Studies on the Turnover of Protein and Glycoprotein Components in Rabbit Kidney Brush Borders

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(Received 27 September 1972)

The kinetics of incorporation of [^3H]lysine and [^{14}C]glucosamine into kidney brush borders were studied *in vivo*. The patterns of incorporation and loss of radioactivity from the brush borders were similar for both radioactively labelled precursors. Maximal labelling occurred 15–20 h after injection of the precursors, and then the radioactivity declined rapidly until 50 h. The radioactivity of brush borders then remained constant until 120 h and thereafter declined slowly. These results are interpreted as indicating that two processes contribute to the turnover of brush-border components, pinocytosis and a slower turnover of the components of the microvilli. Studies of the distribution of radioactivity among glycoprotein components of the brush borders, separated by polyacrylamide-gel electrophoresis, indicated that the membrane components turned over in unison.

The plasma membranes of cells are complex assemblies of lipids and proteins that serve to contain cells mechanically and to maintain their internal environment. Intracellular proteins and lipids, with few exceptions, undergo continual turnover during the lifetime of cells and studies *in vitro* (Warren & Glick, 1968; Baker & Humphreys, 1972; Hughes *et al.*, 1972) and *in vivo* (Ray *et al.*, 1968; Evans & Gurd, 1971; James *et al.*, 1971; Kawasaki & Yamashina, 1971) indicate that plasma membranes also exhibit this phenomenon.

Since relatively little information is available concerning the turnover of plasma membranes of differentiated cells, the present investigation was undertaken to establish the kinetics of formation and degradation of the proteins and glycoproteins of the brush borders from kidney proximal tubule cells.

Materials and Methods

Rabbits (Blue Bevron $\times$ Chinchilla, male, 1.8–2.0 kg) were obtained from the Oxford University Farm. Each animal was injected intravenously with 25 μCi of [^{14}C]glucosamine and 100 μCi of [^3H]lysine dissolved in 2 ml of 0.15 M-NaCl. The radioactive compounds, D-[1- ^{14}C]glucosamine hydrochloride, (specific radioactivity 55 Ci/mol) and L-[4,5- ^3H]lysine monohydrochloride (specific radioactivity 250 Ci/mol), were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. At stated times after injection, the brush-border fraction was isolated from perfused kidneys by the method of Quirk & Robinson (1972); the purity of the fractions obtained in the present study was the same as that reported by these authors. The brush-border suspensions (in 0.5 M-

sucrose) were stored at -20°C before analysis. In all the experiments samples of the initial homogenate of the whole kidney cortex were retained and stored at -20°C for analysis.

In preparation for counting of radioactivity, samples of brush borders and of cortex homogenate were precipitated with trichloroacetic acid (final concn. 5%, w/v) and the precipitates were washed twice with 5% trichloroacetic acid. Lipid was extracted from the residues by washing (3×1 h extractions) with methanol–chloroform–diethyl ether (1:1:2, by vol.). The remaining protein was dissolved in 0.5 M-NaOH at 90°C .

In some experiments lipids were extracted directly from pellets of brush borders obtained by centrifuging the suspensions at 3000 g_{max} for 20 min (Mistral 6L centrifuge, angle rotor 69167). The pellets were extracted with 20 vol. of chloroform–methanol (2:1, v/v) overnight at 20°C , and the resulting residues were extracted twice more with 5 vol. portions of the same solvent. The combined extracts were washed (Folch *et al.*, 1957) in the absence of added salts and both the aqueous and organic phases, together with the residual protein pellet, were retained for counting.

