

Role of Chloride and Intracellular pH on the Activity of the Rat Hepatocyte Organic Anion Transporter

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Abstract

Previous studies in cultured rat hepatocytes revealed that initial uptake of sulfobromophthalein (BSP) was markedly reduced upon removal of Cl^- from the medium. In the present study, unidirectional Cl^- gradients were established in short-term cultured rat hepatocytes and their effect on BSP uptake was determined. These investigations revealed that BSP uptake requires external Cl^- and is not stimulated by unidirectional Cl^- gradients, suggesting that BSP transport is not coupled to Cl^- transport. In contrast, BSP transport is stimulated by an inside-to-outside OH^- gradient, consistent with OH^- exchange or H^+ cotransport. As the presence of Cl^- is essential for but not directly coupled to BSP transport, binding of ^{35}S -BSP to hepatocytes was determined at 4°C . This revealed an ~ 10 -fold higher affinity of cells for BSP in the presence as compared to the absence of Cl^- ($K_a = 3.2 \pm 0.8$ vs. $0.42 \pm 0.09 \mu\text{M}^{-1}$; $P < 0.02$). Affinity of BSP for albumin was Cl^- -independent, and was $\sim 10\%$ of its affinity for cells in the presence of Cl^- . These results indicate that extracellular Cl^- modulates the affinity of BSP for its hepatocyte transporter. (*J. Clin. Invest.* 1991. 87:1496–1502.) Key words: sulfobromophthalein • cultured hepatocytes • transport • albumin binding • chloride transport

Introduction

The organic anions bilirubin and sulfobromophthalein (BSP)¹ circulate tightly bound to albumin (1, 2) and are extracted rapidly from this carrier by hepatocytes (3, 4). After transport into cell cytosol, they bind to soluble proteins, primarily glutathione (GSH)-S-transferases (5, 6) prior to conjugation and excretion into bile. Uptake of bilirubin and BSP has kinetics suggesting carrier mediation. Although several putative transport proteins have been identified (7–11), their function in organic anion transport is not well defined. Previous studies in cultured rat hepatocytes and isolated perfused rat liver have revealed that initial uptake of these organic anions is markedly reduced upon removal of Cl^- from the medium (12). There is no requirement for specific inorganic cations, as initial uptake in the

presence of Na^+ , K^+ , or Li^+ is unchanged (12, 13). The mechanism by which inorganic anions such as Cl^- influence hepatocyte organic anion uptake is not known. A Na^+ -independent Cl^- transport system was previously described in intact rat liver and cultured rat hepatocytes (14, 15), but its possible relationship to Cl^- -dependent organic anion uptake remains unknown.

The present investigation was undertaken to determine whether hepatocyte BSP transport is linked to Cl^- transport. Procedures have been devised to establish transient unidirectional Cl^- gradients in short-term cultured rat hepatocytes and the effect of such gradients on initial uptake of ^{35}S -BSP has been determined.

Methods

Isolation and short-term culture of rat hepatocytes

Rat hepatocytes were isolated from 200–250-g male Sprague-Dawley rats (Taconic Farms, Germantown, NY) after perfusion of the liver with collagenase A (Boehringer Mannheim Biochemicals, Indianapolis, IN). Cells were suspended in medium consisting of Waymouth's 752/1 (Gibco, Grand Island, NY) containing 25 mM Hepes, pH 7.2, 5% heat-inactivated FBS (Hyclone Labs Inc., Logan, UT), 1.7 mM additional CaCl_2 , 5 $\mu\text{g}/\text{ml}$ bovine insulin (Sigma Chemical Co., St. Louis, MO), 100 U/ml penicillin (Gibco), and 0.1 mg/ml streptomycin (Gibco). $\sim 1.5 \times 10^6$ cells in 3 ml were placed in 60-mm Lux culture dishes (Nunc, Inc., Naperville, IL), and cultured in 5% CO_2 atmosphere at 37°C . ~ 2 h later, medium was changed and cells were cultured for an additional 16–18 h (12, 16). Cell viability was $> 90\%$ as judged by Trypan blue exclusion.

Uptake of ^{35}S -BSP by cultured hepatocytes

Uptake of ^{35}S -BSP by overnight cultured hepatocytes was determined as previously described (12). In some experiments, uptake of BSP was determined in cultured cells within 1–2 h of cell isolation. In brief, cells were washed twice with 1.5 ml of modified serum-free medium (SFM), consisting of 135 mM NaCl, 1.2 mM MgCl_2 , 0.81 mM MgSO_4 , 27.8 mM glucose, 2.5 mM CaCl_2 , and 25 mM Hepes adjusted to pH 7.2 with solid Tris base. 1 ml of 0.1% (14.7 μM) BSA (fraction V, Sigma Chemical Co.) in SFM was added to each plate, which was then incubated for 15 min at 4 or 37°C . ^{35}S -BSP (2,500–4,000 mCi/mmol) was prepared as described previously (17) and was dissolved in distilled water. Sufficient unlabeled BSP was added to make an 80- μM BSP stock solution containing $\sim 10^7$ dpm/ml. A 10- μl aliquot of this stock solution was added to 1 ml of albumin-containing medium on each plate and incubation was continued at 4 or 37°C for various periods. After incubation with ^{35}S -BSP, plates were washed twice at 4°C with 1.5 ml of SFM and incubated for 5 min in 1.5 ml of 5% BSA in 20 mM PBS, pH 7.4 at 4°C to displace surface-bound radioactivity. Plates were then washed three times with 1.5 ml of SFM at 4°C . Cells were harvested, and radioactivity was quantified in a RackBeta model 1217 liquid scintillation counter (LKB Instruments, Gaithersburg, MD), after addition of 10 ml of Hydrofluor (National Diagnostics, Inc., Somerville, NJ). Replicate plates were washed and harvested in PBS for determination of cellular protein by the method of Lowry et al. (18), or by the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL) using BSA as standard.

