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OSMOTICALLY REACTIVE PLASMA MEMBRANE VESICLES PREPARED FROM RABBIT KIDNEY TUBULES BY MILD HYPOTONIC LYSIS

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SUMMARY

Plasma membrane vesicles were obtained by hypotonic lysis in an ice-cold medium containing EDTA and $MgCl_2$. The vesicles were isolated by differential centrifugation. Compared to a total kidney homogenate, a 10–12-fold enrichment of trehalase and alkaline phosphatase (marker enzymes for renal brush border), and a 6-fold enrichment of (Na^+-K^+)-stimulated ATPase, (a marker enzyme for the basal and lateral plasma membrane of the tubule cell), was achieved. Contamination by other cell organelles was very low. The plasma membrane vesicles enclosed small amounts of the cytoplasmic enzymes lactate dehydrogenase and malate dehydrogenase, which exhibited full activity only after their release into the medium by sonication.

Electron micrographs of this preparation showed microvilli with drumstick-like expansions, but also spherical vesicles. By measuring the distribution of radioactively labelled compounds of different molecular weight in a packed sediment of the plasma membranes under isotonic conditions, an intravesicular volume of 82% or 9 μl/mg of membrane protein was estimated. The intravesicular volume decreased when the osmolality of the medium was augmented by raffinose. The scattering of light by the vesicular suspension could be used to monitor rapid volume changes. By this method, the following sequence of flux rates was established: glycerol > erythritol > adonitol > mannitol. The fluxes of LiCl, NaCl, and KCl were almost identical, but faster than those of adonitol and mannitol. The data indicate, that a large fraction of the plasma membrane isolated in this preparation have formed vesicles, and also that they have retained, as far as investigated, the permeability characteristics of the plasma membrane.

INTRODUCTION

In the proximal tubule of the mammalian kidney, solutes and fluid are re-absorbed via a trans- and a paracellular route [1]. Solutes re-absorbed by the transcellular way must pass through two membranes: the luminal brush border and the basal part of the tubular cell membrane. Both sites of the cell membrane contribute to the overall transtubular transport of a given substance. Since both cell sites differ

quite markedly in their content of enzymes [2], a difference in their permeability for various solutes would not be surprising and has already been established for electrolytes by electrical measurements [3]. The movement of electrolytes and non-electrolytes through the tubular wall has been studied in vivo by micropuncture techniques [4–6]. Therefore, we are quite well informed about the overall movement of solutes across the tubule cell. But in order to separate the transcellular transfer into its two steps without interference by the paracellular shuntpath, an in vitro approach using the respective cell membranes seemed to be promising. A number of investigators have managed to form vesicles from plasma membranes from other sources and were able to study the fluxes of various solutes through the vesicular membrane independently of cell metabolism and under defined conditions [7, 8]. A quantitative estimate of the amount of vesicles in these preparations and a measurement of an intravesicular concentration of a substance under investigation have not yet been performed.

In two previous studies, a high percentage of vesicles in a crude plasma membrane fraction was obtained when the kidney tubule segments were first disintegrated by mild hypotonic lysis [9, 10]. Using plasma membrane marker enzymes, as well as the quantitative measurement of intravesicular space for the isolation, these vesicles have been further purified in this study. The permeability of these vesicles for various polyalcohols and electrolytes has also been studied. A second paper will describe the separation of vesicles derived from the tubular brush border from those derived from the basal and lateral tubular cell membrane.

METHODS

Preparation of the plasma vesicles

In the following, a method for the preparation of plasma membrane vesicles will be described, which in our hands has been proved to be optimal, and is depicted in Fig. 1. The tubule segments were prepared from rabbit renal cortex according to

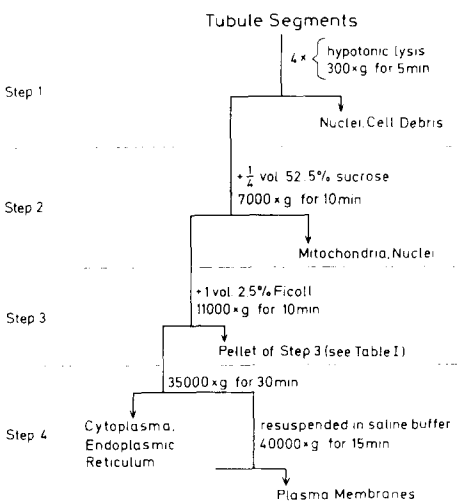

Fig. 1. Scheme for the preparation of plasma membrane vesicles. See Methods for details.

