Influence of Cl^- on Organic Anion Transport in Short-term Cultured Rat Hepatocytes and Isolated Perfused Rat Liver

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Abstract

Transport of ^{35}S-labeled sulfobromophthalein [^{35}S]BSP was studied in short-term cultured rat hepatocytes incubated in bovine serum albumin. At 37°C, initial uptake of [^{35}S]BSP was 5–10-fold that at 4°C, linear for at least 15 min, saturable, inhibited by bilirubin, and reduced by > 70% after ATP depletion or isosmotic substitution of sucrose for NaCl in medium. Replacement of Na^+ by K^+ or Li^+ did not alter uptake, whereas replacement of Cl^- by HCO_3^- or gluconate$^-$ reduced uptake by ~ 40%. Substitution of Cl^- by the more permeant NO_3^- enhanced initial BSP uptake by 30%. Efflux of [^{35}S]BSP from cells to media was inhibited by 40% after ATP depletion or sucrose substitution. To confirm these results in a more physiologic system, transport of [^3H]bilirubin was studied in isolated livers perfused with control medium or medium in which Cl^- was replaced by gluconate$^-$. Perfusion data analyzed by the model of Goresky, revealed 40–50% reductions in influx and efflux with gluconate$^-$ substitution. These results are consistent with existence of a Cl^-/organic anion-exchange mechanism similar to that described by others in renal tubules.

Introduction

The organic anions bilirubin and sulfobromophthalein (BSP),[1] which circulate tightly bound to albumin, are rapidly and efficiently extracted from their albumin carrier by hepatocytes (1, 2). These organic anions are transported into cell cytosol where they bind to soluble proteins, primarily glutathione (GSH)-S-transferases (3, 4), before conjugation and biliary excretion (5, 6). Previous studies in intact animals and isolated perfused liver revealed that uptake of bilirubin and BSP is mutually competitive and saturable (1, 7). Countertransport and a preloading effect have been reported (1), and the uptake mechanism is believed to be one of facilitated diffusion. Although several candidate transport proteins have been proposed (8–11), the identity of the putative carrier is not clear.

Analogous to the liver, the kidney transports several other organic anions including *p*-aminohippurate (PAH) and urate. Recent studies in vesicles derived from renal tubules have revealed that uptake of these organic anions is accompanied by exchange for Cl^- (12–14). An anion exchange mechanism also has been described recently for bile acid transport in intestinal basolateral membrane vesicles (15). The present investigation was performed in short-term cultured rat hepatocytes and isolated perfused rat liver and was designed to examine the relationship of hepatic organic anion transport to cellular ATP levels and [Cl^-] in media.

Methods

Isolation and short-term culture of rat hepatocytes

Rat hepatocytes were isolated from 200–250-g male Sprague-Dawley rats (Marland Farms, Hewitt, NJ) after perfusion of the liver with collagenase (type I, Worthington Biochemical Corp., Freehold, NJ). Cells were suspended in medium consisting of Waymouth's 752/1 (Gibco, Grand Island, NY), 25 mM Hepes, pH 7.2, 5% heat-inactivated fetal bovine serum (Gibco), 1.7 mM additional $CaCl_2$, 5 μg/ml bovine insulin (Sigma Chemical, MO), 100 U/ml penicillin (Gibco), and 0.1 mg/ml streptomycin (Gibco). Approximately 1.5×10^6 cells in 3 ml were placed in 60-mm Lux Contur dishes (Lux Scientific Inc., Newbury Park, CA), and cultured in 5% CO_2 atmosphere at 37°C. Approximately 2 h later, medium was changed and cells were cultured for 16–18 h (16).

Uptake of [^{35}S]BSP by cultured hepatocytes

Cells were washed twice with 1.5 ml of modified serum-free medium (SFM) (16). This consisted 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) bovine serum albumin (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 (138 mCi/mmol on receipt) was obtained from Amersham Corp. (Chicago, IL) and a stock solution of 80 nmol/ml was prepared in distilled water. 10 μl of stock solution was added to 1 ml of media on each plate (0.8 μM final concentration) and incubation was continued at 4 or 37°C for various times. After incubation with [^{35}S]BSP, plates were washed twice at 4°C with 1.5 ml of SFM. In previous studies of binding of [^{35}S]BSP to rat liver cell plasma membrane subfractions, we found that binding was rapidly reversible upon washing with buffer at 4°C (8). There was a small increase in efficiency of removal of membrane-bound [^{35}S]BSP by inclusion of a 100-fold molar excess of unlabeled BSP in the wash solution. We used a similar procedure to maximally displace surface-bound radioactivity from cultured hepatocytes. Plates were incubated for 5 min in 1.5 ml of unlabeled BSP (80 nmol/ml) in 20 mM phosphate-buffered saline, pH 7.4 at 4°C. Plates were then washed three times with 1.5 ml of SFM at 4°C. Cells were scraped into 1 ml of SFM using a rubber policeman and transferred into a scintillation vial, and radioactivity was quantitated in a Beckman model LS7500 liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, CA) after addition of 10 ml of Hydrofluor (National Diagnostics, Inc., Somerville, NJ). Replicate plates were washed three times with 20 mM phosphate-buffered saline at pH 7.4, and extracted with 1 ml of 1 N NaOH. Cellular protein was quantitated in these extracts by the method of Lowry et al. using BSA as standard (17).

Preliminary reports of this work were presented at the 35th and 37th meetings of the American Association for the study of Liver Diseases, Chicago, IL, and appeared in abstract form (1984. *Hepatology.* 4:1075; 1986. *Hepatology.* 6:1199).

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1. *Abbreviations used in this paper:* BSP, sulfobromophthalein; DIDS, 4,4′-diisothiocyano-2,2′stilbene disulfonate; PAH, *p*-aminohippurate; SFM, serum-free medium.

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Additional experiments were performed to insure that further incubation of cells in unlabeled BSP at 4°C would not remove more cell-associated [^{35}S]BSP than a single 5-min incubation. Cells were incubated with radiolabeled ligand at 4 or 37°C for 15 min as above, and then washed twice at 4°C with 1.5 ml of SFM. Plates were then incubated in 1.5 ml of unlabeled BSP (80 nmol/ml) in 20 mM phosphate-buffered saline at pH 7.4 for 1, 3, or 5 min or incubated for two or three 5-min periods. One set of plates was not incubated in unlabeled BSP. All plates were then washed three times with SFM and scraped, and the radioactivity was determined.

Saturation of [^{35}S]BSP uptake

These studies were performed at BSP concentrations of 0.08–1.2 μM, keeping the ratio of albumin to BSP constant at 18.4:1, to minimize changes in the fraction of unbound ligand (18). Uptake of [^{35}S]BSP was determined at 4 and 37°C over the initial 15 min of linear uptake. In these and subsequent studies, the small amount of residual cell-associated radioactivity at 4°C was subtracted as a blank from uptake data at 37°C (see below). Data was analyzed by linear regression of a double reciprocal plot of initial uptake vs. total BSP concentration, and K_m and V_{max} were calculated (19).

