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BINDING OF UNCONJUGATED AND CONJUGATED SULFOBROMOPHTHALEIN TO RAT LIVER PLASMA MEMBRANE FRACTIONS IN VITRO

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Summary

As part of a study of the mechanism whereby organic anionic dyes such as sulfobromophthalein and bilirubin enter hepatocytes, the binding of [^{35}S]sulfobromophthalein and of its glutathione conjugate to two rat liver plasma membrane fractions were studied in vitro. Both fractions reversibly and saturably bound conjugated and unconjugated sulfobromophthalein. Three classes of binding site were necessary to account for the observed sulfobromophthalein binding, their maximal binding capacities being $3.5 \cdot 10^{-11}$, $1.6 \cdot 10^{-7}$ and $5.4 \cdot 10^{-7}$ mol/mg membrane protein. The corresponding association constants were $5.5 \cdot 10^{7}$, $1.5 \cdot 10^{5}$ and $1.3 \cdot 10^{3}$ M^{-1}. Binding of the glutathione conjugate could be accounted for by two classes of binding site only, their association constants being $2.0 \cdot 10^{8}$ and $1.9 \cdot 10^{3}$ M^{-1} and their maximal binding capacities $5.0 \cdot 10^{-11}$ and $2.2 \cdot 10^{-7}$ mol/mg protein, respectively. Conjugated and unconjugated sulfobromophthalein mutually competed for binding, K_I being $7.8 \cdot 10^{-7}$ and $5.5 \cdot 10^{-5}$ M for conjugated and unconjugated sulfobromophthalein, respectively. Similarly, bilirubin and indocyanine green, but not taurocholate, competitively inhibited sulfobromophthalein binding. Treatment with trypsin and phospholipases reduced, while treatment with neuraminidase did not affect binding. Neither changes in pH nor substitution of other cations for Na^+ in the incubation mixture significantly affected sulfobromophthalein binding. Heating the membranes increased binding. This was due to an increase in maximal binding capacity of the low-affinity, high-capacity sites. The descrip-

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tion of saturable binding sites on hepatocellular surface membranes, the affinity of one of the sites exceeding the reported affinities for albumin and ligandin, supports the hypothesis that a membrane-located membrane carrier is responsible for hepatic uptake and biliary excretion of organic anionic dyes. Based on the studies with enzyme treatments, it is speculated that this carrier is a phospholipid-dependent, integral membrane protein.

Introduction

The hepatic uptake and biliary excretion of a variety of endogenous and exogenous organic anionic dyes such as bilirubin, indocyanine green and sulfobromophthalein have been characterized as saturable processes apparently specific for these compounds [1—7]. One possible explanation for these phenomena, at least as regards hepatic uptake, would be passive diffusion across the cell membrane with subsequent binding to cytoplasmic acceptor proteins such as ligandin or Z protein [8]. Alternatively, however, several workers have postulated a membrane-carrier mechanism for the uptake of organic anionic dyes [3—7,9,10]. Convincing evidence for this hypothesis has yet to be developed. To elucidate further whether hepatocellular surface membranes are directly involved in the transport of organic anionic dyes, the binding of sulfobromophthalein to two different plasma membrane fractions of rat liver was studied in vitro.

Materials and Methods

Materials

Male Sprague-Dawley rats, 229 ± 31 (±S.D.) g body wt., were obtained from Taconic Farms, Germantown, N.Y. [^{35}S]Sulfobromophthalein (spec. act. 112 mCi/mmol) and di(*n*-octyl) [^{35}S]sulfide, were purchased from Amersham/Searle Corp., Arlington Heights, IL. Unlabeled sulfobromophthalein was from Sigma Chemical Co., St. Louis, MO, indocyanine green from Hynson, Westcott and Dunning Inc., Baltimore, MD, sodium taurocholate from Calbiochem, San Diego, CA, and unconjugated bilirubin from National Biochemical Co., Cleveland, OH. Trypsin and phospholipases A and C were obtained from Sigma. Neuraminidase was from Grand Island Biological Co., Grand Island, NY. [^{14}C]Erythritol (spec. act. 18.1 mCi/mmol) was from Amersham/Searle Corp. and ^{42}KCl from New England Nuclear, Boston, MA. Sulfobromophthalein-glutathione conjugates were prepared according to the method of Whelan et al. [11]. The conjugate consisted of 93% monoglutathione and 7% diglutathione; no free sulfobromophthalein could be detected by thin-layer chromatography [11]. All other reagents were analytical grade from various sources.

Methods

Liver plasma membrane preparation. Between 30 and 60 g of liver of fed rats were processed per experiment. Fraction 1 was prepared by a modification of the method of Song et al. [12] recently described in detail [13]. Fraction 2

was harvested at the 1.22—1.18 g/ml interface of the discontinuous sucrose gradient described by Song et al. [12], and further purified as proposed by Fisher et al. [14]. The final fractions were suspended in 1 mM $NaHCO_3$ by aspirating them three times through a 23 gauge hypodermic needle.

