Rat Hepatocytes Exhibit Basolateral Na\(^+\)/HCO\(_3^-\) Cotransport

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Abstract

Primary cultures and plasma membrane vesicles were used to characterize Na\(^+\) and HCO\(_3^-\) transport by rat hepatocytes. Na\(^+\) uptake into hepatocytes was stimulated ~10-fold by 25 mM extracellular HCO\(_3^-\). HCO\(_3^-\)-stimulated Na\(^+\) uptake was saturable, abolished by 4-acetamido-4'-isothiocyanato-2,2'-disulfonic acid stilbene (SITS), and unaffected by amiloride or Cl\(^-\) removal. Neither propionate nor acetate reproduced this effect of HCO\(_3^-\). Na\(^+\) efflux from preloaded hepatocytes was similarly increased ~10-fold by an in > out HCO\(_3^-\) concentration gradient. Na\(^+\) efflux was also increased by valinomycin and an in > out K\(^+\) concentration gradient in the presence but not absence of HCO\(_3^-\). Intracellular pH (pH\(_i\)) measured with the pH-sensitive fluorochrome 

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2',7'-\text{bis-(2-carboxyethyl)-5-}
\text{(and 6-)}\text{-carboxyfluorescein (BCECF) decreased at a rate of 0.227}
\text{(±0.074 SEM) pH units/min when extracellular HCO}_3^-\text{ concentration was lowered from 25 to 5 mM at a rate of PCO}_2.\text{ This intracellular acidification rate was decreased 50–60% in the absence of Na}^+\text{ or presence of SITS, and was unaffected by amiloride or Cl}^-\text{ removal. Membrane hyperpolarization produced by valinomycin and an in > out K}^+\text{ concentration gradient caused pH}_i\text{ to fall; the rate of fall was decreased 50–70% by Na}^+\text{ removal or SITS, but not amiloride. An inside positive K}^+\text{ diffusion potential and a simultaneous out > in HCO}_3^-\text{ gradient produced a transient 4,4'-disisothiocyanato-2,2'-disulfonic acid stilbene (DIDS) sensitive, amiloride-insensitive Na}\(^+\) accumulation in basolateral but not canicular membrane vesicles. Rat hepatocytes thus exhibit electronegative basolateral Na}\(^+\)/HCO\(_3^-\) cotransport.

Introduction

The cellular mechanisms whereby hepatocytes secrete bile are not well understood, but there is evidence that active transport of HCO\(_3^-\) plays a role in this process. This evidence includes the observations that removal of perfusate HCO\(_3^-\) (but not Cl\(^-\)) decreases basal bile formation by perfused liver (1–4) and that certain bile acids, such as ursodeoxycholic acid produce a severalfold increase in bile flow and an increase in biliary HCO\(_3^-\) concentration to levels two to three times that present in plasma or perfusate (5–7).

Currently recognized mechanisms for plasma membrane transport of H\(^+\) or HCO\(_3^-\) by hepatocytes include a Na\(^+\)/H\(^+\) exchange mechanism present on the basolateral membrane (8, 9) and a Cl\(^-\)/HCO\(_3^-\) exchange mechanism present on the canicular membrane (10). Recently, we have reported that the HCO\(_3^-\)-rich hypercholerlaemia produced by ursodeoxycholic acid in perfused rat liver is inhibited up to 50% by amiloride or amiloride analogues and virtually abolished (>95% inhibition) by removal of perfusate Na\(^+\) (7, 11). These findings suggest that Na\(^+\)/H\(^+\) exchange plays a role in the choleresis produced by ursodeoxycholic acid. The quantitative discrepancy between the effects of Na\(^+\) substitution and the presumably more specific inhibition of Na\(^+\)/H\(^+\) exchange produced by amiloride and its analogues prompted us to explore the possible existence in hepatocytes of a Na\(^+\)-dependent mechanism for HCO\(_3^-\) transport distinct from Na\(^+\)/H\(^+\) exchange. In this manuscript, we report evidence for the existence in primary rat hepatocyte cultures and basolateral plasma membrane vesicles of electronegative Na\(^+\)/HCO\(_3^-\) cotransport as recently described in several other epithelia, including the renal proximal tubule (12–17), a renal epithelial cell line (18), corneal endothelial cells (19), and gastric parietal cells (20).

We also provide evidence for its localization to the basolateral (sinusoidal/lateral) and not apical (canalicular) membrane.

Methods

Chemicals and radioisotopes. Ouabain, nigericin, 4-acetamido-4'-isothiocyanato-2,2'-disulfonic acid stilbene (SITS), 4,4'-disothiocyanato-2,2'-disulfonic acid stilbene (DIDS), and valinomycin were purchased from Sigma Chemical Co., St. Louis, MO; 2',7'-bis-(2-carboxyethyl)-5-(and 6-)carboxyfluorescein, acetoxyethyl ester (BCECF-AM) and collagenase were purchased from Molecular Probes, Eugene, OR, and Cooper Biomedical, Inc., Malvern, PA, respectively. Amiloride and ethylisopropylamiloride (EIA) were generous gifts of Dr. Edward J. Cragoe, Jr., Merck Sharp & Dohme, West Point, PA, and 22Na was purchased from New England Nuclear, Boston, MA. All other chemicals used were of the highest purification grade commercially available and were obtained from either Sigma Chemical Co. or Fluka Chemie AG (Buchs, Switzerland). Stock solutions of nigericin and valinomycin (each 10 mM in ethanol) and BCECF-AM (1 mg/ml in DMSO) were prepared and stored at −20°C until use. 1 mM amiloride used in studies of 22Na transport and intracellular pH (pH\(_i\)) was dissolved directly by heating in the respective incubation media. 100 mM EIA was dissolved by addition of a slight molar excess of isethionic acid and was added directly to the respective incubation media.

1. Abbreviations used in this paper: BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and 6-)carboxyfluorescein; bLPM, basolateral liver plasma membrane; cLPM, canalicular liver plasma membrane; DIDS, 4,4'-disothiocyanato-2,2'-disulfonic acid stilbene; dH\(^+\)/dt, rate of change in intracellular pH; EIA, ethylisopropylamiloride; NMG, N-methyl-D-glucamine; pH\(_i\), intracellular pH; SITS, 4-acetamido-4'-isothiocyanato-2,2'-disulfonic acid stilbene; TMA, tetramethylammonium.
Cultured hepatocytes. Hepatocytes (> 98% parenchymal cells) were isolated from livers of male Sprague-Dawley rats (250-350 g) by collagenase perfusion, plated on collagen-coated plastic dishes (22Na uptake studies) or collagen-coated plastic cover slips (pH studies), and maintained in modified 199 OR medium supplemented with amino acids, insulin, corticosteroids, and calf serum (1% for dishes, 5% for plates) at 48 h before use, as described previously (21, 22). Cell viability in these monolayer cultures as assessed by trypan blue exclusion is ≥ 98%.

