Brush border membranes

Ciba Foundation symposium 95

1983

Pitman

London

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Chairman's introduction

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Several of the participants at this symposium were also present at Ciba Foundation symposium 50 (Peptide transport and hydrolysis). On that occasion the emphasis was on functional questions such as whether peptides were hydrolysed at the cell surface, in the lumen or inside the cell, and whether hydrolysis preceded transport. Consequently only a minority of the papers were concerned with structure and topology. In the six years that followed that symposium our attitudes and understanding have developed in such a way that we can now concentrate with profit on the molecular aspects of this topic. I believe this is an important development. Major progress in the biological sciences has usually depended on clarifying molecular interactions that were formerly considered to be very mysterious events.

Among the group of people assembled here, some, like me, are mainly concerned with the group of hydrolases in the brush border membrane that face towards the lumen, anchored to the lipids by only a very small portion of the polypeptide chain. During the symposium this group of participants should also try to look below the membrane, into the cytoplasm, and ask what interactions may take place with the cytoskeleton. Others, whom I may refer to as cytoosteologists, and who have for different reasons become enamoured of the brush border, have recently made remarkable progress in defining the proteins of the cytoskeleton. But possibly they may know little about the membrane proteins and may, therefore, be inspired to look outwards towards the cytoplasmic domains of these membrane proteins to ask if any of them interacts with the cytoskeleton, and what this means functionally. I would guess that the *raison d'être* of the microvillus relates to the function of the membrane proteins—the hydrolases, the transport proteins, receptors and so on. Yet microvilli become shapeless vesicles once the cytoskeleton is disorganized. So we do need to ask why the cytoskeleton exists in the form it does, and how its components interact with each other and with the membrane.

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Others here are currently concerned with activities deeper in the cell, in particular the molecular events in the biosynthesis of the brush border enzymes. There is a need to define the primary translation product of the membrane proteins, and to understand how the polypeptide chain becomes associated with a membrane, as well as the details of the post-translational processing and the membrane pathway in the cell through which the precursor forms move. Much has been done in this area in the last few years, and I have no doubt that it is one of the fast moving topics at present.

One topic that some may think is missing from the symposium is a molecular description of the systems concerned with transport of small solutes such as amino acids, sugars and anions. It seems to me that this subject has not really broken out from the confines of its 'black box', and hence it is difficult for us to describe the events in precise molecular terms. Our compromise has been to include in our discussions one well defined transport protein, Na^+,K^+-ATPase (see p 253–272). We must overlook the fact that it is not in the brush border membrane but situated at the opposite pole of the cell. However, it will serve to focus our thoughts on the architecture of a well researched transport protein.

Macromolecules cross the membrane by the process of receptor-mediated endocytosis. We are beginning to learn something about the nature of the molecules involved in, say, the uptake of immunoglobulin across a brush border. Receptors are involved, the cytoskeleton is implicated and questions also arise about the pathway of the coated vesicles through the cell. Again we are concerned with events that are analogous to those concerned with the assembly of newly synthesized or recycled membrane proteins.

I am optimistic that as each section of the symposium develops, it will be the hitherto uncharted border areas between the different approaches that may provide the greatest interest. I hope we shall all try to see where our own area of research links with that of the next person. The apparent differences between the various organs that contain brush borders—the kidney, the intestine and the placenta—will, I hope, become unimportant in these discussions. All microvilli have the same basic architecture in common, and all share some functions, though there are important specializations, too. At this stage in our understanding it may be more profitable to concentrate on the similarities before we try to explain the differences.

Introductory remarks on the brush border

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Abstract The specialized surface of the luminal border of small intestinal epithelial cells was first described by J. Henle in 1837 and for many years controversy raged about the nature of this region. Was it a plate rendered porous by perforating canals or was it composed of an array of rods with their long axes normal to the surface? Because the diameter of the microvilli was below the limits of resolution by optical microscopy, the arguments could not be settled until the region had been observed under the electron microscope in 1950. In 1961, the brush border membrane (also known as the free border or microvillus membrane) was separated for biochemical study and results on transport using vesicles were first described in 1973. The increase in surface area due to the microvilli is about 40 times and the surface : volume 'ratio' for a single microvillus is $4 \times 10^5 \text{ cm}^{-1}$, about 20 times that for an erythrocyte. An important but unresolved question concerns the relationships between transporter proteins in the membrane and proteins that have digestive functions; the physiological role of the glycocalyx is not yet resolved.

