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The kinetics of sulfobromophthalein uptake by rat liver sinusoidal vesicles

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The kinetics of bromo[^{35}S]sulfophthalein (^{35}S-BSP) binding by and uptake across the hepatocyte sinusoidal membrane were investigated using isolated rat liver sinusoidal membrane vesicles containing K^+ as the principal internal inorganic cation. Uptake of ^{35}S-BSP into vesicles was found to be temperature dependent, with maximum uptake between 35 and 40 °C; only binding occurred at or below 15 °C. Uptake at 37 °C was saturable and resolvable by Eadee-Hofstee analysis into two components: one with high affinity (K_m = 53.1 μM) but low capacity, and the second of low affinity (K_m = 1150 μM) but high capacity. By pre- or post-incubation, respectively, with unlabelled BSP, *trans*-stimulation and counter transport of ^{35}S-BSP could also be demonstrated in these vesicles. Uptake was inhibited competitively using 5 μM Rose bengal and 10 μM indocyanine green, and non-competitively using 10 μM DIDS. Taurocholate did not inhibit uptake, and actually enhanced transport at concentrations ≥ 250 μM. Imposition of inwardly directed inorganic ion gradients resulted in the enhancement of ^{35}S-BSP transport when chloride ions were part of this gradient, irrespective of the cation employed whereas there was no apparent cation effect. However, substitution of 10 mM Na^+ for 10 mM K^+ as the internal cation resulted in a significant increase in uptake in the presence of external K^+ as compared to Na^+ gradients. This effect was not observed when 10 mM $Tris^+$ was employed as the internal cation. The kinetics of ^{35}S-BSP uptake by isolated sinusoidal membrane vesicles are indicative of facilitated transport. While the observed inorganic ion effects suggest a possible electrogenic component, the driving forces for hepatic BSP uptake remain uncertain. Isolated sinusoidal membrane vesicles provide a useful technique for studying hepatic uptake processes independent of circulatory or subsequent cellular phenomena.

Introduction

Sulfobromophthalein (BSP) and bilirubin, oleic acid, and taurocholic acid are representatives of three separate classes of organic anions that are efficiently extracted by the liver, in spite of their high affinity for plasma albumin [1–3]. Despite considerable differences in their physicochemical properties and biological roles, there are still many similarities in their hepatocellular uptake processes, which are compatible with carrier-mediated transport in each instance [4–10]. All of these compounds manifest the so-called 'albumin receptor effect' (i.e. accelerated dissociation from albumin at the plasma membrane interface [11–14]) and both specific membrane and cytosolic binding

Abbreviations: BSP, bromosulfophthalein; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid.

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proteins have been isolated for each class [15–24]. Uptake of taurocholate and oleate have both been linked to Na^+ transport and ($Na^+ + K^+$)-ATPase activity [4,6,10,25], but the driving forces for BSP and bilirubin uptake are unknown.

The transport of BSP/bilirubin from plasma to bile is complex, involving uptake at the hepatocyte sinusoidal membrane, interaction with cytosolic proteins and subsequent conjugation, leading to excretion through the canalicular region. Earlier studies of the uptake process have utilized intact animals [7], the isolated perfused liver [26–29] or isolated hepatocytes [30–33]. Each of these systems unfortunately provides only limited information about uptake per se due to difficulties encountered in distinguishing it from circulatory and intracellular effects. The aim of this study was therefore to examine the kinetics of ^{35}S-BSP uptake into isolated rat liver sinusoidal membrane vesicles, a much simpler system than whole cells, in which the separate contributions of binding and transport can be estimated more readily.

This work was presented in poster form at the 35th and 36th meetings of the AASLD in Chicago, November, 1984 and November, 1985.

Materials and Methods

Materials

[^{35}S]Sulfobromophthalein (^{35}S-BSP) (specific activity 207 mCi/mmol) was custom-synthesized by Amersham (Arlington Heights, IL); indocyanine green (Cardio-Green®) was purchased from Hynson Wescott and Dunning (Baltimore, MD) and sodium taurocholate from Cal Biochem (San Diego, CA). Rose bengal, ouabain, choline chloride, Ficoll and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) were obtained from Sigma (St. Louis, MO). All other chemicals were of reagent grade and were purchased either from Fisher (Pittsburgh, PA) or Sigma.

Preparation of rat liver sinusoidal membrane vesicles

Sinusoidal membrane vesicles were prepared from the livers of non-fasted male Sprague-Dawley rats (200–250 g) by the method of Inoue et al. [34]. Following homogenization in a loose fitting Dounce homogenizer at 0°C to give a 25% (w/v) suspension in ice-cold 0.25 M sucrose, containing 10 mM Hepes-KOH buffer (pH 7.4), 10 mM $MgCl_2$ and 0.2 mM $CaCl_2$, the homogenate was filtered through cheesecloth and diluted 3-fold with the same medium. EDTA was added to give a final concentration of 1 mM and the suspension centrifuged for 10 min at $1500 \times g$. The supernatant from this step was then centrifuged at $7600 \times g$ for 10 min, followed by $23\,000 \times g$ for 30 min. The resulting pellet from this last centrifugation step was resuspended in the medium containing EDTA and layered onto a two step density gradient, consisting of 20% (w/v) sucrose over a 23.5% sucrose solution containing 4% Ficoll; all buffered with 10 mM Hepes-Tris buffer (pH 7.4) containing 1 mM EDTA. The gradient was centrifuged at $100\,000 \times g$ for 2 h; the white band at the 20–23.5% interface was then collected, diluted in 4 volumes of medium without EDTA and centrifuged at $65\,000 \times g$ for 40 min. The pellet was resuspended in medium and washed twice more by centrifugation. The final pellet was resuspended in 1–2 ml of the homogenization medium using a syringe with a 25 g needle, and stored at −70°C. All assays were performed on aliquots previously frozen to −70°C for storage and thawed by rapid warming at 40°C before placing on ice. Protein concentration was assayed utilizing Coomassie blue G250 [35], using bovine serum albumin (RIA grade) as the standard.