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1. Abbreviations used in this paper: BSP, sulfobromophthalein; GSH, glutathione; SFM, serum-free medium.

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Effect of valinomycin, salt substitution, and osmolarity on initial uptake of ^{35}S -BSP

Medium was prepared in which NaCl was substituted isosmotically by KCl. Previous studies in which hepatocytes were depolarized in KCl-substituted medium, suggested that BSP uptake was not electrogenic (12). To confirm these findings, cultured hepatocytes were preincubated for 15 min at 37°C in NaCl-containing medium. Initial uptake of ^{35}S -BSP was determined over 5 min in KCl-substituted medium in the presence or absence of 10 μM valinomycin. Uptake in the absence of valinomycin was also determined in choline chloride and NaI-substituted media. Previous studies (12) revealed a marked reduction in initial BSP uptake upon isosmotic substitution of NaCl in medium by sucrose. Chloride-free, sucrose-substituted medium may lead to osmotic shrinkage of cells (19, 20). To determine the influence of cell shrinkage on initial BSP uptake, studies were also performed in cells preincubated for 15 min at 37°C in normal NaCl-containing medium to which 1 M mannitol had been added.

Uptake of ^{36}Cl by short-term cultured rat hepatocytes

H^{36}Cl (19.4 mCi/g) was purchased from New England Nuclear, Boston, MA. Cultured hepatocytes were washed twice at room temperature with 1.5 ml of sucrose-substituted medium and incubated for 30 min at 37°C. They were then washed twice with 1.5 ml of sucrose-substituted medium at 37 or 4°C, and incubated for varied times in 1 ml of NaCl-containing medium with ^{36}Cl (1 $\mu\text{Ci}/\text{ml}$) at 37 or 4°C. The medium was removed, and cells were washed with ice-cold NaCl-containing medium. Preliminary studies were performed to determine the number of washes necessary to remove surface-adsorbed ^{36}Cl . Cells were then harvested, and radioactivity was determined. To insure that cell-associated ^{36}Cl was free and not membrane-bound, cells were disrupted by sonication after washing and centrifuged at 100,000 g for 60 min. Radioactivity was determined in the supernatant and pellet.

Efflux of ^{36}Cl from cultured hepatocytes

Previous studies indicated that incubation of cells (14, 15) or perfused liver (21, 22) in Cl^- -free medium depleted both intracellular and extracellular Cl^- . To deplete intracellular Cl^- , cells were incubated in sucrose-substituted medium for 30 min at 37°C. They were then loaded with ^{36}Cl by incubation for 60 min at 37°C in 1 ml of NaCl-containing medium to which was added 2 μCi of ^{36}Cl . Preliminary studies revealed that cell-associated ^{36}Cl was maximal by this time. Cells were then washed five times with 1.5 ml of ice-cold sucrose-substituted medium. Efflux of ^{36}Cl from these preloaded cells was instituted after incubation at 4 or 37°C for various times in 1 ml of sucrose-substituted medium. Cells were then washed five times with 1.5 ml of ice-cold NaCl-containing medium, harvested, and radioactivity was determined.

Influence of Cl^- gradients on uptake of ^{35}S -BSP

To examine whether chloride gradients influence hepatic organic anion uptake, initial uptake of ^{35}S -BSP was determined under four conditions: (a) normal content of extracellular Cl^- (Cl_e) and intracellular Cl^- (Cl_i); (b) $\text{Cl}_\text{i} > \text{Cl}_\text{e}$; (c) $\text{Cl}_\text{e} > \text{Cl}_\text{i}$; and (d) depletion of both Cl_i and Cl_e . Cultured hepatocytes were washed and incubated in 3 ml of either NaCl-containing or sucrose-substituted medium at 37°C for 30 min. Each group of cells was then washed twice with 1.5 ml of the initial medium, followed by incubation with 0.1% BSA and 0.8 μM ^{35}S -BSP in 1 ml of either NaCl-containing or sucrose-substituted medium at 4 or 37°C for 1 min. After washing twice with 1.5 ml of ice-cold SFM, cells were incubated in 1.5 ml of 5% BSA at 4°C for 5 min. Cells were washed three times with 1.5 ml of SFM at 4°C, harvested, and radioactivity was determined.