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Cock up your beaver, and cock it fu' sprush;
We'll over the border and gie them a brush.
from *Cock up your beaver* (James Hogg 1821)

Some history of the intestinal brush border

The presence of a specialized surface at the luminal border of intestinal epithelial cells has been recognized for nearly 150 years. Henle (he of the loop) described how the free border of intestinal epithelial cells consisted of a refractile lamella, 0.0012–0.0015 lines (3–4 μm) thick, containing fine striations. He demonstrated that the structure disappears on exposure to acetic

* Unable to attend the symposium. Paper read by Dr C. A. R. Boyd.

acid, thereby distinguishing these cells from those of squamous epithelia which are scarcely changed by acetic acid (Henle 1837, 1841), (Fig. 1). The presence of a fibrillar structure at the surface of dog intestinal cells was mentioned by Gruby & Delafond in 1843. Funke (1855a,b) described histological observations on fat absorption and provided illustrations of the epithelial cells which depicted a striated structure of the luminal surface. In the same year an excellent description, with illustrations, of the brush border

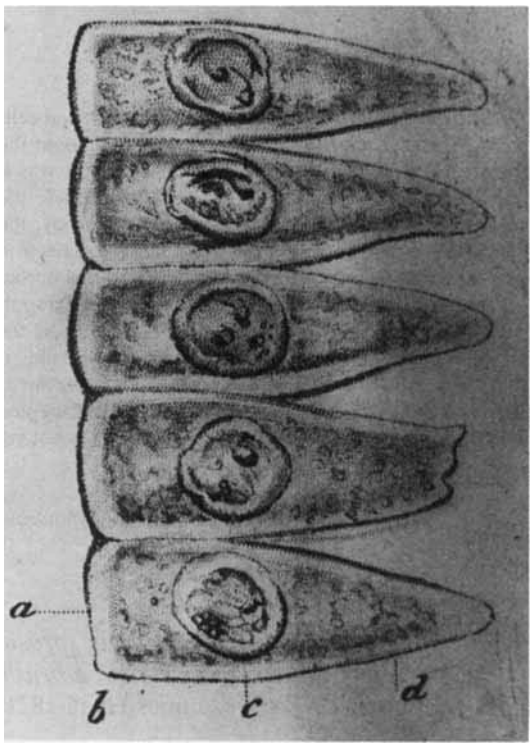

FIG. 1. Epithelial cell of rabbit intestine. (a) the *Freie oberfläche*. From Henle (1841, plate 1, figure 8).

of the cylindrical epithelial cells of mammals, birds and amphibia was provided by Kölliker (1855). He stated that with a good microscope it was possible to recognize a clear striation in the luminal border of the cells, which when viewed from above presented a very fine stippled appearance. Kölliker recognized that when working at the limits of optical resolution of a good microscope it was not possible to decide whether the striations (which he estimated to be equivalent to 0.25–0.45 μm in width) were solid structures or

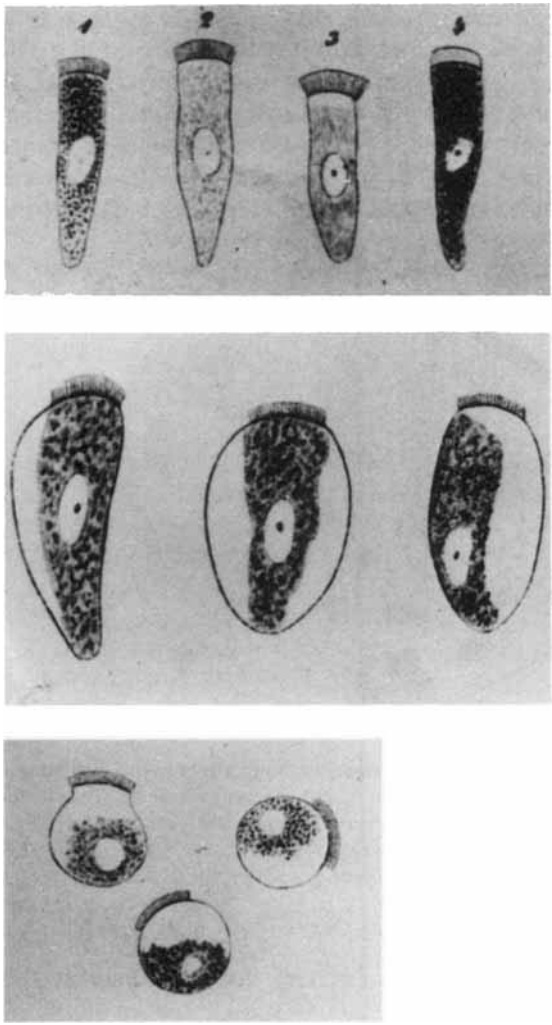

FIG. 2. Intestinal epithelial cell brush border as depicted by Kölliker (1855, plate IV). Upper panel: fresh cells. Centre and lower panels: cells in water, showing separation of the brush border.

little canals. Particularly interesting were his observations on the effects of immersing the epithelial cells in various solutions. In water and in hypotonic solutions the *streifige Zellenwand* becomes swollen and detached from the cell contents (Fig. 2).

