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Bicarbonate stimulation of Na^+ transport in liver basolateral plasma membrane vesicles requires the presence of a transmembrane pH gradient

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The effect of bicarbonate on the uptake of $^{22}Na^+$ into liver basolateral plasma membrane vesicles was studied under conditions where the pH of the medium was controlled by the use of high buffer concentrations. Bicarbonate stimulated the rate of Na^+ uptake only in the presence of a pH gradient (acid inside). The stimulation by bicarbonate was inhibited by both amiloride and DIDS. No evidence for electrogenic Na^+/HCO_3^- symport was found. These results are in part consistent with electroneutral Na^+/HCO_3^- symport, but other explanations cannot be excluded.

Introduction

The recovery of intracellular pH in isolated hepatocytes after intracellular acidification has been shown to be mediated in part via an amiloride-sensitive Na^+/H^+ exchange system similar to those identified in membranes of a number of other cell types [1–3]. The exchanger exhibits a 'set point', i.e., it has very low activity at intracellular pH values greater than 7.2 [4]. The exchanger is activated by phorbol esters [4], epidermal growth factor and cyclic AMP [3] all of which effectors shift the set point to more alkaline values. Na^+/H^+ exchange has been well characterised in liver plasma membrane vesicles [5–7] and is present in basolateral membrane vesicles but absent from canalicular vesicles [7].

It was noted by Henderson et al. [1] that a bicarbonate-dependent system was also involved in pH regulation in intact hepatocytes. On the basis of measurements of intracellular pH [8] and uptake of $^{22}Na^+$ into cultured hepatocytes [2] the existence of a Na^+/HCO_3^- symport system has been postulated. Electrophysiological studies in cultured hepatocytes showed that depletion of bicarbonate from the medium partially depolarised the cell plasma membrane while readdition of bicarbonate caused repolarisation. Similar plasma membrane depolarisation of hepatocytes in suspension in the absence of bicarbonate has been observed previously [9]. Bicarbonate also stimulates electrogenic Na^+-alanine cotransport in hepatocytes – a process which is known to be dependent on the magnitude of the cell membrane potential [10].

In renal basolateral membrane vesicles the presence of an electrogenic Na^+/HCO_3^- symport system with a stoichiometry of 3 HCO_3^-: 1Na^+ has been established [11–14]. It has been suggested that the postulated Na^+/HCO_3^- symport system in hepatocytes is electrogenic and proceeds with the inward net transfer of negative charge [15,2]. Gleeson et al. [8] however found no evidence for electrogenicity of this proposed transport system.

Bicarbonate stimulation of Na^+ uptake has been demonstrated in liver basolateral membrane vesicles [2] but the response of this process to variations in intravesicular and extravesicular pH has not been investigated. In view of the uncertainties about the exact mechanism of bicarbonate-stimulated Na^+ transport in liver cell membranes, it was decided to study Na^+ transport in liver plasma membrane vesicles in more detail. In this paper it is shown that the properties of bicarbonate-stimulated Na^+ uptake into liver plasma membrane vesicles differs significantly from that previously characterised in kidney basolateral membrane vesicles, suggesting that a transport system with different properties may be involved.

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Abbreviations: DIDS, 4,4′-diisothiocyano-2,2′disulphonic acid stilbene; Mes, morpholineethanesulphonic acid; Hepes 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid.

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Materials and Methods

Plasma membrane vesicles were prepared from the livers of normally fed male Wistar rats by the method of van Amelsvoort et al. [16]. The vesicles were routinely resuspended at a concentration of 5–8 mg protein/ml in a medium containing 0.25 M sucrose, 0.2 mM $CaCl_2$ and 10 mM K Hepes at pH 7.4 (sucrose medium). When the vesicles were to be used in experiments in which the intravesicular pH was to be 6, 6.5 or 7.0 the vesicles were given an additional wash in a medium containing 0.25 M sucrose, 0.2 mM $CaCl_2$, 10 mM K Mes at the appropriate pH and resuspended and stored in the same medium. Vesicles were frozen in liquid nitrogen and stored at −20°C until use. These vesicles, which are of predominantly basolateral origin [17] have been fully characterised. Their integrity was routinely assessed by measuring alanine uptake as described by van Amelsvoort et al., [16]. Alanine uptake was Na^+-dependent and exhibited an overshoot (results not shown).