Erythrocyte ghosts were prepared according to the method of Marchesi and Palade [15], microsomes according to that of Schenkman and Cinti [16] and mitochondria according to that of Shephard and Huebscher [17]. The protein concentrations of all the subcellular fractions and of the liver and kidney homogenates were adjusted to approx. 1 mg/ml. The binding assays were performed immediately after preparation of the membrane fractions.

Binding studies. Varying concentrations of [^{35}S]sulfobromophthalein (10^{-10}—10^{-3} M) of known specific activity in 132 mM sodium phosphate buffer, pH 7.6, were incubated with 80—100 μg membrane protein from 1 to 60 min at 2°C. The final concentration of the phosphate buffer was 66 mM. Thereafter, the free ligand was removed from the ligand bound to the membrane fractions by rapid filtration of 50—500 μl (20—500 μg membrane protein) of the incubation mixture through a Whatman GF/C glass-fiber filter (Fisher Scientific Co., Silver Spring, MD) and washed with 5 ml of 66 mM phosphate buffer as described by Accatino and Simon [18]. The binding assay was found to be linear with protein concentration in the range studied. Since the glass-fiber filters retained considerable amounts of radioactivity even in the absence of protein, they were preloaded with 1 ml of cold 1 mM sulfobromophthalein. The preloaded filters retained 0.93 ± 0.48% of [^{35}S]sulfobromophthalein. No protein could be detected in the filtrate. The concentration bound was calculated from the radioactivity retained on the filters after subtraction of the filter blank and was related to mg of protein present in the incubation mixture. The free concentration was calculated as the difference between total and bound concentrations. Since the time-course studies had shown that binding was in equilibrium after 30 min, a 30-min incubation period was used throughout the experiments.

Radioactivity was counted on a Nuclear Chicago Mark II liquid scintillation counter using a scintillation cocktail consisting of 25.6 g 2,5-diphenyloxazole in 0.8 l methoxyethanol and 3.2 l toluene. A quench curve was established by internal standardization with di(*n*-octyl) [^{35}S]sulfide. Efficiency averaged 91.08 ± 0.13% and recovery of ^{35}S was 98.3 ± 1.2%. Counting efficiency was monitored by external standardization using the channel-ratio method.

Reversibility of [^{35}S]sulfobromophthalein binding was tested by adding an excess (100-fold) of unlabelled sulfobromophthalein to the incubation mixture after 30 min. After an additional 30 min, the concentration bound was determined as described above. In the inhibition experiments, varying concentrations of [^{35}S]sulfobromophthalein (10^{-7}—10^{-3} M) were incubated with sulfobromophthalein glutathione, bilirubin, indocyanine green or sodium taurocholate (10^{-7}—10^{-3} M) as inhibitors for 30 minutes. The concentration bound was determined as described above. The effect of detergents was studied using sodium dodecyl sulfate (1%, w/v), Triton X-100 (1%, w/v) and digitonin (0.01, 0.1 and 1%; w/v). Similarly, the effect of a chaotropic agent, 1 M KI, was studied. In another set of experiments, membranes were heated to 56 or 100°C for 5 and 30 min. Trypsin (100 BAEE units/mg membrane protein), trypsin

and soybean trypsin inhibitor (1 mg/mg trypsin), phospholipase A (100 I.U./mg membrane protein), phospholipase C (10 I.U./mg) and neuraminidase (1.0 I.U./mg) were incubated in maleate buffer at a pH optimal for the corresponding enzyme for 30 min at 25°C. A possible effect of products released by the phospholipase treatments was studied by incubating 10 mg membrane proteins with phospholipase A or C. After 1 h, the incubation mixture was centrifuged for 1 h at 100 000 × g and the supernatant used to suspend untreated membranes. In the experiments with detergents, chaotropics and enzymes, paired observations were made on untreated membranes. The amount of dye bound to treated membranes was expressed as the percent of that bound to the corresponding untreated membrane. A possible contribution of dye uptake into vesicles was investigated by measuring [^{14}C]erythritol and ^{42}K space. 20—100 μg of membrane protein were incubated with 100 nCi of [^{14}C]erythritol or 50 nCi of ^{42}K. After incubation periods ranging from 1 to 30 min, aliquots of the incubation mixture were filtered and prepared for liquid scintillation counting. ^{14}C counting efficiency was monitored by internal standardization with [^{14}C]toluene (New England Nuclear) and of ^{42}K by external standardization using the channel-ratio method.

Characterization of membrane fractions. On each membrane fraction, as well as on the corresponding homogenate, the following enzyme activities were determined. Glucagon-stimulated adenylate cyclase (EC 4.6.1.1) was determined as described by Wisher and Evans [19]. 5′-Nucleotidase (EC 3.1.3.5), glucose-6-phosphatase (EC 3.1.3.9) and succinate dehydrogenase (EC 1.3.99.1) were determined using methods described earlier [13]. Protein was determined by using a modification [20] of the technique of Lowry et al. [37]. Sialic acid was determined by the thiobarbituric acid assay [21]. Total sialic acid in membranes was determined by incubating membrane fractions up to 12 h with neuraminidase (10 I.U./mg membrane protein). After 6 h no further sialic acid release could be observed. This value was taken as 100%.