Burg and Orloff [11], and as also previously described [9, 10]. After the tubule segments had been washed free of collagenase, filtered through a cheese cloth, and centrifuged at $1000 \times g$ for 2 min, they were weighed (usually 4–6 g) and ice-cold hypotonic lysing solution was added (1 mM $K_2MgEDTA$, 1 mM $MgCl_2$, 1 mM *N*-acetyl-D-glucosamine, pH 7.4, 8 ml for 1 g of wet tubule segments).

Step 1. The suspension was stirred in an Erlenmeyer flask by a magnetic stirring bar for 5 min at 4 °C. Centrifugation in a refrigerated low speed centrifuge at $300 \times g$ for 5 min, separated the suspension into two layers. The upper layer was removed with a pipette and its volume was replaced by new hypotonic lysing solution. The above procedure, stirring for 5 min, centrifugation, removing the upper layer, and replacing it, was repeated three additional times.

Step 2. The upper layers were combined and the total volume was determined. 0.25 vol. of a sucrose solution was added (52.5% (w/v) in 20 mM Tris–HCl, pH 7.4), so that the concentration of sucrose in the suspension was brought to 10.5%. Centrifugation at $7000 \times g$ for 10 min followed.

Step 3. The resulting supernatant was warmed to 30 °C for 5 min and then cooled to 4 °C in an ice-ethanol bath. The suspension was slowly diluted with one volume of a Ficoll solution (2.5% in 50 mM glycerol, 10 mM Tris–HCl, 2 mM $MgCl_2$ 1 mM *N*-acetyl-D-glucosamine, final pH 7.6). Centrifugation at $11\,000 \times g$ for 10 min followed.

Step 4. After centrifugation the supernatant was taken off and recentrifuged at $35\,000 \times g$ for 30 min. The sediment of this centrifugation was resuspended in an isotonic saline buffer (106 mM NaCl, 25 mM $NaHCO_3$, 5.5 mM KCl, 1 mM $CaCl_2$, 1.37 mM $MgSO_4$, 1.37 mM KH_2PO_4, 13.7 mM Tris–HCl, pH 7.5) and centrifuged at $40\,000 \times g$ for 15 min.

The pellet represented the final plasma membrane preparation and was used for further studies.

Determination of intravesicular volume

For space measurements, membranes containing 0.5–1.5 mg of protein were resuspended in 1 ml of the isotonic saline buffer described above in preweighed plastic centrifuge tubes, warmed to 37 °C for 3 min and incubated with [^{3}H]glycerol and poly[^{14}C]ethyleneglycol (mol. wt 4000) both 0.5 mM for 2 min. After incubation, the membranes were centrifuged for 30 min at $50\,000 \times g$ in a refrigerated Sorvall RC2B centrifuge. The supernatant was removed and aliquots were mixed in a 1:1 ratio with a solution containing 10% trichloroacetic acid, 5 mM glycerol, and saturated with polyethyleneglycol (mol. wt 4000). The last drops of incubation medium were removed from the tubes by a plastic tubing connected to a vacuum pump. After reweighing the centrifuge tubes containing the membrane pellet (5–15 mg wet weight) the bottom of the tube with the membrane pellet was cut off with a razor blade and placed into 0.3 ml of a solution containing 5% trichloroacetic acid, 2.5 mM glycerol and half saturated with polyethyleneglycol (mol. wt 4000) and resuspended by brief sonication. The supernatant incubation medium and the resuspended membrane pellet, both having 5% trichloroacetic acid were centrifuged in an Eppendorf microcentrifuge for 5 min. Aliquots of the supernatants were counted in a Nuclear Chicago liquid scintillation counter. For calculations, the specific gravity was assumed to be 1,

so that radioactivity per mg wet weighed membrane could be compared to radioactivity per microlitre of incubation medium.