Binding of ^{35}S -BSP to the surface of hepatocytes

Binding studies were performed at BSP concentrations of 0.08–3.2 μM at 4°C in the presence of a 15-fold molar excess of BSA, keeping the ratio of albumin to BSP constant to minimize changes in the fraction of unbound ligand (23). Cultured cells were washed three times with 1.5

ml of ice-cold NaCl-containing or sucrose-substituted media, and incubated with 1 ml of the appropriate mixture of ^{35}S -BSP and BSA at 4°C for 1 min. They were quickly washed three times with 1.5 ml of SFM, harvested, and radioactivity was determined.

Effect of Cl^- on binding of ^{35}S -BSP to BSA

Binding of ^{35}S -BSP to BSA was studied by two ultrafiltration techniques. Results from each method were previously demonstrated to agree with those obtained by equilibrium dialysis (24–26), and data obtained by the two methods was identical.

Pressure ultrafiltration. Each chamber of a 22-channel plexiglas apparatus designed by Schramm (24) was loaded with 12 pmol of ^{35}S -BSP with various amounts of unlabeled BSP and 0.1% BSA in 150 μl of NaCl-containing or sucrose-substituted medium. Upper and lower chambers were separated by dialysis membranes with molecular weight cutoff of 8,000 (Spectrum Medical Industries, Inc., Los Angeles, CA). The apparatus was pressurized to 22 psi with N_2 at 4°C and the upper chambers were free of liquid after 8–10 h. The membranes were washed thoroughly with ice-cold ethylene glycol, and radioactivity associated with dialysis membranes and aliquots of the ultrafiltrate was determined.

Centrifugal ultrafiltration. Alternatively, ^{35}S -BSP bound to BSA was determined using a Centrifree Micropartition System (Amicon, Danvers, MA) fitted with a 30,000 mol wt cutoff membrane. 80 pmol of ^{35}S -BSP with various amounts of unlabeled BSP and 0.1% BSA in 500 μl of NaCl-containing or sucrose-substituted medium was loaded onto each Centrifree reservoir, which was fitted into an SS-34 rotor (Sorvall Instruments, Newton, CT) and centrifuged at 4°C at 4,200 rpm for 10 min. Radioactivity in 50- μl aliquots of the ultrafiltrates was determined.

Saturation kinetics of ^{35}S -BSP uptake by cultured hepatocytes

Saturation kinetics of BSP uptake were studied in the presence and absence of chloride. These studies were performed at BSP concentrations of 0.08–1.6 μM as described previously (12) while keeping the ratio of BSA and BSP constant. Initial uptake of ^{35}S -BSP was determined at 4 and 37°C over 5 min in NaCl-containing or sucrose-substituted medium. In these studies, residual cell-associated radioactivity at 4°C was subtracted as a blank from cell-associated radioactivity at 37°C (12). Data was computer analyzed by a nonlinear least squares routine (SigmaPlot version 4.0, Jandel Scientific, Corte Madera, CA) to obtain K_m and V_{max} .

Effect of pH on initial uptake of ^{35}S -BSP and ^{36}Cl by cultured hepatocytes

Modified serum-free medium was prepared as above except that the pH of this Hepes-buffered medium was adjusted to pH 6.0 or 8.0 with 1 N NaOH rather than with Tris base. Cultured cells were washed twice with 1.5 ml of either pH 6.0 or 8.0 medium and incubated for 30 min at 37°C in 1 ml of the corresponding medium. In previous studies, we found that under these conditions, the intracellular pH equilibrated with the extracellular pH (27). Unidirectional pH gradients were formed by rapidly incubating these OH^- -loaded or -depleted cells in medium of the opposite pH. As shown previously, under these conditions, a pH gradient is maintained for several minutes (27). To perform an uptake study at 37°C, the medium was aspirated and 1 ml of medium containing ^{36}Cl or ^{35}S -BSP in 0.1% BSA at the required pH was added and initial uptake determined over 5 min as described above. To determine cell-associated ^{36}Cl or ^{35}S -BSP at 4°C, after the initial 30 min incubation at 37°C, cells were incubated at 4°C for 10 min to insure that they were at the required temperature, and initial uptake was determined as for cells at 37°C.

Statistical analysis

Results are expressed as mean \pm SEM. Significance was determined by Student's paired or unpaired t tests as appropriate (28). Ligand binding

data was analyzed by the LIGAND computer program of Munson and Rodbard (29).