22Na transport by cultured hepatocytes. 22Na uptake studies were performed using minor modifications of a technique previously described (2, 21, 22). Hepatocytes were first preincubated for 30 min in nominally HCO3-free electrolyte solution containing a variable (0-135 mM) sodium concentration (0-135 mM NaCl replaced by choline chloride, N-methyl-d-glucamine [NMG] chloride, or LiCl); 5 mM KCl, 0.8 mM MgSO4; 1.2 mM CaSO4; 0.8 mM KH2PO4; 5 mM glucose; 5 mM ouabain; 10 mM Hepes adjusted to pH 7.4 with KOH/HCl. Measurements of Na+ uptake were then conducted in media containing trace (1-2 μCi) amounts of 22Na. Uptake medium was identical to that used for preincubation except for the presence, in selected incubations, of 25 mM HCO3, inhibitors, and/or varying concentrations of Na+. Namely HCO3-free media were gassed with room air and HCO3-containing media were gassed with 5% CO2 in room air. When used, 1 mM (occasionally 5 mM) SITS was present both in the preincubation and incubation media. 1 mM amiloride was present in incubation medium in selected studies. At various times during incubation in 22Na-containing media, uptake was stopped and extracellular isotope was removed by dipping the dishes for 10 s in each of eight consecutive beakers containing 200 ml of identical ice-cold (4°C) medium without radioisotope. Previous studies have demonstrated that this wash procedure efficiently removes extracellular isotope, while causing minimal loss of intracellular isotope (21, 24). The cells were then scraped from the dishes directly into 2% (wt/vol) Na2CO3 in 0.1 M NaOH. Radioactivity was measured in an aliquot of the scrapings by liquid scintillation counting using external standardization for quench correction, and total cell protein was determined in another aliquot as previously described (21, 22).

This approach to measurement of HCO3-stimulated Na+ uptake is analogous to that used by other investigators (18, 19) and was selected on the basis of preliminary studies in our culture system. A low concentration (5 mM) of Na+ was used in all studies (except those depicted in Fig. 2), because this concentration is well below the apparent Km of Na+/HCO3 symport demonstrated in both other cell types (14, 18, 19) and hepatocytes (see Results) and therefore enhanced detection of HCO3-stimulated Na+ uptake as compared with physiologic Na+ concentration. Moreover, 5 mM ouabain, which maximally inhibits Na+/K+-ATPase in these cultured cells (22), prolongs the initial linear phase of 22Na uptake and presumably minimizes or eliminates differences in transmembrane Na+ concentration or electrical potential differences among the differing preincubation conditions. Initial 22Na uptake rates (up to 3 min) were measured in duplicate or triplicate in each of several batches of cultured cells in the nominal absence or presence of HCO3, amiloride, and SITS, and were expressed as nmol/mg protein/min.

22Na uptake in Cl- free medium was measured as described above, except that Cl- was replaced by gluconate or nitrate in the preincubation and uptake media, EIA was used in place of amiloride (which is available only as a Cl- salt), and the preincubation period was increased to 60 min, which is sufficient to completely deplete cells of intracellular Cl- (reference 21 and unpublished results). 22Na efflux from cultured hepatocytes was measured using a modification of our method previously described for measurement of efflux of fluid phase markers (24). In brief, cells were first preloaded with 22Na in a series of three preincubation media all containing 22Na and consisting of (a) 60 min in HCO3-containing medium (25 mM NaHCO3; 40 mM NaCl; 70 mM KCl; 0.8 mM MgSO4; 1.2 mM CaSO4; 0.8 mM KH2PO4; 5 mM glucose; 10 mM Hepes adjusted to pH 7.4 with KOH/HCl) with 5 mM ouabain for all cells, (b) 30 min in the same HCO3-containing medium with or without 1 mM SITS or amiloride or 30 min in nominally HCO3-free medium (25 mM of NaHCO3 replaced with 25 mM NaCl), and (c) 2 min in the same media used for the previous 30-min preincubation, except for addition of 2 mM KCN (to inhibit metabolic CO2 production, see below). Cells were then washed as described above for uptake studies by dipping for 10 s in each of eight beakers containing ice-cold identical media without 22Na. After being washed, 1 ml of prewarmed (37°C) isotope-free media was added to the culture dish. This isotope-free media was either nominally HCO3-free or contained 25 mM HCO3. It also contained valinomycin (1 or 10 μM) and 5 mM (65 mM of KCl replaced by choline Cl) or 70 mM KCl, and SITS or amiloride as appropriate for the conditions of the experiment. At selected intervals (30 s to 10 min), the media was completely removed for scintillation counting and replaced with identical, isotope-free media. At the end of the last efflux period, cells were scraped and radioactivity and protein measured as described above. Total radioactivity present in the cells at time zero was calculated as the sum of all radioactivity present in efflux media plus residual radioactivity in cells, and radioactivity present in the cells at each time point was calculated as total radioactivity at time zero minus cumulative radioactivity in efflux media (24). These conditions were selected on the basis of extensive preliminary experiments (not shown) that demonstrated that they produced an equivalent degree of Na+ loading in all experimental groups, that complete Na+/K'-ATPase inhibition prolonged the linear phase of Na+ efflux, and that brief incubation in KCN was necessary to inhibit metabolic CO2 production and achieve depletion of intracellular HCO3 (see Results). Moreover, these conditions were very similar to those used to study the effects of membrane hyperpolarization on pH, (except for the KCN preincubation, see below), and they presumably minimized potentially confounding effects of Na+ removal or amiloride (which inhibits Na+/K'-ATPase in these cells [25]) on transmembrane potential difference and/or K+ concentration gradients by clamping intracellular K+ while depolarizing the hepatocytes, thereby permitting independent manipulation of HCO3 concentration gradients and of membrane potential. The concentration of amiloride (1 mM) used in these studies maximally inhibits Na+/H+ both in hepatocyte plasma membranes and in intact hepatocytes (8, 9, 26, 27).

Measurement of intracellular pH: description of the technique. pH was measured fluorometrically (28) using the pH-sensitive carboxyfluorescein derivative BCECF (pKa 6.98). The nonscintillating and membrane permeant acetoxyxymethylester of this compound (BCECF-AM) readily enters cells, where cytosolic esterases cleave the ester bond and form the polyamionic, fluorescent BCECF. In preliminary studies, we observed that cultured hepatocytes secreted up to 50% of the anionic, fluorescent BCECF species within 20-30 min and that accumulating extracellular dye accounted for an unacceptably large and steadily increasing proportion of total fluorescence signal. This represented secretion by hepatocytes rather than leakage from damaged cells, since intact perfused liver also secreted the anionic fluorescent species readily into bile. To circumvent this problem, we devised a system for continuous superfusion (27). This system permitted 95% exchange of cuvette contents within 1 min and effectively eliminated fluorescent signal resulting from extracellular dye (< 3% of the total fluorescence signal at both excitation wavelengths). All measurements were made at 37°C using a water-jacketed cuvette holder, and perfusion solutions (equilibrated with room air [HCO3-free] or 95% room air/5% CO2 [HCO3-containing]) were prewarmed.

After preincubation for up to 60 min at 37°C under conditions appropriate for each experiment (see below), coverslips with adherent hepatocytes were mounted into the cuvette, background fluorescence was measured, and the cells were loaded with dye in the flowthrough mode for 10-15 min with 2.5 μM BCECF-AM in media identical to that used for preincubation. This time period was sufficient to achieve initial fluorescence signals 10-20 times background with excitation at 500 nm and two to three times background with excitation at 450 nm. Fluorescence measurements (done with a SF/330 spectrofluorometer; Varian Instruments, Palo Alto, CA) were made at an emission wave-
length of 530 nm (slit width = 10 nm) after alternate excitation (slit width = 5 nm) at 500 and 450 nm, where fluorescence is highly pH sensitive and relatively pH insensitive, respectively. After correction for background fluorescence, the ratio of the fluorescence intensity at 500 nm to that at 450 nm (ratio of fluorescence unit [RFU] 500/450) was used to calculate pH, from standard curves. The use of this fluorescence ratio provides a measurement of pH, that is unaffected by changes in intracellular dye concentration due to dye leakage and photobleaching (28).