Inhibition of uptake of [^{35}S]BSP by bilirubin

Initial uptake of either 0.8 μM [^{35}S]BSP in 0.1% BSA or 0.4 μM [^{35}S]BSP in 0.05% BSA was determined as described above in the presence or absence of 2.5–10 μM bilirubin. Results were plotted according to the method of Dixon ($1/v$ vs. total bilirubin concentration) (19), and straight lines fit by least squares linear regression (20). The K_i for bilirubin was quantitated as that concentration corresponding to the intersection of the resulting lines (19).

Temperature dependence of [^{35}S]BSP uptake

In these studies, uptake of [^{35}S]BSP was determined over the initial 15 min at several temperatures between 4 and 37°C. At all temperatures, uptake was linear over this time period.

Salt requirements for [^{35}S]BSP uptake

To determine whether uptake of [^{35}S]BSP requires particular monovalent anions or cations, SFM was prepared as above except that NaCl was replaced by isosmotic sucrose or by 135 mM KCl, LiCl, $NaNO_3$, $NaHCO_3$, or sodium gluconate (16). Initial uptake was determined at 4°C and 37°C using one of these modified SFM in place of that containing 135 mM NaCl. In studies to determine divalent cation requirements, SFM containing 135 mM NaCl was prepared without addition of Ca^{2+} or Mg^{2+}.

Effect of ATP depletion on uptake of [^{35}S]BSP

Cultured cells were washed twice with 1.5 ml of SFM at 4°C. To deplete them of ATP, cells were incubated at 37°C for 30 min in 1.5 ml of SFM to which was added 0.1% BSA, 0.1% sodium azide, and 50 mM 2-deoxyglucose. We previously found that these conditions are sufficient to inhibit the endosomal ATP-dependent proton pump (21). After preincubation, cells were either chilled to 4°C in buffer or kept at 37°C for 5 min, after which [^{35}S]BSP was added and uptake was quantitated as described above.

Assay of cell ATP levels

ATP in cultured hepatocytes was quantitated by a modification of the method of Lamprecht and Trautschold (22). In brief, cells were washed three times with 1.5 ml of ice-cold SFM, and 0.5 ml of ice-cold 3% perchloric acid was added to each plate. Plates were scraped with a rubber policeman, extracts were combined and left on ice for 10 min, and then centrifuged at 15,000 rpm for 10 min in a Sorvall SS34 rotor (DuPont-Sorvall, Newtown, CT). Protein was quantitated in the pellet by the method of Lowry et al. (17). The supernatant, containing cell ATP, was removed, 10 μl of universal indicator (Fisher Scientific Co.) was added, and 75 μl of 5 M K_2CO_3 was added to produce a yellow color. The extract was left on ice for 30 min and centrifuged for 10 min at 15,000 rpm in an SS34 rotor, and 0.5-ml aliquots of supernatant were used for the ATP assay as follows.

0.5 ml of the perchloric acid extract was added to a cuvette containing 0.33 ml of 40 mM $MgCl_2$ in 100 mM Tris, pH 7.4, 95 μl of distilled water, 5 μl of 0.1 M glucose, and 67 μl of 10 mM NADP. To start the assay, 15 μl of a mixture of ammonium sulfate suspensions (Boehringer Mannheim, Indianapolis, IN) of hexokinase (sp act 140 U/mg), glucose-6-phosphate dehydrogenase (grade I, sp act 350 U/mg), and 6-phosphogluconic dehydrogenase (sp act 12 U/mg) was added. Blanks contained no enzymes. OD_{340} was determined at 30 min, and ATP was quantitated from a standard curve of ATP in 3% perchloric acid.

Effect of preincubation of hepatocytes with 4,4'-diisothiocyano-2,2' stilbene disulfonate (DIDS) on subsequent uptake of [^{35}S]BSP

DIDS was obtained from Pierce Chemical Co. (Rockford, IL) and was coupled to cultured hepatocytes at room temperature by the method described by Cabantchik and Rothstein (23). Cells were washed three times with 1.5 ml of 20 mM phosphate-buffered saline, pH 7.4, at room temperature. They were then incubated for 30 min in the dark in 1 ml of this buffer alone or containing 100 μM DIDS. To inactivate unreacted DIDS, cells were washed three times with 1.5 ml of 20 mM Tris-buffered saline at pH 7.4 followed by three washes in this buffer containing 0.1% BSA. Uptake of [^{35}S]BSP was then determined as described above.

Uptake of ^{131}I-BSA by cultured hepatocytes

Uptake of [^{35}S]BSP by cells could result from uptake of a complex with albumin. To examine this possibility, cell-associated ^{131}I-albumin after incubation at 4 or 37°C was quantitated. Na^{131}I was obtained from Amersham Corp. (Arlington Heights, IL) and ^{131}I-BSA (2 mCi/mg) was prepared by a chloramine-T procedure (24). Cultured cells were washed twice with 1.5 ml of SFM, followed by addition of 1 ml of 0.1% BSA in SFM. Cells were incubated for 15 min at 4 or 37°C followed by addition of 15 μg of ^{131}I-albumin (~ 55,000 cpm) in 10 μl. Incubation at 4 or 37°C was continued for 30 or 60 min. Cells were then washed twice at 4°C with 1.5 ml of SFM, incubated for 5 min in 1.5 ml of SFM containing 80 nmol of BSP, and washed three times with 1.5 ml of SFM. Cells were scraped into 1 ml of SFM and radioactivity was determined in a Packard gamma scintillation spectrometer (Packard Instrument Co., Downers Grove, IL).

Efflux of [^{35}S]BSP from cultured hepatocytes

Cultured cells were allowed to take up [^{35}S]BSP at 37°C for 15 min as described. They were then washed four times with 1.5 ml of SFM. 1 ml of 5% BSA (fraction V, Sigma Chemical Co.) in SFM was added at 4 or 37°C, and plates were incubated for an additional 15 min. In studies of the Cl^- dependence of [^{35}S]BSP efflux, 5% BSA was dissolved in SFM in which NaCl was replaced by isosmotic sucrose. In studies of the ATP dependence of efflux, a mixture of 0.1% Na azide and 50 mM 2-deoxyglucose was added to SFM containing 5% BSA. At the end of incubation, radioactivity was determined in 0.5 ml of media. Efflux was quantitated as the difference of [^{35}S]BSP in media at 37 and 4°C.