The use of bicarbonate with vesicle preparations involves problems in the maintenance of pH, since bicarbonate-containing solutions become progressively alkaline when exposed to air due to loss of CO_2. This is normally countered by equilibrating the solutions with a gas mixture containing the appropriate partial pressure of CO_2 which is in equilibrium with the required bicarbonate concentration at the particular pH. It is, nevertheless, not easy to ensure that the solution is always in equilibrium with the gas phase, especially during vesicle transport experiments. An alternative approach used here is to perform experiments in the presence of high concentrations of buffer which eliminate pH changes in bicarbonate solutions in the short term. In such solutions, equilibrium of CO_2 with the gas phase occurs relatively slowly. In the experiments described here, the extravesicular medium always contained 50 mM Tris-Mops, and bicarbonate solutions were used within 30 min of preparation. Under these conditions, no pH change in the solutions occurred. Further, there was no measurable loss of CO_2 to the atmosphere during this time as assessed by labelling with $H^{14}CO_3^-$. Both the bicarbonate concentration and the pH of the reaction medium could therefore be considered to be constant thoughout the experiments.

$^{22}Na^+$ uptake was measured using a rapid filtration technique. 0.02 ml of vesicle suspension was rapidly mixed at room temperature (20°C) with an equal volume of reaction medium containing 0.25 M sucrose, 0.2 mM $CaCl_2$, 10 mM K Hepes, 100 mM Tris-Mops, 0.4 mM NaCl and 2 μC/ml $^{22}Na^+$ together with other salts as indicated in the legends at pH 7.5 or 8.2. After mixing, the final concentration of NaCl was 0.2 mM and that of Tris-Mops was 50 mM throughout. The final pH was 7.4 or 8.0. Na^+ uptake was terminated by the addition of 1 ml of ice-cold sucrose medium and the vesicles were immediately separated by filtration through a nitrocellulose filter (0.45 μM). After washing with 4 ml of ice-cold sucrose medium, the filters were placed in 6 ml scintillation fluid and allowed to dissolve before assaying the $^{22}Na^+$ content by liquid scintillation counting. For each vesicle preparation the binding of $^{22}Na^+$ to the membranes and filters was estimated by stopping the transport reaction at zero time by adding the radioactive reaction medium and the ice-cold sucrose medium simultaneously. This value, which varied from 105 to 140 pmol/mg protein for different vesicle preparations was subtracted from the total filter-associated $^{22}Na^+$ to give the uptakes shown in the figures and tables. In order to measure the vesicle protein, vesicles were solubilised by addition of an equal volume of 2.5% (w/v) Triton X-100. Vesicle protein was measured by the method of Bradford [18], using bovine albumin with the approriate amount of Triton X-100 as standard.

Amiloride, DIDS and valinomycin were purchased from the Sigma Chemical Co., Poole, Dorset, U.K. These compounds were dissolved in dimethylsulphoxide. No more than 2 μl of a reagent solution was added to 20 μl of vesicle suspension, and control experiments showed that this volume of dimethylsulphoxide had no effect on $^{22}Na^+$ uptake.

Results

$^{22}Na^+$ uptake into liver plasma membrane vesicles was initially studied under conditions where the internal pH of the vesicles was 6.0 and the external pH was maintained at 7.4 by the presence of 50 mM Tris-Mops. Fig. 1 shows that under these conditions the uptake of $^{22}Na^+$ was faster in the presence of 25 mM $KHCO_3$ than in the presence of 25 mM K cyclamate (a poorly penetrant anion). An overshoot was observed in each case. In the presence of 1 mM amiloride (an inhibitor of Na^+/H^+ exchange), the rate of Na^+ entry was inhibited and the same rate was obtained in the presence of either bicarbonate or cyclamate. Equilibration of the Na^+ ion concentration in the presence of amiloride was almost complete within 3 min. 25 mM choline bicarbonate stimulated $^{22}Na^+$ uptake to the same extent as did 25 mM $KHCO_3$ (results not shown).

