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Proteins of the Kidney Microvillar Membrane

ASYMMETRIC LABELLING OF THE MEMBRANE BY LACTOPEROXIDASE-CATALYSED RADIOIODINATION AND BY PHOTOLYSIS OF 3,5-DI[^{125}I]IODO-4-AZIDOBENZENESULPHONATE

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Two methods were used to label pig kidney microvillar membrane proteins from the luminal and cytoplasmic surfaces of closed membrane vesicles. The first method was lactoperoxidase-catalysed radioiodination. The enzyme reagents, lactoperoxidase and glucose oxidase, were positioned inside the vesicles before sealing or externally after sealing, iodination being initiated by the subsequent addition of glucose and $^{125}I^-$. After resolution of the labelled proteins by electrophoresis in the presence of dodecyl sulphate, asymmetric labelling patterns on radioautographs were observed. However, the major disadvantage of this method is the high degree of intramembrane labelling of the fatty acid chains of membrane lipids, a reaction that undermines any conclusions about the location of the label in that region of the protein supposedly exposed at the surface of the membrane. The second method overcame this disadvantage. A new hydrophilic photoreagent, 3,5-di[^{125}I]iodo-4-azidobenzesulphonate, was synthesized via the intermediate, diazotized 3,5-di[^{125}I]iodosulphanilic acid. It was transported by a Na^+-dependent system into microvillar vesicles, thus permitting labelling from either side of the membrane when the vesicles were photolysed. The labelling of membrane lipids was less than with the first method and was essentially confined to the polar headgroups. The activity of several microvillar peptidases survived the labelling reaction and they could be identified in the immunoprecipitates after resolution of the detergent-solubilized membrane proteins by crossed-immunoelectrophoresis. Treatment with papain converted the detergent-solubilized form of susceptible enzymes into the proteinase-solubilized form, which lacked the intramembrane domain and any portion exposed at the cytoplasmic surface. Radioautography established that aminopeptidases M and A, dipeptidyl peptidase IV and neutral endopeptidase were transmembrane proteins. This novel approach to the investigation of membrane topology may be applicable to other complex membranes.

All the enzymes known to be associated with the microvillar membrane of the kidney proximal tubule are intrinsic proteins, i.e. anchored in the membrane by a hydrophobic domain (for review, see Kenny & Booth, 1978). Their release requires treatment of the membrane with detergents, or a proteinase such as papain. The hydrophobic anchor is a relatively small portion of the molecule, at least in those enzymes so far studied. Thus dipeptidyl peptidase IV (EC 3.4.14.–, or 3.4.21.–) is a dimer with subunits of 130000 mol.wt., but the portion remaining associated with the membrane, after cleavage with papain, is no more than 4000 mol.wt. and includes the *N*-terminal amino acid sequence of the polypeptide chain (Macnair & Kenny, 1979). Other microvillar enzymes from both renal and intestinal brush borders seem to exhibit a similar topology, e.g. aminopeptidase M (EC 3.4.11.2; Maroux *et al.*, 1973; Maroux & Louvard, 1976; Vannier *et al.*, 1976), isomaltase (EC 3.2.1.10; Brunner *et al.*, 1979) and other maltases (Maroux & Louvard,

Abbreviations used: DDISA, diazotized 3,5-di-iodosulphanilic acid; DIABS, 3,5-di-iodo-4-azidobenzenesulphonate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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1976). The intramembrane domains of several kidney microvillar enzymes have been labelled by a hydrophobic photoreactive reagent 1-azido-4-[^{125}I]-iodobenzene (Booth *et al.*, 1979). However, with one exception, it is not known if the polypeptide chain containing the hydrophobic anchor of these enzymes spans the lipid bilayer so as to be accessible from the cytoplasmic surface of the microvillar membrane. Louvard *et al.* (1976), using a macromolecular photolabel, 3-nitrophenyl azide coupled to a human myeloma Fab fragment, were able to show that the intestinal aminopeptidase M was a transmembrane protein. So far, this has been the only microvillar enzyme to be characterized in this way.

In the present paper we have compared two methods, each of which can be exploited to label the microvillar membrane from one or other surface. The first method, lactoperoxidase iodination, has been widely used for labelling plasma-membrane proteins. For example, Hubbard & Cohn (1975) labelled the external surface of mouse L cells, Boxer *et al.* (1974) labelled erythrocyte 'ghost' membranes, Walsh & Crumpton (1977) labelled both surfaces of lymphocyte plasma-membrane vesicles and Schmidt-Ullrich *et al.* (1978) both surfaces of thymocyte plasma membranes. In the case of microvilli, it is possible to label the cytoplasmic surface because the enzyme components of the labelling system can be allowed to penetrate the interior of microvilli before they seal as closed right-side-out vesicles. The second method involves a novel photolabelling reagent, 3,5-di[^{125}I]iodo-4-azidobenzenesulphonate, for which microvilli possess a Na^+-dependent transport system, which enables the reagent to penetrate after the vesicles are sealed. In each method the membrane proteins, after labelling, are analysed electrophoretically, in the presence of dodecyl sulphate in the case of the iodinated proteins and by crossed-immunoelectrophoresis in the case of the photolabelled proteins. The latter method has permitted a positive identification of several antigens as transmembrane proteins and, within limits, is a method of general application to other complex membranes. It has an important advantage over lactoperoxidase iodination in that it does not label components within the membrane. Some preliminary results of lactoperoxidase iodination have been previously reported (Kenny *et al.*, 1977, 1978).

Experimental

Pig kidneys were given by Asda Farmstores, from their slaughterhouse at Lofthousegate, West Yorkshire, U.K., and were used for membrane preparations within 1h of the death of the animal. 2,2'-Azino-di-(3-ethylbenzthiazolinesulphonate) was from BCL, Lewes, East Sussex BN7 1LG, U.K. Sulphanilic acid and BF_3–methanol complex were from BDH Chemicals, Poole, Dorset, BN12 4NN, U.K. Lactoperoxidase was a gift from Dr. C. F. Louis (University of Connecticut Health Centre, Farmington, CT 06032, U.S.A.). The sources of other materials including specific antisera have been given previously (Booth & Kenny, 1976*a*,*b*; Booth, 1977; Booth *et al.*, 1979).

Membrane preparations

Kidney brush borders were prepared by the method of Wilfong & Neville (1970). Microvilli were prepared in two ways: either by the method described previously (Booth & Kenny, 1974), in which case they are referred to as microvilli, or by homogenization of brush borders (Booth & Kenny, 1976*b*), in which case they are referred to as brush-border-derived vesicles. Protein was determined as previously described (Macnair & Kenny, 1979).

^{32}P-labelled membranes

Brush-border-derived vesicles, before being sealed (25mg of protein), were resuspended in 5ml of 0.1M-NaCl/50mM-sodium phosphate, pH7.4. After the addition of 100μl of 1mM-di-isopropyl [^{32}P]phosphorofluoridate (10μCi) in propan-2-ol, the suspension was incubated at 37°C for 1h. The membranes were then pelleted at 26000*g* for 30min in a Sorvall RC-5 centrifuge and washed four times by repeated resuspension in 5ml of 0.1M-NaCl/50mM-sodium phosphate, pH7.4, and centrifugation as above.