Calibration curves were constructed using hepatocytes loaded with dye and superfused with solutions containing 20 mM Hepes or morpholine-ethane-sulfonic acid (adjusted to pH values within the range of 6.3 to 7.8), 130 mM KCl, and 10 mM nigericin, a H+/K+ -exchanging ionophore (28). Under these conditions, pH, presumably equals extracellular pH. In preliminary experiments (not shown), calibration curves performed in the presence and absence of 10 μM valinomycin, a K+ ionophore, were found to be identical, indicating the absence of transmembrane K+ gradients that could potentially drive pH, higher or lower than extracellular pH. Amiloride also did not affect absolute fluorescence or RFU 500/450 under these conditions. Calibration curves were linear over a pH range of 6.30 to 7.80. A calibration was performed on at least one hepatocyte monolayer each study day, immediately after its use in an experiment, and was used to calculate pH, in that monolayer. Because individual calibration of each coverslip was not feasible, pH, in the other monolayers was calculated using the average slope and intercept of all calibration curves, which differed little from day to day (coefficient of variation of slope and intercept = 0.13 and 0.02, respectively). Hepatocyte monolayers at the end of the experiments were intact by light microscopy, and cell viability was further demonstrated by recovery of pH, toward baseline values in selected experiments (see Results).

To determine steady-state pH, rapid fluorescence measurements were made alternatively at excitation wavelengths of 500 and 450 nm, and RFU 500/450 was calculated after background correction. Steady-state pH, was taken to be the average of several such consecutive measurements performed at least 5 min after a change of superfusion solution. Under conditions in which pH, was rapidly changing, fluorescence at 500 nm excitation was continuously recorded on a chart recorder while fluorescence at 450 nm excitation (which is nearly constant despite changing pH,) was measured before and after the change in superfusion medium. The rate of change in pH, (dph/dt) was calculated from a tangent drawn by eye to the tracing at 500 nm, using interpolated values for fluorescence intensity at 450 nm, after correcting for background.

Measurement of pH, during transient lowering of extracellular [HCO3-]. Cells were preincubated for 30 min in Na+-containing media (25 mM NaHCO3; 110 mM NaCl; 5 mM KC1; 0.8 mM MgSO4; 1.2 M CaSO4; 0.8 mM Na2PO4; 5 mM glucose; 10 mM Hepes, adjusted to pH 7.4 with KOH/HCl) or Na+-free media (Na+ replaced by choline). After mounting of the coverslips in the cuvette, cells were loaded with BCECF dissolved in the same media used for preincubation, and pH, measurements were repeated until a stable reading was achieved (typically 5-10 min after loading). The superfusion medium was then rapidly and transiently changed to one in which [HCO3-] was 5 mM instead of 25 mM (20 mM NaHCO3 replaced by 20 mM NaCl and both solutions were equilibrated with 95% air, 5% CO2), while pH, was continuously measured. Studies under Cl- -free conditions were conducted in an identical fashion, except that cells were preincubated for 1 h in media in which Na+ and Cl- were replaced by NMG and gluco- nate, respectively. Cl- -free medium was also used for dye loading and pH, measurements. 1 mM amiloride or 1 mM SITS was present in preincubation and/or superfusion media as appropriate.

Measurement of pH, during hyperpolarization of membrane potential. These studies were conducted in a fashion analogous to those of Na+ efflux (see above). In brief, cells were preincubated for 30 min in Na+-containing media (25 mM NaHCO3; 40 mM NaCl; 70 mM KC1; 0.8 mM MgSO4; 1.2 M CaSO4; 0.8 mM Na2PO4; 5 mM glucose; 10 mM Hepes; adjusted to pH 7.4 with KOH/HCl) or Na+-free media (Na+ replaced by choline). The cells were then loaded with BCECF in the same media used for preincubation and, after stabilization of pH, the superfusion solution was abruptly changed to one containing 10 μM valinomycin and 5 mM K+ (65 mM KCl replaced by 65 mM choline chloride, all other electrolytes remaining unchanged), while pH, was continuously monitored. 1 mM amiloride or 1 mM SITS was present in preincubation and/or superfusion media as appropriate.

Isolation of basolateral (bLPM) and canalicular (cLPM) liver plasma membrane vesicles. bLPM and cLPM vesicles were prepared as previously described from male Sprague-Dawley rats (200-250 g) (29). The vesicles were suspended in a filtered (0.22-μm nitrocellulose filter) buffer medium containing 100 mM tetrathylammonium (TMA), 100 mM gluconate, 50 mM mannitol, 42 mM Hepes, 21 mM TMA/OH, adjusted to pH 7.5, by repeated (10 times) passage through a 25-gauge needle. The vesicle suspension was also treated with 1 μM acetazolamide to inhibit membrane-bound carbonic anhydrase (30) and 100% N2. Aliquots of membrane suspensions (protein concentration > 7.5 mg/ml) were stored frozen in liquid nitrogen for up to 2 wk without loss of transport functions. Protein was determined by the method of Lowry et al. (31) using BSA as standard.

Determination of HCO3- -dependent 22Na uptake into bLPM and cLPM vesicles. 22Na uptake studies were performed by a rapid membrane filtration technique as previously described (32). Frozen vesicle suspensions were quickly thawed in a 37°C waterbath, diluted to a protein concentration of 7.5 mg/ml with membrane suspension buffer containing 1 μM acetazolamide and revesiculated by 20 passages through a 25-gauge needle. The vesicles were treated with 100 μM DIDS or 10 μg/mg protein valinomycin as indicated in the corresponding figure legends. After gassing with 100% N2, 20-μl aliquots of vesicle suspension (150 μg protein) were mixed with 80 μl of incubation media that was either of similar composition as the membrane resuspension buffer or consisted of 100 mM K+, 43 mM gluco- nate, 57 mM HCO3-, 50 mM mannitol, 42 mM Hepes, 21 mM TMA/OH, adjusted to pH 7.5. All incubation media also contained 1 mM Na+ gluconate, 0.17 μM 22NaCl, and 1 μM acetazolamide. Where indicated, 100 μM DIDS or 100 μM amiloride was also added. Micarbonate-free incubation mixtures were gassed with 100% N2, whereas in the presence of a 57 mM out > in HCO3- gradient, gassing was performed with 10% CO2/90% N2. 22Na uptake was routinely determined at 25°C. After the indicated time intervals, the reactions were terminated by adding 3 ml of ice-cold stop solution consisting of 100 mM K+, 100 mM gluconate, 200 mM mannitol, and 20 mM Hepes/KOH, pH 7.5. The incubation mixtures were filtered through nitrocellulose filters (pore size 0.65 μm). The filters were rinsed twice, dissolved in 5 ml of liquid scintillation cocktail (Filter-Count; Packard Instruments, Zurich, Switzerland), and filter-associated (i.e., vesicle) radioactivity was determined by liquid scintillation counting. Nonspecific binding of 22Na to the membranes and/or filters was determined in each experiment by addition of 80 μl cold incubation solutions and 3 ml of cold stop solution to 20 μl of ice-cold membrane preparations. These membrane/filter blanks were subtracted from cell uptake measurements. All determinations were performed in quadruplicate.

