J. Membrane Biol. 27, 83–100 (1976)

Studies on the Brush Border Membrane of Mouse Duodenum

II. Membrane Protein Metabolism

T. Billington and P.R.V. Nayudu

Department of Zoology, Monash University, Clayton 3168, Victoria, Australia

Received 31 January 1974; revised 27 June, 17 December 1975

Summary. Mouse duodenal microvillus membrane protein metabolism was measured using radioactive labelling techniques. Labelled amino acids were introduced into the lumen of ligatured duodena. Following exposure to label, brush border membranes were isolated and analyzed. Experiments measuring the specific activity of protein labelled with a single amino acid revealed that total membrane protein appeared to turnover in about 14 hr. Protein in the mucosal homogenate had a faster turnover rate. Turnover rates of individual proteins were measured with single and dual isotope experiments. Membrane protein was solubilized with sodium dodecyl sulphate (SDS) buffer. Single isotope experiments showed that all polypeptides separated on SDS-gels were maximally labelled at 6 hr after injection. Bands did not incorporate label linearly. Rates of loss (degradation) of label from membrane proteins in the seventeen bands appeared to be related to the estimated molecular size of the proteins. Rates were highest for larger polypeptides. A double isotope technique, in which proteins were allowed to incorporate the same amino acid in two isotopic forms, delivered with a set time interval intervening, revealed that the ratios of the second label to the first in the SDS-separated polypeptides were highest for larger proteins and lowest for smaller polypeptides. Certain assumptions were outlined and the ratios taken as measures of turnover of proteins. Loss of label due to cell sloughing is discussed. A mixture of labelled amino acids (excluding leucine) was used to show that differences in leucine contents of different proteins was not an explanation for the variation in level of leucine radioactivity in different bands. For specific activity measurements throughout, protein in gels was quantitated with reference to the uptake of Coomassie stain. The use of this stain was validated by the finding that, at low protein concentration, the amount of stain taken up was proportional to the amount of bovine serum albumin or membrane protein loaded.

In isotope-labelling experiments of intestinal epithelial cells of the villi, Leblond, Everett and Simmons (1957) found uniform label along the length of villus. Lipkin, Almy and Quastler (1961) obtained autoradiographic evidence, with radioactive leucine, indicating that incorporation of label into proteins mainly took place in the crypt cells. These results led to the interpretation that biosynthesis, and in consequence, the turnover of proteins in the mucosal cells ceased when the cells,

following mitotic divisions, moved up along the villus. More recently, the work of James, Alpers, Gerber and Isselbacher (1971) on the turnover of disaccharidases and that of Alpers (1972 *a*, *b*) on protein turnover in the brush border membrane of the rat intestine, have shown that proteins of the membrane do turn over as the cells move to the tip of the villus, and rapidly, too.

In a previous paper (Billington & Nayudu, 1975), mouse duodenal microvillus membranes were isolated in a reproducibly high state of biochemical and morphological purity. They were shown to be composed of at least seventeen polypeptide species, identified on polyacrylamide gels in the presence sodium dodecyl sulphate. It was also established that there was a considerable heterogeneity in the apparent molecular weights of the protein species.

The purpose of this paper is to report turnover studies on the constituent polypeptides of the microvillus membrane of mouse duodenum. The approach used was to study the rates of degradation of membrane protein as a whole, and then to investigate the rates of synthesis and degradation of the individual protein species of the membrane.

Materials and Methods

Radioactively labelled compounds, tritiated L-leucine (50 mCi/mM), ^{14}C-L-leucine (100–250 mCi/mM), ^{14}C-L-tyrosine, ^{14}C-DL-phenylalanine, and ^{14}C-L-valine, each 10 mCi/mM, were obtained from The Radiochemical Centre, Amersham, U.K. The scintillant used was Instagel, purchased from Packard Instrument Co., Victoria, Australia. The anaesthetic was tribromoethanol, supplied by K and K Laboratory, New York, N.Y.

Methods

Anaesthesia and Surgery. Adult Swiss mice were anaesthetised by the intraperitoneal injection of 5% tribromoethanol in 5% ethanolic solution (0.35 ml per 25 g body weight). A median incision 1.5 cm long was made immediately posterior to the xiphoid cartilage to gain access to the abdominal cavity. A silk ligature was placed around the duodenum at a point just posterior to the junction of the common bile duct. The continuity of the duodenum was traced with a blunt probe and a second ligature placed 5.5 cm from the proximal ligature.

Total Protein Turnover. To investigate total protein turnover in the brush border membrane as a whole, both ^{14}C-L-leucine (20 μCi) and ^{3}H-L-leucine (40 μCi) were injected simultaneously into the ligated segment of duodenum, and after specified intervals of time, groups of mice were sacrificed and microvillus membranes isolated (Billington & Nayudu, 1975). Samples of crude mucosal epithelial cell homogenate were also collected and all tissues frozen for further analysis.

Protein was precipitated from samples by treatment with ten volumes of 15% TCA. The pellet was separated by centrifugation and was washed successively by resuspending it in 7% TCA, ethanol-ether (3:1) and ether. Ether was removed from the final pellet by evaporation. Samples of mucosal homogenate and brush border proteins, precipitated as above, were analyzed for protein (Lowry, Rosebrough, Farr & Randall, 1951) and for radioactivity. Samples for counting were placed in glass vials with 5 ml of Instagel added to each. Vials were gently agitated, capped and placed in a Packard TriCarb Scintillation Spectrophotometer. The counter had a tritium efficiency of 10.1% and a carbon-14 efficiency of 36.8%, and each vial was counted for a minimum of 2,000 counts above background.

Turnover of Individual Proteins. To investigate the turnover of individual proteins/polypeptides in the brush border membrane, two types of experiment were performed using labelled leucine and separate groups of mice.

(1) Single isotope experiment. Phosphate-buffered saline (0.1 ml) containing 20 μCi of ^{14}C-L-leucine was injected into the duodenum just distal to the first ligature. The incision was closed and the animals allowed to regain consciousness. Animals had access to water but not to food. At specified intervals of time following the isotope administrations, groups of five mice were sacrificed and brush border membranes isolated from the duodenal sections between the ligatures.

