Enterohepatic circulation of bile acids requires efficient bile acid absorption by the ileum. The first step in ileal bile acid transport is mediated by a Na\(^+\)-bile acid transporter (ASBT), located at the epithelial cell apical brush border membrane (1). After uptake, the bile acids are transported transcellularly to the basolateral membrane (2) and secreted into the portal circulation by a sodium-independent anion exchange mechanism (3). The apical sodium-bile acid transporter cDNA has been isolated from the hamster (4), rat (5), and human (6) and is homologous to the hepatic Na\(^+\)-coupled bile acid transporter (7). Analysis of mRNA and protein expression revealed that ASBT is also expressed on the apical brush border membrane of renal proximal tubule cells (8) and apical membranes of cholangiocytes lining the large bile duct units (9, 10). Whereas ASBT is known to transport both Na\(^+\) and bile acid, the Na\(^+\)-bile acid stoichiometry and electrogenicity of transport are unknown (2), and previous studies testing for electrogenic transport have produced conflicting results (11–15), although the tentative consensus was that ileal Na\(^+\)-bile acid co-transport is electroneutral (2). Electrogenicity of other transporters, particularly Na\(^+\)-glucose and Na\(^+\)-amino acid, has been definitively demonstrated by measuring the currents associated with transport (16–18). This has not been possible for the bile acid transporters because next flux is small and the detergent properties of the bile acids may activate ion channels (19). We have therefore taken an alternative approach, directly monitoring the movement of a fluorescent bile acid analog in single, voltage-clamped CHO cells transfected with human ASBT. We have for the first time been able to definitely demonstrate electrogenicity and suggest Na\(^+\)-bile acid stoichiometry for a Na\(^+\)-bile acid transporter.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Bile Acid Uptake Assays—**CHO-K1 cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in medium A that consisted of a 1:1 (v/v) mixture of Dulbecco’s modified Eagle’s medium containing 4500 mg/liter D-glucose and Ham’s F-12 medium, 10% (v/v) fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.). Stable overexpression of the hASBT was achieved by cotransfecting the cells with pCMV5-hASBT (6) and pSV3Neo using the calcium phosphate procedure as described (20). Individual colonies were expanded in 24-well plates and screened for hASBT expression using \(^{3}H\)taurocholate uptake assays. Functional expression of the hASBT protein in these cells has been previously demonstrated (21).

\(^{3}H\)Taurocholic acid (2.0–2.6 Ci/mmol) and \(^{2,4-3}H\)icholic acid (27.5 Ci/mmol) were purchased from NEN Life Science Products. Unlabeled taurocholate and cholate were purchased from Sigma. For \(^{3}H\)bile acid uptake assays, CHO-hASBT cells were incubated in medium B, which consisted of a modified Hanks’ balanced salt solution containing 137 mM NaCl or 137 mM choline chloride (6).

**Measurement of Fluorescent Bile Acid Analog Accumulation—**The fluorescent bile acid analog cholylglycylamidofluorescein (CGamF), choleyl-N-neonitrobenzoxadiazol-lysine (C-NBD-L), chenodeoxycholyl-N-neonitrobenzoxadiazol-lysine (CDC-NBD-L), and chenodeoxycholyl-N-neonitrobenzoxadiazol-lysine (C-NBD-L) were generous gifts of Drs. A. Hofmann and C. Schteingart. Uptake specificity and time course of uptake were measured in individual cells with a quantitative fluorescence imaging system, as described previously (22). Cells were superfused at 37 °C with FBA-containing HEPES-buffered Na\(^+\)-Ringer solution (144 mM NaCl, 5 mM KCl, 2 mM NaHPO\(_4\), 1.25 mM CaCl\(_2\), 1 mM MgSO\(_4\), and 10 mM HEPES, pH 7.4), and cell fluorescence was measured at 5–10-s intervals. The total Na\(^+\) concentration was approxi-
mately 150 mM. Where indicated, Na⁺ was quantitatively replaced with tetrathylammonium.

