Biochemical Pharmacology, Vol. 46, No. 5, pp. 925–931, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

DIFFERENCE IN HEPATIC UPTAKE OF TETRA- AND DI-BROMOSULFOPHTHALEIN IN RAT

ROLE OF HYDROPHOBICITY, BINDING TO PLASMA PROTEINS AND AFFINITY FOR PLASMA MEMBRANE CARRIER PROTEIN

ADRIANA M. TORRES,* MARCO STEBEL, JOAQUIN V. RODRIGUEZ,* BRUNO GAZZIN, GIAN CARLO LUNAZZI and CLAUDIO TIRIBELLI†

Centro Studi Fegato, Dipartimento B.B.C.M., University of Trieste, 34100 Trieste, Italy

(*Received* 10 *January* 1993; *accepted* 19 *April* 1993)

Abstract—The relative role of hydrophobicity, binding to plasma proteins and affinity for one of the plasma membrane transport proteins in the hepatic uptake of 3,4,5,6-tetra- (BSP) and 3,6-di- (DBSP) bromosulfophthalein was investigated in the rat. In terms of physicochemical characteristics, the two molecules show different pK_a values and degrees of hydrophobicity, as determined from the *n*-octanol:water partition coefficient. In the intact animal, the plasma clearance and the plasma removal rate after a dose of 1.5 μmol/kg i.v. were significantly ($P < 0.001$) faster for BSP than DBSP, while no difference was found in the plasma distribution volume. The dissociation constant (K_d) of the high affinity binding sites of plasma proteins also differed for the two anions, being significantly lower for BSP than DBSP (0.95 ± 0.02 vs 1.44 ± 0.14 μM, $P < 0.001$). [^{35}S]BSP uptake by liver plasma membrane vesicles was saturable with an apparent K_m of 5.20 ± 0.80 μM, and was competitively inhibited by DBSP (K_i 18.2 ± 1.2 μM) indicating a common uptake system. The K_d value for binding of the organic anions to purified bilitranslocase, a plasma membrane protein involved in the electrogenic transport of pthaleins, was also significantly lower for BSP than DBSP (1.10 ± 0.12 vs 3.02 ± 0.27 μM, $N = 3$, $P < 0.001$), indicating a higher affinity of the former ligand for the carrier protein. No difference was observed in the capacity of the high affinity binding sites (32 ± 3 vs 33 ± 3 nmol/mg protein, BSP and DBSP, respectively). These data indicate that BSP and DBSP are two different cholephilic organic anions which share a common uptake mechanism, at least partly mediated by bilitranslocase. The greater affinity of BSP than DBSP for the carrier protein may account for the faster plasma disappearance rate of BSP observed *in vivo*, in spite of the higher plasma protein binding.

The mechanism by which 3,4,5,6-tetrabromosulfophthalein (BSP‡) is removed from the circulation by the liver has been investigated extensively due to the long-standing clinical use of this dye as a test of hepatic function [1]. The 3,6-dibromo analogue of BSP (DBSP) is identical except for the absence of the two bromide ions in the 4 and 5 positions on the benzenic ring (Fig. 1). The hepatic transport of this compound has been reported to share similarities with BSP with the exception of its biliary excretion [2–4]. While BSP undergoes conjugation with glutathione within the liver cell before biliary secretion [5], more than 90% of DBSP is apparently secreted in the rat bile without metabolic transformation [6]. Both cholephilic dyes are supposed to be taken up by the liver via a common carrier-mediated mechanism [2, 3, 7], although the ATP dependency of hepatic uptake does not seem to be similar for the two compounds [8]. Nevertheless, BSP and DBSP have been used interchangeably to study hepatic uptake in both experimental animals and humans.

In spite of the very similar structure of the two molecules, the lack of two bromines in DBSP should alter considerably the physicochemical properties of the anion. The aim of the present study was to compare BSP with DBSP as to physicochemical properties, including binding to plasma and liver plasma membrane proteins, and hepatic uptake rate. Data presented show significant differences in physicochemical characteristics between the two anions resulting in a difference in efficiency in their removal by the liver. The latter observation agrees with a lower affinity of DBSP than BSP for bilitranslocase, a plasma membrane carrier protein involved in the electrogenic hepatic uptake of the two organic anions.

MATERIALS AND METHODS

Female Wistar rats (60–90 days old) weighing 200–300 g were used throughout the study. Animals were allowed free access to a standard laboratory chow (GLP, Altromin-Rieper, Bolzano, Italy) and tap water. BSP was obtained from Merck AG (Darmstadt, Germany) and DBSP from Serb (Paris, France). Purity of the compounds was checked by TLC (Silica plates G-60, *n*-butanol:acetic

* Permanent address: Farmacologia, Facultad Ciencias Bioquimicas y Farmaceuticas, Universidad Nacional de Rosario, Suipacha 531, 2000 Rosario, Republica Argentina.