Membrane fractions were prepared for electron microscopy by a filtration technique [22]. The pellicles were processed and viewed by Dr. H.O. Bladen, Laboratory of Experimental Pathology, NIAMDD, NIH. Five blocks were cut from each pellicle and 10 pictures taken randomly from each block. The absence of albumin and ligandin from the two fractions was documented by double immunodiffusion on Ouchterlony plates using antibodies against rat albumin as well as against rat liver ligandin.

To determine whether either fraction contained sulfobromophthalein-conjugating enzymes, 1 mg membrane protein was incubated with 1 ml of 10^{-3} M sulfobromophthalein and reduced glutathione (10^{-3} M). Thereafter, the incubation mixture was characterized by thin-layer chromatography [11].

Mathematical and statistical analysis. Initial estimates for the binding parameters were obtained by the methods described in Appendix. Then, the binding data were fitted to the general binding isotherm:

$$c_b = \sum_{i=1}^{n} \frac{P_i \cdot (K_i \cdot c_f)^{h_i}}{1 + (K_i \cdot c_f)^{h_i}} \qquad (1)$$

on a Univac 1008 digital computer using version 27 of the 'Simulation, Anal-

ysis and Modeling' program of Berman and Weiss [23]. In Eqn. 1, c_b represents mol ligand bound/mg membrane protein, c_f the free ligand concentration, K the association constant, P the maximal binding capacity and h the Hill coefficient, while the subscript i indicates the number of the class of binding sites. The competition studies were evaluated similarly, fitting them to the equation:

$$c_b = \sum_{i=1}^{n} \frac{P_i \cdot (K_i \cdot c_f)^{h_i}}{1 + (K_i \cdot c_f)^{h_i} + I/K_I} \quad (2)$$

where K_I describes the dissociation constant of the protein-inhibitor complex and I the inhibitor concentration.

Initial estimates of K_I were obtained graphically according to the method of Dixon and Webb [24]. Regression analysis was performed using the method of least squares [25]. Means of two samples were compared using the Student's t-test after testing the equality of variances by an F-test [25]. When the variances were unequal a modified t-test was applied [26]. $P \leqslant 0.05$ was considered statistically significant. Unless otherwise stated, all results are expressed as mean ± S.D.

Results

The protein content of the liver homogenate was 152 ± 16 mg/g liver. In fractions 1 and 2, 0.30 ± 0.14 and 0.43 ± 0.09 mg of protein per g liver were recovered, respectively. The activities of the different marker enzymes are given in Table I. Neither fraction was contaminated to an appreciable extent by albumin or ligandin, as determined by double immunodiffusion against albumin and ligandin antibodies. When either fraction was incubated in the presence of 10^{-3} M sulfobromophthalein and 10^{-3} M glutathione for 30 min, 4.3 ± 1.6 and 3.9 ± 1.8% of the radioactivity was found to be glutathione conjugates of sulfobromophthalein. This was not significantly different from spontaneous conjugation (4.6 ± 2.0%), in the absence of membrane proteins. Fraction 1 contained mainly structures resembling bile canaliculi as evidenced by Fig. 1. Although fraction 2 also contained some tight junctions, it consisted mainly of vesicular structures (Fig. 1). In both membrane fractions, mitochondria could only

TABLE I

ACTIVITY OF MARKER ENZYMES IN LIVER HOMOGENATE AND THE TWO LIVER PLASMA MEMBRANE FRACTIONS

The activities are expressed as μmol (nmol for adenylate cyclase) of substrate used/h per mg protein. Mean ± S.E. are given (n = 20).

	Homogenate	Fraction 1	Fraction 2
Glucagon-stimulated adenylate cyclase	0.62 ± 0.09	1.78 ± 0.62 *	10.9 ± 2.3
5'-Nucleotidase	2.30 ± 0.21	84.5 ± 2.6 *	32.6 ± 4.6
Glucose-6-phosphatase	6.21 ± 0.34	0.79 ± 0.21 **	0.84 ± 0.2
Succinate dehydrogenase	7.2 ± 0.8	0.32 ± 0.05 *	0.83 ± 0.12

* Significantly different from fraction 2, $P < 0.001$.