**Quantitative Fluorescence Microscopy and Whole Cell Patch Clamp**—Simultaneous whole cell patch clamp and fluorescence microscopy were performed with a photometer-based quantitative fluorescence system as described previously (23). CHO-hASBT cells were grown in medium A on 5 × 5-mm glass coverslips and exposed to sodium butyrate (5 mM) for 16 h. Cells were pre-equilibrated with 1 μM CDC-NBD-L in HEPES-buffered Na⁺-Ringer solution for 30 min. Fluorescent cells were then subjected to whole cell patch clamp in the continued presence of CDC-NBD-L at 37 °C. From an initial holding potential of ~30 mV, intracellular (pipette) voltage was changed at 1–2-min intervals to ~−90, or +30 mV as indicated. Cell fluorescence was measured at 10-s intervals during these voltage steps. The patch pipette was filled with a solution containing 115 mM potassium gluconate, 30 mM KCl, 0.47 mM CaCl₂, 2.1 mM MgSO₄, 2 mM EGTA, 10 mM HEPES, and 3 mM Na₂ATP, pH 7.2. The total Na⁺ concentration was approximately 12 mM. In some experiments the bath solution Na⁺ concentration was reduced by substitution of NaCl with tetrathylammonium chloride.

**Analysis of Stoichiometry**—Stoichiometry was determined by comparing the effects of Na⁺ gradients and voltage on transport. For a cotransport process with n mol of Na⁺ transported for each mol of univalent negatively charged bile acid, the free energy change associated with transport (ΔG) depends on the Na⁺ gradient, the bile acid gradient, and the voltage according to the following relationship.

\[
\Delta G = nRT \ln (Na^+ + Na^-) + RT \ln (B/B_0) + nFV - FV \quad \text{(Eq. 1)}
\]

where n is the number of moles of Na⁺ transported per mol of bile acid, R is the gas constant, T is the absolute temperature, Na⁺, and Na⁻ are the Na⁺ concentrations inside and outside the cell, B and B₀ are the bile acid concentrations inside and outside the cell, F is the Faraday constant, and V is the transmembrane voltage. The reversal potential, \(V_{rev}\), is that voltage at which \(\Delta G\) is zero and is simply as follows.

\[
V_{rev} = RT/F (1 - n) \cdot (ln (Na^+ + Na^-) + ln (B/B_0)) \quad \text{(Eq. 2)}
\]

If Na⁺ is suddenly reduced by a factor of x and assuming that all other parameters initially remain constant, we would predict a change in \(V_{rev}\) according to Equation 3.

\[
\Delta V_{rev} = RT/F (1 - n) \cdot (ln x) \quad \text{(Eq. 3)}
\]

**RESULTS**

**Expression of the Human ASBT in CHO Cells**—The expression plasmid pCMV5-hASBT was stably transfected into CHO cells, and clonal cell lines were selected. As seen in Fig. IA, the uptake of taurocholate and cholate were saturable and Na⁺-dependent. There was little appreciable bile acid uptake in the absence of Na⁺ or in the parental CHO cells (data not shown). At 137 mM Na⁺, analysis of the concentration dependence of bile acid transport revealed apparent \(K_m\) values of 12 and 37 μM for taurocholate and cholate, respectively. Fig. 1B shows that these data fit the Hill equation well with Hill coefficients near 1.0 for both taurocholate and cholate, suggesting that bile acid binding sites are either single or noninteracting in the transporter complex.

**Fluorescent Bile Acid Uptake**—Previous studies of the FBA, CGamF, C-NBD-L, and CDC-NBD-L have shown that uptake is specific for bile acid transporting cells such as hepatocytes and displays Michaelis-Menten kinetics (22). To determine whether these FBA are also substrates for the hASBT, we measured accumulation of these analogs after a 5-min incubation in either parental CHO-K1 or transfected CHO-hASBT cells. As shown in Fig. 2A, CHO-hASBT cells efficiently transported both NBD-labeled bile acids, C-NBD-L and CDC-NBD-L, but failed to take up the fluorescein-labeled bile acid, CGamF. There was no appreciable uptake of any FBA in the parental CHO-K1 cells, and uptake was entirely Na⁺-dependent (data not shown). Initial uptake rates of CDC-NBD-L were saturable with a \(K_m\) of 13.1 μM (Fig. 2B) and a Hill coefficient of 1.07 (Fig. 2C). These kinetic constants were close to those observed for taurocholate.