Brush-border samples were subjected to polyacrylamide-gel electrophoresis to separate the component proteins for radioactivity counting. Sodium dodecyl sulphate (10%, w/v) was added to the brush-border suspensions to give a final concentration of 1%, Bromophenol Blue was added as a tracker dye (final concn. 0.01%) and the resulting mixture was applied to gel columns to give a protein load of 300 μg /column. In some cases 2-mercaptoethanol (final concn. 2.5%, v/v) was added to the sodium dodecyl sulphate and the suspensions were heated at 100°C

for 5 min. Gels were cast in glass tubes (80 mm $\times$ 6 mm internal diam.). The polymerization mixture contained acrylamide (7%, w/v), *NN'*-methylenebisacrylamide (0.09%, w/v), sodium dodecyl sulphate (0.1%, w/v), *NNN'*-tetramethylethylenediamine (0.029%, w/v) and ammonium persulphate (0.07%, w/v) in 0.375 M-Tris-HCl buffer, pH 8.9. The electrophoresis buffer was 0.1 M-Tris-HCl buffer, pH 8.6, containing 0.1% sodium dodecyl sulphate. Electrophoresis was conducted at 4 mA/tube for 5–6 h. The gels were fixed by washing in a large volume of propan-2-ol-acetic acid-water (5:2:13, by vol.) for 18 h. Proteins were stained with 0.1% (w/v) Coomassie Brilliant Blue dissolved in fixing solution and excess of stain was removed by washing in several changes of acetic acid-methanol-water (14:7:79, by vol.). Periodic acid-Schiff base-staining material was detected by bathing gels in periodic acid (1%, w/v, in 3%, v/v, acetic acid) for 90 min, washing in 7% (v/v) acetic acid for 18 h, and placing them in freshly prepared fuschin-sulphite reagent (Fairbanks *et al.*, 1971) for 3 h. The stained gels were washed three times in 0.5% (w/v) sodium metabisulphite in 0.1 M-HCl, 10 min being allowed for each wash.

After staining, the gel patterns were recorded and the gels cut into slices (0.8 mm thick) after freezing in solid CO₂; a multiple razor-blade cutter was used. The slices were retained for counting of radioactivity.

The identities of ¹⁴C-labelled compounds in the brush borders were established by column- and paper-chromatographic analysis (Robinson, 1968).

Radioactivity was determined by dual-isotope-

counting procedures with an Intertechnique SL-30 scintillation counter. Quench corrections were determined by using the external standard channels-ratio method. The scintillator solutions were (A) butyl-PBD [0.8 g of 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole and 8 g of naphthalene in 100 ml of toluene-2-methoxyethanol (3:2, v/v)] and (B) 0.5 g of 2,5-diphenyloxazole and 0.5 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 100 ml of toluene. Trichloroacetic acid extracts were counted in butyl-PBD; lipid extracts were dried and redissolved directly in butyl-PBD; protein solutions were mixed with an equal volume of Hyamine hydroxide (X-100) and counted in butyl-PBD; polyacrylamide-gel slices were dissolved in 30% H₂O₂-aq. NH₃ (sp. gr. 0.88) (99:1, v/v) at 50°C. NCS solubilizer (Searle Ltd., High Wycombe, Bucks., U.K.) was added and the samples were shaken at 37°C until they no longer smelled of NH₃; scintillator solution (B) was then added. This procedure for dissolving the gels gave recoveries of >90% for added [¹⁴C]glucosamine. Other procedures tested gave lower recoveries, apparently because ¹⁴CO₂ was lost from the samples during dissolution.

Protein was determined by the method of Lowry *et al.* (1951), with 3 $\times$ -recrystallized bovine serum albumin used as the standard.

Results

The time-course of the incorporation of radioactivity into the brush borders isolated from rabbits

Fig. 1. Incorporation of radioactivity into brush-border protein

Rabbits were each injected simultaneously with [¹⁴C]glucosamine (25 μ Ci) and [³H]lysine (100 μ Ci). At various times after injection the kidneys were perfused and the proximal-tubule brush borders were isolated (Quirk & Robinson, 1972). The brush-border proteins were precipitated with trichloroacetic acid, lipid was removed by extraction with chloroform-methanol-ether, and the protein residues were counted for radioactivity by using double-isotope-counting procedures. Each point represents the results from one animal. $\bullet$, ¹⁴C radioactivity; $\blacksquare$, ³H radioactivity. The broken line represents a best-fit exponential plot of the results for ¹⁴C from 48 h onward.

