The transport system for inorganic anions has been investigated in hepatocytes and hepatoma tissue culture cells. Sulfate transport in hepatocytes is temperature sensitive and occurs against an electrochemical gradient. Uptake was shown to occur by a sodium-dependent and a sodium-independent route with $K_m$ values of 2.3 and 33 mM and $V_{max}$ values of 2.1 and 10 nmol/mg of protein/min, respectively. An analysis of the sodium dependency indicates a Hill coefficient of 1.05 suggesting an equimolar stoichiometry for sodium and sulfate transport. The transport of sulfate was decreased by metabolic and sodium transport inhibitors. Bicarbonate was shown to affect the transport of sulfate, where uptake was accelerated by intracellular bicarbonate and competitively inhibited by extracellular bicarbonate. In addition, sulfate efflux was stimulated by extracellular bicarbonate. These results suggested that bicarbonate is a substrate for the sulfate transport system and can accelerate uptake and efflux by an anion exchange mechanism. Inhibition of bicarbonate uptake by extracellular sulfate and by the anion transport inhibitor 4,4'-diisothiocyanato-2,2'-stilbene disulfonate demonstrates that bicarbonate does not enter the cell exclusively by CO$_2$ diffusion but can be transported in part as an anionic species. These results are consistent with its role in the sulfate-bicarbonate exchange system. This inorganic anion transport system was shown to be inhibited by approximately 80% in hepatoma tissue culture cells where altered sodium dependency, $K_m$ and $V_{max}$ values reflect possible alterations in the structure and/or membrane content of the carrier.

Hepatocytes have been shown to possess specific transport systems for a wide variety of substrates (1). The uptake and biliary excretion of several anions has been under intensive investigation. The existence of carrier-mediated processes for the uptake of organic anions such as cholic acid and taurocholic acid (2-4), 5-methyltetrahydrofolic acid and methotrexate (5, 6), bromosulphthalein (7), bilirubin (8), and 1-anilinonaphthalene-8-sulfonate (9) has been demonstrated. The transport of inorganic anions such as chloride, bicarbonate, and sulfate, which has been studied extensively in erythrocytes (10, 11), several epithelial cells (12, 13), neurons (14), and muscle (15) has recently been the subject of a number of investigations in perfused liver and isolated hepatocytes (16-18). A membrane carrier for the hepatocyte anion transport system has recently been proposed (18). Anions such as chloride and bicarbonate, which are components of bile, contribute a major fraction of the total osmotic activity (19) of this fluid. Bicarbonate has been postulated to regulate bile salt-independent bile flow (20), a process which is important in the digestion of dietary fat and in the facilitation of cholesterol excretion. This anion has also been shown to stimulate amino acid uptake in isolated hepatocytes (21); however, there is little direct information available concerning its mode of uptake. Sulfate is also involved in various physiological processes in the liver such as the synthesis of sulfated compounds and in detoxification reactions. Several studies have recently demonstrated that the transport capacity for inorganic and organic anions in several hepatoma cell lines is greatly diminished (9, 22-24) when compared to normal quiescent hepatocytes. In this report we present an analysis of the inorganic anion transport system in hepatocytes and hepatoma tissue culture cells.

**Experimental Procedures**

**Cell Preparations**—Isolated hepatocytes were prepared from the livers of male Sprague-Dawley rats (180-250 g) using a collagenase perfusion technique as previously described (25). After washing, the cells were stored in suspension (10 x 10$^6$ cells/ml) in a Krebs-Ringer bicarbonate buffer, pH 7.4, saturated with 95% O$_2$/5% CO$_2$ containing 10 mM glucose. HTC cells were grown as suspension cultures in Sigma's 77 media supplemented with 5% fetal calf serum and 5% calf serum as previously described (26). Cells were washed three times with a Krebs-Ringer bicarbonate buffer, pH 7.4, and then stored in this buffer (1 x 10$^6$ cells/ml). Cell viability was estimated by trypan blue exclusion and cell number determined with a hemocytometer. All studies were carried out on hepatocyte suspensions with viabilities of 85 to 90% and greater than 90% for HTC cells.

