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Bicarbonate transport by basolateral membrane vesicles from rat jejunum

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Fisiologia. — *Bicarbonate transport by basolateral membrane vesicles from rat jejunum* (*). Nota di ALIDE FAELLI, MARISA TOSCO, MARIA NOVELLA ORSENIGO, GIOVANNI ESPOSITO e VITTORIO CAPRARO, presentata (**) dal Corrisp. V. CAPRARO.

RIASSUNTO. — Usando il Percoll è stato possibile purificare le membrane basolaterali da enterociti di digiuno di ratto. In queste membrane, ottenute sotto forma di vescicole, che alla verifica risultavano osmoticamente attive, è stata studiata la diffusione del bicarbonato per mezzo della tecnica di filtrazione rapida.

In recipienti chiusi ed a pH 8,2 l'accumulo del bicarbonato è stato misurato a 18°C per 30 min. Detto accumulo è veloce, sembra inoltre saturabile, stimolato da controtrasporto, indipendente da potenziale di menbrana e poco sensibile a inibitori dello scambio $Cl—HCO_3$.

Questo lavoro dimostra che è sperimentalmente possibile determinare l'accumulo di bicarbonato marcato in vescicole di membrane basolaterali a valori di temperatura e pH non molto lontani dal normale. Da questi primi dati sembra che una via di permeazione del bicarbonato possa essere la diffusione carrier–mediata.

Several hypotheses have been postulated to explain the well-known positive effect of bicarbonate on salts and fluid transport in many epithelia, but the rationale for this effect is still unknown [6]. An active bicarbonate transport due to a plasma membrane bound bicarbonate-stimulated ATPase could explain the enhanced fluid transport, but this enzyme activity, perhaps not different from alkaline phosphatase [9], seems to have a mitochondrial origin [17] and it is not stimulated, at least in rat jejunum, at pH lower than 7.5 [4].

The jejunal tract of the intestine absorbs bicarbonate from the lumen, especially by means of the electroneutral Na^+/H^+ exchange system [16], the CO_2 diffusion across the brush border and its subsequent hydration in the cell. The luminal secretion of H^+ is due to an ATP-dependent process in rat cortical tubules [10]. A bicarbonate diffusion from the enterocyte towards the serosal fluid has been demonstrated in rat jejunum [2] but its mechanism is unknown; the downhill electrochemical gradient across the basolateral membrane (BLM) could facilitate this process [3]. To gain more insight into the mechanism behind the cell-serosal fluid diffusion of bicarbonate we have separated BLM

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vesicles of jejunal enterocytes and bicarbonate uptake has been investigated in this simplified system.

MATERIALS AND METHODS

BLMs were isolated from rat enterocytes and analyzed by a modification of the Percoll-gradient centrifugation procedure described in [14]. A scheme of the isolation process is presented.

Transport of both [^{14}C]bicarbonate (1 mM) and L—[^{3}H]glucose (1 mM) into BLM vesicles was measured at pH 8.2 and at 18°C in triplicate by a rapid filtration technique. It is known that at neutral pH bicarbonate solutions loose CO_2 unless gassed with the proper CO_2 tension; the phenomenon is accelerated at 37°C and in the presence of carbonic anhydrase. In a previous work [4] we have seen that at alkaline pH values (8.1-8.5) ungassed bicarbonate solutions are stable at 37°C probably because the CO_2 tension of the air is enough to keep constant the bicarbonate concentration. To minimize the continuous decrease of labelled bicarbonate specific activity due to dehydration ($H^{14}CO_3^- \rightarrow {}^{14}CO_2$) and hydration ($CO_2 \rightarrow HCO_3^-$) reactions, uptake experiments were performed at 18°C and pH 8.2 taking care that during the incubation period filled vessels were kept tightly closed. According to Magid and Turbeck [12] it is possible to calculate the pseudo first-order rate coefficients of dehydration ($3.1 \times 10^{-4}sec^{-1}$) and hydration ($2.7 \times 10^{-2}sec^{-1}$) at 18°C and pH 8.2: the half-time reactions are 37 min and 0.42 min respectively. The relatively low rate of the dehydration reaction together with the high rate of the hydration reaction, partly with the same $^{14}CO_2$ (closed vessel), makes it possible to perform experiments of bicarbonate uptake. Only spontaneous and not catalyzed (by carbonic anhydrase) reactions were taken into account because this enzymatic activity seems to be present in a low quantity in rat jejunum [13]. Furthermore, this enzyme is likely to have an almost total cytoplasmic origin since in the kidney, which possesses 10 times more carbonic anhydrase than the jejunum [13], 2-5% of total enzymatic activity is membrane bound and only 1% seems to be bound to BLMs [18]. In any case carbonic anhydrase does not appear to play any role in our experiments because uptake rate of bicarbonate does not differ in the presence of 0.5 mM acetazolamide (see results).

