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RECONSTITUTION IN VITRO OF SULFOBROMOPHTHALEIN TRANSPORT BY BILITRANSLOCASE

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Liposomes containing 150 mM KCl and 0.48 mM sulfobromophthalein have been prepared. The internal pH was set at 6.5, a value at which sulfobromopthalein is colorless. When brought to alkaline pH a certain amount of the dye is deprotonated and can be read spectrophotometrically as external sulfobromophthalein. Upon addition of Triton X-100 the membrane is dissolved and all sulfobromophthalein present in the preparation may be measured. Addition of bilitranslocase to such a preparation of liposomes causes the internal sulfobromophthalein to leave the internal compartment. The rate of this phenomenon may be followed directly and shown to be greatly accelerated by the addition of valinomycin. The latter finding indicates that sulfobromophthalein transport occurs in response to a membrane diffusion potential created by permeabilisation to K^+ of liposomes brought about by valinomycin (uniport). The permeability change induced by bilitranslocase is specific and does not reflect an alteration of the normal impermeability of liposomes to small ions such as protons or Ca^{2+}.

Introduction

In the accompanying paper [1] we have reported the isolation and purification of a low molecular weight form of bilitranslocase. This component has been proven to be physiologically involved in hepatic uptake of bilirubin and other organic anions [2—4]. This paper reports the successful reconstitution of sulfobromophthalein transport in liposomes promoted by isolated bilitranslocase. Data will also be presented suggesting that this organic anion movement occurs electrophoretically as a uniport.

Materials and Methods

Liposomes were prepared using phosphatidylcholine (Sigma) from soybean essentially according to Racker [5]. Before use the phospholipids were washed twice with cold acetone. Sodium cholate was purified as described by Schneider et al. [6]. The particles were obtained by dispersing the phospholipids (20 mg/ml) in a buffer solution comprising 0.48 mM sulfobromophthalein (Merck)/150 mM KCl/50 mM Tris-HCl (pH 6.5)/2% sodium cholate. The suspension was subjected to sonic oscillation in aliquots of 4 ml using a Branson Sonifier equipped with a microtip. Sonic irradiation was continued for 2 min under nitrogen bubbling in ice with a 30 s intermission after 1 min. The suspension was then dialysed against the same solution with the exclusion only of cholate. This first dialysis was carried out for 5 h using a volume of dialysing solution at least 100-times larger. The material was dialysed subsequently against a buffer consisting

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of 150 mM KCl/50 mM Tris-HCl (pH 6.5). This dialysis was carried out overnight. A third dialysis was run using either Tris-HCl buffer at pH 6.5 or Tris-glycine buffer at pH 8.4. In both cases the concentration of the buffer was increased to 200 mM in order to compensate for KCl omission. In certain experiments the third dialysis solution contained also 1 g Dowex AG1-X8 in 250 ml.

Measurement of sulfobromophthalein was carried out by diluting the liposome suspension with either 0.1 M NaOH or 0.1 M borate buffer at pH 10.5. The two measurements gave superimposable results, indicating that treatment with 0.1 M NaOH does not appreciably destroy the integrity of liposomes, at least within the few minutes necessary for the reading. To the same cuvette 40 μl 10% Triton X-100 were then added, which results in a net increase in absorbance at 580 nm. An extinction coefficient of 64.0 $\mu mol^{-1} \cdot cm^2$ was used.

Bilitranslocase preparations were obtained as described in the accompanying paper [1].

Calcium movements have been followed by dual-wavelength recording spectrophotometry at 585—565 nm (Phoenix Dual Wavelength Recording Spectrophotometer) using Arsenazo III as a free Ca^{2+} indicator according to Vallieres et al. [7]. The indicator was purified according to Dipolo et al. [8].

The electron microscopy was performed using the standard negative staining technique in the presence of phosphotungstate as a contrasting medium. Protein determination was carried out according to Waddell [9] as modified by Romeo et al. [10].

Results

A classical experiment carried out to assess the quality of a liposomal preparation consists of inducing calcium movements across the artificial membrane by means of a specific ionophore such as A23187. The driving force utilized is usually the diffusion potential of K^+ after permeabilization induced by valinomycin. Fig. 1 shows a typical experiment of this kind. Calcium movements are followed spectrophotometrically using Arsenazo III as a calcium indicator [7]. After addition of calcium, the absorbance remains constant until the calcium ionophore is added. The fact that no net calcium movements occur upon addition of valinomycin clearly indicates that the liposomal membrane is impermeable to calcium. Indirectly the experiment shows also that the membrane is impermeable to potassium ion. In Table I are collected data from a number of experiments carried out under different experimental conditions. Clearly liposomes prepared at pH 6.5 contain two distinct sulfobromophthalein pools, one external, NaOH accessible, and a second one, internal and inaccessible to NaOH. The latter can be measured only after disruption of the membrane by Triton X-100. Interestingly, prolonged dialysis at alkaline pH in the presence of a trap for charged sulfobromophthalein (Dowex AG1-X8) is efficient in removing the dye in the external compartment but it has no effect on the content of the internal one. These experiments indicate that at low pH sulfobromophthalein may be bound to phospholipids and a simple calculation shows that the partition between phospholipids and water favors the former location. Increasing the pH results in decreasing, as expected, the lipophilic nature of sulfobromophthalein.