**Transport Studies**—Hepatocytes and HTC cells were washed and suspended in Krebs-Ringer phosphate buffer, pH 7.4, at a concentration of 5 x 10$^6$ cells/ml. The effect of sodium on sulfate uptake was determined in buffers where NaCl was completely or partially replaced with choline chloride or lithium chloride. Uptake was initiated by the addition of an equal volume of the incubation buffer containing the appropriate concentrations of radioactive substrates. At appropriate times, aliquots (400 µl) of hepatocyte and HTC cell suspensions were rapidly centrifuged through dibutylphthalate or dibutylphthalate-dinonylphthalate (3:1) in a Beckman Instruments Spinco microfuge as previously described (18), and radioactivity in the cell pellet was measured using a scintillation fluid consisting of 16% Bio-Solv BBS-3 and 0.3% Butyl-PBD fluorolucin in toluene. Estimates of trapped and/or bound substrate were made by measuring cell pellet radioactivity at 4 °C at zero time. Experiments were performed in duplicate on two or more cell preparations. The standard error was less than 5%.

**Reagents**—Collagenase (type IV) and 5,5-dimethyl-2,4-oxazolidinedione were obtained from Sigma, dibutylphthalate was from J. T. Baker Chemical Co. $^{35}$SO$_2$- (carrier free) as Na$_2$SO$_4$ and di(buty1)phthalate were from ICN Chemical and Radiosotope Division, Irvine, CA, and Pharmaceutical Division, Plainview, NY, respectively, and NaHCO$_3$ (7.8 mCi/mmol) was from New England Nuclear.

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1 The abbreviation used is: HTC, hepatoma tissue culture.
RESULTS

The uptake of sulfate as a function of time and temperature is shown in Fig. 1. This process was linear up to 30 s. After 10 min at 27 °C the ratio of the intracellular to extracellular concentrations of sulfate was 1.08, using a value of 3.35 µl/10^6 cells for the intracellular water volume (27). Utilizing the Nernst equation and assuming a transmembrane potential of approximately -30 mV (4), a transmembrane distribution ratio of sulfate for passive diffusion is calculated to be 0.1. The observed uptake is almost 11 times greater than the predicted value, indicating that the transport of sulfate occurs against an electrochemical gradient. Sulfate uptake was studied as a function of sulfate concentration as shown in Fig. 2. In the presence of sodium, Lineweaver-Burk analysis (Fig. 3A) afforded a biphasic plot. When sodium was replaced with choline a significant decrease in sulfate uptake was observed (Fig. 2) suggesting that part of the net uptake of sulfate occurs via a sodium-dependent route. This uptake component was calculated by subtracting the values obtained in choline chloride from those observed in sodium chloride. Linear double reciprocal plots of the sodium-dependent and sodium-independent sulfate uptake curves were obtained (Fig. 3, B and C) which afforded V_{max} values of 2.1 and 10 nmol/mg of protein/min and K_{m} values of 2.3 and 33 mM, respectively.

The effect of increasing the external sodium concentration on the initial rate of sodium-dependent sulfate uptake is shown in Fig. 4A. A double reciprocal plot afforded a straight line with a K_{m} for sodium of 15 mM (Fig. 4B). A Hill plot of this data gave a coefficient of 1.05 (Fig. 4C) indicating that one sodium is transported per sulfate ion. Sulfate transport was also shown to be sensitive to various inhibitors (Table I). Significant inhibition was observed in the presence of ouabain, as well as the metabolic inhibitors, carbonyl cyanide m-chlorophenylhydrazone, cyanide, and arsenate, suggesting that sulfate uptake was dependent, in part, on a sodium gradient as well as on metabolic energy.

The transport of sulfate was observed to be highly sensitive to the intra- and extracellular concentrations of bicarbonate. The effect of intracellular bicarbonate on sulfate uptake in the presence and in the absence of sodium is shown in Fig. 5. When hepatocytes were preincubated with increasing concentrations of bicarbonate, a subsequent measurement of initial rates of 1 mM sulfate uptake in bicarbonate-free buffer indicated a significant increase over the values obtained without bicarbonate preincubation. When sulfate uptake was determined in the absence of sodium, the bicarbonate effect was greatly reduced. Preincubation of hepatocytes with the weak acid, 5,5-dimethyl-2,4-oxazolidinedione, had no effect on subsequent sulfate transport (Fig. 5).