Calculations and statistics. Plots of 22Na uptake rate versus extracellular sodium concentration were analyzed by a nonlinear least-squares program; the best-fit function was taken to be that function with the smallest number of parameters that minimized the sum of squares by F test. Initial 22Na efflux rates were determined from a nonlinear least-squares procedure as previously described (24). In brief, sums of exponential functions were fitted to the efflux curves. The best-fit function (the function with the smallest number of parameters associated with the smallest sum of squares by F test), was used to determine initial efflux rates (first derivative at zero time) (24). The effects of the various experimental manipulations were analyzed using unpaired or paired t tests, as appropriate (and as indicated in the table legends). pH calibration curves were analyzed using linear regression analysis. Results are reported as means±SEM and P ≤ 0.05 was considered statistically significant.
Results

Effects of HCO₃⁻ on ²²Na uptake by cultured rat hepatocytes. Sodium uptake, measured as the rate of ²²Na entry after a 30-min preincubation in medium containing 5 mM Na⁺ and 5 mM ouabain (see Methods), was linear to ~3 min in the presence or absence of 25 mM HCO₃⁻, amiloride, and SITS (Fig. 1). As illustrated in Fig. 1 and summarized in Table I, Na⁺ uptake under these conditions was stimulated about four- to five-fold by the presence of extracellular HCO₃⁻, and this stimulation was unaffected by amiloride but was abolished by SITS. Very similar results were obtained when NMG was used instead of choline to partially replace Na⁺, indicating that these findings were not peculiar to a particular impermeant cation (Table I). Of interest, although HCO₃⁻ still stimulated ²²Na⁺ uptake nearly four-fold when Li⁺ was used to replace Na⁺, the absolute rates of ²²Na⁺ uptake were significantly less than in the presence of either choline or NMG (Table I).

Because of the recognized ability of SITS to inhibit a variety of anion transport systems including Cl⁻/HCO₃⁻ exchange, known to be present in hepatocytes (10), the effect of HCO₃⁻ on ²²Na uptake was examined under conditions (60 min incubation in Cl⁻-free medium) in which hepatocytes were depleted of intracellular Cl⁻ and Cl⁻ was also absent from the extracellular medium. As summarized in Table I, SITS-sensitive and EIA-insensitive HCO₃⁻-stimulated ²²Na uptake was still observed when NMG and gluconate were used to replace Na⁺ and Cl⁻, respectively. HCO₃⁻-stimulated Na⁺ uptake was also observed when Na⁺ and Cl⁻ were replaced by Li⁺ and NO₃⁻, respectively. Finally, in four studies, Na⁺ uptake rate did not differ in the presence versus the absence of 1 mM SITS (3.32±0.39 vs. 3.69±0.65 nmol/mg protein per min, respectively; mean±SEM) when HCO₃⁻ was not present in the uptake medium. These findings indicate that SITS at this concentration did not exert a nonspecific/toxic effect on Na⁺ uptake and did not act via inhibition of Cl⁻/HCO₃⁻ exchange.

Effects of weak acids other than H₂CO₃ on ²²Na uptake. To determine whether the salts of other weak acids could substitute for HCO₃⁻, analogous studies were conducted in which the effects of HCO₃⁻ on ²²Na uptake were compared with those of acetate or propionate (all present as K⁺ salts, as dictated by availability and the requirement to maintain [Na⁺] at 5 mM). As summarized in Table II, acetate and propionate both tended to increase ²²Na uptake. However, unlike the increase produced by HCO₃⁻, this increase was not statistically significant in the case of propionate and was inconsistently observed. Also, unlike HCO₃⁻, the stimulation of ²²Na⁺ uptake produced by both acetate and propionate was unaffected by SITS and was completely inhibited by amiloride.

²²Na uptake as a function of extracellular sodium concentration. Initial ²²Na uptake rate was measured at varying (1–135 mM) concentrations of extracellular Na⁺ both in the absence and presence of 25 mM extracellular HCO₃⁻. As is evident from Fig. 2, ²²Na⁺ uptake rate in the presence of HCO₃⁻ exceeded (by up to 10-fold) uptake rate in the absence of HCO₃⁻ over the entire concentration range and was clearly saturable. The apparent Kᵣ for Na⁺ in the presence of HCO₃⁻ was 25.5±2.1 mM.

Effect of HCO₃⁻ on ²²Na efflux. In the first series of studies, the effect of HCO₃⁻ in the preincubation media (with or without inhibitors) was studied under conditions of presumed hyperpolarization (5 mM K⁺ plus valinomycin in the efflux media) and in the absence of HCO₃⁻ in the efflux media. As illustrated in Fig. 3 and summarized in Table III (series a–d), the presence of HCO₃⁻ in the preincubation medium increased the initial rate of ²²Na efflux ~10-fold, and this stimulation was unaffected by amiloride but abolished by SITS. Of interest, in preliminary studies, ²²Na efflux from cells incubated in HCO₃⁻-free media, but without transient exposure to KCN, occurred at a rate similar to that observed with HCO₃⁻-containing preincubation media. This suggests that intracellular HCO₃⁻ generated from metabolic CO₂ production is sufficient to stimulate ²²Na efflux.

In the second series of experiments, the effects of hyperpolarizing conditions (5 mM vs. 70 mM K⁺ plus valinomycin in the efflux media, all cells preincubated in 70 mM K⁺ plus ouabain) and an in > out HCO₃⁻ concentration gradient (presence or absence of HCO₃⁻ in preincubation and/or efflux media) were studied. As shown in Fig. 4 and summarized in Table III, hyperpolarization increased the rate of ²²Na efflux in the presence of an in > out HCO₃⁻ concentration gradient about twofold. No stimulation of ²²Na efflux was produced by hyperpolarization in the absence of intra- and extracellular HCO₃⁻ (series i and j in Table III). Finally, in the presence of HCO₃⁻ in both the preincubation and efflux media (i.e., the absence of an in > out HCO₃⁻ gradient), the rate of ²²Na efflux was significantly reduced ~5 to 10-fold, as compared with an in > out HCO₃⁻ concentration gradient (Fig. 4 and series e–h in Table III). Collectively, these findings indicate that Na⁺ efflux from cultured hepatocytes is stimulated by the presence of an in > out HCO₃⁻ concentration gradient and by an increase in membrane potential difference in the presence but not in the absence of intracellular HCO₃⁻. This increase in Na⁺ efflux
Table I. Effect of HCO\textsubscript{3}\textsuperscript{-} on \textsuperscript{22}Na Uptake by Cultured Rat Hepatocytes