In the absence of bicarbonate ions but in the presence of a pH gradient, Na^+ uptake is accounted for partially by diffusion and partially by the amiloride-sensitive Na^+/H^+ exchange system. The activity of the Na^+/H^+ system is given by the $^{22}Na^+$ uptake in the absence of amiloride minus that in the presence of amiloride. This experiment indicates that bicarbonate could be either increasing the activity of the Na^+/H^+ exchange system or stimulating Na^+ entry by some other amiloride-sensitive mechanism.

The effect of bicarbonate on the rate of $^{22}Na^+$ uptake at an external pH of 7.4 was found to be

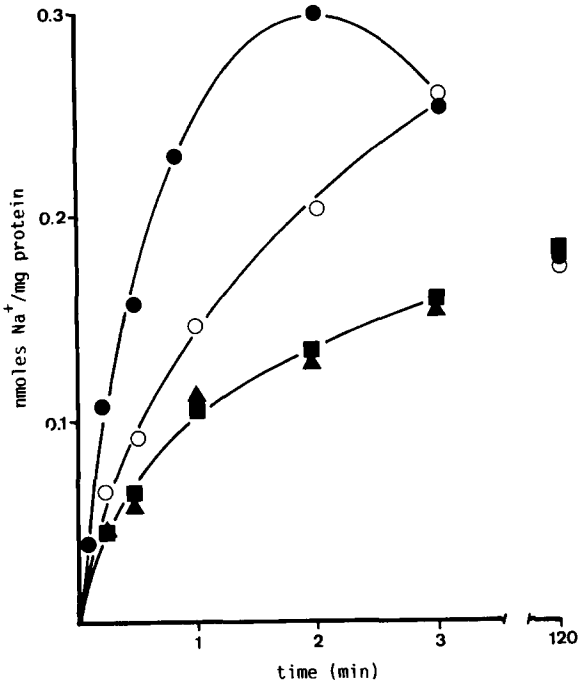

Fig. 1. Time-course of uptake of $^{22}Na^+$ into liver plasma membranes with an internal pH of 6. Vesicles with an internal pH of 6 were prepared as described in the Materials and Methods. The incubation medium contained (final concentrations) 0.25 M sucrose, 5 mM K Mes, 0.2 mM $CaCl_2$, 10 mM K Hepes, 50 mM Tris-Mops, and 0.2 mM $^{22}NaCl$ and further additions as indicated at pH 7.4. Transport was terminated after the time intervals shown. Further additions were 25 mM $KHCO_3$ (●), 25 mM K cyclamate (○), 1 mM amiloride plus 25 mM $KHCO_3$ (■) and 1 mM amiloride plus 25 mM K cyclamate (▲).The results of a single representative experiment are shown.

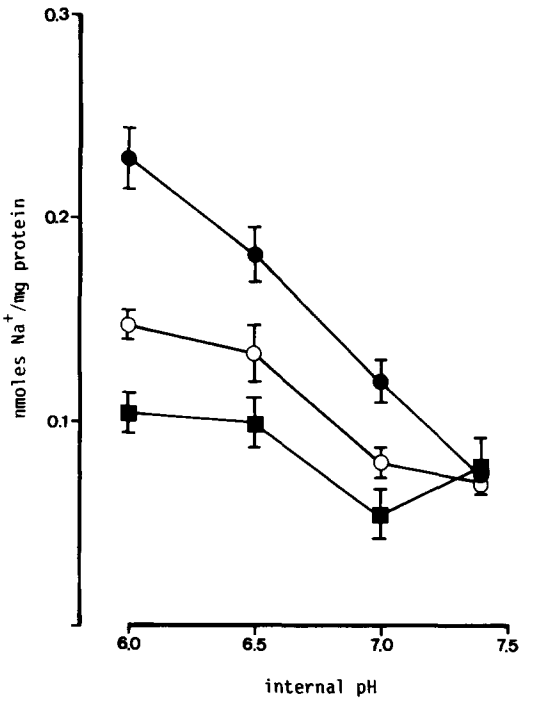

Fig. 2. Effect of intravesicular pH on Na^+ uptake. Vesicles were prepared as described in Materials and Methods with internal pH values of 6, 6.5, 7 or 7.4. Transport was measured in the medium described in Fig. 1 at pH 7.4. The $^{22}Na^+$ taken up in 1 min was determined. The results are the means ± S.E. for 3–5 replicate samples in each case. ●, 25 mM $KHCO_3$; ○, 25 mM K cyclamate; ■, 25 mM $KHCO_3$ plus 1 mM amiloride. Curve ● represents HCO_3^--dependent plus $(H^+)_i$ driven Na^+ uptake; curve ○ represents HCO_3^--independent, solely $(H^+)_i$ driven uptake; curve ■ represents amiloride-insensitive uptake due to diffusion or leakage.

