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Proteins of the Kidney Microvillar Membrane

IMMUNOELECTROPHORETIC ANALYSIS OF THE MEMBRANE HYDROLASES: IDENTIFICATION AND RESOLUTION OF THE DETERGENT- AND PROTEINASE-SOLUBILIZED FORMS

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Antibodies raised in rabbits to detergent-solubilized pig kidney microvillar proteins have been used to investigate the membrane hydrolases by crossed immunoelectrophoresis. Eight enzymes were detected by specific staining methods: aminopeptidase M, dipeptidyl-peptidase IV, neutral endopeptidase, aminopeptidase A, carboxypeptidase P, γ-glutamyl-transferase, trehalase and phosphodiesterase I. The mobility of all these enzymes, with the exception of trehalase and neutral endopeptidase, was increased by treatment of the detergent-solubilized preparation with papain. The difference between the detergent and proteinase forms of these enzymes is attributed to the removal of a small, non-antigenic peptide to which detergent is bound in significant quantities. This interpretation was further supported by experiments in which the microvillus fraction was labelled with an intra-membrane photolabelling reagent, 1-azido-4-[^{125}I]iodobenzene. After photolysis, the radioactivity in the membrane could be solubilized by detergent treatment but not by papain treatment. Radioautography after crossed charge-shift immunoelectrophoresis showed a good correlation between charge-shift (signifying the presence of detergent bound to a hydrophobic domain) and the presence of the label.

Previous studies in this laboratory on the kidney microvillar membrane (Booth & Kenny, 1976; Booth, 1977; Kenny *et al.*, 1978) have used electrophoresis in dodecyl sulphate as the main analytical tool. In this technique the preparation of the samples usually ensures irreversible denaturation of the hydrolases in the membrane. As a consequence, the methods required for the identification of the resolved proteins are necessarily indirect. An analytical system that permits membrane enzymes to be studied in their active state has obvious advantages. Electrophoresis in the absence of dodecyl sulphate of proteins solubilized by either non-ionic detergents or proteinases avoids denaturation, but generally suffers from the drawback of low resolution. However, resolution can be considerably improved by combining this technique with immunoelectrophoresis. This technique, crossed-immunoelectrophoresis, has been used to study relatively few mammalian plasma membranes (for review, see Bjerrum, 1977). Brush-border plasma membranes from pig intestine (Danielsen *et al.*, 1977) and human kidney (Scherberich *et al.*, 1974) have been so examined, but the studies were restricted to the identification of the immunoprecipitates formed by solubilized membrane proteins.

Here we present results of crossed-immunoelectrophoretic analysis of solubilized pig kidney microvillar proteins. We have been able to extend the histochemical staining techniques so that eight distinct hydrolase activities could be detected on the gels. The proteins subjected to analysis need to be solubilized by treatment of the membrane with non-ionic detergents or proteinases. The detergent and proteinase forms of an intrinsic membrane protein differ in the presence and absence of a hydrophobic domain, the anchor or foot, which was formerly associated with the lipid bilayer in the membrane and, on solubilization, will associate with the detergent. By using an intramembrane photolabelling method, combined with crossed charge-shift immunoelectrophoresis, we have been able to carry out comparative studies on the detergent and proteinase forms of several microvillar peptidases. This approach has proved to be valuable for the study of the topology of these enzymes.

Materials and Methods

Pig kidneys were given by Asda Farmstores Ltd., from their slaughterhouse at Lofthousegate, West Yorkshire, U.K. Emulphogen BC 720 (manufactured by G.A.F., Louvres, France) was a gift from Dr. Suzanne Maroux, Centre de Biochimie et de Biologie Moleculaire, CNRS, Marseille, France. Phosphoramidon [*N*-(α-L-rhamnopyranosyloxyhydroxyphosphinyl)-L-leucyl-L-tryptophan] (Suda *et al.*, 1973) was a gift from Professor H. Umezawa, Institute of Microbial Chemistry, Kamiosaki, Shinagawa-ku, Tokyo, Japan. Papain, horseradish peroxidase (type II),

L-amino acid oxidase (type I), glucose oxidase (type V), leucine 2-naphthylamide, Fast Garnet GBC salt, Fast Red TR salt, *o*-dianisidine, naphthol AS-MX phosphate (sodium salt) and thymidine 5′-monophosphate 1-naphthyl ester were from Sigma (London) Chemical Co. Ltd., Poole, Dorset BH17 7NH, U.K. Glycylproline 2-naphthylamide and benzyloxycarbonylprolylmethionine were from Bachem Feinchemikalien A.G., Bubendorf, Switzerland. Insulin B-chain was prepared as previously described (George & Kenny, 1973). Other reagents were from BDH Chemicals Ltd., Poole, Dorset BH12 4NN, U.K.