**Transport during Voltage Clamp**—CHO-hASBT cells were pre-equilibrated for 30 min with 1 μM CDC-NBD-L and subjected to whole cell patch clamp in the continued presence of the FBA. The cells were clamped at three different voltages, cell fluorescence was measured at 10-s intervals, and the rate of change of fluorescence with time was compared at the different clamp voltages. Fig. 3A demonstrates an example of cell fluorescence changes in one cell during this protocol. At ~30 mV, fluorescence was relatively constant, but at ~90 mV the cell fluorescence increased over time and at ~30 mV fluorescence decreased. Control experiments were performed to exclude the possibility that fluorescence changes were due to other voltage-dependent phenomena such as increasing cell volume or fluorophore leak. No voltage-dependent fluorescence changes were observed with either a nontransported fluorescent dye, carboxyfluorescein, or with CDC-NBD-L when the bath temperature was cooled to 10 °C to inhibit activity of the membrane transporter (data not shown). These results demonstrate that voltage-induced fluorescence changes result from either inward or outward transport of CDC-NBD-L. Fig. 3B shows the results from 10 cells clamped under this protocol. CDC-NBD-L movement was bidirectional and voltage-dependent. Increasing inside negative voltage stimulated uptake and inside positive voltage resulted in net efflux. The direction of change was not affected by the order in which the voltage clamps were performed.

To assess stoichiometry of voltage-dependent Na⁺-bile acid cotransport, we examined the relationship between the electri-
cal and chemical driving forces for transport. Voltage dependence was first determined in control bath solution and then again while maintaining the patch clamp configuration in the same cells after reduction of bath Na\(^{+}\) concentration. The shift in the voltage dependence produced by this maneuver results from the additional inward electrical driving force necessary to balance the reduction in the inwardly directed Na\(^{+}\) gradient.

**DISCUSSION**

This study has shown that transport of fluorescent bile acid derivatives by CHO cells stably transfected with hASBT is electrogenic. Previous studies of electrogenicity of ileal bile acid transport reached conflicting conclusions. Authors using similar vesicle techniques reported that Na\(^{+}\)-bile acid cotransport was either electrically (11, 12) or electroneutral (13–15). A major limitation of these prior studies was the need to use an indirect approach to change membrane potential of vesicles. In most cases addition of valinomycin was assumed to hyperpolarize the vesicle interior, but these studies did not validate that the expected voltage changes actually occurred.

In this study we used several approaches to circumvent these problems. We performed whole cell patch clamp to directly voltage clamp and measure the cell membrane potential and used a fluorescent bile acid derivative rather than naturally occurring bile acids. This allowed us to measure transport rates without having to directly measure transport currents. Unlike the case with more hydrophilic substrates such as glucose and amino acids (16–18), bile acid transport currents are extremely difficult to measure because the electrical currents resulting from bile acid transport are small. For the CHO-hASBT cells, the average \(V_{\text{max}}\) was about 2 nmol/min/mg protein, which
predicts transport currents of only 1.5 pA/cell. This compares with greater than 100 nA/cell for the Na+-glucose cotransporter in oocytes (16). A second difficulty is that external bile acids have been shown to affect other conductive pathways (19), and bile acid-induced currents therefore do not necessarily represent current carried by the cotransport proteins.

The hASBT-transfected CHO cells efficiently transported two NBD-labeled bile acids, C-NBD-L and CDC-NBD-L, but not a fluorescein-labeled bile acid, CGamF. Kinetic parameters for CDC-NBD-L transport were similar to that of taurocholate and uptake followed Michaelis-Menten kinetics. Earlier studies of hepatocytes (22) and Ntcp-transfected COS cells (24) had shown specific transport of both CDC-NBD-L and CGamF, and these differences reflect the more restricted substrate specificity of ASBT compared with that of the homologous Ntcp (21). These findings complement a recent report describing the in vivo transport properties of the fluorescent bile acid analogs (25). In that study as well, C-NBD-L but not CGamF was transported by ileum.

Our results clearly demonstrate that transport via the human ASBT is electrogenic and carries positive charge into the cell. The basic principal underlying these measurements is that once a steady-state concentration in a patched cell is reached, changes in cell fluorescence induced by voltage clamping the cell/pipette system result from flux of substrate between the cell and the bath. For this to be the case, there must be no voltage-dependent changes in cell fluorescence such as could result from voltage-dependent changes of the dye quantum yield or cell volume. We examined these possibilities under conditions in which transmembrane transport was abolished either with a nontransported dye or by low temperature. In both of these cases voltage changes had no effect on cell fluorescence.

Another consideration is the possibility that appreciable amounts of dye diffuse from the cell to the pipette, confounding our analysis. Our previous studies have determined that diffusion of bile acids from cell to pipette is small and alters apparent uptake rates by only about 5% (23). The small magnitude of the effect is due to the high degree of intracellular binding of this hydrophobic substrate as well as the relatively small surface area of the pipette (26). Intracellular diffusion coefficients of bile acids are approximately 1000-fold lower than that predicted for simple aqueous diffusion (27). Although diffusion into the pipette is small, it can still affect the voltage at which cell fluorescence is constant. However, there is no voltage gradient between cell and pipette, and therefore changes in cell fluorescence that occur immediately after voltage shift reflect changes in transmembrane transport and not changes in cell to pipette diffusion.