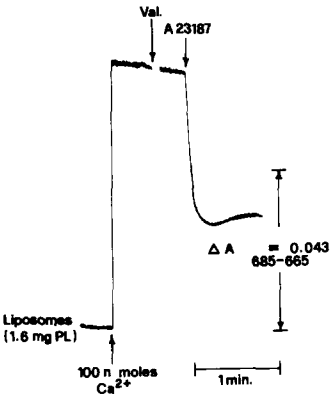
The electronmicrograph presented in Fig. 2 indicates that the preparation of the particles, as seen after negative staining, shows a fairly homogeneous appearance consisting of vesicles mostly surrounded by a single membrane, in agreement with data in the literature [5]. The average size was in the vicinity of 0.4—0.5 μm. The movements of sulfobromophthalein from the inner compartment of liposomes may be followed directly taking advantage of the fact that the dye inside the particles is protonated and colorless. If the medium outside is maintained at pH 8.4 by an appropriate buffer any net efflux of the dye will result in an increase in absorbance at 580 nm.

Fig. 3 shows an experiment in which sulfobromophthalein movements have been followed by dual wavelength spectrophotometry at 580—500 nm. The two traces clearly indicate that when liposomes alone are added with valinomycin the resulting K^+ efflux is virtually without effect on internal sulfobromophthalein content. By contrast, when the liposomes are added with purified and reduced bilitranslocase, addition of valinomycin is followed by a rapid increase in absorbance, suggesting a net efflux of

TABLE I

Influence of the experimental conditions on sulfobromophthalein content of liposomes

Experiment		Sulfobromophthalein (nmol/mg phospholipid)		
		NaOH-accessible	NaOH-inaccessible	Total
1	Dialysis, pH 6.5	32.80	8.65	41.45
2	Dialysis, pH 6.5	32.80	9.00	41.80
3	Dialysis, pH 8.4 + Dowex AG 1-X8	1.91	8.75	10.66



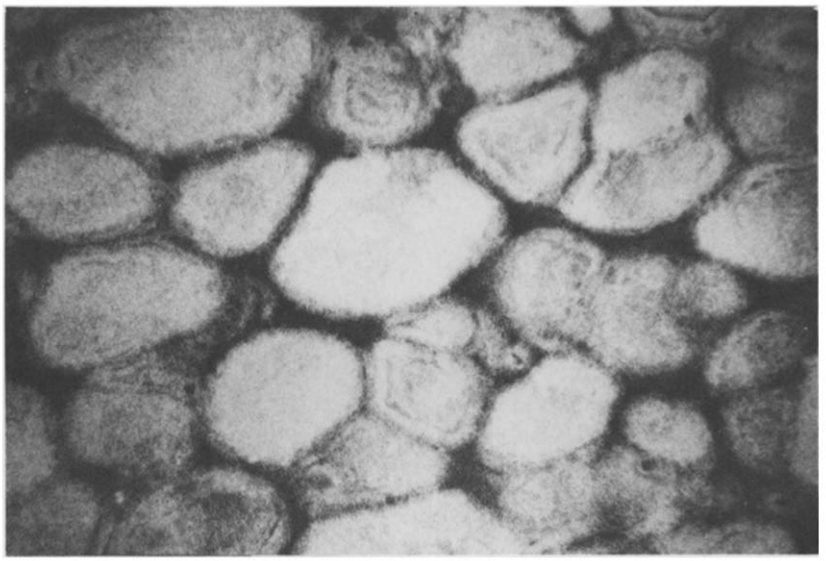


Fig. 1. Calcium transport evoked in sulfobromophthalein-containing liposomes by the calcium ionophore A23187. Experimental conditions: the reaction medium contained in 3 ml: 1.6 mg liposomal phospholipids; 0.3 mM arsenazo III; 50 mM Tris-HCl (pH 6.5); valinomycin (10 μg) (Val.) added in 10 μl of dimethylsulfoxide. 200 nmol A23187 also in 10 μl dimethylsulfoxide were added, where indicated. Phoenix Dual Wavelength Recording Spectrophotometer.