Electron microscopy

Vesicles were examined for orientation by freeze-fracture [36,37]. Samples were fixed in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and cryoprotected with 25% (v/v, final concentration) glycerol at 4°C for 1 h before being frozen in Freon 22, cooled with liquid nitrogen. Specimens were then fractured in a Balzers freeze etching unit at −100°C. Following fracturing, the specimens were shaded with platinum at a 45° angle before being coated with a 50 Å thickness of carbon. After cleansing with hypochlorite solution for 30 min and subsequent washing with distilled water, the specimens were examined by transmission electron microscopy under a JEM CX electron microscope at 60 kV.

Immunological assays

Vesicles were also examined for the presence of

the membrane-associated BSP-binding protein [20] using antisera produced in rabbits by our laboratory by immunofluorescence, Ouchterlony and immunoelectronmicroscopy.

Marker enzyme assays

Alkaline phosphatase activity, a marker for canalicular membranes, was assayed by the method of Walter and Schutt [38]. The mitochondrial marker cytochrome *c* reductase was measured by the method of Omura and Takesue [39], as modified by Duffy et al. [40]. Glucose-6-phosphatase activity, a microsomal marker enzyme, was determined by the method of Baginski et al. [41]. The plasma membrane marker $(Na^+ + K^+)$-ATPase was measured by the method of Scharschmidt et al. [42], and 5′-nucleotidase activity by the method of Gilman [43], using a commercial kit (Boehringer Mannheim GmbH).

Transport assay

Binding and uptake of ^{35}S-BSP by the membrane vesicles was measured by the rapid filtration technique of Lucke et al. [44], using a Millipore vacuum filtration assembly (Millipore, Bedford, MA) and nitrocellulose membranes of pore size 0.45 μm (HAWP, Millipore). All filters were wetted and presaturated with 100 μM unlabelled BSP in running buffer (see below) to minimize non specific filter binding of radioactivity. All incubations were carried out in 0.25 M sucrose, containing 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 10 mM Hepes-Tris (pH 7.4), and varying concentrations of ^{35}S-BSP. Other additions are indicated in the individual experiments, but usually included an inwardly directed salt gradient. The reaction was started by the addition of 25 μl of membrane vesicles (previously thawed rapidly at 40°C and then placed on ice for a minimum of 15 min), containing 125–200 μg protein, to 225 μl of incubation medium, maintained at 37°C, unless otherwise specified. At preset time intervals 20-μl aliquots were removed and diluted to 1 ml with ice-cold stop solution containing 0.25 M sucrose, 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 10 mM Hepes-Tris (pH 7.4), and 100 mM NaCl. The diluted samples were immediately filtered and then washed three times with 3 ml of ice-cold stop solution. Filters were transferred to scintillation vials and dissolved in 1 ml of methoxyethanol. Subsequently 10 ml of scintillant (ACS II, Amersham) was added, the vials chilled to 4°C and counted in a Tracor 6852 mk III liquid scintillation analyzer. Non specific radioactivity binding to filters without membranes was routinely determined, and was less than 0.5% of the total radioactivity applied and less than 10% of the initial uptake values. Preliminary studies established that the filtered solution invariably contained less than 3% of the total membrane protein applied to the filter.

Throughout this study, uptake is reported as nmoles BSP/mg membrane protein per 15 s, after preliminary studies had demonstrated that cumulative uptake curves were consistently linear between 5 and 20 s and usually longer. Use of a 15 s measurement of uptake in vesicle transport studies is also consistent with prior studies employing similar methodology [34,40,44].

Results

1. Characterization of vesicles

(a) Enzyme markers. Both 5′-nucleotidase and $(Na^+ + K^+)$-ATPase specific activities (markers of sinusoidal plasma membranes) were enhanced approximately 14-times in the vesicular fraction, as compared to the liver homogenate (Table I). Alkaline phosphatase activity was only increased 3-fold, indicating a small amount of canalicular material in the pellet, whereas cytochrome *c* reductase was reduced 2-fold, suggesting minimal mitochondrial contamination. Similarly, glucose-

TABLE I

MARKER ENZYME ACTIVITIES OF THE PURIFIED SINUSOIDAL MEMBRANE VESICLES (SMV)

The specific activity is given as μmol substrate per mg protein per h. Values represent the mean ± S.D. of values obtained from five separate vesicle preparations.

	Specific activity		Enrichment
	SMV	Homogenate	
$(Na^+ + K^+)$-ATPase	9.7 ± 0.9	0.7 ± 0.1	(14.5)
5′-Nucleotidase	30.9 ± 4.3	2.2 ± 1.1	(14.0)
Glucose-6-phosphatase	2.0 ± 0.6	4.3 ± 1.3	(0.45)
Alkaline phosphatase	1.1 ± 0.1	0.3 ± 0.1	(3.1)
Cytochrome *c* reductase	16.2 ± 6.4	38.1 ± 7.1	(0.43)