Fig 2. Incorporation of radioactivity into whole-cortex protein

Rabbits were treated as described in Fig. 1 and samples of kidney-cortex homogenate were retained. Protein was precipitated from the homogenates with trichloroacetic acid, lipid was removed by extraction with chloroform-methanol-ether, and the protein residues were counted for radioactivity by using double-isotope-counting procedures. Each point represents the result from one animal. ●, ^{14}C radioactivity, and ■, ^3H radioactivity, of proteins; ○, ^{14}C radioactivity, and □, ^3H radioactivity, of the trichloroacetic acid-soluble fraction.

injected with [^3H]lysine and [^{14}C]glucosamine is shown in Fig. 1. The ^{14}C radioactivity reached a maximum at approx. 20h, and then declined rapidly until 40–50h had elapsed. From 50h to 120h the specific radioactivity remained unchanged but slowly declined from 120h onwards. The pattern of labelling obtained with [^3H]lysine was very similar except that the ^3H radioactivity was less than the ^{14}C radioactivity throughout the experiment.

The trichloroacetic acid extracts of the brush borders contained less than 2% of the total ^3H or ^{14}C . Lipid extracts (chloroform-methanol-ether) contained 8% of the total ^3H and 4% of the total ^{14}C . Chloroform-methanol extracts of intact brush borders contained only 2% of the total radioactivity for both radioactive isotopes, none of which entered the aqueous phase during the Folch method of extraction, but prior treatment of the brush borders with trichloroacetic acid resulted in the extraction of two to three times as much radioactivity. The proportion of radioactivity extracted by lipid solvents varied little during the course of the experiment.

The identity of the ^{14}C -labelled compounds was examined by using brush borders isolated 10, 24, 238 and 285h after injection. Only glucosamine and sialic acid were radioactive; no radioactivity was detected in galactosamine. The glucosamine contained 80% and sialic acid 20% of the total radioactivity. This proportion varied little throughout the experiment.

The time-course of incorporation of [^3H]lysine and [^{14}C]glucosamine into total kidney-cortex protein is shown in Fig. 2. Incorporation of both radioactive isotopes was maximum at about 5h after injection

and then declined exponentially. Throughout the experiment the amounts of ^3H radioactivity were approximately threefold those of ^{14}C radioactivity. The trichloroacetic acid-soluble radioactivity in the cortex declined throughout the experiment (Fig. 2).

Polyacrylamide-gel-electrophoretic analysis of the brush borders showed more than 20 protein components and approx. 12 periodic acid-Schiff base-staining components. Densitometer tracings of gels are shown in Figs. 3 and 4. Treatment of the brush borders with mercaptoethanol before electrophoresis resulted in the appearance of more components migrating rapidly, as compared with untreated gels, presumably because of the reduction of inter- and intra-chain disulphide bridges. Unfortunately the inherently low radioactivity of the brush borders, together with the small quantity of protein that could be applied to the gels without decreasing resolution, resulted in low amounts of radioactivity in the gels. The ^3H was too low to be accurately determined but the ^{14}C could be measured if two or three gel slices were counted in the same vial. Consequently single vials sometimes contained more than one band. Therefore some consecutive bands are listed together in Table 1; this table shows the radioactivity of the compounds expressed as a percentage of the total activity recovered from the gels. Generally more than 90% of the radioactivity applied to gels was recovered in the gel slices. Since only ^{14}C radioactivity was counted, the components listed in Table 1 refer to the periodic acid-Schiff base-staining components observed in the gels. No counts were recorded in regions of the gel containing no periodic

