10 mM glucose-1-phosphate, 1.25–2.5 nCi/μl [^{14}C]glucose-1-phosphate, and 250 μg/ml phosphorylase a. The reaction mixture was incubated at 37°C for the appropriate periods (normally 1–2 h) and the ^{14}C-labeled macromolecules were separated from the low molecular weight compounds by gel filtration column chromatography using a Sephadex G50 spin column [14]. The radioactivity of the fractions was determined by β-scintillation counting.

2.3. Determination of the molecular weight of the glycogen molecules used in these experiments by analytical ultracentrifugation

The molecular weight of the oyster glycogen used as primer for the incorporation of glucose-1-phosphate into glycogen was determined by sedimentation velocity and sedimentation equilibrium experiments using an analytical ultracentrifuge from Beckman (Model E, equipped with a schlieren optical system). First, the sedimentation coefficient was determined by sedimentation velocity experiments, then several sedimentation equilibrium runs were performed in eight-channel cells at 3200 rpm [15]. For the determination of the diffusion coefficient a density meter from Anton Paar model DUA02/c was used.

2.4. Encapsulation of ^{14}C-labeled glycogen into POPC liposomes by the freeze/thaw and extrusion procedure

Radioactive glycogen was synthesized as described above and separated on a Sephadex G50 column from [^{14}C]glucose-1-phosphate. The fractions containing radioactive high molecular weight compounds were collected and a POPC film was dispersed in 25 mM Tris (pH 6.8), 2.5 mM EDTA, 1.25 mM DTT containing 400 μg/ml glycogen with about 25 000 cpm ^{14}C-labeled glycogen, so that the POPC concentration was 120 mM. The lipidic dispersion was frozen/thawed ten times before the suspension was diluted to 40 mM POPC and pressed five times forth and back through two stacked polycarbonate filters with pores of 400 nm in diameter (from Avestin) using a Liposofast (from Avestin). To separate the unentrapped high molecular weight glycogen from the encapsulated one, the liposomal suspension was subjected to an enzymatic digestion for 150 min using 600 μg/ml amyloglucosidase. For the removal of the nonentrapped radioactivity from the entrapped ^{14}C-labeled glycogen, a BioGel A15m spin column chromatography was performed and the radioactivity in the individual fractions could be determined by β-scintillation counting.

To test whether the applied amount of enzyme and the duration of incubation period were sufficient for a thorough degradation of the unentrapped glycogen, a control digestion was carried out. Radioactive glycogen was added to preformed POPC liposomes and a digestion experiment with the same quantity of amyloglucosidase was performed.

2.5. Synthesis of ^{14}C-labeled glycogen in POPC liposomes

All the ingredients necessary for the synthesis of glycogen in liposomes were added to a POPC film and a lipidic dispersion containing 50 mM POPC, 25 mM Tris (pH 6.8), 2.5 mM EDTA, 1.25 mM DTT, 10 mM glucose-1-phosphate, 0.9 nCi/μl [^{14}C]glucose-1-phosphate, and 400 μg/ml glycogen was obtained. The dispersion was subsequently frozen in liquid nitrogen and thawed at 30°C in a water bath for seven times, before the phosphorylase a (250 μg/ml) was added. Again three additional freeze/thaw cycles were carried out before the liposomes were extruded five times through two filters with 400-nm pores in diameter. To the liposomal suspension 600 μg/ml amyloglucosidase were added and the suspension was incubated at 37°C for 2 h. To determine whether radioactive glycogen has been synthesized during this period, the liposomes were incubated for 2 min at 75°C in order to inactivate the enzymes and solubilized by addition of 0.1 vol. 10% deoxycholate (1% (w/v) final concentration). The separation of the radioactive glycogen from the radioactive glucose-1-phosphate was performed on a Sephadex G50 column.

To test whether glycogen was extended within liposomes (and not outside), the same experiment as described above was carried out with one exception, namely that the phosphorylase a was added after the extrusion of the POPC suspension.

2.6. Cholate-induced loading of preformed liposomes with hydrophilic solute molecules from the outside

POPC liposomes were prepared by dispersing a POPC film in aqueous solution and subsequent extrusion through two polycarbonate filters with pore sizes of 100 nm in diameter. To this liposomal suspension (at a concentration of 60 mM POPC) sodium cholate was added (from a 200 mM stock solution) in appropriate amounts and the suspension was immediately treated with a vortex for 30 s before the

solute to be encapsulated was pipetted into the bulk medium. The cholate-induced loading was tested with 100 μM glucose-1-phosphate (and [^{14}C]glucose-1-phosphate), 100 μM dATP (and [^{35}S]dATP), 5 mg/ml myoglobin, and 11.5 mg/ml tRNA. The suspension was allowed to stand for 3.5 h at room temperature. For the determination of the amount of solute which permeated across the bilayer, a gel filtration spin column chromatography was performed (BioGel A15m) and the amount of radioactivity/protein/nucleic acid coeluting with the liposomes was determined either by β-scintillation counting or by UV-VIS measurements.