Experiments lasting less than 30 min were performed. Unless otherwise specified, solution 1 (250 mM sorbitol buffered with 10 mM Hepes/Tris pH 8.2) was used as a basic incubation medium. Due to the low specific activity of sodium [^{14}C]bicarbonate (7.8 μCi/mmol), the unlabelled $NaHCO_3$ was omitted from the incubation medium and the activity of the isotope was checked and arranged to have a final concentration of 1 mM. In some experiments the incubation medium also contained L—[1—^{3}H(N)]glucose (10.7 Ci/mmol) and cold L-glucose to a final concentration of 1 mM. One volume of fresh membrane suspension (2-5 mg/ml protein concentration) was rapidly mixed at zero time with 2 or 8 volumes (see single experiments) of incubation medium kept at 18°C.

20 μl of reaction mixture were taken at selected times and applied to the center of wetted cellulose nitrate filters (0.45 μm pore size) under vacuum (~ 700 torr). The uptake was stopped by immediately rinsing the filters with 5 ml cold stop solution containing 125 mM NaCl, 0.5 mM $HgCl_2$ and 10 mM Hepes/Tris buffer pH 8.2. To take into account the decrease of specific activity of labelled bicarbonate (always lower than 10% at the end of 30 min experiments) samples of the reaction mixture were withdrawn at the same selected times and used as standards. Blanks were obtained by filtering and rinsing 20 μl aliquots of incubation media without membrane suspension. The filters were then dissolved in 10 ml scintillation mixture and assayed for radioactivity in a scintillation counter (Tri-Carb Packard, mod 300). All counts were corrected for background and, in dual-label experiments, for spillover. The radioactivity of the filters containing membranes was at least three times higher than that of the blanks. All kinds of experiments presented in this paper were repeated at least three times and qualitatively similar results were obtained.

For normal behaviour, osmotic and disulphonic stilbenes (SITS and DIDS) effects see captions of figs. 1, 2 and 4.