Conjugation of [^{35}S]BSP in media and cells

Content of GSH-BSP in cell extracts and media was determined at various times of incubation of cells with [^{35}S]BSP. An uptake study was instituted as described above. After the appropriate incubation time, an aliquot of medium was saved for conjugate analysis and the plate was washed twice with 1.5 ml of SFM at 4°C and incubated for 5 min in 1.5 ml of unlabeled BSP at 4°C as above. The plate was then washed three times with 1.5 ml of SFM at 4°C. Cells were then scraped into a mixture of 0.2 ml of 0.1 N NaOH and 1 ml of methanol. The cell extracts were pipetted into 1.5-ml conical centrifuge tubes, and centrifuged for 5 min in an Eppendorf model 5412 microcentrifuge (Brinkmann Instruments, Westbury, NY). Radioactivity was determined in an aliquot of supernatant; the remainder was analyzed for content of GSH-BSP. > 90% of cell-associated radioactivity was recovered in this extract. Media and cell extracts were analyzed for relative content of [^{35}S]BSP and GSH[^{35}S]BSP as follows.

Unlabeled BSP (Sigma) and unlabeled GSH-BSP (prepared by the method of Whelan et al. [5]) were dissolved in distilled water and small amounts of these standards were added to each sample. Three parts of acetone were added to each of the media samples. Samples were then applied to a silica gel G thin-layer chromatography plate which was developed in 1-propanol/acetic acid/water (10:1:5 vol/vol/vol) as previously described (25). Bands corresponding to BSP and GSH-BSP were visualized after incubation of the plate in an ammonia atmosphere and scraped, and radioactivity was determined.

[^{3}H]Bilirubin transport in isolated perfused rat liver

Radiolabeled materials. [^{3}H]Bilirubin (100 mCi/mmol) was purified from rat bile after i.v. administration of [2,3-^{3}H]δ-aminolevulinic acid (60 Ci/mmol; Amersham Corp.) (26). Na^{125}I was obtained from Amersham and ^{125}I-BSA (2.0 mCi/mg) was prepared by a chloramine-T method (24). Gamma activity due to ^{125}I was determined in a Packard Instrument 3002 scintillation spectrometer.

Liver isolation and perfusion. Male Sprague-Dawley rats (200–250 g) were obtained from Marland Farms. After pentobarbital anesthesia (4 mg/100 g body wt i.p.), the portal vein and inferior vena cava were rapidly catheterized (2, 27, 28). The liver was perfused in situ in a temperature-controlled cabinet at 37°C with Krebs-Henseleit buffer containing 0.5% BSA and 0.1% glucose. Perfusate flow was ~ 2 ml/min per g liver. In studies of Cl^- depletion, NaCl and KCl were replaced isosmotically by their gluconate salts. Perfusate contained only 5.1 μM Cl^-, resulting from 2.55 mM $CaCl_2$. Viability of the liver was assessed by gross appearance and perfusate flow, perfusion pressure, and hepatic O_2 consumption (27–29).

Multiple indicator dilution studies. After a 30-min perfusion in the appropriate buffer to allow stabilization and equilibration, a rapid single injection of indicators was administered into the portal vein (27–29). The injection mixture consisted of [^{3}H]bilirubin (0.4 μCi) and the nontransported reference ^{125}I-BSA (0.2 μCi). After the injection, the perfusion circuit was opened on the effluent side to define single-passage dilution curves without recirculation. Data were analyzed according to the flow-limited distribution model of Goresky as previously described (2, 27, 28).

Statistical analysis of data. All results are expressed as mean±standard error of the mean. Statistical significance was calculated by Student's *t* test (20).

Results

Effect of incubation of cells in unlabeled BSP after uptake of [^{35}S]BSP at 4 or 37°C. As seen in Table I, as many as three 5-min incubations of cells in unlabeled BSP (80 μM) at 4°C did not displace more [^{35}S]BSP than a single 5-min incubation. This incubation and wash had little effect on cell-associated radioactivity after uptake of [^{35}S]BSP at 37°C. However, content of radioactivity after incubation with [^{35}S]BSP at 4°C was consistently reduced by incubation in unlabeled BSP as compared with just SFM wash. Cell viability, as judged by trypan blue exclusion remained unchanged by these wash procedures, and cells were > 95% viable. In all further studies, after uptake of [^{35}S]BSP, cells were washed twice in SFM, incubated for 5 min in unlabeled BSP, washed three times in SFM, and scraped and radioactivity was determined, as described in Methods.

Cell accumulation of [^{35}S]BSP. Cell accumulation of [^{35}S]BSP was linear for at least 15 min at 4 and 37°C (Fig. 1 *A*). As compared with results at 37°C, accumulation of ligand at 4°C was markedly reduced (Fig. 1 *A*). Subtraction of this 4°C blank from uptake data at 37°C revealed linear kinetics with a *y*-intercept close to 0 (Fig. 1 *B*), and initial uptake of 1.19±0.07 pmol/min per mg protein (Table II). As described in previous studies (2), [^{35}S]BSP was extracted from albumin before uptake

Table I. Effect of Incubation of Short-term Cultured Rat Hepatocytes in Unlabeled BSP on Cell-associated [^{35}S]BSP

BSP incubations	Duration of each incubation	Cell-associated [^{35}S]BSP after incubation for 15 min at 4°C	Cell-associated [^{35}S]BSP after incubation for 15 min at 37°C
n	*min*	*pmol/mg protein*	
0	0	8.0	22.8
1	1	6.3	21.7
1	3	7.1	23.5
1	5	6.0	19.7
2	5	5.2	23.3
3	5	6.5	21.6

Short-term cultured hepatocytes were incubated for 15 min at 4 or 37°C in 1 ml of medium containing 0.8 μM [^{35}S]BSP and 0.1% BSA. Cells were then washed twice at 4°C with 1.5 ml of SFM and either scraped and radioactivity determined, or in an attempt to minimize residual cell surface radioactivity, incubated in 1.5 ml of unlabeled BSP (80 μM) for 1, 3, or 5 min or for two or three 5-min periods before washing three times and scraping.

(Fig. 2). After a 60-min incubation at 37°C, < 1% of incubated ^{131}I-albumin was cell-associated in contrast to > 8% of incubated [^{35}S]BSP.

These data reveal that at 37°C, [^{35}S]BSP associated with cells increases with time and cannot be washed from cells with buffer or displaced with excess unlabeled ligand. This contrasts with previous findings in rat liver cell membrane preparations (8) and suggests that [^{35}S]BSP is within cells rather than on their surface. Studies of GSH-BSP within cells and in media are consistent with these observations. During a 60-min incubation at 4°C, there was no GSH-BSP in media or cells. At 37°C (Fig. 3), after a 15-min lag, cell content of GSH-BSP increased steadily. Little conjugate appeared in media during the initial 45 min; subsequently, conjugate rapidly appeared in media and attained levels greater than that in cells.

Saturation of [^{35}S]BSP uptake and inhibition by bilirubin. A double-reciprocal plot of initial uptake rate of [^{35}S]BSP vs. concentration of [^{35}S]BSP was linear, indicating saturation kinetics (Fig. 4). In five studies, K_m was 0.19±0.04 μM with V_{max} of 1.69±0.32 pmol/min per mg protein. As seen in Fig. 5, bilirubin inhibited BSP transport. In two studies, K_i was 6.73 and 6.97 μM.

