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Research Article

Primary cultures and plasma membrane vesicles were used to characterize Na+ and HCO3- transport by rat hepatocytes. Na+ uptake into hepatocytes was stimulated approximately 10-fold by 25 mM extracellular HCO3-.HCO3--stimulated Na+ uptake was saturable, abolished by 4-acetamido-4'-isothiocyano-2,2'-disulfonic acid stilbene (SITS), and unaffected by amiloride or Cl- removal. Neither propionate nor acetate reproduced this effect of HCO3-. 22Na efflux from preloaded hepatocytes was similarly increased approximately 10-fold by an in greater than out HCO3- concentration gradient. 22Na efflux was also increased by valinomycin and an in greater than out K+ concentration gradient in the presence but not absence of HCO3-. Intracellular pH (pHi) measured with the pH-sensitive fluorochrome 2',7'-bis-(2-carboxyethyl)-5-(and 6-)carboxyfluorescein (BCECF) decreased at a rate of 0.227 (+/- 0.074 SEM) pH units/min when extracellular HCO3- concentration was lowered from 25 to 5 mM at constant PCO2. This intracellular acidification rate was decreased 50-60% in the absence of Na+ or presence of SITS, and was unaffected by amiloride or Cl- removal. Membrane hyperpolarization produced by valinomycin and an in greater than out K+ concentration gradient caused pHi to fall; the rate of fall was decreased 50-70% by Na+ removal or SITS, but not amiloride. An inside positive K+ diffusion potential and a simultaneous out greater than in HCO3- gradient produced a transient 4,4'-diisothiocyano-2,2' disulfonic acid stilbene (DIDS) sensitive, amiloride-insensitive 22Na accumulation in […]

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'-isothiocyano-2,2'-disulfonic acid stilbene (SITS), and unaffected by amiloride or Cl^- removal. Neither propionate nor acetate reproduced this effect of HCO_3^-. ^{22}Na efflux from preloaded hepatocytes was similarly increased ~ 10-fold by an in > out HCO_3^- concentration gradient. ^{22}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 2',7'-bis-(2-carboxyethyl)-5-(and 6-)carboxyfluorescein (BCECF) decreased at a rate of 0.227 (±0.074 SEM) pH units/min when extracellular HCO_3^- concentration was lowered from 25 to 5 mM at constant P_{CO_2}. This intracellular acidification rate was decreased 50–60% in the absence of Na^+ or presence of SITS, and was unaffected by amiloride or Cl^- removal. Membrane hyperpolarization produced by valinomycin and an in > out K^+ concentration gradient caused pH_i to fall; the rate of fall was decreased 50–70% by Na^+ removal or SITS, but not amiloride. An inside positive K^+ diffusion potential and a simultaneous out > in HCO_3^- gradient produced a transient 4,4'-diisothiocyano-2,2' disulfonic acid stilbene (DIDS) sensitive, amiloride-insensitive ^{22}Na accumulation in basolateral but not canalicular membrane vesicles. Rat hepatocytes thus exhibit electrogenic 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 canalicular membrane (10). Recently, we have reported that the HCO_3^--rich hypercholeresis 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 electrogenic 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'-isothiocyano-2,2'-disulfonic acid stilbene (SITS),[1] 4,4'-diisothiocyano-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, acetoxymethyl 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 ^{22}Na 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 ^{22}Na transport and intracellular pH (pH_i) was dissolved directly by heating in the respective incubation media. 100 μM EIA was dissolved by addition of a slight molar excess of isethionic acid and was added directly to the respective incubation media.

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1. *Abbreviations used in this paper:* BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and 6-)carboxyfluorescein; blLPM, basolateral liver plasma membranes; cLPM, canalicular liver plasma membrane; DIDS, 4,4'-diisothiocyano-2,2'disulfonic acid stilbene; dpH_i/dt, rate of change in intracellular pH; EIA, ethylisopropylamiloride; NMG, *N*-methyl-D-glucamine; pH_i, intracellular pH; SITS, 4-acetamido-4'-isothiocyano-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 (^{22}Na uptake studies) or collagen-coated plastic cover slips (pH_i studies), and maintained in modified 199 OR medium supplemented with amino acids, insulin, corticosteroids, and calf serum (1% for dishes, 5% for plates) for 48 h before use, as described previously (2, 21–23). Cell viability in these monolayer cultures as assessed by trypan blue exclusion is ≥ 98%.

^{22}Na transport by cultured hepatocytes. ^{22}Na uptake studies were performed using minor modifications of a technique previously described (2, 21, 22). Hepatocytes were first preincubated for 30 min in nominally HCO_3^--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 $MgSO_4$; 1.2 mM $CaSO_4$; 0.8 mM KH_2PO_4; 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 ^{22}Na. Uptake medium was identical to that used for preincubation except for the presence, in selected incubations, of 25 mM HCO_3^-, inhibitors, and/or varying concentrations of Na^+. Nominally HCO_3^--free media were gassed with room air and HCO_3^--containing media were gassed with 5% CO_2 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 ^{22}Na-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) Na_2CO_3 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 (2, 21, 22).