Fig. 2. Electron micrograph of a negative-stained specimen of sulfobromophthalein-containing liposomes. Negative staining with 1.5% phosphotungstic acid in distilled water. Electron microscope, Philips M300; magnification $\times$38400.

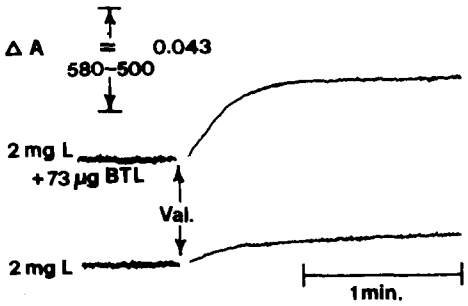


Fig. 3.Sulfobromophthalein movements evoked by bilitranslocase and valinomycin. Experimental conditions: the reaction medium contained in 3 ml: 2 mg liposomal phospholipids (L); when present, 73 μg bilitranslocase (BTL); 200 mM Tris-glycine buffer (pH 8.4). 10 μg valinomycin (Val.) were added, where indicated, dissolved in 10 μl dimethylsulfoxide. Liposomes and bilitranslocase were kept together at room temperature for 2 min before addition of valinomycin. Phoenix Dual Wavelength Recording Spectrophotometer.

sulfobromophthalein from the inner compartment.

Fig. 4 shows the dependence of this phenomenon on the amount of protein added. The transport reaches maximal velocity around 15 μg bilitranslocase added per mg phospholipid. Obviously the rates measured could be interpreted also

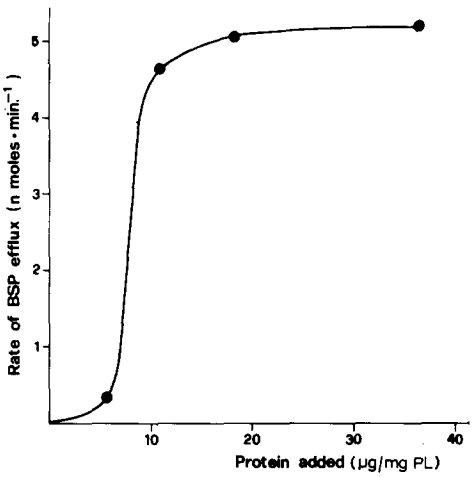


Fig. 4. Influence of bilitranslocase concentration on the rate of sulfobromophthalein (BSP) efflux. Experimental conditions as in Fig. 3. Abscissa, µg bilitranslocase/mg phospholipids (PL) present in the cuvette.

as the result of the detachment of an aliquot of sulfobromophthalein bound superficially to phospholipids. To avoid this criticism, in parallel experiments, liposomes containing sulfobromophthalein were dialysed against a buffer at pH 8.4 either in the presence or in the absence of bilitranslocase. The amounts of sulfobromophthalein in the external and in the internal spaces were measured at time zero and after 2 h. The results of these experiments are reported in Fig. 5. Clearly the dialysis per se removes only that sulfobromophthalein present in the outer compartment, leaving virtually unchanged the dye content in the control liposomes. The sample dialysed in the presence of the protein after 2 h has completely lost its internal content of sulfobromophthalein. The results of these experiments could derive from a nonspecific permeabilisation of liposome membrane, or the effect could indeed be referred to reconstitution of a physiological function. If the effect derives from a nonspecific permeabilisation it is to be expected, how-