<table>
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<tr>
<th>Presence/absence of HCO\textsubscript{3}\textsuperscript{-}</th>
<th>Inhibitor\textsuperscript{II}</th>
<th>Choline/None</th>
<th>NMG/None</th>
<th>Li\textsuperscript{+}/None</th>
<th>NMG/Gluconate</th>
<th>Li\textsuperscript{+}/Nitrate</th>
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<td>1.26±0.25\textsuperscript{III}</td>
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<td>0.79±0.12\textsuperscript{II}</td>
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All results are expressed as the mean±SEM of initial \textsuperscript{22}Na uptake rates measured at 3 min as illustrated in Fig. 1 and described in Methods. The number of different cell batches studied is given in parentheses; measurements were made in triplicate on each cell batch. * Sodium concentration in all media was 5 mM, the remainder being replaced by the indicated cation. \textsuperscript{I} In selected experiments, Cl\textsuperscript{-} was completely replaced by the indicated anion. \textsuperscript{II} HCO\textsubscript{3}\textsuperscript{-} was present at a concentration of 25 mM (+) or absent (−), being replaced by Cl\textsuperscript{-}, except where indicated. \textsuperscript{III} Amiloride was present at a concentration of 1 mM. SITS was used at concentrations of 1 mM (with preincubation) or 5 mM (without preincubation) with equivalent results. \textsuperscript{I} P < 0.05 compared with either choline or NMG substituting for Na\textsuperscript{+}. \textsuperscript{II} P < 0.005. \textsuperscript{III} P < 0.005, respectively, compared with studies in the presence of HCO\textsubscript{3}\textsuperscript{-} and absence of inhibitor under the same conditions of anion and/or cation substitution (vertical row). Ethylisopropyl amiloride (0.1 mM), a potent Na\textsuperscript{+}/H\textsuperscript{+} exchange inhibitor, was used in these studies instead of amiloride, which is available only as a hydrochloride.

Table II. Effect of Weak Acids Other than HCO\textsubscript{3}\textsuperscript{-} on \textsuperscript{22}Na Uptake by Cultured Rat Hepatocytes

<table>
<thead>
<tr>
<th>Weak acid\textsuperscript{*}</th>
<th>Inhibitor\textsuperscript{III}</th>
<th>nmol/mg per min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amiloride\textsuperscript{IV}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SITS\textsuperscript{IV}</td>
<td></td>
</tr>
<tr>
<td>HCO\textsubscript{3}\textsuperscript{-}</td>
<td>2.04±0.27\textsuperscript{IV}</td>
<td>1.85±0.52\textsuperscript{IV}</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td>(3)</td>
</tr>
<tr>
<td>Acetate</td>
<td>1.08±0.24\textsuperscript{IV}</td>
<td>0.59±0.24\textsuperscript{IV}</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>(2)</td>
</tr>
<tr>
<td>Propionate</td>
<td>1.22±0.35</td>
<td>0.54±0.19\textsuperscript{IV}</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>(2)</td>
</tr>
</tbody>
</table>

All results are expressed as the mean±SEM of initial \textsuperscript{22}Na uptake rates measured as described in Methods. The number of different cell batches studied is given in parentheses; measurements were made in triplicate on each cell batch. * The effect of 25 mM HCO\textsubscript{3}\textsuperscript{-} on Na\textsuperscript{+} uptake was compared with 25 mM acetate, 25 mM propionate or Cl\textsuperscript{-} (none), all of which were present as the K\textsuperscript{+} salt. Sodium concentration in all incubations was 5 mM, with choline and Cl\textsuperscript{-} present in the concentration necessary to maintain isomolarity. \textsuperscript{IV} Amiloride was present at a concentration of 1 mM. SITS was present at a concentration of 1 mM. \textsuperscript{IV} P < 0.05 compared with no weak acid (none). \textsuperscript{IV} The effects of amiloride and SITS on HCO\textsubscript{3}\textsuperscript{-}-stimulated \textsuperscript{22}Na uptake were not reexamined in these experiments. The values shown here for comparative purposes are taken from Table I. \textsuperscript{IV} P < 0.05 compared with HCO\textsubscript{3}\textsuperscript{-} and no inhibitor.

Effects of lowering extracellular [HCO\textsubscript{3}\textsuperscript{-}] on pH, of cultured hepatocytes. Resting pH\textsubscript{i} averaged 7.33±0.06 (mean±SEM) in control, HCO\textsubscript{3}\textsuperscript{-}-containing media and tended to be lower in Na\textsuperscript{+}-free medium or media containing amiloride or SITS, although this did not achieve statistical significance (Table IV). Abrupt lowering of extracellular [HCO\textsubscript{3}\textsuperscript{-}] from 25 to 5 mM under control conditions with constant (5%) P\textsubscript{CO\textsubscript{2}}, caused extracellular pH to fall from 7.4 to 6.7 and was associated with a corresponding significant fall in pHi to 6.97±0.08. Raising extracellular [HCO\textsubscript{3}\textsuperscript{-}] to 25 mM caused pHi to return to 7.34±0.06, a value nearly identical to initial resting pHi (Fig. 5). As in control studies, resting pHi was also significantly and reversibly reduced by transiently lowering extracellular [HCO\textsubscript{3}\textsuperscript{-}] from 25 to 5 mM in Na\textsuperscript{+}-free media and in the presence of SITS or amiloride.

The rate of fall in pHi (dpH\textsubscript{i}/dt) after the lowering of extracellular [HCO\textsubscript{3}\textsuperscript{-}] averaged 0.227±0.033 pHi units/min under control conditions. In the presence of SITS or absence of Na\textsuperscript{+}, dpH\textsubscript{i}/dt was significantly (50-60%) reduced, whereas dpH\textsubscript{i}/dt was unaffected by amiloride (Fig. 5 and Table IV). To establish that these changes in pHi were not mediated by Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchange, these same studies were repeated under conditions in which Cl\textsuperscript{-} was replaced by gluconate (with NMG replacing choline). As summarized in Table IV, whereas a resting pHi tended to be higher in Cl\textsuperscript{-}-free medium than in medium containing Cl\textsuperscript{-}, the rate of fall in pHi, upon lowering intracellular HCO\textsubscript{3}\textsuperscript{-} in Cl\textsuperscript{-}-free medium did not differ from that in Cl\textsuperscript{-}-containing medium and was reduced in the absence of Na\textsuperscript{+} or presence of SITS but was unaffected by amiloride. These findings suggest a possible role for Cl\textsuperscript{-} (and presumably Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchange) in maintenance of resting pHi, but also indicate that Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchange does not mediate the fall in pHi after reduction of extracellular HCO\textsubscript{3}\textsuperscript{-} at constant P\textsubscript{CO\textsubscript{2}}. The rate of pHi recovery after return of extracellular HCO\textsubscript{3}\textsuperscript{-} concentration to 25 mM was not systematically analyzed in these experiments because the fluorescence signal-to-noise ratio was con-
Figure 2. $^{23}$Na uptake rate versus extracellular sodium concentration in the presence (c) and absence (●) of 25 mM HCO$_3^-$, Initial uptake rate was measured at 3 min as measured as described in Methods, with varying concentrations (1-135 mM) of NaCl replaced by choline chloride in the preincubation and incubation media.

Figure 3. $^{23}$Na efflux from preloaded hepatocytes measured as described in Methods. Curves depict results without HCO$_3^-$ in the final preincubation media (closed circles; series a in Table III), with HCO$_3^-$ in the preincubation media in the absence of inhibitors (c; series b in Table III), or with HCO$_3^-$ in the preincubation media plus amiloride (●; series c in Table III) or plus SITS (●; series d in Table III). All efflux media contained 5 mM K$^+$ and were nominally free of HCO$_3^-$, Data depicted represent the mean±SEM of studies in three different cell batches, with measurements made in triplicate in each cell batch. Efflux is depicted as a percent of cell Na$^+$ remaining at various time points and are normalized to a starting value of 100 percent. Actual cell Na$^+$ at time zero averaged 140±60 nmol/mg and did not differ significantly among the various groups.