This approach to measurement of HCO_3^--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 K_{Na^+} of Na^+/HCO_3^- symport determined in both other cell types (14, 18, 19) and hepatocytes (see Results) and therefore enhanced detection of HCO_3^--stimulated Na^+ uptake as compared with physiologic Na^+ concentrations. Moreover, 5 mM ouabain, which maximally inhibits Na^+/K^+-ATPase in these cultured cells (22), prolongs the initial linear phase of $^{22}Na^+$ uptake and presumably minimizes or eliminates differences in transmembrane Na^+ concentration or electrical potential difference among the differing preincubation conditions. Initial ^{22}Na 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 HCO_3^-, amiloride, and SITS, and were expressed as nmol/mg protein/per min.

$^{22}Na^+$ 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).

$^{22}Na^+$ 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 ^{22}Na in a series of three preincubation media all containing ^{22}Na and consisting of (*a*) 60 min in HCO_3^--containing medium (25 mM $NaHCO_3$; 40 mM NaCl; 70 mM KCl; 0.8 mM $MgSO_4$; 1.2 mM $CaSO_4$; 0.8 mM KH_2PO_4; 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 HCO_3^--containing medium with or without 1 mM SITS or amiloride or 30 min in nominally HCO_3^--free medium (25 mM of $NaHCO_3^-$ 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 CO_2 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 ^{22}Na. 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 HCO_3^--free or contained 25 mM HCO_3^-. 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 CO_2 production and achieve depletion of intracellular HCO_3^- (see Results). Moreover, these conditions were very similar to those used to study the effects of membrane hyperpolarization on pH_i (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 HCO_3^- 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_i was measured fluorometrically (28) using the pH-sensitive carboxyfluorescein derivative BCECF (pKa 6.98). The nonfluorescent and membrane permeant acetoxymethylester of this compound (BCECF-AM) readily enters cells, where cytosolic esterases cleave the ester bonds and form the polyanionic, 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 [HCO_3-free] or 95% room air/5% CO_2 [HCO_3^--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_i from standard curves. The use of this fluorescence ratio provides a measurement of pH_i 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 morpholino-ethane-sulfonic acid (adjusted to pH values within the range of 6.3 to 7.8), 130 mM KCl, and 10 μM nigericin, a H^+/K^+-exchanging ionophore (28). Under these conditions, pH_i 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_i 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_i in that monolayer. Because individual calibration of each coverslip was not feasible, pH_i 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_i toward baseline values in selected experiments (see Results).

To determine steady-state pH_i, 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_i 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_i 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_i) was measured before and after the change in superfusion medium. The rate of change in pH_i (dpH_i/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_i during transient lowering of extracellular $[HCO_3^-]$. Cells were preincubated for 30 min in Na^+-containing media (25 mM $NaHCO_3$; 110 mM NaCl; 5 mM KCl; 0.8 mM $MgSO_4$; 1.2 M $CaSO_4$; 0.8 mM Na_2PO_4; 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_i 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 $[HCO_3^-]$ was 5 mM instead of 25 mM (20 mM $NaHCO_3^-$ replaced by 20 mM NaCl and both solutions were equilibrated with 95% air, 5% CO_2), while pH_i 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 gluconate, respectively. Cl^--free medium was also used for dye loading and pH_i measurements. 1 mM amiloride or 1 mM SITS was present in preincubation and/or superfusion media as appropriate.

Measurement of pH_i during hyperpolarization of membrane potential. These studies were conducted in a fashion analogous to those of ^{22}Na efflux (see above). In brief, cells were preincubated for 30 min in Na^+-containing media (25 mM $NaHCO_3$; 40 mM NaCl; 70 mM KCl; 0.8 mM $MgSO_4$; 1.2 M $CaSO_4$; 0.8 mM Na_2PO_4; 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_i, 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_i was continuously monitored. 1 mM amiloride or 1 mM SITS was present in preincubation and/or superfusion media as appropriate.

Isolation of basolateral (blLPM) and canalicular (cLPM) liver plasma membrane vesicles. blLPM 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 tetramethylammonium (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 gassed with 100% N_2. 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 HCO_3^--dependent ^{22}Na uptake into blLPM and cLPM vesicles. ^{22}Na 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% N_2, 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 gluconate, 57 mM HCO_3^-, 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 $^{22}NaCl$, and 1 μM acetazolamide. Where indicated, 100 μM DIDS or 100 μM amiloride was also added. Bicarbonate-free incubation mixtures were gassed with 100% N_2, whereas in the presence of a 57-mM out > in HCO_3^- gradient, gassing was performed with 10% CO_2/90% N_2. ^{22}Na 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 ^{22}Na 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 ^{22}Na 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 ^{22}Na 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 $P \leq 0.05$ was considered statistically significant.

Results

Effects of HCO_3^- on ^{22}Na uptake by cultured rat hepatocytes. Sodium uptake, measured as the rate of ^{22}Na 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 HCO_3^-, 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 HCO_3^-, 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 HCO_3^- still stimulated $^{22}Na^+$ uptake nearly four-fold when Li^+ was used to replace Na^+, the absolute rates of $^{22}Na^+$ 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^-/HCO_3^- exchange, known to be present in hepatocytes (10), the effect of HCO_3^- on ^{22}Na 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 HCO_3^--stimulated ^{22}Na uptake was still observed when NMG and gluconate were used to replace Na^+ and Cl^-, respectively. HCO_3^--stimulated Na^+ uptake was also observed when Na^+ and Cl^- were replaced by Li^+ and NO_3^-, 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 HCO_3^- 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^-/HCO_3^- exchange.