Lactoperoxidase-catalysed radioiodination of brush-border-derived membrane vesicles

The iodination system was modified from that described by Hubbard & Cohn (1975). Brush-border membranes were iodinated from their cytoplasmic surface by resuspending brush borders (25mg of protein) in 5ml of a medium containing lactoperoxidase (100μg/ml) and glucose oxidase (2μg/ml) in 0.1M-NaCl/50mM-sodium phosphate, pH7.4. After incubation at 4°C for 18h, the brush borders were homogenized for 1min with an Ultra-Turrax homogenizer (Janke and Kunkel KG, Staufen, West Germany). The homogenate was centrifuged at 1500*g* for 12min to remove any unhomogenized brush borders. The supernatant (containing microvilli) was incubated at 37°C for 30min to produce sealed right-side-out membrane vesicles (Booth & Kenny, 1976*b*). The vesicles, containing entrapped lactoperoxidase and glucose oxidase, were sedimented at 26000*g* for 30min and washed four times with the same NaCl/phosphate medium, free of enzymes. They were finally resuspended in this medium to a protein concentration of 5mg/ml. The iodination reaction was started by

adding carrier-free $Na^{125}I$ (500μCi/ml) and glucose (final concentration, 20mM) to the medium. After incubation at room temperature for 30min, the reaction was stopped by adding NaN_3 to a final concentration of 0.1M. The microvilli were then washed four times with 0.1M-NaCl/50mM-sodium phosphate, pH7.4, and resuspended in the same medium to a protein concentration of 5mg/ml.

The membranes were labelled from their luminal surface by a similar procedure. The initial resuspension medium contained neither lactoperoxidase nor glucose oxidase. These enzymes were added to the medium containing the sealed vesicles immediately before the $Na^{125}I$ and glucose. The membranes were labelled from both sides by following the procedure for labelling the cytoplasmic surface and adding the enzymes again later in the procedure as described for labelling of the luminal surface.

Preparation of [^{125}I]DDISA

This was prepared by a modification of the method described by Helmkamp & Sears (1970). 3,5-Di-iodosulphanilic acid was prepared as described in 90% yield from sulphanilic acid. 3,5-Di-iodosulphanilic acid (8.5mg, 20μmol) was dissolved in 0.5ml of 0.1M-$NaHCO_3$. $Na^{125}I$ was then added (2mCi in 20μl) and the solution was made slightly acid by the addition of 100μl of 0.5M-HCl. The radioactive-isotope-exchange reaction was started by the addition of 50μl of ICl reagent (1.84mM-KI/1.49mM-KIO_3/1.38M-NaCl/1.7M-HCl). After standing at room temperature for 15min, the mixture was cooled to -5°C on ice/salt, and 200μl of 0.8% (w/v) $NaNO_2$ was added in portions of 20μl. Yellow crystals of the internal diazonium salt rapidly formed, and, after 15min, the crystals were sedimented at 0°C, at 1000*g* for 5min and washed twice with 0.5ml portions of ice-cold water. They were finally sedimented again, and 300μl of the supernatant was removed and stored at 4°C. This supernatant was assumed to be a saturated solution, approx. 1.3mM (Sears *et al.*, 1971). The sediment, containing on average 15.5μmol of product, as estimated by the method of Edwards *et al.* (1979), and 75% of the radioactivity, was resuspended in the remaining 200μl of supernatant and used for the preparation of [^{125}I]DIABS.

Preparation of [^{125}I]DIABS

The suspension of [^{125}I]DDISA was transferred on ice to a dark room illuminated with an Ilford ISO 906 safe-light. The diazonium salt was converted to the azide by the addition of 300μl of 0.16M-NaN_3 in portions of 30μl. When the effervescence due to liberated N_2 had subsided, the colourless solution of [^{125}I]DIABS was transferred to a light-proof container and stored at 4°C. The concentration of this solution was taken as 30mM. The preparation of [^{125}I]DIABS from sulphanilic acid is shown in Scheme 1.

Labelling of microvilli with [^{125}I]DDISA

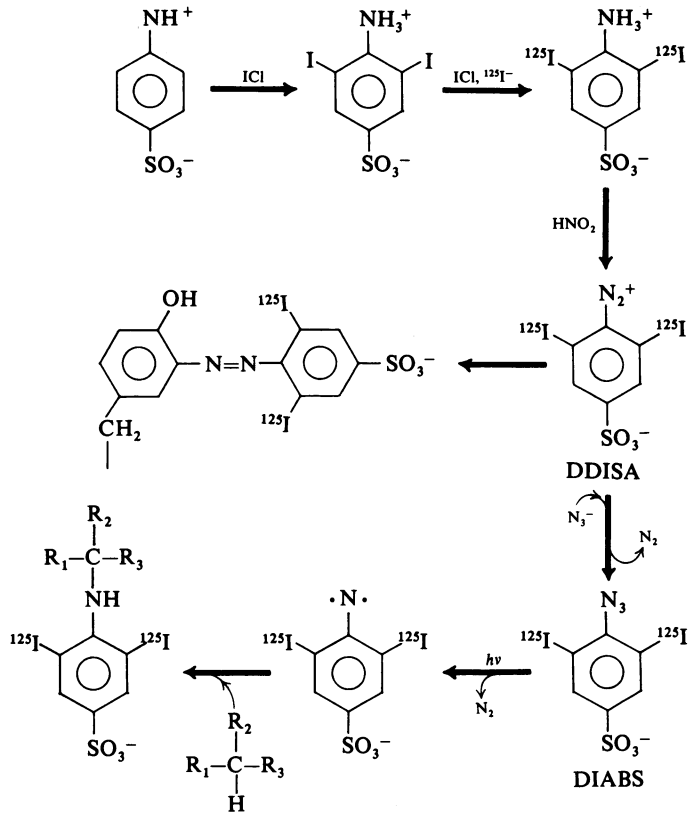
Microvilli (10mg of protein) were resuspended in 2ml of 0.1M-NaCl/10mM-sodium thiosulphate/50mM-sodium phosphate, pH7.4, and kept on ice. [^{125}I]DDISA (100μl of the saturated solution) was added and, after standing on ice for 20min, the suspension was centrifuged for 15min at 25000*g* in a Heraeus Christ Mikrohämatocrit centrifuge. The pellets were washed four times with 2ml of bovine serum albumin (10mg/ml) in 0.1M-NaCl/10mM-sodium thiosulphate/50mM-sodium phosphate, pH 7.4, and resuspended in 0.1M-Hepes/NaOH, pH6.8, to give a final protein concentration of 5mg/ml.

Photolabelling of microvilli with [^{125}I]DIABS

All manipulations before photolysis were performed under the same red safe-light. The membrane was labelled from its cytoplasmic surface by resuspending microvilli (10mg of protein) in 2ml of 0.1M-NaCl/10mM-sodium thiosulphate/50mM-sodium phosphate, pH7.4. [^{125}I]DIABS (200μl of the 30mM solution) was added and the suspension was incubated, in the dark, at 37°C for 90min. After cooling on ice, the microvilli were sedimented at 26000*g* for 30min in a Sorvall RC-5 centrifuge and washed twice with 4ml of ice-cold bovine serum albumin (10mg/ml) in 0.1M-NaCl/10mM-sodium thiosulphate/50mM-sodium phosphate, pH7.4, and finally resuspended in 2ml of this medium. The stirred suspension was illuminated on ice for 1h as previously described (Booth *et al.*, 1979). After photolysis, the microvilli were washed four times with bovine serum albumin (10mg/ml) in 0.1M-NaCl/10mM-sodium thiosulphate/50mM-sodium phosphate, pH7.4, and finally resuspended in 0.1M-Hepes/NaOH, pH6.8, to give a final protein concentration of 5mg/ml. Labelling from the luminal surface of the membrane was achieved by incubating the microvilli (2ml of albumin-free medium containing 10mg of microvillar protein) in the absence of photolabel, at 37°C, for 90min. After cooling the suspension on ice, the [^{125}I]DIABS was added (20μl of the 30mM solution). The suspension was then photolysed, washed and resuspended in Hepes/NaOH buffer as above.