In separate studies, we observed that the rate of fall in pH$_i$ upon lowering extracellular pH from 7.4 to 6.6 in HCO$_3^-$-free, Hepes-buffered medium was significantly ($P = 0.012$) slower (0.075±0.001 pH units/min) than in HCO$_3^-$-containing medium and was unaffected by 1 mM SITS. This indicates that the findings depicted in Fig. 5 and summarized in Table IV are not attributable to a generalized effect of SITS on plasma membrane permeability to H$^+$ or HCO$_3^-$ and further suggest that Na$^+$-coupled transport of HCO$_3^-$ is a predominant mechanism of plasma membrane H$^+$/HCO$_3^-$ transport in these cells.

Effects of hepatocyte hyperpolarization on pH$_i$ (Table V). In preliminary studies ($n = 3$), hyperpolarization was produced by abruptly exposing hepatocytes to 10 μM valinomycin after preincubation in ouabain-free, balanced-electrolyte media with or without SITS or ouabain-free media in which Na$^+$ had been completely replaced by choline. Acidification was consistently observed, and the rate of acidification in Na$^+$-containing media (0.507 pH units/min) was consistently greater than that observed in the absence of Na$^+$ (0.192 pH units/min) or presence of SITS (0.228 pH units/min). Although suggestive of a Na$^+$-dependent and SITS-sensitive electrogenic mechanism for net base efflux, interpretation of these studies was clouded by the potentially confounding effects of prolonged incubation in Na$^+$-free medium on Na$^+$/K$^+$-ATPase, membrane potential, and K$^+$ concentration gradients and hence the magnitude of the hyperpolarization produced by exposure to valinomycin.

These studies were therefore repeated and extended using hepatocytes that had been preincubated for 60 min in the presence of 5 mM ouabain, 65 mM Na$^+$, and 70 mM K$^+$. The purpose of this preincubation was to normalize starting condi-
Table III. $^{22}$Na Efflux from Cultured Rat Hepatocytes

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>[HCO$_3^-$]</th>
<th>[K$^+$]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>% min$^{-1}$ ± SEM</td>
</tr>
<tr>
<td><strong>Series</strong></td>
<td><strong>Final preincubation media</strong></td>
<td><strong>Efflux medium</strong></td>
</tr>
<tr>
<td>a</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>b</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>c</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>d</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>e</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>f</td>
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<tr>
<td>g</td>
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<tr>
<td>h</td>
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<td>25</td>
</tr>
<tr>
<td>i</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>j</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Efflux studies were conducted and initial efflux rates calculated as described in Methods; valinomycin was present in all efflux media. Series a–d were performed simultaneously on the same three cohorts of cultured cells and efflux curves a–d are depicted in Fig. 3. Series e–j were not conducted simultaneously and were performed in varying numbers of cell batches; curves e–h are depicted in Fig. 4. Series b represents a subset of series e. The number of different cell batches studied is given in parentheses; measurements were made in triplicate on each cell batch. *$P < 0.05$ vs. series a and d by unpaired $t$ test. †$P < 0.05$ vs. series f–j. ‡$P < 0.05$ vs. series h–j.

Efflux studies were conducted and initial efflux rates calculated as described in Methods; valinomycin was present in all efflux media. Series a–d were performed simultaneously on the same three cohorts of cultured cells and efflux curves a–d are depicted in Fig. 3. Series e–j were not conducted simultaneously and were performed in varying numbers of cell batches; curves e–h are depicted in Fig. 4. Series b represents a subset of series e. The number of different cell batches studied is given in parentheses; measurements were made in triplicate on each cell batch. *$P < 0.05$ vs. series a and d by unpaired $t$ test. †$P < 0.05$ vs. series f–j. ‡$P < 0.05$ vs. series h–j.

Efflux studies were conducted and initial efflux rates calculated as described in Methods; valinomycin was present in all efflux media. Series a–d were performed simultaneously on the same three cohorts of cultured cells and efflux curves a–d are depicted in Fig. 3. Series e–j were not conducted simultaneously and were performed in varying numbers of cell batches; curves e–h are depicted in Fig. 4. Series b represents a subset of series e. The number of different cell batches studied is given in parentheses; measurements were made in triplicate on each cell batch. *$P < 0.05$ vs. series a and d by unpaired $t$ test. †$P < 0.05$ vs. series f–j. ‡$P < 0.05$ vs. series h–j.

22$^{2}$Na uptake into plasma membrane vesicles. The simultaneous presence of an inside positive K$^+$ diffusion potential and an out $>_{>}$ in HCO$_3^-$ gradient produced transient accumulation of $^{22}$Na within bLPM vesicles (Fig. 6). This HCO$_3^-$ gradient-dependent $^{22}$Na uptake was completely blocked by DIDS. In contrast, amiloride had no effect on the initial rate of HCO$_3^-$ dependent $^{22}$Na uptake, although it exerted weak inhibitory effects at later time points and decreased the magnitude of the overshoot value. In contrast to bLPM vesicles, no stimulation of $^{22}$Na uptake by an out $>_{>}$ in HCO$_3^-$ gradient was detected in cLPM vesicles (Fig. 7).

To more directly evaluate the electrogenic of HCO$_3^-$ dependent $^{22}$Na uptake into bLPM vesicles, the effect of an out $>_{>}$ in K$^+$ gradient was compared in the presence and absence of valinomycin. In each of three separate experiments with different vesicle preparations, valinomycin treatment significantly ($P < 0.05$) increased the effect of the imposed out $>_{>}$ in HCO$_3^-$ gradient on $^{22}$Na uptake (Fig. 8).

In separate experiments with four different vesicle preparations, HCO$_3^-$ dependent $^{22}$Na uptake was studied under K$^+$ equilibrated conditions ([K$^+$] = 100 mM inside and outside) as compared with an in $>_{>}$ out K$^+$ gradient ([K$^+$] = 100 mM inside and 5 mM outside). In the complete absence of HCO$_3^-$, $^{22}$Na uptake was significantly ($P < 0.01$) increased by an inside negative K$^+$ diffusion potential, as would be expected for conductive movement of a cation. By contrast, in the presence of an out $>_{>}$ in HCO$_3^-$ gradient, Na$^+$ uptake rate was significantly ($P < 0.05$) decreased by an inside negative K$^+$ diffusion potential (in $>_{>}$ out K$^+$ gradient). Thus, in the presence of HCO$_3^-$, $^{22}$Na transport by basolateral membrane vesicles, as by intact hepatocytes (Figs. 3 and 4) suggests that Na$^+$ is behaving, in part, as an anion.