Energy dependence of [^{35}S]BSP uptake. Initial uptake of BSP was essentially temperature-independent between 4 and 27°C (Fig. 6). Between 27 and 37°C, there was a rapid increase in the BSP uptake rate, and in three studies, uptake at 37°C was 2.2±0.3-fold that at 27°C. This implies that the temperature coefficient of transport, Q_{10}, is greater than 2, suggesting an energy of activation for transport of > 12,000 cal/mol (19). As these studies suggested that BSP uptake by cultured hepatocytes was energy dependent, experiments were performed to determine the effect on BSP transport of cellular ATP depletion, resulting from a 30-min preincubation in a mixture of sodium azide and 2-deoxyglucose. ATP content of three groups of control cells was 14.5±2.6 nmol/mg protein. Incubation in azide/deoxyglucose for 30 min reduced ATP content by 49±2.6% ($P < 0.03$).

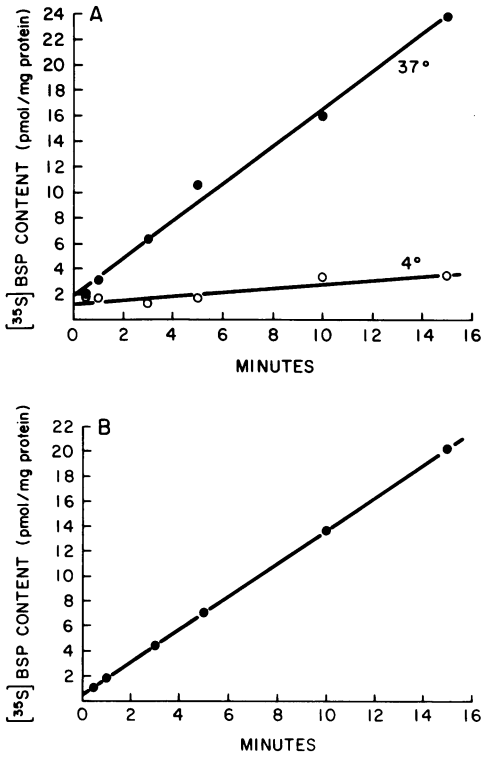

Figure 1. Initial uptake of [^{35}S]BSP by short-term cultured rat hepatocytes. Rat hepatocytes were isolated after collagenase perfusion and cultured in plastic dishes for 16–18 h. At the time of transport studies, cells were washed twice in SFM and then incubated at 4 or 37°C for 15 min. in 1 ml of SFM containing 0.1% BSA. 10 μl (0.8 nmol) of a solution of [^{35}S]BSP was added to each culture dish and incubation was continued at 4°C (*open circles*) or 37°C (*closed circles*). After this incubation, cells were washed with SFM at 4°C and incubated for 5 min at 4°C in unlabeled BSP (80 nmol/ml) to displace surface-bound radioactivity. After washing plates again in SFM, cells were scraped and radioactivity was determined. (*A*) Cell accumulation of [^{35}S]BSP was linear for at least 15 min at 4 and 37°C. As compared with results at 37°C, accumulation of ligand at 4°C was markedly reduced. (*B*) The small amount of residual cell-associated radioactivity at 4°C was subtracted as a blank from uptake data at 37°C. This revealed linear kinetics with a *y*-intercept close to zero.

After this preincubation, we found no effect on cell viability as judged by trypan blue exclusion, but BSP uptake at 37°C was markedly depressed (Fig. 7). There was no effect on association

Table II. Effect of Isosmotic Substitution of NaCl in Media on Initial Uptake of [^{35}S]BSP

Media	Experiments	Initial uptake of [^{35}S]BSP
	n	*pmoles/min per mg protein*
NaCl	22	1.19±0.07
Sucrose	6	0.20±0.04*
KCl	3	1.37±0.08
LiCl	3	1.00±0.17
$NaHCO_3$	4	0.62±0.07*
$NaNO_3$	4	1.58±0.03‡
Na gluconate	3	0.45±0.09*

* Reduced as compared with NaCl ($P < 0.001$).
‡ Increased as compared with NaCl ($P < 0.02$).

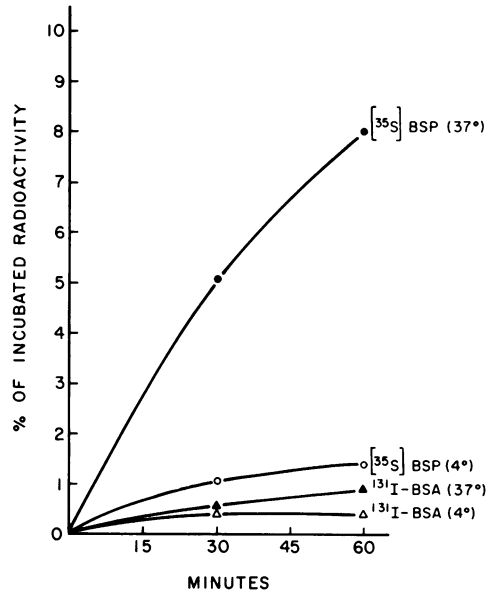

Figure 2. Comparison of uptake of [^{35}S]BSP and ^{131}I-BSA by short-term cultured rat hepatocytes. Uptake of [^{35}S]BSP by cells could result from uptake of a complex with albumin. Uptake of [^{35}S]BSP was determined as in Fig. 1, and compared with uptake of ^{131}I-albumin, quantitated similarly. < 1% of ^{131}I-albumin was associated with cells after 60 min of incubation at 37°C; > 8% of [^{35}S]BSP was within cells at this time, indicating that uptake of BSP could not be explained simply by uptake of albumin.

of BSP with cells at 4°C (Fig. 7). Quantitation of initial temperature-dependent uptake revealed a significant reduction as compared to control (0.36±0.11 pmol/min per mg protein, $n = 3$, $P < 0.001$).