Determination of the distribution of ^{125}I radioactivity in labelled membranes

Radioactively labelled microvilli or brush-border-derived membrane vesicles (5mg of protein in 1ml of either 0.1M-Hepes/NaOH, pH6.8 or 0.1M-NaCl/50mM-sodium phosphate, pH7.4) were shaken with 19ml of chloroform/methanol (2:1,



Scheme 1. *Preparation of [^{125}I]DDISA and [^{125}I]DIABS from sulphanilic acid and their reactions with amino acid residues in proteins*

v/v). After centrifugation to separate the phases, samples of the aqueous and organic layers were carefully removed without disturbing the protein precipitated at the interphase. The protein was removed, washed with chloroform, dissolved in 2ml of 10% (w/v) sodium dodecyl sulphate and counted in a Packard Auto-Gamma spectrometer. The aqueous layer contained no protein and very little radioactivity and was discarded. After counting a portion, the organic layer was dried down under a stream of N_2 and the lipids were subjected to methanolysis as described by Morrison & Smith (1964). The dried lipids were dissolved in 1.5ml of BF_3–methanol and the container was sealed under N_2. Methanolysis was allowed to proceed at 100°C for 2h, after which the container was cooled and opened. Pentane (3ml) and water (1.5ml) were added, the mixture was shaken briefly and then centrifuged to separate the phases. Samples of the organic layer (containing fatty acid methyl esters) and the aqueous layer (containing the components of the lipid headgroups) were assayed for ^{125}I radioactivity.

Membrane transport of [^{125}I]DIABS

All manipulations were performed under a red safe-light. Microvilli were resuspended in 100mM-mannitol/20mM-Tris/Hepes, pH 7.4, or the same buffer containing 100mM-NaCl. After equilibration at 37°C for 30min, the suspension was kept at 22°C until use. Transport of the photolabel was studied by using the apparatus described by Kessler *et al.* (1978). The method used was that previously described for the transport of L-leucine and D-glucose by microvilli (Kenny & O'Halloran, 1979), except that the reaction mixture contained a final concentration of 200μM-[^{125}I]DIABS instead of the amino acid or glucose and the wash solution contained 200μM-3,5-di-iodosulphanilic acid.

Electrophoresis of membrane proteins in organic base dodecyl sulphates

Brush-border-derived membrane vesicles in 0.1M-NaCl/50mM-sodium phosphate, pH 7.4, were solubilized by the addition of an equal volume of 0.14M-4-picolinium dodecyl sulphate/0.14M-4-pico-

line containing 2% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol. After incubation at 100°C for 3 min, the samples were cooled and applied to polyacrylamide gels, containing organic base dodecyl sulphates, previously described as the second dimension of a two-dimensional electrophoretic system (Booth, 1977).

Crossed-immunoelectrophoresis

The methods for preparation of Emulphogen BC 720-solubilized microvillar proteins, their treatment with papain and their resolution by crossed-immunoelectrophoresis have been described previously (Booth *et al.*, 1979). The detection of enzyme activity on the gels was also as described in that paper, except that 2,2′-azino-di-(3-ethylbenzthiazolinesulphonate) was substituted for *o*-dianisidine.

Radioautography

This was performed with Kodak Kodirex X-ray film.

Assay of radioactivity

Samples containing ^{32}P were counted in the ^{3}H channel of a Beckman LS 230 liquid-scintillation counter by means of its Čerenkov radiation in water. ^{125}I was counted in a Packard Auto-Gamma spectrometer.

Results and Discussion

When establishing whether or not a membrane protein spans the lipid bilayer, it is very important to show that the labelling system used produces no labelling at the face of the membrane opposite from the one supposedly being labelled, i.e. that there is essentially no contramembrane labelling. Similarly, there should be no labelling within the lipid bilayer of the membrane, i.e. no intramembrane labelling. If the method permits the occurrence of either contra- or intra-membrane labelling, then proteins located exclusively on one side of the membrane or proteins that impinge into the lipid bilayer, but do not span it, will appear, artifactually, to be transmembrane in nature.

Membrane labelling by lactoperoxidase-catalysed radioiodination

Lactoperoxidase-catalysed radioiodination produces little contramembrane labelling at low concentrations of iodide. Brush-border-derived membrane vesicles were examined by two different approaches after lactoperoxidase-catalysed radioiodination from either surface of the membrane.

In the first type of experiment, the active site of the microvillar serine enzyme, dipeptidyl peptidase IV, was affinity-labelled by incubating the membranes with di-isopropyl [^{32}P]phosphorofluoridate. After radioiodination, the membrane proteins were solubilized with either Lubrol WX or by treating the membrane vesicles with papain. The solubilized dipeptidyl peptidase IV was precipitated with a specific antibody. The immunoprecipitate was resolved into its components by electrophoresis in organic base dodecyl sulphates and the stained band containing the dipeptidyl peptidase IV (subunit mol.wt. 130000) was excised from the gels and counted for ^{32}P and ^{125}I. Dipeptidyl peptidase IV, like several other microvillar enzymes, is anchored into the membrane by a short hydrophobic region of the polypeptide chain located at the *N*-terminus (Macnair & Kenny, 1979). The remainder of the molecule, including the active site, is located on the luminal surface of the membrane. Papain cleaves the hydrophilic portion from the hydrophobic anchor peptide, so that in this experiment, the $^{125}I/^{32}P$ ratio of the papain-solubilized enzyme will give a measure of the incorporation of ^{125}I into that part of the molecule exposed at the luminal surface of the membrane, and the $^{125}I/^{32}P$ ratio of the detergent-solubilized enzyme will give a measure of the incorporation of ^{125}I into the whole molecule.

The results of two such experiments are shown in Table 1. When the membrane was radioiodinated at the luminal surface, there was little difference between the $^{125}I/^{32}P$ ratios of the detergent- and papain-solubilized forms of the enzyme. However, when the radioiodination was performed at the cytoplasmic surface of the membrane the $^{125}I/^{32}P$ ratio of the papain-solubilized form was only about 5% of that of the detergent-solubilized form. This value can be taken as the maximum amount of contramembrane labelling obtained when the membrane was labelled at its cytoplasmic surface. Alternatively, if one assumes that the labelling species cannot migrate across the membrane, then this figure represents the maximum proportion of either 'inside-out' or unsealed vesicles in the preparation.

In the second type of experiment, the proteins of radioiodinated brush-border-derived vesicles were subjected to electrophoresis in organic base dodecyl sulphates, followed by radioautography of the dried gels. The vesicles were labelled from the luminal surface, the cytoplasmic surface and both surfaces of the membrane, as described in the Experimental section, except that cytochrome *c* (final concentration 100μg/ml) was added as an external marker protein, together with the glucose and $Na^{125}I$, after the vesicles were sealed. The vesicles were not washed after the radioiodination reaction, but were immediately solubilized and applied to the gels.

