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Cellular localization of sulfobromophthalein transport activity in rat liver

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The movement of sulfobromophthalein is measured in rat liver plasma-membrane vesicles by direct dual-wavelength spectrophotometry. The technique is based on the principle that the dye, when entering a more acidic compartment, changes its absorption in the visible region. From this study it may be concluded that, among the different cellular subfractions, only liver plasma-membrane vesicles can catalyze electrogenic transport of sulfobromophthalein. Plasma membranes from erythrocytes are unable to perform such a function. The movement follows the distribution pattern of $(Na^+ + K^+)$-ATPase and it is therefore concluded that this process occurs exclusively at the sinusoidal membrane level. Inhibition studies confirm that the process is catalyzed by bilitranslocase.

Introduction

It is generally accepted nowadays that bilirubin and a number of dyes, including sulfobromophthalein, are taken up at the sinusoidal membrane level as the result of a carrier-mediated process (see for recent reviews on the subject, Refs. 1–4). At the molecular level, the carrier has been identified as a protein isolated in our laboratory and named bilitranslocase [5,6]. This protein could be incorporated into liposomes [7] and an electrogenic transport of sulfobromophthalein could be reconstituted in vitro. The technique established for this reconstitution took advantage of the pH-indicator properties of sulfobromophthalein which, inside liposomes, was colorless at pH 6.5 and outside was purple-colored at pH 8.4. The advantage of using a dye derives from the fact that the kinetics of the process can be monitored continuously by dual-wavelength spectrophotometry. We attempted to apply this technique to a more complex system such as plasma membrane vesicles, under the assumption that the movement of the dye from a slightly alkaline medium into a less alkaline internal space could result in a detectable change in absorbance. The extension of the technique to biological preparations could be an efficient tool for answering a number of open questions concerning the competence of the different membranes to catalyze the transport, the specific localization of the translocator in certain domains of the hepatocyte plasma membrane and, possibly, the understanding of the mechanism by which the accumulation of the dye takes place in vivo as the first step of sulfobromophthalein plasma clearance.

Materials and Methods

Sulfobromophthalein was purchased from Merck; ATP, AMP from Pro.Bio. Sint. (Varese,

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Italy); valinomycin, lactate dehydrogenase, pyruvate kinase, ouabain, glucose 6-phosphate, NADH and rotenone, from Sigma Chem. Co. (St. Louis, U.S.A.). All other reagents were commercially available analytical-grade chemicals. Plasma-membrane vesicles were obtained from Wistar albino rat livers according to Van Amelsvoort et al. [8].

Human erythrocyte ghosts were prepared from venous blood samples taken from healthy volunteers, following the technique described by Fairbanks et al. [9]. Resealing of the ghosts was performed according to Steck et al. [10] and was checked photometrically by osmotic swelling experiments. Microsomal preparations were made up in 0.25 M sucrose solution as reported by Sottocasa et al. [11]. Linear sucrose density gradients were prepared by using a mechanical gradient-forming device in the range indicated in the figures. The subfractionation of the gradient was performed by inserting a syringe needle into the bottom of the tube connected via Tygon tubing to a peristaltic pump and a fraction collector. At the end of the run, each fraction was checked for sucrose content using an Abbe refractometer (Officine Galileo, Milano). Rotenone-insensitive NADH–cytochrome *c* reductase, succinate-cytochrome *c* reductase and glucose-6-phosphatase were measured as previously reported [11]; ATPase activities were measured both from the inorganic phosphate appearance according Shoener et al. [12] and by the enzymatic method reported by Scharschmidt et al. [13]. 5'-Nucleotidase was determined according to Ipata [14]. L-γ-Glutamyltransferase was determined according to Persijh [15] (Monotest 10, Boehringer) sulfobromophthalein movements were followed essentially as previously described [7] by means either of a Dual Wavelength Phoenix or a Sigma ZWS II recording spectrophotometer, operated at the wavelength pair 580/540 nm. A differential extinction coefficient of 5.50 $\mu mol^{-1} \cdot cm^2$ was used.