** Not significantly different from fraction 2.

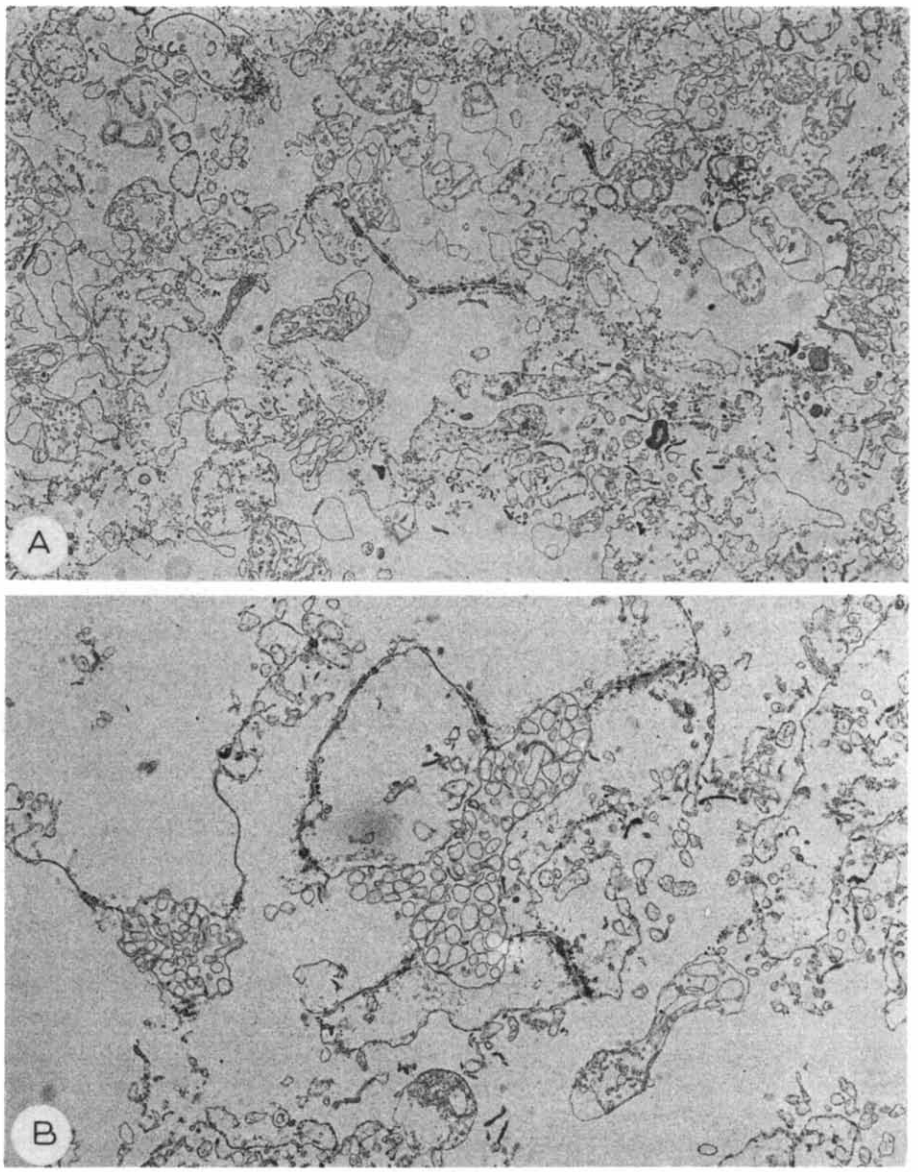

Fig. 1. Electron micrographs of plasma membrane (A) and canalicular enriched plasma membrane fractions (B). Magnification, ×8700.

rarely be seen. Fraction 2, however, contained rough endoplasmatic reticulum in almost every picture taken.

The [^{14}C]erythritol space averaged 1.23 ± 0.13%/mg protein. This value was reached after 10 min of incubation. ^{42}K reached a maximum of 5.48 ± 0.43%/mg at 3 min; thereafter it was slowly released, 1.48 ± 0.43% being retained after 30 min.

[^{35}S]Sulfobromophthalein binding reached equilibrium at 15 min (Fig. 2).

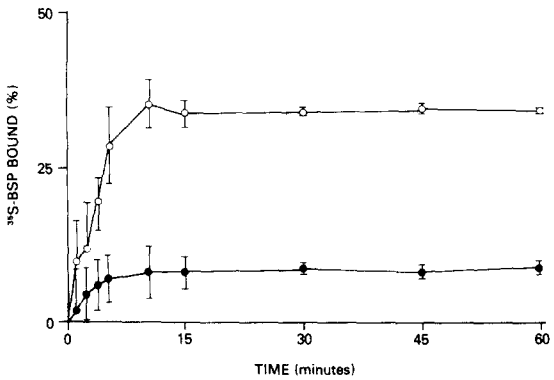

Fig. 2. Time course of 10^{-7} (○) and 10^{-3} M (●) [^{35}S]sulfobromophthalein (^{35}S-BSP) binding to liver plasma membrane fraction 2. Mean ± 1 S.D. of triplicate experiments are given.