(2) Dual isotope experiment. Following the pulse labelling technique of Arias, Doyle and Schimke (1969), this involved injecting ^{14}C-L-leucine and then after a set period, administrating ^{3}H-L-leucine. ^{14}C-L-leucine (20 μCi per mouse) was injected as above. After 9 hr, an injection of tritiated leucine (40 μCi per mouse) was administered. Animals were allowed only drinking water for 8 hr after the second injection. Animals were sacrificed and brush border membranes isolated. At the end of 8 hr, all labelled proteins were assumed to be in a process of isotopic decay.

Solubilization of Membranes. Solubilization of both collections of membranes was accomplished using the solvent outlined by Billington and Nayudu (1975).

Gel Electrophoresis. Samples of clear supernatant, containing about 160 μg of protein were loaded onto 7% polyacrylamide gels. Electrophoresis was performed as described in the above reference, and then gels were stained for protein and scanned densitometrically.

Counting Procedures. Gels were sliced into discs of 1 mm thickness (Iandolo, 1970), and the discs placed individually in scintillation vials. Discs were solubilized by overnight heating in the presence of 0.1 ml each of 0.1 M HCl and 30% H_2O_2. Instagel was then added to each vial, and counting commenced.

For the single isotope, the gain settings were adjusted to give maximal counts. For the counting of dual isotopes, the gain controls were set so as to allow about 4% of tritium counts to appear in the ^{14}C window, and to permit only 3% of the carbon-14 counts to appear in the tritium window.

Determination of Patterns of Incorporation of ^{14}C-L-leucine into Proteins at Positions along the Villus. 40 μCi of the labelled amino acid was injected into the ligatured duodena of eight groups of six mice. After specified intervals groups of six mice were sacrificed, their duodena excised, slit longitudinally and cut into 1 mm squares. Each of these was frozen onto a cryostat chuck so that the long axis of the villi pointed directly away from the face of the chuck. Sections of 10 μ thickness were cut as described by Nordstrom, Dahlqvist and Josefsson (1967), from the tip of the villus to just above crypt level. Protein was precipitated from the cut sections using 10% trichloroacetic acid (TCA). The precipitated protein from each level was solubilised in 0.8 M sodium hydroxide and samples were assayed for protein and radioactivity.

Determination of Patterns of Incorporation of Both Isotopes into the Proteins of Brush Border Membranes Isolated from Different Levels along the Villus. A double labelling experiment was repeated as described above, except that the mucosae were scraped lightly to collect cells from the upper half of the villi, and were then scraped more heavily to obtain cells from the lower half. The levels of scraping were carefully monitored by observation with a Leitz binocular microscope. Crypt material was not included in the scrapings from the lower villus levels. Brush border membranes were isolated from the two scrapings, then solubilized for sodium dodecyl sulfate gel electrophoresis. Gels were stained for protein, scanned, and sliced for liquid scintillation counting of both isotopes.

Incorporation Studies Using an Amino Acid Mixture. To investigate whether differences in incorporation of ^{14}C-L-leucine into membrane proteins may be due to dissimilar leucine content of the proteins relative to one another, an amino acid mixture was used. The mixture consisted of equal amounts of ^{14}C isotopic forms of L-tyrosine, L-phenylaline and L-valine, prepared in phosphate-buffered saline. A sample (0.2 ml) of this buffer containing 18 μCi of activity was injected into each ligatured duodenum. After specified intervals of time, groups of mice were sacrificed and brush borders isolated. They were solubilized and electrophoresed on gels. Gels were stained, scanned and then sliced for counting.

Protein Quantitation Based on Coomassie Blue Uptake. Using the densitometric tracings of gels on which membrane proteins had been run, the amount of protein in each band was estimated. A sample of brush border membranes was solubilized as described previously (Billington & Nayudu, 1975). Protein which had been solubilized into a clear supernatant was determined by the method of Lowry et al. (1951).

Known amounts of microvillus membrane protein were run on separate 7% acrylamide gels. Gels were stained and traced densitometrically. Scans were made from gels on which 50, 80, 100, 160 and 200 μg of protein had been electrophoresed.

For smaller amounts of protein, bovine serum albumin (BSA) was used and gels carrying 2, 5, 10, 20, 30, 35, 40, 45 and 50 μg of BSA were scanned densitometrically. From each of these experiments, a relationship was established between amount of protein on gels and the area under the peaks on the scans. The relationship was linear from zero to 45 μg of BSA. Such data were used to calculate the amounts of protein in each band on a gel on which 160 μg of membrane protein had been electrophoretically separated.

Results

Turnover of Total Brush Border Membrane Protein

The plot of counts per minute versus time of incorporation into total membrane protein and into total mucosal epithelial cell protein is shown in Figure 1. Membrane protein specific activity reached a maximum at about 6 hr, then decreased with an approximate half-life of 14 hr. Maximum specific activity occurred slightly earlier in the homogenate protein, decreasing with an apparent 8-hour half-life. Thus it would appear that proteins in the brush border membrane are synthesized and degraded at quite different rates, as compared with the average for the whole cell.

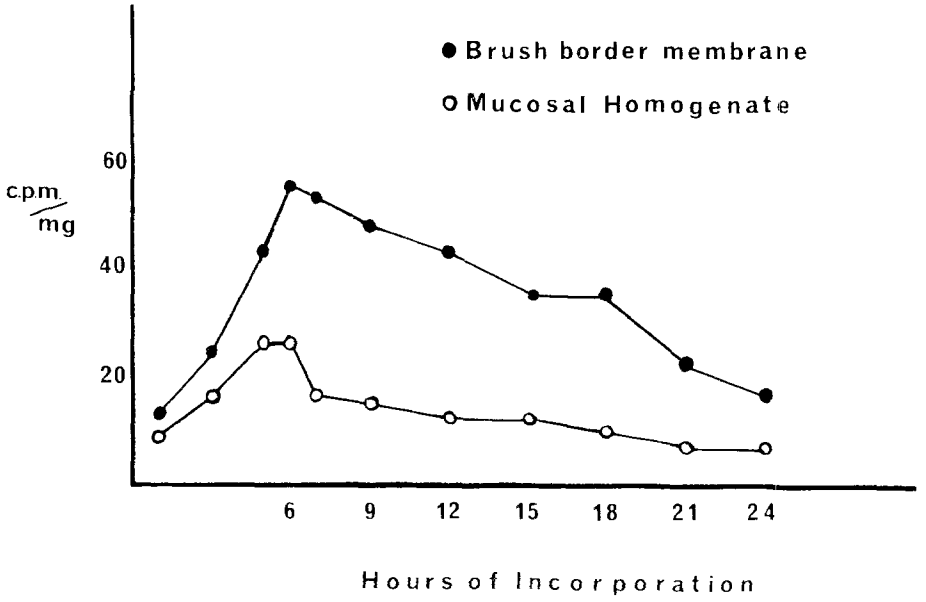

Fig. 1. Incorporation and loss of ^{3}H-L-leucine specific activity with time. This figure shows the change in specific activity in total brush border membrane proteins (●) and in proteins of the whole mucosal homogenate (○). Points are the means of seven to 10 measurements differing from the means by 7–11%