Two controls were included in this experiment. The first was to check that the labelling enzymes did not adsorb on to the luminal surface of the membrane. If this occurred it could produce con-

Table 1. *Radioactivity in dipeptidyl peptidase IV after lactoperoxidase-catalysed radioiodination of brush-border-derived vesicles*

See the Experimental and Results and Discussion sections for details. Dipeptidyl peptidase IV was affinity-labelled with di-isopropyl [^{32}P]phosphorofluoridate. Thus ^{32}P radioactivity gives a measure of the recovery of this enzyme. After radioiodination at either the cytoplasmic or luminal surfaces, the membrane proteins were solubilized with Lubrol WX or by papain. The latter treatment releases the bulk of the enzyme, including the affinity-labelled active site, by cleaving close to the luminal surface of the membrane. The results are shown as the ratio ^{125}I/^{32}P in the 130000-mol.wt. band (i.e. dipeptidyl peptidase IV) after immunoprecipitation and polyacrylamide-gel electrophoresis in the presence of dodecyl sulphate.

	^{125}I/^{32}P			
	Cytoplasmic		Luminal	
Experiment	Lubrol	Papain	Lubrol	Papain
1	1.6	0.11	7.1	7.7
2	0.52	0.025	2.8	2.8

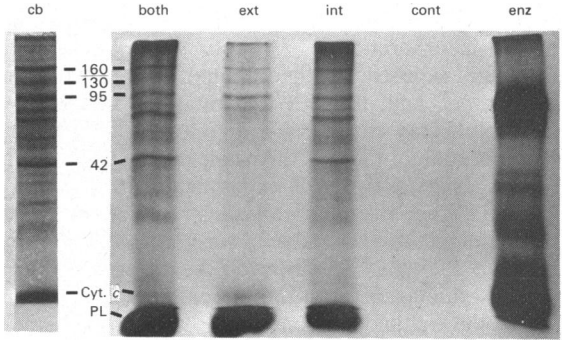


Fig. 1. *Radioautograph of lactoperoxidase-catalysed ^{125}I-labelled microvillar membrane proteins after polyacrylamide-gel electrophoresis in the presence of dodecyl sulphate*

See the Experimental and Results and Discussion sections for details. From left to right the tracks are: cb, the pattern of membrane proteins revealed by staining with Coomassie Blue; the numbers indicate mol.wt. ($\times 10^{-3}$); band 42 is actin; Cyt *c* indicates cytochrome *c* added as an external marker protein. The remaining tracks are revealed by radioautography; both, labelling at cytoplasmic and luminal surfaces; ext, labelling at the luminal surface; int, labelling at the cytoplasmic surface; cont, control (labelling procedure as for ext, but a washing step inserted between addition of enzymes and substrates); enz, autolabelled glucose oxidase, lactoperoxidase and cytochrome *c* (membrane vesicles omitted); PL indicates radioactivity probably associated with membrane lipids.

tramembrane labelling when the membrane was supposedly being labelled from the cytoplasmic surface. A sample of vesicles was sealed, as described in the Experimental section, and then incubated with lactoperoxidase and glucose oxidase. After washing, the vesicles were exposed to glucose, Na^{125}I and cytochrome *c*. After incubation at room temperature, NaN_3 was added and the vesicles were prepared for electrophoresis. The second control was designed to identify the positions on the gels of labelled cytochrome *c* and self-labelled lactoperoxidase and glucose oxidase. This was simply achieved under the same conditions as the labelling of the luminal surface of the membrane, but in the absence of membranes.

The results of such an experiment are shown in Fig. 1. The labelling of two proteins, actin and cytochrome *c*, support the conclusion that negligible contramembrane labelling occurred during this experiment. Actin (subunit mol.wt. 42000) is the major component of the kidney microvillar core (Booth & Kenny, 1976*a*). Since the brush-border-derived vesicles used in this experiment were sealed and 'right-side-out', the actin they contain may be taken as a marker protein for the vesicle interior. The cytochrome *c* added to the medium may be taken as a marker protein for the vesicle exterior. The track labelled 'int' shows that when the membrane was labelled from the cytoplasmic surface, radioactivity was found in the microvillar actin, but not in the cytochrome *c*. Similarly, when the membrane was labelled from the luminal surface ('ext'), radioactivity was found in the cytochrome *c*, but not in the actin. Clearly, contramembrane labelling did not occur to any detectable extent. As expected, when both surfaces of the membrane were labelled ('both'), radioactivity was detected in both marker proteins. The track containing vesicles exposed to the labelling enzymes, followed by washing and exposure to glucose, Na^{125}I and cytochrome *c* ('cont') contained no radioactivity. This confirmed the efficiency of the washing procedure and also showed that non-enzymic radioiodination did not occur. Preliminary experiments

had earlier shown some degree of contramembrane labelling of actin (Kenny *et al.*, 1977, 1978). However, most of the contramembrane-labelled actin was shown to be on the luminal surface, since it was susceptible to attack by thrombin. In the present experiments we avoided the use of sucrose-containing media for the washing steps, and this modification appeared to have minimized the artifact. Several membrane proteins, including aminopeptidase M (subunit mol.wt. 160000; Booth & Kenny, 1976*a*), were radioiodinated from either side of the membrane. However, the transmembrane nature of these proteins could not be taken as established from these experiments because, as shown below, the radioiodination method used produced considerable intramembrane labelling.

Lactoperoxidase-catalysed radioiodination produces considerable intramembrane labelling at low concentrations of iodide. When brush-border-derived vesicles were radioiodinated from either side of the membrane, considerable amounts of radioactivity were detected on radioautographs of polyacrylamide gels, running slightly ahead of the dye front (Fig. 1). This radioactivity could not be dismissed as being due to free $^{125}I^-$, as the dye front in this electrophoretic system moves with a mobility slightly less than that of dodecyl sulphate micelles (Booth, 1977). Hence free iodide from the sample, having an electrophoretic mobility considerably greater than the detergent micelles, crosses the moving boundary at the dye front and rapidly runs off the gel into the lower buffer reservoir. Furthermore, this radioactive material must be microvillar in origin, as it was not present in the control sample from which the vesicles were omitted (Fig. 1, 'enz'). From its mobility, this material was tentatively identified as mixed micelles of dodecyl sulphate and membrane lipids.

To examine whether membrane lipids were labelled during the radioiodination reaction, vesicles previously labelled at their luminal surface were extracted with chloroform/methanol as described in the Experimental section. It was found that 57% of the membrane-bound radioactivity was extracted into the organic solvent (Table 2). Further treatment of the extracted lipids with BF_3–methanol showed that 93% of the radioactivity bound to these lipids was located in their non-polar regions. Clearly, under the conditions used, considerable intramembrane labelling occurred.