† Corresponding author. Tel. (39) 40 399-4927/399-4545; FAX (39) 40 399-4546.

‡ Abbreviations: BSP, 3,4,5,6-tetrabromosulfobrophthalein; DBSP, 3,6-dibromosulfobrophthalein; RSA, relative specific activity.

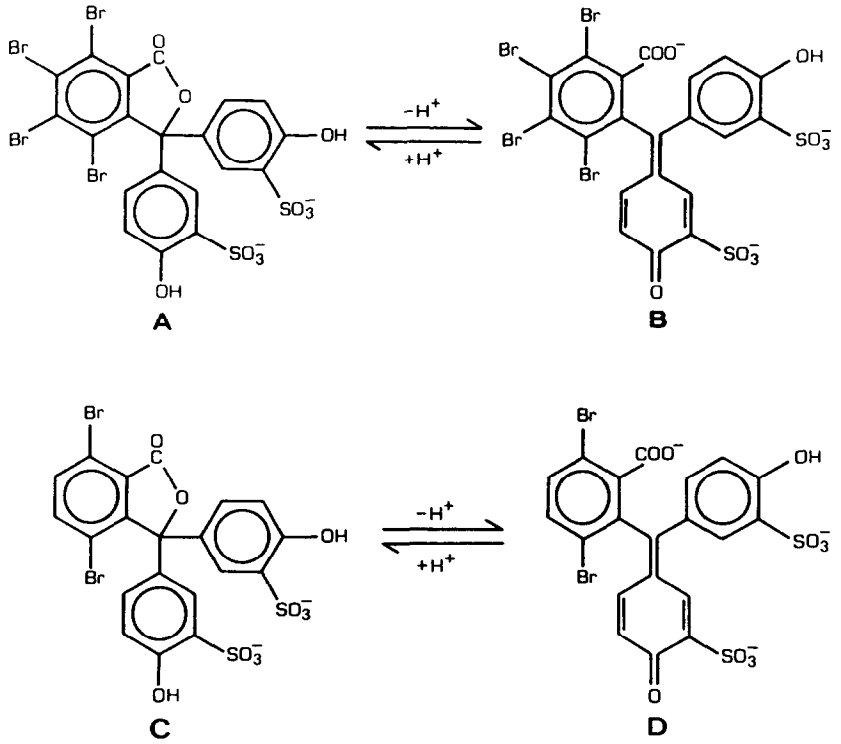

Fig. 1. Structural formulas of protonated (A) and deprotonated (B) BSP, and protonated (C) and deprotonated (D) DBSP.

acid:water, 40:10:10, v/v/v) and found to be higher than 98%. Other reagents were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.) or from Merck AG (Darmstadt, Germany) and were analytical grade pure.

In vivo *clearance studies*. Plasma disappearance rate and plasma clearance of the two organic anions were measured according to Orzes *et al.* [9]. Briefly, the animals were anesthetized with sodium pentobarbital (50 mg/kg body wt). Body temperature was continuously monitored with a deep rectal probe and maintained at 37–37.5° with an infrared lamp. The femoral vein and artery were cannulated with a PE-50 tubing (Clay Adams, Division of Becton, Dickinson and Co., Parrsipanny, NJ, U.S.A.). BSP or DBSP 1.5 μmol/kg body wt dissolved in saline solution in a final volume of 0.1 mL/100 g was injected as a bolus i.v. Blood samples were taken from the femoral artery at 15-sec intervals for 120 sec and collected into 1.5 mL polyethylene tubes (LP, Milano, Italy) containing 5 U heparin. Samples were spun at 12,000 rpm for 4 min in an Eppendorf centrifuge (model 5412), and the dye concentration was assessed spectrophotometrically after alkalinization, as below. Data were plotted on a semi-logarithmic scale, and the initial plasma disappearance rate (k_1, min^{-1}), that mainly reflects hepatic uptake, calculated by least-square fit. Plasma removal rate (μmol/min/kg) and plasma clearance (mL/min) were determined as described [3, 9]. The primary distribution volume of the dye (mL/kg) was calculated from the ratio dose/C_0, where C_0 is the extrapolated plasma concentration at 0 time.

