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A MODIFIED PROCEDURE FOR THE RAPID PREPARATION OF EFFICIENTLY TRANSPORTING VESICLES FROM SMALL INTESTINAL BRUSH BORDER MEMBRANES

THEIR USE IN INVESTIGATING SOME PROPERTIES OF D-GLUCOSE AND CHOLINE TRANSPORT SYSTEMS

MARKUS KESSLER, ORESTE ACUTO *, CARLO STORELLI **, HEINI MURER ***, MARTIN MÜLLER and GIORGIO SEMENZA

Laboratorien für Biochemie und für Zellbiologie (Laboratorium für Elektronenmikroskopie I) der Eidgenössischen Technischen Hochschule, Universitätsstr. 16 and 2, CH-8092 Zürich (Switzerland)

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Summary

We have worked out a simplification of the procedure described by Schmitz et al. (Biochim. Biophys. Acta (1973) 323, 98—112) for the preparation of brush border membranes from small intestine. The procedure ultimately adopted is simple, rapid, does not necessarily require scraping and can be started from fresh or frozen material. It can be scaled up easily, allowing a quick production of large amounts of brush border membrane vesicles. These vesicles prove to be excellently suited for transport studies, as suggested by our measurements of D-glucose transport.

Using these vesicles, the mode of choline transport across the brush border membrane was also investigated. Choline transport was found to occur by a saturable component with a K_m of 83 ± 4 μM (at 20°C) and by a non-saturable component. It is independent of the presence of Na^+ and appears to be non-electrogenic.

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* On leave of absence from the Istituto di Fisiologia Generale Facoltà di Scienze, Università di Roma, Città Universitaria, Roma, Italy.

** Present address: Istituto di Fisiologia Generale, Università degli Studi di Bari, Via Amendola 165 A, I-70126 Bari, Italy.

*** Present address: Max-Planck-Institut für Biophysik, Kennedy-allee 70, D-6000 Frankfurt a/M -70, G.F.R.

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Introduction

The first procedure to isolate brush borders from small intestine [1] was followed by a number of methods for preparing highly purified brush border membranes from this tissue. To date, at least the following have been reported: (i) the Tris disruption procedure [2], followed by density gradient centrifugation; (ii) the hypotonic EDTA procedure, originally introduced by Miller and Crane [1] and later modified by others [3,4]. This procedure also has been complemented with density gradient centrifugations [5,6]; (iii) the Ca^{2+} precipitation procedure, originally developed by Schmitz et al. [7], a simplification and characterisation of which is described in the following; (iv) isotonic homogenisation and density gradient centrifugation [8]; (v) isolation of enterocytes, homogenisation and density gradient centrifugation [9] or free flow electrophoresis [10].

The purpose of the present paper is 3-fold: (i) To present a simplification and characterisation of the Ca^{2+} precipitation procedure [7], which has been in use in our laboratory for 3 years and has proved remarkably simple, rapid (approx. 3 h) and efficient. It can be scaled up to considerable volumes, so that it is now advantageous to use this modified procedure as the first step in the purification of components of the small intestinal brush border membrane. Furthermore, the procedure yields vesicles which, due to their excellent stability, have proved of considerable value in membrane transport studies [11—17] * and may well be to-date the preparation of choice of brush border membrane vesicles. (ii) To test this procedure for Na^+-dependent D-glucose transport, both as an example of a "secondary active" transport system and with the goal of presenting new observations about its properties (e.g. a comparison between D-glucose unidirectional flux and D-glucose-stimulated Na^+ unidirectional flux); (iii) To use this procedure in the study of another, quite different, transport system, i.e. that of choline, for which contradictory reports have been published (from work on intact tissue) as to its Na^+ dependence. It will be shown in the following that choline transport in these vesicles is not electrogenic and is not (at least specifically) dependent on the presence of Na^+.

Methods and Materials

(A) Enzymes and other assays. Sucrase activity was determined using the Tris/glucose oxidase-peroxidase reagent [18] or glucose dehydrogenase [19]. Alkaline phosphatase [9], DNA [20], lactate dehydrogenase [21], cytochrome *c* oxidase [22], neutral K^+-stimulated phosphatase [23], β-glucuronidase [24] and protein [25] were assayed by established procedures. Ca^{2+} was measured by atomic absorption.

(B) Transport measurements. The uptake of substrates by brush border membrane vesicles was determined by the Millipore filtration technique as described by Hopfer et al. [4]. Incubations were performed at 20°C in a buffer containing 100 mM mannitol, 1 mM HEPES/Tris and 0.02% NaN_3 at pH 7.5;

* In previous publications in which this procedure was used [11—17] it was quoted as "Storelli et al., in preparation".

further additions or modifications are indicated in the legends to the figures. Aliquots of 20 μl (about 100 μg membrane protein) were taken at selected times, and diluted in 1.5 ml ice-cold stop solution of the following composition: 150 mM NaCl and 1 mM HEPES/Tris, pH 7.5, in D-glucose transport measurements; 200 mM NaCl, 10 mM HEPES/Tris, pH 7.5, and 100 mM mannitol in the choline uptake experiments *. The diluted sample was immediately transferred onto a Sartorius filter (0.6 μm pore size) and rapidly rinsed with 5—8 ml ice-cold stop solution.

Each point is the average of 2—3 determinations with the same preparation. Large differences between different preparations were never observed, the results being highly reproducible. Corrections for the unspecific retention of radioactivity by the filters were done using the following control: membrane vesicles, in the same amount used for uptake experiments were diluted in 1.5 ml of ice-cold stop solution, radioactivity was added in the same quantity as used for the uptake and immediately collected on a filter and treated as usual. In general, the radioactivity of these controls amounted to <10% of that of the test samples.

(C) Electron microscopy. Negative staining: A formvar-coated copper grid was allowed to float on a drop of the vesicles suspension (approx. 1 mg protein/ml) for 5 min. Without removing excess liquid, the grid was then brought in contact with an aqueous solution of uranyl acetate (1%) for 30 s. The grids were then blotted dry and examined in a Philips 301 electron microscope.

Thin sectioning: A pellet was fixed in 0.1 M sodium cacodylate buffer containing 0.15% acrolein and 0.15% glutaraldehyde, pH 7.4, for 2 h at 4°C. After dehydration [26] it was embedded in araldite/Epon. The sections were stained with uranyl acetate and lead citrate [27].

(D) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The membrane samples were solubilized for 3 min at 95°C in a solution of 58 mM Tris · HCl, pH 6.8, 0.71 M mercaptoethanol and 2% SDS. The electrophoresis was run on a discontinuous slab gel system [28] having the following characteristics. Length of the running gel: 10 cm; thickness: 1 mm. Composition of the sampling gel: (3.6 × 2.7), 58 mM Tris · HCl, pH 6.8, 0.1% SDS. Running gel: (8.4 × 2.7), 380 mM Tris · HCl buffer, pH 8.8, 0.1% SDS. (In the brackets, the first figure indicates the total concentration of acrylamide monomers, the second one the percentage of bis-acrylamide to acrylamide (ref. 29).) The current applied was 16 mA/cm^2.