Microvilli

Microvillus fractions were prepared from pig kidney cortex as previously described (Booth & Kenny, 1974).

Detergent-solubilized microvillar proteins

Microvilli were resuspended in 0.1M-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH, pH 6.8, to give a protein concentration of 5 mg/ml. Emulphogen BC 720 was added to 5% (v/v) and the suspension was incubated at 37°C for 1 h. Iodoacetamide was added to 1 mg/ml and the suspension was centrifuged for 15 min at 25000*g* in a Heraeus Christ Mikrohämatocrit centrifuge. The supernatant was decanted and stored at −20°C.

Papain-treated detergent-solubilized microvillar membrane proteins

Microvilli were resuspended in 0.1 M-Hepes/NaOH, pH 6.8, and treated with Emulphogen BC 720 as above. To each 1 ml portion of suspension was added 0.1 ml of a solution containing 5 mg of papain/ml in 25 mM-2-mercaptoethanol, 10 mM-EDTA, 0.1 M-Hepes/NaOH, pH 6.8, previously incubated at 37°C for 30 min. After incubating at 37°C for 1 h, iodoacetamide (1 mg/ml) was added and the supernatant was separated as above.

Papain-solubilized microvillar proteins

Microvilli were treated with papain as above, but the treatment with Emulphogen BC 720 was omitted.

Immunoglobulin fractions

Microvilli were resuspended in 0.15M-NaCl to a protein concentration of 2 mg/ml. Emulphogen BC 720 was added to 2% (v/v). For immunization, this suspension was emulsified with an equal volume of Freund's complete adjuvant. Rabbits were injected intracutaneously with 500 μg of protein every second week and were bled 1 week after the fourth injection. Immunoglobulin fractions were isolated from sera by $(NH_4)_2SO_4$ fractionation and ion-exchange chromatography (Harboe & Inghild, 1973). They were stored as solutions containing 10 mg of protein/ml in 0.15 M-NaCl/10 mM-sodium phosphate, pH 7.4, with 0.3% (w/v) NaN_3 as preservative.

Iodine monochloride reagent

The iodine monochloride reagent used in the isotope-exchange radiolabelling of 4-iodobenzene described below was prepared as described by Helmkamp & Sears (1970). KI (20.8 mg) and KIO_3 (13.4 mg) were dissolved in 10 ml of 1 M-HCl containing 2M-NaCl. A sample of this solution (1 ml) was mixed with 1.2 ml of 3.3 mM-KIO_3, 0.9 ml of 11.7M-HCl and 3.7 ml of 2M-NaCl. This reagent was stored at 4°C.

1-Azido-4-[^{125}I]iodobenzene

Commercial 4-iodoaniline (10 g) was purified by dissolving it in 100 ml of chloroform/methanol (2:1, v/v) and treating the solution with activated charcoal. After filtering the solution, more activated charcoal was added to the filtrate. This was repeated until the solution had been treated four times and the filtrate was almost colourless. The solvent was removed by rotary evaporation. The yield of purified 4-iodoaniline was 63%. A portion (4.4 mg, 20 μmol) was dissolved in 0.5 ml of 0.5M-HCl. $Na^{125}I$ (2 mCi in 20 μl) was added, followed by 50 μl of the iodine monochloride reagent. The isotope-exchange reaction was allowed to proceed at room temperature for 15 min. The solution was then cooled to −5°C in an ice/salt bath. Then 0.2 ml of 0.8% (w/v) $NaNO_2$ was added in 50 μl portions. After 15 min, 0.1 ml of 2.5% (w/v) urea was added. Still on ice, the solution was transferred to a darkroom and the following steps were performed under a red safe-light (Ilford ISO 906). The diazonium salt was converted into the insoluble azide by the addition of 0.3 ml of 0.16M-NaN_3 in 50 μl portions. When the effervescence due to the liberated N_2 had subsided, the precipitate was centrifuged down at 25000*g* for 15 min. The supernatant was removed and the 1-azido-4-[^{125}I]iodobenzene was dissolved in 0.5 ml of ethanol. It was stored at 4°C in the dark. More than 95% of the radioactivity was recovered in the product.

Kidney microvillar peptidases

Pig kidney microvillar aminopeptidase M, purified after solubilization with Emulphogen BC 720, was a gift from Dr. Suzanne Maroux. Pig kidney microvillar dipeptidyl peptidase IV was purified after solubilization with Triton X-100 [see the preceding paper (Macnair & Kenny, 1979)].

