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A Rapid Method for the Preparation of Microvilli from Rabbit Kidney

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A simple method for the isolation of microvilli from kidney brush border is described. The method depends on the preferential aggregation of other subcellular structures by bivalent metal ions. $MgCl_2$ is added to a homogenate of cortical tissue prepared from frozen rabbit kidneys. Aggregated material is removed by a low-speed centrifugation and the supernatant centrifuged at 15000*g* to yield a pellet enriched in microvilli. This is resuspended and given a second treatment with Mg^{2+}. The purified preparation is obtained after four short differential centrifugations. The six brush-border enzymes that were monitored were enriched 11–17-fold compared with the original homogenate and were obtained in about 10% yield. Marker enzymes for other subcellular components showed the preparation to be essentially free of mitochondria and to be less contaminated with endoplasmic reticulum and baso-lateral plasma membranes than are conventional brush-border preparations. The main contamination was of lysosomal origin, about half of which was attributable to adsorbed acid hydrolases rather than to intact lysosomes. The aggregated components in the low-speed pellet bound less Mg^{2+} than did the microvillus fraction. A possible mechanism for the role of Mg^{2+} is discussed.

There are now several proven methods for the preparation of kidney brush border. Thuneberg & Rostgaard (1968) were the first to prepare brush borders and showed, by electron microscopy, their preparation to be rich in curled strips of apical plasma membrane of the proximal tubule with attached microvilli. The preservation of this type of structure requires gentle disruption of the tissue, for routine homogenization shears the microvilli from the apical plasma membrane and these smaller particles sediment in the heavy microsomal fraction. So far, all the successful methods have aimed at brush border rather than microvillus preparations (in the present paper we shall use these terms to distinguish between the two types of product, even though the enzymology of the two preparations might be expected to be essentially similar). Brush borders have been prepared and enzymically studied by Kinne & Kinne-Saffran (1969), Wilfong & Neville (1970), Berger & Sacktor (1970), Quirk & Robinson (1972) and George & Kenny (1973). Enzymes primarily located in the microvilli are enriched about 15–20-fold in the brush-border fraction compared with the homogenate of the kidney cortex. Each of these preparations depends on a combination of many differential centrifugations (as many as 16) and often an additional rate-zonal step in a sucrose density gradient. Such schemes tend to be time-consuming and the scale is determined by the capacity of the rotor used for the rate-zonal step.

Attempts to prepare a greatly enriched microvillus preparation have been thwarted by the similarity in density of microvilli and other microsomal vesicles. Schmitz *et al.* (1973) have reported a method for preparing brush-border membranes from human intestinal mucosa. Theirs is essentially a microvillus membrane preparation, in which they make use of the observations of Kamath *et al.* (1971) and Schenkman & Cinti (1972) that Ca^{2+} aggregates microsomal fractions so that they sediment in a low-speed pellet. In the present paper we describe a novel method, utilizing the same principle, to prepare kidney microvilli. Two treatments with Mg^{2+} and four short centrifugations gave a product that was comparable with brush-border preparations in enrichment and yield of marker enzymes. Our method utilizes frozen kidneys, can be completed in 2–3h and, since no rate-zonal step is required, may be easily scaled up or down depending on the size of the angle rotor used.

Methods

Rabbit kidneys

Frozen kidneys were purchased from Honee-Bun Farm Products Ltd., Bideford, Devon, U.K. Fresh kidneys were obtained from young adult male New Zealand White rabbits.

Preparation of brush-border membrane

After the frozen kidneys had been allowed to thaw at room temperature, the cortical tissue was

carefully dissected. Cortex (20g) was homogenized in 10mM-mannitol–2mM-Tris–HCl, pH7.10, at 4°C, in a Kenwood blender (model A 956A) run at full speed for 2min. The total volume of the homogenate was 200ml. In a few experiments the homogenate was centrifuged for 2min at 200*g* (1500rev/min) to remove unbroken cells etc., before proceeding to the Mg^{2+} treatment.

Solid $MgCl_2,6H_2O$ was added to the homogenate to give a concentration of 10mM and then stirred occasionally for 15min in an ice bath. Subsequent steps are shown in Scheme 1. An International HR1 centrifuge with an 8×50 rotor (no. 856) maintained at 4°C was used for each step.