Results

Effects of inorganic cation and anion substitution on initial uptake of ^{35}S -BSP. As previously reported (12), initial uptake of ^{35}S -BSP was unaffected by isosmotic substitution of NaCl by KCl, LiCl, choline Cl, or NaI (Fig. 1), but was significantly reduced in NaHCO_3 and Na gluconate. Isosmotic replacement of NaCl in medium by sucrose resulted in an 80% reduction in initial uptake of ^{35}S -BSP by short-term cultured hepatocytes. An identical effect was seen in hepatocytes cultured for only 1–2 h after preparation with an initial BSP uptake of $25 \pm 12\%$ of control with sucrose substitution as compared to that in NaCl medium ($n = 3$, $P < 0.03$). Because sucrose substitution could lead to cell shrinkage, initial uptake of BSP was determined in cells preincubated in NaCl-containing medium to which 1 M mannitol had been added. In this high osmolarity medium, initial uptake of BSP was $80 \pm 4\%$ of control ($n = 3$, $P > 0.1$), as compared to a reduction to $25 \pm 3\%$ of control with sucrose substitution ($n = 3$, $P < 0.01$). We have previously demonstrated that, as expected, short-term cultured rat hepatocytes are depolarized in KCl-substituted medium (12). Normal BSP uptake in this situation suggests that the process is electroneutral. In confirmation of these findings, initial ^{35}S -BSP uptake in KCl-substituted medium containing $10 \mu\text{M}$ valinomycin was $90 \pm 8\%$ ($n = 3$, $P > 0.05$) of initial uptake under control conditions.

Uptake of ^{36}Cl by cultured hepatocytes. After a 30-min incubation in $135 \text{ mM Na}^{36}\text{Cl}$ at 37°C , extracellular and cell-surface adsorbed ^{36}Cl were maximally removed with five 1.5-ml washes in NaCl-containing medium at 4°C (Fig. 2). There was no reduction in residual cell-associated radioactivity with as many as nine total washes. In all subsequent ^{36}Cl studies, cells were washed five times at 4°C . Initial uptake of ^{36}Cl by cells preincubated in NaCl-depleted medium for 30 min at 37°C

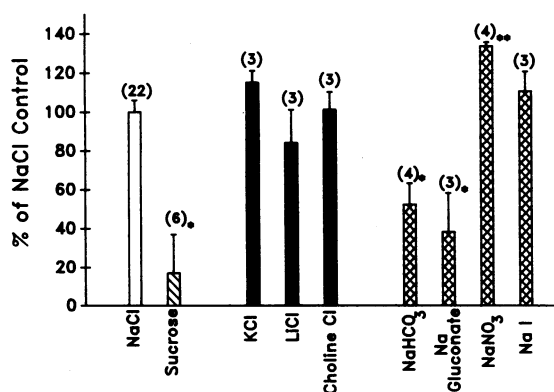


Figure 1. Effects of ion substitution on initial uptake of ^{35}S -BSP by short-term cultured rat hepatocytes. Short-term cultured rat hepatocytes were preincubated at 37°C in control medium containing 135 mM NaCl or medium in which NaCl was isosmotically substituted by the indicated compound. Initial uptake of ^{35}S -BSP ($0.8 \mu\text{M}$) in the presence of an 18-fold molar excess of BSA was then determined as described in Methods and expressed as a percent of that in NaCl medium. Data represent mean \pm SEM. The number of studies is in parentheses. This figure represents a compilation of data presented in reference 12 and new data from this study. * $P < 0.001$. ** $P < 0.002$.

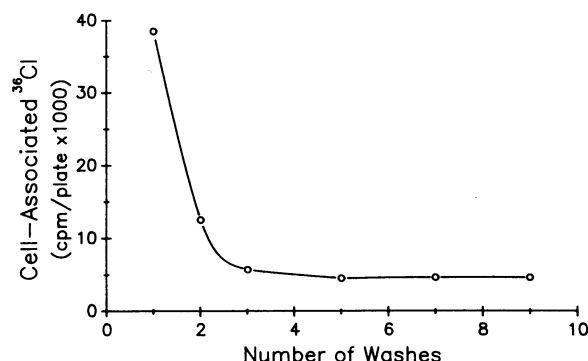


Figure 2. Effect of number of washes on cell-associated ^{36}Cl . Cultured hepatocytes were washed with sucrose-substituted medium and incubated for 30 min in $135 \text{ mM Na}^{36}\text{Cl}$ at 37°C . After removal of the medium, cells were washed with ice-cold NaCl-containing medium. Extracellular and cell-surface adsorbed ^{36}Cl were maximally removed with five 1.5-ml washes. There was no reduction in residual cell-associated radioactivity with as many as nine total washes.

was temperature dependent with markedly reduced uptake at 4°C as compared to 37°C (Fig. 3 A). Equilibrium cell content of ^{36}Cl was attained by 30 min (Fig. 3 B), indicating relatively slow influx of this anion. In four studies, influx of ^{36}Cl over the initial 10 min at 37°C averaged $3.9 \pm 0.2\%$ of equilibrium Cl^- content per minute. This indicates that, over the first minute after incubation of Cl^- -depleted cells in Cl^- -containing medium, there is little change in cellular Cl^- content (Fig. 3 B). After incubation for 30 min in ^{36}Cl , disruption of cells by sonication and centrifugation at $100,000 g$ revealed that $> 95\%$ of radioactivity was present in the supernatant.

Efflux of ^{36}Cl from preloaded cultured hepatocytes. After 60 min preloading of Cl^- -depleted cells with ^{36}Cl , subsequent efflux was found to be temperature dependent, with little efflux at 4°C as compared to 37°C (Fig. 4). Similar to uptake, efflux was a relatively slow event, and at 1 min, over 75% of ^{36}Cl remained in hepatocytes (75 and 85% in two studies). This indicates that over the first minute after incubation of Cl^- -loaded cells in Cl^- -depleted medium, a large inside to outside Cl^- gradient is maintained.