2.7. Glycogen extension inside cholate-permeabilized POPC liposomes

To a POPC film an aqueous solution containing 25 mM Tris (pH 6.8), 4.3 mM EDTA, 2.1 mM DTT, 711 μg/ml glycogen, and 17.1 mM glucose-1-phosphate was added, and treated by a vortex for 30 s. The concentration of the POPC lipids was 107 mM. The dispersion was frozen/thawed for seven times, then 427 μg/ml phosphorylase a were added and again the dispersion was frozen/thawed for three times. Afterwards the lipidic dispersion was diluted with 25 mM Tris to 60 mM POPC and an extrusion through two polycarbonate filters with 400-nm pores was carried out. The liposomes containing undigested glycogen outside the liposomes were diluted again to 40 mM lipid (with a buffer containing 25 mM Tris (pH 6.8)) and subjected to amyloglucosidase treatment (final concentration 600 μg/ml) for 1.5 h at 37°C. After the incubation period sodium cholate was added (final concentration 13.3 mM) by pipetting forth and back. The mixture was vortexed for 10 s and [^{14}C]glucose-1-phosphate (1.33 nCi/μl) was added and incubated for 10 min at 37°C. The reaction mixture was subjected to 75–80°C for 2 min to inactivate the enzymes and the liposomes were solubilized by addition of 0.1 vol. 10% (w/v) deoxycholate. The determination of the amount of radioactive glycogen and its separation from unreacted [^{14}C]glucose-1-phosphate was performed using a Sephadex G50 spin column and subsequent β-scintillation counting of the single fractions.

In order to check whether glycogen was synthesized within the POPC/cholate liposomes, two control experiments were carried out. The former was done in the same way as described above unless that glycogen was added after the extrusion of the liposomes, so that glycogen – which is too large for the permeation through the bilayer even after cholate treatment – was present solely outside the liposomes. Then the amyloglucosidase was added and all steps were carried out as described above. The latter experiment had the intention to simulate an eventual release of undigested glycogen because of the cholate addition. Therefore, after the 90 min incubation of glycogen outside the liposomes by amyloglucosidase, cholate and [^{14}C]glucose-1-phosphate were added as indicated above, then 7.5 μg of additional glycogen were added and the incorporation of radioactivity was measured after a 10-min incubation period.

2.8. Cholate-induced pancreatic DNase I permeability

120-bp DNA was prepared by PCR using the plasmid DNA pSP64-JE and the oligonucleotides A5 (5′-TGCAAGGTGTGGATCCATTT-3′) and A3S (5′-CAATACCTTGGAATCT-3′). The conditions of the PCR were the same as previously described [11]. The in vivo labelling of the DNA was carried out by PCR using [^{35}S]dATP and the labeled 120-bp DNA was separated from the free nucleotides by gel filtration column chromatography (Sephadex G50 spin column). The purity of radioactive macromolecules was checked by trichloroacetic acid (TCA) precipitation.

Approximately 10 000–15 000 cpm (TCA precipitable) ^{35}S-labeled 120-bp DNA were entrapped in POPC liposomes as previously described [14]. In brief, the ^{35}S-labeled DNA (in 50 mM Tris (pH 8.0), 5 mM $MgCl_2$) was added to a POPC film, and the suspension was frozen/thawed ten times. Then the lipidic aggregates were diluted to 60 mM POPC using the same buffer and extruded through filters with 400-nm pores. The liposomes were separated from the unentrapped DNA by gel filtration spin column chromatography (BioGel A15m). It is noteworthy to indicate that the separation of the liposomes from the 120-bp DNA was perfect and no DNase I digestion of the DNA outside was necessary (in contrast to our previously published results with 369-bp DNA [14]). To the liposomes harboring the DNA in their aqueous pool (POPC

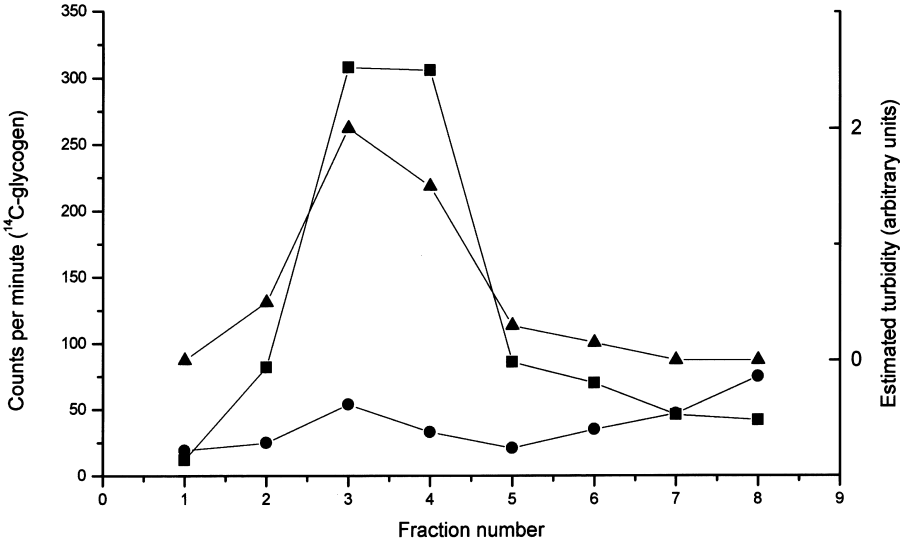

Fig. 1. Glycogen encapsulation in POPC liposomes by freeze/thaw and subsequent extrusion. ^{14}C-labeled glycogen was synthesized and purified by Sephadex G50 column chromatography. Approximately 25 000 cpm of radioactive glycogen were entrapped in liposomes (method of freeze/thaw with subsequent extrusion). The nonentrapped glycogen was digested with 600 μg/ml amyloglucosidase. The efficiency of the digestion by amyloglucosidase was tested by adding the same amount of enzyme to preformed liposomes and incubation for the same period. Then about 40% of the digested material was loaded onto a BioGel A15m column and the radioactivity and the turbidity of the fractions was determined. Squares show the entrapped glycogen, triangles the estimated turbidity of the eluted fractions, and circles show the result of the control experiment, where the same amount of glycogen was added to preformed liposomes.