The binding of sulfobromophthalein to both membrane fractions showed saturation and exhibited two maxima and minima on the semilogarithmic Scatchard plot (Fig. 3). This suggests the presence of at least two classes of binding site with marked cooperativity and/or heterogeneity. In fact, using the analytical methods described in Appendix, three classes of binding site were both necessary and sufficient to describe the data. The calculated binding parameters are given in Table II. Similarly to unconjugated sulfobromophthalein, the glu-

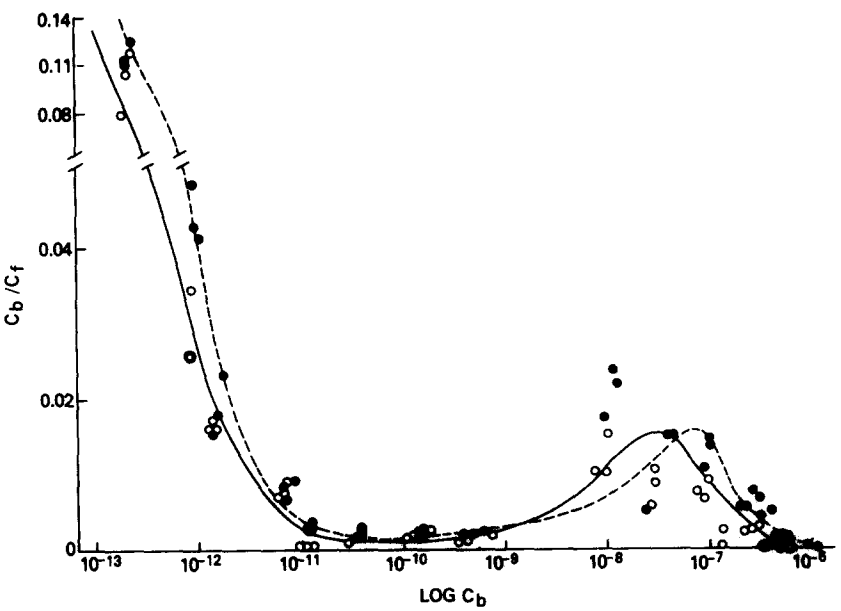

Fig. 3. Semilogarithmic Scatchard plot of [^{35}S]sulfobromophthalein binding to plasma membrane fractions 1 (●) and 2 (○). Each point represents the mean of a triplicate assay. The solid line represents the theoretical binding curve to plasma membrane and the dashed line that to canalicular enriched membrane fractions as calculated from the binding parameters given in Table II.

TABLE II

PARAMETERS OF SULFOBROMOPHTHALEIN BINDING TO LIVER PLASMA MEMBRANE FRACTIONS

K, association constant; *P*, maximal binding capacity; *h*, the Hill coefficient. For heated membranes, only *P* is given since significant changes occurred in this parameter only.

	K (M^{-1})			P (mol/mg protein)			h			F ($n = 45$)
	K_1	K_2	K_3	P_1	P_2	P_3	h_1	h_2	h_3	
Fraction 1										
Native	$5.5 \cdot 10^7$	$1.7 \cdot 10^5$	$1.5 \cdot 10^3$	$3.0 \cdot 10^{-11}$	$1.6 \cdot 10^{-7}$	$1.4 \cdot 10^{-6}$	0.52	1.95	1.08	22.70
Heated				$1.8 \cdot 10^{-11}$	$9.0 \cdot 10^{-8}$	$3.6 \cdot 10^{-6}$				6.94
Fraction 2										
Native	$5.5 \cdot 10^7$	$1.5 \cdot 10^5$	$1.3 \cdot 10^3$	$3.5 \cdot 10^{-11}$	$1.6 \cdot 10^{-7}$	$5.4 \cdot 10^{-7}$	0.58	1.70	1.30	13.70
Heated				$3.8 \cdot 10^{-11}$	$3.5 \cdot 10^{-7}$	$5.5 \cdot 10^{-7}$				6.83

tathione conjugate saturably bound to both membrane fractions, but only two classes of binding site were necessary to account for binding (Fig. 4, Table III). The displacement of [^{35}S]sulfobromophthalein by the addition of increasing amounts of cold ligand is shown in Fig. 5. A dissociation constant of $3 \cdot 10^{-7}$ M can be estimated from this graph.

No saturation in the binding of sulfobromophthalein was observed to different structures presumably not involved in its transport such as erythrocyte ghosts, liver and kidney homogenates, liver microsomes and mitochondria. Sulfobromophthalein and its glutathione conjugate mutually competed for binding to both membrane fractions, K_I being $9.8 \cdot 10^{-7}$ and $5.5 \cdot 10^{-5}$ M, for conjugated and unconjugated sulfobromophthalein, respectively. Other organic anionic dyes such as bilirubin (K_I $6.8 \cdot 10^{-6}$ M) and indocyanine green (K_I $0.1 \cdot 10^{-4}$ M) also competitively inhibited sulfobromophthalein binding. Taurocholate showed no competition to either of the membrane fractions studied.

Detergents and chaotropic agents inhibited sulfobromophthalein binding to fraction 2 to different degrees as shown in Table IV. The only exceptions were digitonin at 0.01% (w/v) and in some instances at 0.1% (w/v), where a slight increase in binding was observed. The effects of the different treatments on [^{14}C]erythritol space are also given in Table IV. The different agents had similar effects on fraction 1.