Results

Fig. 1 shows the pH dependence of sulfobromophthalein absorption at 580 nm on the pH in the range 7.2–8.2. The experiment was so carried out that the conditions (ionic strength, temperature and salt composition) could be perfectly compared

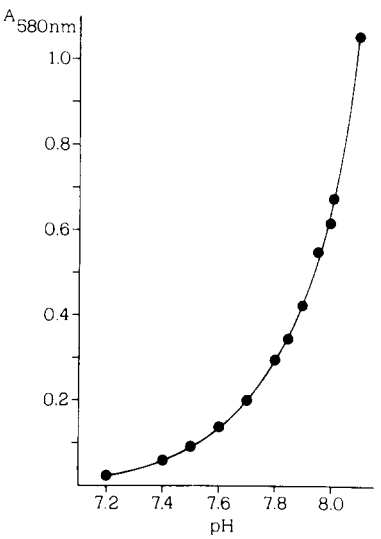

Fig. 1. pH-dependence of absorbance of sulfobromophthalein in aqueous solutions. The medium consisted of 0.25 mM sulfobromophthalein, 100 mM potassium phosphate buffer, 50 mM KCl at the different pH values indicated. Final volume, 3 ml. Measurements were carried out at room temperature using a computer-assisted Carlo Erba recording spectrophotometer Mod. Spectracomp 601.

to those subsequently used with the biological material. From this curve it can be inferred that if sulfobromophthalein moves from a medium at pH 8.1 to another compartment at a pH in the vicinity of 7.4, it becomes virtually colorless and any further pH decrease has no practical effect on the absorbance at 580 nm. It is also expected, on the other hand, that any change in the pH of the outside medium around 8.1 may have great influence on the absolute absorbance, being in this region steepest the curve. The pK of sulfobromophthalein is approx. 8.6 and at pH 8.1 about one-tenth of the molecules are in the quinoid, deprotonated, colored form.

Fig. 2 collates the results of an experiment carried out with isolated plasma membrane vesicles prepared in a medium comprising 10 mM Hepes (pH 7.4)/0.25 M sucrose. The assay medium consisted of a sulfobromophthalein solution strongly buffered at pH 8.1 with potassium phosphate. The system is monitored spectrophotometrically at the wavelength pair 580/540 nm. A decrease in absorbance is expected to reflect the entry of the dye into a compartment whose pH is lower than that

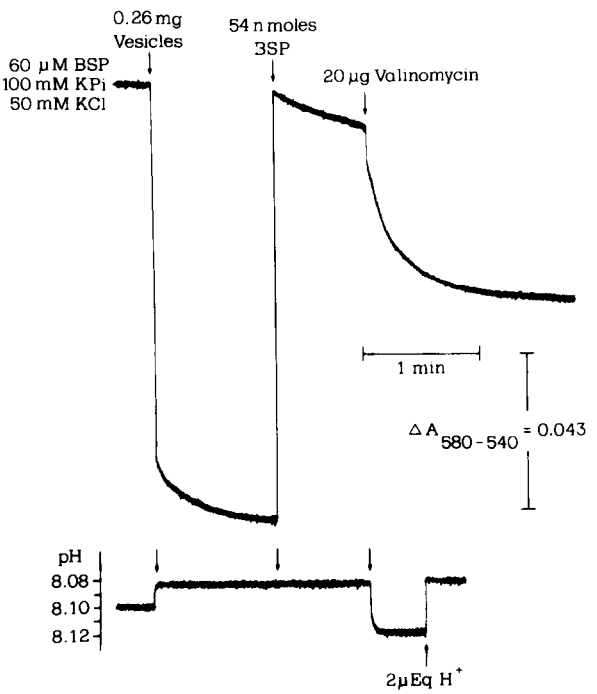

Fig. 2. Sulfobromophthalein movements in rat liver plasma membrane vesicles. Phoenix dual wavelength recording spectrophotometer operated at 20°C; final volume, 1 ml; medium and additions at the arrows as specified in the panel. Lower trace, Metrohm pH meter Model E 300, glass electrode recording.