concentration was estimated to be 50 mM after spin column chromatography) sodium cholate and $MgCl_2$ were added (final concentration 10 mM each) and allowed to stand for 30 min. Then 5 U of pancreatic DNase I were added and aliquots were removed at appropriate time points. The samples were precipitated with TCA and the radioactivity retained on a glass filter was determined by β-scintillation counting. For the control experiment the same procedure was carried out except that the cholate addition was omitted.

100 U of DNase I (2000 U/mg) were entrapped in POPC liposomes in the same way (as described above for DNA encapsulation) and the liposomes were separated from the unentrapped enzyme by spin column chromatography (BioGel A15m). The POPC concentration of the resulting suspension was estimated to be 60 mM. To this suspension, 120-bp DNA (containing approximately 1500 TCA precipitable cpm) and $MgCl_2$ (final concentration 10 mM) were added, so that the POPC concentration was about 35 mM. Then the sodium cholate was added (final concentration 6 mM), aliquots were removed at appropriate time points, and the amount of TCA precipitable material was determined by filtration/β-scintillation counting. In the control experiment the addition of cholate was omitted.

3. Results

It has been known for over 30 years that glycogen or starch can be synthesized enzymatically in vitro using phosphorylase a [16,17], an enzyme which is normally responsible for the glycogen degradation in mammalian cells [18]. If the molar ratio of glucose-1-phosphate to glycogen is relatively high, then phosphorylase can use starch or glycogen as the primer and glucose-1-phosphate units are added in order to extend the length of the appropriate polymer. This extension can be observed using different methods, for example, by using ORD or CD spectroscopy [19], by detecting of the phosphate released [20] or by detecting of the incorporated radioactivity using radioactive glucose-1-phosphate and performing of a gel filtration column chromatography. In the following experiments we used the method of radioactive labelling combined with gel filtration column chro-

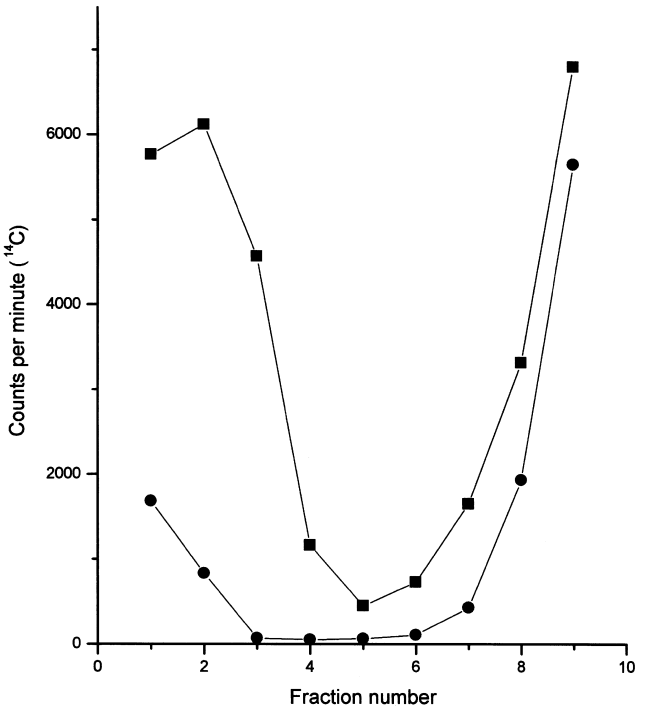

Fig. 2. Elongation of glycogen in liposomes. All ingredients required for synthesis in liposomes were entrapped by freeze/thaw and subsequent extrusion (squares show the experiment with phosphorylase a entrapped; for the control experiment (circles) phosphorylase a was added after the extrusion of the liposomes). Then amyloglucosidase (600 μg/ml) was added outside the liposomes and the dispersion was incubated at 37°C for 2 h. For the analysis of the produced ^{14}C-labeled glycogen, liposomes were treated for 2 min at 75–80°C and treated with 1/10 vol. 10% deoxycholate. The resulting micellar solution was subjected to a Sephadex G50 spin column chromatography and the radioactivity of the individual fractions was determined by β-scintillation counting. For details see Section 2.

matography to show that radioactive glycogen has been synthesized.

In a first series of experiments we aimed at showing whether elongated glycogen was produced and what amount of glucose-1-phosphate could be incorporated into macromolecular components used as primers. It could be clearly demonstrated that under the chosen conditions (after an incubation period of 1 h) 40–50% of the added radioactive glucose-1-phosphate could be eluted in the fractions number one and two obtained after a Sephadex G50 column chromatography. This indicates that these glucose-1-phosphate molecules were incorporated into glycogen used as primer molecules. It was calculated that in a reaction volume of 100 μl containing 40 μg glycogen as primer, about 110 μg of glycogen were newly synthesized (what corresponds to a yield of about 45%). The determination of the molecular weight of the glycogen molecules used as primers by analytical centrifugation revealed that the mean size of the oyster glycogen molecules was in the range of 2800 kDa (and, therefore, as expected for glycogen [21]). And it can be calculated that the mean molecular weight of the ^{14}C-labeled glycogen molecules was in the range of 10 000 kDa.