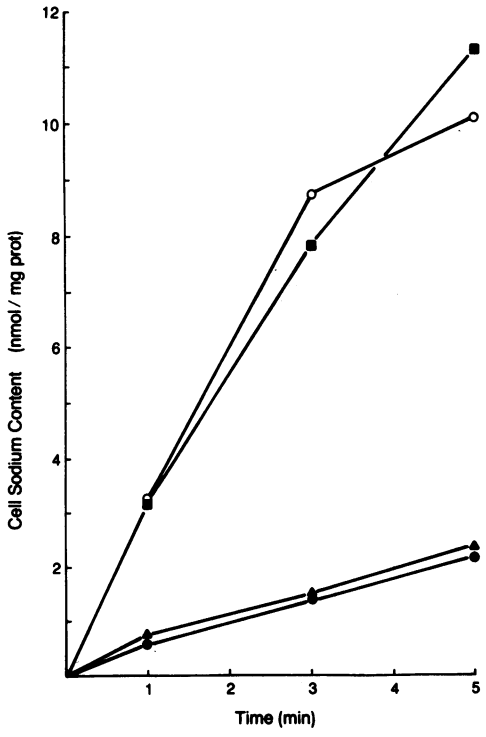

Figure 1. Representative study of ^{22}Na uptake in the absence of HCO_3^- (●), in the presence of 25 mM HCO_3^- (○), in presence of 25 mM HCO_3^- plus 1 mM amiloride (■), or in presence of 25 mM HCO_3^- plus 1 mM SITS (▲). Studies were performed as described in Methods, with choline replacing all but 5 mM Na^+.

Effects of weak acids other than H_2CO_3 on ^{22}Na uptake. To determine whether the salts of other weak acids could substitute for HCO_3^-, analogous studies were conducted in which the effects of HCO_3^- on ^{22}Na 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 ^{22}Na uptake. However, unlike the increase produced by HCO_3^-, this increase was not statistically significant in the case of propionate and was inconsistently observed. Also, unlike HCO_3^-, the stimulation of $^{22}Na^+$ uptake produced by both acetate and propionate was unaffected by SITS and was completely inhibited by amiloride.

^{22}Na uptake as a function of extracellular sodium concentration. Initial ^{22}Na uptake rate was measured at varying (1–135 mM) concentrations of extracellular Na^+ both in the absence and presence of 25 mM extracellular HCO_3^-. As is evident from Fig. 2, $^{22}Na^+$ uptake rate in the presence of HCO_3^- exceeded (by up to 10-fold) uptake rate in the absence of HCO_3^- over the entire concentration range and was clearly saturable. The apparent K_m for Na^+ in the presence of HCO_3^- was 25.5±2.1 mM.

Effect of HCO_3^- on ^{22}Na efflux. In the first series of studies, the effect of HCO_3^- 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 HCO_3^- in the efflux media. As illustrated in Fig. 3 and summarized in Table III (series *a–d*), the presence of HCO_3^- in the preincubation medium increased the initial rate of ^{22}Na efflux ~ 10-fold, and this stimulation was unaffected by amiloride but abolished by SITS. Of interest, in preliminary studies, ^{22}Na efflux from cells incubated in HCO_3^--free media, but without transient exposure to KCN, occurred at a rate similar to that observed with HCO_3^--containing preincubation media. This suggests that intracellular HCO_3^- generated from metabolic CO_2 production is sufficient to stimulate ^{22}Na 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 HCO_3^- concentration gradient (presence or absence of HCO_3^- in preincubation and/or efflux media) were studied. As shown in Fig. 4 and summarized in Table III, hyperpolarization increased the rate of ^{22}Na efflux in the presence of an in > out HCO_3^- concentration gradient about two-fold. No stimulation of ^{22}Na efflux was produced by hyperpolarization in the absence of intra- and extracellular HCO_3^- (series *i* and *j* in Table III). Finally, in the presence of HCO_3^- in both the preincubation and efflux media (i.e., the absence of an in > out HCO_3^- gradient), the rate of ^{22}Na efflux was significantly reduced ~ 5 to 10-fold, as compared with an in > out HCO_3^- 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 HCO_3^- concentration gradient and by an increase in membrane potential difference in the presence but not in the absence of intracellular HCO_3^-. This increase in Na^+ efflux

Table I. Effect of HCO_3^- on ^{22}Na Uptake by Cultured Rat Hepatocytes

Presence/absence of		Cation substituted for Na^+*/Anion substituted for Cl^-‡				
HCO_3^-§	Inhibitor‖	Choline/None	NMG/None	LI^+/None	NMG/Gluconate	Li^+/Nitrate
+	None	2.02±0.24 (7)	1.84±0.14 (5)	0.87±0.17¶ (3)	1.63±0.19 (4)	1.39±0.15 (3)
−	None	0.42±0.05** (6)	0.47±0.05** (5)	0.23±0.10‡‡ (3)	0.45±0.03** (5)	0.23±0.06¶§§ (3)
+	Amiloride	1.85±0.52 (3)	1.75±0.18‖‖ (5)		1.26±0.25‖‖ (4)	
+	SITS	0.47±0.09** (5)	0.93±0.25‡‡ (3)		0.79±0.12‡‡ (5)	

All results are expressed as the mean±SEM of initial ^{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. ‡ In selected experiments, Cl^- was completely replaced by the indicated anion. § HCO_3^- was present at a concentration of 25 mM (+) or absent (−), being replaced by Cl^-, except where indicated. ‖ 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. ¶ $P \leq 0.05$ compared with either choline or NMG substituting for Na^+. ** $P < 0.0005$, ‡‡ $P \leq 0.05$, §§ $P < 0.005$, respectively, compared with studies in the presence of HCO_3^- and absence of inhibitor under the same conditions of anion and/or cation substitution (vertical row). ‖‖ Ethylisopropyl amiloride (0.1 mM), a potent Na^+/H^+ exchange inhibitor, was used in these studies instead of amiloride, which is available only as a hydrochloride.

seen in the presence of a HCO_3^- concentration gradient and increased potential difference is abolished by SITS but is unaffected by amiloride.