(E) Materials. All reagents were of highest purity available. *N,N'*-Dimethylethanolamine was from FLUKA AG, Switzerland, Phlorizin (from ICN/K and K, Plainview, N.Y.) in water was heated in the presence of charcoal, filtered and recrystallized. Cytochrome *c* was type III from Sigma.

L-[1-^{14}C]Glucose and [*methoxy*-^{3}H]methoxy-inulin were from New England Nuclear, Boston, Mass. D-[1-^{3}H]Glucose, D-[U-^{14}C]glucose, [*Me*-^{14}C]choline chloride and ^{22}NaCl were purchased from Amersham Radiochemical Centre,

* When the ice-cold stop solution contained, in addition to NaCl, D-mannitol, or D- or L-glucose, or D-fructose (at varying or constant osmolarities) the uptake values for either D- or L-glucose were found to be higher by a constant amount, which was independent of the length of the incubation. At the overshoot peak the "extra uptake" of D-glucose amounted to less than 5% of the total.

Ltd., Bucks. When ^{22}Na was used as thiocyanate, $^{22}NaCl$ was diluted with a 1000-fold excess of unlabeled NaSCN and used as such.

For the mechanical vibration during the preparation of the brush border membrane vesicles a Vibro-Mixer, Model El from Chemap AG, Männedorf ZH, Switzerland, was used. Sonication was carried out in a round bath sonicator (Ultrasonic Tank from Laboratory Supplies Co., Hicksville, N.Y., U.S.A.).

Results

Unless stated otherwise, the results reported were obtained using rabbit small intestine.

(A) The procedure to prepare vesicles from small intestinal brush borders (see Fig. 1)

(1) Starting material and homogenisation. Either frozen or fresh small intestine can be used. Essentially the same results were obtained with either material. Routinely, however, it was found practical to prepare rabbit brush border vesicles from frozen material kept at −20°C, whereas brush border membrane vesicles from rat, hamster (or guinea pig, see ref. 15) were usually prepared from fresh material.

(a) If fresh intestine was used as the starting material, the animal was killed by a blow in the neck, the small intestine removed as soon as possible, rinsed with cold saline, everted, rinsed again with cold saline and blotted with hard toilet paper for the complete removal of mucus also. The mucosa was scraped off gently with a glass slide, attention being paid of not taking along fat tissue also.

Mucosal scrapings from 6 to 20 hamsters or rats were suspended in 360 ml of ice-cold 50 mM mannitol plus 2 mM Tris/chloride buffer, pH 7.1, and homogenised in the cold in a mixer of the waring blender type at the maximum speed for 2 min.

(b) Preparation from frozen starting material *. The processing of rabbit small intestines will be described as the example. The small intestines from approx. 100 rabbits (approx. 5 kg of fresh tissue) were obtained fresh from a rabbit slaughterhouse, in which the animals were killed by a gun shot in the head. The small intestines were quickly perfused with ice-cold physiological saline, everted and washed again with ice-cold saline. After removal of the excess fluid, the tissue was frozen on solid CO_2. It was then kept at −20°C until shortly before use. For the daily preparation of brush border vesicles, an aliquot of approx. 20 g of frozen small intestine was cut into pieces of approx. 1 g each. The 20 g of small intestine pieces were then immersed into 60 ml of 300 mM mannitol and 12 mM Tris/chloride buffer, pH 7.1, in a 250 ml beaker. After the thawing was complete, the resulting "goulash" was subjected to vibration (100 Hz) by the vibromixer fitted with a 2 cm diameter vibrating perforated plate for 1.5 min at maximum speed (see Materials). This treatment

* For unexplained reasons, the vesicles prepared from frozen rat small intestine showed lesser and more variable solute uptake and osmotic space.

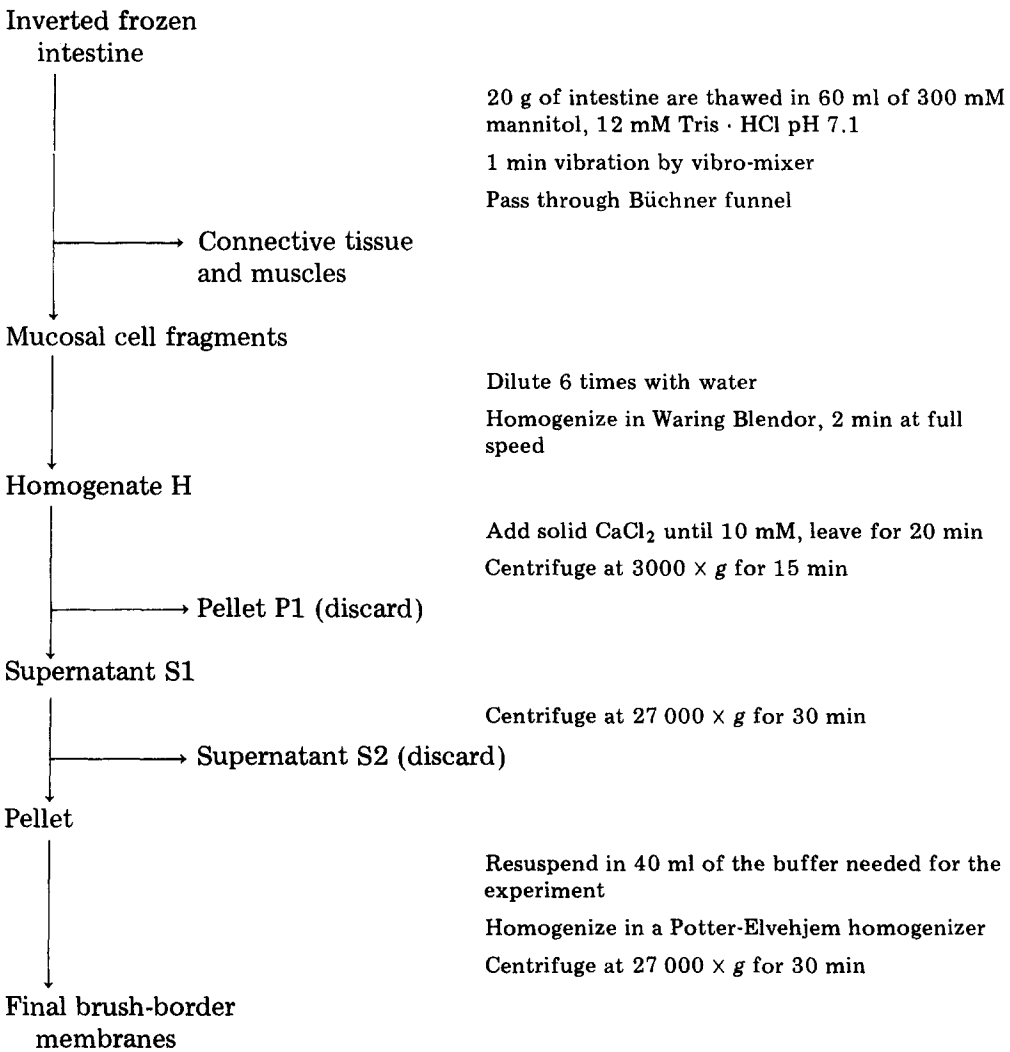

Fig. 1. Flow sheet of the procedure for the preparation of small intestinal brush border membrane vesicles.

released the mucosal cells and other soft components of the tissue into the medium almost completely. The suspension was passed through a Büchner funnel with 1-mm holes; the gross pieces of connective tissue were retained by the funnel. The filtrate was diluted 1 : 6 with ice-cold distilled water and homogenised in a mixer of the waring blender type at the maximum speed for 2 min.