**Discussion**

In these studies, we have explored the existence in hepatocytes of a Na$^+$/HCO$_3^-$ symport system as recently described in certain HCO$_3^-$ transporting epithelia (12–20). Operational criteria for the existence of this symport mechanism, as studied in
Figure 4. $^{22}$Na efflux from preloaded hepatocytes measured as described in Methods. Curves depict results in the presence of an in > out HCO$_3^-$ gradient under presumed hyperpolarizing (in > out K$^+$ gradient plus valinomycin) conditions (c; series e in Table III), in the presence of an in > out HCO$_3^-$ gradient under nonhyperpolarizing conditions (c; series f in Table III), or in the absence of a HCO$_3^-$ gradient (HCO$_3^-$ present in preincubation and efflux media) under hyperpolarizing (c; series g in Table III) or nonhyperpolarizing (c; series h in Table III) conditions. Data depict the mean±SEM of studies in four to six cell batches, with measurements made in triplicate in each cell batch. Efflux is depicted as a percent of cell Na$^+$ remaining at various time points and are normalized to a starting value of 100%. Actual cell Na$^+$ at time zero averaged 140±60 nmol/mg and did not differ significantly among the various groups.

Table IV. Effects of Lowering Extracellular [HCO$_3^-$] in the Presence of Constant CO$_2$ on pH$_i$ of Cultured Rat Hepatocytes

<table>
<thead>
<tr>
<th>Conditions</th>
<th>pH$_i$ (25 mM HCO$_3^-$)</th>
<th>pH$_i$ (5 mM HCO$_3^-$)</th>
<th>Recovery pH$_i$ (25 mM HCO$_3^-$)</th>
<th>dpH$_{id}$/dt</th>
<th>pH$_i$ (25 mM HCO$_3^-$)</th>
<th>pH$_i$ (5 mM HCO$_3^-$)</th>
<th>Recovery pH$_i$ (25 mM HCO$_3^-$)</th>
<th>dpH$_{id}$/dt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.33±0.06</td>
<td>6.97±0.08†</td>
<td>7.34±0.06</td>
<td>0.227±0.033</td>
<td>7.49±0.07</td>
<td>7.20±0.06†</td>
<td>7.44±0.07</td>
<td>0.194±0.041</td>
</tr>
<tr>
<td>(5)</td>
<td></td>
<td></td>
<td>(5)</td>
<td>(5)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
</tr>
<tr>
<td>Na$^+$-free‡</td>
<td>7.15±0.09</td>
<td>6.81±0.07‡</td>
<td>7.12±0.01</td>
<td>0.101±0.026§</td>
<td>7.31±0.03</td>
<td>7.10±0.04‡</td>
<td>7.34±0.02</td>
<td>0.083±0.018†</td>
</tr>
<tr>
<td>(5)</td>
<td></td>
<td></td>
<td>(5)</td>
<td>(5)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
</tr>
<tr>
<td>Amiloride (1 mM)</td>
<td>7.22±0.07</td>
<td>6.85±0.03‡</td>
<td>7.13±0.12</td>
<td>0.228±0.046</td>
<td>7.61±0.07</td>
<td>7.39±0.05‡</td>
<td>7.60±0.01</td>
<td>0.183±0.040</td>
</tr>
<tr>
<td>(4)</td>
<td></td>
<td></td>
<td>(4)</td>
<td>(4)</td>
<td>(3)</td>
<td>(3)</td>
<td>(3)</td>
<td>(3)</td>
</tr>
<tr>
<td>SITS (1 mM)</td>
<td>7.18±0.09</td>
<td>6.87±0.10‡</td>
<td>7.08±0.19</td>
<td>0.092±0.018§</td>
<td>7.40±0.07</td>
<td>7.20±0.08§</td>
<td>7.49±0.04</td>
<td>0.057±0.020†</td>
</tr>
<tr>
<td>(4)</td>
<td></td>
<td></td>
<td>(4)</td>
<td>(4)</td>
<td>(2)</td>
<td>(2)</td>
<td>(2)</td>
<td>(2)</td>
</tr>
</tbody>
</table>

Extracellular [HCO$_3^-$] was lowered from 25 mM (pH 7.4) to 5 mM (pH 6.7) in the presence of constant PCO$_2$ (5%), and pH$_i$ was monitored fluorometrically using BCECF as described in Methods and illustrated in Fig. 5. Data represent means±SEM, and the number of experiments is indicated in parentheses. * Cells were incubated in Cl$^-$-free, gluconate-containing medium for 1 h before BCECF loading and also studied in Cl$^-$-free medium. † P < 0.01 compared with initial pH$_i$ in 25 mM HCO$_3^-$ under the same conditions by paired t test. ‡ Cells were incubated in Na$^+$-free, choline-substituted (Cl$^-$ containing) or NMG-substituted (Cl$^-$ free) medium for 1 h before BCECF loading and studied in similar Na$^+$-free medium. § P < 0.05 compared with dpH$_{id}$/dt in respective (Cl$^-$ containing or Cl$^-$ free) control medium by unpaired t test. ¶ In Cl$^-$-free incubations, 0.1 mM EIA was used instead of amiloride, which is available only as a hydrochloride.
in \(^{22}\text{Na}^+\) efflux produced by membrane hyperpolarization or an in-to-out HCO\(_3^-\) concentration gradient was eliminated by SITS but unaffected by amiloride. Collectively, these findings indicate that a HCO\(_3^-\) concentration gradient stimulates influx as well as efflux of \(^{22}\text{Na}^+\) in rat hepatocytes via a mechanism that is SITS sensitive, amiloride insensitive, Cl\(^-\) independent, and electrogenic.

To assess HCO\(_3^-\) or H\(^+\)/OH\(^-\) movement in this same cell system, pHi was measured fluorimetrically using the pH-sensitive fluorochrome BCECF. Our technique was analogous to that used by others in different cell types (12, 13, 28), and continuous superfusion was used to continuously remove secreted dye (27). HCO\(_3^-\) efflux, assessed as the rate of fall in pHi after lowering extracellular [HCO\(_3^-\)] while keeping P\(_{\text{CO}_2}\) constant, was found to be inhibited by 50–60% by depletion of intra- and extracellular Na\(^+\) and exposure to SITS, but was unaffected by amiloride. As with \(^{22}\text{Na}^+\) uptake, the HCO\(_3^-\) efflux was also unaffected by prolonged incubation in Cl\(^-\)-free

### Table V. pHi of Cultured Rat Hepatocytes during Hyperpolarization of Membrane Potential

<table>
<thead>
<tr>
<th></th>
<th>Initial pHi (preincubation media)</th>
<th>dpH/dt</th>
<th>pHi units/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.35±0.07</td>
<td>0.63±0.10</td>
<td></td>
</tr>
<tr>
<td>Na(^+)-free*</td>
<td>7.19±0.12</td>
<td>0.20±0.05(^f)</td>
<td></td>
</tr>
<tr>
<td>Amiloride(^g)</td>
<td>7.26±0.14</td>
<td>0.64±0.08</td>
<td></td>
</tr>
<tr>
<td>SITS(^h)</td>
<td>7.24±0.12</td>
<td>0.30±0.08(^i)</td>
<td></td>
</tr>
</tbody>
</table>

Hepatocytes were preincubated for 1 h in medium containing 70 mM K and 5 mM ouabain and then abruptly exposed to medium containing 5 mM K\(^+\) and 10 \(\mu\)M valinomycin, while pHi was monitored fluorimetrically using BCECF as described in Methods. Data represent means±SEM, and the number of experiments is indicated in parentheses.

* No Na\(^+\) was present in preincubation and experimental media for these studies, whereas media for control studies contained 65 mM Na\(^+\). In both instances, choline was used to maintain isosmolarity.