Table II. Effect of Weak Acids Other than HCO_3^- on ^{22}Na Uptake by Cultured Rat Hepatocytes

	Inhibitor		
Weak acid*	None	Amiloride‡	SITS§
	nmol/mg per min		
None	0.68±0.14 (3)	—	—
HCO_3^-	2.04±0.27‖ (4)	1.85±0.52¶ (3)	0.47±0.09** (5)
Acetate	1.08±0.24‖ (3)	0.59±0.24** (2)	1.80±1.14 (2)
Propionate	1.22±0.35 (3)	0.54±0.19** (2)	1.12±0.12 (2)

All results are expressed as the mean±SEM of initial ^{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_3^- on Na^+ uptake was compared with 25 mM acetate, 25 mM propionate or Cl^- (none), all of which were present as the K^+ salt. Sodium concentration in all incubations was 5 mM, with choline and Cl^- present in the concentration necessary to maintain isosmolality.

‡ Amiloride was present at a concentration of 1 mM.

§ SITS was present at a concentration of 1 mM.

‖ $P < 0.05$ compared with no weak acid (none).

¶ The effects of amiloride and SITS on HCO_3^--stimulated ^{22}Na uptake were not reexamined in these experiments. The values shown here for comparative purposes are taken from Table I.

** $P < 0.05$ compared with HCO_3^- and no inhibitor.

Effects of lowering extracellular [HCO_3^-] on pH_i of cultured hepatocytes. Resting pH_i averaged 7.33±0.06 (mean±SEM) in control, HCO_3^--containing media and tended to be lower in Na^+-free medium or media containing amiloride or SITS, although this did not achieve statistical significance (Table IV). Abrupt lowering of extracellular [HCO_3^-] from 25 to 5 mM under control conditions with constant (5%) P_{CO_2}, caused extracellular pH to fall from 7.4 to 6.7 and was associated with a corresponding significant fall in pH_i to 6.97±0.08. Raising extracellular [HCO_3^-] to 25 mM caused pH_i to return to 7.34±0.06, a value nearly identical to initial resting pH_i (Fig. 5). As in control studies, resting pH_i was also significantly and reversibly reduced by transiently lowering extracellular [HCO_3^-] from 25 to 5 mM in Na^+-free media and in the presence of SITS or amiloride.

The rate of fall in pH_i (dpH_i/dt) after the lowering of extracellular [HCO_3^-] averaged 0.227± −0.033 pH units/min under control conditions. In the presence of SITS or absence of Na^+, dpH_i/dt was significantly (50–60%) reduced, whereas dpH_i/dt was unaffected by amiloride (Fig. 5 and Table IV). To establish that these changes in pH_i were not mediated by Cl^-/HCO_3^- exchange, these same studies were repeated under conditions in which Cl^- was replaced by gluconate (with NMG replacing choline). As summarized in Table IV, whereas a resting pH_i tended to be higher in Cl^--free medium than in medium containing Cl^-, the rate of fall in pH_i upon lowering intracellular HCO_3^- in Cl^--free medium did not differ from that in Cl^--containing medium and was reduced in the absence of Na^+ or presence of SITS but was unaffected by amiloride. These findings suggest a possible role for Cl^- (and presumably Cl^-/HCO_3^- exchange) in maintenance of resting pH_i, but also indicate that Cl^-/HCO_3^- exchange does not mediate the fall in pH_i after reduction of extracellular HCO_3^- at constant P_{CO_2}. The rate of pH_i recovery after return of extracellular HCO_3^- concentration to 25 mM was not systematically analyzed in these experiments because the fluorescence signal-to-noise ratio was con-

