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Proteins of the Kidney Microvillus Membrane

IDENTIFICATION OF SUBUNITS AFTER SODIUM DODECYL SULPHATE/POLYACRYLAMIDE-GEL ELECTROPHORESIS

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The proteins of microvilli prepared from pig kidney were analysed by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. The typical pattern stained for protein revealed five major bands, four of which also stained for carbohydrate, and about 15 minor bands. For descriptive purposes the bands were designated numerically by their apparent molecular weights ($\times 10^{-3}$). Well-characterized proteins were identified with four of the five major bands. Dipeptidyl peptidase IV, a serine proteinase that may be specifically labelled with di-isopropyl [^{32}P]phosphorofluoridate, was assigned to band 130. Aminopeptidase M was assigned to band 160, though when released from the membrane by a proteinase, this protein comprises three polypeptides each of lower apparent molecular weight than the native enzyme. Neutral endopeptidase can be assigned to band 95 and actin to band 42. The fifth major band (180) is an extrinsic glycoprotein that has not been identified with any microvillus enzyme activity. These four proteins contribute 21% of the microvillus-membrane protein. Kidney microvillus actin was characterized by a variety of properties and was similar to muscle actin. A computer analysis of the gel pattern indicates that it comprises 9.0% of the microvillus protein. Myosin is not present in the microvillus, but another protein associated with band 95, with properties that distinguish it from neutral endopeptidase, was tentatively identified as α-actinin. Alkaline phosphatase was identified as a monomeric polypeptide with an apparent molecular weight of 80000; it is a minor protein of the microvillus and is not discernible as a discrete band in the gel pattern. These and other results permit a model of the organization of the microvillus protein to be suggested. The computer program used has been deposited as Supplementary Publication SUP 50070 (12 pages) at the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms given in *Biochem. J.* (1976) **153**, 5.

The microvilli that line the proximal tubule of the mammalian kidney represent a highly specialized form of the plasma membrane. A variety of enzyme activities are known to be located in this membrane (see, e.g., George & Kenny, 1973; Booth & Kenny, 1974). Most of the enzymes identified in the microvillus membrane are hydrolases capable of attacking peptides, disaccharides or phosphate esters. Other proteins may be associated with transport functions, for example the phlorrhizin-binding protein (Thomas, 1972, 1973). In addition to these membrane-associated proteins, electron micrographs show structures within the core of the microvillus, including microfilaments which Rostgaard & Thuneberg (1972) have suggested are composed of actin.

The membrane proteins of the kidney microvillus may be solubilized by sodium dodecyl sulphate and their component polypeptide chains resolved by electrophoresis in polyacrylamide gels. Using this technique Neville & Glossmann (1971) were able to resolve more than 30 polypeptides from preparations of rat kidney brush border; 11 of these bands also stained with basic Fuchsin after periodate oxidation. Several of the kidney microvillus peptidases have been purified and a number of these have now been shown to be glycoproteins. Aminopeptidase M (EC 3.4.11.2) contains about 20% carbohydrate (Wacker *et al.*, 1971); neutral endopeptidase (EC 3.4.24.-) contains a similar proportion of carbohydrate (Kerr & Kenny, 1974*b*) as does γ-glutamyltransferase (EC 2.3.2.2) (Szewczuk & Baranowski, 1963; Orlowski & Meister, 1965). Dipeptidyl peptidase IV contains about 10%

carbohydrate (A. J. Kenny, unpublished work) and kidney alkaline phosphatase (EC 3.1.3.1) has also been shown to be a glycoprotein (Binkley, 1961). It seemed to us opportune to attempt to correlate the electrophoretic pattern with at least some of the known microvillus proteins. Where specific affinity-labelling techniques existed these were applied; in other cases the identification has depended on comparisons with purified proteins. Some membrane-bound enzyme activities are known to survive treatment with sodium dodecyl sulphate and this property has been used by Maestracci *et al.* (1975) and Critchley *et al.* (1975) to identify some of the intestinal microvillus enzymes.

However, it appears that enzyme activity is detectable only if the sample is not reduced by treatment with thiol before electrophoresis, a condition that must raise some doubt as to the oligomeric state of these proteins. In view of this uncertainty, we have preferred to use fully reduced samples in our studies. In one instance the residual activity of one enzyme in a non-reduced sample was attributable to the dimer.

Materials and Methods

Tissues

Pig and rabbit kidneys were obtained as described previously (Booth & Kenny, 1976), and microvilli were prepared from pig kidneys by the method of Booth & Kenny (1974); enrichment values for marker enzymes and the yields were similar to those we reported for microvilli prepared from rabbit kidneys. Crude brush-border fractions were prepared by the method of George & Kenny (1973), except that the rate-zonal-centrifugation step was omitted.

Chemicals

The sources of materials not otherwise specified were those detailed by Kenny *et al.* (1976). N^{τ}-Methylhistidine, ouabain, oligomycin, sodium *p*-nitrophenyl phosphate-5′-thymidylate were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K. Sodium *p*-nitrophenyl phosphate and ATP (disodium salt) were obtained from BDH Chemicals, Poole, Dorset BH12 4NN, U.K. $^{32}P_i$ (code PBS1) was obtained from The Radiochemical Centre, Amersham, Bucks. HP7 9LL, U.K.

Methods

Treatment of microvillus fractions with papain. The method was that previously described (Kenny *et al.*, 1976), except that incubation of each sample (1.5mg of protein in 2ml) was terminated by cooling in liquid N_2. Each sample was then mixed with 2ml of a solution containing 4% (w/v) sodium dodecyl sulphate and 2% (v/v) 2-mercaptoethanol and heated to 100°C for 5min.

Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate, and radioautography of the dried gels. The methods, including staining for protein, carbohydrate and the calibration of the gels, have been described (Kenny *et al.*, 1976).

Preparation of actin from rabbit muscle. This was prepared from the dorsal muscle by the method of Spudich & Watt (1971).

Preparation of kidney microvillus actin. The initial steps were identical with those for muscle actin. The starting material was 150g of pig kidney cortex. Microvilli were prepared and these yielded about 1g of acetone-treated powder, the subsequent treatment being scaled-down accordingly. However, after three cycles of polymerization and depolymerization, the actin was impure as judged by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. Further purification was achieved as follows. $(NH_4)_2SO_4$ was added to the G-actin solution to 25% saturation, after which the pellet obtained by centrifugation at 26000*g* for 1h was redissolved in 1ml of Buffer A (Spudich & Watt, 1971) containing 0.2mM-$CaCl_2$, 0.2mM-ATP, 0.5mM-2-mercaptoethanol, 2mM-Tris/HCl, pH8.0 (25°C), and the solution dialysed overnight against the same buffer. This sample of G-actin was then applied to a column (100mm×9mm) of Sepharose 6B equilibrated and developed with the same buffer. Fractions (1ml) were collected, monitored for protein at E_{280} and those fractions that corresponded to a peak of retarded protein were pooled. G-actin in the sample was converted into F-actin by adding KCl (0.6M) and $MgCl_2$ (2mM) and stirring gently at 4°C for 2h. The pellet obtained by centrifugation at 100000*g* for 3h was resuspended in 1ml of Buffer A now containing 50mM-KCl and 2mM-$MgCl_2$. This solution was left at 4°C for 16h before being applied to the same Sepharose 6B column now equilibrated with Buffer A, containing KCl and $MgCl_2$ as above. Fractions were collected and monitored as before, but on this run the protein excluded from the gel was collected and pooled. The sample of F-actin thus obtained was stable when stored at −15°C over a period of some weeks. ^{125}I-labelled actin was prepared by a modification of the chloramine-T method described by Glover *et al.* (1967); 0.5mg of actin was iodinated with 5μmol of KI containing 20μCi of ^{125}I.

Heavy meromyosin. Myosin was prepared from rabbit dorsal muscle by the method of Tonomura *et al.* (1966). Heavy meromyosin was prepared from myosin by the method of Lowey *et al.* (1969) and further purified by $(NH_4)_2SO_4$ fractionation as described by Palevitz *et al.* (1974).

Decoration of brush-border actin filaments by

heavy meromyosin. This was performed after treatment with glycerol as described by Ishikawa *et al.* (1969). Material for electron microscopy was processed and examined as previously described (Booth & Kenny, 1976).