Iodine produced during lactoperoxidase-catalysed radioiodination can cause contramembrane labelling. The intramembrane labelling must be caused by $^{125}I_2$, produced during the labelling reactions, which diffuses into the membrane where it labels unsaturated lipids. Presumably, the lack of contramembrane labelling must be due to these lipids acting as a 'sink' for the $^{125}I_2$. However, if the $^{125}I_2$

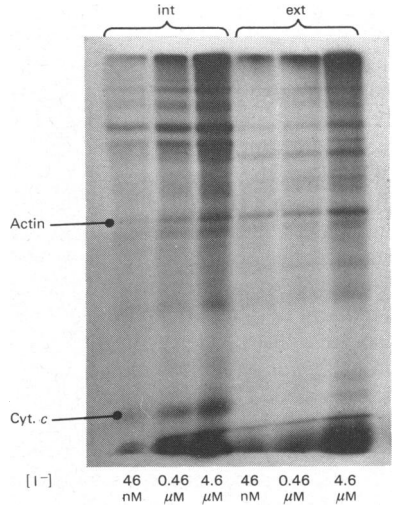


Fig. 2. *Radioautograph of lactoperoxidase-catalysed ^{125}I-labelled microvillar membrane proteins showing the effect of different concentrations of iodide*

The experiment is similar to that shown in Fig. 1, except that $Na^{127}I$ was added to the iodination mixture to give the concentrations of I^- shown. ext, labelling at the luminal surface; int, labelling at the cytoplasmic surface; bands corresponding to actin and cytochrome *c* (Cyt. *c*) are indicated.

Table 2. *Distribution of membrane-bound label* (%) *in kidney microvilli after photolabelling with [^{125}I]DIABS or lactoperoxidase-catalysed radioiodination*

See the Experimental section for details.

Fraction	Method of labelling ...	Distribution of label: DIABS, after photolysis	Distribution of label: Lactoperoxidase-catalysed radioiodination
Protein		78.4	42.9
Lipid (polar fraction)		19.9	4.1
Lipid (non-polar fraction)		1.7	53.0
Total lipid		21.6	57.1

were to be produced in sufficient quantity, then the lipid 'sink' might become filled. In this case $^{125}I_2$ would be able to diffuse across the membrane and produce contramembrane labelling.

This possibility was examined by varying the concentration of iodide in the labelling medium. Vesicles were radioiodinated from either side of the membrane, in the presence of cytochrome *c*, for 30 min. Carrier $Na^{127}I$ was added to the reaction medium to give total iodide concentrations of 46 nM, 0.46 μM or 4.6 μM. The labelled membranes were examined by electrophoresis in organic base dodecyl sulphates followed by radioautography of the dried gel. The result is shown in Fig. 2. Contramembrane labelling of actin and cytochrome *c* was observed under these conditions and increased with the total concentration of iodide in the labelling medium.

Photolabelling of microvilli with [^{125}I]DIABS

Although lactoperoxidase-catalysed radioiodination fails to satisfy the criteria required for a method capable of asymmetric labelling of membranes (i.e. no intramembrane and contramembrane labelling), it does, however, possess an important and useful feature. The labelling reaction is not started until after the reagent enzymes have been positioned on either or both sides of the membrane. The labelling system is inert until it is 'switched on' by the addition of glucose and $Na^{125}I$. Aromatic azides also have this property. In the dark they are stable and inert, but on illumination a highly reactive nitrene is generated. The nitrene has a lifetime in the range 0.1–1 ms (Reiser *et al.*, 1968) and will insert into carbon–hydrogen bonds. Hence the photolabelling of a membrane protein does not depend on the presence of given amino acid residues in the section of protein exposed to the label.

[^{125}I]DDISA, which has been shown to be a non-penetrant labelling reagent for the erythrocyte membrane (Sears *et al.*, 1971, 1977; Edwards *et al.*, 1979), is easily and cheaply made at high specific radioactivity (Helmkamp & Sears, 1970). Conversion of this compound to the corresponding azide has enabled us to investigate a new hydrophilic photolabelling reagent for membranes.

DIABS labels microvilli without significant loss of enzyme activities. In a preliminary experiment, [^{125}I]DIABS was prepared as described in the Experimental section and added in the dark, to a stirred suspension of microvilli. After various times of photolysis, samples were removed and kept on ice in the dark until the last sample had been removed. After washing, the incorporation of radioactivity into each membrane sample was determined. The results are shown in Fig. 3. Under the conditions used, the radioactivity incorporated into the vesicles increased with the time of illumination. After 1 h, the microvilli that had been illuminated contained 21

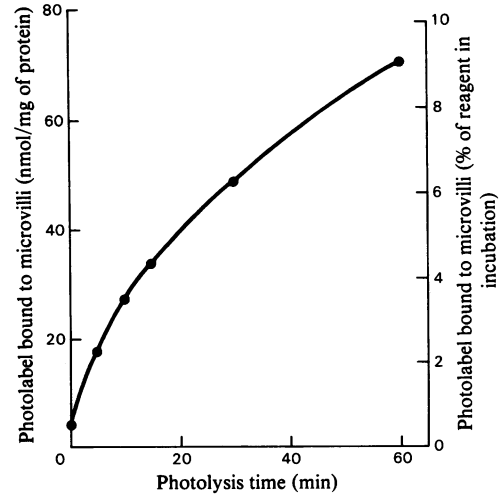


Fig. 3. *Incorporation of radioactivity into microvilli during photolysis of [^{125}I]DIABS*
See the Experimental and Results and Discussion sections for details. In this experiment [^{125}I]DIABS at a concentration of 3 mM was used.

times the radioactivity of an equivalent unilluminated sample. When the samples were assayed for aminopeptidase M, aminopeptidase A and dipeptidyl peptidase IV, it was found that the activities of these enzymes were unaffected by the photolysis reaction.

[^{125}I]DIABS produces relatively little intramembrane labelling. Microvilli were photolabelled as in the previous experiment, with 3 mM-[^{125}I]DIABS and then extracted with chloroform/methanol. The lipids were treated with BF_3–methanol as described in the Experimental section. The distribution of radioactivity in the fractions obtained is shown in Table 2. The proportion of the membrane-bound label incorporated into lipids (22%) was considerably less than that observed after lactoperoxidase-catalysed radioiodination (57%). Furthermore, only 8% of this lipid-bound radioactivity was found in the non-polar regions of the lipids. Hence, unlike lactoperoxidase-catalysed radioiodination, photolabelling with [^{125}I]DIABS produces relatively little intramembrane labelling. This distribution of label correlates well with the hydrophilic nature of the photolabel. The partition coefficient for DIABS between heptane and 0.1 M-NaCl, 50 mM-sodium phosphate, pH 7.4 is 0.014 at 25°C.

[^{125}I]DIABS is transported across the microvillar membrane by a Na^+-dependent system. Despite the evidence against any significant intramembrane labelling, contramembrane labelling, i.e. labelling of actin, was apparent when the proteins of

photolabelled microvilli were analysed by electrophoresis in dodecyl sulphate (result not shown). The microvilli used in these studies were prepared directly from cortex tissue (Booth & Kenny, 1974) without the intermediate step of preparing brush borders. Such preparations contain microvillar vesicles that are sealed and 'right-side-out' (Haase *et al.*, 1978). In order to produce contramembrane labelling, the photolabel must traverse the membrane. The sulphonate group might be expected to prevent its passage through the lipid bilayer, and indeed, the very low amount of intramembrane labelling (Table 2) supports this supposition. We therefore deduced that the transfer of the photolabel across the membrane was facilitated by a transport system.