Enzymes

Trehalase was assayed according to Sacktor [12] at 25 °C. The glucose liberated was determined with a Boehringer test kit for measuring blood sugar by the glucose oxidase method. Since the sucrose, which was used during the differential centrifugation, contained measurable quantities of glucose, a control containing no substrate was always subtracted. Alkaline phosphatase was also assayed with a Boehringer test kit for this enzyme. 4-Nitrophenol was used as a standard. Mg^{2+}-dependent and (Na^+–K^+)-stimulated ATPase were determined according to Jørgensen [13]. The enzymes were activated for at least 2 h at 4 °C in a 50 mM imidazole, 3 mM EDTA buffer, pH 7.4, containing 0.05% deoxycholate. The inorganic phosphate liberated was determined according to Lindberg and Ernster [14]. Glucose-6-phosphatase was assayed according to Hübscher and West [15] in a modification described by Berger and Sacktor [16]. In this assay, the inorganic phosphate liberated was determined according to Bartlett [17].

Succinate dehydrogenase was assayed according to Veeger et al. [18]. The concentration of $K_3F_3(CN)_6$, which was used as electron acceptor, was changed to 1 mM, and succinate was added as the last component. The decrease in absorbance was recorded at 436 nm.

Lactate dehydrogenase and malate dehydrogenase were assayed according to Rodbell [19], DNA according to Giles and Myers [20], and protein according to Lowry et al. [21].

RESULTS

Hypotonic lysis and isolation

Step 1. The hypotonic lysing solution was similar to that used in the previous studies [9, 10], except that Mg^{2+} was added, which we assumed increased the yield of closed plasma membrane vesicles. Replacing K_2MgEDTA in the lysing solution by 1 mM $CaCl_2$ reduced the yield of plasma membranes in the final preparation to about half. After the first hypotonic lysis and subsequent centrifugation, about 20% of the total protein was found in the upper layer, which contained the smaller plasma membrane fragments and vesicles. By repeating the lysis three additional times, the yield could be increased by an additional 20%. The ratio of DNA to protein was three to four times higher in the lower layer than in the upper layers. The specific activity of the brush border enzyme trehalase in the upper layers did not differ from the lower layer (data not shown).

Step 2. A broad range of sucrose concentrations and of "*g*-forces" were tried in the next centrifugation step. A sucrose concentration 0.3 M and $7000 \times g$ for 10 min proved to remove the mitochondria optimally (data not shown).

Step 3. Warming the supernatant of the last centrifugation to 30 °C for 5 min gave a slightly better yield and a higher specific activity of trehalase in the subsequent separation. During this heat treatment, fibrous material became visible in the suspension, which could be sedimented in the next centrifugation. In the following, a centrifugation under reduced osmotic pressure was tried, which should change the density

TABLE I

CHARACTERIZATION OF TWO MEMBRANE FRACTIONS OBTAINED BY STEP 3 OF DIFFERENTIAL CENTRIFUGATION

The supernatant of Step 2 was diluted with one volume of a 2.5% Ficoll solution and centrifuged at 11 000 × *g* for 10 min, to give the pellet and the supernatant of Step 3. Centrifugation of the supernatant of Step 3 at 35 000 × *g* for 30 min, resulted in the pellet of Step 4. All enzyme activities are expressed as nmoles product formed/min per mg of protein. For details see Methods. For comparison see also the specific activities of the same enzymes in the starting material as depicted in Table II.

	Yield (mg protein)	Succinate dehydrogenase	Mg^{2+}-ATPase	(Na^+-K^+)-ATPase	Trehalase	Intravesicular volume	
						As percent of wet weight	As μl/mg protein
Pellet of Step 3	3.80	107	647	1561	340	68.1	8.02
Pellet of Step 4	4.47	22	383	2345	774	80.1	9.10

TABLE II

SPECIFIC ACTIVITIES OF MARKER ENZYMES IN THE TOTAL KIDNEY, THE KIDNEY CORTEX, THE ISOLATED TUBULE SEGMENTS AND IN THE PLASMA MEMBRANES

Enzyme activities are expressed as nmoles product formed/min per mg of protein. In the fifth column, the enrichment of the enzymes in the plasma membrane fraction ± S.D. of at least five observations is shown.