Effect of Cl^- gradients on BSP uptake by hepatocytes. The preceding results indicate that transient unidirectional Cl^- gradients can be established in cultured hepatocytes. Initial uptake of ^{35}S -BSP was quantified at 4 or 37°C over 1 min under conditions in which extracellular Cl^- (Cl_e) and intracellular Cl^- (Cl_i) were varied as described above. Under control conditions, with normal content of Cl_e and Cl_i , initial uptake of ^{35}S -BSP by hepatocytes was $8.13 \pm 2.65 \text{ pmol/mg protein/min}$ ($n = 4$). As seen in Fig. 5, in the absence of extracellular Cl^- , initial uptake of ^{35}S -BSP was significantly reduced under conditions of normal ($n = 4$) or depleted intracellular Cl^- ($n = 4$) as compared with control ($P < 0.01$ and $P < 0.001$, respectively). Initial uptake of ^{35}S -BSP with $\text{Cl}_e > \text{Cl}_i$ did not differ significantly from control ($n = 4$, $P > 0.08$). These results indicate that there is no stimulation of BSP uptake by transmembrane Cl^- gradients, suggesting that BSP transport is not coupled to chloride transport.

Chloride-dependent binding of ^{35}S -BSP to the surface of cultured hepatocytes. To determine whether extracellular Cl^- might directly modulate the affinity of BSP for its hepatocyte

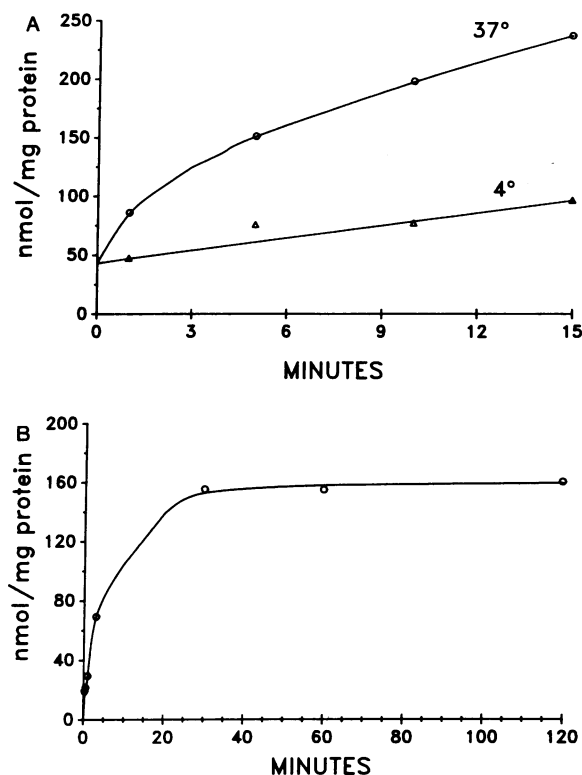


Figure 3. Uptake of ^{36}Cl by short-term cultured rat hepatocytes. Short-term cultured rat hepatocytes were incubated in sucrose-substituted medium for 30 min at 37°C. Cells were then incubated in 1 ml of 135 mM Na^{36}Cl (1 μCi). At the indicated times, medium was removed and cells were washed rapidly five times with 1.5 ml of ice-cold NaCl-containing medium. Cells were then harvested, and radioactivity was determined. (A) Initial uptake of ^{36}Cl at 4 and 37°C reveals temperature dependence. This is a representative study of four that were performed. (B) Prolonged study reveals that equilibrium was attained by 30 min. Initial influx of ^{36}Cl was relatively slow, and averaged $3.9 \pm 0.2\%$ of equilibrium Cl^- content per minute. This is a representative study of four that were performed.

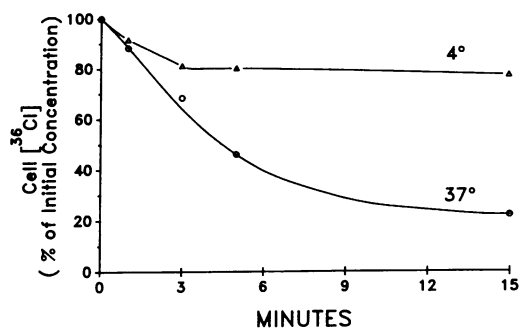


Figure 4. Efflux of ^{36}Cl from preloaded cultured hepatocytes. Cells were incubated in sucrose-substituted medium for 30 min at 37°C. They were then incubated for 60 min at 37°C in 1 ml of 135 mM Na^{36}Cl (2 μCi). They were washed with 1.5 ml of ice-cold sucrose-substituted medium five times and incubated for various times in this medium at 4 or 37°C. The fraction of initial cell-associated ^{36}Cl remaining in cells over time is plotted, and reveals that at 1 min, over 75% of ^{36}Cl remained in hepatocytes. This is a representative study of two that were performed.