Turnover of Protein in Individual Bands

Figure 2 shows the result of using a single isotope and then at specified intervals of time after administration, measuring the extent of its incorporation into individual protein species. In a preliminary experiment designed to determine the time when maximum specific activity was reached, it was evident that this time was approximately 6 hr. Figure 3 compares bands as to the rate of loss of specific activity. This loss seemed to follow first-order kinetics as judged from the fact that the points define a linear relationship only if the ordinate has a logarithmic scale. There were marked differences in degradation rates between bands. Larger molecular weight bands lost label the most quickly. The reverse seemed to be true for bands with smaller molecular weights. Bands 9 and 10 appear, however, to be an exception to this trend and bands 15, 16 and 17 were degraded so slowly that it was impossible to assign reliable turnover times to them. Table 1 outlines the approximate turnover times of the 17 protein bands, as determined solely from their rates of loss of labelled amino acid.

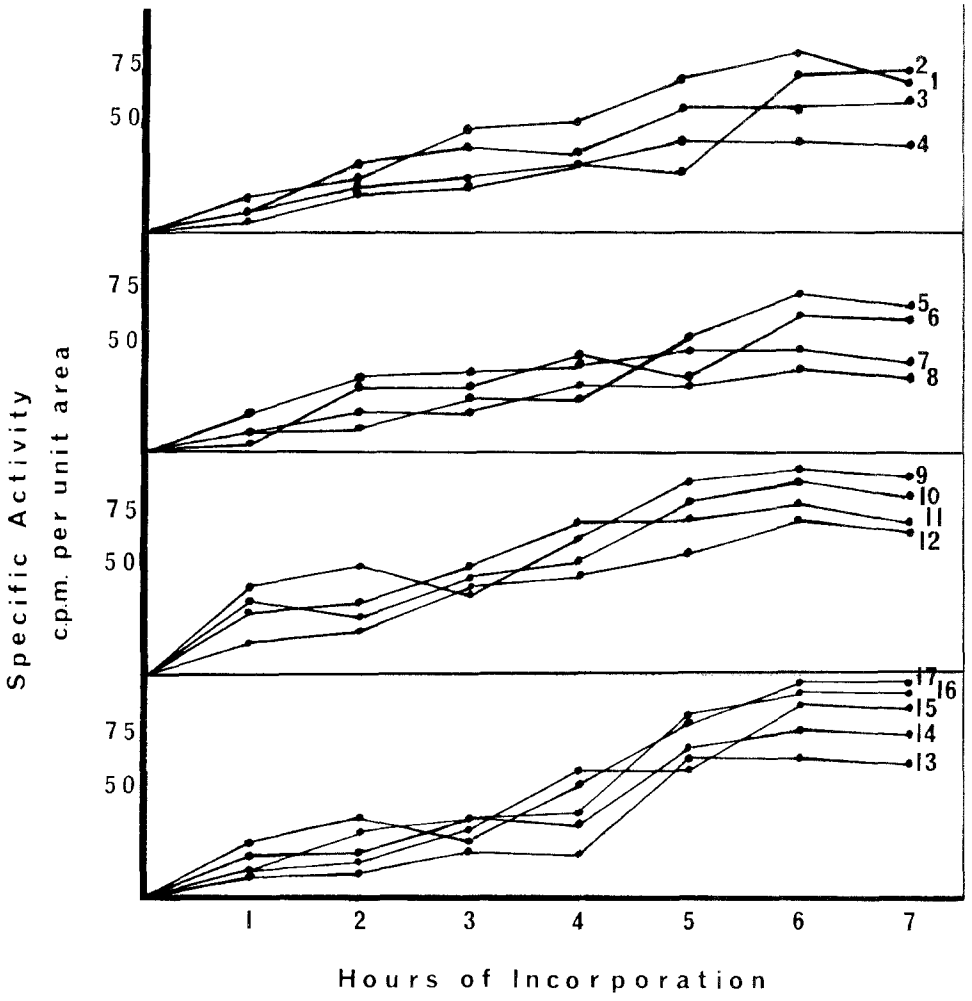

Fig. 2. Time-course of incorporation of ^{3}H-L-leucine into polypeptides in individual bands of microvillus membrane protein separated on sodium dodecyl sulphate polyacrylamide gels. The specific activities were measured as counts per minute per unit area under the peaks in the densitometric tracings, corresponding to polypeptide bands stained with Coomassie Brilliant Blue. The bands are numbered on the right of the figure. Points are the means of sets of 12 measurements

Turnover of Individual Proteins—Double-Labelling Technique

A sequential administration of two isotopic forms for the same amino acid was used to gain additional information about the dynamics of protein metabolism in the microvillus membrane. Figure 4 shows that there was a decline in the ratio of the second label to the first for bands along the gel. Higher molecular weight proteins had the larger ratios, while proteins of lower molecular size exhibited smaller ratios.