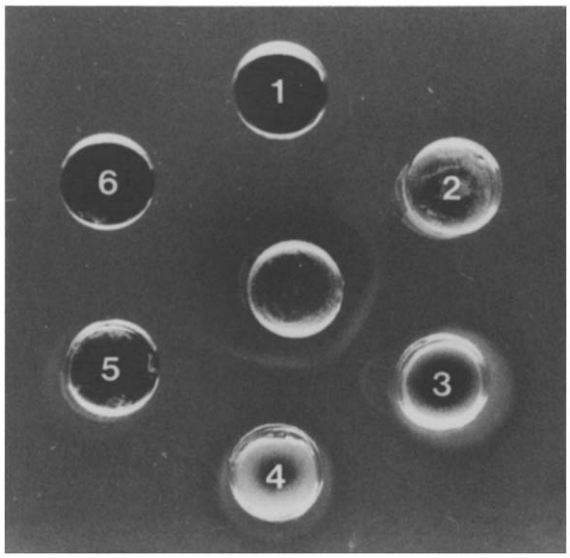

Fig. 1. Ouchterlony analysis of sinusoidal membrane vesicles for sulfobromophthalein binding protein. Wells were punched in a 1% agarose gel, prepared in phosphate buffered saline, filled with 10 μl of appropriate test solutions and allowed to diffuse overnight at room temperature. Membrane preparations were solubilized in 1% sodium deoxycholate. Well 1 contained rat serum, well 2 – sinusoidal membrane vesicles (final protein concentration 2.8 mg/ml), well 3 – sinusoidal membrane vesicles (protein concentration 3.4 mg/ml), well 4 – liver sinusoidal plasma membranes (4.7 mg protein/ml), well 5 – a liver canalicular membrane enriched fraction (5.6 mg protein/ml) and well 6 – red blood cell ghosts membrane protein (4.4 mg/ml). The center well contained 10 μl of monospecific antisera to the rat bromosulfophthalein binding protein produced in rabbits in our laboratory [20].

6-phosphatase activity was reduced 2-fold, indicating little microsomal material in the preparations (Table I). The 5′-nucleotidase activity in the vesicle preparations was enriched, suggesting some canalicular contamination. However, since the canalicular region accounts for less than 10% of the plasma membrane surface, we believe total canalicular contamination to be too small to modify the experimental results appreciably.

(b) Electron microscopy. Electron microscopic examination of sinusoidal membrane vesicle preparations demonstrated predominantly a collection of vesicles heterogeneous with respect to size, but largely free of particulate contamination with microsomal structures or mitochondria, or of identifiable canalicular structures.

Estimates of vesicle orientation of the fracture face [36,37] were made on randomly selected electron photomicrographs of numerous vesicle preparations independently by at least two observers. Inter-observer variability was < 10%. On average, 63% of the sinusoidal membrane vesicles were oriented right side in and only 36% inside out in reasonable agreement with earlier studies [34,40].

(c) Immunochemical determinations. Plasma membrane BSP binding protein [20] was demonstrated in the vesicles by immunofluorescence, immunoprecipitation (Fig. 1) and immunoelectron microscopy (Fig. 2) with specific antisera produced in our laboratory.

(d) Uptake studies. Preliminary studies showed

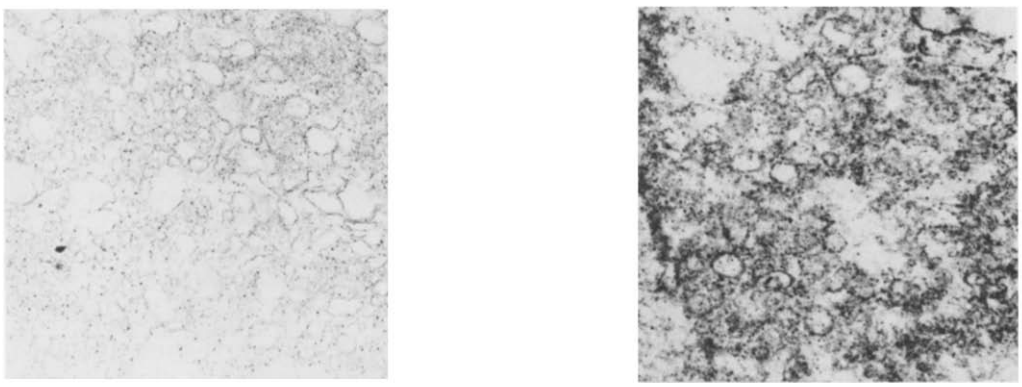

Fig. 2. Immunoelectron micrographs of representative vesicle preparations incubated with pre-immune rabbit serum (left) and rabbit anti-rat LPM BSP/BR binding protein (right) [20], followed in both cases by incubation with immunoperoxidase conjugated goat anti-rabbit $F(ab')_2$ fragment of IgG (Cappel-Worthington, Malvern, PA). Original magnification × 20750.

that vesicles, prepared in this manner, rapidly transported taurocholate, oleate and BSP. In confirmation of previous reports [10,34], the initial uptake rates at 15 s (V_i) of 10 μM taurocholate and 10 μM oleate were observed to be sodium dependent in the presence of an inwardly directed 100 mM nitrate gradient. V_i was 50% less in the presence of potassium nitrate than in the presence of sodium nitrate (Table II). No difference was observed in the V_i for 10 μM BSP when K^+ was substituted for Na^+ under the same conditions.

2. BSP uptake: Transport vs. binding

The initial association of ^{35}S-BSP with the vesicles, in the presence of an inwardly directed 100 mM $NaNO_3$ gradient at 37°C, is rapid (Fig. 3), achieving a maximum by 2 min, with only a very minor 'overshoot' under these conditions. Equilibrium is reached by 10 min; there being no significant change in vesicle-associated radioactivity between 10 and 30 min. That there is actual transport into the vesicle, as well as membrane binding, can be deduced in several ways:

(a) Temperature-dependence studies. Fig. 3 demonstrates the effect of temperature on vesicle-associated ^{35}S-BSP. There was no difference in the initial association rate (15 s) of BSP between 0°C and 15°C, but association increased with increasing temperature thereafter to a maximum between 37°C and 40°C. At 45°C, and beyond, initial vesicle-associated ^{35}S-BSP diminished (data not shown). Although ligand binding may be partially temperature dependent under some circumstances [45], we tentatively construed the differences in initial vesicle-associated radioactivity observed at 37°C and that seen at 0–15°C as indicative of transport. On this basis, the differences in membrane associated radioactivity at 15 s, (2.24 ± 0.25 nmol BSP/mg vesicle protein at 37°C vs. 1.10 ± 0.22 nmol/mg protein at 0°C) and at equilibrium (10 min) (4.31 ± 0.17 nmol/mg at 37°C vs. 2.38 ± 0.19 nmol/mg at 0°C) suggest that between 49 and 55% of the measured vesicle associated radioactivity at 37°C is accounted for by membrane binding, the remainder representing ligand internalized within the vesicles.