	Total kidney	Kidney cortex	Tubule segments	Plasma membranes	Enrichment of plasma membrane enzyme as compared to	
					Tubule segments	Total kidney
Trehalase	71.2	81.2	142.8	862	6.03 ± 1.23	12.1
Alkaline phosphatase	123	149	244	1306	5.35 ± 1.61	10.6
Mg^{2+}-ATPase	327	245	794	443	0.558 ± 0.186	1.35
$(Na^{+}-K^{+})$-ATPase	392	374	698	2254	3.23 ± 1.70	5.75
Succinate dehydrogenase			206	22.2	0.108 ± 0.083	
Glucose-6-phosphatase			80.5	67.3	0.84 ± 0.34	
Lactate dehydrogenase			181	7.5	0.041 ± 0.015	
Lactate dehydrogenase after sonication			212	46.4	0.219 ± 0.080	
Malate dehydrogenase			497	34.3	0.069 ± 0.070	
Malate dehydrogenase after sonication			658	110.3	0.186 ± 0.193	
DNA as μg/mg protein			23.5	n.d.*	< 0.02	

* In plasma membranes containing 2 mg of protein, no DNA was detectable. The assay was sensitive for even 0.5 μg DNA.

and the size of all osmotically reactive particles. But the dilution of the centrifugation medium to lower osmolality is limited, since a 0.2 M sucrose solution no longer gives an optimal separation. Therefore, Ficoll's reagent was added, which kept the density and the viscosity high, while the osmolality was diluted to almost half. This centrifugation removed 80% of the remaining mitochondria into the sediment as shown by the measurement of succinate dehydrogenase (Table I, 2nd column). It also proved to sediment some non-vesicular materials as demonstrated in the sixth and seventh column of Table I, where the intravesicular volume of the resulting two fractions were compared .A separation of brush-border vesicles from vesicles containing (Na^+-K^+)-stimulated ATPase, which are derived from the basal and lateral site of the tubular cell membrane [2], was not achieved (Table I).

Step 4. Centrifugation of the superantant of the last step at 35 000 $\times g$ for 30 min and a subsequent wash with an isotonic saline buffer, separated the plasma membranes from cytoplasmic proteins and from most of the endoplasmic reticulum (data not shown).

Purity of the plasma membranes

In Table II, the specific activities of various enzymes, used as markers for different cell fractions in the course of separation were compared. There are only minor differences between total kidney homogenate and kidney cortex with respect to the plasma membrane enzymes trehalase, alkaline phosphatase, and (Na^+-K^+)-stimulated ATPase. However, the tubule segments had an almost twice as high specific activity of these enzymes. The four steps of differential centrifugation which followed, increased the specific activities of the brush-border enzymes trehalase and alkaline phosphatase by an additional factor of 5–6. The (Na^+-K^+)-stimulated ATPase was also three times more concentrated, which suggests that a part of the final plasma membrane preparation is derived from the basal and lateral sites of the tubule cell. All other markers tested in this study, were diluted in the final preparation as compared to the starting material. DNA, used as a marker for nuclei, succinate dehydrogenase, a marker for mitochondria, and lactate dehydrogenase, a marker for the cytoplasm, (these three cell fractions contribute 80–90% of all cellular protein) were diluted 10-fold or more as compared to the tubule segments. However, when the plasma membrane preparation was subjected to 30 min of sonication at 170 mA, the activity of lactate dehydrogenase increased 6-fold, an activity, which is still one-fifth of the activity in the tubule segments. Malate dehydrogenase, which is also mainly a cytoplasmic enzyme, behaved similarly. This finding will be discussed later in detail. Mg^{2+}-dependent ATPase, which is an enzyme of the plasma membrane and also of the mitochondria, was reduced to 55%, glucose-6-phosphatase, a marker for the endoplasmic reticulum, was reduced to 84% of its specific activity in the tubule segments.

Electron microscopy

Electron micrographs of this preparation showed microvilli of different length with drumstick-like expansions, but also large spherical vesicles (0.5–1.0 μm), whose origin could not be identified by electron microscopy (Fig. 2). These spherical vesicles could be either derived from the expansions of the microvilli or from the basal and lateral tubular cell membrane.