(2) Calcium precipitation and centrifugations. Solid $CaCl_2$ was added to the homogenate, final concentration 10 mM. After standing in the cold for 15 min, the suspension was spun down at 3000 × *g* for 15 min. The pellet which con-

tained nuclei, mitochondria, most of the basolateral membranes and other cell debris was discarded.

The supernatant was then spun down at 27000 × *g* for 30 min. This time the supernatant was discarded. The pellet was then resuspended in 40 ml of 50 mM mannitol plus 10 mM HEPES/Tris buffer, pH 7.5 (or 10 mM Tris/chloride buffer, pH 7.1) and spun down once more at 27000 × *g* for 30 min. This pellet contained almost pure vesicles from brush border membranes which could be used as such for transport and other studies. Occasionally further purification steps such as a $MgSO_4$ wash [4,11] or density gradient centrifugation were used. The additional purification thereby achieved (e.g. the contamination by K^+-activated phosphatase could be reduced by a factor 2—3) was, however, small, and therefore these steps were not usually carried out.

(3) Characterisation of the vesicles throughout the preparation. Table I reports the enrichment or the disappearance of various marker enzymes as well as nucleic acids. It is apparent that the preparation finally obtained is essentially free of mitochondrial, cytosolic, nuclear and microsomic contaminants. The specific activity of a typical brush border enzyme, namely sucrase (which was determined after removal of Tris, an inhibitor of this enzyme), is equal to that in other preparations of vesicles from intestinal brush borders (e.g. refs. 3, 4, 7 and 9).

As expected, brush border vesicles prepared by the present procedure contained rather large amount of bound calcium which could be only partially removed by repeated washing in the absence of chelating agents (typically, 120—130 nmol Ca^{2+} per mg protein). Treatment of these vesicles with EDTA at 37°C for 30 min does decrease the calcium content, but does not remove it completely.

Fig. 3 reports SDS-polyacrylamide gel electrophoresis of vesicles prepared from rabbit small intestine. The pattern can be compared with the one reported by Schmitz et al. [7] for their own preparations from hamster small intestine. The question of how many of the vesicles are closed cannot be answered at the moment. However, there are indications that those vesicles which are closed, are oriented right side out: hydrolysis of sucrose was not increased, when the brush border vesicles were lysed with Triton X-100 (sucrase activity was assayed at room temperature during 30 s under conditions identical to those under which D-glucose transport was measured). Thus, all sucrase molecules had access to the substrate in the intact vesicles. In so far as sucrase active sites are totally confined to the outer surface of the membrane [16,17], this indicates that essentially all vesicles are right side out. This is compatible with the morphology of the vesicles as determined with the electron microscope (Fig. 2).

Tightness and stability of the vesicles: All low molecular weight compounds tested so far (L-glucose, D-mannitol, D-galactitol) equilibrated across the membrane within 1—2 h. In contrast, methoxyinulin uptake did not exceed 25% of the value determined for D-glucose, not even after an incubation as long as 19 h (Fig. 7). Therefore these vesicle membranes seem to be almost completely impermeable for this high molecular weight compound. Attempts to bring medium components into normal-sized vesicles by sonication, or by ways other than transport or "diffusion" across the membrane were unsuccessfull so far.

TABLE I

RECOVERIES OF ENZYMES AND DNA DURING THE PREPARATION OF BRUSH BORDER VESICLES

The amounts of protein varied from one preparation to another, probably due to the heterogeneity of the different rabbits in respect to breed, feeding etc. (see first line). Therefore, recoveries were first calculated as percents for each experiment and then averaged. The table shows the means and the S.E. Number of experiments is given in parentheses. The enrichment factor is the ratio of the specific activities in the final brush border membranes and the homogenate.

	Protein (7)	Sucrase (4)	Alkaline phosphotase (2)	Cytochrome *c* oxidase (3)	K^+-stimulated phosphatase (4)	Lactate dehydrogenase (3)	β-Glucuronidase (2)	DNA (5)
Amount found in homogenates of different preparations	450—1260 mg	27—72 units	30—32 units	400—600 units	2.6—6.4 units	1100—1600 units	230—245 units	
Homogenate (H)	100%	100%	100%	100%	100%	100%	100%	100%
Pellet 1 (P1)	50.6 ± 2.4	22.1 ± 3.7	19 ± 1	92.3 ± 1.8	66.7 ± 4.1	2.4 ± 0.4	19 ± 2	100 ± 10.2
Supernatant 2 (S2)	43.0 ± 2.2	5.1 ± 1.7	8 ± 6	0.5	9	95.5 ± 3.5	75 ± 3	0
Final brush border membranes	2.3 ± 0.2	47.5 ± 5.4	30	0.14 ± 0.08	8.5 ± 1.3	0.6 ± 0.1	0.4	0.3 ± 0.1
Specific activity in the final brush border membranes		1.22 ± 0.06 units/mg	0.94 ± 0.24 units/mg	9.0 ± 2.9 munits/mg	18.8 ± 4.6 munits/mg	0.32 ± 0.03 units/mg	0.06 ± 0.02 units/mg	
Enrichment factor		22 X	15 X	0.07 X	2.2 X	0.19 X	0.2 X	0.14 X