Until 1950 controversy raged over the nature of the brush border. Was it a plate rendered porous by perforating canals (Baker 1942a,b) or was it composed of an array of rods with their long axes normal to the surface? The early published work and that on the controversy was reviewed by Baker (1942a) who also reported studies of his own which, alas, convinced him that what he called the 'free border' was composed of a continuous substance traversed by canals perpendicular to the plane of its surface (Fig. 3). At this time Baker also described the 'intercellular band' (terminal bar) and the

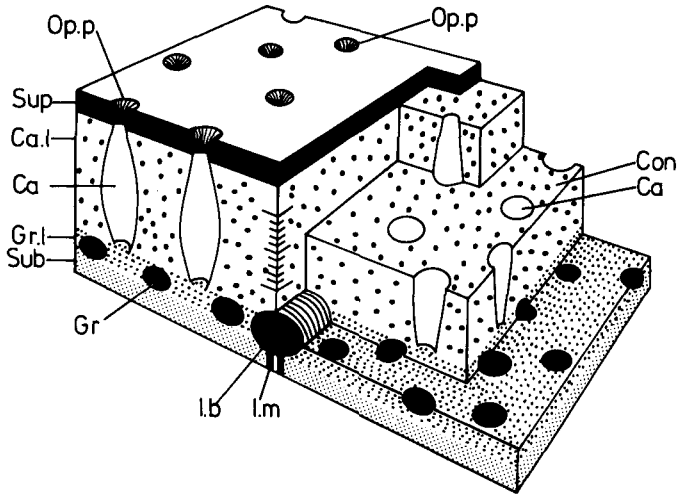

FIG. 3. Diagram from Baker (1942a) representing his views on the structure of the 'free border' of the intestinal epithelial cell of vertebrates. The central vertical scale represents 1 μm divided into tenths. The dimensions are those for newts (*Triturus vulgaris*). *Abbreviations*: Ca, canal; Ca.l, canal layer; Con, continuous substance of canal layer; Gr, granule; Gr.l, granular layer; I.b, intercellular band; I.m, intercellular membrane; Op.p, open pore; Sub, sub-granular layer; Sup, superficial layer.

'intercellular membranes' (basolateral membranes) that terminate at this 'band' (Baker 1942a).

The limit of resolution of a microscope, as first shown by Abbé (see Carpenter & Dallinger 1901, Beck 1938) is given by $R = 0.6\lambda/N.A.$, where R is the distance resolved, λ the wavelength of incident radiation and $N.A.$ the numerical aperture of the objective. Thus, with visible light of, say, 500 nm and with an objective of $N.A. = 1.30$ perfectly illuminated, the theoretical resolution should be 230 nm (0.23 μm). In fact, various practical problems prevent this limit being achieved, and the early arguments between microscopists were concerned with the structure of what proved to be an array of objects 1 μm or more in length and about 100 nm in diameter, that is, well

below the limit of resolution of the optical microscope in the visible spectrum. It was not until 1950 that B. Granger & R. F. Baker produced the first electron microscope pictures that revealed the true structure of the refractile *streifige Zellenwand* of Kölliker.

In the second half of the nineteenth century microscope objectives with numerical apertures of 1.30–1.50 were available from manufacturers such as Zeiss (in Germany) and Powell and Lealand (in England); from 1883 onwards, superbly corrected apochromatic objectives of high *N.A.* were produced by the same makers, those from Zeiss being designed by Abbé (see Bradbury 1967). With the increasing use of such high-quality objectives appropriately illuminated, it is not surprising that some details of cellular ultrastructure became revealed even if that of the 'free border' did not. Thus, the T-system of striated muscle was clearly described in the 1880s, but was then forgotten, to be rediscovered much later by electron microscopists (see Huxley 1977).

Nomenclature

The specialized border that we now call 'brush border'—Baker (1942a) hyphenates the two words—has been given many names. In addition to Kölliker's term mentioned above it has been called *bürsten*, cuticle, *cuticula*, free border, *freie Oberfläche*, microvillus membrane, *Stäbchensaum*, *Stäbchenorgan* and *Stäbchen cuticula* (Baker 1942a). According to the Oxford English Dictionary the use of the word 'brush' in a biological connection to mean a brush-like bunch or comb goes back to 1581—the tail of a fox—while in 1828 a brush-like organ was described 'on the legs of bees etc'.

Recent interest in the brush border

A search of the published work (MEDLARS; Medical Literature Analysis and Retrieval System) for papers in which the term 'brush border' appears in the title shows a great increase in such publications after 1976 (Table 1). The

TABLE 1 Citations in MEDLARS (Medical Literature Analysis and Retrieval System) of articles that contain in the title the term 'brush border'

Period	*Titles per month*
1969–1971	1.25
1972–1974	2.58
1975–1976	3.40
1977–1978	18.5
1979	20.2
1980	32.5
Jan.1.1981–Oct.1.1981	31.0

paper of Miller & Crane, which first described the separation of brush border membranes for biochemical study, was published in 1961, and accounts of the preparation of vesicles of brush border membrane and their use for studies of transport began to appear from 1973 onwards (Hopfer et al 1973, Murer & Hopfer 1977).