The effects of the various enzymatic treatments are summarized in Table V. The supernatant of phospholipase A-treated membranes did not affect binding, while phospholipase C supernatant inhibited dye binding by 42.4, 31.7 and 36.4% at 10^{-4}, 10^{-6} and 10^{-8} M, respectively. Neuraminidase released 46.8 ± 10.1% of total membrane sialic acid.

Heating the membrane fractions to 56°C for 10 min led to binding of consistently and statistically significant higher amounts of sulfobromophthalein. This was mainly due to an increase in the maximal binding capacity of the second or

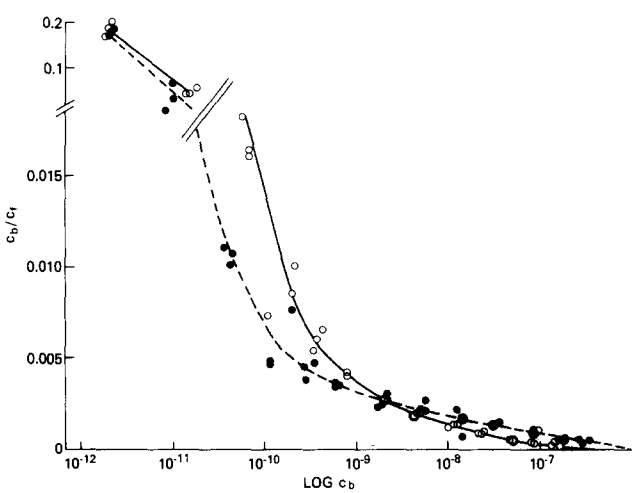

Fig. 4. Semilogarithmic Scatchard plot of [^{35}S]sulfobromophthalein glutathione binding to liver plasma membrane fractions 1 (•) and 2 (○). Each point represents the mean of a triplicate assay. The solid line represents the theoretical binding curve to fractions 1 and 2, respectively, calculated from the binding parameters given in Table III.

TABLE III

PARAMETERS OF GLUTATHIONE SULFOBROMOPHTHALEIN BINDING TO LIVER PLASMA MEMBRANE FRACTIONS

K, association constant; P, maximal binding capacity; h, the Hill coefficient.

	K_1 (M^{-1})		P (mol/mg protein)		h		F (n = 39)
	K_1	K_2	P_1	P_2	h_1	h_2	
Fraction 1	$3.5 \cdot 10^8$	$2.1 \cdot 10^3$	$3.2 \cdot 10^{-11}$	$4.2 \cdot 10^{-7}$	0.52	0.83	46.69
Fraction 2	$2.0 \cdot 10^8$	$1.9 \cdot 10^3$	$4.0 \cdot 10^{-11}$	$2.2 \cdot 10^{-7}$	0.58	0.72	32.68

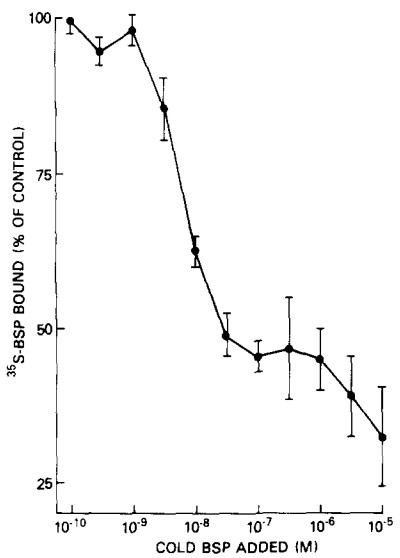

Fig. 5. Displacement of 10^{-8} M [^{35}S]sulfobromophthalein (^{35}S-BSP) from membrane fraction 2 by the addition of increasing amounts of unlabeled (cold BSP) dye. Results are expressed as percent of control. Mean ± 1 S.D. are given. The dissociation constant, K_d, estimated graphically, was $3 \cdot 10^{-7}$ M.

TABLE IV

THE EFFECT OF VARIOUS DETERGENTS AND A CHAOTROPIC AGENT ON SULFOBROMOPHTHALEIN BINDING BY LIVER PLASMA MEMBRANE FRACTION

The results express the mean of duplicate determinations in two independent experiments and are given as percentage of sulfobromophthalein bound compared to the amount of dye bound to paired, untreated membranes.

	Sulfobromophthalein concentration (M)			[^{14}C]Erythritol space
	10^{-4}	10^{-6}	10^{-8}	
Sodium dodecyl sulfate (1%, w/v)	0.0	0.3	0.0	10.8
Triton X-100 (1%, w/v)	0.8	2.3	15.2	72.4
Digitonin (0.01%, w/v)	108.8	108.0	113.2	98.6
Digitonin (0.1%, w/v)	59.7	55.4	63.7	97.4
Digitonin (1%, w/v)	16.9	12.9	14.1	102.1
KI (1 M)	61.5	56.1	47.8	56.4

TABLE V

THE EFFECT OF VARIOUS ENZYMATIC TREATMENTS ON SULFOBROMOPHTHALEIN BINDING BY LIVER PLASMA MEMBRANE FRACTIONS

The results express the mean of duplicate determinations and are given as the percentage of sulfobromophthalein bound compared to paired, untreated membranes.