The stoichiometry of Na+-bile acid cotransport was assessed by comparing the effects of voltage changes and Na+ gradient changes on the zero net flux voltage ($V_{rest}$). Provided that only transmembrane flux is changed by voltage clamp, the immediate change in $V_{rest}$ after sudden reduction of extracellular Na+ is described by Equation 3. It predicts that for any given Na+ gradient change, the magnitude of $\Delta V_{rest}$ is greatest for $n = 2$ and decreases as $n$ increases. This can be understood because at higher coupling stoichiometry the transported complex has a greater charge and thus requires less of a voltage change to balance the chemical free energy component of the altered Na+ gradient.

The observed $\Delta V_{rest}$ for a 3-fold reduction in bath Na+ was −52 mV. The theoretical value predicted from Equation 3 is −58.7 mV for $n = 2$ and −44 mV for $n = 3$. These theoretical values are expected to be upper limits for the observed change because the internal bile acid concentrations (B) may have decreased as a result of outward transport resulting from the reduction of bath Na+ concentration. A slight reduction of B gives rise to an inward bile acid gradient and thus reduces the magnitude of the negative voltage required to re-establish the zero flux condition. Such non-ideal effects would tend to decrease the observed $\Delta V_{rest}$. Because the observed value is greater than the upper limit for an $n = 3$ coupling and only slightly less than that for $n = 2$, we conclude that the most likely coupling ratio is 2 Na+ per 1 bile acid, but we cannot definitively exclude a 3:1 ratio. This finding is consistent with kinetic measurements of the Hill coefficient for Na+ in these cells of approximately 2 (21) and the Hill coefficient for bile acid of approximately 1 (Figs. 1 and 2).

The apical Na+-bile acid cotransporter cDNA encodes a polytopic membrane glycoprotein that shares considerable amino acid identity and remarkable structural similarity to Ntcp, the major Na+-dependent liver bile acid transporter (28). Considerable evidence has now accumulated suggesting that Ntcp is also electrogenic including direct measurements of bile acid transport currents (29, 30) as well as voltage dependence of fluorescent bile acid transport in hepatocytes (23). Kinetic analysis of the Ntcp transporter also shows a Hill coefficient of 1.9 for Na+ and 1.0 for bile acid (28). Considering the similarities in sequence and predicted structure for the Na+-bile acid transporters, it is likely that they share a common electrogenic mechanism.

Previous ileal bile acid transporter modeling studies relied upon substrate binding properties and assumed an electroneutral transport mechanism. Based in part on this assumption, a model for the substrate binding site was proposed that encompassed a closely positioned negatively charged group for interaction with a single sodium ion (31, 32). The previous model will have to be re-evaluated in light of the present study demonstrating electrogenicity.

Electrogenicity confers two significant advantages for ileal bile acid transport. First, as described by Kimmich (33), the driving force for uptake sets the upper limit for the concentrating ability of the system, but in practice gradients achieved are significantly lower due to leak pathways. In the case of intestinal bile acid transport, basolateral exit occurs by a passive transporter. Therefore, electrogenic uptake with a high Na+: bile acid coupling ratio at the apical membrane results in a much higher intracellular bile acid concentration and thus increases net transcellular transport. Secondly, electrogenicity of uptake provides a means for regulation of transport. Fasting (34), aldosterone (34), and 5-hydroxytryptamine (35) have all been shown to alter intestinal electrical gradients in the direction of hyperpolarization of the brush border membrane. These physiologic changes may provide the ileal intestinal cells with a mechanism to regulate bile acid uptake rates for optimal bile acid recycling and cytoprotection.

In summary, this study is the first unequivocal demonstration of an electrogenic mechanism for a cloned Na+-bile acid transport protein. These results will be critical in modeling the structure-function relationship for this important but poorly understood class of Na+-coupled transport proteins.

Acknowledgments—We thank Drs. A. Hofmann and C. Schteingart for kindly providing the fluorescent bile acid analogs and Drs. L. Reuss and S. King for helpful comments. We also acknowledge the excellent technical assistance of Lori Showalter and Ann L. Craddock.