Densitometric scanning of the Coomassie Blue-stained gels. Stained gels were scanned in a Unicam SP.1800 spectrophotometer, equipped with an SP.1809 scanning densitometer attachment, at a wavelength of 560nm.

Computer analysis of the gel scans. Scans of gels containing microvillus proteins showed that the bands were incompletely resolved from one another. A method using the University of Leeds ICL 1906A computer was developed to resolve mathematically each peak on the scan. The scans were first converted, by manual measurement, into x and y co-ordinates and input to a computer program. This program (DH13AGB) has been deposited with the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K. as Supplementary Publication SUP 50070. The program uses the assumption that each band observed on these scans may be described by the equation for a Gaussian curve, and hence the whole scan by an equation of the form:

$$y = k_3 e^{-1/2\left(\frac{x-k_1}{k_2}\right)^2} + k_6 e^{-1/2\left(\frac{x-k_4}{k_5}\right)^2} + \cdots + k_{3n} e^{-1/2\left(\frac{x-k_{3n-2}}{k_{3n-1}}\right)^2}$$

where each term represents a band on the scan, there being n bands. The program requires the following data: the number of constants in the overall equation ($3n$); the number of co-ordinates describing the scan (usually no less than $6n$); rough estimates of the mobilities of the bands (k_1, k_4, k_7 etc.); rough estimates of the standard deviation of each band (k_2, k_5, k_8 etc.); rough estimates of the height of each band as a peak on the scan (k_3, k_6, k_9 etc.); the error allowed; the number of iterations allowed; and the values of the co-ordinates. The program uses an iterative procedure to generate an equation, of the form shown above, which describes the scan within the allowed error. The program outputs co-ordinates of the equation for comparison with the original co-ordinates, the constants for each band, the area under each peak on the scan, the co-ordinates for each resolved peak on the scan and the variance and coefficient of multiple correlation between the original co-ordinates of the scan and their computer-fit equivalents. A calibration curve was obtained by using known weights of muscle actin run on similar gels, stained, scanned and input to the computer program.

Myosin and actomyosin. A microvillus preparation (150 mg of protein) was resuspended in 48.5ml of a medium comprising 0.3M-KCl, 0.2mM-ATP, 0.5mM-2-mercaptoethanol and 50mM-histidine/HCl buffer, pH6.8 (4°C). Butan-1-ol (1.5ml) was added, and the mixture stirred at 4°C for 1h. After centrifugation at 26000g for 1h at 4°C the supernatant was diluted tenfold with water and left for 48h at 4°C. Under these conditions myosin might be expected to sediment.

A similar amount of a microvillus preparation was resuspended in 48.5ml of a medium containing 0.6M-KCl, 0.5mM-2-mercaptoethanol and 15mM-Tris/HCl, pH7.8 (4°C). Butan-1-ol (1.5ml) was added and after shaking briefly the suspension was left for 16h at 4°C. After centrifugation (details as above) the supernatant was diluted eightfold with 2mM-$MgSO_4$ and the pH adjusted with 0.1M-HCl to 6.8. It was left for 48h at 4°C, conditions under which actomyosin would be expected to sediment.

Localization of enzyme activity in gels after electrophoresis in the presence of sodium dodecyl sulphate. Provided that the samples to be treated with sodium dodecyl sulphate were neither boiled nor reduced with 2-mercaptoethanol, alkaline phosphatase and phosphodiesterase I activities could be detected as follows. For alkaline phosphatase, the gel was incubated at 37°C in a test tube containing 5.5mM-sodium *p*-nitrophenyl phosphate, 0.5mM-$MgCl_2$ and 50mM-glycine/NaOH, pH10.5, until a yellow-stained band was visible, usually about 5min. For phosphodiesterase I, the gel was treated similarly in a medium containing 2.5mM-sodium *p*-nitrophenyl phosphate-5′-thymidylate and 50mM-Tris/HCl, pH9.0 (37°C). The activities of these enzymes are unexpectedly stable in the presence of sodium dodecyl sulphate. After incubation of a microvillus preparation for 30min at 37°C with 2% (w/v) sodium dodecyl sulphate, 95% of the initial activity of alkaline phosphatase and 64% of the initial activity of phosphodiesterase I remained.

Affinity labelling of alkaline phosphatase with $^{32}P_i$. The method of Engström (1961*b*) was followed. A microvillus preparation (1mg of protein in 1ml of 0.1M-sodium acetate/acetic acid buffer, pH5.0) was treated with 0.1ml of sodium [^{32}P]phosphate (about 100μCi). After 1min at 0°C, 1ml of 25% (w/v) trichloroacetic acid was added. The precipitate was sedimented by centrifugation, resuspended in 2ml of the same acetate buffer and the precipitation repeated. After two such washes the pellet was redissolved by boiling in 1ml of the buffer used for electrophoresis, containing 2% (w/v) sodium dodecyl sulphate and 1% (v/v) 2-mercaptoethanol, subjected to gel electrophoresis and radioactivity was assayed in the intact gel (Kenny *et al.*, 1976).

Purification of aminopeptidase M. The starting material was 5mg of a commercial sample of aminopeptidase M (Rohm and Haas, Darmstadt, German Federal Republic) in 10ml of 5mM-Tris/HCl, pH7.9 (4°C). It was applied to a column (9mm×50mm)

of DEAE-cellulose equilibrated with the same buffer, a linear gradient (100ml) of NaCl from 0 to 400mM in the same buffer being used to elute the activity. The pooled active fractions were dialysed against the starting buffer, concentrated on a column (9mm × 20mm) of DEAE-cellulose by elution with 500mM-NaCl and applied to a column (25mm × 1000mm) of Sepharose 6B and eluted with the same buffer. The pooled fractions were concentrated as before and dialysed against the same buffer. About 1mg of enzyme was obtained.

Enzyme assays. Alkaline phosphatase and aminopeptidase M were assayed as described by George & Kenny (1973). Phosphodiesterase I (EC 3.1.4.1) was assayed by the method of Aronson & Touster (1974). ATPase* (EC 3.6.1.3) activity in heavy meromysin was determined as follows. The incubation mixture (2.0ml) contained 2.5mM-ATP, 15mM-KCl, 2.5mM-$MgCl_2$, 25mM-Tris/HCl, pH7.6 (37°C) and 0.3mg of heavy meromyosin. After 15min at 37°C, the reaction was stopped by the addition of 1ml of 25% (w/v) trichloroacetic acid, and, after centrifugation, P_i was determined in the supernatant by the method of Fiske & SubbaRow (1925). ATPase activity in microvilli was assayed as follows. The basic incubation mixture (1.5ml) contained 3mM-ATP, 20mM-Tris/HCl, pH7.6 (37°C), 1mM-ouabain and 3μg of oligomycin. Incubation and P_i determinations were as described above. Myosin ATPase activity was assayed in the presence of 0.6M-KCl and 5mM-$CaCl_2$; actomyosin ATPase activity was assayed in the presence of 5mM-$MgCl_2$.

Extraction of extrinsic proteins from microvilli. The methods were based on those used to extract erythrocyte membranes (Mitchell & Hanahan, 1966; Marchesi & Steers, 1968). Microvilli (20mg of protein) were suspended in 5ml of either 5mM-EDTA/5mM-2-mercaptoethanol/20mM-Tris/HCl, pH8.5 (4°C) or 0.5M-NaCl/5mM-2-mercaptoethanol/20mM-Tris/HCl, pH8.5 (4°C). The suspensions were left for 16h at 4°C then centrifuged at 105000g for 90min in a Beckman SW 39L rotor maintained at 4°C.

Other methods. The methods for the determination of protein, radioactivity and the technique of negative staining for electron microscopy have been described (Kenny *et al.*, 1976). The amino acid composition of kidney microvillus actin was determined on 1.5 mg of protein (hydrolysed for 48h in 6M-HCl at 110°C) by using a Biocal BC200 amino acid analyser equipped with a short column of Aminex A5 resin. A standard of N^{τ}-methylhistidine was used for calibration. High-voltage electron microscopy (1MV) was carried out on the AEI EM7 at the Swinden Laboratories of the British Steel Corporation, Rotherham, S. Yorks., U.K.