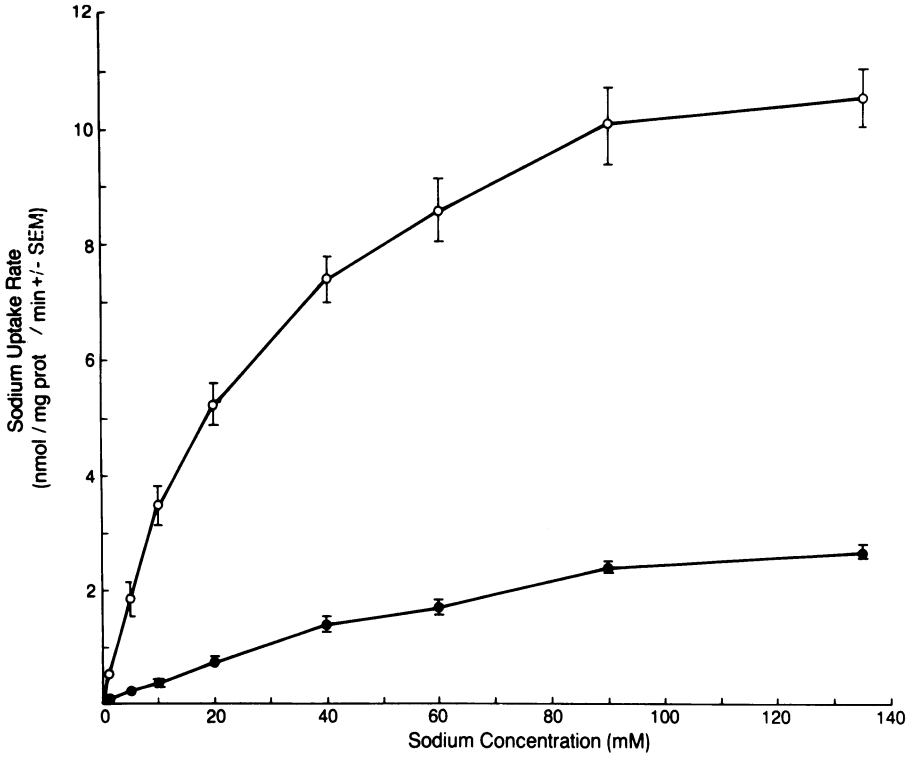

Figure 2. ^{22}Na uptake rate versus extracellular sodium concentration in the presence (○) and absence (●) of 25 mM HCO_3^-. Initial uptake rate was measured at 3 min as measured as described in Methods, with varying concentrations (1–135 mM) of NaCl replaced by choline chloride in the preincubation and incubation media.

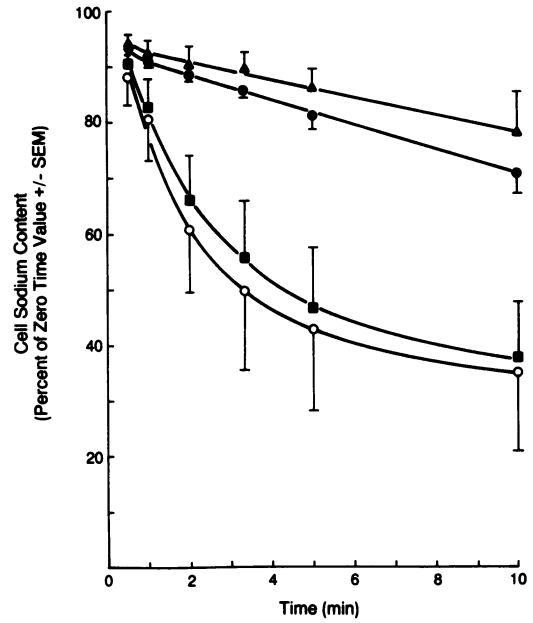

Figure 3. ^{22}Na efflux from preloaded hepatocytes measured as described in Methods. Curves depict results without HCO_3^- in the final preincubation media (closed circles; series *a* in Table III), with HCO_3^- in the preincubation media in the absence of inhibitors (○; series *b* in Table III), or 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.

siderably lower, due to dye extrusion, than earlier in the course of the study.

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

Series	Experimental conditions				Inhibitor	Initial efflux rate
	$[HCO_3^-]$		$[K^+]$			
	Final preincubation media	Efflux medium	Preincubation media	Efflux medium		
	mM					*% min^{-1} ± SEM*
a	0	0	70	5	None	2.7±0.5 (3)
b	25	0	70	5	None	37.8±14.5* (3)
c	25	0	70	5	Amiloride	34.0±11.9* (3)
d	25	0	70	5	SITS	1.5±0.3 (3)
e	25	0	70	5	None	37.9±6.7‡ (6)
f	25	0	70	70	None	14.8±4.6§ (4)
g	25	25	70	5	None	8.3±3.8 (4)
h	25	25	70	70	None	3.7±0.2 (4)
i	0	0	70	5	None	3.6±0.6 (7)
j	0	0	70	70	None	4.0±0.6 (4)

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*.

tions so far as possible by controlling for the effects of prolonged incubation in Na^+-free or amiloride-containing medium on Na^+/K^+-ATPase and membrane potential (25). Hyperpolarization of membrane potential produced by abruptly lowering extracellular $[K^+]$ to 5 mM in the presence of 10 μM valinomycin, under control conditions, caused pH_i to fall from 7.35±0.07 to 7.20±0.06 at an initial rate of 0.629 pH units/min. As was true for the effects of lowering extracellular $[HCO_3^-]$ on pH_i, the rate of fall in pH_i during hyperpolarization was significantly reduced under Na^+-free conditions or by SITS, but was unaffected by amiloride (Table V).

^{22}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 blLPM 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 blLPM 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 electrogenicity of HCO_3^--dependent ^{22}Na uptake into blLPM 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

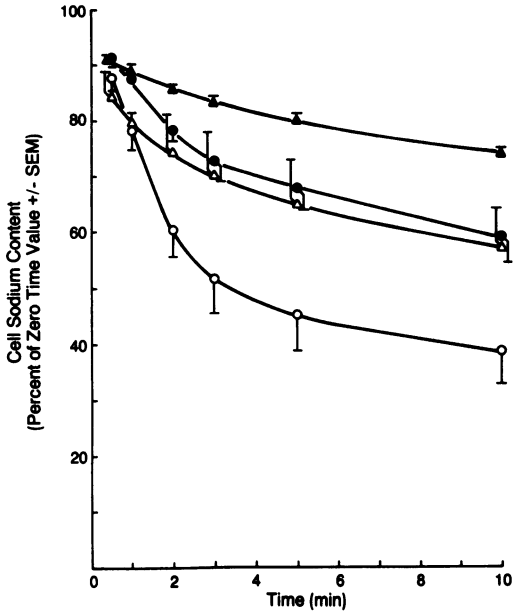

Figure 4. ^{22}Na efflux from preloaded hepatocytes measured as described in Methods. Curves depict results in the presence of an in > out HCO_3^- gradient under presumed hyperpolarizing (in > out K^+ gradient plus valinomycin) conditions (○; series *e* in Table III), in the presence of an in > out HCO_3^- gradient under nonhyperpolarizing conditions (●; series *f* in Table III), or in the absence of a HCO_3^- gradient (HCO_3^- present in preincubation and efflux media) under hyperpolarizing (△; series *g* in Table III) or nonhyperpolarizing (▲; 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.