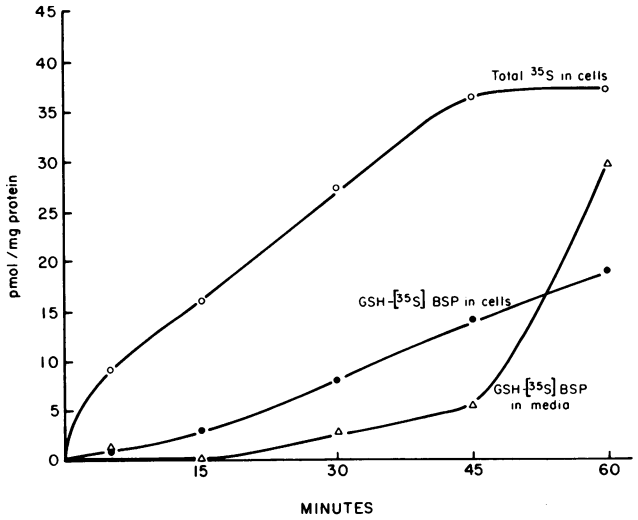

Figure 3. Conversion of [^{35}S]BSP to GSH-[^{35}S]BSP by short-term cultured rat hepatocytes. An uptake study of [^{35}S]BSP at 37°C was instituted as described in Fig. 1. Media were saved for conjugate analysis and cells were extracted in alkaline methanol. [^{35}S]BSP and GSH-[^{35}S]BSP content of cell extracts and media was determined by thin-layer chromatography. Cell content of GSH-[^{35}S]BSP progressively increased. Media contained little conjugate in the initial 45 min; subsequently conjugate rapidly appeared in media and attained levels greater than that in cells.

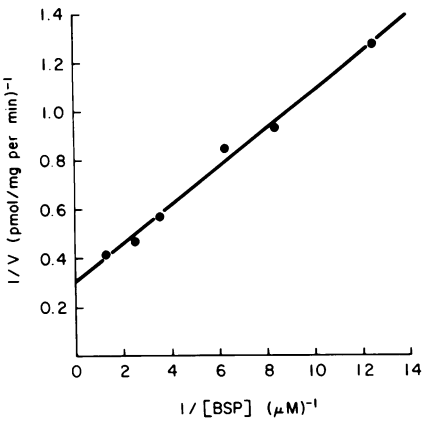

Figure 4. Saturation of initial [^{35}S]BSP uptake by short-term cultured rat hepatocytes. Initial uptake of varied concentrations of [^{35}S]BSP (0.08–1.2 μM) was determined as in Fig. 1, keeping the ratio of albumin to BSP constant at 18.4:1, to minimize changes in the fraction of unbound ligand. Results were analyzed by linear regression of a double-reciprocal plot of uptake vs. concentration. In this representative study, K_m was 0.28 μM and V_{max} was 3.3 pmol/mg protein per min.

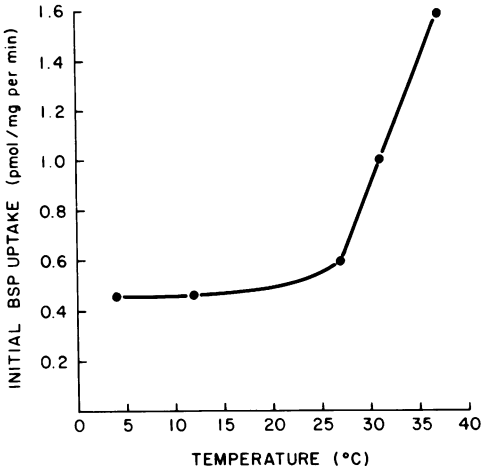

Figure 6. Temperature dependence of [^{35}S]BSP uptake. Uptake of BSP was determined over the initial 15 min at temperatures between 4 and 37°C. At all temperatures, uptake was linear over this time period. Initial uptake of BSP was essentially temperature independent between 4 and 27°C. Between 27 and 37°C, there was a greater than two-fold increase in the BSP uptake suggesting an energy of activation for transport of > 12,000 cal/mol.

Salt requirements for BSP uptake. Whether NaCl in media was required for uptake of [^{35}S]BSP by cultured hepatocytes was determined in media in which NaCl was isosmotically replaced by sucrose. Although previous studies (16) revealed that internalization and endosomal acidification of asialoorosomucoid by cultured hepatocytes was normal in this modified media, there was little uptake of [^{35}S]BSP (Fig. 8). As seen in Table II, initial uptake of BSP was reduced by > 80% in sucrose substituted media as compared with control ($P < 0.001$). To determine whether this was due to a requirement for extracellular Na^+, NaCl was substituted isosmotically by KCl or LiCl. These substitutions had no effect on initial uptake of BSP (Table II) or cell accumulation of BSP for 30 min at 37°C (Fig. 9). Elimination of Mg^{2+} and Ca^{2+} from NaCl media also had no effect on [^{35}S]BSP transport (data not shown). Thus, these studies suggested a BSP uptake requirement for NaCl, KCl, or LiCl, with no evidence for cation specificity. Additional studies were performed to determine if extracellular Cl^- was required for BSP uptake. Substitution of NaCl by $NaNO_3$ consistently augmented cell accumulation of BSP at 37°C (Fig. 10). This was reflected in a 30% increase in initial uptake rate (Table II) ($P < 0.02$). Substitution of NaCl by $NaHCO_3$ markedly reduced cell accumulation of BSP at 37°C (Fig. 10), and initial uptake in $NaHCO_3$ substituted media (Table II) was reduced by almost 50% ($P < 0.001$). Because HCO_3^- can modify intracellular pH, experiments were also performed in which NaCl was substituted isosmotically by sodium gluconate. As seen in Fig. 11, cell accumulation of BSP at 37°C in gluconate$^-$-substituted media was

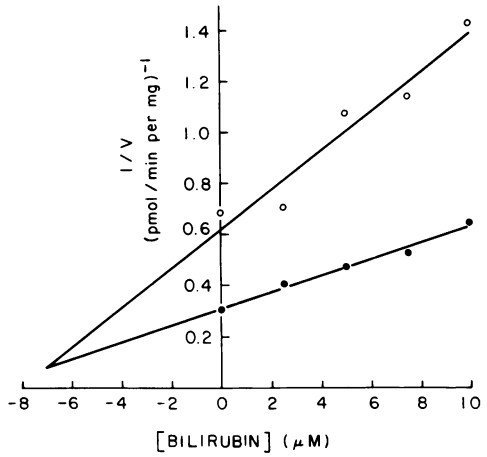

Figure 5. Inhibition of uptake of [^{35}S]BSP by bilirubin. Initial uptake of either 0.8 μM [^{35}S]BSP in 0.1% BSA (*closed circles*) or 0.4 μM [^{35}S]BSP in 0.05% BSA (*open circles*) was determined as in Fig. 1 in the presence or absence of 2.5–10 μM bilirubin. Results are plotted according to the method of Dixon. The K_i for bilirubin was quantitated as that concentration corresponding to the intersection of the resulting lines. In this representative study, K_i was 6.73 μM.