of the outside medium. In the same figure, a glass electrode trace is also reported, to monitor in parallel the pH changes in the medium. Clearly the addition of the particles causes a slight (less than 0.02 pH units) change in pH and a very marked decrease in absorbance at the wavelength pair employed. The pH drop due to addition of the vesicle suspension is expected to contribute also to the absorbance decrease found. This contribution is however marginal, accounting roughly for 1/50 of the total deflection. To compensate for this first drop in absorbance, 54 nmol sulfobromophthalein are added to the mixture. This operation brings the signal back to the starting value. In this way the free sulfobromophthalein concentration in the external medium is restored to the original level (before the addition of the particles). If at this point valinomycin is added to the system, the vesicle membrane is made permeable to potassium, which creates a diffusion potential of this ion present in the medium. In response to this potential, a second drop in absorbance ensues. This phenomenon could be the result of a number of events, including proton extrusion from the vesicles. This can be immediately ruled out on the basis of the lower trace in the figure, which shows that, upon addition of valinomycin, a slight alkalinization occurs. This experiment deserves some comments concerning both the first and the second drop in absorbance observed. In addition to the already mentioned slight drop in pH, the former may be the result of (1) absorption to and/or solubilization of sulfobromophthalein in the phospholipids of the membrane and (2) entry into the internal space of the vesicles; the latter drop, in turn, may derive from (1) absorption onto a polarized membrane and/or (2) entry into the inner space. To rule out possible artifacts and to confer specificity to the activity observed, we have carried out the same experimentation using also other membrane preparations devoid of bilitranslocase [16].

Fig. 3 collates the results obtained using erythrocyte ghosts before and after resealing treatment. Both in the case of unsealed and resealed erythrocyte envelopes, the addition to sulfobromophthalein solution causes only a minor deflection of the signal, indicating that the interaction sulfobromophthalein-phospholipids per se contributes only marginally to the first rapid drop in absorbance measured in Fig. 2 with hepatocyte membrane vesicles. As expected, on the other hand, in

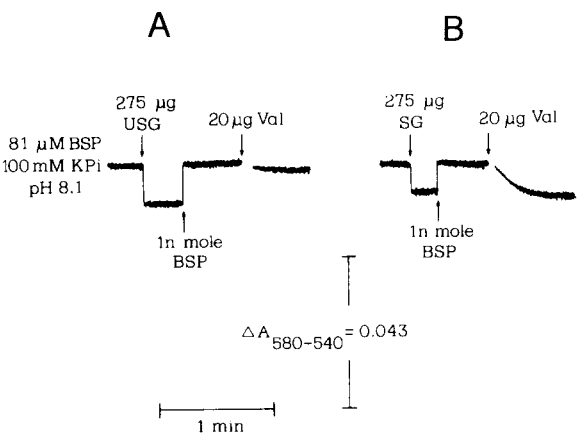

Fig. 3. Sulfobromophthalein movements in unsealed and resealed erythrocyte ghosts. Experimental conditions as in Fig. 2; (A) unsealed ghosts (USG in the panel); (B) sealed ghosts (SG in the panel).

unsealed ghosts (trace A) valinomycin is without effect. In other words, any major contribution in the deflection of the trace due to solubilization of sulfobromophthalein in phospholipids and/or physical absorption of the dye on the surface of vesicles can be excluded. After resealing, the change in absorbance evoked by the antibiotic is very slow in rate and limited in extent (trace B) if compared to that obtained with rat-liver plasma-membrane vesicles. In conclusion, the results obtained with erythrocyte ghosts are consistent with the interpretation that, with rat liver plasma-membrane vesicles, the absorbance changes seen are to be attributed to sulfobromophthalein movements into the internal space. This conclusion is reenforced by the the experiment presented in Fig. 4, in which the amount of sulfobromophthalein taken up per mg protein during the first fast deflection of the optical signal is plotted versus the reciprocal of the osmolarity in the outside medium. The latter parameter is modulated by changing the concentration of sucrose in the sulfobromophthalein solution of the cuvette, maintaining all the other parameters strictly constant. The straight line in the figure indicates that sulfobromophthalein is taken up in an osmotically active space.