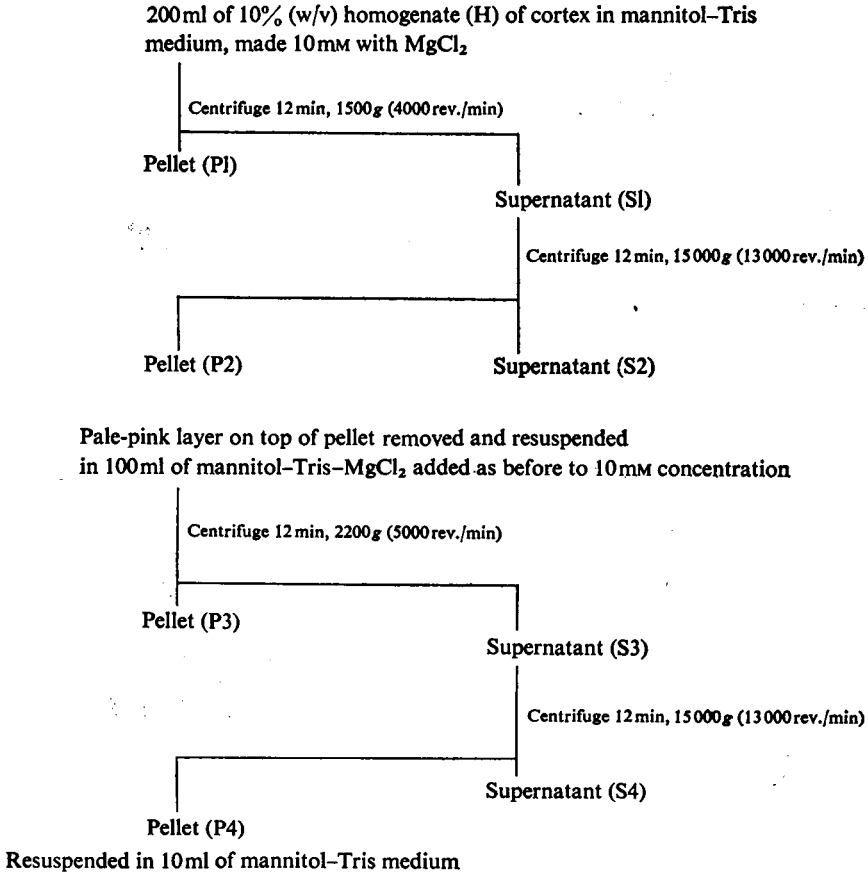
Enzyme assays

Neutral endopeptidase (Kerr & Kenny, 1974*a*,*b*), acid peptidase, ATPases* (EC 3.6.1.3), alkaline phosphatase (EC 3.1.3.1), aminopeptidase M (EC 3.4.11.2), aminopeptidase A, γ-glutamyltransferase (EC 2.3.2.2) and cathepsin C (EC 3.4.14.1) were assayed as previously described (George & Kenny, 1973). Dipeptidyl aminopeptidase IV (Hopsu-Havu *et al.*, 1968) was assayed by the same fluorimetric method as aminopeptidase M except that glycyl-L-proline 2-naphthylamide was the substrate and the pH was 8.0. NADPH–cytochrome *c* reductase (EC 1.6.2.4) was assayed by the method of Masters *et al.* (1967) with 2,6-dichlorophenol-indophenol as acceptor, and succinate dehydrogenase (EC 1.3.99.1) was assayed by the method of Veeger *et al.* (1969). Enzyme units are expressed as nmol of substrate converted/min at 37°C. Specific activity is defined as units/mg of protein.

* Abbreviation: ATPase, adenosine triphosphatase.

Protein

This was determined as previously described (George & Kenny, 1973).



Scheme 1. *Preparation of microvillus fraction from kidney cortex*

Magnesium

This was determined by atomic-absorption spectrophotometry in a Unicam SP.90A Series 2 instrument fitted with an SP.95 integrator.

Electron microscopy

Samples for electron microscopy were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M-sodium phosphate buffer (pH 7.5) containing 0.5% NaCl, washed with the same buffer, post-fixed with buffered 2% (w/v) OsO_4 (pH 7.5), dehydrated through graded alcohols and propylene oxide, and embedded in Epon. Ultrathin sections, stained with lead citrate, were examined in a Philips EM 300 electron microscope operating at 80 kV.