![Image 1](http://www.jbc.org/...)

**Fig. 1.** Uptake of sulfate by hepatocytes. Hepatocytes (2.5 x 10^6 cells/ml) were suspended in Krebs-Ringer phosphate buffer at the indicated temperature. The cells were incubated for the indicated times at 4 °C (■) and 27 °C (▲) with 2 µCi of 1 mM Na_2SO_4. The uptake was terminated by centrifugal filtration through dialysis tubing, and the cell-associated radioactivity was measured as described under "Experimental Procedures."

![Image 2](http://www.jbc.org/...)

**Fig. 2.** The effect of sulfate concentration and sodium on the initial rates of sulfate uptake. Hepatocytes (2.5 x 10^6 cells/ml) were incubated with the indicated concentrations of sulfate in Krebs-Ringer phosphate buffer containing 137 mM NaCl (■) or 137 mM choline chloride (▲). Transport was measured for 30 s at 27 °C, and the cells were filtered and counted as described under "Experimental Procedures." The sodium-dependent uptake component (▲) was determined by subtracting the uptake in the choline chloride buffer from that obtained in the NaCl buffer.

![Image 3](http://www.jbc.org/...)

**Fig. 3.** Double reciprocal plots of the concentration dependence of sulfate uptake as shown in Fig. 2. **A,** sulfate transport measured in the presence of 137 mM NaCl. **B,** sulfate transport in the presence of 137 mM choline chloride. **C,** sodium-dependent transport of sulfate.
The effect of extracellular concentrations of bicarbonate on sulfate uptake is demonstrated in Fig. 6, A and B. At low concentrations of sulfate (0.5 to 5 mM) in the presence of sodium, concomitant addition of bicarbonate resulted in the inhibition of sulfate uptake. A Lineweaver-Burk plot indicated that bicarbonate appeared to be a competitive inhibitor of sulfate uptake with a $K_i$ of 23 mM (Fig. 6A). Similar results were obtained at high sulfate concentrations (10 to 40 mM) in the absence of sodium where a $K_i$ of 24 mM was calculated (Fig. 6B). Further evidence to support the hypothesis that sulfate and bicarbonate may utilize a common carrier was derived from efflux studies as shown in Fig. 7. Sulfate efflux

**TABLE 1**

| Reagent     | $SO_4^{2-}$ uptake | Inhibition |
|-------------|--------------------|------------|
| Control     | 1.08 ± 0.05        | 0          |
| Ouabain (0.5 mM) | 0.51 ± 0.01    | 53        |
| CCCP* (40 mM)   | 0.68 ± 0.02        | 37        |
| KCN (2 mM)    | 0.54 ± 0.03        | 50        |
| Arsenate (5 mM) | 0.68 ± 0.05    | 37        |

*CCCP, carbonyl cyanide m-chlorophenylhydrazone.

**Fig. 4.** The effect of sodium concentration on sodium-dependent sulfate uptake. A, hepatocytes (2.5 × 10^6 cells/ml) were incubated with increasing external concentrations of sodium using various combinations of NaCl and choline chloride. The sodium-dependent uptake rate was calculated by subtracting the rate in choline chloride from that observed in the presence of the indicated amounts of NaCl. Transport was measured for 30 s at 27 °C and analyzed as described under "Experimental Procedures." B, double reciprocal plot of the sodium-dependent uptake rate from which a $K_m$ for sodium of 15 mM was calculated. C, Hill plot of the sodium-dependent sulfate uptake data.

**Fig. 5.** The effect of intracellular bicarbonate or 5,5-dimethyl-2,4-oxazolidinedione (DMO) on sulfate uptake. Hepatocytes (2.5 × 10^6 cells/ml) were incubated with the indicated concentrations of sodium bicarbonate for 30 min at 27 °C in Krebs-Ringer phosphate buffer, pH 7.4. Following the preloading procedure, cells were centrifuged and resuspended in Krebs-Ringer buffer. Transport of 1 mM sulfate was measured for 30 s at 27 °C, and uptake was evaluated as described under "Experimental Procedures" in the presence of 137 mM NaCl (○) or 137 mM choline chloride (■). Hepatocytes were also preincubated with the indicated concentrations of 5,5-dimethyl-2,4-oxazolidinedione as described for bicarbonate and sulfate transport evaluated in the presence of 137 mM NaCl (Δ).