A question which remained to be answered concerned the nature of the forces involved in the uptake by the vesicles both before and after the addition of valinomycin. One of the obvious

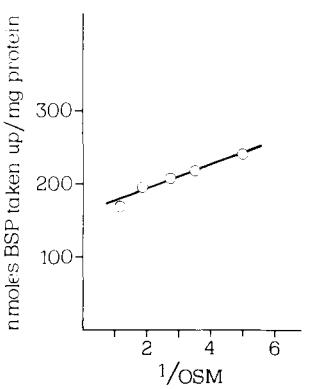

Fig. 4. Dependence of sulfobromophthalein uptake by plasma membrane vesicles on the osmolarity of the medium. Experimental conditions as in Fig. 2 except that the medium in the cuvette contained, in addition, variable concentrations of sucrose. On the ordinate the extent of sulfobromophthalein disappearance following the addition of the vesicles is plotted in terms of dye taken up per mg protein.

parameters to be taken into account is the concentration gradient of the dye across the membrane of the vesicles. The effect of this parameter alone, however, is expected to elicit only minor changes in the optical measurement, in view of the great difference in size between internal and external volume. A second parameter which may contribute substantially to sulfobromophthalein uptake during the first rapid phase is the pH difference existing across the membrane. It is expected that deprotonated molecules enter the vesicles and, once inside, are protonated. The result of this process would be the maintenance of a concentration gradient of the deprotonated form between outside and inside. This statement implies that the transported species is only the deprotonated, colored form and, if this is the case, the extent of the uptake should be influenced by the buffering capacity of the inner space. To check this hypothesis the experiment presented in Fig. 5 has been carried out. The vesicles were presonicated in a medium at constant pH but at different buffer concentrations. The different buffer solutions were made iso-osmolar by appropriate additions of sucrose. The sonic irradiation insures an equilibration of the inside buffer composition with a comparatively large volume of the chosen buffer system. After the treatment the vesicles were spun down and taken up in a minimum volume of the same buffer. The figure shows that the amount of sulfobromophthalein taken up progressively increased as the internal buffer concentration is increased. The upper line relates to the extent reached after the addition of valinomycin. It should be observed that the two lines are parallel, indicating that the amount of sulfobromophthalein taken up after the addition of valinomycin is constant and independent of the buffering capacity inside the vesicles. As for the forces involved in the uptake promoted by valinomycin, It is reasonable to assume that these are related to the creation of a membrane potential positive inside or, alternatively, to reversal of a negative inside membrane potential created by the entry of sulfobromophthalein. It must be borne in mind that sulfobromophthalein, even when colorless, is not completely protonated, having two sulfonic groups whose pK is very low. With these premises we decided to investigate further the valinomycin-in-

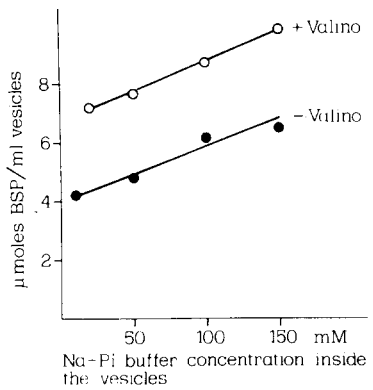

Fig. 5. Dependence of the extent of sulfobromophthalein uptake, before and after the addition of valinomycin, on the concentration of the buffer inside the plasma membrane vesicles. Aliquots of 50 µl of plasma membrane vesicles suspensions at 13 mg protein per ml were diluted to 3.5 ml with the different buffer solutions indicated in the figure and subjected to sonic irradiation in an ice-cooled SW 39 Spinco rotor tube, using a Branson Sonifier equipped with a microtop, operated at 3.5 A. The sonication time was 1 min per sample, in four 15 s periods with intermissions of equal duration. Each sample was then centrifuged in a 50Ti Spinco rotor at 105000 × *g* for 30 min. The pellet after very painstaking removal of the supernatant and drying of the inner surface of the tube by filter paper, was taken up with 50 µl of the buffer used during sonication. The resuspension of the pellet was effected by inserting a small Teflon pestle into the centrifuge tube to insure homogeneous distribution of the particles in the suspension. 20 µl of the samples so prepared were then assayed spectrophotometrically under the conditions described in Fig. 2.