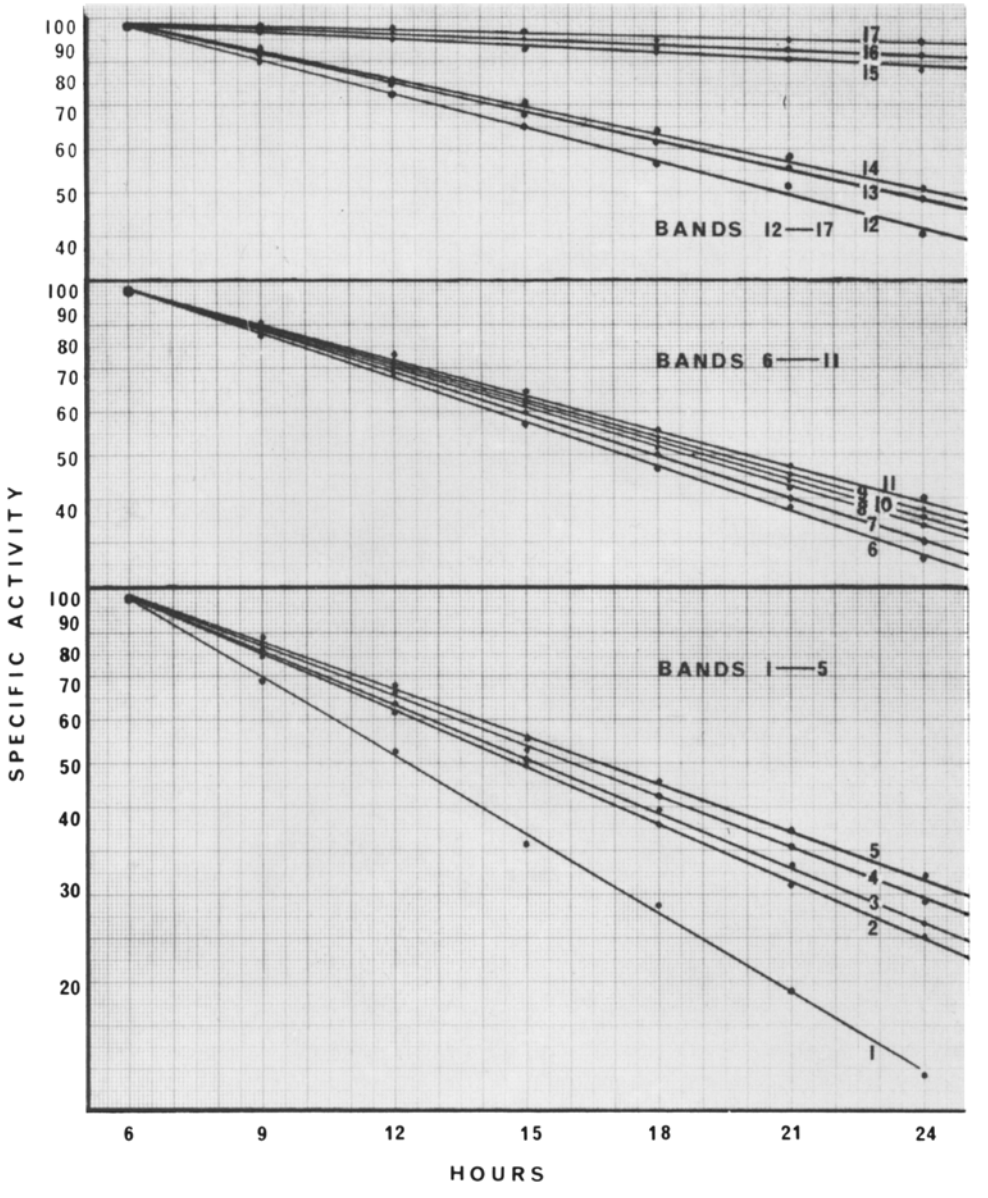

Fig. 3. Time-course of loss of ^{3}H-L-leucine from polypeptides in individual bands of microvillus membrane proteins fractionated on sodium dodecyl sulphate-polyacrylamide gels. The horizontal axis indicates time after injection of labelled amino acid. The vertical axis is a logarithmic scale delineating percentage maximum specific activities. Points are the means of 12 measurements

Table 1. Turnover times for the microvillus membrane protein species in each Coomassie stained band, estimated from the rates of loss of a single label from bands, this being assumed to be closely related to rate of protein degradation

Band	1	2	3	4	5	6	7	8	9
Approx. turnover time (h)	6.3	8.8	8.8	9.2	10.0	10.7	11.3	12.0	12.8
Band	10	11	12	13	14	15	16	17	
Approx. turnover time (h)	13.5	13.0	14.0	14.8	17.4	18.3	very	large	

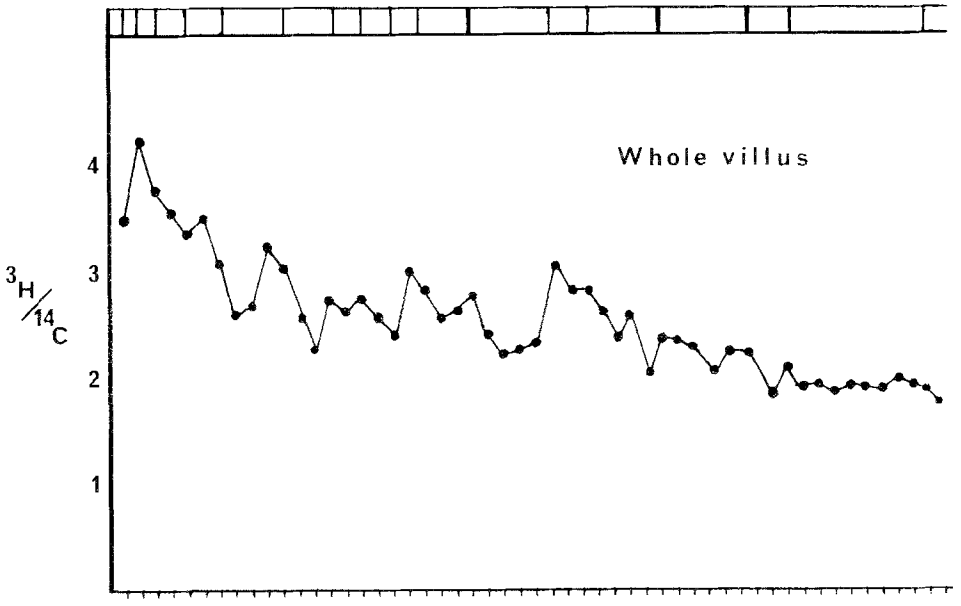

Fig. 4. Ratios of the second label to the first in bands of membrane proteins measured in dual isotope experiments. The microvillus membranes were isolated from epithelial cells at all levels along the villus. Points are the means of sets of 10 values. The centers of the bands are shown at the top of the figure. The marks on the horizontal axis indicate the positions of slices cut from gels used to construct the figure. Gel origin is on the left

Administration of a Mixture of Labelled Amino Acids

The results (not shown) demonstrated that a mixture of labelled amino acids was taken up to about the same extent as was labelled leucine (Fig. 2).