Measurement of the dissociation constants of BSP and DBSP for rat plasma proteins. Plasma samples were obtained from six non-fasted female rats by addition of EDTA 3 mM. Increasing amounts of plasma proteins (50–1000 μg/mL) obtained from a single pool of the plasma of six animals were added to 10 μM solution of either BSP or DBSP, in 0.1 M potassium phosphate buffer, pH 7.4. Final volume was 1.0 mL. Protein concentration was measured by the bicinchoninic acid protein assay according to Smith *et al.* [10]. After an equilibration time of 15 min at 37°, the unbound dye fraction was separated by ultrafiltration in a Centrifree micropartition system (Amicon, Danvers, MA, U.S.A) equipped with a YMT membrane (cut-off 30 kDa of molecular mass). Control experiments showed that similar results were obtained by using a YMT membrane with a cut-of 10 kDa molecular mass. The recovery of both BSP and DBSP in the absence of protein was 98 ± 1% indicating absence of non-specific binding of the dyes to the membrane. The ultrafiltrate (0.3 mL) was assayed for BSP and DBSP content spectrophotometrically upon alkalinization (2.7 mL of NaOH 0.1 M) at an emission wavelength of 580 nm (E_{580} = 64 $mM^{-1}.cm^{-1}$ and E_{580} = 67 $mM^{-1}.cm^{-1}$ for BSP and DBSP, respectively). Dissociation constant (K_d) was calculated by plotting the data according to Scatchard assuming a model with two categories of binding sites.

Determination of the pK_a *values.* The pK_a value of the two dyes was determined by taking advantage of the fact that the two compounds are colored in the deprotonated form, while they become colorless upon protonation. Titration curves were done by addition of increasing amounts of HCl 0.1 M to a 50 μM solution of the anion dissolved in NaOH 0.1 M, pH 13. Absorbance was determined spectrophotometrically at 580 nm. The pK_a value was derived from the best-fitting curve by the Enzfitter program software (Enzfitter, Sigma).

Determination of the octanol:water partition coefficient. The partition ratio of the dyes was measured by adding 4 mL of a dye solution (1 mM) in potassium phosphate buffer 0.1 M, pH 7.4 to an equal volume of *n*-octanol. The mixture was shaken (80 strokes/min) for 10 min at room temperature and centrifuged for 10 min at 3500 rpm. The two phases were separated and the concentration of the dye determined spectrophotometrically in the aqueous phase after alkalinization with 0.1 M NaOH. The concentration of the dye in the aqueous phase was corrected for the volume change observed (8% due to the solubility of water in *n*-octanol). The concentration of the dye in the organic phase was derived by difference, and the ratio between the two phases gave the apparent partition coefficient.

Measurement of transport activity in liver plasma membrane vesicles. Liver plasma membrane vesicles were prepared according to van Amelsvoort *et al.* [11] in a medium containing Hepes 10 mM, sucrose 0.25 M, pH 7.4, and stored in liquid nitrogen until use (within 4 weeks). The degree of purity of liver vesicles was determined by measuring the activity of Na^+,K^+-ATPase [12], glucose-6-phosphatase and succinate-cytochrome *c* reductase [13], and 5'-nucleotidase [14]. The release of inorganic phosphate was measured according to Widnell [15]. Protein concentration was determined as above [10]. In agreement with previous data from our laboratory [16–18], the relative specific activity (RSA) of Na^+,K^+-ATPase and 5'-NT was enriched 13 and five times, respectively, over the homogenate. Conversely, the contamination of either the endoplasmic reticulum (RSA of glucose-6-phosphatase 0.3) or mitochondria (RSA of succinate-cytochrome *c* reductase 0.2) was low.

[^{35}S]BSP was prepared by Dr Stremmel (University of Düsseldorf, Germany) according to Kurisu *et al.* [19] with a specific activity of 37 Ci/mmol. BSP uptake by the plasma membrane vesicles was measured by the rapid filtration technique using a Millipore vacuum filtration assembly (Millipore, Bedford, MA, U.S.A.) and nitrocellulose membranes of pore size 0.45 μm (Millipore). All filters were wetted and pre-saturated with 10 μM unlabeled BSP in the incubation buffer to minimize the non-specific filter binding of radioactive BSP. BSP transport was determined by measuring uptake at different concentration of unlabeled BSP (3–20 μM) in a medium containing (mM) 50 sucrose, 10 Hepes–Tris, pH 7.4, 100 KCl, 0.2 CaCl, 10 MgCl, 10 μCi [^{35}S]BSP and valinomycin dissolved in methanol (final concentration 50 μg/mg protein). The reaction was started by the addition of 20 μL (80–100 μg protein) of rapidly thawed (37°) vesicles to 80 μL of incubation medium at 37°. After 15 sec, 1.0 mL of ice-cold stop solution containing (mM) 50 sucrose, 10 Hepes–Tris, pH 7.4, 100 KCl, 0.2 CaCl, 10 MgCl was added. The diluted sample was immediately filtered and the filter washed three times with 3 mL ice-cold stop solution. Radioactivity of the filter was measured in a β-counter (Kontron). Non-specific binding to the membrane was determined in each experiment and at each BSP concentration by the addition of ice-cold incubation solution and ice-cold stop solution to 20 μL of vesicles kept at 4°. This value was subtracted from all the determinations. Unless otherwise indicated, all incubations were performed in triplicate and all the observations repeated with two or more separate plasma membrane vesicle preparations.