All our attempts to replace glycogen with other primers having a lower molecular weight and a higher solubility (such as maltooligosaccharide or dextran) failed and no synthesis of radioactive high molecular weight compounds could be observed (data not shown). For our final goal – the synthesis of glycogen in liposomes – this result meant that two macromolecular components, namely the enzyme catalyzing the reaction and the glycogen as primer as well as the substrate molecules had to be present within the same liposome in order to perform the reaction.

To the best of our knowledge, such a high molecular weight compound (as the ^{14}C-labeled glycogen used for our experiments with a mean molecular weight of about 10 000 kDa) has never been entrapped in liposomes. Therefore, we aimed at showing first whether radioactively labeled glycogen could be entrapped at substantial amounts in extruded liposomes. For this reason, radioactive glycogen was synthesized in aqueous solution and it was determined whether radioactive glycogen produced in such a way could be entrapped in POPC liposomes by our freeze/thaw procedure. The freeze/thaw procedure was selected because it has previously been shown that it was the most efficient one for such entrapment experiments [14]. The entrapment results clearly demonstrated (see Fig. 1) that encapsulation of these large molecules was possible: about 6–8% of the radioactive glycogen could be entrapped in POPC liposomes extruded through filters with pores of 400 nm in diameter.

For one of our goals, namely the synthesis of glycogen in POPC liposomes, one major problem was that the glycogen, which had not been entrapped during the freeze/thaw cycles, had to be removed (by enzymatic degradation of the polymer and the degraded units by column chromatography) in order

to prevent the extension of the glycogen primers outside the liposomes. Experiments with liposomes and externally added glycogen demonstrated clearly that the BioGel A15m spin columns were not efficient enough to ensure a proper separation of glycogen from the liposomes. For this reason, all following experiments were planned without column chromatography, but with a glycogen degrading enzyme added externally to the liposomes after the process of entrapment. Therefore, a POPC film was dispersed in the presence of glucose-1-phosphate, glycogen, and rabbit muscle phosphorylase a. Then the encapsulation efficiency was increased by repeated freeze/thawing and liposomes were formed by extrusion of the suspension through two filters with pore sizes of 400 nm in diameter. To this liposomal suspension a second enzyme, namely amyloglucosidase, was added, which was responsible for both the degradation of the glycogen outside the liposomes as well as of the newly synthesized radioactive glycogen during the process of freeze/thaw. Control experiments were performed in the same way except that the phosphorylase a was added after extrusion of the suspension together with the amyloglucosidase. The obtained results show that the amount of radioactivity eluted in fractions number one to four was significantly higher than in the control experiment (see Fig. 2). Thus, it can be assumed that about 20 000 cpm were incorporated into glycogen inside the POPC liposomes. It is noteworthy to mention that these 20 000 cpm correspond to an increase in the mean molecular weight of about 2800 to about 7000 kDa.

For our main goal, namely the loading of liposomes with substrate molecules from the bulk medium, we selected the method of detergent-induced liposome loading that has been described in the literature [12,13]. Those results were mainly obtained with egg PC (containing various fatty acid moieties) and at various lipid/detergent ratios. Therefore, to determine what amount of cholate was necessary for a cholate-induced loading of the POPC liposomes with substrate molecules, POPC liposomes (60 mM final lipid concentration) were prepared and cholate was added. After a brief treatment with a vortex, the solute to be permeated across the bilayer was added and the reaction was allowed to stand for 3.5 h. The cholate-induced 'feeding' was tested with 100 μM glucose-1-phosphate, 100 μM dATP, 5 mg/ml myoglobin, and 11.5 mg/ml tRNA added outside to the liposomes. For the separation of the nonentrapped material, a gel filtration spin column chromatography was carried out and the amount of radioactivity or the amount of A_{260} absorption coeluted with the liposomes (the fractions containing the liposomes can easily be determined because of the turbidity of the suspension at such high concentrations) was determined.

As shown in Fig. 3, each solute tested seemed to have an optimal cholate/POPC ratio; for instance, glucose-1-phosphate can be 'fed' from the outside in the most efficient way at 14.5 mM cholate, whereas dATP requires 19 mM to obtain the best loading.