dependent on the pH difference across the vesicle membranes under conditions where the inwardly-directed bicarbonate gradient was constant. Fig. 2 shows that in the absence of bicarbonate, the amiloride-sensitive entry of Na^+ via Na^+/H^+ exchange decreased as the pH gradient decreased, in accordance with previous work of others. The stimulatory effect of bicarbonate on Na^+ entry similarly decreased as the pH gradient decreased. When the internal pH was 7.4, so that no pH gradient existed across the membrane, bicarbonate did not affect the rate of Na^+ uptake, and the rate of Na^+/H^+ exchange was also zero. The effect of bicarbonate was abolished by 1 mM amiloride at all pH values.

Table I shows the effects of 1 mM amiloride and of 1 mM DIDS (an inhibitor of bicarbonate-dependent transport systems in other membranes) on the rate of Na^+ uptake in the presence of 25 mM bicarbonate in vesicles where the internal pH was 6.0 and the external pH was 7.4. 1 mM amiloride abolished the effect of bicarbonate, while 1 mM DIDS inhibited Na^+ uptake in the presence of bicarbonate to a lesser extent. A titration of the effects of amiloride and DIDS on Na^+ uptake in the presence of bicarbonate is shown in Fig. 3. Both compounds inhibited Na^+ uptake, but with

TABLE I

Effect of inhibitors on bicarbonate-stimulated Na^+ uptake

Vesicles with an internal pH of 6 were prepared as described in Materials and Methods and preincubated for 1 min with the appropriate concentration of inhibitor. The incubation medium contained (final concentrations) 0.25 M sucrose, 0.2 mM $CaCl_2$, 5 mM K Mes, 10 mM K Hepes, 50 mM Tris-Mops, 0.2 mM $^{22}NaCl$ and either 25 mM $KHCO_3$ or 25 mM K cyclamate at pH 7.4. The transport was terminated after 1 min. The results show the mean ± S.E. of the number of replicate determinations shown. Significance was estimated by Student's *t*-test.

Additions	$^{22}Na^+$ uptake after 1 min (pmol/mg)
25 mM $KHCO_3$	194 ± 11 (4)
25 mM $KHCO_3$ + 1 mM amiloride	100 ± 12 (5) [a]
25 mM $KHCO_3$ + 1 mM DIDS	128 ± 2 (3) [b]
25 mM $KHCO_3$ + 1 mM DIDS + 1 mM amiloride	92 ± 18 (3) [b]
25 mM K cyclamate	149 ± 8 (4) [c]
25 mM K cyclamate + 1 mM amiloride	115 ± 16 (3) [b]

[a] $P < 0.001$.
[b] $P < 0.01$.
[c] $P < 0.05$ vs. 25 mM $KHCO_3$.