**Fig. 6.** The effect of extracellular bicarbonate on sulfate transport. A, hepatocytes (2.5 × 10^6 cells/ml) were incubated with the indicated concentrations of sulfate in the absence (■) and in the presence of 10 mM (△) and 30 mM (□) bicarbonate in Krebs-Ringer phosphate buffer containing 137 mM NaCl. Transport was measured for 30 s at 27 °C and evaluated as described under "Experimental Procedures." The results are analyzed on a double reciprocal plot. B, hepatocytes (2.5 × 10^6 cells/ml) were incubated with the indicated concentrations of sulfate in the absence (●) and in the presence of 15 mM (■) and 30 mM (□) bicarbonate in Krebs-Ringer phosphate buffer containing 137 mM choline chloride. Transport was analyzed as above.
from preloaded hepatocytes was greatly stimulated by the addition of extracellular sulfate, presumably by a process of anion exchange. A significant enhancement of efflux was also observed upon the addition of extracellular bicarbonate.

An understanding of these bicarbonate effects on sulfate transport required further information on the nature of bicarbonate transport in hepatocytes. Since bicarbonate is in equilibrium with CO$_2$ transport could occur via a bicarbonate ion and/or by CO$_2$ diffusion. Cells were incubated with [14C]bicarbonate, and the uptake of total 14C (bicarbonate + CO$_2$) was measured as shown in Table II. When bicarbonate uptake was measured in the presence of 20 mM sulfate or with cells that had been pretreated with the anion transport inhibitor, 4,4'-diisothiocyano-2,2'-stilbene disulfonate, a significant inhibition of 14C transport was observed. Inhibition was also observed in the absence of sodium or in the presence of ouabain and cyanide.

The uptake of sulfate was also measured in HTC cells and shown to be linear for at least 5 min (data not shown). Sulfate uptake as a function of concentration (Fig. 8A) indicated that the rate was only 20% of that observed in normal hepatocytes in the presence of sodium (Fig. 2). The omission of sodium from the incubation buffer appeared to have no effect on the sulfate uptake properties of HTC cells, in contrast to the sodium dependency observed in normal hepatocytes (Fig. 2). A double reciprocal plot (Fig. 8B) afforded a $K_m$ value of 8 mM and a $V_{max}$ of 1.0 nmol/mg of protein/min.

**DISCUSSION**

In this report we have described the properties of the transport system for inorganic anions in hepatocytes and HTC cells. In contrast to erythrocytes (10) hepatocytes transport sulfate against an electrochemical gradient utilizing a sodium-dependent as well as a sodium-independent route (Fig. 2). Whether these two uptake systems utilize the same carrier which has separate binding sites for sulfate and sodium remains to be elucidated. At low sulfate concentrations such as are observed in plasma (0.3 mM), most of the transport is mediated by the sodium-dependent high affinity pathway. An analysis of the sodium dependency of sulfate uptake (Fig. 4) indicated an equinolar stoichiometry for sodium and sulfate transport. In addition to this sodium dependency, sulfate uptake was sensitive to cellular energy supplies as assessed by the effects of several metabolic inhibitors (Table I). These properties closely resemble the characteristics of the hepatocyte transport systems for organic anions such as cholic acid and taurocholic acid (2, 3, 16). The relationship between the inorganic and organic transport system remains to be defined.

The rate of sulfate transport was observed to be highly sensitive to the presence of bicarbonate, where stimulation or inhibition of this process was a function of bicarbonate compartmentation. Preloading hepatocytes with bicarbonate resulted in a 2-fold stimulation of subsequent sulfate uptake (Fig. 5). In contrast, when extracellular bicarbonate was added concomitantly with sulfate, a significant inhibition of transport was observed. These results suggest that sulfate and bicarbonate may both be substrates for the same transport system. The stimulation of sulfate transport by intracellular bicarbonate could thus result from an exchange of one bicarbonate ion for one sulfate and one sodium ion, since maximal stimulation by intracellular bicarbonate is dependent on extracellular sodium (Fig. 5). The above stoichiometry would result in an electroneutral process. Intracellular bicarbonate was also demonstrated to stimulate the uptake of 40 mM sulfate in the absence of sodium (data not shown), suggesting that sodium-independent anion exchange occurs at high sulfate concentrations.