(b) Hypotonic lysis. Replacing the normal stop solution with ice-cold deionized water to lyse the vesicles reduced both the initial and equilibrium vesicle-associated radioactivity values at 37°C to values similar to those observed at 0°C with normal stop solution (Fig. 4). No significant differences were observed in membrane-associated radioactivity as a result of increasing the time the

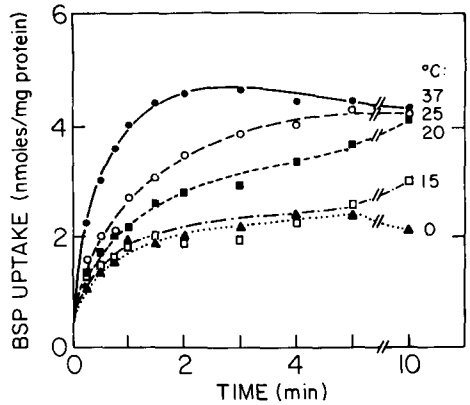

Fig. 3. Temperature dependence of ^{35}S-BSP uptake and binding. All experiments were performed in the presence of a 100 mM inwardly directed $NaNO_3$ gradient using 10 μM ^{35}S-BSP. Each point represents the mean of three experiments on a single vesicle preparation (experimental variation was < 5% for each for each value). (▲) 0°C, 4°C, 10°C; (■) 15°C; (□) 20°C; (○) 25°C, (●) 37°C, 40°C.

TABLE II

CHARACTERIZATION OF THE INITIAL UPTAKE AND BINDING (AT 15 s) OF ORGANIC ANIONS BY RAT LIVER SINUSOIDAL MEMBRANE VESICLES

Vesicles were incubated at 37°C in standard incubation buffer in the presence of either a 100 mM $NaNO_3$ or a 100 mM KNO_3 inwardly directed gradient and the appropriate organic anion at a final concentration of 10 μM. The reactions were terminated after 15 s incubation by dilution into ice-cold stop solution and rapid vacuum filtration. Each value represents the mean (nmol/mg protein) ± S.D. of five observations. The vesicles used were from a single preparation. n.s., not significant.

	Inwardly directed 100 mM gradient	
	Na^+	K^+
[^{14}C]Taurocholate	0.952 ± 0.061	0.540 ± 0.060 *
[^{3}H]Oleate	0.248 ± 0.041	0.125 ± 0.025 *
[^{35}S]Sulfobromophthalein	2.00 ± 0.16	2.17 ± 0.18 **

* $P < 0.001$, Na^+ vs. K^+.
** P = n.s., Na^+ vs. K^+.

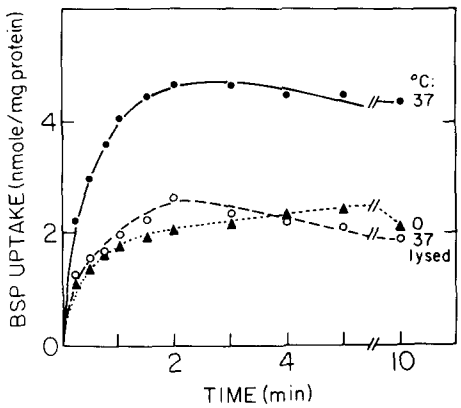

Fig. 4. Uptake and binding of 10 μM ^{35}S-BSP by rat liver sinusoidal membrane vesicles, effect of hypotonic lysis. Vesicles were incubated with BSP in the presence of an inwardly directed 100 mM $NaNO_3$ gradient at either 37 °C or 0 °C. The reaction was terminated at each time interval using either ice-cold stop solution or ice-cold distilled water, followed by rapid vacuum filtration. Each point represents the mean of three determinations on a single vesicle preparation (variability < 7%). (●) 37 °C, normal stop solution; (○) 37 °C hypotonic lysis; (▲) 0 °C, normal stop and hypotonic lysis.

vesicles were left in ice-cold deionized water for up to 30 min, suggesting that this latter value in fact represents membrane bound ^{35}S-BSP.

(c) Osmolarity studies. Fig. 5 shows the effect of increasing osmolarity in the incubation medium on vesicle-associated radioactivity following 30 min incubation with 10 μM ^{35}S-BSP. Vesicle associated ^{35}S-BSP diminished with increasing osmolarity, indicating that BSP was being transported into an osmotically active intravesicular space. Extrapolation to infinite osmolarity (1/osm = 0), theoretically representing zero intravesicular space, indicated that approx. 49% of the BSP uptake could be accounted for by membrane binding. Hence, three independent methods (temperature, lysis, osmotic variation) suggest that approx. 50% of vesicle-associated radioactivity under standard incubation conditions has been transported into the vesicles. This justifies our subsequent use of the term 'uptake' to describe such radioactivity in the studies which follow.

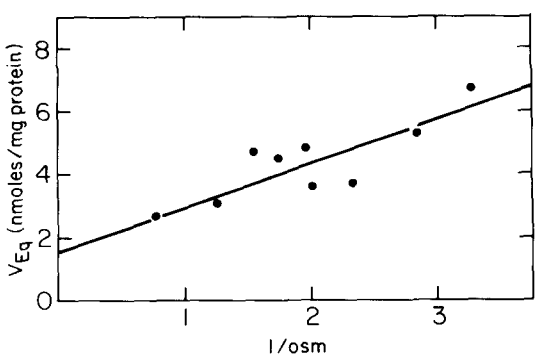

Fig. 5. Effect of osmolarity on BSP uptake. 10 μM ^{35}S-BSP was incubated with the vesicles in the presence of an inwardly directed 100 mM $NaNO_3$ gradient for 30 min before termination of the reaction by dilution into ice-cold stop solution and rapid vacuum filtration. Osmolarity (expressed as reciprocal osM) was altered by the addition of sucrose to the incubation medium. Each point represents the mean of five observations from at least three vesicle preparations (variability < 8%, r = 0.8173, P < 0.01).