* Abbreviation: ATPase, adenosine triphosphatase.

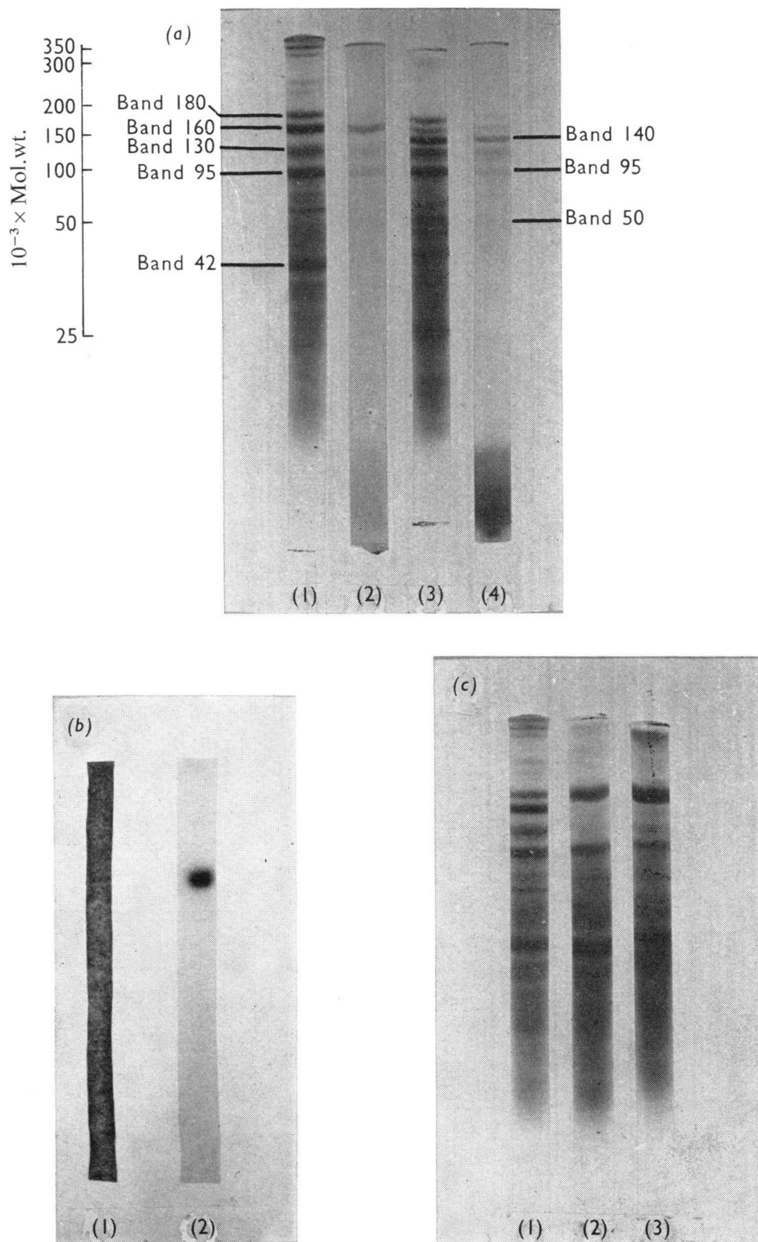
Results and Discussion

Polypeptides of the microvillus membrane as resolved by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate

Plate 1(*a*) shows the pattern of bands observed when the gels were stained with Coomassie Blue. About 20 bands were consistently observed. Of these, five were considered to be major bands in that they stained more densely than the others. The gels were calibrated by using suitable marker proteins as described previously (Kenny *et al.*, 1976), and included in Plate 1(*a*) is a scale showing the apparent molecular weights. For convenience each band will be referred to by a number derived from the apparent molecular weight $\times 10^{-3}$. Thus the five major bands have been designated 180, 160, 130, 95 and 42, indicating that these bands have apparent mol.wts. of 180000, 160000 etc. Among the minor bands there are two high-molecular-weight polypeptides (greater than 350000mol.wt.) and other bands that have been designated 240, 220, 90, 75, 70, 65, 55 and 50. In addition there are about six incompletely resolved bands of molecular weight less than 40000 which do not at present warrant a numerical designation. Plate 1(*a*) also shows the appearance of a gel stained for carbohydrate. Six bands can be seen, two of high molecular weight (greater than 350000) and other bands identical in mobility with those previously designated as 180, 160, 130 and 95. Of these, band 160 appears to carry most of the carbohydrate. In addition there is some diffusely staining material that migrates with the sodium dodecyl sulphate front.

Dipeptidyl peptidase IV

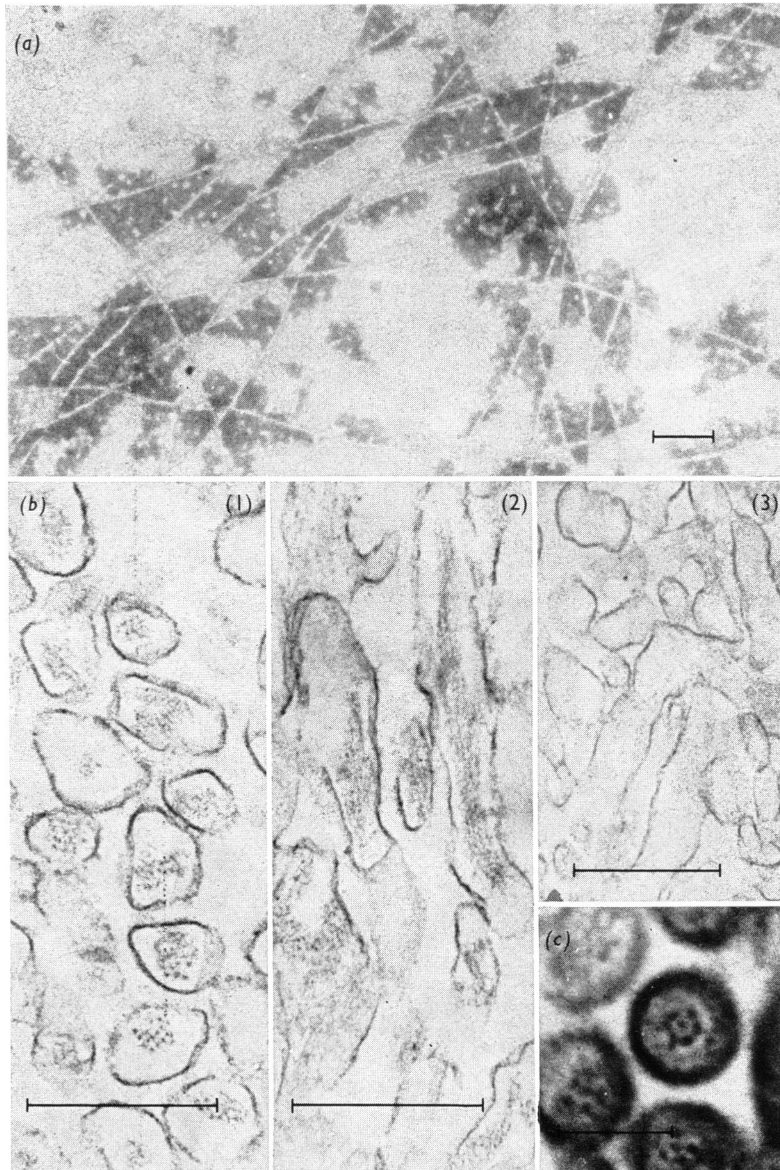
This enzyme is a serine proteinase that may be conveniently labelled with di-isopropyl [^{32}P]phosphorofluoridate. This technique has shown that only one major component of the microvillus membrane is so labelled and that this corresponds to band 130. The evidence for this conclusion has been presented previously (Kenny *et al.*, 1976). Comparison of the specific enzyme activities and specific radioactivities of the membrane preparation and the purified enzyme has shown that this protein comprises 4–5% of the microvillus-membrane protein. The enzyme is an intrinsic protein of the microvillus membrane in that it cannot be released from the membrane by simple salt-extraction methods. When released by treatment of microvillus-membrane preparations with papain, the soluble radioactive protein co-chromatographed with a sample of purified active enzyme. The similar electrophoretic mobilities suggested that the solubilized enzyme did not differ in molecular weight from the native (membrane-bound) enzyme by more than about 6500.