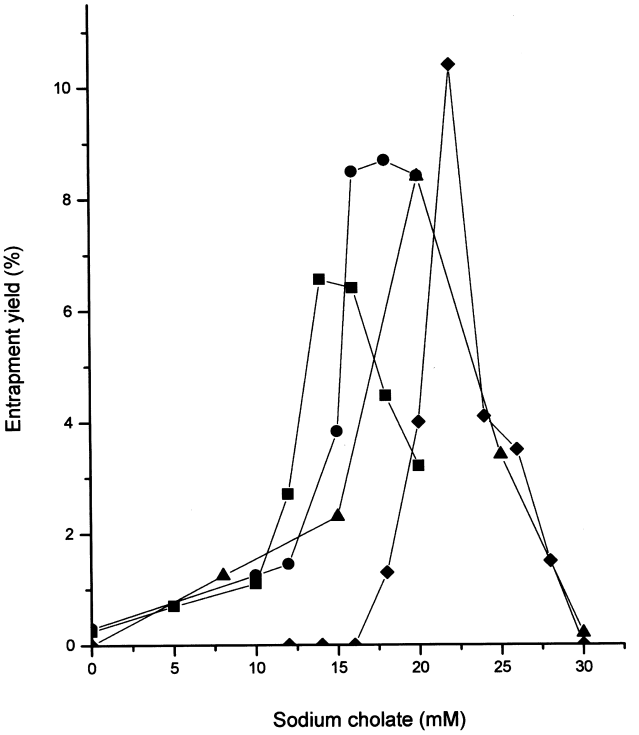

Fig. 3. Sodium cholate-induced liposome loading with solute molecules. POPC liposomes were formed by dispersing a lipid film in buffer solution before sodium cholate was added. To test the cholate-induced permeability of the bilayers, solute molecules were added (squares show the entrapment with 100 μM glucose-1-phosphate, circles 100 μM dATP, triangles indicate 5 mg/ml myoglobin (≅300 μM) and diamonds show 11.5 mg/ml tRNA (≅450 μM)). After incubation for 3.5 h, the liposomes were separated from the unentrapped solute molecules by spin column chromatography (BioGel A15m) and the amount of entrapped solute was determined by β-scintillation counting or UV-VIS spectroscopy.

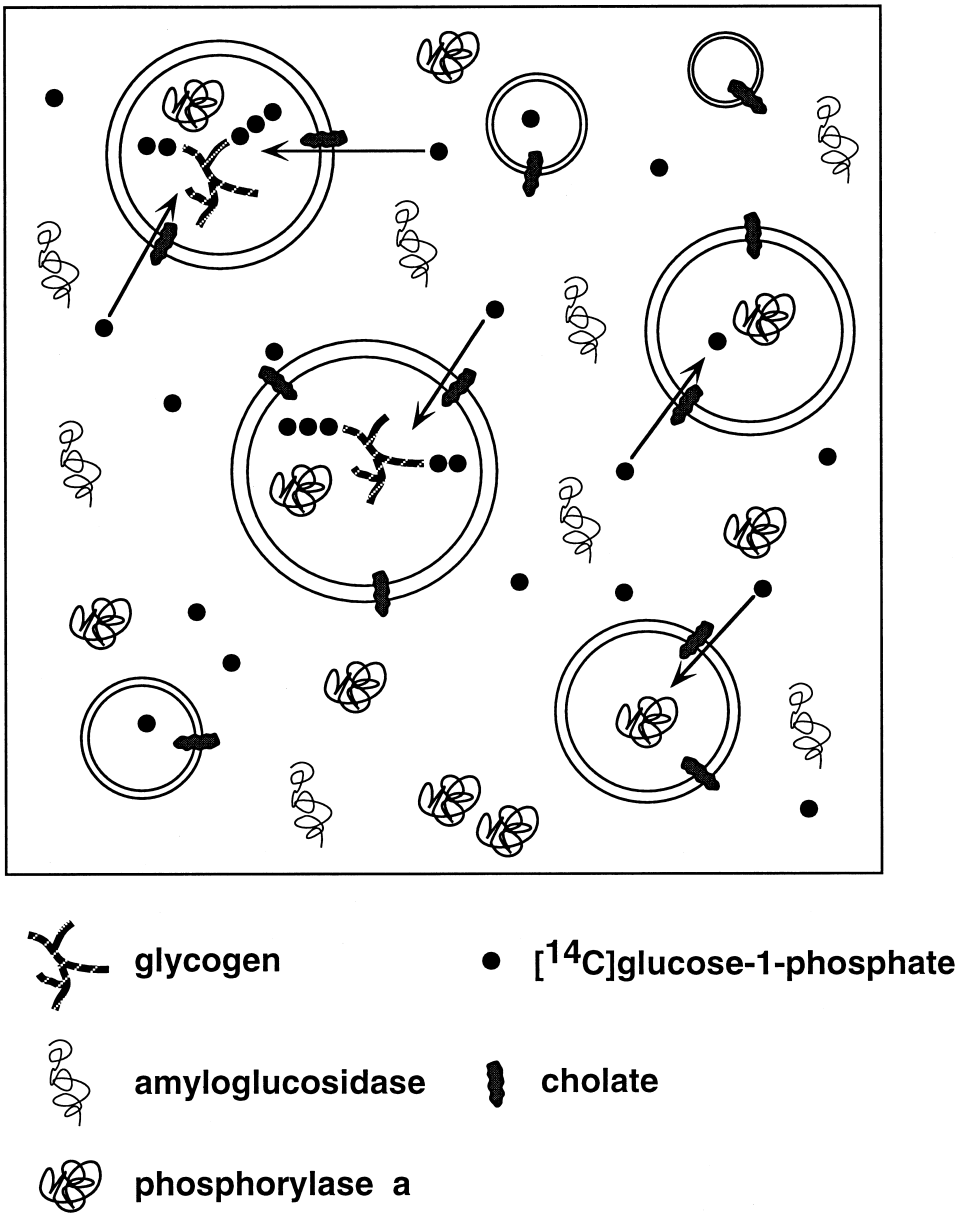

Fig. 4. Schematic illustration of the elongation of glycogen by phosphorylase a in POPC/cholate liposomes with [^{14}C]glucose-1-phosphate loaded from the outside.