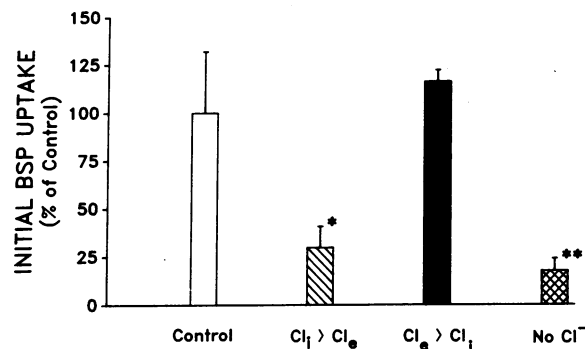


Figure 5. Effect of transmembrane Cl^- gradients on initial ^{35}S -BSP uptake by short-term cultured rat hepatocytes. Initial uptake of ^{35}S -BSP was determined over 1 min under four conditions: (a) control, normal content of extracellular Cl^- (Cl_e) and intracellular Cl^- (Cl_i), (b) $\text{Cl}_i > \text{Cl}_e$, (c) $\text{Cl}_e > \text{Cl}_i$, and (d) depletion of both intracellular and extracellular Cl^- (no Cl^-). Under control conditions with normal content of Cl_e and Cl_i , initial ^{35}S -BSP uptake was 8.13 ± 2.65 pmol/mg protein per min. In the absence of extracellular Cl^- , uptake of ^{35}S -BSP was reduced under conditions of normal or depleted intracellular Cl^- . Initial uptake of ^{35}S -BSP with $\text{Cl}_e > \text{Cl}_i$ did not differ significantly from control. There was no evidence for stimulation of BSP uptake by Cl^- gradients suggesting that BSP transport was not coupled to chloride transport. Data is presented as mean \pm SEM of four studies. * $P < 0.01$ as compared with control. ** $P < 0.001$ as compared with control.

transporter, binding of ^{35}S -BSP to hepatocytes was determined at 4°C in the presence of a 15-fold molar excess of albumin in NaCl-containing or sucrose-substituted media. Data was computer analyzed by the method of Munson and Rodbard (27) and, in both media, the fit was optimal when a single class of binding sites was used. Nonspecific binding is determined directly by this method, and was $0.11 \pm 0.06\%$ of total ligand in the presence of NaCl, and $0.07 \pm 0.04\%$ in sucrose-substituted medium ($P > 0.6$). The affinity of BSP for hepatocytes was significantly higher in the presence of Cl^- than in sucrose-substituted medium ($K_a = 3.2 \pm 0.8 \mu\text{M}^{-1}$ vs. $0.42 \pm 0.09 \mu\text{M}^{-1}$; $n = 5$, $P < 0.02$) (Fig. 6). Under these conditions, there was no difference in the number of binding sites (10.2 ± 1.7 vs. 12.4 ± 0.9 pmol/mg protein; $n = 5$, $P > 0.2$).

Equilibrium binding studies of ^{35}S -BSP to BSA. Changes in apparent affinity of ^{35}S -BSP to the cell surface could reflect altered affinity of this ligand for BSA. For this reason, binding of ^{35}S -BSP to BSA was quantified to determine whether Cl^- influences the affinity of albumin for BSP. No significant difference was observed in the affinity of BSA for BSP between NaCl-containing and sucrose-substituted media with K_a of $0.33 \pm 0.06 \mu\text{M}^{-1}$ and $0.59 \pm 0.08 \mu\text{M}^{-1}$, respectively ($n = 4$, $P > 0.1$).

Saturation kinetics of ^{35}S -BSP uptake. As seen in Fig. 7, in NaCl-containing medium, initial uptake of ^{35}S -BSP displayed saturation kinetics as previously described (12). With isosmotic substitution of NaCl by sucrose, there was little cell uptake at any concentration of ^{35}S -BSP. That is, the transport mechanism for BSP was functionally inactive.

Influence of cellular pH on uptake of ^{36}Cl and ^{35}S -BSP. Unidirectional pH gradients were formed to determine the effect of cellular pH on initial uptake of ^{36}Cl . There was no effect of inwardly or outwardly directed gradients of pH (6–8) on initial uptake of ^{36}Cl (26.0 ± 1.0 vs. 27.0 ± 5.0 nmol/min per mg pro-