Sulfobromopthalein concentration (M):	Fraction 1			Fraction 2		
	10^{-4}	10^{-6}	10^{-8}	10^{-4}	10^{-6}	10^{-8}
Trypsin	13.3	7.9	8.4	25.0	22.7	12.7
Trypsin and trypsin inhibitor	106.6	96.1	101.7	106.0	112.2	85.3
Phospholipase A	62.7	63.1	62.2	45.3	78.2	91.5
Phospholipase C	17.2	21.9	41.3	16.5	28.7	30.8
Neuraminidase batch A	75.4	81.9	53.2	76.3	62.4	59.6
Neuraminidase batch B	97.1	105.3	92.4	103.8	109.8	89.9

third classes of binding site in fraction 2 and 1, respectively (Table II). The first class of binding site was unaffected in fraction 2 and its binding capacity even decreased in fraction 1. Heating to 100°C or extending the treatment to 30 min did not further alter the binding parameters. Heating had similar effects on binding of sulfobromophthalein glutathione to both membrane fractions.

Lowering the pH to 5.0 led to a small but statistically insignificant increase in binding, whereas raising the pH to 9.0 did not affect binding. Substitution of sodium in the incubation mixture by tris(hydroxymethyl)aminomethane had no effect on sulfobromophthalein binding by either of the membrane fractions studied.

Discussion

The mammalian liver appears to contain a highly efficient mechanism for removing endogenous and exogenous organic anionic dyes such as bilirubin [2,3,5,7], indocyanine green [4—6] and sulfobromophthalein [3,5] from the portal circulation and excreting them into bile [1—3]. Two basically different mechanisms could explain the saturation phenomena observed in vivo: passive diffusion with subsequent binding to an intracellular acceptor protein such as ligandin or Z protein [8,11], or carrier-mediated transport. Based on the finding that albumin has a higher affinity for sulfobromophthalein than does ligandin [27,28], the necessity of an active participation of the hepatocellular surface membrane in dye transport has been postulated [9].

Extensive binding of sulfobromophthalein by hepatocellular surface membranes has been demonstrated [10]. Sulfobromophthalein binding to albumin has been described to be due to three classes of binding site, the high-affinity class exhibiting a dissociation constant of $1.7 \cdot 10^{-7}$ M [29]. This corresponds to an association constant of $6 \cdot 10^{6}$ M^{-1}, almost an order of magnitude lower than the association constant of the high-affinity class of binding site in liver plasma membranes described in the present study. This supports the hypothesis

that the liver plasma membrane plays an active role in the transport of organic anionic dyes. This contention is further corroborated by the striking parallelism of the competition experiments with the in vivo studies. Thus, the excretory transport maximum is higher for conjugated than for unconjugated sulfobromophthalein [11]. Moreover, it has been shown by several authors that bilirubin, indocyanine green and sulfobromophthalein mutually compete for uptake [3,5] and excretion [1]. When the K_I values determined in the present investigation are arranged in increasing order, the relative affinities for the different dyes appear to be: sulfobromophthalein glutathione > sulfobromophthalein > bilirubin > indocyanine green. This is consistent with data on transport in vivo [5]. It has been well established that bile salts share a pathway clearly different from that employed by organic anionic dyes [1,5—7]. Similarly, sulfobromophthalein and taurocholate showed no competition in the present experiments.

The low [^{14}C]erythritol space argues against a significant contribution of uptake to our observations on dye binding. In addition, there was no correlation between the effects of detergents on reduction in sulfobromophthalein binding and [^{14}C]erythritol space (Table IV). It is unlikely that the observed binding phenomena are due to a contamination of the membrane fractions with albumin or ligandin, since neither of these proteins could be demonstrated in the membrane fractions. Consistent with the latter finding, no appreciable sulfobromophthalein-conjugating capacity of either membrane fraction could be demonstrated.

The similarity of the binding parameters calculated for both membrane fractions is probably due to a poor separation of sinusoidal and canalicular membranes by the presently used technique, since the enrichment of the respective marker enzymes, 5′-nucleotidase and adenylate cyclase, was only 3- and 5-fold (Table I). Both membrane fractions contain appreciable amounts of lateral membranes since tight junctions were present in both fractions (Fig. 1).

The relevance of the finding of more than one class of binding site remains to be established. The second and third classes of binding site showed affinities considerably lower than the affinity reported for albumin [29]. This presumably precludes their participation in dye transport unless there are driving forces for transport other than the affinities to 'carrier' proteins such as albumin and ligandin and the membrane carrier. The binding parameters of the two low-affinity, high-capacity binding sites are similar to those described for the adsorption of sulfobromophthalein by isolated hepatocytes, a phenomenon presumably unrelated to transport [30].