3. Concentration dependence of BSP uptake

Fig. 6 shows the concentration dependence of both the initial and equilibrium uptake values in the presence of an inwardly directed 100 mM $NaNO_3$ gradient at 37°C. This study demonstrates saturation of the uptake mechanism above 1 mM. Eadie-Hofstee analysis for V_i (Fig. 7) resolves these data into two separate components: a

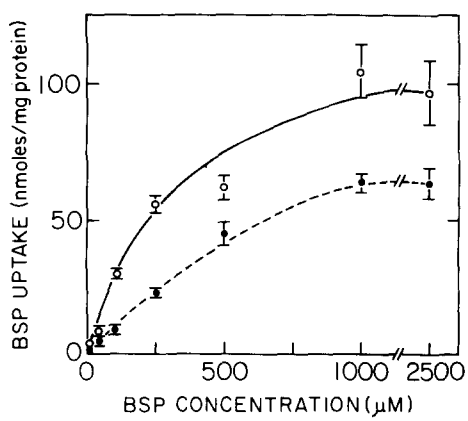

Fig. 6. Concentration dependence of ^{35}S-BSP uptake by rat liver sinusoidal membrane vesicles. All experiments were carried out at 37 °C in the presence of an inwardly directed 100 mM $NaNO_3$ gradient and the reaction terminated at either 15 s (●) or 30 min (○). Each point represents the mean ± S.D. of five determinations from two vesicle preparations. Several additional data points obtained at concentrations < 50 μM have been omitted from this figure for the sake of clarity. These data were used, however, in the Eadie-Hofstee analysis which appears in Fig. 7.

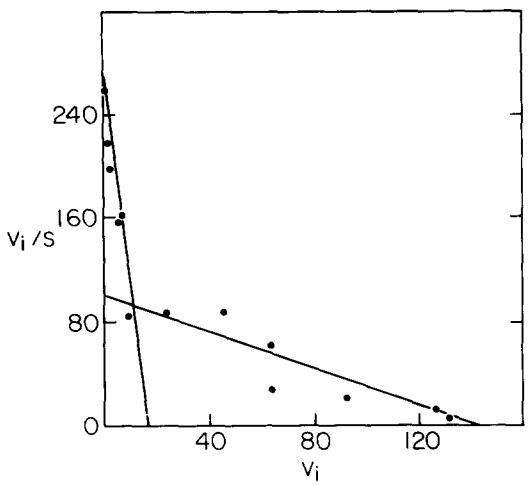

Fig. 7. Eadie-Hofstee analysis of BSP uptake. V_i, initial uptake at 15 s (nmol/mg per 15 s). S, BSP concentration (mM). The results shown here are obtained by subtraction of the hypotonic lysis values, representing membrane binding, from those obtained with normal stop solution, representing binding and uptake.

high-affinity, low-capacity one with an apparent K_m of 53.1 μM and a V_{max} of 13.5 nmol/mg per 15 s, and a second, low-affinity, high-capacity component with K_m of 1150 μM and a V_{max} of 136 nmol/mg per 15 s. It is possible that this second K_m reflects a diffusion component of BSP flux into these vesicles.

4. Transstimulation and countertransport

Preloading the vesicles by prior incubation at 37°C for 30 min with 50 μM unlabelled BSP stimulated the initial uptake of ^{35}S-BSP by 96% (Fig. 8), compared to control membrane vesicles not pre-loaded. Preincubation of the vesicles for 30 min in the presence of 50 μM ^{35}S-BSP prior to diluting them in incubation buffer in the absence of additional BSP resulted in a transient efflux of approx. 20% of the membrane associated radioactivity within the first minute before equilibrium values were regained (Fig. 9). The addition of 0.1% bovine serum albumin to the media under these conditions results in an apparent BSP efflux of more than 75% at equilibrium. Lysis in the presence of albumin shows that approx. 50% of the radioactivity remaining is tightly bound to the plasma membrane.

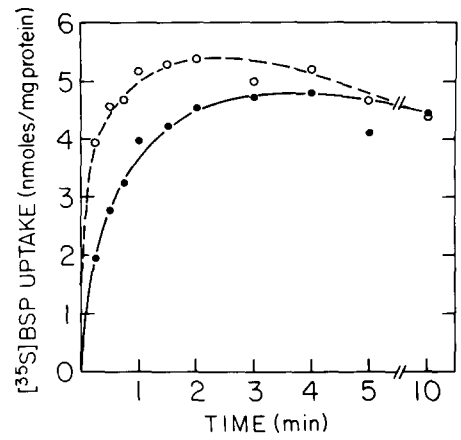

Fig. 8. Effect of preloading on BSP uptake by sinusoidal membrane vesicles. Vesicles were pre-incubated with 50 μM unlabelled BSP for 30 min at 37°C, before addition (dilution 1:10), to the incubation mixture containing 10 μM ^{35}S-BSP and 100 mM $NaNO_3$ (pH 7.4) (○). Controls (●) were pre-incubated with buffer alone prior to addition to an incubation mixture containing 15 μM BSP.

Incubation of the vesicles with 10 μM ^{35}S-BSP for 1 min, followed by the addition of a bolus of 1 mM (final concentration) of unlabelled BSP, resulted in the countertransport back into the medium, against the concentration gradient of 90% of the ^{35}S-BSP taken up into the intravesicular space (Fig. 10).