Results and Discussion

The efficiency of the preparative method can be assessed on three criteria: first the enrichment of microvillus enzymes in the purified fraction, secondly the elimination of contaminating structures, judged enzymically and morphologically, and thirdly the percentage yield of microvilli.

Enrichment of microvillus enzymes

Six enzymes known to be comparably enriched in brush-border preparations were assayed in these experiments. The specific activities of these enzymes are shown in Table 1. Five of the group (neutral endopeptidase, aminopeptidases A and M, γ-glutamyl transferase and alkaline phosphatase) have been shown to be brush-border enzymes (see, e.g., George & Kenny, 1973). The sixth, dipeptidyl aminopeptidase IV (to use the nomenclature suggested by McDonald *et al.*, 1971), has also been demonstrated to be a brush-border peptidase (S. G. George & A. J. Kenny, unpublished work). This group of enzymes showed enrichments (ratio of activity in fraction P4/activity in fraction H) in the range 11.0–17.2. These values are slightly lower than the values reported by George & Kenny (1973) for the brush-border preparations, which were in the range 15–25. On this criterion the microvillus fraction may not be quite as homogeneous as the brush-border preparation.

Contamination by other subcellular components

Marker enzymes for mitochondria, lysosomes, endoplasmic reticulum and plasma membrane were also assayed. The preparative procedure was highly successful in removing mitochondria: the enrichment value for succinate dehydrogenase in fraction P2 was 0.04 and 0.02 in fraction P4. There was also very little contamination by endoplasmic reticulum as judged by the low enrichment (0.46 in fraction P4) of NADPH–cytochrome *c* reductase. In assaying ATPase the total, Mg^{2+}-activated and

Table 1. *Specific activities of brush-border enzymes and other markers in fractions prepared from frozen rabbit kidney cortex*

See the Methods section for details (the fractions are those defined in Scheme 1). –, indicates not determined. Specific activities are expressed as units/mg of protein. Values are means ± S.E.M. with the numbers of preparations shown in parentheses.

Enzyme	Fraction	Specific activity								Relative specific activity (enrichment)	
		...	H	P1	S2	P2	P3	S4	P4	P2/H	P4/H
Neutral endopeptidase		1.76±0.17 (6)	1.62±0.12 (5)	0.64±0.06 (5)	18.6±0.5 (6)	16.5±0.8 (6)	13.7±2.2 (5)	28.8±2.0 (6)	10.6	16.4
Alkaline phosphatase		144±13 (6)	–	–	–	–	–	1672±133 (6)		11.6
Aminopeptidase M		153±5.0 (6)	–	–	–	–	–	2631±187 (6)		17.2
Aminopeptidase A		60.8±4.0 (6)	–	–	–	–	–	955±61 (6)		15.7
γ-Glutamyl transferase		73.2±3.2 (6)	–	–	–	–	–	809±34 (6)		11.0
Dipeptidyl aminopeptidase IV		70.8±4.2 (6)	–	–	–	–	–	1031±44 (6)		14.6
Total ATPase		279±27 (5)	–	76.4±1.8 (5)	314±13 (5)	404±21 (5)	168±28 (4)	402±25 (6)	1.12	1.44
$(Na^+ + K^+)$-activated ATPase		162±15 (5)	–	8.8±1.4 (5)	121±10 (5)	162±11 (5)	74.1±25.0 (4)	123±4 (6)	0.75	0.76
Mg^{2+}-activated ATPase		118±12 (5)	–	67.6±2.5 (5)	193±11 (5)	242±18 (5)	75.7±8.7 (4)	279±25 (6)	1.64	2.37
Cathepsin C		3.99±0.49 (3)	–	9.64±0.65 (3)	12.05±1.73 (3)	8.62±0.38 (3)	–	8.57±0.58 (3)	3.02	2.15
Acid peptidase		0.32±0.02 (4)	–	0.78±0.14 (5)	0.73±0.16 (6)	0.57±0.02 (6)	0.91±0.19 (5)	0.59±0.05 (6)	2.28	1.84
Succinate dehydrogenase		3.29±1.2 (4)	–	–	0.13±0.05 (4)	–	–	0.07±0.02 (4)	0.04	0.02
NADPH–cytochrome *c* reductase		4.57±0.12 (4)	–	0.64±0.11 (3)	2.44±0.25 (4)	2.80±0.14 (4)	2.88±0.59 (3)	2.12±0.22 (4)	0.54	0.46