**Table II**

Transport of [14C]bicarbonate by hepatocytes

| Conditions   | 14C Uptake | Inhibition |
|--------------|------------|------------|
|              | nmol/mg prot/30 s | %         |
| Control      | 2.27 ± 0.03  | 0          |
| Sulfate (20 mM) | 1.14 ± 0.02  | 50         |
| DIDS (40 µM)  | 1.39 ± 0.01  | 39         |
| KCN (2 mM)    | 1.36 ± 0.05  | 41         |
| Ouabain (0.5 mM) | 1.64 ± 0.04  | 28         |
| Choline chloride (137 mM)* | 0.81 ± 0.04  | 65         |

* No sodium included.
Incubation of hepatocytes with bicarbonate could lead to an increase in intracellular bicarbonate by direct transport of this anion and/or by rapid diffusion of CO₂ which, at pH 7.4, comprises approximately 10% of the total material. After intracellular hydration, ionization would lead to the formation of a proton and a bicarbonate ion with a resulting acidification of the cytoplasmic compartment, effectively transferring one bicarbonate ion into the cell. An increase in the transmembrane pH gradient has been demonstrated as a result of cytoplasmic acidification following the treatment of hepatocytes with bicarbonate (21). Thus the stimulation of sodium-dependent sulfate uptake by pretreatment with bicarbonate could possibly also result from a sodium-proton exchange as suggested by McGivern for the mechanism of amino acid transport stimulation in hepatocytes (21). Acidification of the cytoplasmic compartment could also lead to the protonation of the anion carrier system, converting it to a more active form. Incubation with the weak acid, 5,5-dimethyl-2,4-oxazolidinedione (pK₆ 6.1) has been used as an alternate method for obtaining intracellular acidification in liver (20). Preincubating hepatocytes with this reagent, however, had no effect on the subsequent transport of sulfate, suggesting that the bicarbonate effect may, in fact, be mediated though an anion exchange system. In contrast to the stimulatory effect of intracellular bicarbonate on sulfate uptake, extracellular bicarbonate added concomitantly with sulfate was shown to be a competitive inhibitor of this process (Fig. 6, A and B), suggesting that bicarbonate was also interacting with the inorganic anion carrier for sulfate, a conclusion further supported by the stimulation of sulfate efflux by extracellular bicarbonate (Fig. 7).

Despite the considerable complexities in measuring bicarbonate uptake in hepatocytes, attempts were made to define definitively the transport properties of this anion. As shown in Table II, bicarbonate uptake was significantly inhibited by 4,4'-disothiocyanato-2,2'-stilbene disulfonate and extracellular sulfate, results which further suggest that bicarbonate can be transported, in part, as an anionic species as well as by CO₂ diffusion. This result is consistent with its demonstrated capacity as a competitive inhibitor of sulfate uptake and its involvement in a sulfate-bicarbonate exchange system. Bicarbonate uptake was also shown to be dependent on sodium (Table II). This requirement could result from both a bicarbonate-sodium co-transport system as observed for sulfate or from a sodium-proton exchange where the proton is generated as a result of CO₂ diffusion, hydration, and ionization.

When compared to hepatocytes, the rate of sulfate uptake by HTC cells was only 20% as great, which is in contrast to the elevated rates of sugar and amino acid transport observed in many transformed cells (28–30). The transport of organic anions, such as taurocholic acid, by HTC cells was also observed to occur at 10% of the levels observed in normal hepatocytes. The modification in transport capacity could result from an alteration in the membrane content of the carrier due to changes in the synthesis or processing of this protein or in the structural properties of the carrier. Studies are currently underway to characterize the hepatocyte anion carrier and the molecular basis of the transport defect in HTC cells.

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