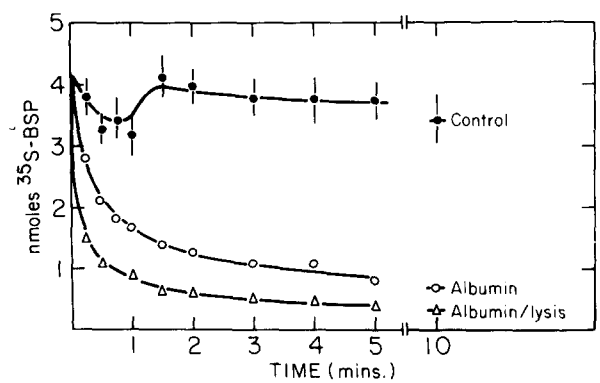

Fig. 9. Efflux of ^{35}S-BSP from sinusoidal membrane vesicles. Vesicles were pre-incubated with 50 μM ^{35}S-BSP for 30 min before addition (dilution 1:5) to the incubation medium, containing 100 mM $NaNO_3$ and 0.1% bovine serum albumin. The reaction was then terminated at preset time intervals using either conventional stop solution (○) or ice-cold distilled water (△). Control SMV (●) were added to incubation medium without albumin.

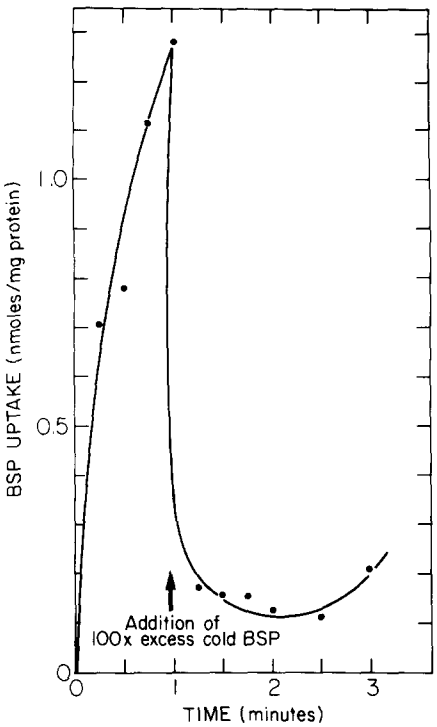

Fig. 10. ^{35}S-BSP uptake and counter-transport in rat liver sinusoidal membrane vesicles (SMV). SMV were incubated at 37°C for 1 min with 10 μM ^{35}S-BSP in an incubation medium containing 100 mM $NaNO_3$, before the addition of excess unlabelled BSP (final concentration 1 mM) to the mixture. They thus provide a measure of ^{35}S-BSP internalized with the vesicles.

5. Inhibition of uptake

The specificity of uptake was examined by the addition of various unlabelled compounds to the incubation medium, in the presence of an inwardly directed 100 mM $NaNO_3$ gradient and 10 μM ^{35}S-BSP. Both binding and uptake of BSP were significantly inhibited by 10 μM indocyanine green and by 5 μM Rose bengal (Table III). Taurocholate, however, had no significant effect at low concentrations and tended to enhance uptake at high concentrations (> 250 μM), possibly by its detergent action increasing vesicle permeability.

The addition of 10 μM DIDS to the normal incubation mixtures reduced the initial BSP uptake at 15 s by circa 53% and at equilibrium by 44%, without affecting membrane binding (Table IV). Increasing the DIDS concentration to 1 mM reduced initial ^{35}S-BSP uptake by 82% and initial binding by 51%. However, at equilibrium, membrane binding values had returned to control levels, while uptake was reduced by 75%.

6. Effect of ion gradients

The effect on uptake of substitution of other salts for $NaNO_3$ in the external medium was investigated (Table V). With conventionally prepared vesicles containing 10 mM K^+ as the internal cation, the respective initial and equilibrium ^{35}S-BSP uptake velocities observed in the presence of 100 mM external concentrations of the sodium salts of chloride, nitrate, thiocyanate, phosphate, bicarbonate, sulfate or iodide were unaltered if Na^+ was replaced by potassium, lithium, ammonium or choline as the external cation. The addition of 1 mM ouabain to an inwardly directed $NaNO_3$ or NaCl gradient similarly had no inhibitory effect. By contrast, irrespective of the external cation (Na^+, K^+, Li^+, NH_4^+, Choline$^+$), V_i in the

TABLE III

EFFECTS OF COMPETITIVE INHIBITORS ON BSP UPTAKE AND BINDING BY SINUSOIDAL MEMBRANE VESICLES

Values (nmol BSP/mg protein) represent the mean ± S.D. of five experiments on vesicles from two separate preparations.

	V_i (15 s)		V_{eq} (30 min)	
	uptake	binding	uptake	binding
Controls	1.178 ± 0.166	1.087 ± 0.239	1.738 ± 0.292	1.834 ± 0.459
5 μM Rose bengal	0.439 ± 0.202 *	0.483 ± 0.223 *	0.631 ± 0.184 *	1.457 ± 0.227
10 μM Indocyanine green	0.675 ± 0.181 *	0.636 ± 0.236 *	0.863 ± 0.291 *	1.280 ± 0.346
250 μM taurocholate	1.243 ± 0.227	1.348 ± 0.273	2.632 ± 0.408 *	2.968 ± 0.355 *

* $P < 0.001$.

TABLE IV

EFFECT OF DIDS ON ^{35}S-BSP UPTAKE AND BINDING BY SINUSOIDAL MEMBRANE VESICLES (SMV)

Values (nmol BSP/mg SMV protein) represent the mean ± S.D. of five experiments on vesicles from a single preparation. Incubations were carried out using 10 μM ^{35}S-BSP in the presence of an inwardly directed 100 mM $NaNO_3$ gradient (pH 7.4).

	V_i (15 s)		V_{eq} (30 min)	
	uptake	binding	uptake	binding
10 μM DIDS	0.436 ± 0.125 *	0.965 ± 0.114	1.086 ± 0.237 *	1.747 ± 0.250
1000 μM DIDS	0.163 ± 0.050 *	0.488 ± 0.045 *	0.488 ± 0.164 *	1.771 ± 0.110
Controls	0.924 ± 0.105	0.990 ± 0.061	1.947 ± 0.096	1.682 ± 0.101

* $P < 0.001$.

presence of chloride was significantly increased compared to that observed with any of the other inorganic anions tested (Table V). Thus, ^{35}S-BSP V_i increased from 1.99 ± 0.16 nmol/mg per 15 s to 2.63 ± 0.08 nmol/mg per 15 s ($P < 0.01$) with no alteration in membrane binding as measured by hypotonic lysis when 100 mM $NaNO_3$ was replaced by 100 mM NaCl in the external medium. This effect did not appear to be concentration dependent over an external salt concentration range of 20–250 mM.