EXPLANATION OF PLATE 1

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

See the Materials and Methods section for details. (*a*) Untreated and papain-treated kidney microvillus preparations. (1) Untreated preparation, stained for protein; (2) untreated preparation stained for carbohydrate; (3) preparation treated with papain for 1 h stained for protein; (4) as (3) but stained for carbohydrate. Apparent molecular weights and band numbers are shown. (*b*) Demonstration of alkaline phosphatase by radioautography. (1) Kidney microvillus preparation after labelling with $^{32}P_i$ (dried gel); (2) radioautograph of gel (1). (*c*) Proteins of kidney microvillus preparations extracted by media containing NaCl or EDTA. (1) Untreated microvillus preparation; (2) proteins extracted by 0.5M-NaCl; (3) proteins extracted by 5mM-EDTA.



EXPLANATION OF PLATE 2

Electron microscopy

(*a*) Kidney microvillus F-actin negatively stained with uranyl acetate. Bar represents 100nm. (*b*) Glycerinated brush borders incubated with heavy meromyosin. (1) and (2) show transverse and longitudinal sections of microvilli exhibiting filaments decorated with heavy meromyosin. Many microvilli have fused and contain more than the usual seven filaments. (3) Microvilli from an experiment similar to that shown in (1) and (2) except for the presence of 1mM-ATP during incubation with heavy meromyosin. No decoration of actin filaments can be seen. Bar represents 500nm. (*c*) A kidney microvillus seen in transverse section. This micrograph was obtained from a section 1 μm thick observed with an accelerating voltage of 1 MV. A typical 1+6 arrangement of the core filaments can be seen. Bar represents 100nm.

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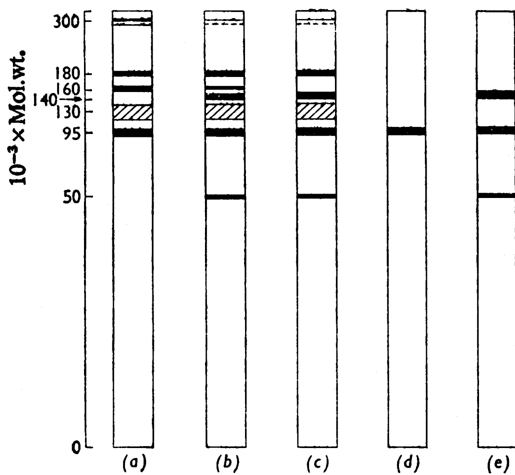

Fig. 1. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of microvillus preparations and purified aminopeptidase M*

The gels are stained with basic Fuchsin after periodate oxidation. The bottom of the gel represents the dye front. See the Materials and Methods section for details. (*a*) Untreated microvillus preparation; (*b*) microvillus preparation after treatment with papain for 1h; (*c*) 100000*g* supernatant obtained from a papain-treated microvillus preparation; (*d*) 100000*g* pellet from papain-treated microvillus preparation; (*e*) purified aminopeptidase M.

Aminopeptidase M

This enzyme is known to comprise about 5% of the kidney brush-border protein and to be released by treatment with papain (George & Kenny, 1973). No suitable affinity label is available for this enzyme, and the activity of the enzyme does not survive the treatment with sodium dodecyl sulphate and 2-mercaptoethanol. Hence its location in the gel pattern has depended mainly on comparison with samples of the purified enzyme. Purified pig aminopeptidase M may be resolved by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate into three bands (Wacker *et al.*, 1971; Wacker, 1974). The purification of this enzyme has so far required the use of proteolytic enzymes to release it from the membrane. A partially purified preparation is commercially available. When this was examined by electrophoresis, the three main bands had apparent mol.wts. of 140000, 95000 and 50000 with some other polypeptide components that were eliminated by purification of this preparation by DEAE-cellulose chromatography and gel filtration on a column of Sepharose 6B (Fig. 1*e*). The mobilities of the two smaller polypeptide chains differ slightly from those observed by Wacker *et al* (1971) and this may well be attributable to slight differences in the carbohydrate content (Wacker, 1974). Although the intact microvillus membrane contains polypeptide chains corresponding to apparent mol.wts. of 95000 and 50000, there is no band visible corresponding to that of the 140000. It seemed probable that this last band, at least, was produced during the proteolytic release of the enzyme from the membrane. This view was supported by an experiment in which the microvillus membrane was treated with papain. Plate 1(*a*) shows that papain diminished band 160 and produced a new band designated 140. Staining the gels for carbohydrate revealed two new glycoproteins, one corresponding to band 140 and the other with the same mobility as band 50 (Plate 1*a* and Figs. 1*a* and 1*b*). After centrifugation of the papain-treated membrane preparation at 100000*g* for 30min, the band pattern in the supernatant, after staining for carbohydrate, showed bands of mobility identical with bands 180, 140, 130, 95 and 50 (Fig. 1*c*). Band 130 may be attributed to dipeptidyl peptidase IV, since this enzyme is known to be released by papain (Kenny *et al.*, 1976). Band 180 is an extrinsic glycoprotein and cannot be attributed to any of the known microvillus enzyme activities (see below). Band 95 in the papain supernatant can be distinguished from a polypeptide of the same mobility, which has been attributed to neutral endopeptidase (see below), since the latter enzyme is not released from the membrane by treatment with papain (George & Kenny, 1973). Band 50 must be a glycoprotein generated by papain treatment, since no glycoprotein with this mobility is detectable in the untreated membrane. Thus the supernatant after papain treatment contains glycoproteins of mobilities similar to those found in the purified preparations of aminopeptidase M. Band 140 can clearly be attributed to degradation of band 160 in the intact membrane, but the source of bands 95 and 50 cannot be ascertained with the same degree of certainty. At present, we cannot exclude the possibility that band 50 may have arisen from degradation of another band on treatment with papain. Nevertheless, we may conclude that band 160 can be identified with the monomer of aminopeptidase M in the intact membrane. When purified, the enzyme exists as a dimer of mol.wt. 280000 (Wacker *et al.*, 1971).

Neutral endopeptidase

This microvillus-membrane enzyme is a zinc-containing metallopeptidase. It has been purified by a method that involves release from the membrane by using toluene and trypsin, and shown to be a glycoprotein with a single polypeptide chain, mol.wt. 93000 (Kerr & Kenny, 1974*a*,*b*). The identification of neutral endopeptidase on polyacrylamide gels of microvillus

preparations must depend on a comparison of properties, since no affinity label is at present available. Previous results indicate that the neutral endopeptidase is a major component of the microvillus membrane. This calculation may be performed on the basis of a comparison of specific activities of the purified enzyme and microvillus membrane or by a comparison of the enrichment factors for the preparation of the enzyme and that of the purified membrane (Kerr & Kenny, 1974*a*; Booth & Kenny, 1974). Such calculations give values of 9.6 and 6.5% respectively. However, assays for this enzyme in all preparations other than the purified enzyme are likely to be distorted by the presence of aminopeptidase M (Kerr & Kenny, 1974*a*). This is because, although aminopeptidase M does not initiate the release of trichloroacetic acid-soluble radioactivity from the substrate, [^{125}I]iodoinsulin B chain, it amplifies the release once the endopeptidase has initiated its attack on the substrate. It may be calculated that the activity of aminopeptidase M in the microvillus preparation and in the homogenate increases the hydrolysis of the iodoinsulin B-chain substrate by a factor of about two. Applying this correction, the contribution of neutral endopeptidase to the microvillus-membrane protein is in the range of 3.3–4.8%. In this respect the enzyme is comparable with dipeptidyl peptidase IV and aminopeptidase M, in that it corresponds to one of the major bands in the gel pattern. Neutral endopeptidase is neither released nor inactivated during treatment of the microvillus membrane with papain (George & Kenny, 1973). The relevant properties of the enzyme to be considered in the identification are that (*a*) it should be a major band staining positively for carbohydrate, (*b*) it should have a mobility corresponding to a molecular weight greater than or equal to 93000 and (*c*) it should remain in the membrane after prolonged treatment with papain. These criteria are based on information obtained with preparations from rabbit kidney (Kerr & Kenny, 1974*a*,*b*); nevertheless, we have found no discrepancies in repeating these experiments with preparations obtained from pig kidney. Only one band in the gel pattern fits these criteria, band 95, a major band (Plate 1) which persists after treatment with papain (Fig. 1*d*); neutral endopeptidase has therefore been assigned to this band. However, this enzyme is not the only peptide contributing to band 95, since an extrinsic protein (not containing carbohydrate) also contributes to this band (see below).