Crossed immunoelectrophoresis

Electrophoresis was performed in a Shandon Southern U77 flat-bed electrophoresis apparatus using 1.0mm thick 1% (w/v) agarose gels on either 1.5×100×100mm or 1.5×110×90mm glass plates. The electrode buffer used was 37.5mM-Tris/0.1M-glycine, pH8.7, containing 0.3% NaN_3. In addition, the gels contained 1% (v/v) Emulphogen BC 720. Usually 10μl of each sample containing 10–50μg of protein was applied to the gel. Electrophoresis in the first dimension was carried out at 5V/cm for 1h; in later experiments this was extended to 2h. The gel was removed from the apparatus and 'windows' were cut for the second-dimension gel. This had the same composition as the surrounding gel, but contained, in addition, 10–50μg of immunoglobulin/cm^2. The samples were then electrophoresed into the second-dimension gel at 1V/cm for 18h.

Crossed charge-shift immunoelectrophoresis

This technique was performed as described by Bhakdi *et al.* (1977), except that Emulphogen BC 720 was used as the non-ionic detergent. Electrophoresis times and voltage gradients were as described above.

Fused 'rocket' immunoelectrophoresis

This was performed in gels of the same composition as the 'second-dimension gel' described above. The sample wells were cut in pairs separated by 1mm of gel. The samples were applied and allowed to diffuse for 1h before electrophoresis was started. This was performed at 1V/cm for 18h.

Staining methods

The gels were pressed for 10min (Weeke, 1973), rehydrated in 0.15M-NaCl for 10min, pressed again, rehydrated in distilled water for 10min and then stained as follows.

Protein. The gels were pressed again and thoroughly dried on to the glass plates with a hair drier. The plates were stained with 0.5% (w/v) Coomassie Brilliant Blue R250 dissolved in ethanol/acetic acid/water (9:2:9, by vol.) for 3min, destained in ethanol/acetic acid/water (5:2:9, by vol.), and finally dried with a hair drier. Enzymes hydrolysing 2-naphthylamide substrates were stained by a modification of the method of Louvard *et al.* (1975).

Aminopeptidase M (*EC* 3.4.11.2). Leucine 2-naphthylamide (5mg) was dissolved in 0.5ml of dimethylformamide. This was mixed with 10ml of 0.2M-Tris/HCl, pH7.0, at 37°C, containing 20mg of Fast Garnet GBC salt, and poured over the gel. When a precipitate was seen to be adequately stained, the gel was washed with water, pressed and dried.

Aminopeptidase A (*EC* 3.4.11.7). As above, but α-glutamic acid 2-naphthylamide in the presence of 1.25mM-$CaCl_2$ was used.

γ-Glutamyltransferase (*EC* 2.3.2.2). As above, but γ-glutamyl 2-naphthylamide in the presence of 3mM-glycylglycine was used.

Dipeptidyl peptidase IV (*EC* 3.4.14.–). As above, but glycylproline 2-naphthylamide was used.

Carboxypeptidase P (*EC* 3.4.12.–). Horseradish peroxidase (4mg), L-amino acid oxidase (60mg) and benzyloxycarbonylprolylmethionine (20mg) were dissolved in 20ml of 0.2M-Tris/HCl, pH7.0, at 37°C. *o*-Dianisidine (0.5ml of a 10mg/ml solution in dimethylformamide) and $MnCl_2$ (1ml of a 30mM solution) were added to the solution, which was then poured over the gel. Carboxypeptidase P was specifically stained brown.

Neutral endopeptidase (*EC* 3.4.24.–). After washing, the gels were pressed and rehydrated in 10ml of 0.2M-Tris/HCl, pH7.0, containing 1.5mg of reduced *S*-carboxymethylated insulin B-chain. After incubation at 37°C for 30min, the surface of the gel was rinsed with distilled water. The gel was then immersed in 10% (w/v) trichloracetic acid for 30min at room temperature. It was then pressed, rehydrated in distilled water, pressed again, dried and stained with Coomassie Blue.

Trehalase (*EC* 3.2.1.28). Glucose oxidase (2mg), horseradish peroxidase (2mg) and trehalose (75mg) were dissolved in 10ml of 0.1M-sodium phosphate buffer, pH6.0, and 0.5ml of 2% (w/v) *o*-dianisidine in dimethylformamide was added. After mixing, this mixture was poured over the gel.

Alkaline phosphatase (*EC* 3.1.3.1). Naphthol AS-MX phosphate sodium salt (2mg) was dissolved in 5ml of distilled water (Naphthol AS-MX is 3-hydroxy-2-naphtho-2,4-xylidide). This solution was mixed with 5ml of 0.2M-Tris/HCl, pH8.5, containing 30mg of Fast Red TR salt, poured over the gel and incubated at 37°C for 1h.

Phosphodiesterase I (*EC* 3.1.4.1). This was stained in a similar manner to alkaline phosphatase, except that thymidine 5′-monophosphate naphthyl ester was used as substrate.