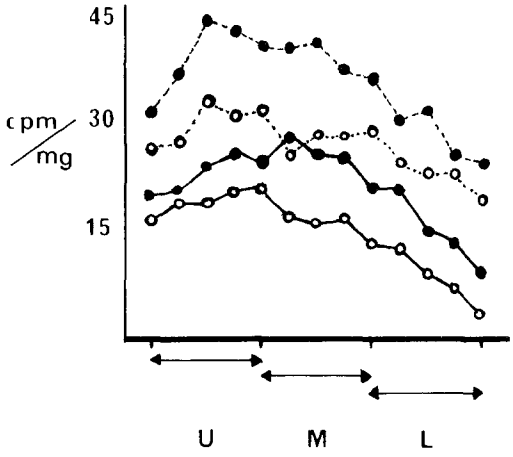

Fig. 5. Incorporation of a single label into protein along the villus of the mouse duodenum. The time dependence is indicated as: ●---●, 15 hr; ○——○, 18 hr. Positions along the villus are: *U*, upper villus, *M*, middle villus, *L*, lower villus. Points are the means of sets of nine values

Appearance of Labelled Leucine in Proteins of the Brush Border Membrane

Figure 2 shows the rates of appearance of tritiated leucine in the protein species. There were some variations between bands in the first 6-hr interval after injection, and the overall incorporation rate for each band was only approximately linear.

Location of Labelled Amino Acid with Respect to Villus Architecture

Figure 5 shows clearly that the incorporation pattern along the villus did not vary to any great extent over the stated interval and that the number of counts gradually diminished relatively evenly over the whole villus length.

Incorporation of Two Labels into Microvillus Membranes at Different Levels on the Villus

Figure 6 shows the analysis of the ratios of counts in proteins from membranes at two different levels on the villus.

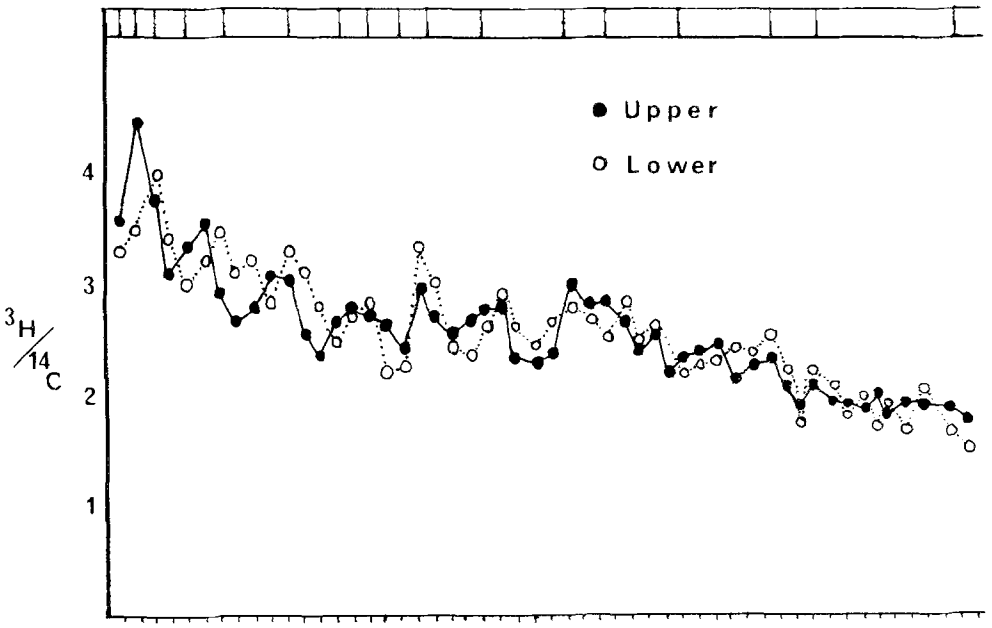

Fig. 6. Ratios of the second label to the first in individual bands of protein solubilized from microvillus membranes isolated from epithelial cells at two extreme positions along the villus by differential scraping as described in the text. Centers of stained polypeptide bands and positions of gel slices are shown in the figure. Points are the means of sets of nine values

Protein Quantitation

Figure 7(*a*) shows that up to and including 40 μg of BSA, there is a linear relationship existing between area and amount of protein. As can be seen, the amount of stain bound to amounts greater than 40 μg, becomes variable. Figure 7(*b*) outlines the extent of the linear relationship for total membrane protein. For amounts of membrane protein greater than 160 μg, the pattern of staining of bands relative to one another becomes variable.

Discussion

Protein metabolism appears to be an important cellular process, in determining the dynamic steady state of cell function. Rechcigl (1971) and Schimke (1973) have reported that there seem to be characteristic rates at which particular protein molecules in cells are broken down