Alkaline phosphatase

Alkaline phosphatase is an unusual enzyme in that most of the activity survives treatment with sodium dodecyl sulphate. This has been observed for both the rabbit kidney and the human intestinal enzymes (Kerr, 1973; Maestracci *et al.*, 1975). It is therefore possible to identify the position of the active enzyme after electrophoresis in sodium dodecyl sulphate provided that the sample is neither boiled nor reduced with 2-mercaptoethanol. By this technique it was established that active alkaline phosphatase migrated with a mobility corresponding to an apparent mol.wt. of 160000. A comparable technique using the substrate for phosphodiesterase I (sodium *p*-nitrophenyl phosphate-5′-thymidylate) yielded an apparent mol.wt. of 120000 for this enzyme. Cathala *et al.* (1975) studied a purified preparation of bovine kidney alkaline phosphatase that had been solubilized by the treatment with butan-1-ol. It was a dimer of mol.wt. 172000, which lost enzyme activity when converted into the monomeric form. In our experience, no activity can be demonstrated in polyacrylamide gels that would correspond to the monomeric form of the pig kidney enzyme.

An alternative approach to identification of this enzyme involves affinity labelling with $^{32}P_i$ (Engström & Ågren, 1958; Engström, 1959, 1961*a*,*b*,*c*). This approach, which was developed with respect to the purified calf intestinal enzyme, has proved to be equally applicable to the microvillus membrane. Plate 1(*b*) shows the appearance of the dried gel and the radioautograph obtained from it. A single radioactive band was observed whose mobility corresponded to a polypeptide of mol.wt. 80000, a value that suggests that the active form of this enzyme is the dimer. The position of this radioactive band lies between band 90 and band 75 in the gel pattern and does not correspond to a stained band. Engström (1961*b*,*c*) found that radioactive phosphate incorporated into the native enzyme at pH5 is stable, but is rapidly released at higher pH values. He studied a number of factors that affected the labelling of the alkaline phosphatase and use of these has enabled us to show that the labelled protein can be identified with alkaline phosphatase (Table 1). In the absence of any effectors, the extent of labelling appears to reach a maximum, since no increase could be achieved by the addition of $MgCl_2$. EDTA inhibited the incorporation of the label as did the presence of the substrate, *p*-nitrophenyl phosphate or several inhibitors, e.g. 5′-AMP, Na_2HPO_4, phenylalanine and $ZnCl_2$. Our results are therefore similar to the findings of Engström (1961*b*,*c*) with respect to the purified enzyme. The absence of a stained band corresponding to the monomeric form of alkaline phosphatase (mol.wt. 80000) is not surprising bearing in mind the small contribution that alkaline phosphatase makes to the total microvillus protein. The calculated value of 5.15×10^{-12}g-mol of phosphate incorporated per mg of microvillus protein is equivalent to 0.04% of the microvillus protein, assuming 1 mol of phosphate per subunit of mol.wt. 80000. This is two orders of magnitude less than major components such as

Table 1. *Incorporation of radioactivity into microvillus protein after incubation with $^{32}P_i$*

See the Materials and Methods section for details. The effectors were present at the concentrations shown before addition of $^{32}P_i$. The results of Engström (1961*b,c*) in similar experiments with purified alkaline phosphatase are shown for comparison. ND, Not determined.

Addition	^{32}P incorporated into kidney microvilli ($10^{14}\times$ g-atoms/mg of protein)	^{32}P incorporated into kidney microvilli (% relative to control)	^{32}P incorporated into intestinal alkaline phosphatase (Engström, 1961*b,c*) (% relative to control)
None (control)	515	100	100
EDTA (10mM)	12.3	2.4	2.0
p-Nitrophenyl phosphate (10mM)	16.4	3.2	ND
AMP (10mM)	12.3	2.4	ND
Na_2HPO_4 (10mM)	8.3	1.6	9.2
Phenylalanine (50mM)	251	49	ND
$MgCl_2$ (10mM)	518	101	99
$ZnCl_2$ (10mM)	218	42	46

aminopeptidase M and neutral endopeptidase. This conclusion is consistent with published values of enrichment factors for the purified kidney enzyme (Mathies, 1958; Binkley, 1961; Cathala *et al.*, 1975).

Preparation of kidney microvillus actin

Ishikawa *et al.* (1969) first demonstrated the presence of actin in intestinal microvilli by an electron-microscopic technique in which glycerinated chick-embryo intestinal-mucosa tissue was shown to bind heavy meromyosin. Further proof of the presence of actin in intestinal microvilli came when it was isolated and partially characterized by Tilney & Mooseker (1971). Rostgaard & Thuneberg (1972) noted that the core filaments of kidney brush borders appeared to be beaded and showed a strong resemblance to actin filaments isolated from skeletal muscle. Rostgaard *et al.* (1972) demonstrated that heavy meromyosin was bound by filaments in the basal region of the proximal-tubule cell, but did not demonstrate any binding to the core filaments of the brush border. The binding of fluorescein-labelled human smooth-muscle autoantibody to the apical region of the kidney proximal-tubule cell (Gabbiani *et al.*, 1973) has been taken to indicate that the microvilli probably contained an actin-like protein. Band 42 has the mobility expected for actin and is not released or degraded when the microvilli are treated with papain (Plate 1*a*). However, in view of the uncertainty with regard to the presence of actin in the kidney microvillus core, it seemed desirable to attempt to characterize this protein from microvillus preparations. A procedure based on that for the preparation of muscle actin yielded an inhomogeneous preparation, which required further steps in which first G-actin and then F-actin were fractionated on a column of Sepharose 6B. The final product appeared to be homogeneous in polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. The preparation migrated as a single band with a mobility corresponding to that of a polypeptide of mol.wt. 42000. Rabbit skeletal-muscle actin has a mol.wt. of 42000 as calculated from the amino acid sequence (Elzinga *et al.*, 1973). Moreover, kidney microvillus actin co-electrophoresed with purified skeletal-muscle actin.

Properties of kidney microvillus actin

In the presence of 50mM-KCl and 2mM-$MgCl_2$ the microvillus actin formed long polymers similar in appearance to skeletal-muscle F-actin (Plate 2*a*); indeed, the principle of the method used in the preparation of microvillus actin depends on repeated polymerization and depolymerization. Muscle actin is known to be depolymerized by KI; accordingly we attempted to see if microvillus F-actin would respond in the same way. The results with these experiments using a column of Sepharose 6B to resolve the G and F forms are shown in Fig. 2. Microvillus F-actin is eluted in a volume that corresponds to the void volume determined by Dextran Blue. Re-running the sample of actin in 0.6M-KI showed this medium to be partially effective in depolymerizing the F-actin, two peaks of material being seen in the elution pattern, one corresponding to the void volume and the other to a species of smaller molecular size. A preparation of [^{125}I]iodo-actin prepared from muscle was found to remain in the G form and, since it could not be polymerized under any conditions, it was therefore a suitable marker with which to compare the microvillus G-actin. The labelled muscle actin and the microvillus G-actin were eluted in the same volume.