Intramembrane photolabelling with 1-azido-4-[^{125}I]-iodobenzene

All manipulations before photolysis were performed under a red safe-light. Microvilli (28.5mg of protein) were resuspended in 5ml of 0.15M-NaCl, 10mM-sodium phosphate, pH7.4, containing 0.3% (w/v) NaN_3, and 50μl (200μCi) of 40mM-1-azido-4-[^{125}I]iodobenzene in ethanol was added. After mixing, the suspension was cooled in ice for 5min. The microvilli were washed twice by centrifugation at 25000*g* for 15min followed by resuspension in 5ml of 0.15M-NaCl/10mM-sodium phosphate, pH7.4. The suspension was stirred in a 25ml glass beaker on ice and illuminated from above for 1h. The lamp used was a Leitz 50W mercury-vapour lamp in a Leitz

100Z lamp housing equipped with a heat filter. The output of this lamp below 300nm wavelength was tested and found to be negligible. The focused beam was reflected on to the surface of the stirred suspension by a mirror. After photolysis, the microvilli were washed four times with the phosphate-buffered NaCl solution containing 1% (w/v) bovine serum albumin and finally resuspended in 5ml of 0.1M-Hepes/NaOH, pH6.8. Detergent-solubilized and papain-treated detergent-solubilized proteins were prepared as described above and these preparations were subjected to crossed charge-shift immunoelectrophoresis. The gels were dried on to a large glass plate and the radioactivity was located by radioautography with Kodak Kodirex X-ray film.

Enzyme assays

These were performed as previously described (Kenny, 1977).

Assay of radioactivity

Samples containing ^{125}I were counted in a Packard Auto-Gamma spectrometer.

Results and Discussion

Immunoglobulin fractions

The immunoglobulin fractions used in these studies were tested for their ability to precipitate two purified microvillar peptidases. It was found that 1mg of immunoglobulin would precipitate 15–50μg of aminopeptidase M and 25–100μg of dipeptidyl peptidase IV. Both enzymes retained full activity in the immunoprecipitates produced by the immunoglobulins obtained from four immunized rabbits. Control immunoglobulins from non-immunized rabbits did not precipitate these peptidases and gave no precipitates when used in immunoelectrophoresis with solubilized microvillar proteins as antigens.

Crossed immunoelectrophoresis

Detergent-solubilized proteins. At least 12 Coomassie Blue-stained precipitates were seen after crossed immunoelectrophoresis (Fig. 1*a*).

Aminopeptidase M, aminopeptidase A, dipeptidyl peptidase IV and γ-glutamyltransferase were stained by established techniques (Louvard *et al.*, 1975) that make use of their ability to hydrolyse amino acid or peptide 2-naphthylamides to release 2-naphthylamine, which is detected by reaction with Fast Garnet GBC salt. Carboxypeptidase P, a microvillar carboxypeptidase (Kenny *et al.*, 1977) specific for peptides of the form X-Pro-Y, was detected by oxidizing the methionine released by this enzyme from benzyloxycarbonyl-Pro-Met. The H_2O_2 produced by L-amino acid oxidase was detected with horseradish peroxidase and *o*-dianisidine.

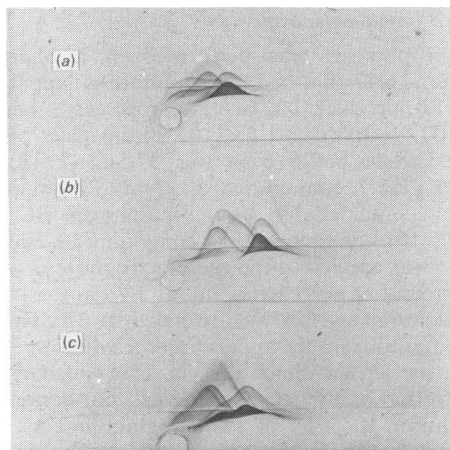

Fig. 1. *Crossed immunoelectrophoresis of solubilized microvillar proteins*

Electrophoresis was performed from left to right at 5V/cm for 2h and in the second dimension from bottom to top at 1V/cm for 18h. In each case the second-dimension gel contained 10μg of immunoglobulin/cm². The samples applied were: (*a*) detergent-solubilized proteins (30μg); (*b*) papain-treated detergent-solubilized proteins (30μg); (*c*) a mixture of samples (*a*) and (*b*) (30μg of each). The precipitates are shown after staining with Coomassie Blue. See the Materials and Methods section for details.

Neutral endopeptidase was identified by its ability to hydrolyse the B-chain of insulin into trichloroacetic acid-soluble fragments. The activity on the gels was revealed as a clear area surrounding an immunoprecipitate in a background of Coomassie Blue-stained insulin B-chain (Fig. 2). The specificity of this assay was confirmed by a control in which phosphoramidon (1μM) was added to the substrate mixture. Phosphoramidon is a specific inhibitor of the endopeptidase (Kenny, 1977). Trehalase was detected with glucose oxidase, and phosphodiesterase I activity was located with the specific substrate thymidine 5′-monophosphate 1-naphthyl ester. The naphthol released was detected by using Fast Red TR salt.