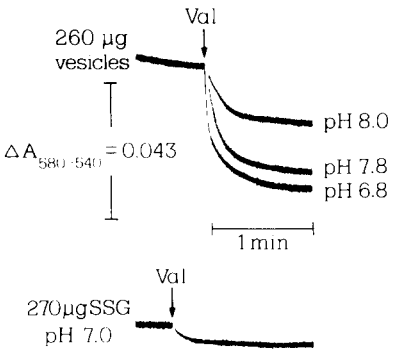

Fig. 6. Dependence of valinomycin-induced sulfobromophthalein uptake by rat liver plasma membrane vesicles and human erythrocyte ghosts on the pH of the internal medium. Experimental conditions were similar to those described in the previous figure with the exception that the buffer in the sonication medium was 100 mM sodium phosphate at the pH indicated in the panel. The lower curve refers to an experiment in which resealed erythrocyte ghosts (SSG) were treated in parallel exactly as rat liver plasma membrane vesicles, at pH 7.0. All traces refer to experiments in which the level of the optical signal after the addition either of the vesicles or of the ghosts was reestablished first by addition of NaOH to adjust the pH exactly to 8.1 and then by small additions of sulfobromophthalein as in Fig. 2.

duced, electrogenic transport of sulfobromophthalein. It was important to show its dependence on the pH inside the vesicles. The experiment reported in Fig. 6 shows the behavior of vesicles pre-equilibrated by sonication at different pH values. Clearly the initial rate of optical change and the extent of it is greatly influenced, as expected, passing from pH 8.0 to pH 7.8. On the other hand, below pH 7.8 only marginal changes are seen both in terms of initial rate and total extent. For comparison, in the same figure a parallel experiment is also reported in which the resealed erythrocyte ghosts are preequilibrated under the same experimental conditions as hepatocyte plasma-membrane vesicles at pH 7.0. The results obtained represent a neat confirmation of the experiment reported in Fig. 3. On the other hand, this experiment provides further evidence that erythrocyte ghosts are unable to carry out any efficient sulfobromophthalein transport; this is most probably related to the absence of bilitranslocase in that plasma membrane.

The data so far presented suggest the idea that the initial rate of sulfobromophthalein disappearance evoked by the addition of valinomycin could be taken as a quantitative measure of bilitranslocase in the vesicles. Prerequisite for this application was, of course, that the system was operating at saturation, i.e., was not rate-limited by the availability of the substrate. The experiment reported in Table I clearly indicates that already at a concentration as low as 0.35 µM, the rate measured is maximal.

Fig. 7 shows the dependence of the initial rate on the amount of the vesicle protein added. The measure is linear in the range of 0–300 µg per sample. On these grounds we decided to consider this rate of transport as if it were a classical enzyme activity.

Table II collates some of the data obtained during a purification scheme of the plasma-membrane vesicles. As a reference, the activities of a crude preparation (second pellet in table) are also

TABLE I

DEPENDENCE OF INITIAL RATE OF ABSORBANCE CHANGE INDUCED BY VALINOMYCIN ON THE FREE SULFOBROMOPHTHALEIN CONCENTRATION OUTSIDE VESICLES

Experimental conditions: variable concentrations of sulfobromophthalein in 0.1 M potassium phosphate (pH 8.1) buffer were added with 25 μl vesicle suspension at 10 mg protein/ml. The outside sulfobromophthalein concentration was readjusted by addition of variable amounts of a 6 mM sulfobromophthalein solution to the starting optical level. At this moment the slow rate of entry is greatly accelerated by the addition of 20 μg valinomycin dissolved in absolute ethanol (1 mg/ml) and the initial rate is recorded. Sigma ZWS-11 dual-wavelength spectrophotometer operated at room temperature at the wavelength pair 580/540 nm.

Initial [sulfobromophthalein] in medium (μM)	Initial rate ($\Delta A_{580-540}$/min)
0.35	0.110
0.70	0.100
1.40	0.120
3.00	0.110
6.00	0.135
12.00	0.110
22.00	0.110
64.00	0.110

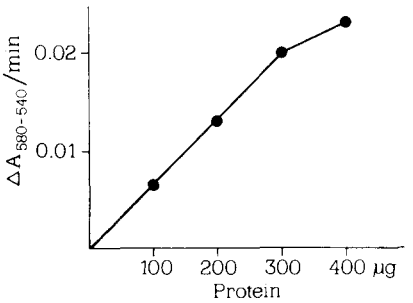

Fig. 7. Dependence of the initial rate of valinomycin-induced sulfobromophthalein uptake on the amount of vesicles added. Experimental conditions as in Fig. 2.

reported. We consider this comparison more reliable than that with the starting homogenate where most of the activities cannot be safely determined.