$(Na^+ + K^+)$-activated enzymes were determined. The $(Na^+ + K^+)$-activated ATPase is a plasma-membrane enzyme which, in the proximal tubule cell, is concentrated in the region of the basal infoldings. Mg^{2+}-activated ATPase is distributed more uniformly over both basal and apical regions (Schmidt & Dubach, 1971). The enrichments in the microvillus fraction were 1.44 for total ATPase, 0.76 for $(Na^+ + K^+)$-activated ATPase and 2.37 for Mg^{2+}-activated ATPase. These values indicate much less contamination of fraction P4 by plasma-membrane fragments than in the conventional brush-border preparations. The specific activity of the $(Na^+ + K^+)$-activated ATPase in the homogenate is four times higher than that reported by George & Kenny (1973). This is attributed to the activation of this enzyme by freezing, as noted by Møller (1971), and it does not invalidate the enrichment values.

A peptidase assayed at pH3.5 with [^{125}I]iodo-insulin B chain as substrate, and probably identical with cathepsin D, was used as a routine as a lysosomal marker (Wong-Leung *et al.*, 1968). In a few experiments another lysosomal enzyme, cathepsin C, was also assayed. The enrichment factor for acid peptidase in fraction P4 was 1.84, and that for cathepsin C was 2.15, values that indicate greater contamination of the microvillus preparation with lysosomal enzymes than in the brush-border preparation, in which values of 0.01–0.2 were observed.

The use of frozen tissue homogenized in a very hypo-osmotic medium makes it likely that many of the lysosomes were ruptured at an early stage in the procedure. Some of the contamination may therefore be due to the binding of released lysosomal enzyme on to membranes (of lysosomal or brush-border origin) in fraction P4. This view was supported by an experiment, shown in Fig. 1, in which nearly half of the cathepsin C and acid peptidase activities were removed by washing fraction P4 in a medium containing 0.1M-NaCl. However, only an insignificant amount of protein was removed by this treatment and the specific activity of neutral endopeptidase was not increased.

A typical micrograph of a thin section of pellet P4 is shown in Plate 1. Elongated structures (0.5–1.0μm long) predominate. There are also small vesicles (0.2–0.5μm diam.). Both are typical of microvilli in different degrees of vesicularization and sectioned in various orientations. A lysosome is also visible.

Yield of microvilli

The distribution of protein and enzyme activity in the various fractions is shown in Table 2. Pellet P4 contained 0.77% of the protein in the homogenate, or, in absolute terms, about 35mg of protein from 20g of cortical tissue. The protein yield in brush-border preparations was 0.57% (George & Kenny, 1973). In terms of microvillus protein, the two yields are probably similar, bearing in mind the slightly higher enrichment values for the brush-border fraction. Indeed the percentage yield of brush-border enzyme activities were very similar (brush-border values in parentheses): neutral endopeptidase 13.0 (10.2), aminopeptidase M 12.8 (14.2), aminopeptidase A 11.7 (11.4), γ-glutamyltransferase 8.2 (10.2), dipeptidyl aminopeptidase IV 10.8, alkaline phosphatase 8.2 (8.5). Among the non-brush-border enzymes, the distribution of acid peptidase is noteworthy. Most of the activity (76%) remained in the supernatant fraction S2. This supports the conclusion that many of the lysosomes were burst by the combination of freezing and osmotic shock.

The value of the second Mg^{2+} treatment may be seen by comparing the enzyme activity of fraction P2 with that of fraction P4. Although the yield of the microvillus enzyme (neutral endopeptidase) is halved (down to 46%) there is a much greater decrease in yield of acid peptidase (24%), NADPH–cytochrome *c* reductase (24%) and $(Na^+ + K^+)$-activated ATPase (33%). The removal of these contaminating structures is largely responsible for the increase in relative specific activity of neutral endopeptidase from 10.6 in fraction P2 to 16.4units/mg in fraction P4.