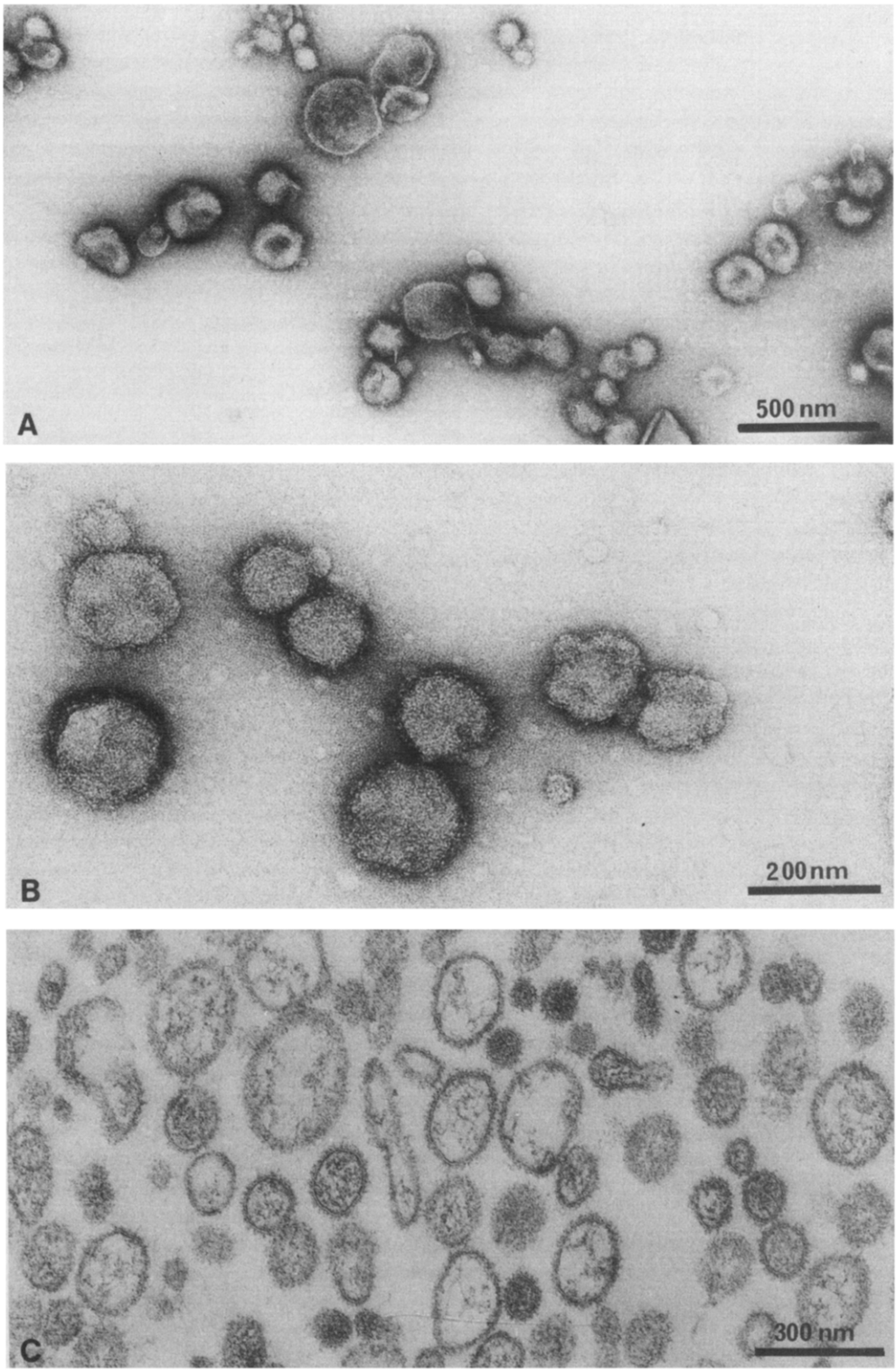

Fig. 2. Electron microscopic appearance of the final vesicle preparation, at negative staining (A, B) or thin sectioning (C). Note the rather uniform size and shape, the fuzzy contours of the outer surface at negative staining (B) and the small amounts of dark material of varying density inside many vesicles (C).

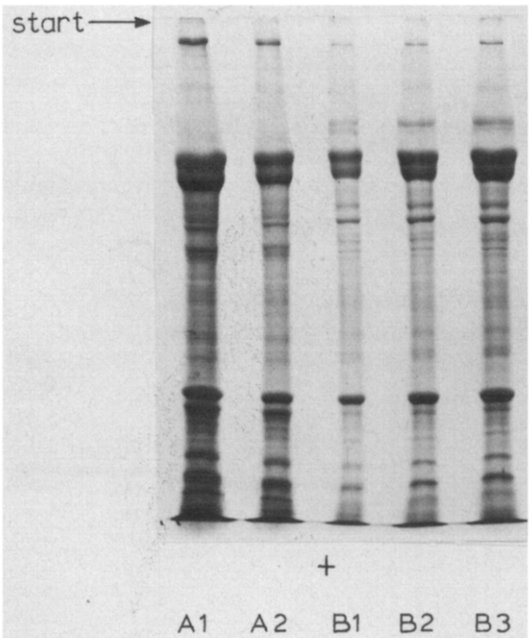

Fig. 3. SDS-polyacrylamide gel of brush border membrane vesicles from rabbit small intestine. (A) Vesicles prepared according to the present procedure (A1, 100 μg protein; A2, 50 μg protein). (B) Vesicles prepared by the EDTA procedure [4] (B1, 35 μg protein; B2, 50 μg protein; B3, 70 μg protein). For the conditions of the electrophoresis, see Methods and Materials.

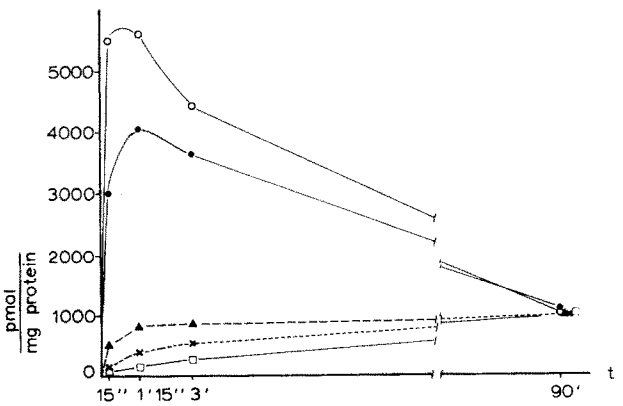

Fig. 4. Uptake of D- and L-glucose by brush border membrane vesicles under different conditions. D-Glucose uptake (1 mM): X———X, in the presence of an initial gradient of choline chloride (100 mM outside, 0 inside) and in the absence of Na^+; ▲———▲, before adding the substrates, the vesicles had been equilibrated with 100 mM NaCl; ●———●, in the presence of an initial gradient of NaCl (100 mM outside, 0 inside); ○———○, in the presence of an initial gradient of NaSCN (100 mM outside, 0 inside). L-Glucose uptake (□———□) was identical under all the conditions specified above. The buffer was in all cases 10 mM Tris/HEPES, pH 7.5, 100 mM D-mannitol [4].

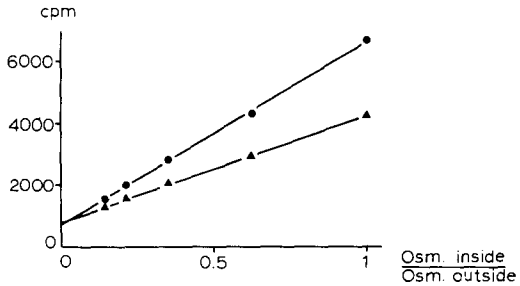

Fig. 5. D-Glucose (●———●) and L-glucose (▲———▲) associated with brush border membrane vesicles after 60 min incubation, as a function of the osmolarity of the incubation medium. Buffer at both sides of the membrane: 1 mM Tris/HEPES, pH 7.5. The vesicles contained, at the start of the incubation, 100 mM cellobiose. The incubation was started by adding together D-glucose, L-glucose (1 mM each), 10 mM NaSCN plus varying concentrations of cellobiose. The uptake values at 10 and 30 min, respectively, could also be extrapolated to the same intercept on the *y*-axis.