Another characteristic property of muscle actin is the activation of the ATPase activity of heavy meromyosin. Fig. 3 shows the activation curves for both microvillus and skeletal-muscle actins; although not superimposable the two actins give comparable curves. Muscle actin activates about

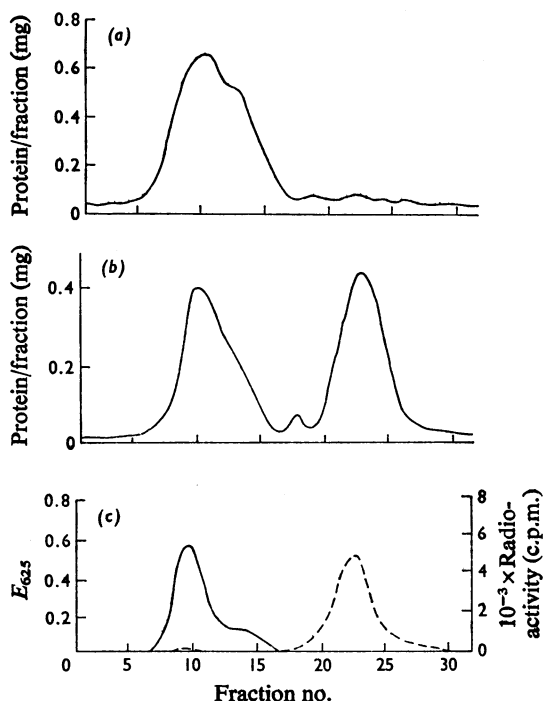

Fig. 2. *Gel filtration on Sepharose 6B of kidney microvillus actin*

The column (80mm×8mm) was loaded with the samples and eluted with the media indicated below, and fractions (100μl) were collected. (*a*) Kidney microvillus actin (3mg) suspended in 0.2ml of 0.6M-KCl/5mM-Tris/HCl, pH7.0 (20°C) and eluted with same medium; (*b*) 3mg of kidney microvillus actin suspended in 0.2ml of 0.6M-KI/40mM-$Na_2S_2O_3$/5mM-Tris/HCl, pH7.0 (20°C); (*c*) 0.3mg of Dextran Blue 2000 with 10μg of [^{125}I]iodoactin (muscle) (specific radioactivity, 4μCi/mg) in 0.2ml of the KI medium described above. ——, E_{625} (Dextran Blue 2000); ----, radioactivity.

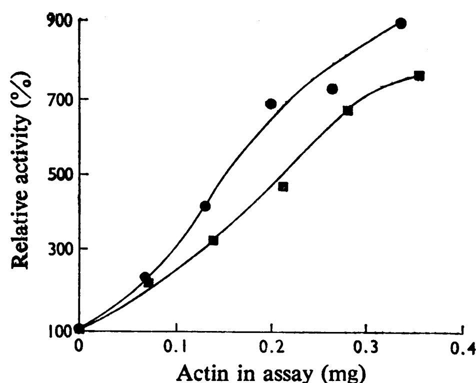

Fig. 3. *Activation by kidney microvillus and muscle actins of Mg^{2+}-activated ATPase of heavy meromyosin*

The assay mixture (2ml) contained 0.3mg of heavy meromyosin, 2.5mM-ATP, 15mM-KCl, 2.5mM-$MgCl_2$, 25mM-Tris/HCl, pH7.6 (37°C). After incubation at 37°C for 15min the reaction was stopped by the addition of 1ml of 25% (w/v) trichloroacetic acid; P_i was determined after removal of protein. ●, Muscle actin; ■, kidney microvillus actin.

ninefold when assayed in the presence of an equal weight of heavy myosin, and microvillus actin activated about sevenfold in the same conditions.

The amino acid compositions of a number of actins have now been reported. Table 2 shows the compositions of human platelet and rabbit skeletal-muscle actins. Also shown is the analysis made of our microvillus actin; the similarity to other actins is clearly demonstrated, in particular the presence of N^{τ}-methylhistidine (a residue characteristic of actin and myosin, and amounting to one residue per 42000 mol.wt. in actin) is in agreement with the other analyses.

We have also investigated the electron-microscopic appearance of partially purified brush-border preparations after the addition of heavy meromyosin. This technique, developed by Ishikawa *et al.* (1969), involves initial treatment of the preparation with glycerol to render the core filaments accessible to heavy meromyosin. In these conditions heavy meromyosin decorates actin filaments to give a characteristic arrowhead appearance, a phenomenon that does not occur in the presence of ATP, which prevents the binding of the heavy meromyosin to actin. Plate 2(*b*) shows the appearance of glycerinated brush borders incubated with heavy meromyosin in the presence and absence of ATP. The decoration of the core filaments confirms their identity with actin, and confirms that the preparation described of microvillus actin had been derived from the core filaments.

It has been possible to quantify the microvillus content of several enzymes by calculation based on specific activities. With respect to actin another approach was needed. This has depended on quantifying the densitometric trace of the Coomassie Blue-stained gels of microvillus preparations. However, most bands are not fully resolved, including band 42, which has been attributed to actin. A computer program was therefore developed capable of resolving the peaks and determining the area of each. Fig. 4 shows the densitometric trace of a typical gel. The region shown between the two arrows in Fig. 4(*a*) was used for computer resolution. The computer-generated approximation of this scan is shown in Fig. 4(*b*) and the computer-generated resolution of

Table 2. *Amino acid composition of kidney microvillus actin compared with actins from some other sources*

The values are expressed as g-mol/42000g of protein. ND, Not determined; C, corrected for losses during hydrolysis. Analyses for methionine, cystine and tryptophan have been omitted. The value for N^{τ}-methylhistidine in kidney microvillus actin is the mean of two analyses (0.79 and 1.57) on one sample. References: (1) the present study; (2) Booyse *et al.* (1973); (3) Johnson & Perry (1968).

	Pig kidney microvillus (1)	Human platelet (2)	Rabbit skeletal muscle (3) (C)
Aspartic acid	33.1	33.2	30.5
Threonine	20.6	25.8	23.3
Serine	17.0	25.1	20.0
Glutamic acid	38.9	46.3	38.3
Proline	20.9	20.2	16.6
Glycine	28.3	32.4	25.4
Alanine	29.8	32.7	27.0
Valine	17.2	16.4	18.2
Isoleucine	26.3	22.8	25.9
Leucine	26.7	30.1	23.7
Tyrosine	15.4	11.8	13.6
Phenylalanine	14.7	13.3	10.6
Lysine	24.2	18.7	18.3
Histidine	9.5	8.9	6.9
Arginine	20.9	17.6	16.1
N^{τ}-Methylhistidine	1.18	0.88	0.89

it is shown in Fig. 4(*c*). This enabled the area under the peak corresponding to band 42 to be accurately calculated; it corresponded to 9.0% of the microvillus protein.

Myosin

The presence of actin in microvilli led us to search for myosin in such preparations. Attempts to extract myosin or actomyosin-like proteins by methods established for muscle and other sources were totally unsuccessful. No protein resembling myosin in its solubility characteristics was obtained by these procedures and further, no myosin- or actinomyosin-like ATPases were identified in the extracts. The search was then extended to the unextracted microvillus preparations. Some ATPase activity, which is stimulated by Ca^{2+} and Mg^{2+} ions, could be demonstrated in microvillus preparations (Table 3). Both activities were only slightly decreased in the presence of 0.6M-KCl. If the activities were attributable to actomyosin one would expect the Mg^{2+}-activated ATPase to be strongly diminished in the presence of 0.6M-KCl. Further, mersalyl, a potent inhibitor of actomyosin ATPase (Hoffmann-Berling, 1956), had no effect on the microvillus ATPase activities. On the other hand, EDTA in the presence of 0.6M-KCl was a strong inhibitor, a finding that differs clearly from the reported behaviour of myosin ATPase (Friess, 1954). We conclude that myosin is not a component of our microvillus preparations.