Measurement of the classical markers of endoplasmic reticulum, mitochondria and plasma membrane are reported. The data are consistent with published values of vesicular preparations in other laboratories [17]. The contamination of mitochondria or submitochondrial particles in our case seems to be even lower than that reported in literature [17]. The table summarizes, in addition, the data of valinomycin-supported sulfobromophthalein transport. This activity is concentrated approximately 17-fold in the purified vesicles as compared to the starting second pellet. Interestingly, $(Na^+ + K^+)$-ATPase activity is purified to the same extent. In view of the fact that the

TABLE II

MARKER ENZYMES AND SULFOBROMOPHTHALEIN TRANSPORT ACTIVITIES IN RAT-LIVER PLASMA-MEMBRANE VESICLES

The second pellet was obtained according to Van Amelsvoort et al. [8] and constitutes the imput-to-density-gradient. 5'-Nucleotidase, Mg^{2+}-ATPase, $(Na^+ + K^+)$-ATPase are expressed as μmol/mg protein per h; Cytochrome *c* reductase activities as μmol cytochrome *c* reduced/mg protein per min. BSP transport, sulfobromophthalein transport expressed as nmol sulfobromophthalein/mg protein per min. RSA, relative specific activity.

	Second pellet activity/mg	Vesicles		
		activity/mg	% recovery	RSA
5'-Nucleotidase	2.81 ± 0.52	11.51 ± 1.31	10.2 ± 2.8	4.1
NADH-cyt.*c* reductase (rotenone insensitive)	0.845 ± 0.210	2.5 ± 0.44	7.4 ± 2.3	3
Succinate-cyt.*c* reductase	0.104 ± 0.060	0.015 ± 0.007	0.33 ± 0.06	0.1
Mg^{2+}-ATPase	6.8 ± 0.63	12.3 ± 1.75	4.7 ± 2	1.8
$(Na^+ + K^+)$-ATPase	0.33 ± 0.16	5.99 ± 0.48	45 ± 18	18.1
BSP transport	24.8 ± 11.5	439 ± 170	39 ± 9	17.7
Protein (%)	100		2.5 ± 1	

plasma-membrane vesicle preparations we used are reported to originate in part from the endoplasmic reticulum [17] (as shown also by their enzyme composition), it was important to rule out the possibility that the sulfobromophthalein-transporting activity was a reflection of microsomal contamination. On the other hand, it is well known that plasma-membrane vesicles are consistently a contaminant of the microsomal fraction [18]. On these grounds it is expected that a microsomal fraction will demonstrate sulfobromophthalein transport when tested in our system. This was found to be the case. If the activity measured in this microsomal preparation is indeed the reflection of the existence of a population of plasma-membrane vesicles in the microsomal suspension, it is expected also that this activity may be concentrated by subfractionation of the particles by density gradient centrifugation at a density value adequate to separate the plasma-membrane. Table III collates the results of this experimentation and shows that the sulfobromophthalein transport activity follows the distribution pattern of the plasma-membrane enzyme activities. On the contrary, the fraction containing the bulk of sulfobromophthalein transport activity does not show any appreciable increase in NADH- and NADPH-cytochrome *c* reductase activity, marker enzymes of endoplasmic reticulum.

At this stage we were not in the position, however, to decide which sector of the plasma membrane contained specifically the translocator. The