DBSP inhibition was studied by measuring [^{35}S]-BSP uptake at two different concentrations (5 and 20 μM) in the presence of different amounts of DBSP to a final concentration of 2, 4, 8, 16 and 24 μM DBSP.

Measurement of the dissociation constant (K_d) *of BSP and DBSP for bilitranslocase.* Purified preparations of bilitranslocase were obtained starting from liver plasma membrane fraction as described previously [20, 21]. Protein homogeneity was investigated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) performed as described by Laemmli [22]. Samples (80 ng protein) were boiled for 3 min in the presence of 1% 2-mercaptoethanol and 1% SDS. Staining was performed with Comassie brilliant blue. Electrophoretic blotting was carried out according to Towbin *et al.* [23]. Polyclonal monospecific anti-bilitranslocase antibodies were obtained from New Zealand rabbits as described previously [20, 24]. BSP and DBSP K_d was measured by incubating 65 μg purified bilitranslocase for 15 min at 37° with increasing concentrations (0.5–20 μM) of BSP or DBSP in 20 mM Tris–HCl buffer, pH 8.0. Final volume was 1.0 mL. The unbound dye fraction was separated by centrifugation as reported for plasma proteins with a YMT membrane with a cut-off of 10 kDa of molecular mass. BSP and DBSP content was measured spectrophotometrically in the ultrafiltrate upon alkalinization, as indicated for plasma proteins. The K_d value of the high affinity, low capacity binding site was determined according to Scatchard assuming a model with two categories of binding sites by the Enzfitter program (Sigma).

Statistical analysis. Data are expressed as means ± SD. Student's *t*-test was used to compare differences between different values. P value less than 0.01 was considered statistically significant.

RESULTS

Figure 1 shows the molecular structure of the two dyes studied in their protonated (colorless) and deprotonated (colored) forms. Though very similar in molecular structure, the two molecules differ in terms of pK_a of the carboxylic group (8.6 vs 9.2 for BSP and DBSP, respectively), and apparent octanol:water partition coefficient (0.283 ± 0.03 vs 0.118 ± 0.02, N = 6, BSP and DBSP, respectively,

Table 1. Plasma clearance (Cl), plasma removal rate (V), plasma distribution volume (V_d) and dissociation constant with plasma proteins of BSP and DBSP

	BSP	DBSP
Cl (mL/min)	57 ± 6*	38 ± 4
V (μmol/min/kg)	2.02 ± 0.16*	1.49 ± 0.11
V_d (mL/kg body wt)	42 ± 2	38 ± 6
K_d (μM)	0.95 ± 0.02*	1.44 ± 0.14

Each number refers to six experiments. Data are expressed as means ± SD.
* $P < 0.001$.

$P < 0.001$). Thus BSP is more hydrophobic than DBSP.

Table 1 reports the plasma clearance (Cl), plasma removal rate (V), plasma distribution volume (V_d) and dissociation constant of the high affinity, low capacity binding sites for plasma proteins for BSP and DBSP. While the distribution volume was comparable for the two anions, both plasma clearance and removal rate were significantly higher ($P < 0.001$) for BSP than for DBSP. Conversely, the K_d for plasma proteins was lower ($P < 0.001$) for BSP than for DBSP, indicating a greater affinity of BSP for plasma proteins. As these values are very similar to that reported for rat albumin using a different technique [25], it may be concluded that in plasma, BSP and DBSP bind primarily to albumin.

Figure 2 shows that the kinetics of the BSP uptake sensitive to membrane potential (electrogenic) in liver plasma membrane vesicles follows a saturable, carrier-mediated process. By plotting the data according to the Lineweaver–Burk plot (inset), an apparent K_m of 5.20 ± 0.80 μM and a V_{max} of 1.10 ± 0.10 nmol/mg protein/15 sec were derived. The K_m value obtained agrees with data previously obtained both with freshly isolated rat hepatocytes [26] and liver plasma membrane vesicles by using a dual wavelength spectrophotometric technique [16].