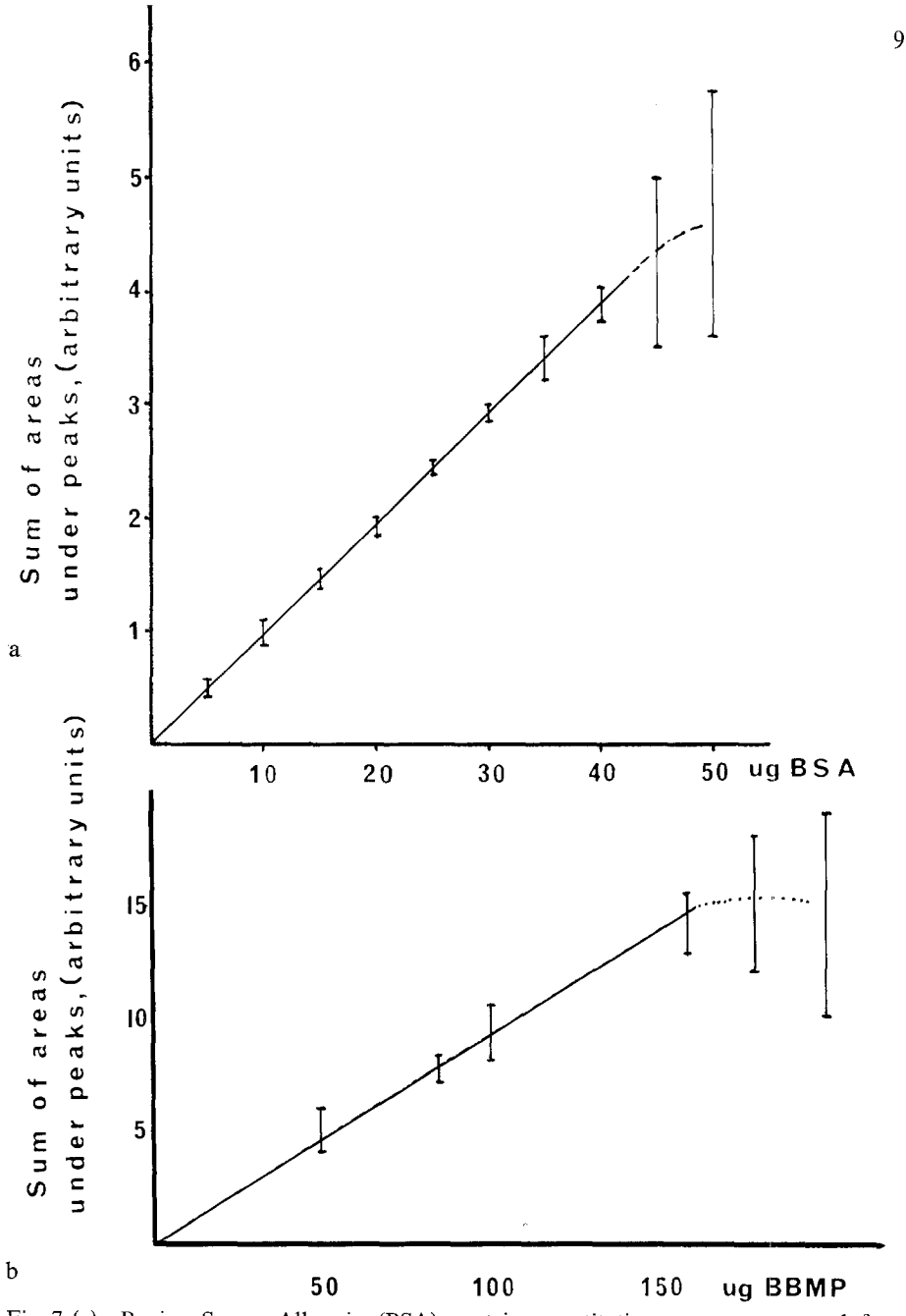

Fig. 7. (*a*) Bovine Serum Albumin (BSA) protein quantitation curve constructed from total areas under the peaks of the densitometric tracings of Coomassie-stained gels, on which known amounts of BSA had been electrophoretically fractioned. The straight line joins the means of eight to 10 values for each point, extreme values being shown by horizontal bars. (*b*) Brush border membrane protein (BBMP) quantitation curve constructed from total areas under the peaks of the densitometric tracings of Coomassie Blue-stained gels on which known amounts of membrane protein had been electrophoresed. The straight line joins the means of eight to 10 values for each point, extreme values being shown by horizontal bars

to their constituent peptides and amino acids. Siekevitz (1963) demonstrated that there were many biochemical reactions occurring at or on the endoplasmic reticulum of rat hepatocytes. Hormonal and nutritional stimulation studies (Ashmore, Hastings, Nesbett & Renold, 1956; Freedland & Harper, 1959) lent support to the idea that this membrane is structurally dynamic, with enzyme levels in a state of flux. Omura, Siekevitz and Palade (1967) used isotopic tracer technology to show that protein and lipid molecules of the mammalian endoplasmic reticulum are being rapidly synthesized and degraded with a heterogeneity of rates.

Plasma membrane proteins have characteristic turnover rates (Dehlinger & Schimke, 1971; Gurd & Evans, 1973). Further, there appears to be a rough correlation between turnover rate and molecular size, in which high molecular weight fractions tend to turn over most rapidly, while proteins of small molecular size seem to be synthesized and degraded at low rates.

When preliminary trials were conducted to determine the best route of administration of labelled amino acid, it was observed that injection into the coccygeal vein led to low incorporation into proteins of the epithelial cell homogenates, but no label was ever observed in the brush border membrane fraction. The parenteral route was thus abandoned, despite its apparently successful use in the studies of James et al. (1971).

The intraluminal mode of administration (Alpers, 1972*a*) was used in the present studies, because it gave far more efficient incorporation of isotope into membrane protein. In addition, longer time intervals required for the incorporation of parenterally-supplied label into brush border membrane proteins, could permit the loss of label from proteins with short half-lives, leading to difficulties in the interpretation of turnover data.

The specific activity of isotope in total membrane protein increased approximately linearly to reach a maximum at 6 hr after administration. A drop in specific activity was observed by the 7th hr and the decrease continued, to show a 50% reduction by the 20th hr, indicating a half-life of total membrane protein of 14 hr. James *et al.* (1971) made similar measurements in rat small intestine and demonstrated that 10 hr was required for maximal labelling of brush border membrane protein. A half-life of 18 hr was observed in rat. Specific activity in mucosal homogenate proteins showed a half-life of 31 hr in rat, which is very different from our findings in the mouse. It is likely that the cause of this difference is that in the work on rat, the authors were observing the results of a marked reutilization of label. This would be expected to lead to an

over-estimation of turnover time. It may be conversely argued that our studies in mouse have under-estimated the turnover due to all the available label having been consumed at a time when more may have been required to achieve maximal labelling, under steady-state condition. It seems, however, that this lack of label is very unlikely, since at the time of sacrifice there were always considerable amounts of label remaining in the lumen.

Considering the incorporation of label into total proteins of the brush border membrane, we observed that there was a 4-hr difference between the times for maximal labelling in rat and mouse. An explanation for this difference could be that there exists a disparity between mouse and rat in the rate of transfer of labelled membrane precursors from a cytoplasmic to a membrane location.