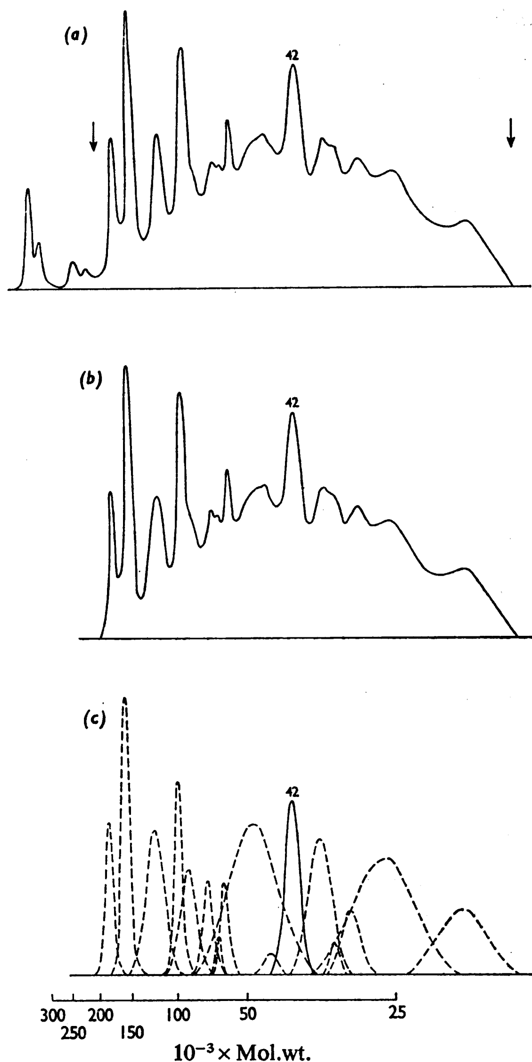

Fig. 4. *Computer resolution of the densitometric trace of kidney microvillus proteins in a sodium dodecyl sulphate/polyacrylamide gel*

See the Materials and Methods section for details. The gel scanned in (*a*) had been stained with Coomassie Blue. The region of the trace between the two arrows was used for computer resolution. (*b*) Computer-generated approximation to the input data. (*c*) Computer generated resolution of (*b*), the actin peak (band 42 in each trace) is shown as a continuous line.

Extrinsic proteins

The term extrinsic is used to designate those proteins that were readily extractable by either 0.5M-NaCl or 5mM-EDTA, both containing 5mM-2-mercaptoethanol, 20mM-Tris/HCl, pH 8.5. Extraction for 16h at 4°C with the NaCl medium released 14% of the microvillus protein; EDTA in the same conditions extracted 23% of the total protein. None of the microvillus-membrane enzyme activities could be demonstrated in either extract. When the extracts were examined by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate three major bands could be seen (Plate 1*c*) and these have the same mobilities as bands 180, 95 and 42. EDTA seemed to be particularly effective in extracting a polypeptide corresponding to band 180, which was the only one of the three staining for carbohydrate in both extracts. The failure of band 95 to stain for carbohydrate clearly distinguishes it from neutral endopeptidase, which is known to be a glycoprotein. This establishes that band 95 in the intact microvillus-membrane pattern must contain two proteins, one a glycoprotein and the other an extrinsic protein. The extrinsic protein of this mobility has been tentatively identified with α-actinin (Booth & Kenny, 1976). We have observed in negatively stained samples, prepared from NaCl extracts of microvilli, filaments, all of which are laterally aggregated into bundles; no free filaments have been observed, an appearance attributable to an actin–α-actin complex.

A model of the kidney microvillus

Sufficient knowledge has now accumulated for a fairly detailed view of the molecular architecture of the kidney microvillus to be proposed. Several uncertainties still remain, not least the extent of penetration of most of the membrane proteins into the lipid bilayer of the membrane. We may assume that the presence of carbohydrate, now established for many of the membrane proteins, implies that part of these proteins is exposed on the luminal surface. Since none of the known microvillus enzymes has been purified without the use of a proteolytic step early in the procedure we may also assume the existence of a hydrophobic foot penetrating into the lipid bilayer. But we have no information to indicate which, if any, traverse the whole structure and present an internal face to the core of the microvillus. An operational classification of the membrane proteins permits some simple propositions: (*a*) proteins extracted by simple salt media (extrinsic proteins) are located on a surface (not necessarily luminal) accessible to the medium and held in place by electrostatic forces; (*b*) proteins released by treatment with papain are also superficial but with a part of the polypeptide chain penetrating the bilayer; (*c*) the third class are those components that resist release by papain because they are mainly within the lipid bilayer or within the inaccessible core of the microvillus. The characteristics of some of the kidney microvillus proteins are shown in Table 4. It is possible to ascribe over 20%

Table 3. *ATPase activity in kidney microvilli*

See the Materials and Methods section for details of the basic assay. The concentrations refer to the assay mixture.

Addition	Activity relative to control (%)
None (control)	100
5mM-$CaCl_2$	241
5mM-$CaCl_2$, 0.6M-KCl	198
5mM-$CaCl_2$, 1mM-mersalyl	234
5mM-$MgCl_2$	172
5mM-$MgCl_2$, 0.6M-KCl	123
5mM-$MgCl_2$, 1mM-mersalyl	167
1mM-EDTA, 0.6M-KCl	25
10mM-EDTA, 0.6M-KCl	1.3

Table 4. *Kidney microvillus proteins*

Information on glycosylation is derived both from data on purified proteins and staining of gels. The class and location of the proteins is explained in the text. The values for percentage of total microvillus protein have been derived from experimental data, except for that of α-actinin which has been calculated as explained in the text; extrinsic glycoprotein is a major component, but has not been quantified. ND, Not detectable.

Protein	Subunit mol.wt.	Band	Glycosylation	Class	Location	Solubilization by papain	Proportion of microvillus protein (%)	Molecules per microvillus
Extrinsic glycoprotein	180000	180	+	Extrinsic	Membrane	+	—	—
Aminopeptidase M	160000	160	+	Intrinsic	Membrane	+	4.1	450
Dipeptidyl peptidase IV	130000	130	+	Intrinsic	Membrane	+	3.9	530
Neutral endopeptidase	95000	95	+	Intrinsic	Membrane	0	4.1	760
α-Actinin	95000	95	0	Extrinsic	Core	0	1.7–2.5	310–470
Alkaline phosphatase	80000	ND	+	Intrinsic	Membrane	0	0.04	10
Actin	42000	42	0	Extrinsic	Core	0	9.0	3800

of the microvillus protein to four components: actin, aminopeptidase M, dipeptidyl peptidase IV and neutral endopeptidase. It is also clear that the extrinsic glycoprotein (band 180) and the other extrinsic protein contributing to band 95, and tentatively identified with α-actinin, are also major proteins, as yet not directly quantified, but perhaps contributing another 5% of the microvillus protein. Even so, more than one-half of the microvillus protein remains unassigned and most of this must be associated with the minor bands or with unresolved material seen in the gel-electrophoresis pattern.

Actin is the major protein of the microvillus. Electron micrographs of transverse sections usually show a core of seven filaments arranged in a 1+6 pattern [Plate 2c; see also, Rostgaard & Thuneberg (1972)]. In the kidney the polarity has not been defined, but Mooseker & Tilney (1975) have shown unequivocally that in intestinal microvilli (where there are about 20 filaments/microvillus) the polarity is such that a myosin molecule would move in a direction from the tip of the microvillus towards its base, The half-pitch of the actin double helix is 36nm and contains between 13 and 14 actin subunits (Moore *et al.*, 1970; O'Brien *et al.*, 1975). The average length of a microvillus is about 1500nm, from which we may calculate that there are some 3800 molecules of actin/microvillus. We know that these contribute 9% of the microvillus protein and so it follows that each microvillus contains 2.94×10^{-15}g of protein. From this value, and the percentage values for the other microvillus proteins, we may calculate the number of monomers of each present in a typical microvillus. After actin, neutral endopeptidase is the most abundant protein (760 molecules/microvillus) and aminopeptidase M and dipeptidyl peptidase IV contribute 450 and 530 molecules/microvillus respectively. Alkaline phosphatase, which has an activity in microvilli comparable with that of these two enzymes contributes only 10 molecules/microvillus, an indication of its high turnover number.