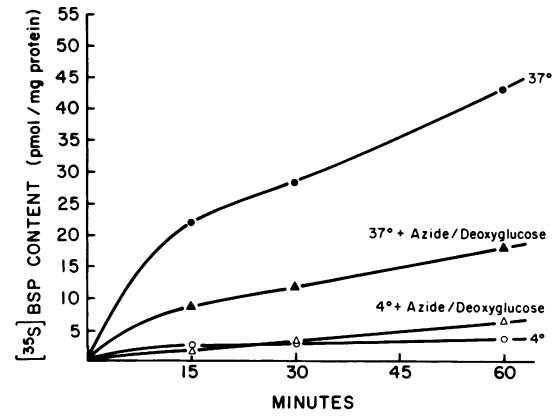

Figure 7. Effect of ATP depletion on uptake of [^{35}S]BSP by short-term cultured rat hepatocytes. Uptake of [^{35}S]BSP was determined at 4 and 37°C as in Fig. 1 with or without a 30-min preincubation at 37°C in a mixture of 0.1% sodium azide and 50 mM 2-deoxyglucose. This preincubation reduced cell ATP content by ~ 50% and resulted in marked inhibition of BSP uptake for 60 min. Quantitation of initial uptake revealed a 70% reduction as compared with control.

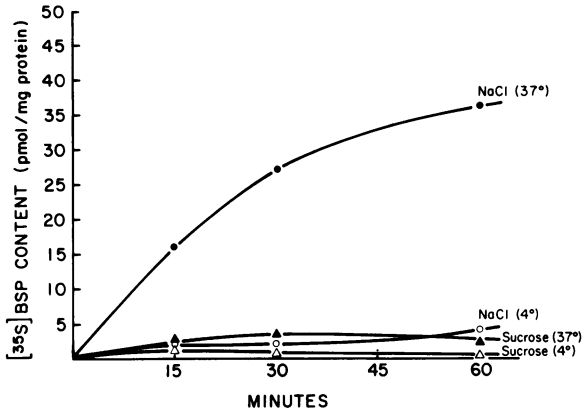

Figure 8. Effect of isosmotic substitution of NaCl in media by sucrose on uptake of [^{35}S]BSP by short-term cultured rat hepatocytes. Cultured hepatocytes were incubated in SFM containing 135 mM NaCl or isosmotic sucrose and uptake of [^{35}S]BSP was determined at 4 and 37°C as in Fig. 1. During the 60 min in the absence of NaCl, there was little uptake of ligand. Quantitation of initial uptake revealed an 80% reduction as compared with control (Table II).

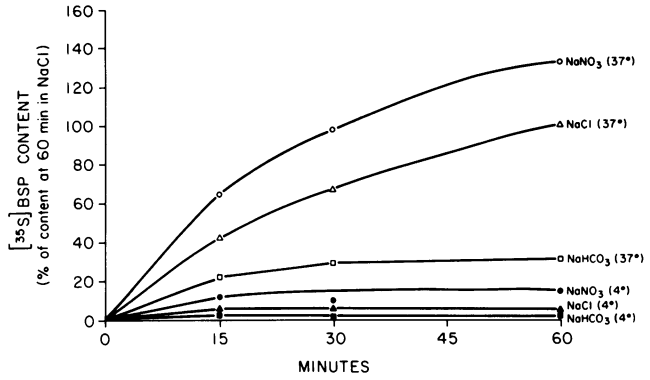

Figure 10. Effects of anion substitution in media on uptake of [^{35}S]BSP by short-term cultured rat hepatocytes. SFM was prepared in which NaCl was substituted by 135 mM $NaNO_3$ or 135 mM $NaHCO_3$. Uptake of [^{35}S]BSP at 4 and 37°C was determined using these media as in Fig. 1. Results are expressed as a percentage of content of [^{35}S]BSP in cells incubated for 60 min in 135 mM NaCl. Although substitution of NaCl by $NaNO_3$ augmented BSP uptake, substitution by $NaHCO_3$ markedly reduced uptake. Similar results were observed for initial uptake of BSP (Table II).

also depressed. Initial uptake (Table II) was reduced by over 60% ($P < 0.001$). That this gluconate effect did not result from irreversible toxicity to cells was seen in experiments in which gluconate-substituted media was removed and replaced by NaCl media; [^{35}S]BSP uptake returned to normal (Fig. 11).

Previous studies suggested that hepatocytes have a DIDS-sensitive Cl^- channel (29). To determine the effect of DIDS on BSP transport, cultured hepatocytes were preincubated for 30 min in 100 μM DIDS. This had no effect on cell viability as judged by trypan blue exclusion but reduced initial uptake of [^{35}S]BSP by 50±4.2% of control ($P < 0.01$).

Efflux of [^{35}S]BSP from cells to media. Previous studies performed in isolated perfused liver revealed that organic anion transport is bidirectional (2, 27, 28). Similarly, in cultured hepatocytes, cell-associated [^{35}S]BSP is not irreversibly sequestered, as demonstrated by studies in which cells preloaded with ligand for 15 min were incubated in media containing 5% BSA. For an additional 15 min in 5% BSA in NaCl-containing media, 13.7±2.19 pmol/mg protein of [^{35}S]BSP returned to the media ($n = 4$). With sucrose replacement of NaCl, 8.7±1.92 pmol/mg protein of [^{35}S]BSP returned to the media ($n = 4$, $P < 0.003$). Efflux of BSP was similarly reduced by incubation in azide/deoxyglucose to 7.1±1.38 pmol/mg protein ($n = 3$, $P < 0.01$).

Effect of Cl^- substitution by gluconate$^-$ on transport of [^{3}H]bilirubin in isolated perfused rat liver. The studies presented above revealed that, in cultured hepatocytes, removal of external

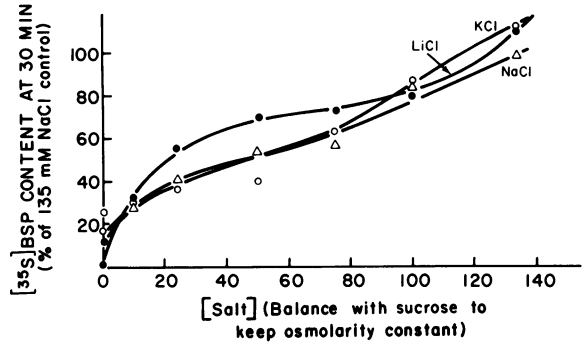

Figure 9. Effect of substitution of NaCl in media by KCl or LiCl on uptake of [^{35}S]BSP. SFM was prepared with 135 mM NaCl, 135 mM LiCl, or isosmotic sucrose. Each of the salt-containing media was mixed with an appropriate volume of sucrose media to vary the salt concentration; osmolarity remained constant. Uptake of [^{35}S]BSP by cultured hepatocytes in these media was determined at 30 min. at 37 and 4°C as in Fig. 1. Results are expressed as a percentage of content of radioactivity in cells incubated in 135 mM NaCl. All three salts gave identical results at both temperatures (4°C data not shown) with optimal uptake at 135 mM, indicating no cation selectively. Quantitation of initial uptake revealed no changes with KCl or LiCl substitution as compared with NaCl (Table II).