Increase in area due to microvilli

In mammals microvilli are about 1 μm long although in lower animals, including amphibia, they may be longer; Baker depicts the 'free border' of the newt intestinal cell to be about 2 μm in length (Fig. 2). For a mammalian microvillus considered as a right cylinder 1 μm high and with an external diameter (excluding the glycocalyx structure) of 100 nm, the external surface is 3.3×10^{-9} cm^2, while the area of the base is only 8×10^{-11} cm^2. Thus, for a structure of these dimensions the microvillar structure increases the external surface by about 40 times. This can be a useful increase in area at a surface where digestive enzymes are deployed. But what are the effects of such an increase on transport? Clearly, more membrane is available for the insertion of copies of transporter proteins, but all the traffic traversing the microvillus membrane nevertheless has to enter the main body of the cell by passing through the *base* of the microvillus. With a membrane thickness of, say, 5 nm the internal diameter in the above example is 90 nm, across a base area of 6×10^{-11} cm^2, ie, only one fiftieth of the external surface. This area may be further reduced by the presence of longitudinal fibrils passing into the cell from the microvillus for attachment to the terminal web.

An important consideration here is the very large surface : volume 'ratio' of microvilli. For the example given above it is about 4×10^5 cm^{-1} compared with about 4.4×10^3 cm^{-1} for a cylindrical cell of radius 5 μm and depth 25 μm (the approximate size of an intestinal epithelial cell) and 1.6×10^4 cm^{-1} for an erythrocyte. The effect of the large surface : volume 'ratio' will be to increase the concentration of the transported substrate on the *trans*-side of the membrane, although the extent to which this occurs will depend on the density (number of proteins per unit area of brush border membrane) and turnover number of the transporter proteins inserted in the microvillus membrane, the 'leakiness' of the membrane and the permeability of the exit pathway from the microvillus into the cell. The relevant transport processes described in the brush border membrane include those for monosaccharides, amino acids, peptides, sodium chloride, short-chain fatty acids, purines and pyrimidines. The entry of micelles of long-chain fatty acids and triglycerides also has to be considered. It is interesting to speculate whether the large surface : volume 'ratio' of brush border membranes is related to the alleged

ability of the microvilli to contract longitudinally (see Mooseker et al, this volume, p 195–215).

How are products of hydrolysis by brush border enzymes captured for transport?

The monosaccharides released by the action of hydrolytic proteins in the brush border membrane on disaccharides have two fates: they are either captured by the monosaccharide transport systems and then move into the cell, or they appear in the bulk phase in the intestinal lumen. At low concentrations of disaccharide the efficiency of capture for transport can be

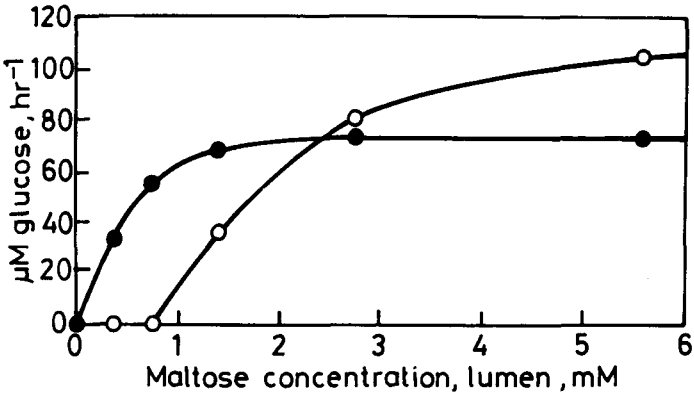

FIG. 4. Rate of transport of glucose into portal venous vasculature (solid circles) and rate of backflux of glucose into bulk phase of intestinal lumen (open circles) measured simultaneously during absorption of maltose at different concentrations by small intestine of *Rana pipiens*. Redrawn from Parsons & Prichard (1968).

remarkably high; for frog intestine at luminal concentrations of maltose up to 1 mM none of the liberated glucose appears in the lumen; only at maltose concentrations above 3 mM does free glucose appear in the lumen at rates in excess of its transport rate into the mesenteric blood (Parsons & Prichard 1968), (Fig. 4). Although these findings were made on amphibian intestine, the small intestinal epithelium of such animals is remarkably similar to that of mammals in both structure and functions (Parsons & Prichard 1968; see also Boyd in General Discussion III, p 318).

Two sorts of explanations have been proposed for such a high efficiency of capture. The membrane-bound oligosaccharidases may form part of the sugar

transporter system in the brush border membrane. If this is so, then in mammals not more than about 10% of hexose derived from disaccharides could be transported in this way; Semenza (1977) has discussed this point and the possible ways in which the sucrase–isomaltase complex may be involved in the transport.

Our own experiments on amphibian small intestine favour the alternative supposition that the hexose molecules derived from disaccharides are liberated into a pool immediately adjacent to the hexose transport system, the pool being also accessible to any free hexose units present in the lumen. We find no evidence for a competition for hydrolysis between maltose and trehalose, yet competition for transport does take place between the hexose units derived from these two disaccharides. Competition for transport is also observed between free hexose added to the intestinal lumen and hexose derived from either maltose or trehalose. These findings although necessary are not sufficient to prove the point; the experiments were done on transport across the epithelium so it is theoretically possible that the competition occurred not outside the brush border for entry into the cell but inside the cell for exit into the plasma. However, we also found that the cation Tris inhibits disaccharide hydrolysis but not monosaccharide transport. The hydrolase and the hexose-transporter system can thus be quite separate protein components of the brush border membrane (see Parsons & Prichard 1971).