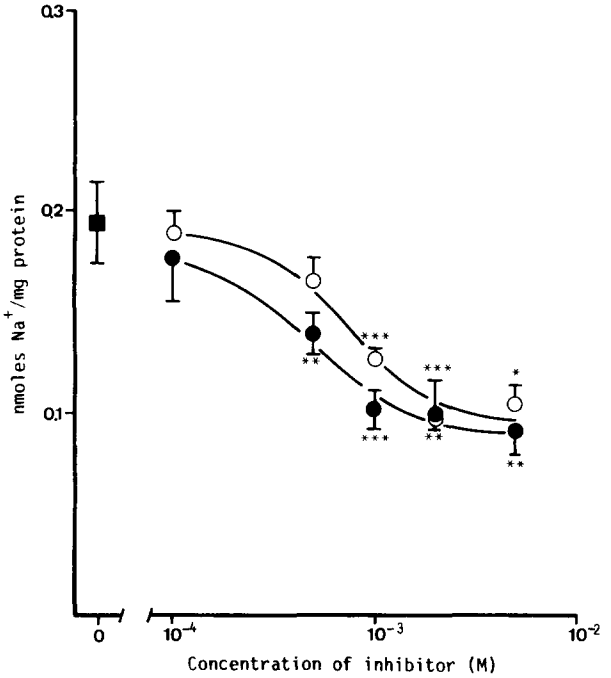

Fig. 3. Inhibition of $^{22}Na^+$ uptake by amiloride and DIDS. Vesicles (internal pH 6) were preincubated for 1 min with inhibitors at the concentrations shown. Transport was measured in the medium described in Fig. 1 in the presence of 25 mM $KHCO_3$ at pH 7.4. Each point is the mean ±S.E. of between 3 and 8 replicate samples, the number of replicates differs for each point. Results which are significantly different from the values in the absence of inhibitors (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by Student's *t*-test) are indicated. ■, 25 mM $KHCO_3$; ●, 25 mM $KHCO_3$ plus amiloride; ○, 25 mM $KHCO_3$ plus DIDS.

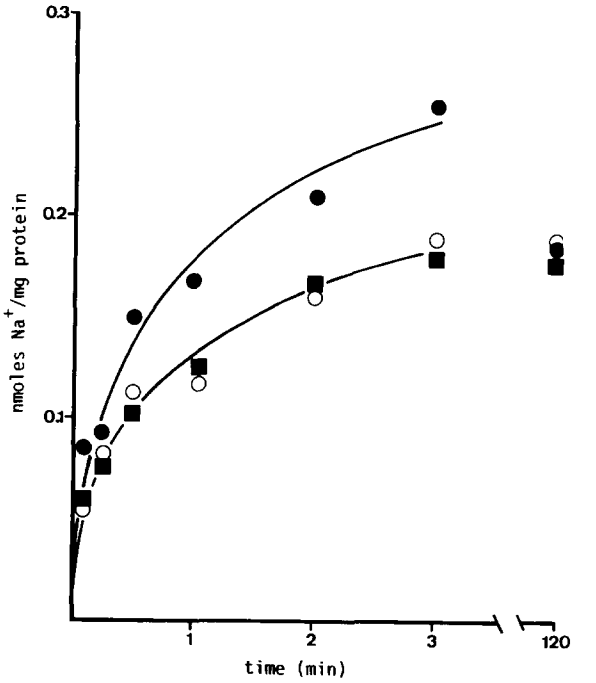

Fig. 4. Time-course of $^{22}Na^+$ uptake at an internal pH of 7.4. Vesicles (internal pH 7.4) were incubated in a medium containing (final concentrations) 0.25 M sucrose, 0.2 mM $CaCl_2$, 10 mM K Hepes, 50 mM Tris-Mops, 0.2 mM $^{22}NaCl$ and further additions as shown at either pH 7.4 or pH 8.0. ●, 50 mM $KHCO_3$, pH 8; ○, 50 mM $KHCO_3$, pH 7.4; ■, 50 mM K cyclamate, pH 7.4. The rate of uptake in the presence of 50 mM K cyclamate at pH 8.0 was the same as that in K cyclamate at pH 7.4 (not shown). Results of a single representative experiment are shown.

different concentration dependences. A half-maximum effect was obtained at 0.5 mM amiloride or 0.9–1 mM DIDS.

Fig. 4 shows the time-course of Na^+ uptake in vesicles with an internal pH of 7.4. When a pH gradient was imposed by holding the external pH at 8.0 using 50 mM Tris Mops, bicarbonate stimulated the rate of Na^+ uptake. Table II shows that this bicarbonate-stimulated uptake was sensitive to 1 mM amiloride. When the external pH was 7.4 so that no pH gradient was present, bicarbonate did not stimulate Na^+ uptake, in agreement with Fig. 2. Similar results were obtained when the extracellular medium was equilibrated with O_2/CO_2 (95 : 5) at pH 7.4 rather than being buffered with 50 mM Tris-Mops (not shown). The uptake in the presence of cyclamate was not significantly different at pH 8 from that at pH 7.4. In kidney basolateral membrane vesicles, valinomycin in the presence of a K^+ gradient stimulates bicarbonate-dependent Na^+ uptake and this finding has been used as evidence for an electrogenic Na^+/HCO_3^- symport. Table II shows that in liver plasma membranes there was consistently no effect of 10 μM valinomycin on Na^+ entry in the presence of bicarbonate plus a K^+ gradient. In a series of experiments valinomycin was without effect in the concentration range 1 μM to 150 μM. Valinomycin also failed to stimulate Na^+ transport in vesicles with an internal pH