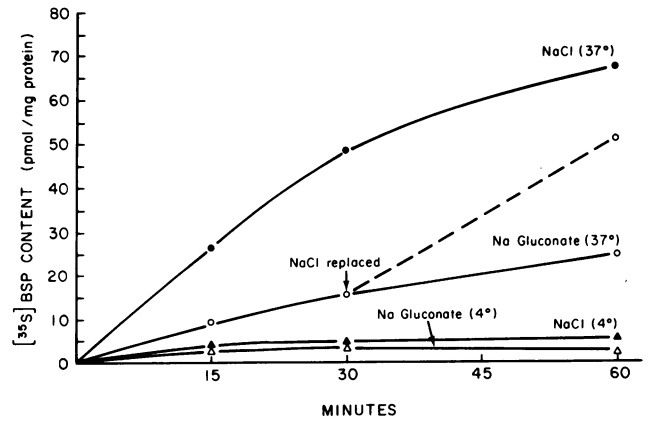

Figure 11. Reversible inhibition of [^{35}S]BSP uptake by substitution of NaCl in media by Na gluconate. SFM was prepared in which NaCl was substituted by Na gluconate. Uptake of [^{35}S]BSP was determined at 4 and 37°C as in Fig. 1, and was depressed in gluconate-containing media. At 30 min at 37°C, as indicated by the arrow, replacement of gluconate by media containing 135 mM NaCl restored uptake to normal. Inhibition of initial BSP uptake by gluconate substitution was also observed (Table II).

Table III. Effect of Cl^- Substitution by Gluconate on Parameters of Bilirubin Transport by the Isolated Perfused Rat Liver

Study	Influx (k_1)	Efflux (k_2)	Sequestration (k_3)	Coefficient of variation of computer fit ($\times 10^{-2}$)
	$s^{-1} \times 10^{-2}$	$s^{-1} \times 10^{-2}$	$s^{-1} \times 10^{-2}$	
Control perfusate				
Cl 1	4.14	2.42	8.25	1.08
Cl 2	4.21	2.07	6.41	0.68
Cl 3	5.37	1.85	6.09	1.62
Cl 4	3.67	2.56	6.04	0.68
Mean±SEM	4.35±0.36	2.23±0.16	6.70±0.52	1.01±0.22
Gluconate-substituted perfusate				
Gluc 1	2.97	0.68	4.71	1.20
Gluc 2	1.86	0.83	2.99	1.31
Gluc 3	2.83	1.78	3.93	1.49
Gluc 4	3.52	0.91	3.22	0.73
Gluc 5	2.59	1.27	7.48	1.00
Mean±SEM	2.75±0.27*	1.09±0.20*	4.47±0.81	1.15±0.13

* $P < 0.005$ as compared with control.

Cl^- markedly depressed initial uptake and efflux of [^{35}S]BSP. To confirm these results in a more physiologic system, transport of [^{3}H]bilirubin was studied in isolated livers perfused with control media or media in which Cl^- was replaced by gluconate$^-$. Parameters of viability during gluconate perfusion were identical to that in control perfusion. Analysis of data by the model of Goresky (Table III) revealed ~ 50% reductions in both influx and efflux ($P < 0.005$). Indicator dilution curves of representative studies are seen in Figs. 12 and 13.

Discussion

Previous studies revealed that hepatic organic anion uptake has carrier-mediated kinetics (1–7). Details of the uptake mechanism have been unclear. There have been several previous studies of BSP transport by isolated hepatocytes (30–33). These investigations were conducted in the absence of albumin and suggested temperature-dependent, sodium-independent uptake. Because of rapid binding of a significant proportion of ligand to the cell surface, however, quantitation of ligand transported into cells was difficult. In the present study, low affinity interaction of BSP with the liver cell surface was minimized by incubating cells with BSP in the presence of a molar excess of BSA. The use of cultured hepatocytes permits easy manipulation of the incubation environment, and was important in elucidation of the pathway of receptor-mediated endocytosis of asialoglycoproteins in earlier studies (16).

The present investigation reveals linear uptake of BSP by cultured hepatocytes over at least 15 min with little formation of its GSH conjugate over this time. This contrasts with a more rapid process that has been described in vivo (1), in freshly isolated hepatocytes (30–33), and in perfused liver (7, 34). Similar slowing of cellular events in cultured hepatocytes as compared with these other systems has also been noted in studies of receptor-mediated endocytosis of asialoorosomucoid. However, this has facilitated quantitation and characterization of several otherwise very rapid steps of the endocytic pathway (16, 35). That [^{35}S]BSP entered cells and was not on their surface was suggested by inability to remove cell associated ligand with repeated washing or by displacement with unlabeled BSP. In previous studies, we found that these procedures rapidly remove ligand bound to liver cell plasma membrane preparations (8). Temperature-dependent accumulation of BSP, suggesting transport, is also not seen in purified plasma membrane (8). Additionally, binding of BSP to isolated plasma membrane is rapid, and maximal by 1 min in contrast to our findings in cultured cells at 37°C (8).

The marked temperature dependence of BSP uptake by cultured hepatocytes suggests that this is an energy-requiring process. In support of this is the finding that reduction of cellular ATP levels by preincubation in a mixture of Na azide and 2-deoxyglucose reduces initial uptake of BSP by 70%. The site at which BSP transport may be coupled to energy utilization is not known. The present studies do not necessitate that BSP transport itself is active and it is possible that the energy requirement may be indirect. Previous studies by Van Dyke et al. (36) revealed that uptake of GSH-BSP by isolated rat hepatocytes and perfused liver was not accompanied by increased oxygen consumption. However, the high background level of oxygen consumption in hepatocytes may preclude accurate quantitation of changes produced by transport of this compound. Van Bezooijen et al. (31) also described an energy requirement for BSP uptake by isolated hepatocytes. This was not seen in studies by Schwenk et al. (30). This apparent discrepancy may be due to differences in metabolic inhibitors, as has been described in studies of GSH-BSP uptake by isolated rat hepatocytes (33).

As previously demonstrated in isolated perfused liver (7, 34), transport of [^{35}S]BSP by cultured hepatocytes is bidirectional. Similar to findings for uptake, efflux of BSP was also inhibited by reduced temperature (4°C) and by inclusion of azide and 2-deoxyglucose in the medium. This efflux component does not represent transport of GSH-BSP into medium as little if any of this compound is found in medium over the relatively short time period that efflux studies were conducted.