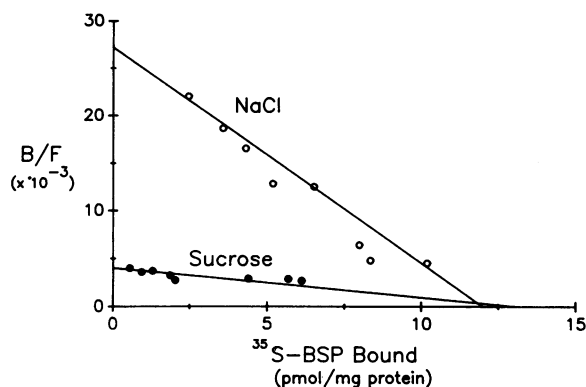


Figure 6. Chloride-dependent binding of ^{35}S -BSP to the surface of short-term cultured rat hepatocytes. Binding assays were performed at varied concentrations of ^{35}S -BSP (0.08–3.2 μM) at 4°C in the presence of a 15-fold molar excess of BSA in NaCl-containing or sucrose-substituted media keeping the ratio of albumin to BSP constant to minimize changes in the fraction of unbound ligand. Results were analyzed by the LIGAND computer program as described by Munson and Rodbard and plotted according to the method of Scatchard. The affinity of BSP for hepatocytes was significantly higher in the presence of Cl^- than in sucrose-substituted medium. Under these conditions, there was no difference in the number of binding sites. In this representative study of five, K_d in the presence and absence of Cl^- was 1.6 vs. 0.17 μM^{-1} , and the number of binding sites was 11.8 vs. 12.6 pmol/mg protein.

tein; $n = 3$, $P > 0.05$). However, initial uptake of ^{35}S -BSP with an inwardly directed H^+ gradient ($n = 4$) (outwardly directed OH^- gradient) was approximately twice that with an outwardly directed H^+ gradient ($n = 4$) (inwardly directed OH^- gradient) ($P < 0.02$) (Fig. 8).

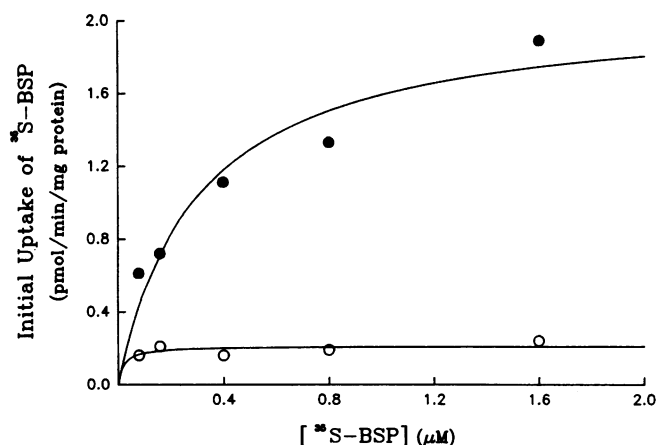


Figure 7. Saturation of initial ^{35}S -BSP uptake by cultured rat hepatocytes. Initial uptake of varied concentrations of ^{35}S -BSP (0.08–1.6 μM) was determined in NaCl-containing or sucrose-substituted media as in Fig. 1, keeping the ratio of BSA to BSP constant. Results were computer fit to a single class of binding sites by a nonlinear least squares regression method. In this figure, the circles represent experimental data, and the lines represent the computer fit to the data. In this representative study of three, in NaCl-containing medium (\bullet — \bullet), K_m was 0.31 μM and V_{\max} was 2.1 pmol/mg protein per min. In sucrose-substituted medium (\circ — \circ), there was little BSP uptake at any concentration, suggesting that the transport mechanism was functionally inactive.

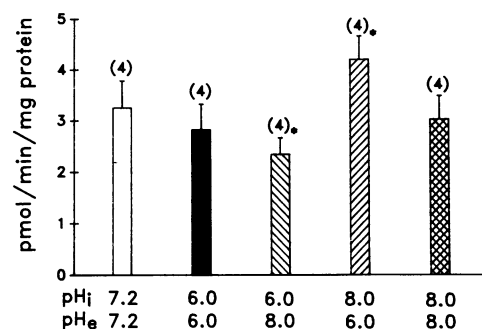


Figure 8. Effect of pH on initial uptake of ^{35}S -BSP by short-term cultured rat hepatocytes. Modified SFM was prepared in which the pH of the medium was adjusted to pH 6.0 or 8.0 with 1 N NaOH. Cells were washed with either pH 6.0 or 8.0 medium and incubated for 30 min at 37°C in the corresponding medium. Unidirectional pH gradients were established by incubating the OH^- -loaded or -depleted cells in medium of the opposite pH. Uptake of ^{35}S -BSP was determined over 5 min. With an inwardly directed H^+ gradient (outwardly directed OH^- gradient), there was an approximately twofold increase in the initial ^{35}S -BSP uptake as compared to an outwardly directed H^+ gradient (inwardly directed OH^- gradient), suggesting that a component of BSP uptake is associated with H^+ cotransport or OH^- exchange. However, indirect linkage to other anion exchangers must be considered. Results are presented as mean \pm SEM. The number of studies performed is in parentheses. * $P < 0.02$.

Discussion

Previous studies revealed that initial uptake of bilirubin and BSP by short-term cultured rat hepatocytes, and isolated perfused rat liver was markedly reduced after substitution of Cl^- by gluconate or HCO_3^- , but was unaltered after substitution of Na^+ in medium by other inorganic cations (12). These investigations suggested a role for inorganic anions, particularly Cl^- , in hepatic organic anion uptake. Hepatocyte uptake of other compounds such as $^{99\text{m}}\text{Tc}$ -iminodiacetic acid derivatives, was found to be independent of Cl^- concentration (30), indicating specificity of this finding for particular organic anions. Although replacement of NaCl in medium by sucrose may osmotically shrink cells (19, 20), this is not the mechanism by which BSP uptake is reduced with sucrose substitution. As initial uptake of BSP is quantified at a time well before steady state has been established (12), one would not expect a large effect upon alteration of cell size. In support of this, shrinkage of cells with 1 M mannitol had little effect on initial uptake of BSP. Although others have suggested that BSP uptake is electrogenic (13, 31), the finding of normal BSP uptake in KCl-substituted medium in the presence and absence of valinomycin suggests that this is not the case.