TABLE II

Bicarbonate-stimulated $^{22}Na^+$ uptake in vesicles with an internal pH of 7.4

Vesicles with an internal pH of 7.4 were prepared as described in Materials and Methods and were incubated in a medium containing (final cocentrations) 0.25 M sucrose, 0.2 mM $CaCl_2$, 10 mM K Hepes, 50 mM Tris-Mops, 0.2 mM $^{22}NaCl$ and 50 mM $KHCO_3$ or 50 mM K cyclamate at either pH 7.4 or 8.0. Transport was terminated after 1 min. The results show the mean ± S.E. of the number of replicate values shown which were derived from experiments on three membrane preparations. Significance was estimated by Student's *t*-test.

Additions	pH of external medium	$^{22}Na+$ uptake after 1 min (pmol/mg)
K cyclamate	7.4	95 ± 6 (14)
K cyclamate	8	115 ± 10 (4)
$KHCO_3$	7.4	84 ± 10 (13)
$KHCO_3$	8	189 ± 7 (10) [a,b]
$KHCO_3$ + 10 μM valinomycin	7.4	75 ± 7 (8)
$KHCO_3$ + 100 μM valinomycin	7.4	82 ± 6 (4)
$KHCO_3$ + 1 mM amiloride	8.0	76 ± 8 (4)

[a] $P < 0.001$ vs. cyclamate at pH 7.4
[b] $P < 0.001$ vs. cyclamate at pH 8.

TABLE III

Anion specificity of stimulation of $^{22}Na^+$ uptake

Vesicles with an internal pH of 7.4 were prepared as described in Materials and Methods and were incubated in a medium containing (final concentrations) 0.25 M sucrose, 0.2 mM $CaCl_2$, 10 mM K Hepes, 50 mM Tris-Mops, 0.2 mM $^{22}NaCl$ and the K salt shown at a concentration of 50 mM. The final pH of the medium was 8.0. Transport was terminated after 1 min. The results show the mean ± S.E. of the number of replicate values shown. The uptake in the presence of $KHCO_3$ is significantly greater than that in all the other K salts; there is no significant difference in between the uptake in the other salts.

K^+ salt (50 mM)	Uptake of $^{22}Na^+$ after 1 min (pmol/mg)
HCO_3^-	216 ± 5 (5)
Mops	104 ± 8 (3)
DMO^- [a]	108 ± 9 (3)
SCN^-	116 ± 10 (3)
Cl^-	110 ± 5 (3)
Acetate	105 ± 12 (5)

[a] 5,5-Dimethyl-2,4-oxazolidinedione.

of 6 and external pH of 7.4 in the presence or absence of bicarbonate (results not shown).

In accordance with previous findings of others in isolated hepatocytes [2] Na^+ uptake was found to be stimulated by bicarbonate specifically. Table III shows that the rate of Na^+ uptake was not stimulated when K^+ cyclamate was replaced by equal concentrations of the K^+ salts of Mops, CNS^-, Cl^-, acetate or DMO. Bicarbonate is therefore not exerting its stimulatory effect by acting simply as a permeant anion or as a weak acid. It was found that the transport of bicarbonate could not be measured directly in these vesicles using $H^{14}CO_3^-$, since $^{14}CO_2$ was lost to the air during the filtration process. In general, no direct evidence for bicarbonate transport has been provided in previous investigations of this type in the literature.

Discussion

In this study, high buffer concentrations have been used to eliminate changes in extravesicular pH in bicarbonate-containing solutions. The major findings of this investigation are that the bicarbonate stimulation of Na^+ uptake into liver plasma membrane vesicles is dependent on the presence of a transmembrane pH gradient, and is sensitive to both amiloride and DIDS under the conditions used. These results differ from those obtained in kidney basolateral membranes [12] in a number of respects (i) no amiloride sensitivity was observed in kidney basolateral membranes at the same Na^+ concentration (ii) bicarbonate clearly stimulated Na^+ uptake into kidney basolateral membrane vesicles in the absence of a pH gradient and (iii) valinomycin increased the bicarbonate stimulation of Na^+ uptake in kidney vesicles in the presence of K^+ gradient, but failed to do so in these experiments on liver plasma membrane vesicles. It is possible that different transport systems for Na^+ occur in these two membrane systems.