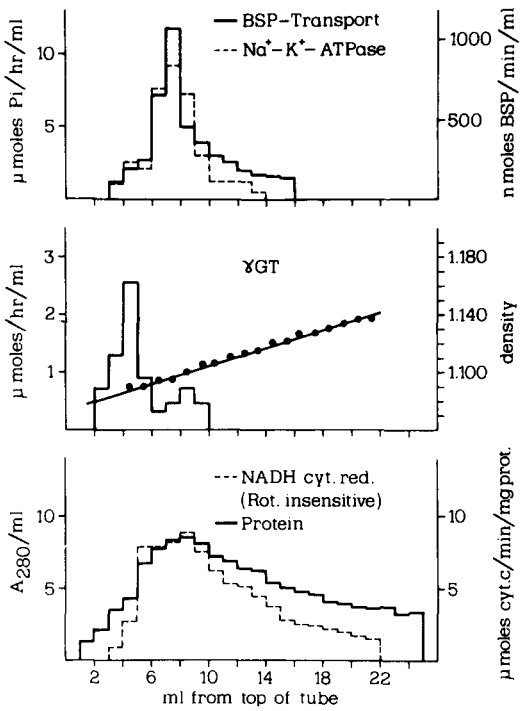

Fig. 8. Subfractionation of a plasma membrane vesicles preparation by isopicnic centrifugation in a linear sucrose gradient. 13 mg plasma membrane vesicles suspension in 1 ml stored in liquid nitrogen were rapidly thawed at 37°C and loaded on a pre-formed continuous sucrose gradient. The system was centrifuged for 4 h with an SW-28 Spinco rotor at 64000 × *g*. The gradient was fractionated and the fractions assayed as indicated in the text.

TABLE III

DISTRIBUTION OF PROTEIN, SOME MARKER ENZYMES AND SULFOBROMOPHTHALEIN TRANSPORT ACTIVITY IN RAT LIVER MICROSOMES AND SUBFRACTIONS DERIVED THEREFROM BY DISCONTINUOUS DENSITY GRADIENT CENTRIFUGATION

Specific activity for cytochrome *c* reductases is defined as μmol cytochrome reduced/min per mg protein; for ATPase as μmol p_i/h per mg protein. BSP transport, sulfobromophthalein transport expressed as nmol/min per mg protein. RSA, relative specific activity: activity of the fraction divided by the activity of starting microsomes.

	Microsomes	Heavy fraction	Light fraction	
Total protein (mg):	120	54	27	
	spec.act.	spec.act.	spec.act.	RSA
NADH-cyt.*c* reductase	1.45	1.17	1.77	1.2
NADPH-cyt.*c* reductase	0.055	0.049	0.071	1.3
$(Na^+ + K^+)$-ATPase	0.5	0.24	1.5	3.0
BSP transport	125	59	434	3.4

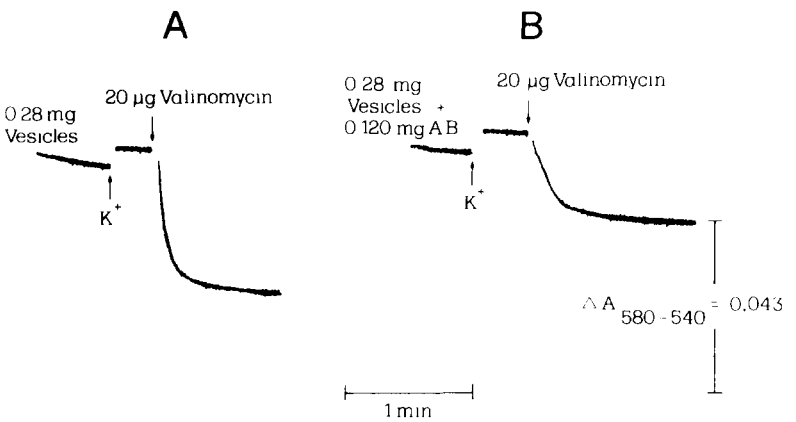

Fig. 9. Effect of Fab fragments of antibilitranslocase antibodies on the rate of sulfobromophthalein uptake by plasma membrane vesicles from rat liver. Trace A, 0.28 mg vesicles in 50 µl were diluted 1 : 1 with saline solution and kept in ice for 30 min; trace B, 0.28 mg vesicles in 50 µl were diluted 1 : 1 with saline solution containing 0.12 mg Fab anti-bilitranslocase antibodies, purified by affinity chromatography (AB in the panel) and kept in ice for 30 min. In both cases the experiment was started immediately at the end of the preincubation time.

possibility could not be excluded that the canalicular pole of the cell might also contribute to the activity measured. To give an answer to this question we carried out the experiment, the results of which are reported in Fig. 8. A typical preparation of plasma membrane vesicles was subjected to isopicnic centrifugation in a sucrose density gradient, and, after fractionation, analyzed for marker enzymes and sulfobromophthalein transport. It appears that $(Na^+ + K^+)$-ATPase, the marker enzyme for the sinusoidal pole, is copurified with the sulfobromophthalein-transporting activity, whereas γ-glutamyltranspeptidase, the marker of the canalicular portion of the cell, is equilibrated at a lower density value.