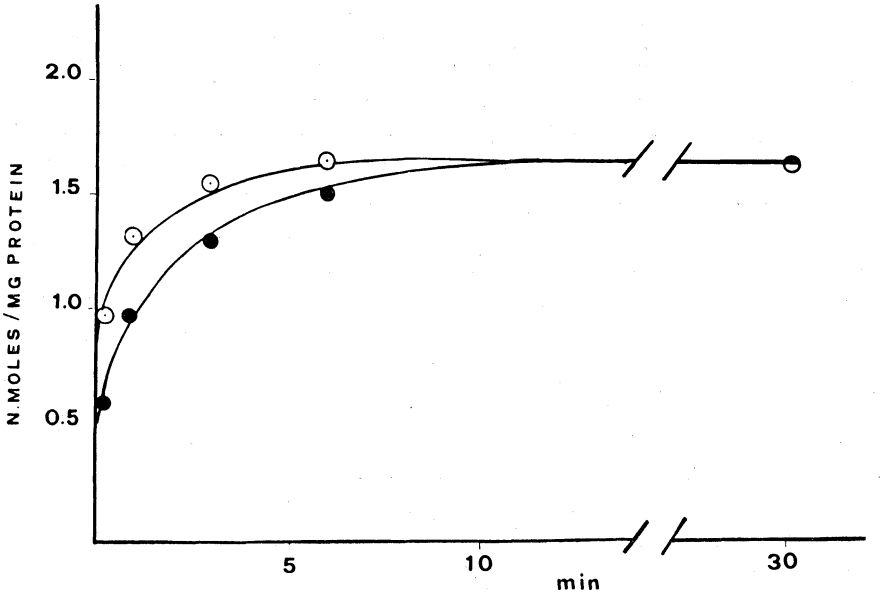

Fig. 1. – Bicarbonate and L-glucose uptake (ordinate, nmoles/mg protein) by BLM vesicles of rat enterocytes (absissa, time of incubation). BLM vesicles were separated in the presence of 250 mM sorbitol, 10 mM Hepes/Tris pH 8.2. 80 μl vesicles (2-5 mg proteins/ml) were mixed with 160 μl of the same solution added with both $NaHCO_3$ (⊙) and L-glucose (●) to a final concentration of 1 mM. Time of incubation = 0.4-1-3-6-30 min.

The rate of bicarbonate uptake was measured in the presence of a membrane potential; in a group of experiments an inside-positive potential was generated by K^+ diffusion in the presence of 0.02% valinomycin: an aliquot of membrane

suspension was equilibrated in the presence of valinomycin and mixed with an incubation medium to give a final composition of 150 mM sorbitol, 50 mM KCl, 10 mM Hepes/Tris pH 8.2, 2% ethanol with (or not = control) 10 mg/ml valinomycin. In another group of experiments an inside-negative potential was created by substituting (or not = control) 160 mM sorbitol with 80 mM KSCN in the incubation medium.

In trans-stimulation experiments, part of BLM vesicles were pre-loaded with 10 mM $NaHCO_3$ for 10 min at room temperature (closed vessels). 10 µl pre-loaded or not (= control) vesicles were mixed with 80 µl of sol. 1 added with 1 mM Na[^{14}C]-bicarbonate at 18°C. Samples were withdrawn at 7 sec, 1 and 15 min. In pre-loaded experiments uptake was calculated taking into account that external bicarbonate was 2.11 mM.

RESULTS AND DISCUSSION

Part of purified BLMs were assayed for enzyme activities; enrichment factors and percent recovery were calculated with respect to the initial homogenate and reported in Table I. Markers of BLMs are 15 fold enriched, on the contrary brush border, endoplasmic reticulum and lysosomes are slightly enriched and mitochondria are reduced. Nine percent of the (Na, K)-ATPase and one percent or less of all other tested enzymatic activities, present in the initial

TABLE I

Enrichment factors and recovery of marker enzymes in the basolateral membrane fraction

Enrichment factors (mean ± S.E.M.) represent the specific activity (m. units/mg protein or sec^{-1}/mg protein for cytochrome *c* oxidase) relative to the activity found in the homogenate. Recovery in % (mean ± S.E.M.) represents the percentage of activity with respect to the initial homogenate.

Marker enzyme	Basolateral membrane (Na, K)-ATPase	Brush Border γ-GT	Mitochondria cytochrome c oxidase	Endoplasmic Reticulum KCN res. NADH ox. red.	Lysosomes acid phosphatase
Enrichment factors .	15.1 ± 1.0	1.5 ± 0.1	0.5 ± 0.2	1.5 ± 0.2	1.2 ± 0.2
Recovery in % . . .	9.4 ± 1.0	0.9 ± 0.1	0.4 ± 0.1	0.8 ± 0.3	0.7 ± 0.2
Number of experiments	13	13	4	4	4

homogenate, were recovered in the BLM fraction. Results of Table I show that in the purified fraction BLMs are 10 times more concentrated than all other membranes. Percoll-gradient centrifugation procedure is a useful tool for separating BLMs; as only a few hours are needed to purify BLMs, it is possible to perform uptake experiments in the same day. Care must be taken since BLMs are concentrated at different levels of the Percoll gradient using Percoll aged or coming from different stocks. Moreover EDTA (0.5 mM) makes possible an easy separation of fluffy layer from dark layer.