(B) The use of these brush border vesicles for studying D-glucose transport

Vesicles prepared by the EDTA procedure have proved of considerable value in providing strong evidence [30] for Crane's [31,32] cotransport mechanism for solute transport. The calcium vesicles prepared by the procedure reported here are essentially free of K^+-stimulated phosphatase (presumably identical with $(Na^+ + K^+)$-ATPase), cytosolic, or mitochondrial contaminants (Table I). When incubated in the absence of metabolites they can thus be used in transport studies under controlled conditions. A typical transport experiment is reported in Fig. 4. Some conclusions are immediately apparent from this experiment, i.e. (i) In the presence of sodium, but in the absence of any sodium gradient at time zero (▲), the uptake of glucose is accelerated, as compared to the experiment in the absence of sodium; however, no accumulation, not even a transitory one, of D-glucose can be observed. (ii) In the presence of a gradient of NaCl across the membrane (out > in, ●) a transient accumulation of D-glucose against its own concentration gradient is observed. (iii) When thiocyanate (○) is substituted for chloride an even higher transient accumulation of D-glucose against its concentration gradient is observed. Thiocyanate, which is more lipophilic than chloride, probably produces a larger membrane potential than chloride (negative inside the vesicles), which increases the driving force for the flow of Na^+ and D-glucose into the vesicles. (iv) Under all conditions tested, L-glucose equilibrated slowly and never produced any detectable overshoot.

These observations therefore confirm those of Hopfer's group [30] and are in excellent agreement with the cotransport mechanism proposed by Crane [31,32] for this system. Fig. 6 compares the uptake of D-glucose with that of Na^+. As expected, the initial uptake of sodium is higher in the presence of D-glucose than in its absence. The difference between the two uptake values of Na^+ vanishes at the same time as the overshoot of D-glucose fades away. (Note that the initial gradient of NaSCN was only 10 mM (out) to 0 (in)). An unequivocal interpretation of the ratio between initial D-glucose flux and initial glucose-dependent Na^+ uptake is hampered by the large binding of Na^+ to these vesicles *. With this limitation in mind and if the initial D-glucose-dependent

* Note that the equilibrium value of Na^+ is about twice as high as that of D-glucose. Na^+ binding to the vesicles depends on the ionic strength: for example it is strongly reduced by 100 mM and totally suppressed by 450 mM choline chloride.

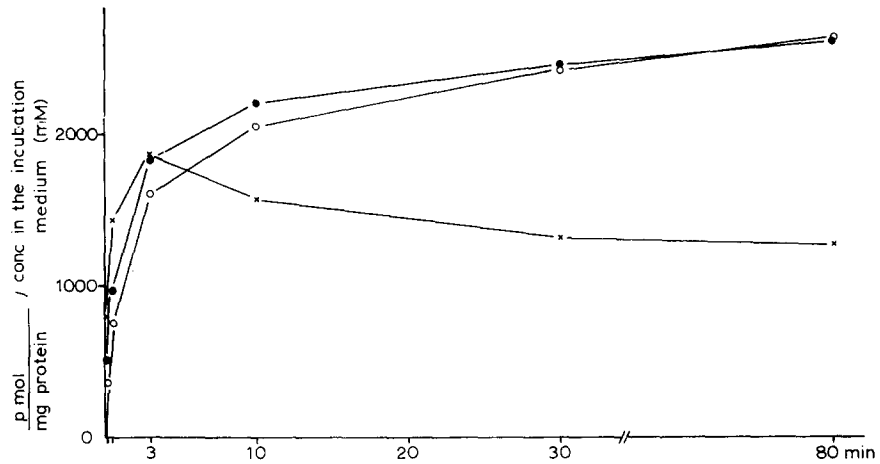

Fig. 6. Uptake of ^{22}Na (as NaSCN, 10 mM) in the presence (•), or in the absence of D-glucose (D-[^{3}H]-glucose, 1 mM) (o). The uptake of D-[^{3}H]glucose (X) in the same experiment as in • is also shown. In addition to NaSCN (± D-glucose), the incubation media contained: 100 mM D-mannitol. 10 mM Tris/HEPES, pH 7.5, and 0.02% NaN_3. Room temperature. The values given are means of 3—7 determinations, the standard deviations being smaller than the size of the symbols used in the figure.

Na^+ uptake does indeed correspond to the initial D-glucose-dependent Na^+ flux, the observed behaviour of the two phenomena corresponds to what is predicted by the cotransport mechanism, as indicated by Crane [31,32].

The uptake of D-glucose in the experiment of Fig. 4 corresponds mainly to a transport in an osmotic space. This is borne out by the data of Fig. 5, in which the amount of substrate associated with the vesicles at equilibrium * is plotted vs. the reciprocal of the medium osmolarity, which is a measure of the size of the osmotic space. If substrate binding is negligibly small in comparison with the uptake into an osmotic space, the amount of substrate associated with the vesicles at equilibrium should extrapolate to zero at infinite medium osmolarity. In the experiment of Fig. 5 this is not so, which is indicative of a small binding of both D- and L-glucose to the vesicles. Similar observations were made for other transporting vesicles [4,33].

(C) Studies on transmembrane transport of choline

Differentiation of transport versus binding. Fig. 8 shows the time courses of uptake of choline, D-glucose and L-glucose in isolated brush border membranes in the presence of a NaSCN gradient. Clearly, the initial uptake velocity of choline is larger than that of L-glucose but smaller than that of D-glucose.

The D-glucose and the L-glucose associated with the brush border membranes at long incubation times are present in an osmotically active space, their binding to the membranes being small. In contrast, the equilibrium value of choline, under the same conditions, amounts to some three times that of either D- or

* In the experiment of Fig. 5 the amount of D-glucose taken up did not increase any further beyond 60 min incubation, whereas that of L-glucose did keep increasing, albeit very little.

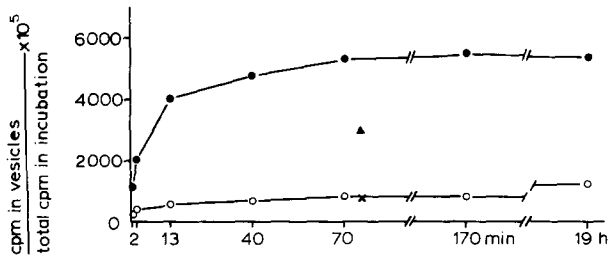

Fig. 7. Uptake of D-glucose (●———●) and of methoxy-inulin (○———○) by brush border membrane vesicles, and effect of sonication. The incubation medium did not contain any Na^+ and was composed of 1 mM D-[^{14}C]glucose, 19 mg % [^{3}H]methoxy-inulin, 100 mM D-mannitol and 5 mM Tris/HEPES, pH 7.5. Room temperature. After 70 min incubation an aliquot of the suspension was submitted to sonication at 4°C for 1 min; after 5 more min, the sonicated aliquot was processed as usual and the amounts of D-glucose (▲) and methoxy-inulin (X) remaining in the filter was measured.

L-glucose (Fig. 8). This was the first indication that at least some choline was bound to the brush border membranes, rather than being present in an osmotically active space.

Whereas the nature of the anion present influenced the uptake of D-glucose (thiocyanate producing a higher overshoot), this was not so for choline uptake, the values in the presence of Cl^- or SCN^- being identical (data not shown).