The particular binding kinetics of sulfobromophthalein to hepatocellular surface membranes deserve some comments. The decision as to which sets of data points are to be used for the analysis of a particular class of binding site is arbitrary [31] and it has to be emphasized that the solution presented here is non-unique. Part of this uncertainty is dealt with by the inclusion of the Hill coefficient in the mathematical analysis [31]. The Hill coefficient smaller than unity calculated for the first class of binding site may indicate that this class is very heterogeneous, or that marked negative cooperativity exists. The Hill coefficient of the higher classes of binding site was greater than unity. Whether this represents real positive cooperativity or the binding of dye aggregates at high ligand concentrations [32] cannot be definitely answered from

the present experiments. In contrast to unconjugated sulfobromophthalein, binding of the glutathione conjugate could adequately be described by two classes of binding site, both having a Hill coefficient smaller than unity.

The apparent heat stability of dye binding raises questions as to the specificity of sulfobromophthalein binding to those sites. Although heat-stable carrier molecules are known from bacterial sugar transport systems [33], no such phenomenon is known to us in mammalian cells. This apparent heat stability could be due to thermostability of the hypothetical carrier molecules or to the opening of additional, unspecific binding sites on heated membranes. The latter hypothesis is supported by the apparent increase in binding capacity of the low-affinity sites and the reduction of binding capacity in the high-affinity sites.

The molecular nature of the hypothetical carrier has not been elucidated by the present experiments. The results of the different enzymatic treatments suggest that the hypothetical carrier is a protein, since aggressive digestion with trypsin almost completely abolished sulfobromophthalein binding. A lipid locus responsible for dye binding, however, cannot be excluded. While products of phospholipase A digestion did not seem to interfere with sulfobromophthalein binding, phospholipase C released products which clearly inhibited dye binding. This did not account for the whole extent of the reduction in binding after phospholipase C treatment. The hypothesis that an integral, eventually phospholipid-dependent membrane protein is responsible for hepatic sulfobromophthalein transport is further supported by our recent demonstration of proteins with apparent molecular weights of 40 000, 46 000 and 60 000 specifically retained on sulfobromophthalein-labeled affinity columns [34]. The hypothetical carrier protein seems not to be dependent on sialic acid, since neuraminidase treatment did not abolish binding.

The pH experiments seem to indicate that interactions other than ionic ones are responsible for sulfobromophthalein binding to liver plasma membrane. Cornelius et al. [10] described increased sulfobromophthalein binding at lower pH which could not be demonstrated as distinctly in the present experiments. The lack of an effect of substitution of sodium by tris(hydroxymethyl)aminomethane is consistent with the finding that organic anionic dye transport into isolated hepatocytes is not dependent on the sodium gradient [30].

Although the exact mechanism whereby organic anionic dyes interact with hepatocellular surface membranes remains to be characterized, the present investigation supports the hypothesis that hepatic plasma membranes play an active role in hepatic uptake and biliary excretion of organic anionic dyes.

Appendix

The separation of the data into different classes of binding site was performed on a trial-and-error basis using the criteria of Pliska [31] and an F test [25]. Initial estimates for the final computation [23] were obtained using two methods. Given the general binding isotherm (Eqn. 1), the h_i terms were ordered so that:

$$0 < h_1 < h_2 < h_3 < h_n \qquad (3)$$

For $h_1 \geqslant 1$, at c_f approaching 0, the general binding isotherm can be approximated by:

$$c_b \simeq P_1 \cdot (K_1 \cdot c_f)^{h_1} \tag{4}$$

By transforming Eqn. 4 to:

$$\ln c_b = \ln(P_1 \cdot {K_1}^{h_1}) + h_1 \cdot \ln c_f \tag{5}$$

h_1 and $P_1 \cdot {K_1}^{h_1}$ can be calculated by linear regression analysis. For $h < 1$, the same procedure was employed, except that Eqn. 5 becomes:

$$\ln c_b = \ln(P_1 \cdot {K_1}^{h_1}) + (1 + h_1) \cdot \ln c_f \tag{6}$$

When c_f tends toward infinity, c_b tends towards ΣP_i. Therefore, at increasing c_f, Eqn. 1 can be approximated by:

$$c_b \simeq \sum_{i=1}^{n} P_1[1 - 1/(K_1 \cdot c_f)^{h_i}] \tag{7}$$

An estimate of $P_1/({K_1}^{h_i})$ is then obtained by transforming Eqn. 7 into:

$$c_b \simeq \Sigma P_i - P_1/({K_1}^{h_1}) \cdot 1/{c_f}^{h_1} \tag{8}$$

Here again, linear regression analysis was employed to obtain the estimates. Simultaneous solution of Eqns. 5 and 8 yields all the binding parameters for the first class of binding site. The contribution of this class was then subtracted from the observed c_b values and the procedure repeated. The procedure described above is similar to the iterative procedure described by Wieker et al. [35] for one class of binding site, but is not dependent on a reliable initial estimate of the maximal binding capacity. The program of Wieker et al. [35] was also used for the calculation of initial estimates. Both methods gave similar results and were finally validated by fitting the data directly to the general binding isotherm as described in Materials and Methods.

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