Since no labeled DBSP is available, thus preventing a direct measurement of its uptake in membrane vesicles, we examined the competition exerted by DBSP on BSP uptake. Addition of increasing concentrations of DBSP reduced the electrogenic BSP uptake as measured at two concentrations (5 and 20 μM). Figure 3 shows the Dixon plot obtained from these experiments. DBSP induced competitive inhibition of BSP uptake with a K_i of 18.2 ± 1.2 μM. The competitive type of inhibition suggests that the two organic anions share a common transport system.

As the electrogenic transport of BSP has been reported to occur via bilitranslocase [20, 27, 28], the affinity of the two phtaleins for this membrane carrier protein was investigated. The apparent dissociation constant (K_d) of both dyes was measured in purified preparations of the plasma membrane protein. The preparation was homogeneous on SDS–PAGE with a single band with an apparent molecular mass of 37 kDa. The band gave a positive reaction when immunoblotted with polyclonal monospecific

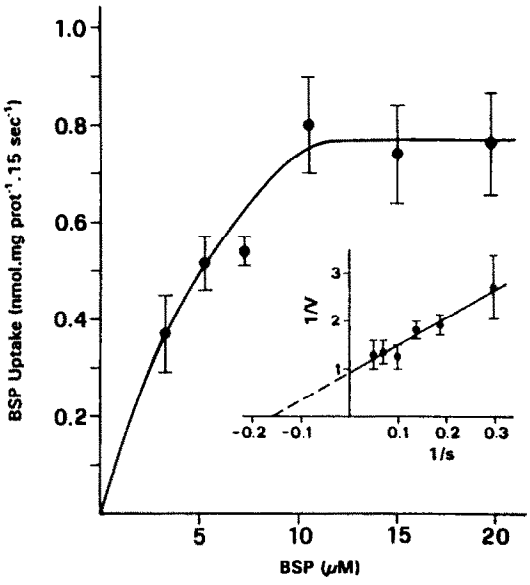

Fig. 2. Uptake rate (V, nmol BSP/mg protein/15 sec) by liver cell plasma membrane vesicles as function of BSP concentration (μM). Inset: double reciprocal plot according to Lineweaver–Burk of the data in the figure. Experimental conditions: vesicles (80 μg protein) were added to a medium containing (mM) 50 sucrose, 10 Hepes–Tris, pH 7.4, 100 KCl, 0.2 CaCl, 10 MgCl, 10 μCi [^{35}S]BSP and different concentrations of unlabeled BSP, and valinomycin (final concentration 50 μg/mg protein). Each point represents the mean ± SD of three experiments.

antibilitranslocase antibodies. Figure 4 shows the Scatchard plot obtained by measuring the binding to bilitranslocase of BSP (left panel) and DBSP (right panel). In both cases, binding was accounted for by two classes of binding sites. The high affinity, low capacity binding site showed an apparent K_d of 1.10 ± 0.12 and 3.02 ± 0.27 μM (N = 3) for BSP and DBSP, respectively ($P < 0.001$). In contrast, the affinity of the low affinity, high capacity sites was comparable for the two anions (30 ± 3 for BSP, and 32 ± 3 nmol/mg protein for DBSP.

DISCUSSION

BSP and DBSP are phthalein dyes which have been extensively used in assessing liver function in humans as well as in the study of hepatic transport of foreign substances in the experimental animal [3]. While in terms of chemical structure they only differ in the number of bromide ions on the benzenic ring, hepatocellular and biliary metabolism are remarkably different for the two organic anions. BSP is conjugated with glutathione before biliary excretion [5] whereas more than 90% of DBSP has been reported to reach the bile without metabolic transformation [6]. In contrast, it is generally agreed with the two substrates share a common uptake system [2, 4, 28]. Recent evidence suggests, however, that the transport of DBSP may also occur via an ATP-dependent mechanism [8, 29]. In particular, in

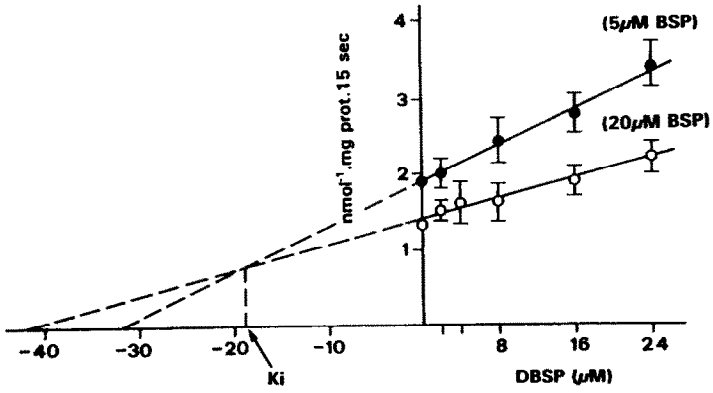

Fig. 3. Dixon plot of the inhibition induced by increasing concentrations of DBSP on BSP uptake by liver plasma membrane vesicles. BSP uptake was measured at two different concentrations: 20 (open circles) and 5 (closed circles) μM. Experimental conditions as in Fig. 2.

the isolated perfused rat liver, the uptake portion seems to be linked to energy supply whereas ATP only marginally affects efflux from the cell to the perfusate [29]. This latter finding indicates, in addition, that two different transport mechanisms are possibly involved in the uptake and efflux of DBSP.