REFERENCES

1. Wilson, F. (1981) Am. J. Physiol. 241, G83–G92
2. Wilson, F. A. (1991) in Handbook of Physiology: The Gastrointestinal System IV (Schultz, T., and Stanley, S., eds) pp. 389–404, Waverly Press, Baltimore, MD
3. Weinberg, S. L., Burckhardt, G., and Wilson, F. A. (1986) J. Clin. Invest. 78, 44–50
4. Wong, M. H., Oelkers, P., Craddock, A. L., and Dawson, P. A. (1994) J. Biol. Chem. 269, 1340–1347
5. Shneider, B. L., Dawson, P. A., Christie, D.-M., Hardikar, W., Wong, M. H., and Suchy, F. J. (1995) J. Clin. Invest. 95, 745–754
6. Wong, M. H., Oelkers, P., and Dawson, P. A. (1995) J. Biol. Chem. 270, 27228–27234
7. Hagenbuch, B., and Meier, P. J. (1994) J. Clin. Invest. 93, 1326–1331
8. Christie, D. M., Dawson, P. A., Thevananther, S., and Shneider, B. L. (1996) Am. J. Physiol. 271, G377–G385
9. Lazaridis, K. N., Pham, L., Tietz, P., Marinelli, R. A., deGroen, P. C., Levine, S., Dawson, P. A., and LaRusso, N. F. (1997) J. Clin. Invest. 100, 2714–2721
10. Lucke, H., Gertraud, S., Kinne, R., and Murer, H. (1978) Biochem. J. 174, 951–958
11. Wilson, F. A., and Treanor, L. (1979) Biochim. Biophys. Acta 554, 430–440
12. Rouse, D. J., and Lack, L. (1979) Life Sci. 25, 45–52
13. Barnard, J. A., Ghishan, F. K., and Wilson, F. A. (1985) J. Clin. Invest. 75, 869–873
14. Barnard, J. A., and Ghishan, F. K. (1987) Gastroenterology 93, 925–933
15. Parent, L., Supplisson, S., Loo, D. D. F., and Wright, E. M. (1992) J. Membr. Biol. 125, 49–62
16. Jauch, P., Petersen, O. H., and Lauger, P. (1986) J. Membr. Biol. 94, 99–115
17. Bergman, J., Zaal, M., and Bergman, C. (1989) J. Membr. Biol. 111, 241–251
18. Wehner, F. (1993) Eur. J. Physiol. 424, 145–151
19. Ridgway, N. D., Dawson, P. A., Ho, Y. K., Brown, M. S., and Goldstein, G. L. (1992) J. Cell Biol. 116, 307–319
20. Craddock, A. L., Love, M. W., Daniel, R. W., Kirby, L. C., Walter, H. C., Wong, M., and Dawson, P. A. (1998) Am. J. Physiol. 274, G157–G169
21. Maglova, L. M., Jackson, A. M., Meng, X.-J., Carruth, M. W., Schteingart, C. D., Ton-Nu, H.-T., Hofmann, A. F., and Weinman, S. A. (1995) Hepatology 22, 637–647
22. Grune, S., Meng, X.-J., and Weinman, S. A. (1996) Am. J. Physiol. 270, G339–G346
23. Boyer, J. L., Ng, O.-C., Ananthanarayanan, M., Hofmann, A. F., Schteingart, C. D., Hagenbuch, B., Stieger, B., and Meier, P. J. (1994) Am. J. Physiol. Gastrointest. Liver Physiol. 266, G382–G387
24. Holzinger, F., Schteingart, C. D., Ton-Nu, H., Eming, S. A., Monte, M. J., Hagey, L. R., and Hofmann, A. F. (1997) Hepatology 26, 1263–1271
25. Weinman, S. A., and Schteingart, C. D., Ton-Nu, H. (1994) J. Biol. Chem. 269, 1340–1347
26. Hagenbuch, B., and Meier, P. J. (1996) Semin. Liver Dis. 16, 129–136
27. Lidefsky, S. D., Fitz, J. G., Weisiger, R. A., and Scherschmidt, B. F. (1993) Am. J. Physiol. 264, G478–G485
28. Weinman, S. A., and Weeks, R. F. (1993) Am. J. Physiol. 265, G73–G80
29. Lack, L. (1979) Environ. Health Perspect. 33, 79–90
30. Kimmich, G. A. (1990) J. Membr. Biol. 114, 1–27
31. Debnam, E. S., and Thompson, C. S. (1984) J. Physiol. 355, 449–456
32. Grubb, B. R., and Bentley, P. J. (1987) Am. J. Physiol. 253, G211–G216
Bile Acid Uptake via the Human Apical Sodium-Bile Acid Cotransporter Is Electrogenic
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J. Biol. Chem. 1998, 273:34691-34695.
doi: 10.1074/jbc.273.52.34691

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