TABLE III

DISTRIBUTION OF [^{3}H]GLYCEROL AND POLY[^{14}C]ETHYLENEGLYCOL (MOL. WT 4000) IN A PACKED SEDIMENT OF THE PLASMA MEMBRANE VESICLES AND THE ESTIMATION OF THEIR INTRAVESICULAR VOLUME AFTER VARIOUS TIMES OF INCUBATION AT 37 °C

Plasma membrane vesicles were incubated in an isotonic saline buffer at 37 °C for 3, 30, and 60 min. Then [^{3}H]glycerol and poly[^{14}C]ethyleneglycol (mol. wt 4000) were added for 2 min, and the spaces were estimated as described under Methods. Each experiment is the mean of three observations.

Incubation time (min)	Distribution as percent of the wet weight for		Intravesicular volume	
	[^{3}H]Glycerol	Poly[^{14}C]ethyleneglycol, mol. wt 4000	As percent of the wet weight	As μl per mg protein
3	95.3	15.2	80.1	9.05
30	98.1	14.5	83.6	8.80
60	98.3	15.4	82.9	9.31

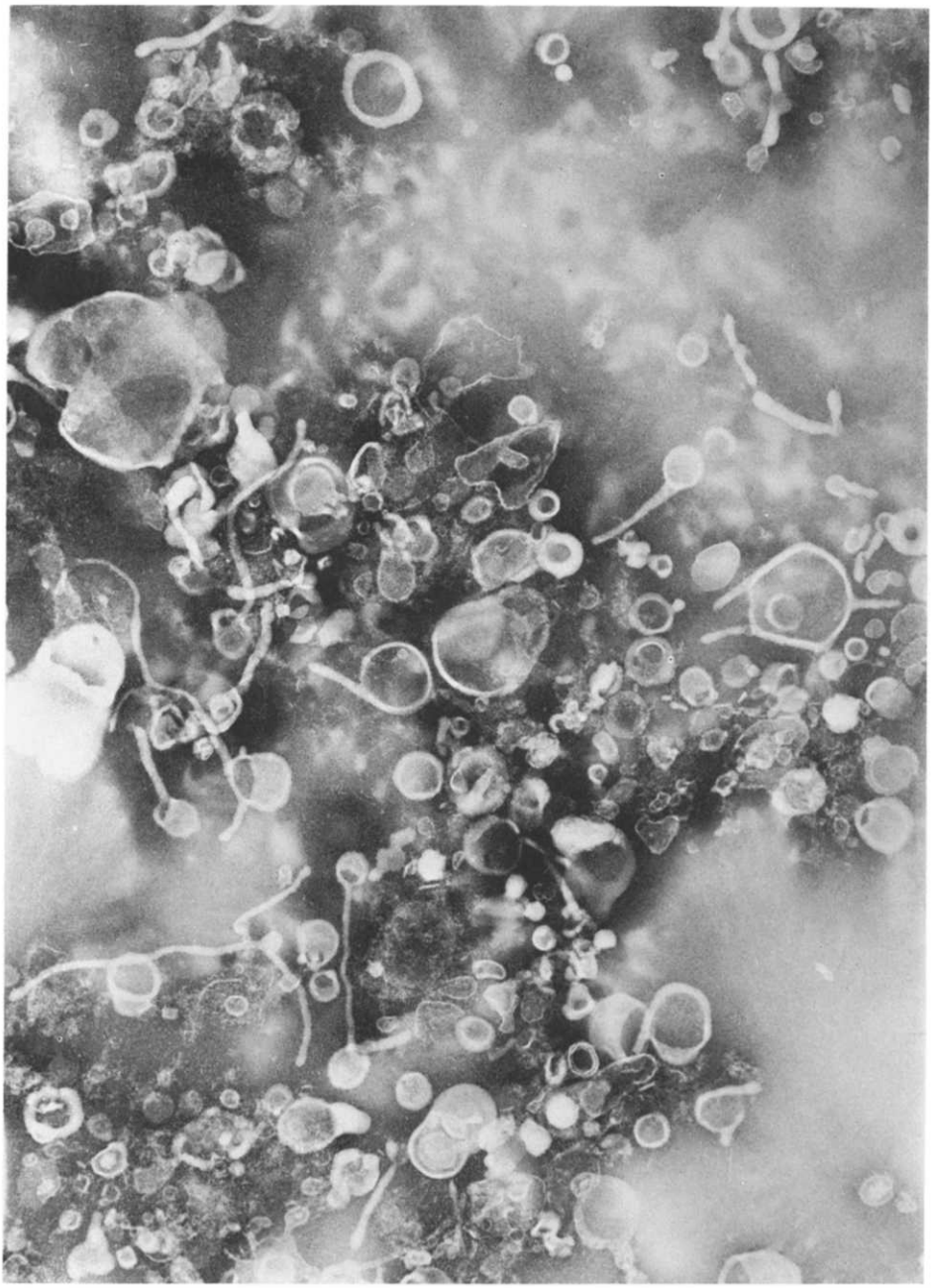

Fig. 2. Electron micrograph of the plasma membranes after negative staining in 2.5 % ammonium molybdate, pH 5.

Space measurements

Since it is very difficult to obtain a quantitative aspect of the amount of closed vesicles within a membrane preparation by electron microscopy, the distribution of radioactively labelled compounds of different molecular weight was measured in the membrane pellet. The penetration of poly[^{14}C]ethyleneglycol (mol. wt 4000) was only 15%, where as [^{3}H]glycerol penetrated 98% of the pellet volume. Increasing the time of incubation with poly [^{14}C]ethyleneglycol and [^{3}H]glycerol from 2 to 30 min, did not change the