The measurement of turnover rates of individual proteins in the mouse brush border membrane was approached using both single and dual isotope labelling techniques.

Singel isotope experiments revealed that each polypeptide species rapidly incorporated labelled amino acid after intraluminal injection, which is consistent with the idea that intestinal proteins may be synthesized preferentially from luminal rather than blood-borne amino acids (Hirschfield & Kern, 1969). It was surprising to find that at the 6-hr time point, each of the 17 Coomassie-stained bands had incorporated label maximally. With one exception, measurements made at the 7-hr point revealed that each band had begun to lose label. As was noted in the Results section, there was a nonlinear uptake of label (synthesis) for the bands separated on gels. A nonlinear synthetic rate for a particular band of protein may be the result of several rates occurring simultaneously but all being isolated in one band whose overall rate of synthesis we can estimate. This is because sodium dodecyl sulphate electrophoresis separates polypeptides on the basis of molecular weights only. Thus two or more protein species of about the same molecular weight may migrate to the same position on the gel, but may have completely different rates of synthesis.

From the single isotope studies reported here, the rate of loss of label from the 17 bands appears to be related to the molecular sizes of the proteins in those bands. It is evident that the rate of degradation (label loss) is high for proteins of large molecular size and low for fast-migrating, small molecular weight polypeptides. There are no other reports of studies of metabolic aspects of mouse intestinal brush border membrane proteins. Alpers (1972*b*) reported on the half-lives of intestinal

brush border membranes of rat. This report on mouse duodenal microvillus membranes has not only revealed a similar heterogeneity in the rates of degradation of membrane protein species, but it has also become evident that there is a comparable inverse relationship between molecular size and half-life of the membrane proteins.

The major drawback of the single isotope experiments is that label may be reutilized in successive rounds of protein synthesis. To check on this possibility, a double isotope method was used, based on certain assumptions outlined by Arias *et al.* (1969). The assumptions are:

(1) At time of sacrifice, all labelled proteins are in a process of isotopic decay. To be assured that the isotopic ratios obtained in this study were a measure of degradation rates, animals were sacrificed 8 hr after administration of the second isotope. This was assumed to be long enough after the injection to ensure that all labelled proteins were in a process of isotopic decay.

(2) The isotope is not metabolized to products which can be incorporated into the protein fraction used for determination of radioactivity. A simple experiment was performed in which labelled brush border membrane protein was characterized as to its ratio of tritium to carbon-14. Then lipid was extracted and the ratio measured again. Very few counts were recovered in the lipid fraction, suggesting that only negligible amounts of labelled amino acid were channelled into the tricarboxylic acid cycle and thence to Acetyl CoA for fat synthesis. The ratios were found to differ by only 9%, averaged over eight experiments. Slightly more than 90% of counts were recovered in the protein fraction, on each trial, presumably as leucine.

(3) The proteins follow exponential decay kinetics: Data expressed in Figure 3 suggest that this is a valid assumption, since a logarithmic scale for the ordinate was found to be necessary if a linear relationship was to be shown between specific activity and time after attainment of maximum specific activity.

(4) The rates of synthesis of protein constituents of the membrane are the same at both times of isotope administration. Alpers (1972*a*) has shown that there is no diurnal variation in the rate of incorporation of amino acid into brush border membranes of rats. No assumptions about such variation were made with mouse and to overcome the problems of differential synthesis related to time of day, injections and sacrifices were always made at the same time of day. The possibility was also entertained that nutritional status may have a bearing on protein

synthetic rates. Uniformly optimum nutritional status was assumed from the fact that mice were allowed water and ration cubes *ad libitum* before each set of experiments. This last assumption is the most difficult to apply to the mouse experiments since it is a problem of major dimensions to measure the rate of protein synthesis at the two injection times.

When this double labelling technique was used, it was observed that there is a gradual decline in the isotopic ratio in the protein bands as one proceeds from the origin of the gel to the tracking dye position. Higher molecular weight proteins have the larger ratios, while lower ratios were observed in small molecular weight bands. If the above assumptions are made, then one may take the isotopic ratio to be a measure of the turnover rate of the protein concerned. A high ratio would indicate that the second isotope was being actively incorporated at time of sacrifice, that is the polypeptide was being synthesized rapidly. A protein exhibiting a low ratio may be assumed to be synthesized only slowly since it retained high counts from the first isotope.

We appear to have confirmatory support of the previous finding that proteins of higher molecular weight turn over at greater rates than proteins of lower molecular weight. Alpers (1972*b*) reported a similar relationship for the proteins of the brush border membranes in rat. Gurd and Evans (1973) have established that in isolated mouse liver plasma membranes, high molecular weight protein components were more rapidly degraded than those of low molecular weight. Previously, this same relationship had been established for rat hepatocyte plasma membrane proteins.

Clarke (1973) has provided evidence to suggest that, in mouse, 36 hr elapse between the emergence of the epithelial cell from the mouths of the crypts and the sloughing off of this cell into the lumen at the villus tip. Thus, the time span of the experiment represents a significant fraction of the maturation time for epithelial cells. Turnover data may have been complicated because a fraction of labelled cells would be sloughed off thereby providing a route for the loss of label other than by protein degradation. If a significant fraction, that is, a quarter of the labelled cells were sloughed off, then the second isotope would be labelling a different set of brush border membranes from the first. If the above situation was obtained, then one would predict differences in the pattern of incorporation along the villus between the 6th and 18th hr.

However, the incorporation pattern along the villus did not vary significantly over the 12-hr interval. Thus, at the time of the second injection,

there was still a reasonable amount of the first label remaining in the epithelial cells throughout the villus length. Therefore, the two labels may be expected to be labelling the same brush border membranes.

A major shortcoming of the isolation technique is that one does not know from which level along the villus the brush border membranes are being isolated. If there is differential synthesis of membrane proteins along the villus then the interpretation of turnover becomes complicated. Thus in a double isotope experiment, if the rate of synthesis differed along the length of the villus, one would expect isotopic ratios to differ significantly. Figure 6 clearly shows that the ratios did not differ noticably in proteins along the membranes isolated from lower and upper villus levels. Thus, differences observed between ratios are related to the variation in incorporation of the second isotope, and not to having isolated membranes from different heights up the villus.