Similarly, alteration of the vesicle internal ion composition affected uptake (Table VI) with predominantly right-side out vesicles (> 65%). Substitution of 10 mM Na^+ for the more physiologic K^+ as the internal cation resulted in cation-dependent uptake, i.e. there was a significant increase in BSP V_i in the presence of an inwardly directed 100 mM KNO_3 gradient compared to that with a 100 mM $NaNO_3$ gradient (V_i = 3.17 ±

TABLE V

EFFECT OF INWARDLY DIRECTED INORGANIC ION GRADIENTS ON ^{35}S-BSP UPTAKE AND BINDING BY RAT LIVER SINUSOIDAL MEMBRANE VESICLES

Vesicles were incubated with 10 μM ^{35}S-BSP in standard incubation medium containing the appropriate inorganic salt (100 mM final concentration). Results represent the mean ± S.D. of five experiments on at least three membrane preparations. For a given cation, BSP uptake is significantly greater in the presence of chloride ions than with any of the other anions studied.

	Salt		V_i (nmol/mg per 15 s)
Na^+	NO_3^-	(n = 18)	1.99 ± 0.16
	Cl^-	(n = 5)	2.63 ± 0.08 *
	SCN^-	(n = 2)	2.22 ± 0.12
	HCO_3^-	(n = 2)	2.21 ± 0.04
	I^-	(n = 2)	2.04 ± 0.14
	SO_4^{2-}	(n = 5)	2.22 ± 0.12
	P_i (pH 7.0)	(n = 2)	2.19 ± 0.08
Cl^-	Li^+	(n = 5)	2.77 ± 0.35
	Na^+	(n = 5)	2.63 ± 0.08
	K^+	(n = 5)	2.57 ± 0.16 **
NO_3^-	Na^+	(n = 18)	1.99 ± 0.16
	K^+	(n = 5)	2.17 ± 0.16

* $P < 0.001$, NaCl vs. $NaNO_3$.
** $P < 0.01$, KCl, vs. KNO_3.

TABLE VI

EFFECT OF ALTERATION OF THE INTERNAL CATION ON ^{35}S-BSP UPTAKE BY SINUSOIDAL MEMBRANE VESICLES.

Vesicles were incubated in the standard incubation medium containing either 100 mM $NaNO_3$ or 100 mM KNO_3. Vesicles were prepared using 10 mM K^+, 10 mM Na^+ or 10 mM $Tris^+$ as the internal cation. Values expressed as mean ± S.D. (nmol/mg protein per 15 s) for five experiments from three separate preparations.

Internal cation	External salt	Initial uptake and binding *
K^+	$NaNO_3$	1.99 ± 0.16
	KNO_3	2.17 ± 0.15
Na	$NaNO_3$	2.17 ± 0.22
	KNO_3	3.17 ± 0.18 *
$Tris^+$	$NaNO_3$	1.67 ± 0.47
	KNO_3	1.91 ± 0.23

* $P < 0.01$ for $NaNO_3$ vs. KNO_3.

TABLE VII

EFFECT OF A pH GRADIENT ON BSP UPTAKE BY RAT LIVER SINUSOIDAL MEMBRANE VESICLES

Values represent the mean ± S.D. of five experiments, in terms of nmol BSP/mg vesicle protein. All experiments were performed on a single vesicle preparation using 10 μM BSP and a 100 mM inwardly directed sodium nitrate gradient.

pH		V_i (15 s)	V_{eq} (30 min)
Internal	External		
7.4	7.4	1.985 ± 0.158	3.596 ± 0.278
6.0	7.4	2.383 ± 0.156 *	3.574 ± 0.111
8.5	7.4	2.242 ± 0.089 *	4.233 ± 0.255 *

* $P < 0.01$ vs. no pH gradient.

0.18 nmol/mg per 15 s for KNO_3 vs. 2.17 ± 0.22 nmol/mg per 15 s for $NaNO_3$, $P < 0.001$). Substitution of internal K^+ with 10 mM $Tris^+$, however, did not alter the initial uptake rates from those of control values ($V_i = 1.67 \pm 0.47$ nmol/mg per 15 s for $NaNO_3$ and 1.91 ± 0.23 nmol/mg per 15 s for KNO_3, P = NS). Imposition of a proton gradient (about 1 pH unit) on vesicles containing 10 mM K^+ in the presence of a 100 mM inwardly directed sodium nitrate gradient did, however, increase the initial uptake of ^{35}S-BSP slightly ($P < 0.01$), irrespective of which direction the pH gradient was imposed (Table VII).

Discussion

Considerable controversy has arisen over whether the mechanism of transport of BSP, bilirubin and related substances into the hepatocyte is passive or facilitated [30,46–48]. While much evidence suggests the latter, the driving forces for uptake remain virtually unknown. Other unresolved questions include whether or not the uptake pathway is shared with bile acids [32,49–52] and what influence albumin has on the transport of such compounds from the plasma into the cell [11,29,53–57]. However, most of these studies have been carried out either in isolated perfused livers, or in isolated hepatocytes, where it is possible that the metabolic state of the cells affected the kinetics. For example, Laperche and co-workers [33] have observed changes in BSP uptake between hepatocytes isolated from fasted animals and those from non-fasted controls. It is because of the numerous problems in interpretation encountered in these earlier investigations that we have undertaken this study, using vesicle techniques similar to those successfully employed in studies of bile acid uptake [34,40].