distribution of these compounds (data not shown). The "polyethyleneglycol space" subtracted from the "glycerol space" gave us the intravesicular space. Because the extra- and intravesicular spaces, when expressed as percent of total volume, change markedly depending upon the preceding centrifugation which packs the vesicles more or less tightly, it is more usefull to express the intravesicular volume in relation to the protein content. Table III shows the distribution of [^{3}H]glycerol and poly[^{14}C]ethyleneglycol (mol. wt 4000) in the plasma membrane sediment, and demonstrates that the plasma membrane vesicles are stable at 37 °C for at least 1 h.

Osmotic behaviour

Raffinose, which does no penetrate the cell membrane in vivo, decreased the intravesicular volume when added to the incubation medium. In Fig. 3, the intravesicular volume is plotted against the reciprocal ideal osmolality of a medium containing raffinose. The decrease of the intravesicular volume is approximately linear. The line

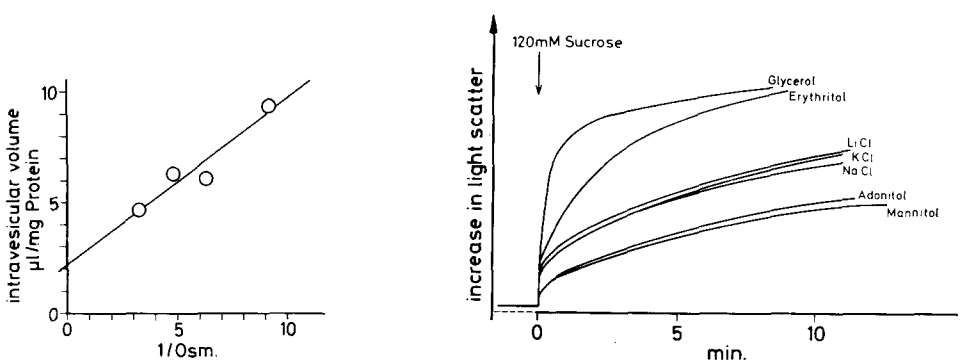

Fig. 3. Dependence of the intravesicular volume upon the ideal osmolality of a medium containing increasing concentrations of raffinose. Plasma membranes were resuspended in a saline buffer (see Methods), in which 100 mM NaCl was omitted or replaced by 50, 100 or 200 mM raffinose. After 40 min of incubation at 37 °C, [^{3}H]glycerol and [^{14}C]polyethyleneglycol (mol. wt 4000) were added and the spaces were estimated as described in Methods.