In the present study, BSP transport has been determined in cells in which inside vs. outside pH gradients have been established. These studies suggest that a component of BSP uptake is associated with H^+ cotransport or OH^- exchange. However, indirect linkage to other anion exchangers (e.g., hydroxyl/sulfate) must be considered. DIDS, an inhibitor of various anion exchangers, is an effective inhibitor of hepatocyte BSP uptake (12). BSP transport has also been determined in cells in which inside vs. outside Cl^- gradients have been established. Initial uptake of BSP in the absence of extracellular Cl^- is significantly decreased regardless of the concentration of intracellular Cl^- . In contrast to results with pH gradients, there was no stimula-

tion of initial uptake of BSP with inwardly or outwardly directed Cl^- gradients. This suggests that BSP transport is not coupled to Cl^- transport. Although nonspecific alterations in cell surface membrane function may be considered with Cl^- and OH^- manipulation, binding and internalization of asialo-orosomucoid are not affected under similar conditions (16, 27).

Several other Cl^- -dependent organic anion transport systems (e.g., urate transport by kidney [32]) are characterized by stimulation of organic anion uptake by inside to outside Cl^- gradients. The present study reveals that uptake of BSP by hepatocytes does not involve Cl^- /organic anion exchange or Cl^- /organic anion cotransport. Rather, Cl^- modulates the affinity of BSP for its hepatocyte transporter. The affinity of BSP for hepatocytes is ~ 10 -fold higher in the presence of extracellular Cl^- than in sucrose-substituted medium. The number of cell surface binding sites is unaffected. The affinity of BSP for cells is also ~ 10 -fold that of its affinity for albumin in Cl^- -containing medium. This may help to explain the rapid, saturable transfer of albumin-bound BSP to hepatocytes. In the absence of extracellular chloride, this difference in affinities of BSP for cells and albumin is lost. As albumin is in excess of cell surface binding sites, little BSP is transferred to cells. Determination of saturation kinetics reveals functional inactivation of the transport mechanism. It must be considered that because these cell-surface binding studies were performed in the presence of albumin, alterations in apparent affinity of ligand to the cell surface could actually represent altered affinity of ligand to albumin. That this is not the case is seen from equilibrium binding studies which revealed no significant difference in the affinity of BSA for BSP in the presence or absence of Cl^- . This is in agreement with previous results of Blauer and associates (33) who found no influence of Cl^- on bilirubin binding to human serum albumin as determined by circular dichroism. In contrast to results with bilirubin and BSP, Cl^- competes for the high-affinity binding site of medium-chain fatty acids to human serum albumin (34). This finding is in agreement with the demonstration of different binding sites on albumin for bilirubin/BSP and fatty acids (35). A similar chloride effect on hepatocyte transport has been described by several other investigators. Duffy et al. (36) found maximal taurocholate uptake into mixed liver plasma membrane vesicles in the presence of chloride as compared to results in the presence of more permeant and less permeant anions. Meier et al. (37) found highest initial uptake rates of taurocholate into rat canalicular membrane vesicles in the presence of chloride. Novak et al. (38) made similar observations in studies of taurocholate transport by human liver basolateral plasma membrane vesicles. Whether this stimulation of taurocholate transport by chloride represents altered interaction with its membrane carrier is unknown.

A number of other Cl^- -dependent organic anion transport systems which do not involve Cl^- /organic anion exchange have been described. In pigeon red blood cells, Cl^- increases the affinity of Na^+ for the glycine transporter with subsequent enhancement of glycine uptake (39, 40). Selhub et al. (41) described anion-induced stabilization of endogenous folate-binding protein complex in brush border membranes from rat kidney, with chloride as the most effective anion. In rat brain synaptosomes, substitution of Cl^- by anions such as acetate, isethionate, and sulfate reduced the high-affinity uptake of neurotransmitters such as dopamine, norepinephrine, serotonin, and gamma-aminobutyric acid (42). It was suggested that Cl^-

might modulate the affinity of these neurotransmitters for their transporter (42–44).

The present study indicates that extracellular Cl^- modulates the affinity of BSP for its hepatocyte transporter. This effect of Cl^- on BSP uptake does not correlate with transport of Cl^- nor with altered interaction of albumin with the organic anion. These results are consistent with binding of Cl^- to the liver cell organic anion transporter with consequent conformational change in its organic anion binding domain. This hypothesis, however, is speculative. Identification of the liver cell plasma membrane protein(s) comprising the organic anion transporter and functional reconstitution in vitro may be necessary for the ultimate elucidation of the role of Cl^- in this process.

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