Possible roles of glycocalyx

Models based on the existence of a pool external to the transporter systems in the brush border can be devised, which predict the relationships between the disaccharide concentration in the bulk phase of the lumen and the ultimate fate of the products of hydrolysis, as depicted in Fig. 4. Such models require the presence of a diffusive resistance superficial to the site of membrane transport (Hamilton & McMichael 1968) and also require that this region of diffusive resistance has the capacity to adsorb monosaccharides in such a fashion that they are eventually available for transport into the microvillus core (Prichard 1969). The glycoproteins of the brush border membrane that constitute the *glycocalyx* (polysaccharide cell coat) are obvious candidates for the physical basis of the barrier postulated in such models.

As well as acting as a barrier as described above, the glycocalyx may play an indirect role in digestion as a surface on which digestive enzymes of exogenous origin (e.g. from the pancreas) are adsorbed and act on substrates moving inwards from the lumen towards the brush border.

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Microvillar endopeptidase, an enzyme with special topological features and a wide distribution

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Abstract The endopeptidase present in the kidney microvillar membrane (EC 3.4.24.11) has been purified by immunoadsorbent chromatography from the pig. Three physically different forms have been obtained. The toluene–trypsin solubilized form has hydrophilic properties. The detergent and detergent–trypsin forms are amphipathic. Only a small change in apparent relative molecular mass of the subunit is produced by trypsin, indicating that little of the polypeptide is removed by the proteinase. Although apparently immunologically identical, the intestinal form has slightly different molecular properties, possibly attributable to differences in glycosylation. In spite of the failure of papain and other proteinases to release the endopeptidase from the membrane, reconstitution of the purified enzyme in liposomes has shown that it is a stalked, dimeric protein, thus resembling other hydrolases in this membrane. In addition to its main locations in kidney and intestinal microvilli, there is clear evidence from inhibitor and immunological studies that the enzyme has a wide distribution including membrane fractions prepared from spleen, lung aorta and myocardium.

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Kidney microvilli contain an endopeptidase that hydrolyses peptides, such as insulin B chain (George & Kenny 1973). The rabbit kidney enzyme was the first example to be purified and fully characterized (Kerr & Kenny 1974a,b), and it hydrolyses peptide bonds involving the amino groups of hydrophobic amino acids. An interesting feature of this Zn^{2+}-metalloenzyme (EC 3.4.24.11) is that it resembles the microbial group of chelator-sensitive endopeptidases in its mode of attack and, in particular, in its sensitivity to inhibition by phosphoramidon (Kenny 1977), a reagent that is specific for this group of peptidases. For several years the kidney endopeptidase was the only mammalian example of this type of enzyme. Recently endopeptidases with similar characteristics have been shown to be present in intestinal brush

borders (Danielsen et al 1980), spermatozoa (McRorie et al 1976) pituitary (Orlowski & Wilk 1981), pancreas (Mumford et al 1980) and in brain (Almenoff et al 1981, Fulcher et al 1982). In this paper we wish to describe some recent molecular and topological studies on the purified endopeptidase from pig kidney and intestine and to report the presence of a similar enzyme in a variety of other pig tissues.

Purification of endopeptidase from pig kidney

The rabbit kidney enzyme was solubilized by treatment of the membrane pellet with toluene, followed by incubation with trypsin (Kerr & Kenny 1974a). This method yielded a hydrophilic form of the enzyme in which the polypeptide chain had been subjected to limited proteolysis, thus cleaving the hydrophilic protein from its hydrophobic anchor (see e.g. Kenny & Maroux 1982). The detergent form, representing the intact amphipathic protein, has only recently been purified (Kenny et al 1981, Mumford et al 1981). Column chromatography of detergent forms of membrane proteins is often associated with poor resolution, compared with resolution of the corresponding proteinase forms. Hence an affinity step was sought. In this case it depended on the production of a specific antiserum, achieved in a step-wise fashion, as outlined in the scheme shown in Fig. 1. A partially purified form was first obtained and electrophoresed into an agarose gel containing antibodies raised to the whole membrane. The immunoprecipitate corresponding to the endopeptidase was excised and the electrophoresis was repeated until a sufficient quantity of the immune precipitate had been accumulated for use as an antigen for injecting into a rabbit. The antiserum thus obtained was used to prepare an immunoadsorbent column. The column could bind endopeptidase from relatively crude extracts of either kidney or intestine and the activity could be eluted in an almost pure form. Some traces of other microvillar hydrolases could be removed by a second immunoadsorbent column, prepared from an antiserum raised to the whole membrane. The final product was homogeneous in sodium dodecyl sulphate (SDS)/polyacrylamide gel electrophoresis (Fig. 2). The kidney enzyme is seen as a single band, stained with Coomassie blue, (lane 2) and corresponding to the faster moving band in the 90 000–95 000 relative molecular mass (M_r) region of kidney microvilli (lane 1). The intestinal form has a slightly lower mobility (lane 3) and appears to correspond to a minor band in intestinal microvilli (lane 4).