Anion transport systems exist in the kidney brush-border membrane. Phosphate (Hoffmann *et al.*, 1976) and sulphate (Lücke *et al.*, 1979) are transported across the kidney brush-border membrane by Na^+-dependent systems. Presumably an analogous system was responsible for the transport of $^{125}I^-$ across the membrane to permit the radioiodination of the cytoplasmic face. The passage of [^{125}I]DIABS into kidney microvilli, in the presence and absence of an inward-directed Na^+ gradient, is shown in Fig. 4. The uptake of the photolabel was Na^+-dependent, showing an overshoot of accumulation of the reagent into the microvilli which subsided when the Na^+-gradient dissipated. The accumulation of label did not occur in the absence of an initial Na^+ gradient. It is interesting that another anionic photolabelling reagent, *N*-(4-azido-2-nitrophenyl)-2-aminoethyl[^{35}S]sulphonate is known to penetrate the erythrocyte membrane via an anion-transport protein (Staros *et al.*, 1975).

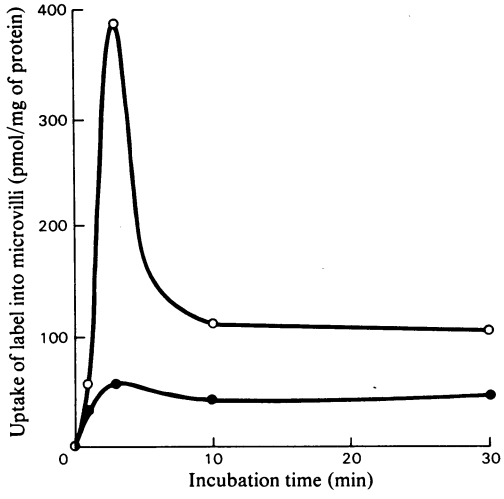


Fig. 4. *Uptake of [^{125}I]DIABS by sealed microvilli in the presence and absence of a Na^+ gradient*

See the Experimental section for details. O, 100mM-NaCl present externally at start of experiment; ●, microvilli preloaded by incubation for 60min with 100mM-NaCl; 200μM-[^{125}I]DIABS was used.

Transmembrane enzymes of the kidney microvillar membrane

The transport of [^{125}I]DIABS across the microvillar membrane is presumably a temperature-dependent process. We have observed that the contramembrane labelling produced when sealed microvilli are exposed to the reagent can be diminished by performing the photolysis on ice. Alternatively, the non-penetrant reagent, [^{125}I]DDISA, can be used to label the luminal surface of sealed microvilli.

The transport of DIABS across the membrane was used as the basis of a method for labelling the interior of sealed microvilli. Microvilli were incubated in the dark, at 37°C, with a high concentration of [^{125}I]DIABS (3mM). When the reagent had equilibrated across the membrane, the suspension was cooled to 0°C, washed in the dark and photolysed in the presence of an excess of bovine serum albumin. The microvillar interior was labelled by the entrapped DIABS, and contramembrane labelling caused by any efflux of DIABS from the microvilli was prevented by the inclusion of albumin in the photolysis medium.

The details of the three labelling methods are given in the Experimental section. The concentrations of the labelling reagents were chosen so that the amounts of radioactivity incorporated into the microvilli after washing were approximately the same in each case. The labelled microvillar proteins were examined by crossed-immunoelectrophoresis in non-ionic detergent. Compared with electrophoresis in dodecyl sulphate, this analytical technique has the advantage that enzymic activities are retained and the immunoprecipitates can be identified by histochemical staining techniques (Danielsen *et al.*, 1977; Booth *et al.*, 1979).

A preliminary experiment was designed to examine the fidelity of the three labelling methods, i.e. labelling the external (luminal) microvillar membrane surface with [^{125}I]DIABS or [^{125}I]DDISA and labelling of the internal (cytoplasmic) surface with [^{125}I]DIABS. Microvilli were labelled as described in the Experimental section and then solubilized with Emulphogen BC 720. A portion of the solubilized material was then treated with papain. The six samples thus generated were run in crossed-immunoelectrophoresis against a monospecific antibody directed against pig kidney aminopeptidase M. After staining, the gels were dried down and radioautographed. The result is shown in Fig. 5. The three precipitates containing the detergent-solubilized en-

zyme contained radioactivity, indicating the transmembrane nature of kidney aminopeptidase M.

Papain treatment cleaves the hydrophilic portion, originally exposed at the luminal surface and containing all the antigenic determinants, from the hydrophobic anchor and any contramembrane

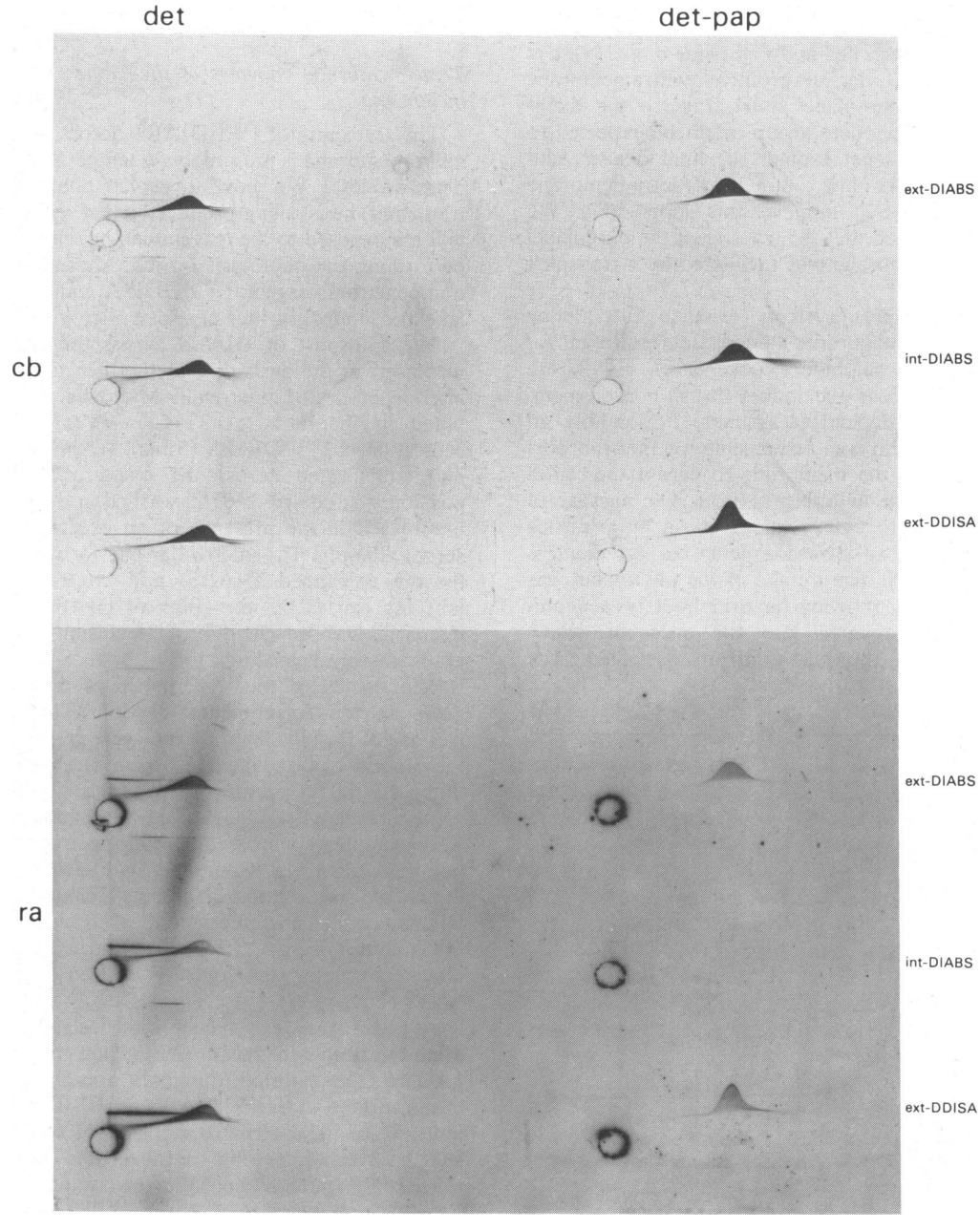


Fig. 5. *Crossed-immunoelectrophoresis of* [^{125}*I*]*DIABS- and* [^{125}*I*]*DDISA-labelled proteins using anti-(aminopeptidase M) antibody*