Direct evidence for choline binding is provided by Fig. 9 which presents the equilibrium values of choline uptake as a function of the size of the osmotically active vesicular space. A part of the choline uptake depends on the size of the osmotic space, but under the conditions of the experiments of Figs. 8 and 9, much choline was bound to the membranes, since extrapolation to infinite osmolarity (i.e. at zero osmotic space) still yielded a very sizeable value for choline associated with these membrane vesicles (the intercept is highly signifi-

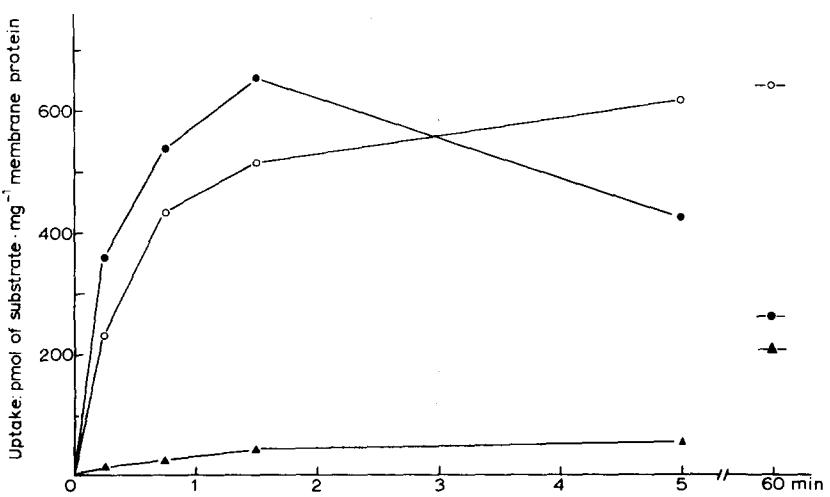

Fig. 8. Uptake of choline, D-glucose and L-glucose by brush border membrane vesicles in the presence of a NaSCN gradient at zero time. To the standard incubation medium (see Methods and Materials) following solutes were added to start the incubation: 100 mM NaSCN and 0.25 mM [^{14}C]choline (○); or 100 mM NaSCN, 0.25 mM D-[^{3}H]glucose (●) and 0.25 mM L-[^{14}C]glucose (▲).

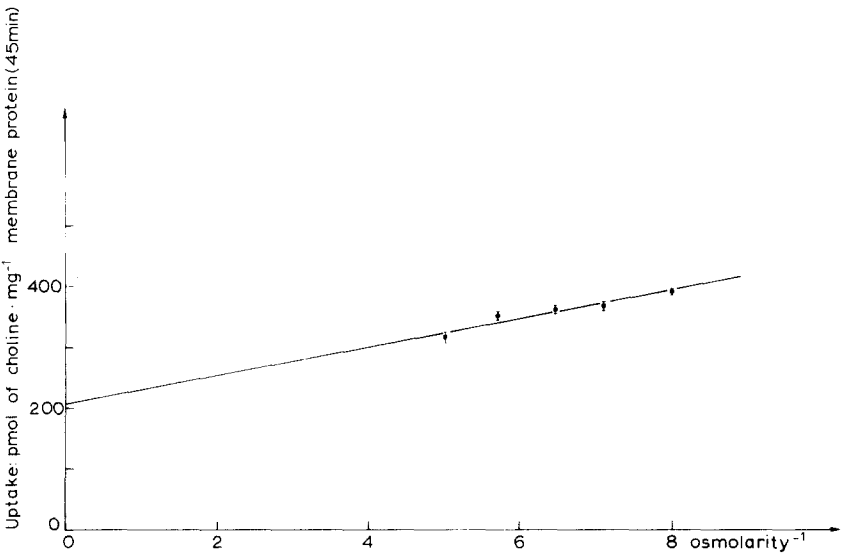

Fig. 9. Effect of medium osmolarity on choline uptake. Brush border membrane vesicles were prepared with cellobiose (100 mM) instead of D-mannitol, and incubated for 45 min in the standard medium (see Methods and Materials), to which 0.25 mM [^{14}C]choline and varying amounts of cellobiose were added. The reciprocal values of the osmolarities obtained are given on the abscissa.

cantly different from 0, $P << 0.001$). We concluded that the membrane vesicle preparation both binds and transports choline under the conditions used.

Since choline is a cation we considered the possibility that its binding could be mainly electrostatic in nature and thus be reduced in the presence of high ionic strength. We thus preincubated the membrane vesicle preparation for 1 or 2 h at room temperature in the presence of NaCl or KCl at concentrations equal to or higher than 0.1 M. This treatment did not detectably affect the integrity of the vesicles as shown by essentially normal uptake of D-glucose and not preincubated, and 0.1 M NaCl was added at the beginning of the incubation along with radioactive choline (as given in Fig. 8 for NaSCN), the high ionic strength did not prevent choline binding to the brush border membranes. We concluded that choline binding either was faster than that of Na^+, or took place primarily at the inner face of the vesicles (but this would require choline mem-L-glucose. However, the equilibrium concentration of choline taken up by vesicles which were exposed to this high ionic strength both during preincubation and during incubation, was drastically reduced (Table II). If the vesicles were not preincubated, and 0.1 M NaCl was added at the beginning of the incubation along with radioactive choline (as given in Fig. 8 for NaSCN), the high ionic strength did not prevent choline binding to the brush border membranes. We concluded that choline binding either was faster than that of Na^+, or took place primarily at the inner face of the vesicles (but this would require choline membrane transport being faster than Na^+ transport). Conversely, Na^+ binding to brush border membrane vesicles was inhibited by choline (see footnote on page 145).

Fig. 10 shows that the velocity of uptake of choline by brush border membrane vesicles which have been preincubated in 0.1 M NaCl and incubated in

TABLE II

DIFFERENCES BETWEEN THE EQUILIBRIUM VALUES OF CHOLINE UPTAKE AND L-GLUCOSE UPTAKE, AT VARIOUS CONCENTRATIONS OF NaCl

The vesicles were preincubated in the standard incubation medium (see Methods and Materials) containing various concentrations of NaCl, for 2 h at room temperature. Incubation was carried out under the same conditions, after addition of either [^{14}C]choline or L-[^{14}C]glucose (0.25 mM each), for 1 h. Preliminary experiments had shown that after 1 h incubation no further increase in the uptake (i.e. transport plus binding) of either choline or L-glucose took place.

NaCl (M)	Equilibrium uptake values (pmol ± S.E./ mg membrane protein)		Differences between the equilibrium uptake values (pmol/mg membrane protein)
	Choline	L-glucose	
0.4	102.3 ± 6.1	64.3 ± 2.3 *	38
0.2	182 ± 1.5	132 ± 8.5	50
0.1	188.3 ± 5.6	124 ± 5.7	64.3
0	670 ± 9.6	137 ± 20	533

* The reason for the reduced osmotic space at this high concentration of NaCl was not investigated. It can be attributed to either shrinkage or leakiness, or both.

the same medium is several times faster than the uptake of L-glucose. That this uptake of choline is indeed due to a transmembrane transport and not due to binding is corroborated by the finding, that *N,N'*-dimethylethanolamine, a choline analogue known to act as competitive inhibitor in chicken small intestine [34], strongly reduces choline uptake during the first minutes without affecting the equilibrium value of choline (Fig. 10).