intact cells or epithelia (12, 16, 18, 19) or plasma membrane vesicles (12, 15, 17), have included the demonstration of Na^+-dependent HCO_3^- transport and/or HCO_3^--dependent Na^+ transport that is electrogenic, inhibited by certain disulfonic acids such as DIDS or SITS, and unaffected by amiloride. These findings, summarized below, provide strong evidence that such a mechanism exists in hepatocytes.

Na^+ uptake by hepatocytes, under the conditions of these experiments, was increased ~ 4- to 10-fold in the presence of extracellular HCO_3^- as compared with its absence. HCO_3^--stimulated $^{22}Na^+$ uptake was eliminated by SITS, was unaffected by amiloride or EIA at concentrations (1 mM or 100 μM, respectively) that block Na^+/H^+ exchange in rat hepatocyte plasma membranes (8, 9) as well as in intact hepatocytes (26, 27), and was saturable with respect to extracellular Na^+ with an apparent K_{Na^+} of ~ 25 mM. HCO_3^--stimulated Na^+ uptake was observed when either NMG or Li^+ was used instead of choline to partially replace Na^+, although the absolute rate of ^{22}Na entry was reduced in the presence of Li^+. The explanation for this finding is unknown; competition between Li^+ and Na^+ for the putative transporter represents one possibility. HCO_3^--stimulated $^{22}Na^+$ uptake was also observed after prolonged incubation in Cl^--free (gluconate or nitrate substitution) media, indicating a lack of dependence on Cl^-/HCO_3^- exchange. Moreover, acetate and propionate did not appear to substitute for HCO_3^- with respect to stimulation of $^{22}Na^+$ uptake. Whereas $^{22}Na^+$ uptake tended to be increased in the presence of these other weak acids, this increase was not inhibited by SITS but was abolished by amiloride. These findings suggest that the increase in $^{22}Na^+$ uptake caused by these other weak acids was mediated via Na^+/H^+ exchange, perhaps stimulated by intracellular acidification.

To determine whether HCO_3^- stimulates both $^{22}Na^+$ efflux and uptake, cells were preloaded with $^{22}Na^+$ and the rate of $^{22}Na^+$ efflux measured under various conditions. As with uptake, $^{22}Na^+$ efflux was increased ~ 5- to 10-fold in the presence as compared with the absence of an intra- to extracellular HCO_3^- concentration gradient, and membrane hyperpolarization produced by an out-to-in K^+ concentration gradient plus valinomycin increased $^{22}Na^+$ efflux in the presence, but not in the absence of HCO_3^-. Moreover, as with uptake, the increase

Table IV. Effects of Lowering Extracellular [HCO_3^-] in the Presence of Constant CO_2 on pH_i of Cultured Rat Hepatocytes

Conditions	Cl^--containing medium				Cl^--free medium*			
	Initial pH_i (25 mM HCO_3^-)	pH_i (5 mM HCO_3^-)	Recovery pH_i (25 mM HCO_3^-)	dpH_i/dt	Initial pH_i (25 mM HCO_3^-)	pH_i (5 mM HCO_3^-)	Recovery pH_i (25 mM HCO_3^-)	dpH_i/dt
				pH units/min				*pH units/min*
Control	7.33±0.06	6.97±0.08‡	7.34±0.06	0.227±0.033	7.49±0.07	7.20±0.06‡	7.44±0.07	0.194±0.041
	(5)	(5)	(5)	(5)	(4)	(4)	(4)	(4)
Na^+-free§	7.15±0.09	6.81±0.07‡	7.12±0.01	0.101±0.026‖	7.31±0.03	7.10±0.04‡	7.34±0.02	0.083±0.018‖
	(5)	(5)	(5)	(5)	(4)	(4)	(4)	(4)
Amiloride (*1 mM*)¶	7.22±0.07	6.85±0.03‡	7.13±0.12	0.228±0.046	7.61±0.07	7.39±0.05‡	7.60±0.01	0.183±0.040
	(4)	(4)	(4)	(4)	(3)	(3)	(3)	(3)
SITS (*1 mM*)	7.18±0.09	6.87±0.10‡	7.08±0.19	0.092±0.018‖	7.40±0.07	7.20±0.08‡	7.49±0.04	0.057±0.020‖
	(4)	(4)	(3)	(4)	(4)	(4)	(2)	(4)

Extracellular [HCO_3^-] was lowered from 25 mM (pH 7.4) to 5 mM (pH 6.7) in the presence of constant P_{CO_2} (5%), and pH_i was monitored fluorimetrically 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_i/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.