Surprisingly, the high molecular compounds myoglobin and tRNA can be entrapped with efficiencies similar to the low molecular weight compound dATP. But it should be kept in mind that these results correspond to the material still entrapped after the isolation of the liposomes by spin column gel filtration chromatography. During this procedure, a part of the entrapped solute molecules could leak out. Therefore, this apparent best cholate/POPC ratios may not reflect the conditions allowing an optimal uptake of the solute molecules.

The main goal of the present work was the implementation of a microreactor which has its macromolecular components trapped within its water pool and receives its substrate molecules from the bulk medium (see Fig. 4). The advantage in comparison to the simpler model described above – where all ingredients were added from the beginning – is that the

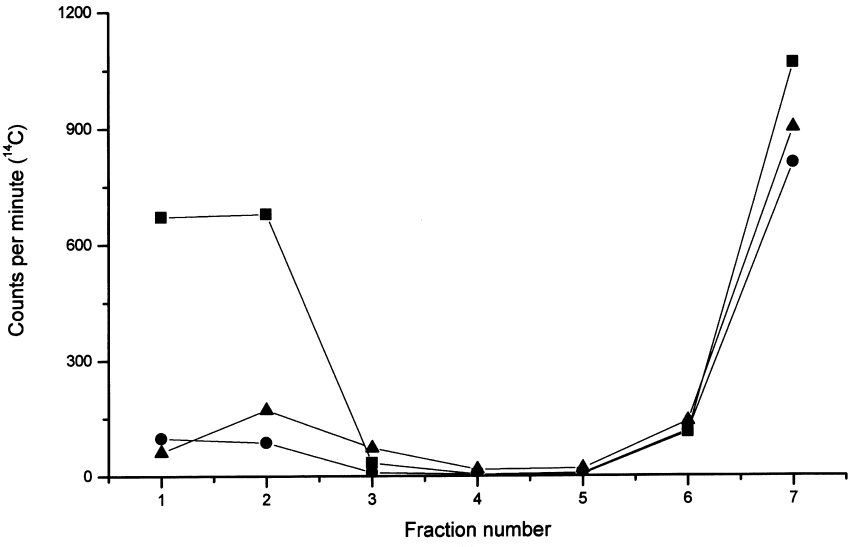

Fig. 5. Glycogen synthesis in liposomes loaded from the outside with [^{14}C]glucose-1-phosphate. All nonradioactive ingredients required for glycogen synthesis in liposomes were entrapped in POPC liposomes (final concentration 40 mM). The suspension was treated with amyloglucosidase for 1.5 h to digest the nonentrapped glycogen. Then cholate was added (final concentration 13.3 mM), and the suspension was briefly vortexed before the radioactive [^{14}C]glucose-1-phosphate was also added. After an incubation period of 10 min, the liposomes were incubated for 2 min at 75–80°C before solubilized by addition of 10% (w/v) deoxycholate and the micellar solution was subjected to a spin column chromatography (Sephadex G50). The glycogen is eluted in the fractions number one to three. Squares show the amount of radioactive glycogen produced in cholate-permeabilized POPC liposomes in 10 min. The circles show the same experiment with glycogen addition after extrusion of the liposomes (i.e. glycogen only outside the liposomes). The triangles show the second control experiment where 7.5 μg of glycogen were added before the addition of the radioactive glucose-1-phosphate in order to simulate an eventual release of glycogen due to cholate-induced solubilization of individual liposomes.

addition of the substrate molecules and thereby the incubation period for the enzymatic reaction of the amyloglucosidase can be precisely controlled. In such a way it became possible to form liposomes by extrusion and to digest the nonentrapped glycogen outside the liposomes completely before the substrate molecules were added. In fact, our results demonstrate (Fig. 5) that such an implementation of a 'feedable' microreactor is possible; it could be shown that [^{14}C]glucose-1-phosphate added from the outside could permeate across the cholate-destabilized POPC bilayer where it was enzymatically incorporated into glycogen. The elution profile of a Sephadex G50 spin column chromatography demonstrated that the fractions number one to three contained substantial amounts of radioactive glycogen. With glycogen added only after the production of the liposomes, the fractions number one to three showed almost no radioactivity. Therefore, it becomes clear that the newly produced radioactive glycogen must have been formed in the liposomes and not outside, where it would have been degraded by the abundant amyloglucosidase. To rule out the possibility that a glycogen release due to the addition of the sodium cholate caused the synthesis of radioactive glycogen, 7.5 μg of glycogen and the same amount of detergent were simultaneously added to preformed liposomes and the number of incorporated [^{14}C]glucose-1-phosphate was determined. As demonstrated in Fig. 5, no substantial amount of radioactivity could be found in the fractions number one to three. It should be kept in mind that the added amount of glycogen corresponded to about 40% of the glycogen entrapped within liposomes and, therefore, it is not conceivable that such a release caused a false-positive result.