The results presented here are in part consistent with the existence of an electroneutral Na^+/HCO_3^- symport system in liver basolateral membranes. Electroneutral influx of Na^+ plus bicarbonate would tend to neutralise a preexising gradient (acid inside), and the presence of such a pH gradient would be expected to stimulate Na^+/HCO_3^- cotransport. However, this interpretation of the results is not entirely satisfactory. It is not clear why bicarbonate consistently failed to stimulate Na^+ transport in the presence of a bicarbonate gradient but absence of a pH gradient. Further, Na^+/HCO_3^- symport systems are in general not inhibited by amiloride, and there is no direct evidence in the literature that bicarbonate ions can be transported across the liver plasma membrane. The presence of other bicarbonate-stimulated Na^+ transport systems cannot be ruled out.

The major discepancy between these results on liver plasma membrane vesicles and work on intact hepatocytes concerns the amiloride sensitivity of the bicarbonate effect. 1 mM amiloride did not abolish the effect of bicarbonate on pH recovery in single isolated hepatocytes [8] and did not abolish bicarbonate-stimulated $^{22}Na^+$ uptake in cultured hepatocytes [2]. The reason for this discrepancy is not clear, but it is possible that the amiloride is competitive with Na^+ ions and does not inhibit bicarbonate-stimulated Na^+ entry at the Na^+ concentrations used in whole cell experiments which are considerably higher (5–150 mM) than the concentrations used here (0.2 mM). This possibility could not be adequately tested using this vesicle system, since it was not possible to obtain accurate values for initial rates of Na^+ transport at high Na^+ concentrations. This is due to the low specific activity of Na^+ available at high concentrations, to the very rapid rate of uptake at these concentrations and to the difficulty of distinguishing binding from transport under these conditions. Alternatively, amiloride may not be able to penetrate easily to the active site of the transport system in intact cells whereas this site may be more readily accessible in vesicles.

The present work provides no evidence for electrogenic Na^+/HCO_3^- cotransport in liver basolateral membranes. Valinomycin failed to increase bicarbonate-stimulated Na^+ uptake under any conditions used. It is in principle possible that the vesicle membranes used were permeable to K^+ ions and that movement of K^+ might charge-compensate an electrogenic Na^+/HCO_3^- symport. This is unlikely for two reasons: (i) in preliminary experiments (not shown) choline chloride stimulated Na^+ uptake to the same extent as did an equal concentration of $KHCO_3$ and (ii) others have shown that this vesicle preparation is relatively imper-

meable to K^+. Thus a 100 mM K^+ diffusion gradient did not stimulate Na^+-alanine cotransport in these vesicles in the absence of valinomycin [17]. However, postulation of an electrogenic Na^+/HCO_3^- cotransport system is not necessary to explain the effect of bicarbonate ions on the liver cell membrane potential. The effects of bicarbonate can be explained by any mechanism of bicarbonate-stimulated electroneutral Na^+ entry. An increase in the rate of electroneutral Na^+ entry would lead to activation of the electrogenic Na^+/K^+ ATPase thus causing membrane hyperpolarisation [10]. Effects on cell plasma membrane potential (or on intracellular pH) involving both Na^+ and HCO_3^- ions do not necessarily therefore provide evidence for an electrogenic Na^+/HCO_3^- symport.

The physiological importance of membrane hyperpolarisation by bicarbonate [9,15] is illustrated by the fact that bicarbonate stimulates electrogenic Na^+/alanine cotransport [19], a process important in the regulation of gluconeogenesis [20]. The mechanism by which this hyperpolarisation occurs is still unclear. The present results on bicarbonate-stimulated Na^+ uptake in liver plasma membrane vesicles do not support electrogenic Na^+/HCO_3^- symport. It is more likely that HCO_3^- ions stimulate the electroneutal entry of Na^+ either via Na^+/HCO_3^- symport or by some other mechanism. Membrane hyperpolarisation is then mediated by a consequent increase in electrogenic Na^+/K^+-ATPase activity.

Acknowledgements

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