BSP is transported across the sinusoidal plasma membrane via a carrier-mediated mechanism, and putative plasma membrane proteins have been identified for the uptake process [28, 30, 31]. Among them, bilitranslocase has been characterized [21], and experimental evidence was provided for its involvement in accounting for the electrogenic portion of the hepatic uptake of BSP [16, 20, 27] and related compounds [18]. Monospecific antibodies against bilitranslocase inhibit BSP transport in plasma membrane vesicles obtained from either the liver [20] or renal tubules [24]. Bilitranslocase has also been shown to be involved in the hepatic uptake of DBSP as, in isolated perfused rat liver, antibodies against this membrane protein inhibited the

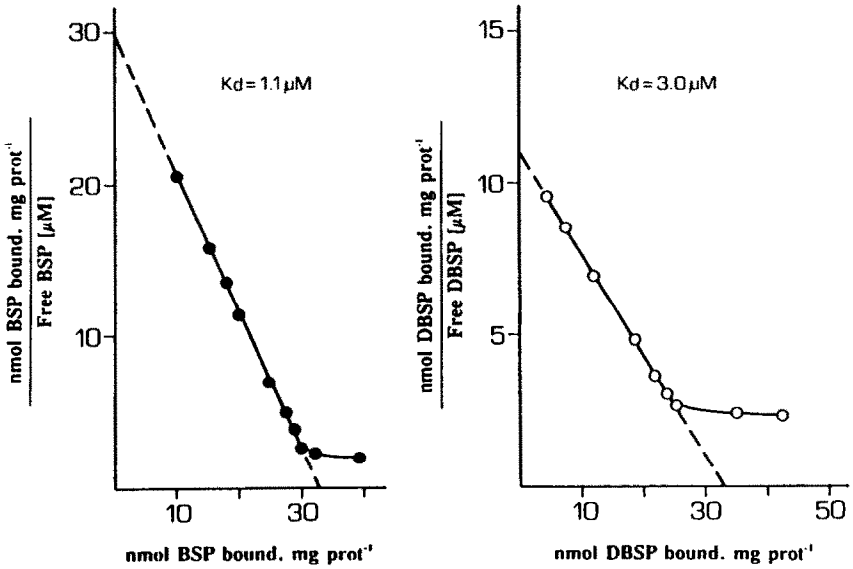

Fig. 4. Scatchard plot of binding to purified preparations of bilitranslocase of BSP (left panel) and DBSP (right panel). Calculation of the dissociation constant (K_d) and capacity (nmol/mg protein) for the high affinity, low capacity binding site was derived by the best-fitting curve with the Enzfitter program for a model assuming two categories of binding site. BSP and DBSP K_d was measured by incubating for 15 min at 37° 65 μg purified bilitranslocase with increasing concentrations (0.5–20 μM) of BSP and DBSP in 20 mM Tris–HCl buffer, pH 8.0. The unbound dye fraction was separated by centrifugation, and the dye content measured spectrophotometrically in the ultra-filtrate upon alkalinization. Please note the difference in *y*-axis scale between BSP and DBSP.

hepatocytic uptake and efflux, but not the biliary excretion of the cholephilic dye [7]. On this basis, it was concluded that BSP and DBSP share a common uptake system mediated, at least in part, by bilitranslocase. Data reported further support this conclusion, with the demonstration of a competitive inhibition of DBSP on the electrogenic portion of BSP uptake.

The efficiency of the transport, however, seems to be different for the two anions. *In vivo*, the plasma clearance is significantly lower for DBSP than BSP, in spite of a higher concentration of free DBSP as indicated by the lower affinity (higher K_d) of the former dye for plasma proteins. The finding of more rapid BSP plasma removal may be accounted for by a higher affinity (lower apparent K_d) of this organic anion for bilitranslocase, a plasma membrane carrier protein involved in the electrogenic transport of BSP and related cholephilic anions [18] which apparently compensates for the higher plasma protein binding. The different affinity for the purified transport protein agrees with the different affinity observed in liver plasma membrane vesicles, as indicated by the K_i value. Of notice is the comparable difference existing between both the K_d of purified bilitranslocase and K_m/K_i value of the electrogenic transport in liver plasma membrane vesicles.