Fig. 3. Densitometric scans of brush-border proteins separated by electrophoresis on polyacrylamide gels

Samples of radioactive brush borders were dissociated by using 1% (w/v) sodium dodecyl sulphate and subjected to electrophoresis on polyacrylamide-gel columns in the presence of this detergent with Tris-HCl buffer, pH 8.6 (details are given in the text). The gels were stained with Coomassie Brilliant Blue or periodic acid-Schiff base reagent and the bands were scanned by using a densitometer (J. Loeb, mark III C.S.). The upper trace was obtained with Coomassie Blue stain and the lower trace with periodic acid-Schiff base stain. The numbers represent the division of the periodic acid-Schiff base-staining bands employed when the gels were sectioned for counting of radioactivity. The first peak of the lower trace comprised three distinct but closely situated bands.

acid-Schiff base-staining material. The relative radioactivities of the carbohydrate-containing components did not alter appreciably during the course of the experiment (Table 1).

Discussion

The results with [^{14}C]glucosamine, show that the loss of radioactivity from the brush borders does not follow a single exponential function (Fig. 1). After reaching a maximum value, the specific radioactivity declined rapidly until 50 h after injection and then the rate of decline markedly diminished, indicating a biphasic process. The exact form of the slowly declining phase of the curve is difficult to determine because of variation between animals; Fig. 1 shows two alternative forms, namely a plateau up to 120 h followed by an exponential decline, and a best-fit exponential decay after 50 h. Statistical comparison of these alternatives with a Snedecor F test (Quandt, 1960) indicated that the plateau representation was

more probable than the exponential decay ($F_{2,1} = 217.4$, $F_{1,2} = 4.35$; $F = 3.18$ at the 10% probability level).

The results with [^3H]lysine were similar to those with [^{14}C]glucosamine, radioactivity declining rapidly up to 50 h and then more slowly. Again statistical analysis indicated that the curve representing a plateau followed by an exponential decline was a better fit than an exponential decay from 50 h onwards ($F_{1,2} = 31.95$, $F_{2,1} = 2.72$ and $F = 3.18$ at the 10% probability level) but the results are less convincing than those obtained with glucosamine.

Studies of the turnover of isotopically labelled compounds are prone to various errors. Reutilization of the labelled precursors may obscure the actual rate of decay of the labelled products. [^{14}C]Glucosamine was not extensively reutilized in rats (Robinson, 1969) and the very small amounts of trichloroacetic acid-soluble ^{14}C -labelled compounds found in kidney-cortex homogenate 50 h after injection (Fig. 2) indicates that reutilization of this precursor was un-

Fig. 4. Densitometric scans of proteins from brush borders dissociated in the presence of mercaptoethanol and separated by electrophoresis on polyacrylamide gels

Details were as given for Fig. 3 except that the brush-border samples were dissociated in the presence of 2.5% (w/v) mercaptoethanol in addition to sodium dodecyl sulphate and were heated at 100°C for 5 min.

likely to influence the results markedly. However, reutilization of [^3H]lysine may be more marked. Small amounts of trichloroacetic acid-soluble ^3H -labelled material were detected in kidney cortex throughout the duration of the experiments (Fig. 2), but were low compared with the initial pulse of radioactive material, and the kinetics with [^3H]lysine were very similar to those with [^{14}C]glucosamine, supporting the view that reutilization did not markedly influence the results.

A further source of error would derive from contamination of the brush borders by adsorbed protein. The kidneys were perfused before removal from the animals to minimize possible adsorption of plasma protein. Further, antisera to rabbit kidney brush borders showed no cross-reaction with plasma protein (M. Kramers, unpublished work). Alternatively, the brush borders could adsorb protein after homogenization but the disproportionate ratio of ^3H radioactivity to ^{14}C radioactivity in the whole homogenates (Fig. 2) as compared with brush borders (Fig. 1) indicates that this is unlikely unless adsorption of a few specific proteins occurs.