Each of the peptidases and γ-glutamyltransferase corresponded to a Coomassie Blue-stained precipitate. The precipitates are listed in decreasing order of intensity as follows: aminopeptidase M > dipeptidyl peptidase IV > neutral endopeptidase > aminopeptidase A > carboxypeptidase P > γ-glutamyltransferase. Aminopeptidase M was the major antigen detected. It is known to comprise 8% of the membrane protein as assessed by quantitative immunoelectrophoresis calibrated with the detergent-solubilized pig kidney enzyme (A. G. Booth, unpublished work). Dipeptidyl peptidase IV and

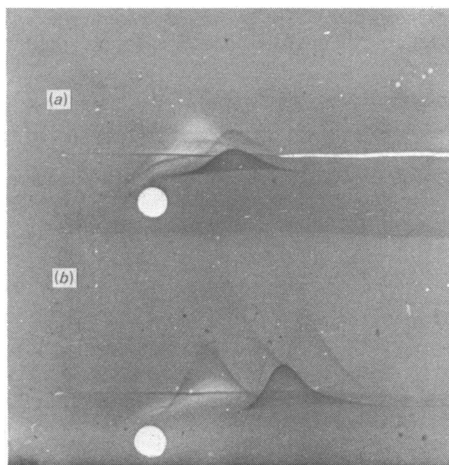

Fig. 2. *Detection of neutral endopeptidase activity after crossed immunoelectrophoresis*

Conditions of electrophoresis were as described for Fig. 1. The samples applied were (*a*) detergent-solubilized proteins (30μg); (*b*) papain-treated detergent-solubilized proteins (30μg). Neutral endopeptidase activity was detected as described in the Materials and Methods section. The gel is shown after staining with Coomassie Blue. The dark background is due to the staining of unhydrolysed insulin B-chain. The neutral endopeptidase activity is revealed as clearer areas in this background. This activity could not be detected in the presence of 1μM-phosphoramidon.

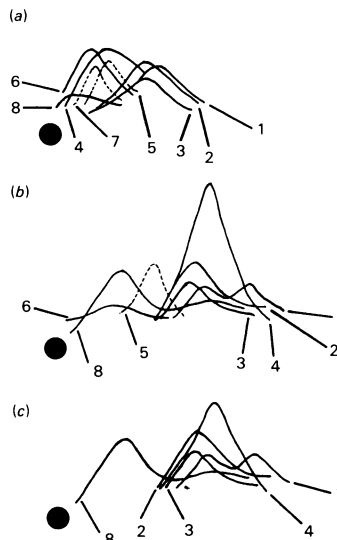

Fig. 3. *Identification of immunoprecipitates with microvillar enzymes*

The positions of the identified immunoprecipitates are shown diagrammatically. Electrophoresis in the first dimension was from left to right and in the second dimension from bottom to top. ● Denotes the origin. The samples illustrated are: (*a*) detergent-solubilized proteins; (*b*) papain-treated detergent-solubilized proteins; (*c*) papain-solubilized proteins. The identified precipitates were: 1, carboxypeptidase P; 2, aminopeptidase A; 3, aminopeptidase M; 4, γ-glutamyltransferase; 5, phosphodiesterase I; 6, neutral endopeptidase; 7, trehalase; and 8, dipeptidyl peptidase IV. Solid lines denote precipitates that could be detected by enzymic activity and by staining with Coomassie Blue. Broken lines denote precipitates that could only be detected by their enzymic activities. Unidentified precipitates have been omitted for clarity. See the Materials and Methods section for details of the detection of enzymic activities.

neutral endopeptidase each comprise about 4% of the membrane protein (Booth & Kenny, 1976). Trehalase and phosphodiesterase I activities did not correspond to stained precipitates; presumably their precipitates were present in quantities below the detection limit of Coomassie Blue. A similar observation has been reported for intestinal dipeptidyl peptidase IV (Danielsen *et al.*, 1977), which is much less abundant in this tissue (Kenny *et al.*, 1976). Alkaline phosphatase activity could not be detected on the gels. This enzyme comprises only 0.04% of the membrane protein (Booth & Kenny, 1976). Either no antibodies were elicited to this enzyme, or antibodies were produced that inhibited the enzyme.

The precipitates containing the identified enzymes, shown in Fig. 3(*a*), were all slow-moving (relative to the proteinase forms, see below) in the first dimension. Their electrophoretic mobilities are listed in Table 1. Although the heights of the precipitates relative to each other depended on the animal used as the source of the immunoglobulins, they were always lower than those produced by the same amount of enzyme activity in preparations that had been exposed at some time to papain. The precipitates showed that all the enzymes in this preparation existed as single species.