Fresh kidneys

The application of the procedure to fresh rather than frozen tissue was examined in one experiment. In general, the results were comparable, although

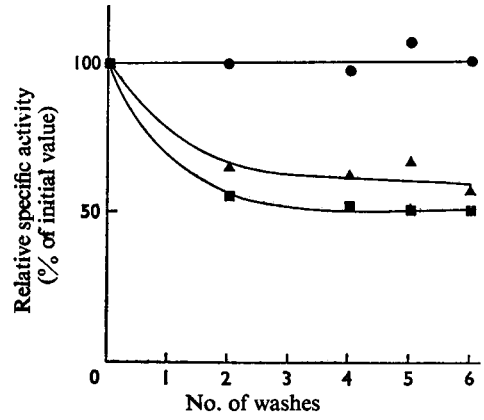
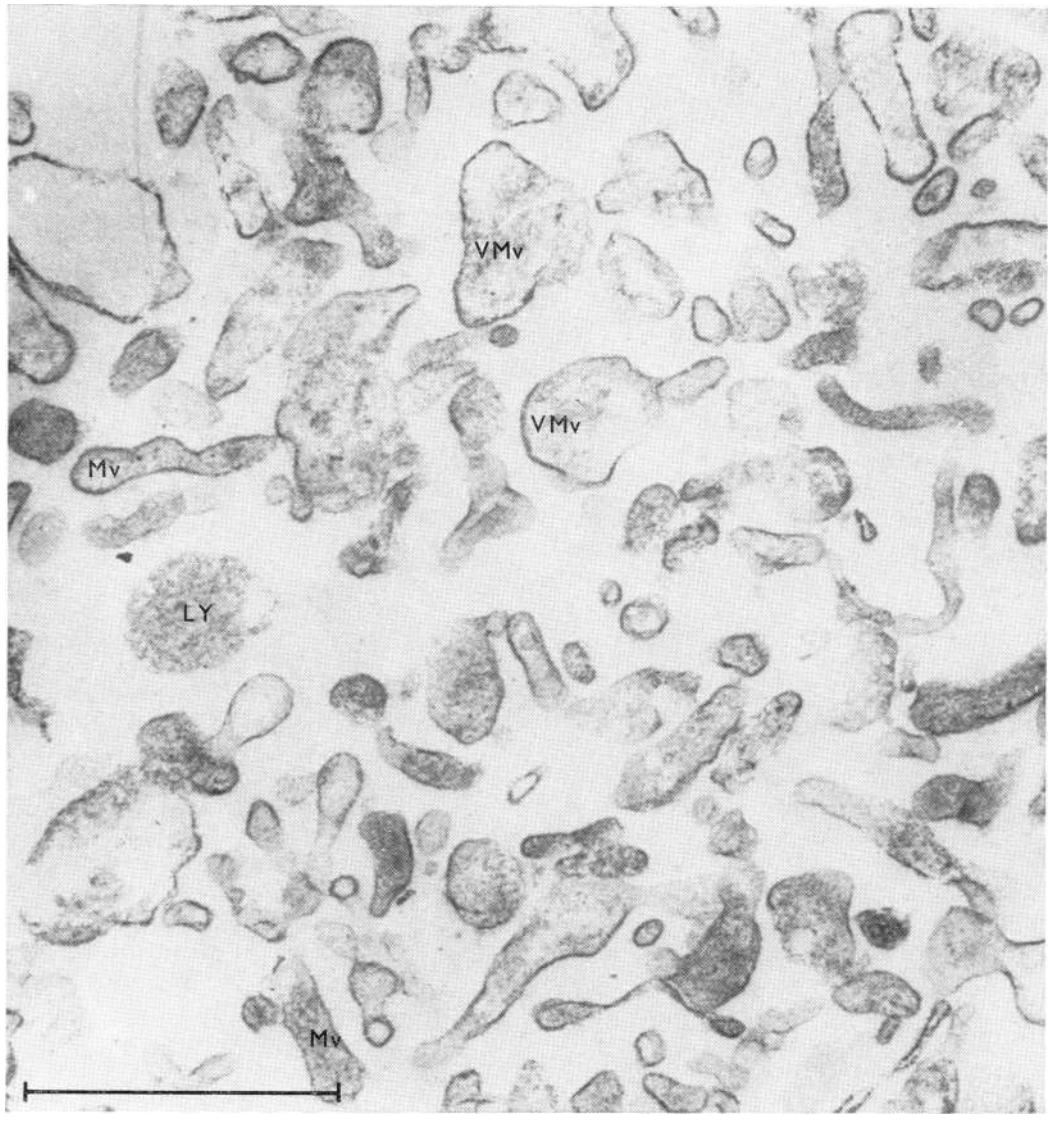