To establish whether the system described above – namely the cholate-induced permeation of solute molecules across a POPC bilayer membrane – could also be used for experiments in which an enzyme is the species permeating through the POPC bilayer, a relatively short DNA (120-bp long) was produced by PCR and purified by spin column chromatography (Sephadex G50 column). In a first series of experi-

ments the 120-bp DNA was entrapped in the POPC liposomes and the unentrapped DNA was removed by gel filtration spin column chromatography from the liposomes (a procedure which was previously shown to be sufficient using these relatively short DNA species). Then, cholate and DNase I were added and it was clearly shown that the 120-bp DNA in the liposomes was digested (see Fig. 6A). The control experiment, in which the cholate addition was omitted, demonstrated that there was no unentrapped DNA outside the liposomes and therefore the amount of DNA precipitated by trichloroacetic acid remained constant for 30 min. The fact that the $t=0$ value in the presence of cholate gave a slightly lower amount of TCA-precipitable DNA is not surprising because it took approximately 30 s to 1 min between addition of cholate and the removal of an aliquot for the TCA precipitation. To test whether the DNase I or the 120-bp DNA molecules permeated across the POPC bilayer, the 120-bp DNA was entrapped again and incubated at various cholate concentrations without detecting any DNA-permeation through the POPC bilayer (data not shown). Therefore, it can be concluded that it was the DNase I which permeated across the POPC bilayer. Similar experiments with entrapped pancreatic DNase I in the POPC liposomes and addition of cholate/DNA from the outside gave substantially the same results (Fig. 6B). The cholate-induced DNase-permeation led to a rapid degradation of the 120-bp DNA molecules outside the liposomes.

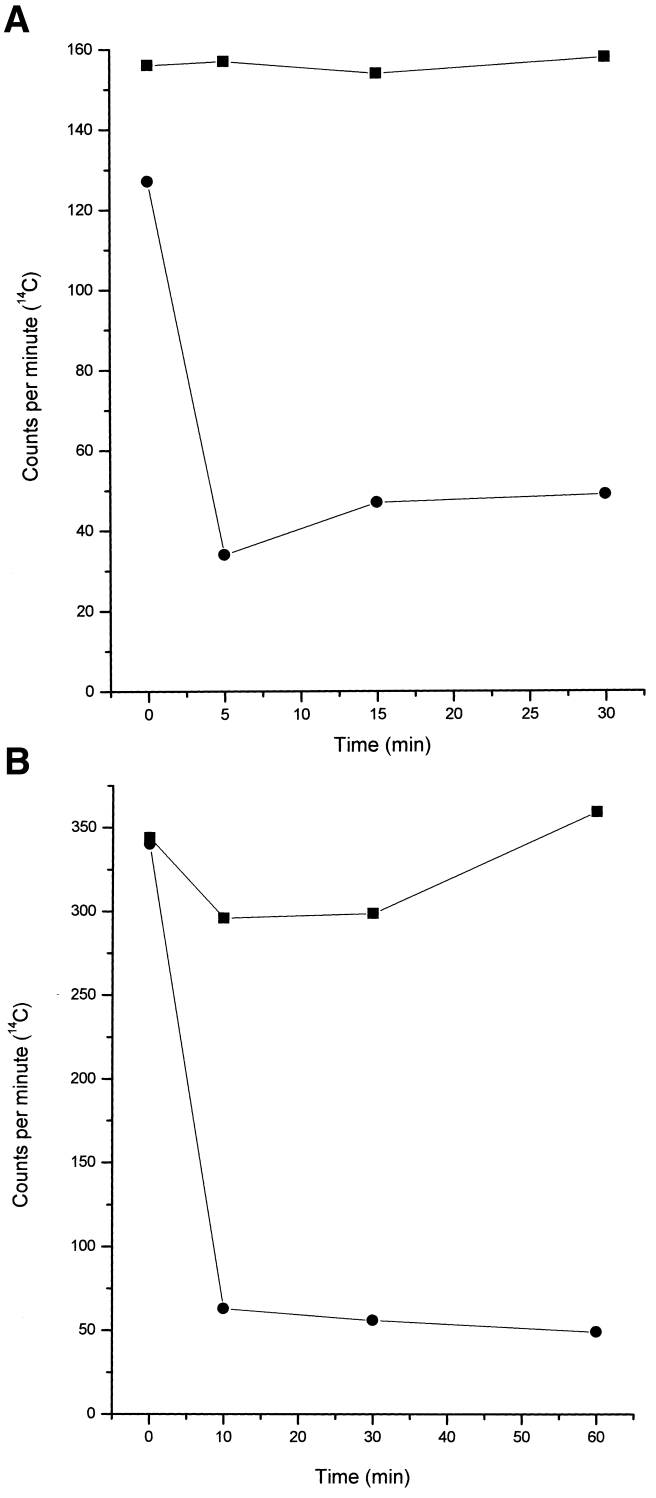

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Fig. 6. Cholate-induced permeability of pancreatic DNase I. (A) ^{35}S-labeled 120-bp DNA (about 12 500 cpm) was entrapped in POPC liposomes and the liposomes were purified by spin column chromatography (BioGel A15m). The final POPC concentration was about 50 mM. Then cholate was added (final concentration 10 mM) and the liposomes were allowed to stand for 30 min. Then 5 U of pancreatic DNase I were added, aliquots were removed at appropriate time points and the DNA was precipitated by TCA 10% (circles). The radioactivity retained on glass filters was determined by β-scintillation counting. (B) 100 U of DNase I were entrapped in POPC liposomes and purified by BioGel A15m spin column chromatography. In this experiment first the ^{35}S-labeled 120-bp DNA was added before the cholate. Again aliquots were removed at appropriate time points and subjected to TCA precipitation (circles). The control experiments in Fig. 5A and B (squares) show the same experiment without addition of cholate to ensure a proper purification of the liposomes.