Effect of papain treatment of the detergent-solubilized proteins

Again, more than 12 precipitates were visible after staining with Coomassie Blue (Fig. 1*b*). All the hydrolases except trehalase could be detected on the gels. The electrophoretic mobilities of the identified enzymes were all increased relative to the untreated detergent-solubilized enzymes (Table 1). Furthermore, all the enzymes except neutral endopeptidase penetrated further into the antibody-containing gel. Another interesting feature was that papain treatment generated bimodal precipitates in the cases of dipeptidyl peptidase IV and carboxypeptidase P, both peaks migrating faster and one very much faster than the single peak of the detergent form. Both peaks of each enzyme contained activity and

Table 1. *Electrophoretic mobilities of the different forms of microvillar enzymes detected after crossed-immunoelectrophoresis*
Samples were prepared as described in the Materials and Methods section. ND indicates that the enzyme could not be detected.

	Electrophoretic mobility ($cm^2 \cdot V^{-1} \cdot h^{-1}$)		
Enzyme Form ...	Detergent-solubilized	Papain-treated detergent-solubilized	Papain-solubilized
Carboxypeptidase P	0.13	0.19, 0.25	0.19
Aminopeptidase A	0.12	0.18	0.18
Aminopeptidase M	0.12	0.17	0.17
γ-Glutamyltransferase	0.10	0.20	0.20
Phosphodiesterase I	0.08	0.13	ND
Neutral endopeptidase	0.07	0.08	ND
Trehalase	0.06	ND	ND
Dipeptidyl peptidase IV	0.03	0.09, 0.19	0.09

showed reactions of complete identity. In the case of dipeptidyl peptidase IV the fastest moving peak was about 10% of the total; in the case of carboxypeptidase P, the two peaks were more nearly equal.

Precipitation pattern given by papain treatment of the microvillar membrane

Not surprisingly, in view of the selective solubilization of membrane enzymes by papain, fewer precipitates were visible than when detergent-solubilized membrane preparations were studied. Neutral endopeptidase, trehalase and phosphodiesterase I activities could not be detected. These enzymes are not released from the pig kidney microvillar membrane by papain (Kenny & Booth, 1978). γ-Glutamyltransferase was present in smaller amounts; this enzyme is only slowly released by papain. The mobilities of the enzymes present were identical with those of their papain-treated detergent-solubilized counterparts, except that the faster-migrating peaks of dipeptidyl peptidase IV and carboxypeptidase P were sometimes absent. The nature of these faster-migrating forms is unknown, but they clearly indicate a difference in susceptibility to cleavage by papain between the membrane-bound and the Emulphogen-solubilized forms of these two enzymes. The precipitation pattern of all three preparations is shown diagrammatically in Fig. 3.

Resolution of the detergent and proteinase forms of the enzymes

When samples of the detergent-solubilized and papain-treated detergent-solubilized proteins were mixed, the two forms of several enzymes could be resolved by crossed immunoelectrophoresis (Fig.1*c*). These enzymes were those that showed the greatest increases in mobility after treatment with papain, i.e. aminopeptidase M, aminopeptidase A, dipeptidyl peptidase IV, γ-glutamyltransferase and carboxypeptidase P. The relative amounts of the two forms of these enzymes could be estimated by this technique.

The difference in mobility of the two forms of neutral endopeptidase (Table 1) was too small to allow their resolution and indeed, this small difference may not be significant. The two forms of phosphodiesterase I gave a single fused precipitate whose mobility was intermediate between those of the two individual components.

Fused 'rocket' immunoelectrophoresis. The increased penetration of the proteinase forms of all the peptidases except neutral endopeptidase into the antibody-containing gel might arise in three ways. First, these peptidases might degrade some of the immunoglobulins. This would effectively decrease the antibody concentration in the gel, giving rise to increased penetration of the antigens. This explanation seems unlikely, because it would imply that although the detergent forms of these peptidases cannot cleave immunoglobulins, papain treatment alters their specificity so that the proteinase forms possess this ability. Such an effect has not been reported. The possibility that any papain activity surviving iodoacetamide treatment is responsible for cleavage of the antibodies can be dismissed because, at pH8.7, papain is cationic and does not enter the second dimension gel. Furthermore, there are no reports that the combined activities of the pig kidney microvillar peptidases can degrade polypeptides larger than about 4000mol.wt. Secondly, treatment with papain might remove some antigenic determinants from these peptidases. In this case, some of the antibodies in the gel would no longer be able to combine with their antigens. Again, this would have the effect of diminishing the antibody concentration in the gel. Thirdly, the increased penetration might be merely a reflection of the increased electrophoretic mobilities (see, e.g., Weeke, 1968). The enzymes, moving faster, would be able to penetrate further into the gel before being precipitated by their specific antibodies.