The K_d value and the number of moles bound observed for the BSP–translocator complex agrees with those reported previously [25]. The difference between the two dyes involves the high affinity, low capacity binding sites, since a comparable value was observed in the low affinity, high capacity sites.

BSP and DBSP are two very closely related molecules differing only by the absence of two bromine ions in the 4 and 5 positions on the benzenic ring in the case of DBSP (Fig. 1). The lack of two bromines apparently exerts important effects on the physicochemical characteristics of the ligand. In addition to a different pK_a value for the carboxylic group (8.6 and 9.2 for BSP and DBSP, respectively), the higher degree of hydrophilicity and the lower affinity for both albumin and bilitranslocase found for DBSP support this conclusion.

The difference in degree of hydrophobicity of the two molecules may play a role in both binding to albumin and plasma membrane carrier protein(s). It has been reported that, at comparable substrate concentrations, a linear correlation exists between liposolubility of the various organic anions and the efficiency of hepatic uptake [32]. This finding may explain further why, in spite of a lower plasma concentration of the free anion, the BSP plasma removal rate is more efficient.

From these data we conclude that BSP and DBSP are taken up by the liver via a common transport system, mediated at least in part by bilitranslocase. The contribution of other transport systems, and their possible dependence on ATP, should be considered also. The efficiency of the uptake is, however, different for the two cholephilic anions. BSP is removed from plasma significantly faster, in spite of a lower concentration of the free anion. The higher affinity for the plasma membrane carrier molecule for BSP probably accounts for the difference in the overall process. In addition, these data indicate also that apparently small differences in chemical structure between two structurally related molecules may considerably affect their hepatic transport and metabolism.

Acknowledgements—A.T. and J.R. were recipients of a fellowship from the Third World Academy of Sciences. J.R. was partially supported by a grant from Fondo Studio Fegato, Trieste, Italy while A.T. was recipient of a research fellowship from Consorzio Sviluppo Internazionale Università di Trieste. Supported by grants from Ministero Università e Ricerca Scientifica (60%), Roma, Italy.

REFERENCES

1. Rosental SM and White EC, Clinical application of the bromosulphthalein test for hepatic function. *J Am Med Assoc* **84**: 1112–1114, 1925.
2. Blom A, Keulemans K and Meijer DKF, Transport of dibromosulphtalein by isolates rat hepatocytes. *Biochem Pharmacol* **30**: 1809–1816, 1981.
3. Meijer DKF and Groothuis GMM, Hepatic transport of drugs and proteins. In: *Oxford Textbook of Clinical Hepatology* (Eds. McIntyre N, Benhamou JP, Bircher J, Rizzetto M and Rodes J), pp. 40–78. Oxford University Press, Oxford, 1991.
4. Schwenk M, Burr R, Schwarz L and Pfaff E, Uptake of sulfobromophthalein by isolated liver cells. *Eur J Biochem* **4**: 189–197, 1976.
5. Wheeler HO, Meltzer JI and Bradley SE, Biliary transport and hepatic storage of sulfobromophthalein. *J Clin Invest* **39**: 1131–1144, 1970.
6. Javitt NB, Phenol 3,6-dibromophthalein disulfate, a new compound for the study of liver disease. *Proc Soc Exp Biol Med* **119**: 254–257, 1964.
7. Tiribelli C, Meijer DKF, Lunazzi GC, Hardonk MJ and Sottocasa GL, Specific inhibition of the hepatic uptake of dibromosulphthalein by anti-bilitranslocase antibody. *Hepatology* **2**: 749, 1982.
8. Yamazaki M, Suzuki H, Sugiyama Y, Iga T and Hanano M, Uptake of organic anions by isolated rat hepatocytes. A classification in terms of ATP dependency. *J Hepatol* **14**: 41–46, 1992.
9. Orzes N, Bellentani S, Aldini R, Simoni P, Ferretti I, Lunazzi GC, Sottocasa GL and Tiribelli C, Sex difference in the hepatic uptake of sulphobromophthalein in the rat. *Clin Sci* **69**: 587–583, 1985.
10. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klein DC, Measurement of protein using bicinchoninic acid. *Anal Biochem* **150**: 76–85, 1985.
11. van Amelsvoort JMM, Sips HJ and van Dam K, Sodium-dependent alanine transport in plasma membrane vesicles from rat liver. *Biochem J* **174**: 1083–1086, 1978.
12. Schoner W, Von Ilberg C, Kramer R and Seubert W, On the mechanism of Na^+ and K^+-stimulated hydrolysis of adenosine triphosphate. *Eur J Biochem* **1**: 334–343, 1967.
13. Sottocasa GL, Kuieleastierna B and Ernster L, An electron-transport system associated with the outer membrane liver mitochondria. *J Cell Biol* **32**: 415–438, 1967.
14. Emmelot P, Bos CJ, Benedetti EL and Rumke PH, Studies on plasma membranes: chemical composition and enzyme content of plasma membranes isolated from rat liver. *Biochim Biophys Acta* **90**: 126–145, 1964.
15. Widnell CB, Purification of rat liver 5′-nucleotidase as a complex with sphingomyelin. *Methods Enzymol* **32**: 368–374, 1974.