Evidence is presented here to support the hypothesis that BSP is taken up by sinusoidal membrane vesicles by a facilitated process, extending earlier work from our laboratory indicating that BSP uptake has the kinetics of carrier-mediated transport in the intact rat [7] and isolated hepatocyte suspensions [58] and by several groups demonstrating the existence of (a) specific membrane binding protein(s) for BSP [15,18–20]. Using rat liver sinusoidal membrane vesicles we have shown that BSP transport across the sinusoidal plasma membrane meets most of the criteria characteristic of facilitated transport: the initial uptake velocity is saturable, temperature dependent, selectively competitively inhibitable, capable of both *cis*-inhibition and *trans*-stimulation and operates against the concentration gradient.

In studies employing ^{35}S-BSP at various concentrations, the BSP uptake velocity into the vesicles was clearly saturable, and resolvable into two components in agreement with work using isolated hepatocytes [30,32]. Although the vesicles are only 63% 'right side out', transstimulation experiments suggest that the transport process works efficiently in both directions and therefore should not lower the uptake rates significantly. The finding of several kinetically resolvable uptake components is also consistent with membrane binding studies, which have shown two classes of binding sites for dibromsulfophthalein [48] and three for BSP [59]. Three BSP binding proteins have also been isolated from rat liver sinusoidal enriched plasma membranes [15,18–20], of which those described by Stremmel et al. [20] and Wolkoff and Chung [19] appear to be related. An antibody to one of these proteins selectively inhibits hepatocellular uptake of BSP and bilirubin by a high-affinity, low-capacity transport process [58]. The physiologic significance of the low-affinity, high-capacity membrane binding sites and transport components remains to be established.

The process by which BSP enters these

sinusoidal membrane vesicles is temperature dependent, being maximal at temperatures around 37°C; the data suggest that membrane binding alone occurs at or below 15°C. Whilst some workers have suggested that ligand binding to membranes may also be temperature dependent [45,60,61], uptake rather than temperature-dependent binding is more likely to explain the differences in vesicle-associated radioactivity at 37°C and 0–15°C, as evidenced by the hypotonic lysis studies, as well as studies demonstrating osmotic sensitivity and, by implication, the existence of an osmotically sensitive intravesicular space. The use of a hyperosmolar external medium, as well as controlling uptake rates, also minimizes the leakiness of these vesicles.

BSP transport into these vesicles is markedly inhibited by DIDS, even at low concentrations (≤ 10 μM), both initially and at equilibrium. At these low concentrations membrane binding was not affected, suggesting that the values observed for binding may reflect less specific sites which are not associated with the uptake process. This marked inhibition of uptake, however, implies that BSP uptake is linked to anion movement, as DIDS is a potent inhibitor of chloride and sulphate transport. This is supported by the observation that BSP uptake is enhanced in the presence of an inwardly directed chloride gradient. Since neither valinomycin nor ouabain significantly alter BSP transport rates into the vesicles under the conditions employed in this study, the implication is that BSP uptake is not highly cation dependent. However, cations may play some part in its transport; alteration of the vesicle internal cation clearly alters the BSP transport rates, indicating that a more complex electrogenic process is involved.

Selective competitive inhibition of transport by related compounds is also characteristic of a specific transport mechanism. Rose bengal produced typical competitive inhibition of BSP uptake into the vesicles. Indocyanine green, another cholephilic dye which competitively inhibits BSP uptake in vivo [7], also inhibited its uptake into the vesicles. This inhibition of V_i was concentration dependent for Rose bengal, but more complex for indocyanine green. The reduction in equilibrium values in the presence of these compounds, however, suggest a more complicated relationship which may involve *trans*-stimulation, or inhibition based on binding to the transporter molecules. More detailed investigation of this phenomenon is required to further elucidate this observation. At low concentrations, taurocholate, for which the predominant uptake pathway in vivo or into isolated hepatocytes is different from that of BSP [32], produced no inhibition of BSP uptake into sinusoidal membrane vesicles, as predicted. However, taurocholate appeared to stimulate BSP uptake into the vesicles in a concentration-dependent manner above 250 μM. The basis of this phenomenon is unclear, but may reflect taurocholate induced changes in vesicle permeability [62].

Both *cis*-inhibition and *trans*-stimulation of BSP uptake by the vesicles were demonstrable in the present studies. Uptake of BSP is enhanced by preloading, and counter-transport can be demonstrated against a 100-fold BSP concentration gradient. Efflux of ^{35}S-BSP at equilibrium can only be seen when albumin is present in the external medium. It is possible that more efficient efflux may occur from intact cells, in which the BSP is conjugated to glutathione. Certainly glutathione efflux from similar sinusoidal membrane vesicles has been demonstrated [63], and shares a common pathway with conjugated glutathiones.

These studies have shown that the flux of BSP across the liver cell sinusoidal plasma membrane exhibits all the kinetic characteristics of facilitated transport, in a simple system where only the effects of membrane processes are likely to be evident. Uptake studies with vesicles avoid complications introduced by intracellular metabolism, binding to cytosolic proteins, and even such phenomena as circulatory effects, which may be important modifiers of kinetic observations in vitro [64].

Although BSP uptake is clearly a facilitated process, the driving forces remain obscure. Unlike uptake of both bile acids [34] and free fatty acids [10], BSP uptake does not appear to be driven by a transmembrane sodium gradient established by $(Na^+ + K^+)$-ATPase. The very preliminary data presented above on the role of various inorganic anions, and especially chloride, in modifying BSP uptake, suggest an electrogenic component to the process in sinusoidal membrane vesicles, and analogous effects have recently been observed in a

preliminary study with isolated hepatocytes [65]. However, these authors suggest a mechanism involving a HCO_3^-/Cl^- antiport. Our data do not support this and suggest a more complicated electrogenic mechanism, possibly involving Cl^- together with K^+_{in}/Na^+_{out}. Despite these recent observations the driving forces for BSP remain poorly defined at present.

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