See the Experimental and Results and Discussion sections for details. After labelling microvilli with either [^{125}I]DIABS or [^{125}I]DDISA, the membrane proteins were solubilized with Emulphogen BC720 (det). A portion was further treated with papain (det-pap). Samples were then subjected to crossed-immunoelectrophoresis using a monospecific antibody raised to pig kidney aminopeptidase M. The gels were stained with Coomassie Blue (cb) and after drying a radioautograph was obtained (ra); ext, labelling from luminal surface; int, labelling from cytoplasmic surface.

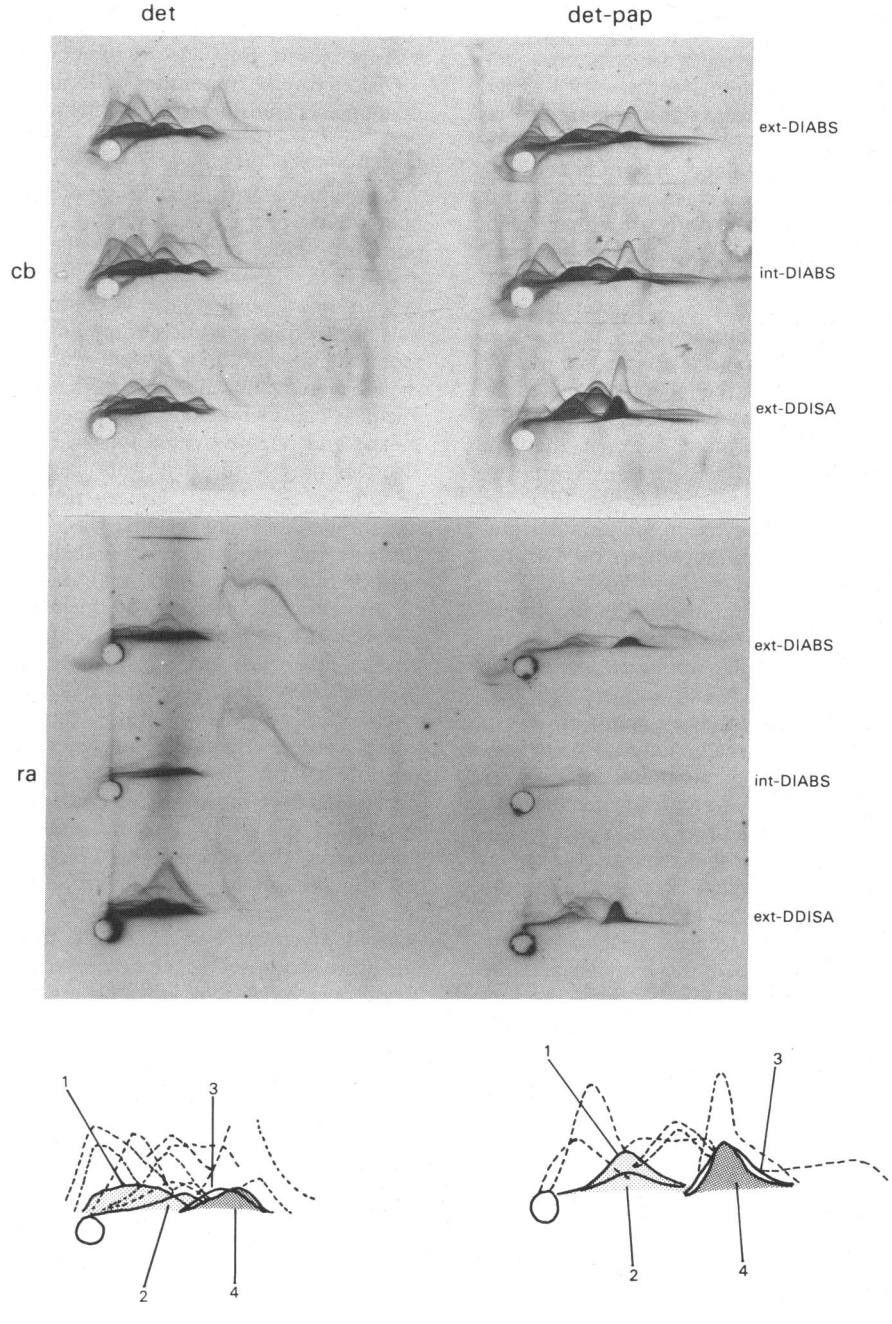


Fig. 6. *Crossed-immunoelectrophoresis of* [^{125}I]*DIABS- and* [^{125}I]*DDISA-labelled proteins using antibodies raised to total microvillar proteins*

See the legend to Fig. 5 for details of the experiment. The identified precipitates are shown in the pair of line drawings. They are: 1, dipeptidyl peptidase IV; 2, neutral endopeptidase; 3, aminopeptidase A; 4, aminopeptidase M. Other precipitates are shown in dotted lines.

portion of the protein (Booth *et al.*, 1979). Hence the precipitates containing the papain-treated enzyme contain only the antigenic portion of the enzyme exposed at the luminal surface of the membrane. The radioautographs show that the only radioactive precipitates were from those membrane samples that had been labelled at the luminal surface with either DIABS or DDISA. However, the precipitate from the sample that had been photolabelled at the cytoplasmic surface contained no radioactivity. Clearly, in this case, the label was situated solely on that portion of the protein lost after treatment with papain. This shows that the addition of albumin prevented any contramembrane labelling. Since photolabelling with [^{125}I]DIABS appeared to produce little intramembrane labelling, and, in this case produced no contramembrane labelling, we believe that the result indicates that the label was located on a portion of the protein originally exposed at the cytoplasmic surface of the membrane, i.e. that pig kidney aminopeptidase M is a transmembrane protein. In this context it is of interest that the immunologically related enzyme in the pig intestinal brush border has also been shown to be a transmembrane protein (Louvard *et al.*, 1976).

The substitution of an immunoglobulin fraction of an antiserum raised to the total microvillar proteins in place of the monospecific antibody allows this method to be used to detect any antigen, provided that it can be recognized on the gels and is present in sufficient quantity for the radioactivity in its precipitate to be detected. The minimum criterion for such an enzyme to be considered transmembrane is that the detergent-solubilized form should be radioactive after labelling from each side of the membrane. If the enzyme is susceptible to papain in the same way as aminopeptidase M, then it should be possible to use the papain-treated form to confirm the transmembrane nature of the enzyme and the lack of contramembrane labelling. A typical result of such an experiment is shown in Fig. 6. Several other enzymes have been demonstrated to be transmembrane. These are listed in Table 3, and comprise aminopeptidases M and A, dipeptidyl peptidase IV and neutral endopeptidase. The transmembrane nature of dipeptidyl peptidase IV was also demonstrated by using a monospecific antibody in the same manner as described for aminopeptidase M (result not shown). Since neutral endopeptidase is not susceptible to cleavage by papain (Booth *et al.*, 1979), only the minimum criterion for its classification as a transmembrane protein is satisfied.