Immunoelectrophoresis is a more rigorous criterion of purity. The endopeptidase is less immunogenic than many other microvillar enzymes. Hence, minor contaminants in the preparation are readily revealed by crossed

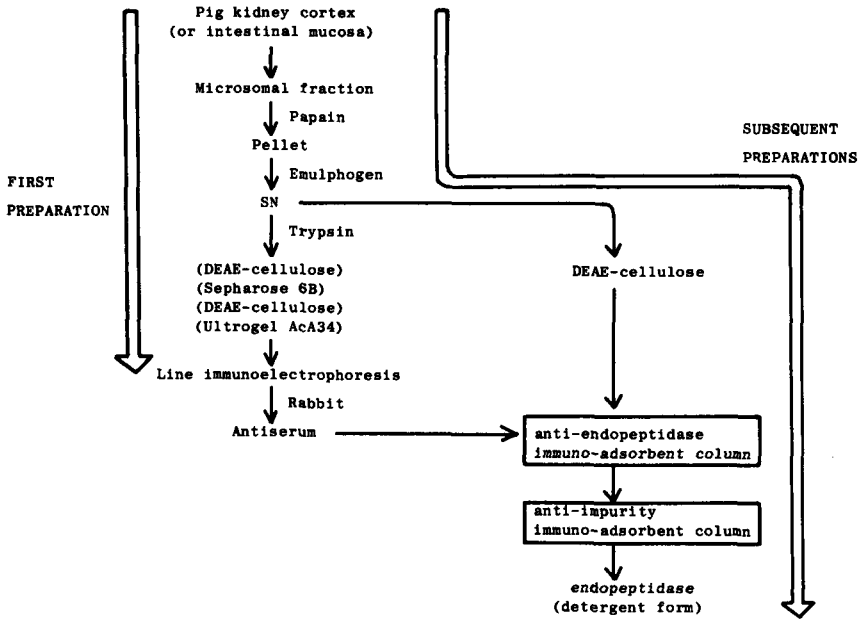

FIG. 1. Scheme showing the purification of the detergent form of the endopeptidase from pig kidney and intestine. The first purification, employing conventional steps, produced only a partially pure preparation. Immunoelectrophoresis against an antiserum to the whole membrane was successful in resolving the endopeptidase from most of its contaminants. This immunoprecipitate was used as an antigen to raise a specific antiserum. Subsequent preparations exploited the immunoadsorbent column prepared from this antibody. Traces of other microvillar hydrolases were removed by a small second immunoadsorbent column which contained antibodies to the whole membrane. SN, supernatant.

immunoelectrophoresis of the purified enzyme into an antiserum raised to the whole membrane. This has consistently revealed only one precipitate attributable to the endopeptidase. The positive identification of the antigen has been achieved by a novel histochemical stain (Kenny et al 1981), using glutaryl-glycylglycylphenylalanyl-2-naphthylamide as substrate. It is hydrolysed by the endopeptidase thus: Glutaryl–Gly–Gly–Phe–2-NNap → Glutaryl–Gly–Gly + Phe–2-NNap. The addition of aminopeptidase N (EC 3.4.2.11) to the staining mixture releases free 2-naphthylamine (2-NNap) which can be visualized by a diazo reaction. A control, containing phosphoramidon, can be used to confirm the identity of the enzyme that hydrolyses the Gly–Phe bond (results not shown).

Our standard assay uses [^{125}I]iodo-insulin B chain as substrate. The apparent activity in crude samples is markedly affected by the presence of aminopeptidases, since they can rapidly generate some smaller trichloroacetic