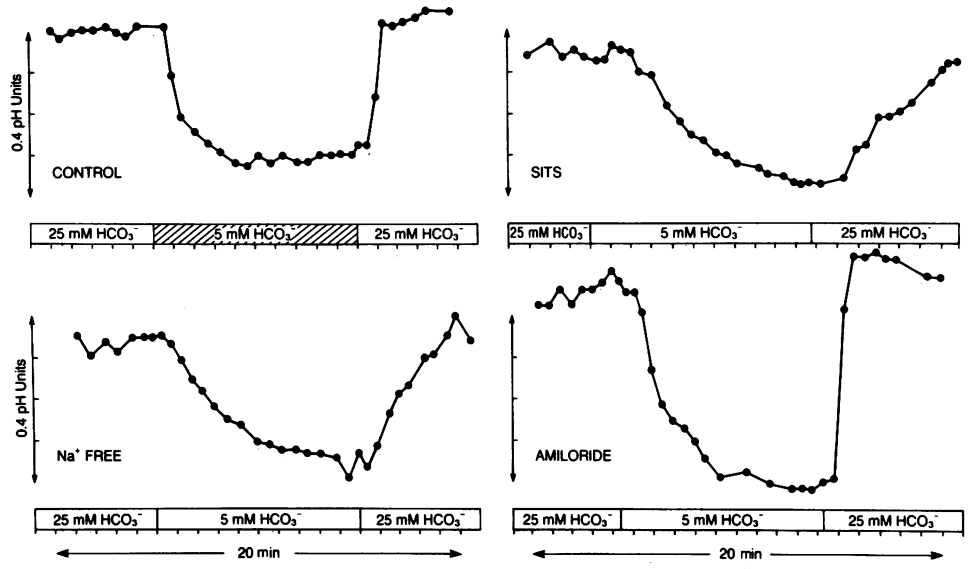

Figure 5. Representative studies of the effect of changing extracellular [HCO_3^-] from 25 mM (pH 7.42) to 5 mM (pH 6.70) in the presence of constant (5%) P_{CO_2} on hepatocyte pH_i under control conditions (*upper left*), in Na^+-free medium (*lower left*), or in medium containing 1 mM SITS (*upper right*) or 1 mM amiloride (*lower right*). The rate of fall in pH_i 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 pH_i before lowering extracellular [HCO_3^-] ranged from 7.19 to 7.23 in the hepatocyte monolayers depicted.

in $^{22}Na^+$ 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 $^{22}Na^+$ 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, pH_i 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 pH_i after lowering of extracellular [HCO_3^-] while keeping P_{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 $^{22}Na^+$ uptake, the HCO_3^- efflux was also unaffected by prolonged incubation in Cl^--free

Table V. pH_i of Cultured Rat Hepatocytes during Hyperpolarization of Membrane Potential

	Initial pH_i (preincubation media)	dpH_i/dt
		pH units/min
Control	7.35±0.07 (5)	0.63±0.10 (5)
Na^+-free*	7.19±0.12 (4)	0.20±0.05‡ (4)
Amiloride§	7.26±0.14 (4)	0.64±0.08 (4)
SITS§	7.24±0.12 (4)	0.30±0.08‖ (4)

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 μM valinomycin, while pH_i 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.

‡ $P < 0.01$ compared with dpH_i/dt under control conditions by unpaired "t" test.

‖ $P < 0.05$ compared with dpH_i/dt under control conditions by unpaired "t" test.

§ Amiloride and SITS were both present at a concentration of 1 mM in preincubation and/or experimental media.

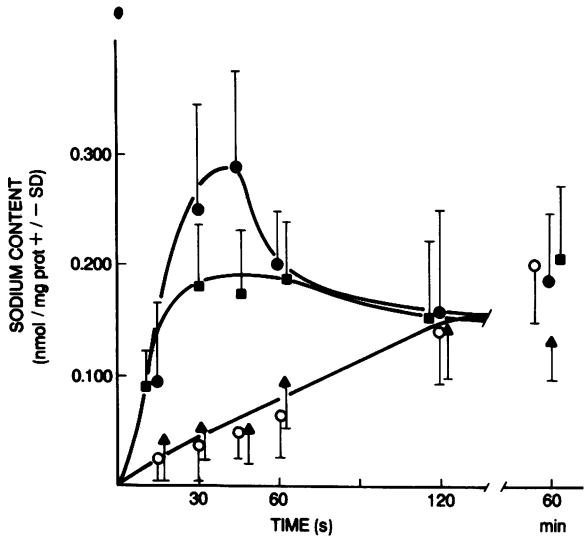

Figure 6. HCO_3^--dependent Na^+ uptake into blLPM vesicles. Vesicles were resuspended in a HCO_3^-- and K^+-free TMA buffer (see Methods) supplemented with 1 μM acetazolamide (inhibition of membrane-bound carbonic anhydrase). All vesicle suspensions were treated with valinomycin and a separate sample also with 100 μM DIDS. 20-μl aliquots of vesicle suspension were incubated with 80 μl incubation medium with a composition identical to the membrane resuspension buffer (▲) or consisting of 100 mM K^+, 43 mM gluconate, 57 mM HCO_3^-, 50 mM mannitol, 42 mM Hepes, 21 mM TMA/OH, pH 7.5, and 1 μM acetazolamide without inhibitors (●), plus 100 μM amiloride (■), or plus 100 μM DIDS (○). Data represent the means±SD of 12 determinations in three different membrane preparations.