To distinguish between these two last possibilities, fused 'rocket' immunoelectrophoresis was used in an attempt to detect the antigenic determinants postu-

lated to be removed by papain. In this technique, two different samples are applied close together on the gel. They are allowed to diffuse towards each other and then electrophoresed into a gel containing antibodies raised to one of them. The proteins, if identical, produce fused precipitates with no evidence of spurring. If the first form contains antigenic determinants not found on the second form (the antibodies having been raised to the first), these determinants will produce visible spurs. When this technique was performed with detergent-solubilized and papain-treated detergent-solubilized microvillar proteins (the antibodies were raised to the former preparation), all the identified precipitates showed reactions of complete identity between the two forms. Clearly, the material removed by papain from the detergent-solubilized proteins does not bear detectable antigenic determinants. However, it is the presence of this non-antigenic material that is responsible for the relative retardation of the 'detergent forms'.

Intramembrane photolabelling and crossed charge-shift immunoelectrophoresis

Several well-documented microvillar hydrolases have been shown to be inserted into the membrane lipid bilayer by relatively short hydrophobic regions usually located at the *N*-terminal region of each enzyme (for review, see Kenny & Booth, 1978). In each case the anchoring peptide is removed by papain. After solubilization with non-ionic detergents, the hydrophobic peptides become associated with detergent, possibly with micelles of about 90000 mol.wt. Treatment with papain removes both the small peptides and their associated detergent. It would therefore seem reasonable to propose that the increases in mobility of the peptidases produced by papain could be caused by the removal of such peptides and associated detergent.

To examine this possibility, the microvillar proteins were radioactively labelled with 1-azido-4-[^{125}I]iodobenzene. This compound partitions into the membrane lipid bilayer and, on illumination, a highly reactive nitrene is generated that reacts unspecifically with components located in the apolar core of the membrane (Klip & Gitler, 1974). Our method of preparation of the reagent yielded a product of high specific radioactivity (>95% of the label was recovered in the product), and the use of the isotope-exchange reaction eliminated the hazard of iodinating aniline directly with $^{125}I_2$.

When microvilli were mixed with 1-azido-4-[^{125}I]-iodobenzene, as described in the Materials and Methods section, 97% of the radioactivity remained associated with the membrane vesicles after washing and before photolysis. After photolysis, and further washing, the labelled microvilli were treated with either Emulphogen BC 720 or papain. Samples of the suspensions, together with a sample of untreated labelled microvilli were assayed for radioactivity and dipeptidyl peptidase IV activity. After centrifugation at 25000g for 15 min, the supernatants were reassayed. The results are shown in Table 2. After correcting for the controls, Emulphogen BC 720 was found to solubilize 75.5% of the radioactivity and 78.4% of the peptidase activity. However, papain solubilized only 0.3% of the radioactivity while solubilizing 72.1% of the peptidase activity. This result is consistent with the location of the photolabel within the apolar core of the lipid bilayer where it can label the intramembrane regions of intrinsic membrane proteins, as reported by Klip & Gitler (1974).

Crossed charge-shift immunoelectrophoresis has been shown by Bhakdi *et al.* (1977) to be a reliable method for distinguishing between amphiphilic proteins that bind non-ionic detergents from hydrophilic proteins that do not. The method, which is derived from that originally published by Helenius & Simons (1977) relies on the fact that if a protein binds a micelle of non-ionic detergent, then its electrophoretic mobility may be altered by incorporating an anionic (deoxycholate) or cationic (cetyltrimethylammonium bromide) detergent into the micelle. This 'charge-shift' is not exhibited by proteins that do not bind non-ionic detergents.

When photolabelled detergent-solubilized microvillar proteins were examined by this technique, most of the detected enzymes showed charge-shifts. Radioautography showed that these enzymes contained considerable amounts of the label. After treatment with papain, the charge-shifts were abolished and the amount of radioactivity in the precipitates was drastically diminished (Fig. 4). Neutral endopeptidase is a notable exception. Its immunoprecipitate retained radioactivity and also displayed charge-shift after treatment with papain.

These results are consistent with our proposal that the increases in electrophoretic mobility of the peptidases produced by papain are caused by the removal of hydrophobic peptides to which are bound substantial quantities of detergent. Neutral endopeptidase cannot be converted into the proteinase

Table 2. *Solubilization of radioactivity and dipeptidyl peptidase IV activity from microvilli photolabelled with 1-azido-4-[^{125}I]iodobenzene*

The solubilization methods were as described in the Materials and Methods section.