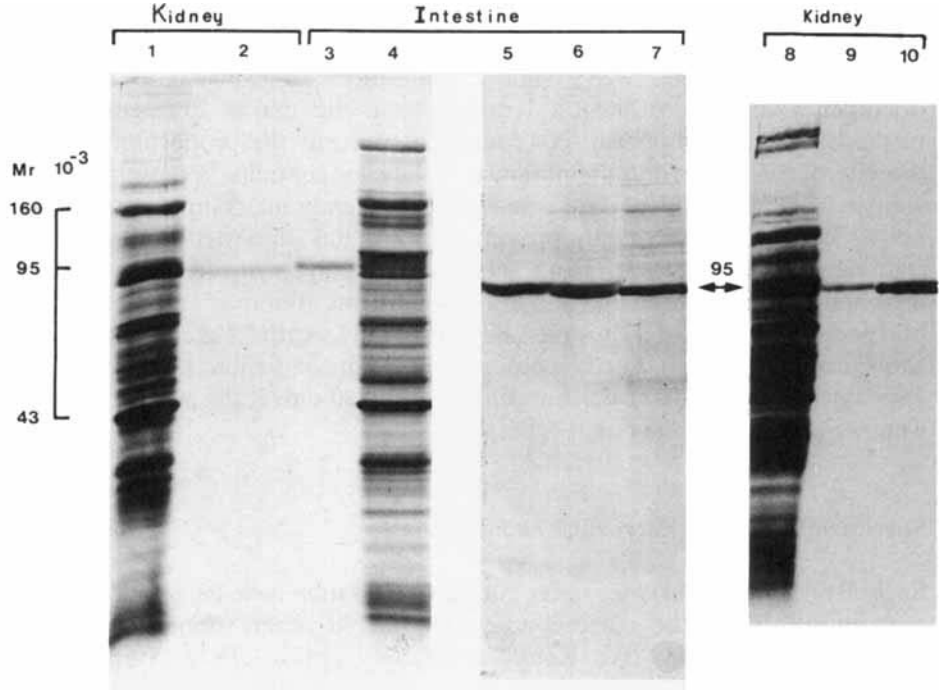

FIG. 2. Sodium dodecyl sulphate/polyacrylamide gel electrophoresis of microvilli and purified endopeptidase. 7–17% acrylamide gradient gels were used and the polypeptides were revealed by Coomassie blue. Lanes: 1, kidney microvilli; 2, kidney d-form (detergent-solubilized); 3, intestine d-form; 4, intestine microvilli; 5, intestine d-form; 6, intestine d-form + dt-form (d-form, trypsin-treated); 7, intestine dt-form; 8, kidney microvilli; 9, kidney dt-form; 10, kidney d-form.

acid (TCA)-soluble fragments from the initial cleavage products of the endopeptidase. The pure enzyme releases only half the substrate radioactivity in a TCA-soluble form. Crude extracts containing exopeptidases can achieve total conversion. This limitation of the assay affects the calculation of the enrichment factor in the purification. The rabbit kidney enzyme was purified 250-fold over the homogenate. The purification of the pig kidney enzyme has given a value of 135, which, if corrected for the effect of aminopeptidases in the homogenate, should be 1.5–2.0 times higher, i.e. a value in the range 200–270. Our results contrast sharply with the purification factor of 4200 obtained by Mumford et al (1981). Their assay substrate was a fluorogenic compound, the succinyl–Ala–Ala–Phe derivative of 7-amino-4-methyl-coumarin (a compound that is comparable to the peptide naphthyl-amide that we have used for histochemical purposes). They also reported a

recovery of 870%, which they attributed to unspecified inhibitors in the cruder fractions. We have never observed this phenomenon in any purification (now numbering twelve) and cannot offer an explanation for the discrepancy, except to suggest it arises from the use of different assay methods. A more important consideration concerns the proportion of the proteins of the microvillus membrane that the endopeptidase represents. Our view is that the endopeptidase is a significant membrane component contributing 3–5% of the microvillus protein, a view that is supported by the strong staining of the 93 000 M_r band seen in SDS-polyacrylamide gels of the membrane. Extraction by non-ionic detergents removes most of the polypeptides migrating in this region, leaving only a cytoskeletal polypeptide (presumed to be villin). Such an observation is not compatible with a genuine enrichment value of 4000 which would be observed only if the endopeptidase were a very minor component.

Species differences in microvillar endopeptidases

Several microvillar enzymes so far purified from rabbit have been found to be monomeric, while the corresponding enzyme in other species has been dimeric (for examples, see Kenny & Maroux 1982). The endopeptidase follows the same rule. However, a more significant difference is that some species possess a second endopeptidase. In rat microvilli, only half the endopeptidase activity (towards [^{125}I]insulin B chain) can be inhibited by phosphoramidon (Kenny et al 1981). This contrasts with microvilli from the kidneys of pig, rabbit and human. Mouse kidney microvilli also contain a phosphoramidon-insensitive endopeptidase (Kenny et al 1981). Indeed, the metalloendopeptidase purified from mouse kidneys (Beynon et al 1981) is

TABLE 1 Enrichment values of peptidases in microvillus preparations from rat and mouse kidneys

Enzyme	*Rat*	*Mouse*
Aminopeptidase N (EC 3.4.11.2)	12.9	15.8
Dipeptidyl peptidase IV (EC 3.4.14.5)	12.3	13.3
Endopeptidase I (EC 3.4.24.11, phosphoramidon-sensitive)	11.2	18.5
Endopeptidase II (phosphoramidon-insensitive)	9.2	15.4

The microvillus fraction was prepared and the peptidases were assayed as previously described (Booth & Kenny 1974). For endopeptidase II the assays were done in the presence of 1 μM-phosphoramidon.

most likely to be of microvillar origin. Both endopeptidases I and II have enrichment values comparable to other microvillar enzymes found in preparations of microvilli from rat and mouse kidneys (Table 1). The phosphoramidon-insensitive enzyme (endopeptidase II) is readily released from rat kidney microvilli by a papain treatment, and we have achieved a partial purification (100 times) of the enzyme after solubilization by this means. We found that at each chromatographic step, the hydrolysis of azocasein followed that of the [^{125}I]insulin B chain assay. At present we are in the process of obtaining a specific antiserum, by the same approach that we have used for the pig endopeptidase.