16. Baldini G, Passamonti S, Lunazzi GC, Tiribelli C and Sottocasa GL, Cellular localization of sulfobromophthalein transport activity in rat liver. *Biochim Biophys Acta* **856**: 1–10, 1986.
17. Miccio M, Orzes N, Lunazzi GC, Gazzin B, Corsi R and Tiribelli C, Reversal of ethinylestradiol-induced cholestasis by epomediol in rat. *Biochem Pharmacol* **38**: 3559–3563, 1989.
18. Torres AM, Rodriguez JV, Lunazzi GC and Tiribelli C, Carrier-mediated transport of tetrabromosulfonephthalein by rat liver plasma membrane vesicles. *Am J Physiol (Gastrointestinal Liver Physiol)* **263**: G338–G344, 1992.
19. Kurisu H, Nilprabhassorrn P and Wolkoff AW, Preparation of ^{35}S-sulfobromophthalein of high specific activity. *Anal Biochem* **179**: 72–74, 1989.
20. Miccio M, Baldini G, Basso V, Gazzin B, Lunazzi GC, Tiribelli C and Sottocasa GL, Bilitranslocase is the protein responsible for the electrogenic movement of sulfobromophthalein in plasma membrane vesicles from rat liver: immunochemical evidence using mono- and poly-clonal antibodies. *Biochim Biophys Acta* **981**: 115–120, 1989.
21. Sottocasa GL, Lunazzi GC and Tiribelli C, Isolation of bilitranslocase, the anion transporter from liver plasma membrane for bilirubin and other organic anions. *Methods Enzymol* **174**: 50–57, 1989.
22. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
23. Towbin H, Stheilin T and Gordon J, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350–4354, 1979.
24. Elias MM, Lunazzi GC, Passamonti S, Gazzin B, Miccio M, Stanta G, Sottocasa GL and Tiribelli C, Bilitranslocase localization and function in basolateral plasma membrane of renal proximal tubule in rat. *Am J Physiol (Renal Fluid Electrolyte Physiol)* **259**: F559–F564, 1990.
25. Gentile S, Bajema BL, Baldini G, Lunazzi GC, Groothuis GMM, Tiribelli C, Meijer DKF and Sottocasa GL, Measurement of the association of cholephilic organic anions with different binding proteins. *Biochem Pharmacol* **34**: 2439–2444, 1985.
26. Persico M, Bellentani B, Marchegiano P, Orzes N, Lunazzi GC, Sottocasa GL and Tiribelli C, Sex steroids modulation of the hepatic uptake of organic anions in the rat. *J Hepatol* **6**: 343–349, 1988.
27. Tiribelli C, Determinants in the hepatic uptake of organic anions. *J Hepatol* **14**: 385–390, 1992.
28. Tiribelli C, Lunazzi GC and Sottocasa GL, Biochemical and molecular aspects of the hepatic uptake of organic anion transport. *Biochim Biophys Acta* **1031**: 261–275, 1990.
29. Nijssen HMJ, Pijning T, Meijer DKF and Groothuis GMM, Mechanistic aspects of uptake and sinusoidal efflux of dibromosulfophthalein in the isolated perfused rat liver. *Biochem Pharmacol* **42**: 1997–2002, 1991.
30. Berk PD, Potter BJ and Stremmel W, Role of plasma membrane ligand-binding proteins in the hepatocellular uptake of albumin-bound organic anions. *Hepatology* **7**: 165–176, 1987.
31. Wolkoff AW and Chung CT, Identification, purification and partial characterization of an organic anion binding protein from rat liver cell plasma membrane. *J Clin Invest* **65**: 1152–1161, 1980.
32. Tokumo H, Aoyama N, Busch N, Mancuso DJ and Holzbach RT, Hepatic extraction of organic anions in the rat depends upon ligand hydrophobicity. *Hepatology* **13**: 62–67, 1991.