These calculations depend crucially on the validity of our value for the actin content of microvilli. This could be obtained only by use of a computer program to resolve and quantify the actin peak in the densitometer trace of microvilli and of known amounts of purified muscle actin. A purified preparation of actin, or any other protein under consideration, must be used to calibrate the densitometer trace, since the intensity of staining varies from protein to protein. Bray & Thomas (1975), for example, have observed that muscle actin stained with only 67% of the intensity of bovine serum albumin. Even so our result depends on other assumptions, e.g. that each peak on the trace can be adequately described by the equation for a Gaussian curve and that the resolved actin peak is derived from a homogeneous protein. A calculation using independent results suggests that these assumptions are justified. The surface area of a microvillus 1500nm in length and 80nm diameter is $3.69\times10^{-13}m^2$ and, from our data, the distribution of protein is $8.0\times10^{-3}g/m^2$. If these values are compared with those for the erythrocyte membrane of surface area $1.4\times10^{-10}m^2$ (Steck, 1974) and containing 6.6×10^{-13}g of protein (Dodge *et al.*, 1963) the distribution of protein amounts to $4.7\times10^{-3}g/m^2$, i.e. 0.59 of that calculated for the the microvillus membrane. This difference should be reflected in the ratio of lipid/protein in the two membranes, or more simply, the ratio of (phospholipid plus cholesterol)/protein, since these are the main lipid constituents. The erythrocyte membrane contains 490μg of phospholipid and 230μg of cholesterol/mg of protein (Dodge *et al.*, 1963) and the corresponding values for rabbit kidney brush border are 301μg and 97μg (Quirk & Robinson, 1972). The ratio of brush border/erythrocyte-membrane lipid is 0.55, a value that agrees well with that predicted from the computer-derived data.

The molecular organization of the microvillus core deserves comment. We have reported on the cross-bridges that link the actin microfilaments to the membrane and have suggested that these are composed of α-actinin (Booth & Kenny, 1976), a postulate that explained the temperature-dependent vesiculation process in microvilli *in vitro*. Mooseker & Tilney (1975) have independently reached the same conclusion from some very elegant electron-microscopic studies of intestinal brush borders. In muscle, α-actinin is localized to the Z-lines of the sarcomeres (Masaki *et al.*, 1967; Stromer & Goll, 1972) but it has also been demonstrated by immunofluorescent techniques in non-muscle cells, including intestinal epithelial cells, where it occurs at the tips of the microvilli and along the length of the actin filaments (Schollmeyer *et al.*, 1974; Lazarides & Burridge, 1975; Lazarides, 1976). α-Actinin has a mol.wt. of 95000 (Ebashi *et al.*, 1972) and is an elongated molecule 30nm×2nm (Podlubnaya *et al.*, 1975). Mooseker & Tilney (1975) observed in electron micrographs that the cross-bridges attached to demembranated actin filaments, derived from intestinal microvilli, have a periodicity of 33nm along the length, a value similar to the half-pitch of the F-actin helix. They found that the length of the cross-bridges varied from 15 to 30 (mean 20)nm, implying that this protein is elastic in nature, a property not previously reported for α-actinin.

Band 95 contains two components, an intrinsic glycoprotein (neutral endopeptidase) and an extrinsic protein devoid of carbohydrate. This latter protein co-sediments with F-actin obtained from salt extracts of kidney microvilli. Electron miscroscopy of such

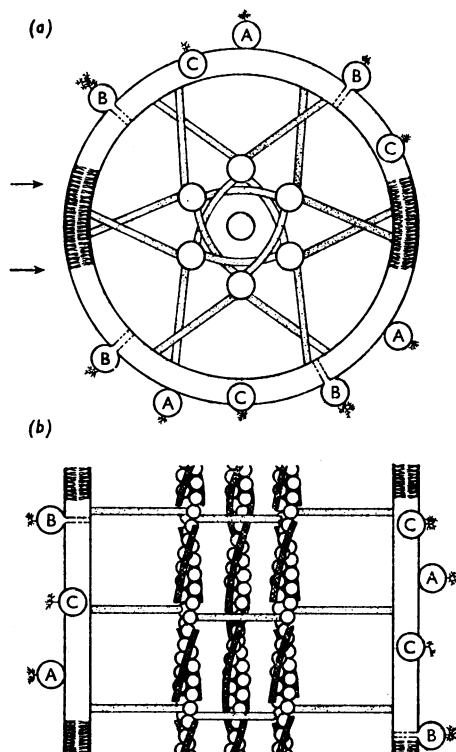

Fig. 5. *Hypothetical model for the structure of a kidney microvillus*

(*a*) A projection of the view that would be seen by looking down a length of a microvillus. Not all of the cross-bridges shown would be seen in a transverse section thinner than 80nm. This is clearer from the longitudinal view shown in (*b*). All the α-actinin cross-bridges (stippled) are drawn about 30nm in length and are shown as if arising from the centre of an actin subunit. The peripheral actin–membrane cross-links seen in (*a*) occur in pairs and, if unresolved in an electron micrograph of a transverse section, would give the appearance of six spokes (see Plate 2*c*). The thickness of the lipid bilayer is 7nm and the diameter of the microvillus is 100nm. The three classes of membrane proteins are indicated: A, extrinsic proteins; B, intrinsic proteins susceptible to release by papain; C, intrinsic proteins not accessible to papain. In (*a*) no attempt has been made to depict the subunits of actin, the double helix being represented by a circle of the same diameter. (*b*) A projection of a longitudinal section of the central region indicated by the arrows on the left of (*a*). Three actin filaments are depicted showing α-actinin linking between actin filaments and between the membrane and the filaments with a periodicity of 36nm. Tropomyosin is shown shaded. It is possible that α-actinin molecules interact with intrinsic membrane proteins (e.g. B), but in this model they are not so depicted.

preparations suggests that the actin filaments are cross-linked into bundles (Booth & Kenny, 1976), similar in appearance to those of F-actin–α-actinin complexes observed by Podlubnaya *et al.* (1975). We therefore suggest that band 95 contains α-actinin as well as neutral endopeptidase. In intestinal microvilli, the molar ratio of actin/α-actinin is 8:1, assuming similar staining properties (Mooseker & Tilney, 1975). If this ratio applies to kidney microvilli, one would expect α-actinin to contribute 2.5% of the microvillus protein, i.e. about 470 molecules/microvillus. This assessment may also be tested by a theoretical approach. It is possible for one actin subunit in every seven to be available to bind a molecule of α-actinin. This is based on the finding that non-muscle tropomyosins are smaller than those from muscle (30000mol.wt. compared with 35000mol.wt.; Fine & Blitz, 1975) and are therefore likely to associate with only six rather than seven actin subunits (Spudich *et al.*, 1972). Tropomyosin prevents the binding of α-actinin to actin (Goll *et al.*, 1972), hence if non-muscle tropomyosins bind to actin with the same periodicities as in muscle, the maximum number of α-actinin molecules associating with actin can be calculated as one-seventh of the number of actin subunits, giving 540 α-actinin molecules in a single microvillus. This is the maximum number (and equivalent to 2.9% of the microvillus protein), since it excludes actin-to-actin attachment of α-actinin in our model (Fig. 5). We suggest that one-third of the α-actinin molecules are involved in this way. Further, the central actin filament may not bind α-actinin, hence more appropriate values may be 310 molecules and 1.7% respectively. The same assumptions predict that tropomyosin would contribute about 1% of the microvillus protein.

The model shown in Fig. 5 incorporates the assumptions discussed above. The drawing, which is approximately to scale, shows the three classes of membrane-associated proteins together with the core structures. Some of the microvillus enzymes are known to be dimers when purified, e.g. dipeptidyl peptidase IV. These, however, may be artifacts produced by the treatment required to release the enzyme from the membrane; we have no evidence on the oligomeric state of the enzyme *in situ*. The arrangement of the α-actinin cross-links is also arbitrary. In this model we have chosen to accommodate slightly flexible cross-links of a fixed length (30nm) rather than to assume that they possess elasticity. We also preferred an actin helix containing 28 subunits in 13 turns as described by O'Brien *et al.* (1975), since this permits all the α-actinin-binding sites on one helix to be aligned vertically. Six of the seven actin filaments are braced by α-actinin cross-bridges in such a way that the central region of the core is empty except for a single actin filament, a feature that can be noted in some electron micrographs (e.g. Plate 2*c*). One-third of the α-actinin molecules in a microvillus are involved in actin–actin cross-bridges, the rest in actin–membrane links. The precise inter-

action of α-actinin with the membrane is unknown. It may be via an intrinsic membrane protein, which, if the same protein is involved in the interaction along the length of a microvillus, must be stoicheiometrically equivalent, i.e. at least 300 molecules/microvillus. It is tempting to assign such a function to one of the three major intrinsic proteins (Table 4), but if a smaller intrinsic protein of, say 20000 mol.wt. were involved it could correspond to one of the minor bands as yet unidentified.

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