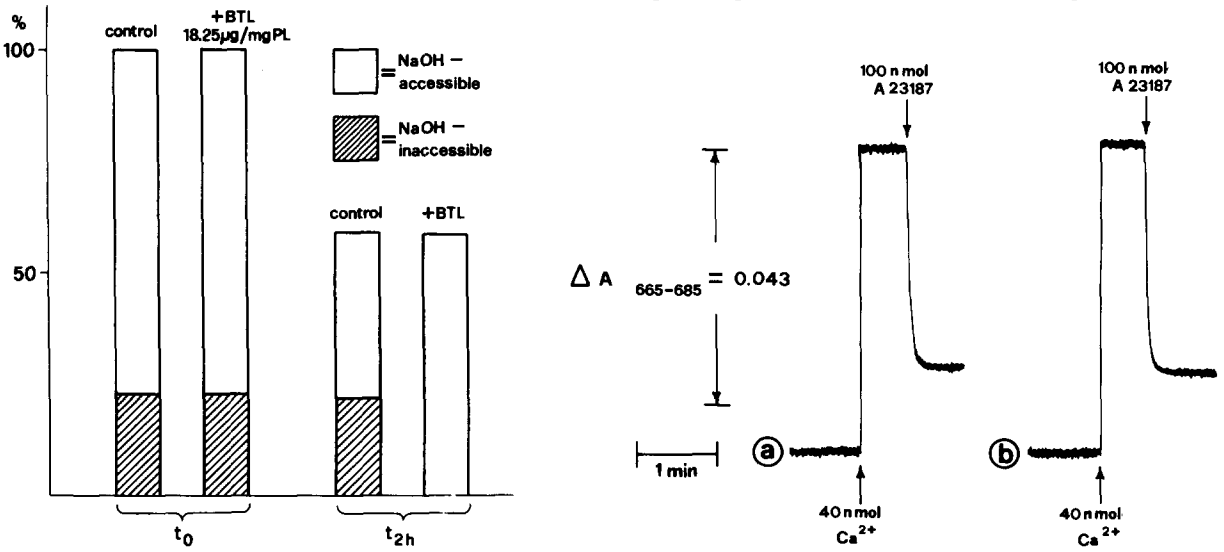


Fig. 5. Influence of bilitranslocase on the sulfobromophthalein content of the inner compartment of liposomes. Experimental conditions: 10 mg liposomal phospholipids (0.5 ml) were diluted with an equal volume of 200 mM Tris-glycine buffer (pH 8.4) in the presence of 18.25 µg bilitranslocase/mg phospholipids and in its absence. 20 µl of the two samples were immediately assayed for NaOH-inaccessible sulfobromophthalein. The samples were then dialysed at 0°C for 2 h against 200 ml 200 mM Tris-glycine buffer (pH 8.4). At the end of the dialysis samples were again assayed for NaOH-accessible and NaOH-inaccessible sulfobromophthalein contents.

Fig. 6. Calcium movements evoked by A23187 in liposomes at the end of the experiment in Fig. 5. Experimental conditions: as in Fig. 1 with the exception of the buffer which, in this case, is 200 mM Tris-glycine (pH 8.4). Sample a corresponds to liposomes dialysed in the presence of bilitranslocase (18.25 µg/mg phospholipids); sample b corresponds to liposomes dialysed in the absence of the protein.

ever, that liposomes of the sample dialysed in the presence of bilitranslocase after complete loss of their sulfobromophthalein content have also lost their proton content and have become permeable to Ca^{2+}.

Fig. 6 shows an experiment carried out using particles obtained at the end of the dialysis in the presence (curve a) and absence (curve b) of bilitranslocase. Clearly, upon addition of Ca^{2+} in both cases the absorbance remains constant, indicating that the membrane is still impermeable to the ion. By adding the calcium ionophore, Ca^{2+} is almost completely taken up and the level reached is in both cases the same. In this case, the pH in the external medium being 2 units higher than in the internal space, the extent of calcium uptake is a measure of the pH gradient between the inner and the outer compartment. It is remarkable that although sample a has lost completely its sulfobromophthalein content, no appreciable difference is noticed with respect to sample b. It may be concluded that the permeabilisation of the membrane brought about by the addition of a small amount of bilitranslocase is specific and not accompanied by an alteration of the impermeability properties to proton or Ca^{2+}. A similar conclusion, i.e., specificity of the transport induced, may be derived from the experimental data with old bilitranslocase preparations which have lost their reconstitutive capacity (not shown).

Discussion

Data presented in this paper show that sulfobromophthalein trapped in liposomes may be released from the internal compartment by addition from outside of a purified, mercaptoethanol-reduced preparation of bilitranslocase. The effect is present also in preparations of the same protein obtained in the presence of deoxycholate [11]. In this case, however, the presence of the detergent complicates remarkably the interpretation of the data. The finding that valinomycin greatly stimulates the rate of sulfobromophthalein movements by creating a positive outside membrane potential is a strong indication in favor of the view that the dye is transported electrophoretically. It could be argued that the external addition of a sulfobromophthalein sequestering agent such as bilitranslocase could act as a trapping system favoring sulfobromophthalein efflux without the intervention of a real carrier mechanism. The evidence coming from the experiment carried out in the presence of an ion-exchange resin which did not alter at all the sulfobromophthalein content of the inner compartment speaks against this view. On the other hand, the amount of bilitranslocase capable of promoting total depletion of the inner compartment is orders of magnitude below that necessary for a simple complexation. Of special interest is the observation that the transport reconstituted in vitro is specific and not accompanied by gross alteration of the permeability properties of the liposomes.

In conclusion we think that the model system set up will prove useful in the future to study the physiological function of organic anions uptake in liver at the molecular level.

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