Concluding remarks

Our aim was to study the topology of a group of microvillar peptidases without the necessity of purifying each one in turn. This required the asymmetric labelling of the membrane using a system that deposited label at the membrane surface but not within the membrane interior. A second requirement was for a system capable of resolving the labelled membrane proteins that also allowed the unequivocal identification of their detergent-solubilized and papain-treated forms. Crossed-immunoelectrophoresis satisfied this latter requirement (Booth *et al.*, 1979). However, the choice of a membrane-labelling system was far from clear. Perhaps the ideal membrane-labelling reagents are antibodies capable of discriminating between one set of antigenic determinants of a membrane protein on one side of a membrane from another set presenting on the other side (see e.g. Cotmore *et al.*, 1977). Unfortunately, the antigenic determinants on the microvillar peptidases under study were located entirely on the luminal side of the membrane, i.e. any portions of these peptidases exposed at the cytoplasmic surface of the membrane were not antigenic (Booth *et al.*, 1979). In the analogous intestinal membrane, this problem was elegantly solved for aminopeptidase M by Louvard *et al.* (1976) by attaching an artificial antigen, nitrophenylated human myeloma Fab fragment, to the portion of the protein exposed at the cytoplasmic surface of the membrane by a photolabelling reaction. This technique was not applicable to our system for two reasons. First, several azidonitrophenylated proteins were found to bind very tightly to the luminal surface of kidney microvilli, even in the dark (A. G.

Table 3. *Peptidases demonstrated to be transmembrane in the kidney microvillar membrane*
See the text for details.

Enzyme	EC no.	Confirmation	
		By monospecific antibody	By treatment with papain
Aminopeptidase M	3.4.11.2	Yes	Yes
Aminopeptidase A	3.4.11.7	No	Yes
Dipeptidyl peptidase IV	3.4.14.– or 3.4.21.–	Yes	Yes
Neutral endopeptidase	3.4.24.11	No	No

Booth, unpublished results). This meant that the cytoplasmic surface of the kidney microvillar membrane could not be unequivocally labelled. Second, the method lacks general application, since a monospecific antibody is necessary for each membrane protein to be investigated.

Lactoperoxidase-catalysed radioiodination is regarded by many workers as the method of choice for membrane-labelling studies. In the presence of iodide, an enzyme–substrate complex is formed, and the iodine is then transferred directly to exposed tyrosine or histidine residues of the protein (Morrison, 1974). However, as pointed out by Morrison (1974), if the enzyme is not saturated with the acceptor substrate, it will catalyse the production of free $^{125}I_2$, which will label not only tyrosine and histidine residues in proteins, but will also diffuse into the membrane and label unsaturated fatty acid chains. Our results extend those of Louvard *et al.* (1976) in showing that most of the radioactivity was found in the membrane lipids, predominantly in the hydrocarbon region of the lipids. Intramembrane labelling of proteins is always possible when lactoperoxidase-catalysed radioiodination is used to identify transmembrane proteins and such data should be treated with caution unless confirmed by an independent technique.

An alternative method required a reagent that could be positioned on either side of the membrane before its activation, that was sufficiently hydrophilic to prevent intramembrane labelling and would leave the electrophoretic properties of the membrane proteins undisturbed. Hydrophilic aromatic azides, the precursors of aromatic nitrenes, have the required properties. One such compound, *N*-(4-azido-2-nitrophenyl)-2-aminoethyl[^{35}S]sulphonate, has been used to study erythrocyte-membrane proteins (Staros & Richards, 1974; Staros *et al.*, 1974, 1975). Although a useful reagent, its preparation is expensive and achieves, at best, a 30% yield (Staros *et al.*, 1975). Our reagent was prepared from sulphanilic acid in four efficient steps: the first three steps, iodination, radioactive-isotope exchange and diazotization, have been described by Helmkamp & Sears (1970) as the synthesis of [^{125}I]DDISA, a proven non-penetrant label for the erythrocyte membrane (Sears *et al.*, 1971, 1977; Edwards *et al.*, 1979). In the fourth step, [^{125}I]-DIABS was produced quantitatively from [^{125}I]DDISA by treating it with an excess of NaN_3. It could be positioned on either side of the membrane. The reactive nitrene generated during illumination adds across C–H bonds, so that labelling is not dependent on the presence of certain amino acid residues. The application of this method to the study of the topology of other membranes is clearly possible. The essential requirements are: (*a*) a preparation of sealed vesicles of known orientation, (*b*) the existence of an anion-transport system capable of equilibrating the reagent across the membrane, (*c*) an antiserum to the membrane proteins and (*d*) means for the identification of the antigens in the immunoprecipitates.

Our current view of the topology of the microvillar peptidases is shown in Fig. 7. Aminopeptidases M and A and dipeptidyl peptidase IV are anchored to the membrane by a relatively small region of the polypeptide chain, part of which is also exposed at the cytoplasmic surface. Close to the luminal surface of the membrane is a region susceptible to attack by proteinases, such as papain. The peptidases are shown as dimers in the membrane, a result consistent with cross-linking studies (A. P. Waters & A. G. Booth, unpublished results). Neutral endopeptidase is not released by papain and may not conform to this model. Other known microvillar hydrolases may also be transmembrane. Insofar as Table 3 lists only four examples, it reflects the limit of detection of radioactivity in their

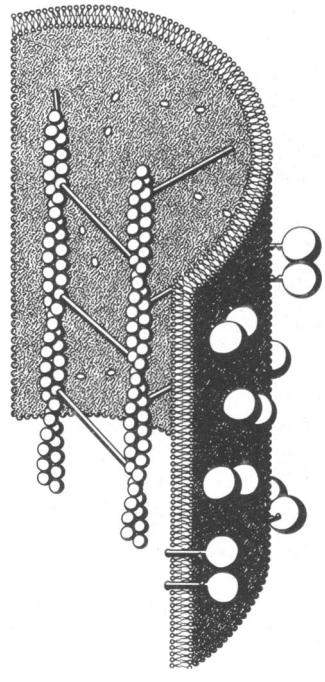


Fig. 7. *Proposed topology of kidney microvillar membrane peptidases*

The enzymes are indicated as dimers in the membrane, a small part of the polypeptide chain of each monomer being involved in anchoring the enzyme to the membrane and also presenting at the cytoplasmic surface. The notches near the luminal surface represent regions susceptible to attack by papain.

immunoprecipitates by our method. Furthermore there are other transmembrane proteins detectable in Fig. 6, but these immunoprecipitates have not yet been identified.

It is interesting that the major antigens of the kidney microvillar membrane, which are glycoproteins with peptidase activity, all appear to be transmembrane. This may reflect a common mode of insertion into the membrane. It also gives some support to the proposition that all intrinsic membrane glycoproteins have a transmembrane topology (Bretscher & Raff, 1975).

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