Solubilization method	Percentage in 25000g supernatant: Radioactivity	Dipeptidyl peptidase IV
No treatment	8.1	8.3
Emulphogen BC 720	83.6	86.7
Papain	8.4	80.4

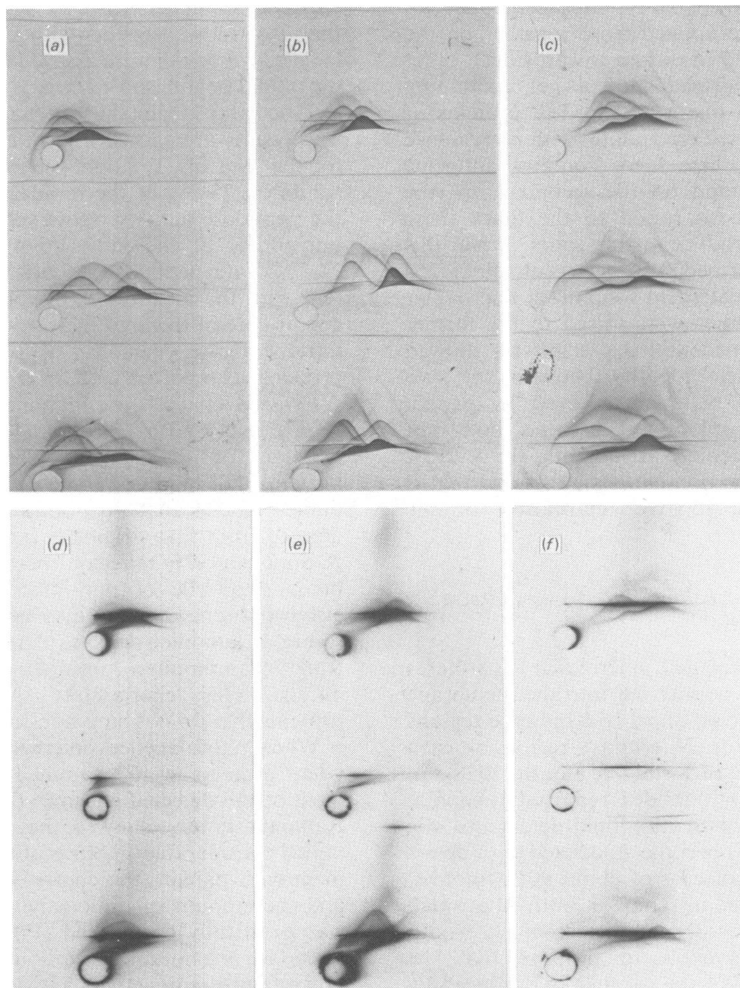

Fig. 4. *Crossed charge-shift immunoelectrophoresis of microvillar proteins solubilized after intramembrane photolabelling with 1-azido-4-[^{125}I]iodobenzene*

The microvillar proteins were solubilized after intramembrane photolabelling as described in the Materials and Methods section. In each photograph the three samples applied are: upper, detergent-solubilized proteins (30 μg); middle, papain-treated detergent-solubilized proteins (30 μg); lower, a mixture of detergent-solubilized and papain-treated detergent-solubilized proteins (30 μg of each). Electrophoresis in the first dimension was performed at 5 V/cm for 2 h. The gel and electrode buffers (for electrophoresis in the first dimension only) contained: (*a*) and (*d*) 0.5 % (v/v) Emulphogen BC 720 plus 0.0125 % (w/v) cetyltrimethylammonium bromide; (*b*) and (*e*) 0.5 % (v/v) Emulphogen BC 720 alone; (*c*) and (*f*) 0.5 % (v/v) Emulphogen BC 720 plus 0.2 % (w/v) sodium deoxycholate. Electrophoresis in the second dimension was performed at 1 V/cm for 18 h in gel containing 10 μg of immunoglobulin/cm^2 as described in the Materials and Methods section for crossed immunoelectrophoresis. (*a*), (*b*) and (*c*) show the patterns of Coomassie Blue-stained precipitates, and (*d*), (*e*) and (*f*) show radioautographs of (*a*), (*b*) and (*c*) respectively.

form by treatment of either the membrane or the detergent form with papain. It presumably does not possess a papain-sensitive region close to its membrane-anchoring hydrophobic peptide.

The reason why papain removes no detectable antigenic determinants while producing gross changes in electrophoretic mobility is now clear. The bulk of the material removed by papain is detergent, with only a minor contribution from the hydrophobic peptides themselves. These peptides may be too small to be antigenic, or more likely, they are masked from any antibodies by the covering of detergent.

Concluding remarks

Cross immunoelectrophoresis is a very sensitive high-resolution technique for the study of kidney microvillar enzymes. It has allowed the unequivocal identification of several hydrolases on the basis of their enzymic activity and has shown that the major microvillar antigens can be identified with the intrinsic peptidases. It has also distinguished quantitatively between the detergent forms and proteinase forms of those enzymes that can be released from the membrane by papain. In combination with suitable membrane-labelling techniques, it is therefore capable of providing information on the topology of the microvillar hydrolases.

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