The simplest explanation of the results is to assume that there are two kinetic components involved in the removal of proteins from the brush borders and that

the experimental results represent a summation of these two processes. One component is stable, remaining constant for the next 100h after maximum radioactivity is reached and decaying from 120h onwards; the second is a labile component exhibiting an exponential decay. These two processes are represented in Fig. 5 for the results obtained with glucosamine; the assumed values for the total radioactivity of the stable component up to 50h are subtracted from a curve representing total radioactivity in the brush borders, and the difference has been plotted for each time-point. The resulting function has an exponential decay with a half-life of 8h; similar analysis of the results obtained with [^3H]lysine yielded an exponential function with a half-life of 10h. Since the two components are presumed to be derived from a common precursor pool, the maximum radioactivity of the rapid component should be reached at the time when the point of inflexion occurs in the line representing the increase of radioactivity of the slow component. The variation with time of the total radioactivity of the rapid component will partly reflect the turnover of the precursor, so no inferences can be made concerning the actual behaviour of this component.

It may be reasoned that these two kinetic phases of

Table 1. *Relative radioactivity of glycoproteins from kidney brush borders at various times after administration of [¹⁴C]glucosamine*

Kidney brush borders were isolated from rabbits injected with [¹⁴C]glucosamine (25 μ Ci). The brush borders were dissociated with sodium dodecyl sulphate (a) in the presence and (b) in the absence of mercaptoethanol, and the component proteins and glycoproteins were separated by polyacrylamide-gel electrophoresis (details are given in the text). The gels were frozen and then sliced and the ¹⁴C radioactivity in the slices was determined. The radioactivity in the slices was compared with the periodic acid-Schiff base-staining components observed in duplicate gels, allowance being made for gel swelling during the fixing and staining procedures. The numbers refer to the numbered bands in Figs. 3 and 4. The radioactivity in the bands was expressed as a percentage of the total radioactivity recovered from each gel. The recovered radioactivity averaged 95% of the applied radioactivity.

(a) Brush borders dissociated in the presence of mercaptoethanol

Staining band no.	Time after injection (h) ...	5.3	16	33	63	120	167
		% of total radioactivity					
1-3		14	8	12	8	8	11
4		3	4	2	7	8	4
5-6		33	28	25	18	26	24
7-8		45	41	50	50	52	52
9-10		5	12	10	13	5	9

(b) Brush borders dissociated in the absence of mercaptoethanol

Staining band no.	Time after injection (h) ...	10	16	63	120	167
		% of total radioactivity				
1-3		12	11	13		13
4		9	6	3	13	5
5-6		25	28	26	31	28
7-8		38	41	46	44	45
9		8	10	8	7	3
10		5	3	0	6	2
11		3	0	0	0	3

the decay represent the independent breakdown of two groups of molecules of different identities. However, the similarity of distribution of radioactive isotopes among the glycoproteins at different times during the experiment (Table 1) argues against this hypothesis. Indeed these results suggest that all the components are degraded at the same rate throughout the experiment.

Since proximal tubules exhibit pinocytosis at the base of the microvilli (Maunsbach, 1966) the newly synthesized components may follow two paths, as portrayed in Fig. 6. A proportion of these components may be incorporated into pinocytotic vesicles and so be removed rapidly from the brush borders, while the remainder move away from the site of pinocytosis into the microvillus to remain there for prolonged periods. This mechanism would result in the complex turnover kinetics observed. The plateau shape of the curve for the slowly declining component indicates that little of the material entering the microvillus membrane is lost before 120 h has elapsed; pos-

sibly this material moves slowly up the microvillus and is lost only as it approaches the end. On the other hand if the slow decay is assumed to follow an exponential function loss would be random with a half-life of 150 h. This turnover is unlikely to represent a loss of cells since the proximal tubule epithelial cells are replaced only very slowly; the turnover time of these cells in mice is 190 days (Cameron, 1971). The ultimate fate of the microvillus components is not known but since brush-border antigens and enzymes have been detected in urine (M. Kramers, unpublished work) it appears that they may be lost in part to the urine. Studies *in vitro* have demonstrated that membrane components are lost to the external environment (Hughes *et al.*, 1972).