Fig. 1. *Effect of washing fraction P4 with* 0.1M-*NaCl on specific activities of neutral endopeptidase, cathepsin C and acid peptidase*

The initial specific activities (≡ 100) in units/mg were: neutral endopeptidase (●), 27.6; cathepsin C (▲), 8.04; acid peptidase (■), 0.78. The pellet, P4 (20mg of protein), was resuspended for each wash in 10ml of 0.1M-NaCl and centrifuged for 20min at 15000*g*.



EXPLANATION OF PLATE 1

Electron micrograph of fraction P4 prepared from frozen rabbit kidney cortex

For details see the Methods section. The scale line represents 1 μm. The pellet contained many elongated structures typical in appearance of microvilli (Mv). The other predominant structures had the characteristic appearance of microvilli in various stages of vesicularization (VMv). A few lysosomes (LY) were also observed.

Table 2. *Distribution of brush-border enzymes and other markers in fractions prepared from frozen rabbit kidney cortex*

See the Methods section for details (the fractions are those defined in Scheme 1). Results are expressed as the percentage of the total activity or protein (H = 100%) in each fraction. Values are means ± S.E.M. with the numbers of preparations shown in parentheses.

Enzyme	Fraction	Distribution in fractions (as % of homogenate)				
	... P1	S2	P2	P3	S4	P4
Protein	67.7±1.7 (5)	29.8±0.6 (5)	2.5±0.1 (5)	0.66±0.08 (5)	1.05±0.05 (5)	0.77±0.05 (5)
Neutral endopeptidase	62.4±1.1 (5)	10.8±0.9 (5)	28.2±1.5 (6)	6.40±0.30 (6)	8.10±1.30 (5)	13.0±0.9 (6)
Alkaline phosphatase						8.2±0.3 (6)
Aminopeptidase M						12.8±0.9 (6)
Aminopeptidase A						11.7±0.7 (6)
γ-Glutamyl transferase						8.2±0.4 (6)
Dipeptidyl aminopeptidase IV						10.8±0.5 (6)
Total ATPase	89.0±2.3 (5)*	8.19±0.27 (5)	2.79±0.11 (5)	1.02±0.05 (5)	0.60±0.08 (4)	1.15±0.07 (6)
$(Na^+ + K^+)$-activated ATPase	96.5±0.2 (5)*	1.61±0.28 (5)	1.85±0.12 (5)	0.71±0.06 (5)	0.45±0.13 (4)	0.61±0.04 (6)
Mg^{2+}-activated ATPase	78.8±0.8 (5)*	17.2±0.6 (5)	4.09±0.28 (5)	1.44±0.08 (5)	0.67±0.11 (4)	1.88±0.15 (6)
Cathepsin C	20.5±1.6 (3)*	72.0±5.6 (3)	7.55±0.98 (3)	1.42±0.42 (3)		1.67±0.72 (3)
Acid peptidase	18.1±4.7 (5)*	76.0±4.6 (5)	6.20±0.60 (6)	1.24±0.07 (6)	3.15±0.73 (5)	1.50±0.11 (6)
Succinate dehydrogenase			0.19±0.08 (4)			0.11±0.03 (6)
NADPH–cytochrome *c* reductase	94.9±0.8 (3)*	3.84±0.65 (3)	1.20±0.10 (4)	0.37±0.01 (4)	0.60±0.12 (3)	0.29±0.03 (4)

* Indicates that the value for fraction P1 is calculated (P1 = H − P2 − S2); this was necessary because aggregated material in fraction P1 sometimes gave erratic results in enzyme assays.

the enrichment values for the neutral endopeptidase in fractions P2 (6.63) and P4 (12.1) were below the mean values given in Table 1 for frozen tissue. The specific activities of neutral endopeptidase and cathepsin C did not differ significantly in homogenates of fresh tissue compared with frozen tissue. The distribution of cathepsin C differed in that less of the activity (52% compared with 72%) was in the supernatant, S2, suggesting that the fresh homogenate contained a greater proportion of intact lysosomes.