Molecular properties of the endopeptidase from pig kidney and intestine

In all species examined the endopeptidase is unusual, although not unique, in resisting release from the microvillus membrane by treatment with proteinases. The rabbit enzyme was obtained in a hydrophilic form only after prolonged stirring with toluene followed by a lengthy incubation with trypsin, and it was a monomeric glycoprotein, of M_r 93 000, which showed no amphipathic properties. Now that we have purified the pig kidney enzyme after detergent solubilization it is possible to define the differences between it and the proteinase-treated forms. We have now studied three structurally distinct forms of the active enzyme from kidney: (1) the detergent-solubilized form (d-form); (2) the detergent form also treated with trypsin (dt-form); and (3) a form that was solubilized by the use of toluene and trypsin (tt-form). The d- and dt-forms have many physical properties in common. Both require the presence of detergent to prevent aggregation, both show hydrophobic binding to octyl-Sepharose (Table 2) and phenyl-Sepharose (not shown) and both have generally similar M_r values by gel filtration (Fig. 3) and SDS-polyacrylamide gel electrophoresis (Fig. 2). The d- and dt-forms of the kidney enzyme yielded mean M_r values of 330 000 on Ultrogel AcA 34, but there was

TABLE 2 Hydrophobic chromatography of different forms of pig kidney endopeptidase

Form of the enzyme	*Binding to octyl-Sepharose 4B*	
	Bound and eluted (%)	*Unbound (%)*
Detergent-solubilized (d)	85	15
Detergent–trypsin-solubilized (dt)	74	26
Toluene–trypsin-solubilized (tt)	10	90

Enzyme samples were loaded in 150 mM-NaCl, 10 mM-sodium phosphate buffer at pH 7.4, with 0.1% (w/v) Triton X-100 and washed with the same medium without detergent. Elution medium was 1 mM-sodium phosphate at pH 7.4, with 2.5% (w/v) Triton X-100.

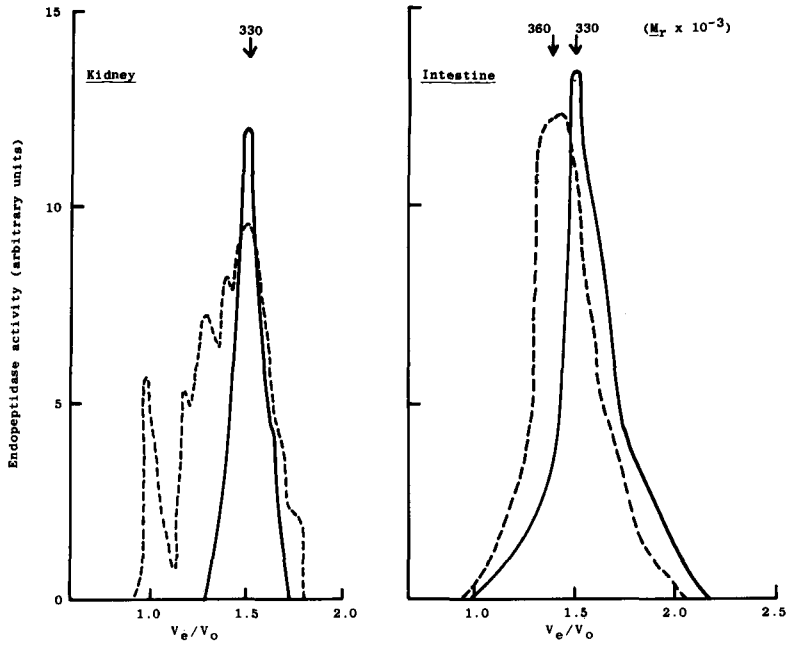

FIG. 3. Gel filtration on Ultrogel AcA 34 of endopeptidase from kidney and intestine. Dotted line, d-form; solid line, dt-form. V_e, elution volume; V_o, void volume.

a marked change in peak width after trypsin treatment. The d-form was polydisperse in its behaviour; the dt-form seemed to be monodisperse. We have observed this effect of trypsin repeatedly with different batches. Yet trypsin has little effect on the mobility in SDS-polyacrylamide gel electrophoresis (Fig. 2, lanes 9, 10). Thus, if there is a reduction in size as a result of trypsin treatment, it is not easily quantifiable, nor is it possible to resolve the two forms when they are run in the same track (not shown). Trypsin may cleave a small fragment from one end of the polypeptide chain. This fragment is presumably strongly hydrophobic and is capable of binding variable amounts of detergent. The estimated M_r of 330 000 is consistent with a dimeric protein that binds Triton X-100, thereby contributing about 150 000 to the M_r—a value which is higher than necessary for a simple micelle (Helenius & Simons 1975).

The intestinal form of the enzyme has a slightly different response to trypsin treatment (Fig. 3). The d-form has an M_r of 360 000 and the dt-form is significantly smaller (330 000). More surprisingly, the apparent M_r on SDS-polyacrylamide gel electrophoresis is demonstrably changed by trypsin and,