Because of the possibility that bicarbonate can be transformed into related forms (CO_2, $CO_3^{=}$), or transported as OH^- (or H^+ in the opposite direction) $+ CO_2$, it is not easy to work with this anion. A direct measurement of bicarbonate transport across BLMs has been performed; as demonstrated in the « Materials and Methods » section, 30 min experiments are possible at pH 8.2 and at 18°C only if vessels are kept tightly closed during the incubation period.

In fig. 1 the uptake of bicarbonate and L-glucose into BLM vesicles is plotted against time. Uptake of bicarbonate seems to be a rapid phenomenon if compared with many other uptake kinetics shown by brush border membrane: in fact more in than 50% equilibration is reached within 15 sec. Bicarbonate uptake seems to be faster than L-glucose uptake, especially if BLM vesicles have been obtained and equilibrated in the presence of 20 mM KCl (sorbitol 210 mM). In this case the equilibration often takes place at higher values. The fast bicarbonate uptake is not surprising since other anions of higher molecular weight such as L-lactate and p-aminohippurate are rapidly transported across BLM vesicles [1, 8, 15]. Furthermore, in the proximal tubule of rat kidney the very rapid depolarization on removal of serosal bicarbonate demonstrates that peritubular cell membrane is highly permeable to bicarbonate [5].

The influence of the medium osmolarity on the equilibrium uptake of both bicarbonate and L-glucose is shown in fig. 2 in which the equilibration values are plotted against the ratio between the inner and outer osmolarities. Cellobiose was used as a solute of low permeability but raffinose (not reported) gives similar results. It seems that for both substances the extrapolation to infinite osmolarity yields practically zero uptake i.e. no binding seems to be involved. Unlike what happened in preliminary experiments the available intravesicular space (1.8 µl/mg protein) is practically the same for bicarbonate and L-glucose

From Henderson-Hasselbalch equation we can calculate that less than 1% HCO_3^- is present as CO_2 at pH 8.2 Due to the low rate of uncatalyzed dehydration reaction (see above) it is difficult to explain the rapid equilibration of bicarbonate uptake (fig. 1) as due to CO_2 uptake. In the presence of carbonic anhydrase the catalyzed dehydration of bicarbonate may be taken into account. To rule out the possibility that our results were due in part to CO_2 uptake, experiments were carried out in the presence of 0.5 mM acetazolamide: the uptake kinetics did not vary from controls performed by using the same BLM vesicles without the inhibitor. These results seem to exclude that we are in the presence of a massive CO_2 uptake.