4. Discussion

Microcompartmentation is of great interest because it allows to mimic the compartmentalization of biological systems with their distinct aqueous compartments within a single cell.

In the first part of the present work it could clearly be shown that even such large molecules such as glycogen from oyster with an estimated molecular weight of 2800 to 10 000 kDa can be encapsulated in liposomes with a mean radius of approximately 150 nm in diameter. This finding was similar to that found with linearized DNA where a linearized 3368-bp plasmid DNA (having an estimated length of about 1100 nm) was entrapped quite efficiently within liposomes produced in the same way [14]. With such a linearized plasmid DNA (molecular weight of about 2200 kDa) an entrapment yield of 27% was obtained with pure POPC liposomes; indicating that the entrapment yield obtained with the ^{14}C-labeled glycogen (MW of about 10 000 kDa) of about 6–8% might be reasonable. These findings show that relatively small compartments as those of 100 to 200-nm liposomes are sufficient to harbor molecules being much longer in their extended form. It is noteworthy to indicate that freeze/fracture electron micrographs taken from liposomes after the entrapment procedure of glycogen showed no significant difference to those liposomes prepared without glycogen (T. Oberholzer, unpublished results).

The extension of glycogen inside POPC liposomes showed that this reaction can be carried out in liposomes quite efficiently. This finding was rather surprising, because all our attempts to perform enzymatic reactions in liposomes resulted in relatively low efficiencies [11,22]. In those cases the likelihood to combine all ingredients within one single compartment was very low and therefore the enzymatic reaction could only rarely occur. (Furthermore, freeze/thaw as the most efficient procedure for encapsulation was not possible in the case of Qβ replicase because this method decreased its activity dramatically.) These difficulties could be eliminated in the present systems: all ingredients were present at sufficiently high concentrations and the enzymes remained – at least in part – active even after several cycles of freeze and thaw. This is quite surprising when taken into account that the heterotrimeric Qβ replicase lost almost all its activity during freeze/thaw in the presence of liposomes whereas the homodimeric phosphorylase a seems to be unaffected by these procedures.

In a first series of experiments, glycogen was synthesized in liposomes with all ingredients being present during the process of entrapment. The ratio 'reaction in liposomes' to 'control experiment' showed that a relatively high amount of glycogen was radioactively labeled outside the liposomes. Obviously, the amyloglucosidase could not efficiently degrade the whole amount of radioactively labeled glycogen. This ratio was significantly improved in the experiments in which the [^{14}C]glucose-1-phosphate was added from the outside and permeated in a cholate-dependent manner into the interior of the liposomes where it was polymerized to glycogen. In these experiments, glycogen could be digested by the amyloglucosidase prior to the addition of the radioactive glucose-1-phosphate and, therefore, the 'background radioactivity' was very low. As shown by the second control experiment, even smaller amounts of released glycogen did not lead to a change of the results. When 7.5 μg of glycogen were added from the outside – to simulate an eventual release of glycogen because of the cholate addition – there was no synthesis of radioactive glycogen outside the liposomes.

The glycogen experiments described above were all performed at a POPC concentration of 40 mM and a cholate concentration of 13.3 mM. However, as seen in Fig. 3, this ratio (POPC/detergent) of three does not seem to be the best ratio for an efficient entrapment of glucose-1-phosphate into POPC/cholate liposomes. We think that these values in Fig. 3 do not really reflect the best conditions for uptake of solute molecules from the outside, they solely reflect the situation after the entrapment and subsequent isolation of the liposomes. In the experiments with glycogen synthesis in liposomes and glucose-1-phosphate loading from the outside there is a totally different situation. In these experiments the solute to be loaded from the outside had not to be removed by gel filtration chromatography and therefore other conditions as shown in Fig. 3 were selected.

The last series of experiments demonstrated that also macromolecules are able to permeate across a POPC/cholate bilayer and still retain their activity.

DNase I (MW of about 29 kDa) could permeate across the POPC/cholate bilayer and digested the 120-bp DNA on the other side of the bilayer. A DNA-permeation, even with relatively short pieces of DNA (MW of about 60 kDa, T. Oberholzer, unpublished results), could not be observed, whereas the tRNA molecules (MW of about 25 kDa) could be loaded in liposomes in measurable amounts (as shown in Fig. 3). Interestingly, also Schubert et al. showed that short-chain poly(U) molecules were able to permeate across POPC/detergent bilayers [13,23]. As suggested by Schubert, there seems to be a critical molecular weight above which permeation of macromolecules is not possible; according to his results, this critical molecular weight is around 70 kDa. Our results with DNase I and the proteins required for the synthesis of glycogen in liposomes seems to confirm his observation. DNase I could clearly permeate across the cholate-treated POPC bilayers, whereas no permeation of phosphorylase or amyloglucosidase could be observed. (It should be kept in mind that a amyloglucosidase permeation in substantial amounts would have destroyed all glycogen inside the liposomes and therefore, no glycogen synthesis in liposomes with [^{14}C]glucose-1-phosphate loading from the outside would have been observed.)

For a further development of such systems it seems to be necessary to determine in detail the conditions required for detergent-induced loading in phospholipid bilayers. Which characteristics of the solute are important, is it mainly the molecular weight or the charge of the species? How do the bilayer charge and charge density influence the cholate-triggered permeation of hydrophilic compounds? Can other detergents be found with which the permeation of positively charged molecules becomes also possible?

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