Role of Mg^{2+} in the purification of microvilli

Schmitz *et al.* (1973) noted that Ca^{2+} and Mg^{2+} were equally effective for the purification of intestinal brush-border membranes. We preferred Mg^{2+} simply because $MgCl_2$ is less deliquescent than $CaCl_2$. Both gave identical results, at least to the stage of fraction P2. The mechanism of the selective effect of these cations is to some extent speculative. Schmitz *et al.* (1973) argued that despite the negatively charged glycocalyx, the intestinal brush-border-fragment surface is more positively charged than the surfaces of other components, which therefore react preferentially with the cation. We are sceptical in applying this interpretation to our results for three reasons. First, there is evidence that most of the microvilli pelleting in fraction P1 do so because they are aggregated by Mg^{2+}. This is apparent if unbroken cells are first removed from the homogenate by a preliminary centrifugation for 2 min at 200*g*. About 30% of the neutral endopeptidase is present in this 'debris' pellet. When Mg^{2+} is then added to the supernatant, 35% of the neutral endopeptidase pellets in fraction P1. Without Mg^{2+} only 9% of this marker enzyme pellets in fraction P1, this percentage representing the brush borders that were not further fragmented during homogenization. The difference (26%) is attributed to Mg^{2+}-aggregated microvilli pelleting in fraction P1.

Secondly, we have determined Mg in both fractions P1 and P4. These results (Fig. 2) were obtained from pellets washed repeatedly with the mannitol–Tris medium. About 70 ng-atoms of Mg were bound/mg of protein in fraction P4 and about 30 ng-atoms/mg of protein in fraction P1. This shows that more Mg^{2+} was bound by the microvillus-rich fraction than by fraction P1. In both pellets the Mg^{2+} was bound electrostatically, since washing with 0.1 M-NaCl decreased the bound Mg^{2+} to very low values.

Thirdly, we have observed that when fraction P4 is resuspended in a Mg^{2+}-free mannitol–Tris medium and frozen overnight, spontaneous aggregation of microvilli slowly occurs in the thawed suspension. After keeping overnight at 4°C, the aggregated microvilli fall to the bottom of the tube. When fraction P4 was resuspended in a medium containing 10 mM-$MgCl_2$ or 20 mM-NaCl, aggregation did not develop. This effect was dependent