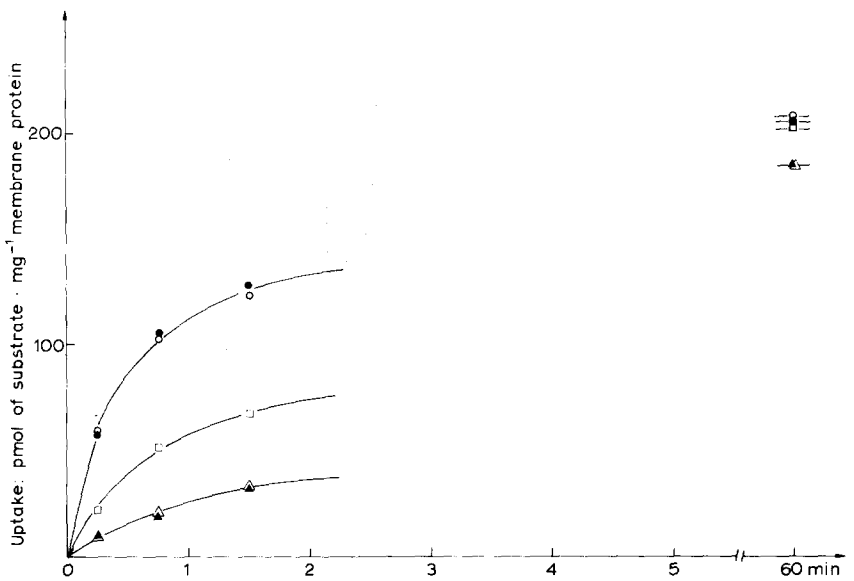

Fig. 10. Uptake of choline and L-glucose by brush border membrane vesicles after preincubation in the presence of NaCl or KCl. Inhibition of choline uptake by *N,N'*-dimethylethanolamine. The vesicles were preincubated for 2 h at room temperature in the standard incubation medium (see Methods and Materials) containing either 0.1 M NaCl (●, ▲) or 0.1 M KCl (○, △). Incubation (all other components being present at the same concentrations as in the preincubation) was started with the addition of 0.25 mM [^{14}C]-choline (●, ○), or 0.25 mM L-glucose (▲, △), or 0.25 mM [^{14}C]choline plus 10 mM *N,N'*-dimethylethanolamine · HCl (□).

In so far as the uptake of L-glucose can be taken as a measure of diffusion or of a system of facilitated diffusion with low capacity and high K_m, the much higher uptake of choline under these conditions (i.e. under conditions in which binding was effectively reduced) shows that choline transmembrane transport is not merely due to diffusion. The same figure also shows that the transport of choline is the same in sodium or potassium as the major cation.

The equilibrium values of choline uptake, however, were still somewhat higher than those of D- or L-glucose (e.g. Fig. 10 and Table II). This indicated a very limited but still present binding of choline even in the presence of 0.1 M NaCl or KCl.

Saturable and non-saturable components of transmembrane choline transport in brush border membrane vesicles. Up to 10 s, choline uptake (after preincubation in either 0.1 M KCl or NaCl) deviated little from linearity, (data not shown). The uptake values at 10 s incubation were thus taken as a close approximation of the unidirectional flux. Choline influx was measured as a function of choline concentration (Fig. 11) over the range of 0.011—1.1 mM. The measurements were carried out with vesicles pre-incubated in 0.1 M NaCl which inhibits, nearly completely, the binding. As reported for the intact tissue [34—36], saturation was partially masked by a large non-saturable component. The K_m value for the saturable part of the influx could be obtained by correcting graphically for the linear component. A Lineweaver-Burk plot of the corrected data gave a K_m of 82 μM (S.E. ± 4) (correlation coefficient 0.999) with a V of 33.3 ± 2.2 μmol · 10 s^{-1} · mg^{-1} membrane protein (averages of six determinations). The K_m value obtained agrees well with those of literature for intact tissue (hamster, 160 μM, [35]; chicken, 110 μM [34]). For guinea pig the K_m reported is 600 μM [36]). We conclude that choline is transported by

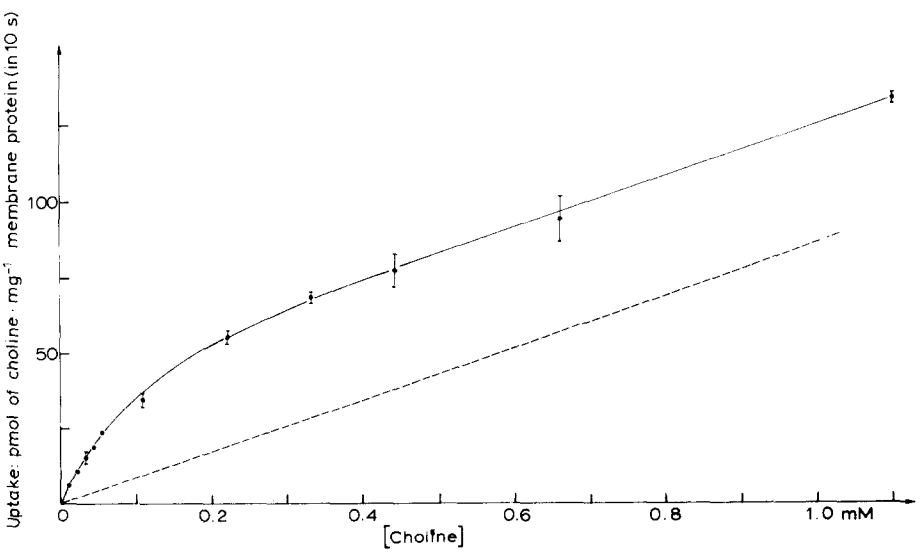

Fig. 11. Choline influx as a function of choline concentration. The vesicles were preincubated in 0.1 M NaCl, as in the experiment of Fig. 10. [^{14}C]Choline was added to the medium at the start of the incubation, which lasted 10 s. The dotted line gives the graphical estimation of the non-saturable component of choline entry. The bars indicate the S.D.

one saturable and one non-saturable agency. Due to the slight binding still present it is not possible to precisely assess the relative contributions of each mechanism.

Is choline transport across brush border membrane vesicles Na^+ dependent and/or electrogenic? Fig. 10 shows the uptake of choline by vesicles equilibrated in 100 mM NaCl or 100 mM KCl. The substitution of K^+ for Na^+ as the major cation obviously did not affect choline uptake.

If choline movement across these vesicles is not balanced by the movement of an other ion (anion or cation), the transport process is electrogenic and should, therefore, be influenced by a potential difference across the membrane. In the experiment of Fig. 12, the vesicles were first preequilibrated in 100 mM KCl to keep choline binding low; then, choline uptake was measured in the presence of a transmembrane potential, produced by a gradient of NaSCN across the membrane (a potential is built up across the membrane, negative inside, which should accelerate choline influx into the vesicles, if it were electrogenic (see above)). Under these conditions, choline is not accumulated inside the vesicles, as it is found for D-glucose. A comparison of Fig. 12 with curves in Fig. 10 shows, that no acceleration of uptake at all is produced by applying a transmembrane potential.