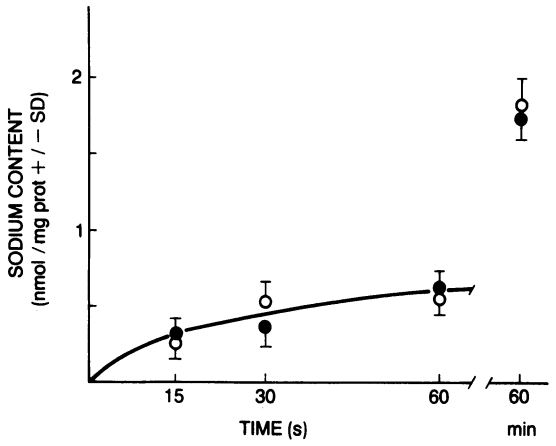

Figure 7. $^{22}Na^+$ uptake into cLPM vesicles in the absence (○) and presence (●) of an out > in HCO_3^- gradient. The incubation conditions were similar to shown in the legend to Fig. 5, the only exception being that the effects of amiloride and DIDS were not evaluated because no HCO_3^--dependent portion of ^{22}Na uptake could be detected. Data represent the means±SD of eight determinations in two 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_i 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 HCO_3^--dependent and SITS-sensitive Na^+ conductance, a Na^+-dependent and SITS-sensitive conductive pathway for HCO_3^- efflux, Na^+-dependent Cl^-/HCO_3^- exchange [33]), collectively, they appear to be best explained by the existence of electrogenic Na^+/HCO_3^- symport in hepatocytes. The observation that hepatocyte hyperpolarization stimulated both Na^+-dependent HCO_3^- efflux and Na^+ efflux in the presence, but not absence of HCO_3^- (Figs. 3 and 4, Table III) provides particularly strong evidence for the cotransport of HCO_3^- 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 HCO_3^- transport by rat hepatocytes in primary culture is electrogenic (34).

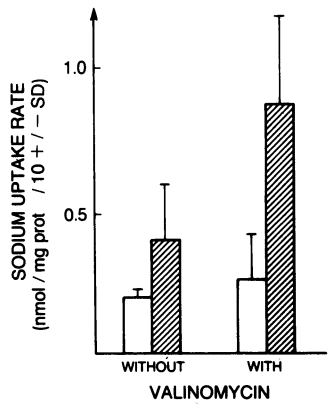

Figure 8. Electrogenicity of HCO_3^--dependent ^{22}Na uptake into blLPM vesicles. Vesicles were resuspended in HCO_3^-- and K^+-free TMA buffer supplemented with 1 μM acetazolamide. Half of the vesicles were treated with valinomycin. The vesicles were incubated either in 100 mM K^+, 100 mM gluconate, 50 mM mannitol, 42 mM Hepes, 21 mM TMA/OH, pH 7.5 (gassed with 100% N_2; *open bars*) or in 100 mM K^+, 43 mM gluconate, 57 mM HCO_3^-, 50 mM mannitol, 42 mM Hepes, 21 mM TMA/OH, pH 7.5 (gassed with 10% CO_2/90% N_2; *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 ^{22}Na uptake in the presence, but not absence of an out-to-in HCO_3^- gradient. Data represent the means±SD of triplicate determinations in one representative vesicle preparation.

The results of the studies with plasma membrane vesicles are also consistent with the presence of an electrogenic Na^+/HCO_3^- 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 HCO_3^- concentration gradient accelerated the rate of $^{22}Na^+$ entry into blLPM and caused a transient overshoot. $^{22}Na^+$ entry into cLPM was not stimulated under these conditions. In blLPM, valinomycin accelerated HCO_3^--dependent ^{22}Na uptake into blLPM 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 $^{22}Na^+$ uptake in the absence of HCO_3^-, but inhibited $^{22}Na^+$ uptake in the presence of HCO_3^-. 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^+/HCO_3^- cotransport at the sinusoidal surface domain.

Assuming that these findings do indicate the presence in hepatocytes of Na^+/HCO_3^- 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 (HCO_3^-, OH^-, H^+, CO_3^{-2}) carried by the transporter (12, 18, 19). Our preliminary observation that Na^+ efflux is minimally affected by simple removal of extracellular HCO_3^- from the preincubation medium, unless the cells are also exposed transiently to KCN, suggests that metabolically produced CO_2/HCO_3^- is sufficient to drive the transporter. Because KCN had no effect on Na^+ efflux in the presence of extracellular HCO_3^-, this is unlikely to represent a toxic effect. Our observations also indicate that the putative symporter is reversible, because it appears to mediate both $^{22}Na^+$ 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 HCO_3^-. A similar finding has recently been reported in proximal tubular cells (35).

Second, the basolateral location of Na^+/HCO_3^- symport in hepatocytes was unexpected. By analogy with other epithelial cells, such as parietal cells or proximal tubular cells, in which Na^+/HCO_3^- symport appears localized to the basolateral membrane and is presumed to mediate HCO_3^- exit from the cell, we anticipated that Na^+/HCO_3^- symport, like Cl^-/HCO_3^- antiport, would be localized to the canalicular membrane. Its location on the basolateral membrane, and not the canalicular membrane across which HCO_3^- presumably enters the canaliculus, raises the possibility that Na^+/HCO_3^- symport in hepatocytes may mediate HCO_3^- 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 HCO_3^- exit mediated via a negatively charged Na^+/HCO_3^- cotransport mechanism. The present studies provide no direct information regarding the direction in which the

symporter operates in hepatocytes, and the question clearly merits further study.

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