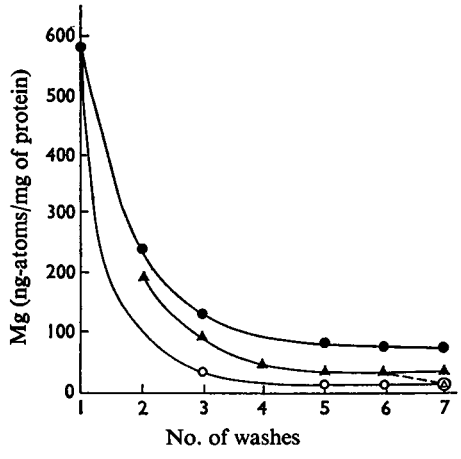


Fig. 2. *Mg bound to fractions P1 and P4*

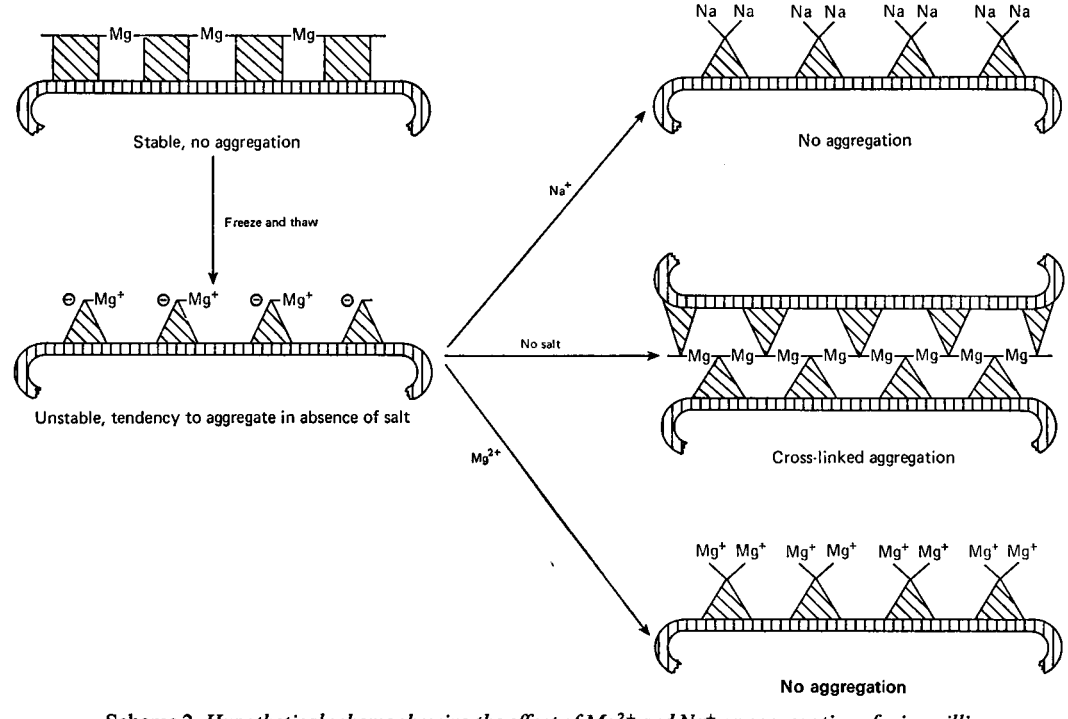
Fractions P1 and P4 were repeatedly suspended in 10ml of either mannitol–Tris medium or 0.1M-NaCl and centrifuged for 20min at 15000*g*. ●, Fraction P4 washed with mannitol–Tris medium; ○, fraction P4 washed with NaCl; ▲, fraction P1 washed with mannitol–Tris medium; △, fraction P1 washed with NaCl. Curves are means of two experiments.

on the concentration of microvillus protein. At high concentrations (8mg/ml) 55% of the protein aggregated even in the presence of salt. At concentrations between 2 and 4mg/ml aggregation could be wholly or partly prevented by salts (Table 3).

Table 3. *Spontaneous aggregation of microvillus membranes in fraction P4*

Fraction P4 was resuspended in the three media and at the dilutions indicated. Each tube, containing 1ml of the suspension, was frozen at −15°C for 1h, thawed and, after mixing, a sample (0.1ml) was removed from each for determination of protein (A). They were then left overnight at 4°C and samples (0.1ml) were carefully removed from the top of the suspension for determination of protein (B). Aggregation = 100×(A−B)/A (%).

	Aggregation (%) in:		
[Protein] (mg/ml)	Mannitol–Tris	Mannitol–Tris–10mM-$MgCl_2$	Mannitol–Tris–20mM-NaCl
8	57	49	31
4	55	34	25
2.7	55	22	0
2.0	56	0	0



Scheme 2. *Hypothetical scheme showing the effect of Mg^{2+} and Na^{+} on aggregation of microvilli*

■, Glycocalyx; ▥, microvillus membrane.

We suggest that aggregation of membranous components occurs when bivalent cations establish cross-links between membranes. Some microvilli are aggregated in this manner and pellet in fraction P1, but the rest avoid this fate because cross-linking by Mg^{2+} is established between contiguous anionic sites on the same microvillus. This may be possible because the glycocalyx on the microvillus membrane is richer in sialic acid than are the membranes of other components, with the exception of lysosomes (Glossmann & Neville, 1971; Quirk & Robinson, 1972; Li *et al.*, 1965; Henning *et al.*, 1970), thus accounting for the greater amount of Mg^{2+} bound to fraction P4 compared with fraction P1. A change, as yet undefined, occurs on freezing and thawing that permits cross-linking to develop slowly between microvilli, leading to aggregation. This process can be prevented if the bivalent cation is replaced by a univalent ion (Na^+) or if the concentration of Mg^{2+} is high enough to saturate all the anionic sites thus preventing the tendency to cross-link. This hypothesis is shown diagrammatically in Scheme 2. If kidney lysosomes are comparable with liver lysosomes in their high sialic acid content, this might explain the contamination by this organelle of the microvillus fraction.

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