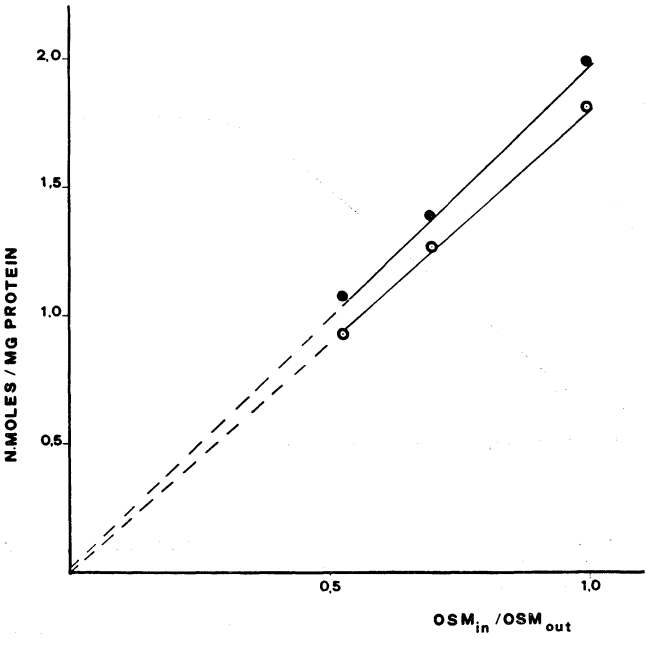

Fig. 2. – Equilibrium uptake of bicarbonate and L-glucose (ordinate, nmoles/mg proteins) as a function of the osmolarity of the incubation medium (abscissa). BLM vesicle were prepared in 100 mM cellobiose, 20 mM KCl, 10 mM Hepes/Tris pH 8.2. Same solution with 100 mM, 200 mM and 300 mM cellobiose plus $NaHCO_3$ (⊙) and L-glucose (●) to a final concentration of 1 mM were used as incubation media. Time of equilibration = 15 min.

The dependence of bicarbonate uptake on its concentration in the incubation medium is presented in fig. 3. The uptake of bicarbonate into BLM vesicles is linear in a wide range of concentrations; only above 50 mM concentration does the uptake become non-linear. These data seem to suggest a carrier-mediated transport of bicarbonate only saturable at high bicarbonate concentration.

The effect of a sodium concentration gradient on the uptake of both bicarbonate and L-glucose was tested by substituting 40 mmoles sorbitol with 20 mmoles NaCl in the incubation medium (Sol. 1). The time courses of both bicarbonate and L-glucose uptake (not reported) are very similar to those presented in fig. 1; the classical « overshoot » phenomenon is absent and the uptake seems to be sodium independent for both substances as happens for many other transport systems located in the BLM such as the one of lactate [1, 15] and p-aminohippurate [8],

The possibility that bicarbonate flux could be electrogenic has been investigated by creating electrical potential differences (inside positive or negative) across the BLM. In both cases the uptake rate (not reported) did not vary from controls for both bicarbonate and L-glucose. These results seem to exclude an involvement of membrane potential in the uptake of bicarbonate. Alternatively a rapid dissipation of electrical gradients, due to a large passive

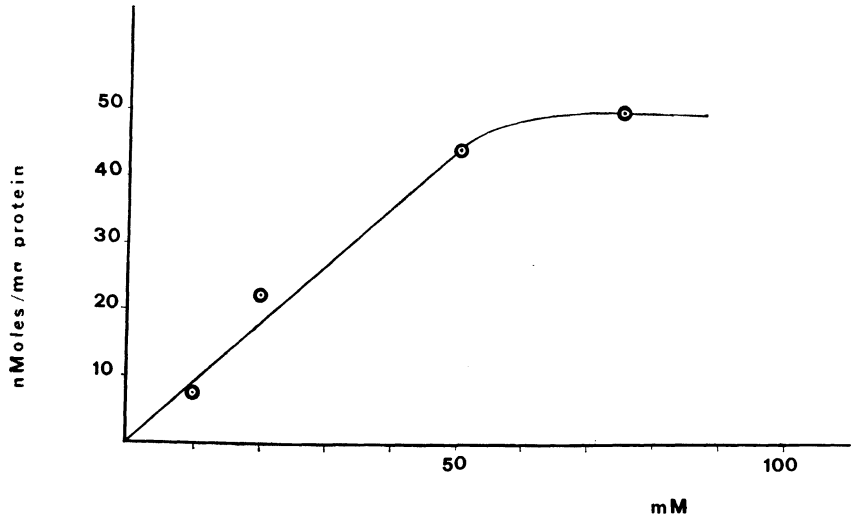

Fig. 3. - Concentration dependence of bicarbonate uptake into BLM vesicles. After 7 sec incubation, uptake of labelled bicarbonate (ordinate, nmoles/mg protein) into BLM vesicles was measured at different medium bicarbonate concentrations (abscissa, mM). In these experiments cold bicarbonate was added to the incubation medium (210 mM sorbitol, 20 mM KCl, 10 mM Hepes/Tris pH 8.2) to reach final concentrations of 10, 25, 50 and 75 mmoles/litre.

permeability of BLM vesicles, could explain the lack of effect on bicarbonate diffusion.