Fig. 4. Osmotic shrinking of plasma membrane vesicles in solutions of various polyalcohols and salts. Plasma membranes containing 0.6 mg of protein were resuspended in 1 ml of 60 mM Tris–HCl, 6 mM $MgCl_2$, 6 mM $CaCl_2$, pH 7.4. 0.1 ml of this suspension was added to 1.1 ml of solutions containing 120 mM of different polyalcohols or 60 mM of different chloride salts, and incubated at 37 °C for 30 min. Then, the scattering of light with a wavelength of 450 nm was recorded in a fluorimeter (thermostated at 37 °C), in which the secondary light-filter was omitted. Air was blown onto the surface of the suspension through a plastic tube and provided constant stirring. Injection of 0.1 ml of 53 % sucrose solution induced the shrinking of the vesicles and the efflux of their low molecular content. This figure shows one typical example out of four experiments.

extended to infinite osmolality does not reach zero. Osmotically reactive particles should also give a change in the scattering of light, when subjected to an osmotic change in the surrounding medium. Fig. 4 shows the increase of light scattering, when sucrose was added to the vesicular suspension, which had previously been equilibrated with various low molecular substances. The increase in light scattering was used as a parameter for the efflux of solutes from the vesicles. The speed of efflux of various polyalcohols decreased in the following order: glycerol>adonitol>mannitol. The effluxes of LiCl, NaCl and KCl were almost identical, but faster than those of adonitol and mannitol.

Latency of lactate dehydrogenase and malate dehydrogenase

Sonication of the plasma membrane preparation for 30 min at 170 mA increased the activity of lactate dehydrogenase and malate dehydrogenase 6- and 3-fold, respectively (Table II), whereas the activities of trehalase and alkaline phosphatase were identical before and after sonication (data not shown). This striking effect of sonication upon the activity of lactate dehydrogenase and malate dehydrogenase led us to suspect that these enzymes might be trapped inside the vesicles. As shown in Fig. 5, lactate dehydrogenase could be washed out of the vesicles after sonication. As a control, the solubilization of alkaline phosphatase by the sonication was also observed.

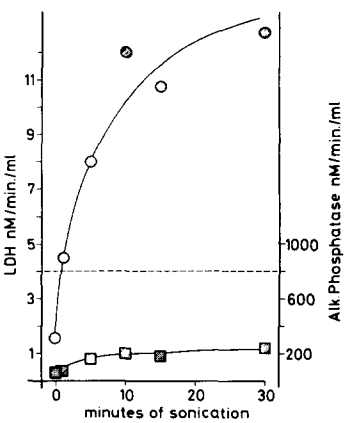

Fig. 5. Activities of lactate dehydrogenase and of alkaline phosphatase in the supernatant of the plasma membrane preparation after various times of sonication. Plasma membranes were resuspended in a saline buffer (see Methods), in which 100 mM NaCl was omitted. After they had been sonicated for various times at 170 mA at 4 °C, they were centrifuged at 50 000 × *g* for 2 h in a refrigerated centrifuge. The supernatant was assayed for the two enzymes. The circles represents the activity of lactate dehydrogenase, the squares the activity of alkaline phosphatase. The broken line represents the activity of both enzymes in the membrane suspension before sonication. Note, that even without sonication (at time zero), small fractions of the enzymes were recovered in the supernatant due to the handling of the membrane suspension during centrifugation.

DISCUSSION

Preparation of plasma membrane vesicles

The aim of this work was to obtain closed plasma membrane vesicles, which could be used later to study the permeability of plasma membranes for various solutes in

vitro, and hopefully to separate vesicles derived from the luminal side of the tubular cell membrane from those derived from the basal and lateral side, because they are claimed to have totally different functions in the overall transport of solutes across the tubule cell [22, 23]. In order to get undamaged membrane vesicles, it is crucial to avoid any vigorous homogenization [24]. Therefore, a mild hypotonic lysis was chosen to break up the cells. This lysis was also found to support the formation of vesicles. To avoid unnecessary resuspension, a differential centrifugation was designed in which the desired fraction was in the supernatant, whenever possible.