Studies on the turnover of membrane components of intestinal brush borders with labelled glucosamine (Forstner, 1968) and labelled amino acids (James *et al.*, 1971) each showed that loss of label follows an exponential function with, in the latter case, a half-life of 18 h. James *et al.* (1971) compared the turnover

of a disaccharidase-rich fraction (half-life 11 h) with that of whole brush border, and concluded that the membrane components exhibited a differential rather than a concerted turnover. The evidence obtained in the present study, i.e. that the relative radioactivity of the various glycoproteins does not alter during the experiment (Table 1) and that the kinetics of [^3H]lysine incorporation are very similar to those for [^{14}C]glucosamine, indicates that the kidney brush-border membrane components turn over in unison.

The turnover of liver plasma membranes is exponen-

tail, with a half-life of 37 h (Kawasaki & Yamashina, 1971). That study and that of Evans & Gurd (1971) indicated that the protein and glycoprotein components of liver plasma membranes undergo a concerted synthesis and turnover, although this conclusion has been disputed by Simon *et al.* (1970). Evans & Gurd (1971) also reported that the glycolipids of liver plasma membranes contained radioactivity when [^{14}C]glucosamine was used as the precursor. Little radioactivity was detected in lipid extracts from kidney brush borders; lipid extracts contained some

Fig. 5. *Theoretical analysis of the incorporation of glucosamine into brush-border protein on the assumption that the experimental results represent a summation of two kinetic components*

For simplicity total radioactivities rather than specific radioactivities are employed in the figure.

The total brush-border radioactivity must follow a curve identical with that for specific radioactivity except that the numerical values of the ordinate will be multiplied by some number representing the total brush-border protein. One kinetic component is assumed to be stable, reaching a plateau, whereas the other is unstable, exhibiting rapid turnover. Both components are presumed to be derived from a common precursor pool so that the maximal radioactivity of the rapid component should occur at the same time as, or shortly after, the point of inflexion in the line representing the increasing radioactivity of the stable component. Summation of the total activities of the two theoretical components at any time will equal the total radioactivity in the brush borders at that time. Since the proportion of the total protein in each component is not known no relative specific radioactivities can be ascribed to them. The upper curve represents the total brush-border radioactivity and the lower curves the total radioactivities of the two hypothetical components. The points indicate the experimental values for total brush-border ^{14}C radioactivity as shown in Fig. 1.

Fig. 6. *Schematic representation of the two possible pathways for turnover of brush-border protein*

Newly synthesized protein is assumed to enter the brush borders at the base of the microvilli. A proportion of these proteins form pinocytotic vesicles and are lost from the brush borders; the remainder enter the microvilli, moving outwards from the base.

^3H in addition to ^{14}C , possibly indicating the presence of some lipoprotein. It is not known why chloroform-methanol extracted more radioactivity from trichloroacetic acid-treated brush borders than from native brush borders. Possibly prior denaturation of some lipoproteins rendered them more readily soluble in the solvent mixture.

Membrane turnover has been studied in cells maintained *in vitro* but it is not known to what extent the behaviour of these cells resembles that of non-dividing differentiated cells. The results of Warren & Glick (1968) and Hughes *et al.* (1972) indicate that the loss of labelled material from the membranes of cells incubated *in vitro* is more rapid than that observed for kidney brush borders *in vivo*.

S. J. Q. is indebted to the Medical Research Council for a Scholarship for Training in Research Methods. We thank Dr. R. W. Hiorns and Dr. T. Quirk for assistance with statistical analyses; we are grateful to the Cancer Research Campaign for financial support.

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