Similar conditions were produced by preincubating the vesicles in high potassium plus valinomycin and diluting the outer potassium concentration at the start of the incubation in the presence of choline (data not shown). Under these conditions also, no accelerated influx of choline was produced by the negative membrane potential. If anything, the valinomycin-accelerated K^+ efflux seemed to decrease the velocity of choline uptake.

We conclude therefore, that the uptake of choline under these conditions is an electroneutral process.

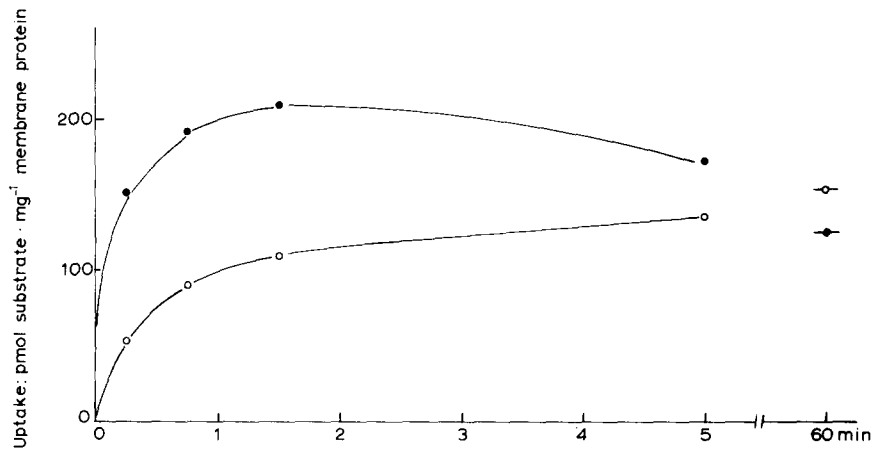

Fig. 12. Effect of a NaSCN gradient on choline and D-glucose uptake by vesicles preincubated in 0.1 M KCl. The incubation was started by addition of 100 mM KCl plus 100 mM NaSCN plus 0.25 mM [^{14}C]-choline (○); or 100 mM KCl plus 100 mM NaSCN plus 0.25 mM D-[^{3}H]glucose (●).

Discussion

(A) Comments on the procedure

One first major advantage of the procedure described here is that it can be applied to frozen material and no scraping is needed. Both points are of importance when one is dealing with large amounts of tissues. In fact, scraping large amounts of small intestine is tedious and time-consuming. Long times also mean reduced yields.

The precipitation with Ca^{2+} for the purification of intestinal brush borders was introduced by Schmitz et al. [7], following the observation [37,38] that it induces precipitation of "microsomes". Booth and Kenny [39] have developed a procedure to prepare microvilli from rabbit kidney by Mg^{2+} precipitation. In the small intestine, if Ca^{2+} is replaced by Mg^{2+}, there is no significant improvement in the product, the final material being perhaps somewhat less pure. However, Ca^{2+} activates small intestinal, brush border-bound phospholipase A [40]. Hydrolysis of some of the phospholipids in the brush border membranes does occur during their preparation in the presence of Ca^{2+} (Hauser, H. et al., unpublished). Whereas this apparently does not affect the stability or the functional properties of the final brush border membrane vesicles, Mg^{2+} should be preferred to Ca^{2+} if partial modification of the membrane lipids is to be avoided. The mechanism whereby either of these divalent cations facilitates the separation of brush border membranes from other cell membranes is poorly understood [7,37—39].

Density gradients, which are an essential step in other preparation procedures (e.g. refs. 5—9) were avoided here, with the goal of allowing the quick processing of larger amounts of material. Nevertheless, the purity of the final preparation was at least comparable with that of membranes obtained by other methods. In addition to being susceptible to scaling up, the procedure as modified here is remarkably simple, reproducible, and rapid; it requires, when starting from frozen material, approx. 3 h for completion.

(B) Suitability for transport studies (in particular, of electrogenic, Na^+-dependent systems)

The transport ability of the "calcium vesicles" studied here should be compared with that of the "EDTA vesicles" (for a preliminary report, see ref. 14). (i) Calcium vesicles constantly produced a longer lasting and higher overshoot peak of D-glucose (3—8 times as high as the equilibrium value with 1 mM D-glucose and 100 mM NaSCN initial outer concentration). Even NaSCN or NaCl gradients (out > in) as low as 10 mM to 0 produced detectable overshoots (Fig. 6 and ref. 13). (ii) These vesicles allowed the unequivocal demonstration of transient accumulation of L-alanine [11], which had proved impossible in EDTA vesicles. (iii) These vesicles are considerably more stable than EDTA vesicles: e.g. after 90 min at 37°C (ref. 13) or after 2—3 days at +4°C calcium vesicles generally showed only a minor decrease in their transport ability. The reason for the higher transport efficiency of the calcium vesicles was not investigated but it is likely to be related to the stabilising effect of divalent cations on phospholipid bilayers (e.g. refs. 41 and 42). This stabilisation may result in a slower collapse of the sodium electrochemical gradient across the membrane.

Be as it may, the favorable transport properties, as well as the ease and rapidity of preparation and of its scaling up are likely to make the calcium vesicles characterized here the preparation of choice in transport studies across small intestinal brush border membranes.

(C) Characterisation of choline transport

Using the preparation described in this paper, we have also investigated some aspects of choline transport in isolated brush border membranes from rabbit intestine. Recent literature has considered the absorption of choline in vivo in the rat [43] and in the dog [44], and in vitro in surviving small intestinal tissue from the rat and hamster [35], from the chicken [34] and from the guinea pig [36].

Confirming, essentially, what has been reported in the intact tissue for the chicken, rat and guinea pig [34—36], we found that the transport of choline across the brush border membrane takes place via two transport agencies, one saturable (for which the K_m was determined) and one unsaturable.

Conflicting results concerning the sodium dependence of choline transport have been reported from the in vitro studies, using whole tissue preparations: choline uptake was found to be dependent on Na^+ in the chicken intestine [34], but not or only minimally depending on this cation in the guinea pig, rat and hamster [35,36]. Surprisingly, ouabain is reported not to inhibit choline uptake in the chicken [34], but to do so in the hamster [35]. This is, of course, opposite to what one would have predicted on the basis of the Na^+ dependence, or Na^+ independence, in the chick or in the hamster, respectively. Our own experiments clearly show, that choline uptake in rabbit brush border vesicles does not specifically require sodium, since it was not affected when K^+ was substituted for Na^+.

The experiments reported here failed to produce any evidence of choline transport being electrogenic. The charge movement associated with choline transport into these vesicles must be compensated by the exit of another cation (perhaps indifferently Na^+, K^+, or H^+), or the entry of an anion, through the same transport agency as choline. Attempts to identify the additional ion movements associated with choline transport are in progress.

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