Finally in our consideration of possible difficulties involved in the interpretation of turnover data, we should dicuss the results of using a mixture of amino acids rather than simply leucine. The labelled mixture was incorporated to about the same extent as was labelled leucine alone, indicating that differences in incorporation levels between bands were due to the dynamics of leucine metabolism in the proteins and not to differences in leucine content. Although leucine content varies from band to band, similar variation is also found for one or more of the amino acids in the mixture, demonstrating that differences in leucine content are not likely to be the cause of the variations observed in the ratios.

With respect to the overall problem of membrane protein metabolism, the observed heterogeneity of turnover rates indicates that the brush border membrane may be synthesized component by component and not by a unit synthesis. This idea may correspond to the suggestion of Omura *et al.* (1967), that a biological membrane is a mosaic of building pieces, each with its own characteristic rate of synthesis and its own biochemical function.

Validity of protein quantitation based on Coomassie blue uptake: Coomassie blue was used as protein stain in preference to Amido Schwarz 10B because it has greater sensitivity than Amido Schwarz (Gorovsky, Carlson & Rosenbaum, 1970). However, unlike Amido Schwarz (Graham, Grieve & Smillie, 1968), Coomassie blue cannot always be relied upon to be taken up by a protein in proportion to the amount of protein. In this study it has been found that over a strictly limited range the optical density due to Coomassie uptake is proportional to the amount of protein in a band. This finding is supported by a similar revelation

for a protein from chloroplasts (Bennett & Scott, 1971). Those authors found linearity of stain uptake up to a maximum of 50 μg of protein. The present study demonstrated that, in the conditions used, linearity began to disappear for amounts of BSA greater than 45μg.

Because it was thought that BSA might have some special properties as regards Coomassie stain uptake, it was necessary to test known amounts of membrane protein to see if a similar relationship existed. That this was found to be the case is demonstrated in Figure 7(*b*). Thus protein quantitation based on Coomassie blue uptake was thought to be valid for the indirect determination of protein in studies of the specific radioactivity of isotope-containing protein.

References

Alpers, D.H. 1972*a*. Protein synthesis in intestinal mucosa: The effect of route of administration of precursor amino acids. *J. Clin. Invest.* **51**:167

Alpers, D.H. 1972*b*. The relation of size to the relative rates of degradation of intestinal brush border proteins. *J. Clin. Invest.* **51**:2621

Arias, I.M., Doyle, D., Schimke, R.T. 1969. Studies on the synthesis and degradation of proteins of the endoplasmic reticulum of rat liver. *J. Biol. Chem.* **244**:3303

Ashmore, J., Hastings, A.B., Nesbett, F.B., Renold, A.E. 1956. Studies on the carbohydrate metabolism in rat liver slices. VI. Hormonal factors influencing glucose-6-phosphatase. *J. Biol. Chem.* **218**:77

Bennett, J., Scott, K.J. 1971. Quantitative staining of fraction 1 protein in polyacrylamide gels using Coomassie Brilliant Blue. *Anal. Biochem.* **43**:173

Billington, T., Nayudu, P.R.V. 1975. Studies on the brush border membrane of mouse duodenum. I. Isolation of membranes and analysis of protein components. *J. Membrane Biol.* **21**:49

Clarke, R.M. 1973. Progress in measuring epithelial turnover in the villus of the small intestine. *Digestion* **8**:161

Dehlinger, P.J., Schimke, R.T. 1971. Size distribution of membrane proteins of rat liver and their relative rates of degradation. *J. Biol. Chem.* **246**:2574

Freedland, R.A., Harper, A.E. 1959. Metabolic adaptations in higher animals. V. The study of metabolic pathways by means of metabolic adaptations. *J. Biol. Chem.* **234**:1350

Gorovsky, M.A., Carlson, K., Rosenbaum, J.L. 1970. Simple method for quantitative densitometry of polyacrylamide gels using fast green. *Anal. Biochem.* **35**:359

Graham, D., Grieve, A., Smillie, R.M. 1968. Phytochrome as a primary photoregulator of the synthesis of Calvin cycle enzymes in etiolated pea seedlings. *Nature* **218**:89

Gurd, J.W., Evans, W.H. 1973. Relative rates of degradation of mouse-liver surface-membrane protein. *Eur. J. Biochem.* **36**:273

Hirschfield, J.H., Kern, F., Jr. 1969. Protein starvation and the small intestine. III. Incoporation of orally and intraperitoneally administered leucine 4,5 ^{3}H into Intestinal mucosal protein of protein-deprived rats. *J. Clin. Invest.* **48**:1224

Iandolo, J.J. 1970. Device for slicing polyacrylamide gels. *Anal. Biochem.* **36**:6

James, W.P.T., Alpers, D.H., Gerber, J.E., Isselbacher, K.J. 1971. The turnover of disaccharidases and brush border proteins in rat intestine. *Biochim. Biophys. Acta* **230**:194

Leblond, C.P., Everett, N.B., Simmons, B. 1957. Sites of protein synthesis as shown by radioautography after administration of ^{35}S-labelled methionine. *Am. J. Anat.* **101**:225

Lipkin, M., Almy, T.P., Quastler, H. 1961. Stability of protein in intestinal epithelial cells. *Science* **133**:1019

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **193**:265

Nordstrom, C., Dahlqvist, A., Josefsson, L. 1967. Quantitative determination of the enzymes in different parts of the villi and crypts of rat small intestine. Comparison of alkaline phosphatase, disaccharidases and dipeptidases. *J. Histochem. Cytochem.* **15**:713

Omura, T., Siekevitz, P., Palade, G.E. 1967. Turnover of constituents of the endoplasmic reticulum membrane of rat hepatocytes. *J. Biol. Chem.* **242**:2389

Rechcigl, M. (Editor) 1971. Enzyme synthesis and degradation in mammalian systems. Karger, Basel

Schimke, R.T. 1973. Control of enzyme levels in mammalian tissues. *Adv. Enzymol.* **37**:135

Siekevitz, P. 1963. Protoplasm: Endoplasmic reticulum and microsomes and their properties. *Annu. Rev. Physiol.* **25**:15