\(^f\) P < 0.01 compared with dpH/dt under control conditions by unpaired "t" test.

\(^g\) P < 0.05 compared with dpH/dt under control conditions by unpaired "t" test.

\(^h\) Amiloride and SITS were both present at a concentration of 1 mM in preincubation and/or experimental media.

---

**Figure 5.** Representative studies of the effect of changing extracellular [HCO\(_3^-\)] from 25 mM (pH 7.42) to 5 mM (pH 6.70) in the presence of constant (5%) P\(_{\text{CO}_2}\) on hepatocyte pHi under control conditions (upper left), in Na\(^+\)-free medium (lower left), or in medium containing 1 mM SITS (upper right) or 1 mM amiloride (lower right). The rate of fall in pHi after lowering extracellular [HCO\(_3^-\)] was determined from tangents drawn to the fluorescence tracings (not shown) at 500 and 450 nm excitation as described in Methods. The basal pHi before lowering extracellular [HCO\(_3^-\)] ranged from 7.19 to 7.23 in the hepatocyte monolayers depicted.

---

**Figure 6.** HCO\(_3^-\)-dependent Na\(^+\) uptake into bLPM vesicles. Vesicles were resuspended in a HCO\(_3^-\)- and K\(^+\)-free TMA buffer (see Methods) supplemented with 1 \(\mu\)M acetazolamide (inhibition of membrane-bound carbonic anhydrase). All vesicle suspensions were treated with valinomycin and a separate sample also with 100 \(\mu\)M DIDS. 20-\(\mu\)l aliquots of vesicle suspension were incubated with 80 \(\mu\)l incubation medium with a composition identical to the membrane resuspension buffer (\(\bullet\)) or consisting of 100 mM K\(^+\), 43 mM glucose, 57 mM HCO\(_3^-\), 50 mM mannitol, 42 mM Hepes, 21 mM TMA/OH, pH 7.5, and 1 \(\mu\)M acetazolamide without inhibitors (\(\bullet\)), plus 100 \(\mu\)M amiloride (\(\circ\)), or plus 100 \(\mu\)M DIDS (\(\circ\)). Data represent the mean±SD of 12 determinations in three different membrane preparations.
media. Finally, hyperpolarization of hepatocytes, produced by abrupt exposure to media containing valinomycin and a low concentration of K+, caused an abrupt fall in pH, that was also inhibited by removal of Na+ and exposure to SITS but not by amiloride.

Although several explanations exist for each of these observations in intact hepatocytes when taken separately (e.g., a HCO3-dependent and SITS-sensitive Na+ conductance, a Na+-dependent and SITS-sensitive conductive pathway for HCO3 efflux, Na+-dependent Cl-/HCO3 exchange [33]), collectively, they appear to be best explained by the existence of an electroneutral Na+/HCO3 symport in hepatocytes. The observation that hepatocyte hyperpolarization stimulated both Na+-dependent HCO3 efflux and Na+ efflux in the presence, but not absence of HCO3 (Figs. 3 and 4, Table III) provides particularly strong evidence for the cotransport of HCO3 and Na+ as part of a negatively charged complex, and is not readily explained by conductance pathways. In separate studies using electrophysiologic techniques to measure membrane potential, we have also observed that Na+-coupled HCO3 transport by rat hepatocytes in primary culture is electrogenic (34).

The results of the studies with plasma membrane vesicles are also consistent with the presence of an electrogenic Na+/HCO3 cotransport system in the basolateral, but not canalicular plasma membrane domain of rat hepatocytes. In particular, the simultaneous presence of an inside positive K+ diffusion potential and an out > in HCO3 concentration gradient accelerated the rate of 22Na entry into bLPM and caused a transient overshoot. 22Na entry into cLPM was not stimulated under these conditions. In bLPM, valinomycin accelerated HCO3-dependent 22Na uptake into bLPM in the presence of an out > in K+ gradient (Fig. 8), and an inside negative K+ diffusion potential, as compared with K+ equilibrated conditions, accelerated 22Na uptake in the absence of HCO3, but inhibited 22Na uptake in the presence of HCO3. These findings provide further evidence of electrogenic cotransport. Thus, the isolated vesicle studies support the conclusions from the intact hepatocyte studies and provide direct evidence for the selective localization of electrogenic Na+/HCO3 cotransport at the sinusoidal surface domain.

Assuming that these findings do indicate the presence in hepatocytes of Na+/HCO3 symport, then two additional points merit emphasis. First, previous studies in membrane vesicles or intact cells have generally not permitted identification of the charged component(s) of the bicarbonate buffer system (HCO3, OH-, H+, CO2) carried by the transporter (12, 18, 19). Our preliminary observation that Na+ efflux is minimally affected by simple removal of extracellular HCO3 from the preincubation medium, unless the cells are also exposed transiently to KCN, suggests that metabolically produced CO2/HCO3 is sufficient to drive the transporter. Because KCN had no effect on Na+ efflux in the presence of extracellular HCO3, this is unlikely to represent a toxic effect. Our observations also indicate that the putative symporter is reversible, because it appears to mediate both 22Na uptake and efflux, and that in hepatocytes, as in renal epithelial (18) or corneal endothelial cells (19), other permeant weak buffers such as acetate do not substitute for HCO3. A similar finding has recently been reported in proximal tubular cells (35).

Second, the basolateral location of Na+/HCO3 symport in hepatocytes was unexpected. By analogy with other epithelial cells, such as parietal cells or proximal tubular cells, in which Na+/HCO3 symport appears localized to the basolateral membrane and is presumed to mediate HCO3 exit from the cell, we anticipated that Na+/HCO3 symport, like Cl-/HCO3 antiport, would be localized to the canalicular membrane. Its location on the basolateral membrane, and not the canalicular membrane across which HCO3 presumably enters the canalculus, raises the possibility that Na+/HCO3 symport in hepatocytes may mediate HCO3 influx as well as or instead of efflux. This possibility is particularly intriguing because hepatocytes exhibit a lower membrane potential than many other epithelial cells (36) and hence less of an electrical driving force for electrogenic HCO3 exit mediated via a negatively charged Na+/HCO3 cotransport mechanism. The present studies provide no direct information regarding the direction in which the

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**Figure 8.** Electrogenicity of HCO3-dependent 22Na uptake into bLPM vesicles. Vesicles were resuspended in HCO3- and K+-free TMA buffer supplemented with 1 mM acetazolamide. Half of the vesicles were treated with valinomycin. The vesicles were incubated either in 100 mM K+, 100 mM gluconate, 50 mM mannitol, 42 mM Heps, 21 mM TMA/OH, pH 7.5 (gassed with 100% N2; open bars) or in 100 mM K+, 43 mM gluconate, 57 mM HCO3, 50 mM mannitol, 42 mM Heps, 21 mM TMA/OH, pH 7.5 (gassed with 10% CO2/90% N2; hatched bars). Final Na+ concentrations were adjusted to 1 mM. In each of three experiments using different vesicle preparations, the addition of valinomycin significantly (P < 0.05) increased 22Na uptake in the presence, but not absence of an out-to-in HCO3 gradient. Data represent the mean±SD of triplicate determinations in one representative vesicle preparation.
sympporter operates in hepatocytes, and the question clearly merits further study.

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