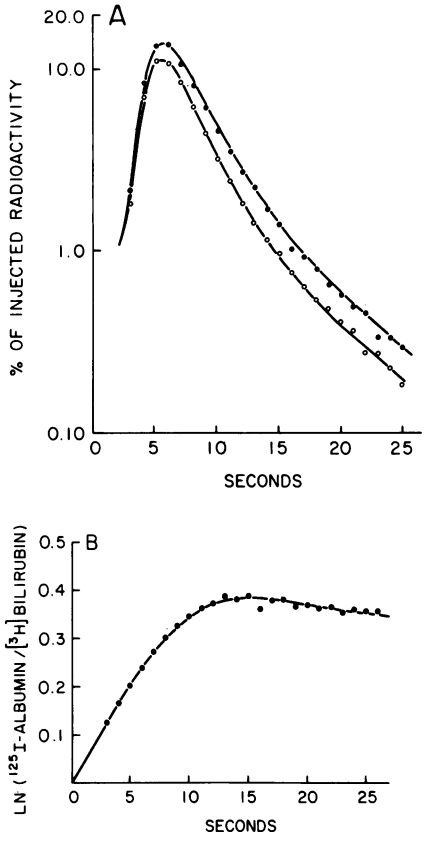

Figure 12. Representative indicator dilution outflow curve for [^{125}I]albumin (*closed circles*) and [^{3}H]bilirubin (*open circles*) in isolated rat liver perfused with control Krebs-Henseleit buffer. Livers were perfused for 30 min to allow equilibration before the transport study was performed. (*A*) Distribution of radioactivity as a percentage of that injected. (*B*) Ratio of ^{125}I-albumin to [^{3}H]bilirubin at each collection point. As described by the Goresky model, influx is represented by the initial slope of this curve. As [^{3}H]bilirubin, which has entered hepatocytes, effluxes back to the [^{125}I]albumin carrier, this line breaks; the greater the change in slope at the break, the greater the efflux rate. The study shown is Cl 1 from Table III.

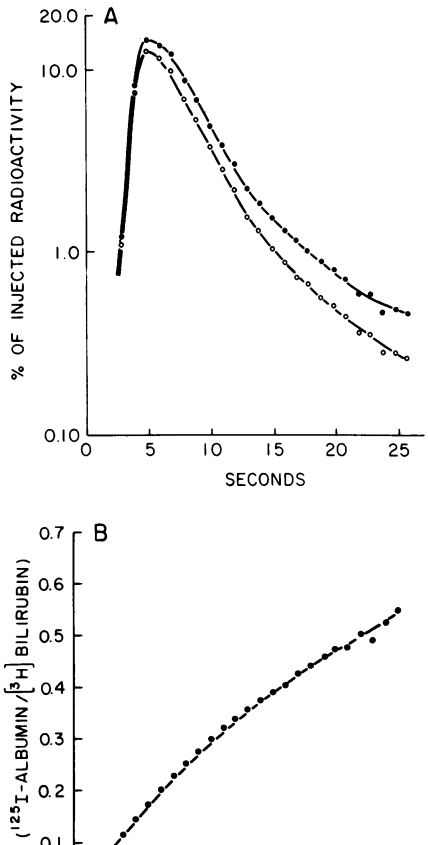

Figure 13. Representative indicator dilution outflow curve for [^{125}I]albumin (*closed circles*) and [^{3}H]bilirubin (*open circles*) in isolated rat liver perfused with Krebs-Henseleit buffer in which NaCl and KCl have been replaced by their respective gluconate salts. Livers were perfused for 30 min to allow equilibration before the transport study was performed. Parameters of viability remained unchanged from control perfusion. (*A*) Distribution of radioactivity as a percentage of that injected. (*B*) Ratio of [^{125}I]albumin to [^{3}H]bilirubin at each collection point. As compared with Fig. 12 *B*, initial slope of this curve is reduced, indicating reduced influx of [^{3}H]bilirubin. More pronounced is the reduction in the degree of break of this curve later in time, indicating reduced efflux. The study shown is Gluc 1 from Table III.

Both influx and efflux of [^{35}S]BSP were depressed by isosmotic substitution of NaCl by sucrose. That this finding does not represent nonspecific toxicity of this modified media on cultured hepatocytes was demonstrated in previous studies of asialoglycoprotein endocytosis (16). A specific cation requirement for BSP uptake is unlikely as uptake was unaffected by substitution of NaCl by KCl or LiCl. However, substitution of Cl^- by HCO_3^- or gluconate$^-$ markedly inhibited BSP uptake. Replacement of Cl^- by the more permeant anion NO_3^- enhanced uptake. The obligate role of Cl^- or NO_3^- for BSP transport may be similar to that for organic anion transport by renal tubules, in which several studies have revealed evidence for Cl^-/organic anion exchange (12–14). In particular, an anion exchanger with affinity for PAH, OH^-, and Cl^- has been described in dog and rat microvillus membrane vesicles (12, 14). In rat basolateral vesicles, an anion exchanger with affinity for urate and Cl^- but not PAH has been described (13). Recent studies in rat intestinal basolateral membrane vesicles have also revealed evidence for inorganic anion/bile acid exchange (15).

Although studies in cultured cells may be valuable models of transport mechanisms in vivo, it is possible that culture conditions may have enhanced a normally minor Cl^--sensitive component. For this reason, confirmatory studies of the relationship of Cl^- to bilirubin transport were conducted in the isolated perfused rat liver. Previous studies have revealed that the perfused liver closely approximates normal hepatic transport physiology, while permitting controlled manipulation of cellular environment without interference by circulatory or hormonal alterations (2, 7, 27, 28, 34). Similar to results in cultured hepatocytes, replacement of Cl^- by gluconate$^-$ inhibited both influx and efflux of bilirubin by ~ 50% without altering parameters of viability. Although these changes in transport kinetics have been quantitated by computer, they can be seen graphically in Figs. 12 *B* and 13 *B* in which the logarithm of the ratio of recovered ^{125}I-albumin to [^{3}H]bilirubin has been plotted at each

collection point. As the albumin and bilirubin outflow curves diverge (Figs. 12 *A* and 13 A), this ratio increases at a rate (seen from the initial slope) that approximates influx (37). Later in time, as [^{3}H]bilirubin which has entered hepatocytes effluxes back to the ^{125}I-albumin carrier, these curves converge, resulting in a break in the ratio curve. The greater the break, the greater the rate of efflux (37).

Whether Cl^--dependent BSP transport utilizes the Na^+ independent Cl^- uptake system recently described by Scharschmidt et al. (38, 39) is not known. Other recent studies performed by Bear and colleagues (29) revealed evidence for a hepatocyte Cl^- channel that was inhibited by DIDS. Although we found inhibition of BSP uptake by DIDS pretreatment of cultured hepatocytes, it is not clear whether this represents inhibition of a Cl^- channel or direct inhibition of the organic anion transport mechanism. In the rat, the related compound 4-acetamido-4′-isothiocyano-2,2′-stilbene disulfonic acid has been found to be transported intact into bile (40).

The present studies are consistent with bidirectional hepatic organic anion transport in exchange for Cl^- or NO_3^-, similar to mechanisms described in kidney and intestine. Incubation of cells (38, 39) or perfused liver (41, 42) in Cl^--free medium depletes both intracellular and extracellular [Cl^-]. Studies of organic anion transport under conditions of unidirectional inorganic anion gradients, best performed in sinusoidal liver cell membrane vesicles, may help to elucidate this mechanism.

Acknowledgments

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