Before performing a lysis in a hypotonic medium, cells or small cell complexes had to be isolated and freed of connective tissue. The method of Burg and Orloff [11] provided a good yield of tubule segments. It also enriched the plasma membrane enzymes trehalase, alkaline phosphatase, and (Na^+–K^+)-ATPase by a factor of 2, which is sufficient reason to employ this procedure [25]. In methods for isolating plasma membranes, homogenization and hypotonic lysis are usually designed to break up all cells and to fragment the plasma membrane into particles of homogeneous size. The hypotonic lysis in this work was rather incomplete, because the ratio of the volume of the cells to the volume of lysing solution was kept high (1:8) as compared to other isolation procedures [26]. The only homogenization used in this work was done by a magnetic stirring bar. Therefore, about 60% of the plasma membranes were lost as large-sized fragments or incompletely lysed cells during the first step. But the gentle handling probably represents the reason for the high percentage of closed vesicles in the final preparation. Unfortunately this gentle procedure results in a contamination by cytoplasmic proteins enclosed by the vesicles.

Contamination of the plasma membranes by other cell organelles is very low as shown in Table II. The mitochondria especially were removed quite effectively in the third step of differential centrifugation (Table I). The brush-border microvilli do not contain a (Na^+–K^+)-stimulated ATPase [2]. The (Na^+–K^+)-stimulated ATPase usually found in a brush-border preparation is believed to be localized in plasma membrane fragments derived from the basal and lateral site of the tubule cell. A separation of brush-border membranes from those containing (Na^+–K^+)-stimulated ATPase by differential centrifugation was not achieved in this study, but will be described in a second paper. Using the preliminary data from this second study, we can say that the ratio of brush-border to basal and lateral plasma membranes in this preparation is about 9 to 1.

Space measurements and the permeability of the plasma membrane vesicles

Biological membranes tend to form vesicles under conditions used in isolation procedures, as can be seen in the electron microscopic pictures of many different preparations. In this work, the amount of vesicles formed was estimated quantitatively and the method for isolating the plasma membranes was designed to enrich vesicles.

Polyethyleneglycol 4000 could not penetrate these vesicles and high concentrations of raffinose decreased the volume of these vesicles significantly (Table III and Fig. 3). The activities of lactate dehydrogenase and malate dehydrogenase could be increased by sonication 6- and 3-fold, respectively (Table II). This probably means that NAD, the co-substrate in these enzyme assays, can reach the enzymes enclosed by the vesicles only after sonication.

Under isotonic conditions, the plasma membranes enclosed a space of 9 μl/mg

of protein (Table III), about twice as much as that of mitochondria [27]. The intravesicular volume was inversely proportional to the osmolality of a surrounding medium containing raffinose (Fig. 3). The intercept on the ordinate represents the intravesicular volume at an infinite concentration of raffinose (2 μl/mg of protein). The method described for the measurements of spaces determines only the aqueous volumes, because for calculations the [^{3}H]glycerol space was used, which was identical to the ^3H_2O space (data not shown), although in addition we had always determined the wet weight of the membrane pellet. Therefore, this remaining intravesicular volume, at infinite osmolality of raffinose, represents an aqueous volume, which is permeable for raffinose but not for polyethyleneglycol 4000. Erythrocyte ghosts and vesicles derived from the ghosts retain only partly the characteristics of the permeability barrier of the genuine erythrocyte, and a special treatment is needed to re-seal the ghosts [28]. They are especially leaky for smaller molecules. In order to show that our plasma membrane vesicles have for example a different permeability for glycerol and for erythritol as is true for the genuine plasma membrane, the osmotic shrinking was observed using a light scattering technique. As shown in Fig. 4, the vesicular membrane could distinguish between glycerol and erythritol. The flux rates of different chloride salts were almost identical suggesting that Cl^- is the rate-limiting factor in these fluxes. The plasma membrane was quite permeable for glycerol and erythritol, but not so much for adonitol, which has only one H–C–OH group more than erythritol. The same observation has also been made on the erythrocyte [29]. But this light-scattering technique does not allow a quantitative estimation of the amount of vesicles which participate in the process of shrinking. Therefore, we can only say that at least a part of the plasma membrane vesicles obtained in this preparation are closed in a similar way to the genuine plasma membrane.

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