In the membrane of the erythrocyte and of many other cells a transmembrane glycoprotein regulates the carrier-mediated exchange Cl^-—HCO_3^-; this exchange can be inhibited by SITS and DIDS. The effect of these substances has been checked in efflux experiments represented in fig. 4. SITS and its congener DIDS seem to affect slightly HCO_3^- diffusion from BLM vesicles, thus indicating that the exchange process between Cl^- and HCO_3^- is only slightly involved in bicarbonate diffusion across BLM. Absence of DIDS effect on chloride efflux has also been found in BLM vesicles prepared from rat small intestine and renal proximal tubule of dog [7].

The trans-stimulation (or counter-transport) effect has been checked after pre-loading BLM vesicles with 10 mM unlabelled $NaHCO_3$. In Table II the ratio between 7 sec uptakes into pre-loaded and non pre-loaded vesicles is calculated from esperiments performed in the presence of 250 mM sorbitol alone, 210 mM sorbitol + 20 mM choline-Cl or 210 mM sorbitol + 20 mM KCl. The equilibration values after 15 min uptake are the same in the three cited experimental conditions. Under these experiments the trans-stimulation effect is always present and this is further evidence that bicarbonate is transported by a carrier-mediated mechanism. The magnitude of the trans-stimulation effect is the same in the presence of sorbitol alone or sorbitol + choline-Cl; the lack of uptake difference in the presence or in the absence of chloride seems to exclude an exchange between bicarbonate and chloride in the BLM. On