To conclude unequivocally that the activity measured involved the operation of bilitranslocase we have carried out the experiment reported in Fig. 9, in which the vesicles were preincubated (trace B) in the presence of affinity-chromatography purified Fab fragments of anti-bilitranslocase antibodies [19]. Clearly this treatment results in more than 50% inhibition of the rate of sulfobromophthalein movement as well as in a substantial depression of the extent of the optical change. Not shown in the figure, suitable blanks treated with Fab fragments derived from nonspecific γ-globulins gave negative results.

Discussion

The idea of utilizing the pH-indicator properties of sulfobromophthalein to monitor movements of the dye by direct spectrophotometry is not new. We have already used this approach to follow the phenomenon in a reconstituted system with liposomes and purified bilitranslocase [7]. The advantage of applying this system to biological membranes lies in the insight obtained as to the localization of this membrane function on and within the hepatocyte. A prerequisite to this aim, however, is the identification of a function whose parameters can be strictly controlled and whose activity could be safely measured and treated as a membrane marker. The data presented indicate that the initial rate of electrogenic movement of sulfobromophthalein evoked by addition of valinomycin in the presence of K^+ fulfils the requirements quoted, i.e., (1) it is linearly related to the amount of vesicles added; (2) below 7.4 is virtually independent of the pH inside the vesicles; (3) the rate measured is not influenced by sulfobromophthalein in the medium over a large range of concentrations. Cellular fractions prepared in a medium at pH 7.4 or lower, and in a system buffered precisely at pH 8.1, could be assayed for this transport activity. This allowed a quantitative measurement of the translocator in different portions of the cell. On this basis it is possible to conclude that bilitranslocase is localized exclusively in that sector of the plasma membrane which contains $(Na^+ + K^+)$-ATPase, the enzyme marker of the sinusoidal pole of the cell. If the L-γ-glutamyltranspeptidase can be safely taken as an exclusive marker of the canalicular membrane [20], its distribution will indicate the existence of a population of vesicles, essentially canalicular, whose density is definitely lower than that corre-

sponding to the peak of $(Na^+ + K^+)$-ATPase and sulfobromophthalein transport. The absence of a bimodal distribution of the latter would speak in favor of the interpretation that the excretion process into the bile proceeds via a different transport system. Were this the case, then it would not be surprising to learn that more than one protein with organic anion binding ability has been isolated from liver preparations [21–24]. The finding that bilitranslocase is localized at the sinusoidal plasma membrane level, on the other hand, confirms previous data obtained by immunofluorescent techniques [16], as well as in vivo experiments [19], in which anti-bilitranslocase antibodies, once injected into the portal system, could specifically block the normal uptake of bilirubin. The data reported in this paper, in addition, extend this finding, suggesting the idea that a different translocator could be operating at the biliary pole. A series of additional considerations may arise from this experimentation. The fact that, for instance, protonation of the dye inside the vesicles (even in the presence of a very small ΔpH) may efficiently function as a trapping device for sulfobromophthalein, could reflect a physiological event occurring in vivo. This mechanism would operate either in series or in parallel with glutathione conjugation and binding to cytosolic proteins in maintaining a concentration gradient of the transported species across the plasma membrane. A question which remains still open concerns the possibility that the first drop seen in the experiment reported in Fig. 2, induced by the addition of the vesicles, is due to the operation of other transport systems in addition to bilitranslocase. The possibility cannot be excluded that the vesicle preparations may catalyze also nonelectrogenic sulfobromophthalein movements.

A point which deserves some comment concerns the recent findings that the electrogenic movement of sulfobromophthalein evoked in plasma membrane vesicles by valinomycin and potassium is efficiently inhibited by nicotinate [25] and rifamycin-SV [26]. These data, in combination with the observation that the two substances bind bilitranslocase in vitro, have been considered a good indication that this carrier is involved in vivo in some effects brought about by the administration of the two drugs both in normal subjects and in patients with Gilbert's syndrome.

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