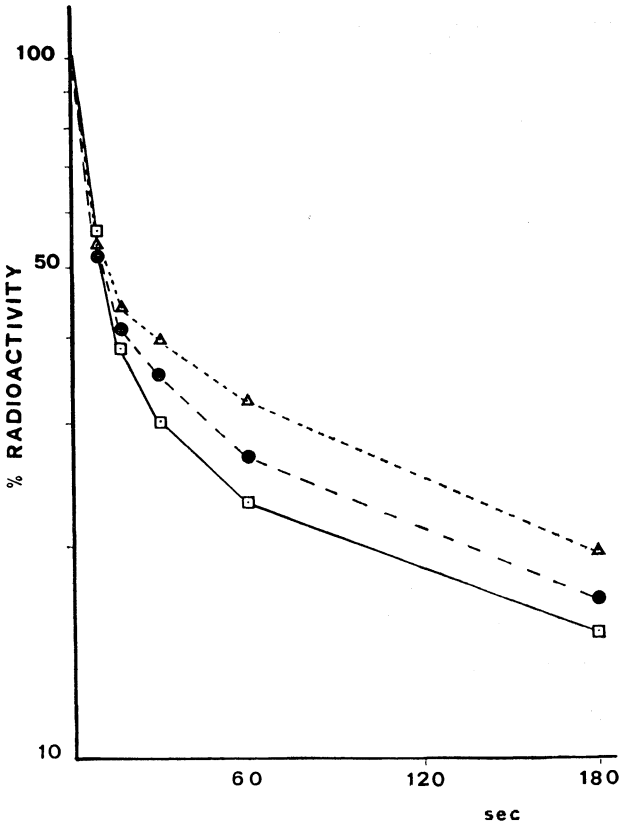

Fig. 4. – Effect of SITS and DIDS on bicarbonate efflux from BLM vesicles. Vesicles were e quilibrated for 10 min at 18°C with 1 mM Na—[^{14}C]-bicarbonate, 210 mM sorbitol, 20 mMKCl, Hepes/KOH buffer pH 8.2 in the absence (□ = control) and the in presence of 1 mM SITS (△) or 100 μM DIDS (●). Samples were 50-fold diluted into a medium of identical composition but not containing bicarbonate and filtered through wetted cellulose nitrate filters. Percent radioactivity remaining in the vesicles (ordinate) was plotted against time (abscissa, 7-, 15-, 30-, 60- and 180 sec).

TABLE II

Trans-stimulation effect on bicarbonate uptake into basolateral membrane vesicles in different experimental conditions

Pre-loaded/not pre-loaded ratio (mean ± S.E.M.) after 7 sec uptake are reported. Four experiments in triplicate for each experimental condition, always in the presence of $NaHCO_3$ 10 mM inside and 2.1 mM outside of vesicles (10 mM Hepes/Tris buffer pH 8.2) were performed. P values with repsect to the first condition (250 mM sorbitol) are also reported.

	250 mM sorbitol	210 mM sorbitol + 20 mM choline Cl	210 mM sorbitol + 20 mM KCl
Pre-loaded/not pre-loaded ratio	1.18 ± 0.12	1.20 ± 0.12	1.55 ± 0.17
P		> .7	> 0.1

Scheme of the isolation of basolateral membrane

Rat jejunum
Jsoosmotic 27 mM Na citrate (pH 7.3) used to free epithelial cells

↓

Dilute to 50 ml with 250 mM sucrose, 0.1 mM phenylmethanesulphonyl fluoride, 0.5 mM EDTA, 10 mM triethanolamine Cl (pH 7.6). Homogenize for 70 sec at ± 11,000 rpm (Sorvall, Omni-Mixer)

↓

Centrifuge for 10 min at 1,500 × g

↓ Discard pellet

↓ Supernatant

Centrifuge for 20 min at 19,400 × g

↓ Discard supernatant

↓ Double pellet

↓ Discard dark layer

↓ Fluffy upper layer

Re-suspend in 35 ml first sol, homogenize (glass-teflon potter), add 10% Percoll, homogenize, centrifuge for 30 min at 48,000 × g

↓

Fractions

Withdraw by a slow rate peristaltic pump the third 8 ml from the bottom (Marker Beads in the contralateral vessel as a density control). Dilute with 250 mM sorbitol, 10 mM Hepes/Tris (pH 8.2). *Sol.* 1. Centrifuge two times for 20 min at 100,000 × g

↓

Vesiculate by sucking through a 27 gauge needle.

the contrary, the presence of potassium increases, even if not statistically, the trans-stimulation effect and this result seems to indicate the possibility of a co-transport with potassium.

Some years ago from electrical and pH measurements, an exchange between K^+ and H^+ was postulated at the serosal pole of jejunum rat intestine [11]. Due to the presence of CO_2 in the intestine, this mechanism is only apparently different from a co-transport of K^+ and HCO_3^-. The existence of an electroneutral co-transport of potassium and bicarbonate is supported by the lack of response to either positive or negative potential difference across the BLM but, unfortunately, we never detected the « overshoot » phenomenon in the presence of potassium.

In conclusion, results of this work demonstrate that it is possible to measure bicarbonate fluxes across BLM vesicles in proper experimental conditions, even if a direct chemical measurement of bicarbonate concentration is not feasible. A facilitated diffusion mechanism appears to be present at the serosal border of the jejunal enterocyte allowing bicarbonate transport from the cell towards the serosal fluid but we cannot exclude other mechanisms such as simple diffusion or gated channels. Chemical rather than electrical gradients seem to drive bicarbonate across BLM in the " in vitro " perfused intestine, because the carrier is likely to diffuse as a neutral molecule.

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