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Research Article

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The route of administration of precursor (-leucine) is important in determining which part of the villus incorporates the label into protein. After intravenous administration, protein from cells near the villus-crypt junction is most heavily labeled, whereas after intraluminal administration protein from cells near the villus tip is most heavily labeled. The pattern of proteins most heavily labeled by radioactive precursor is different in the villus when compared with proteins from the crypt cells. Smaller molecular weight membrane-bound proteins are preferentially labeled in the crypt cells, whereas on the villus the pattern of labeling is more evenly distributed among the various proteins. Moreover, intraluminal leucine is utilized for protein synthesis to a greater extent than that in the blood, when the concentration in both compartments is similar. Thus, intraluminal and intravenous injections of labeled precursor are not equivalent. Both routes should be considered in data for experiments measuring intestinal protein synthesis.

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A B S T R A C T All cells in the intestinal villus of the rat are capable of synthesizing protein from amino acid precursors (L-leucine). Moreover, polyribosomes from both crypts and villi are equally able to incorporate L-leucine into protein. Unlike other tissues, e.g. liver, there is no diurnal variation of protein synthesis in the intestine of the unfed rat, whether leucine is administered intraluminally or intravenously.

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INTRODUCTION

The intestinal mucosa is a tissue which rapidly incorporates amino acids into protein (1). Leblond, Everett, and Simmons (2) first demonstrated that protein was synthesized primarily in cells undergoing division (in the crypts) or in postmitotic cells (in the crypt-villus junction). These findings were soon supported by the

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work of others (3–5). However, some protein synthesis occurred along the villus itself. The junctional zone between the crypt and villus is the region where cells differentiate and produce enzymes critical for digestive and absorptive functions. These data have been interpreted as consistent with the concept that cells at the villus-crypt junction are in some way specialized to make protein at a rate more rapid than that of cells in other anatomic positions along the villus.

In all of the above studies, the amino acid label was given parenterally. Recently, the suggestion has been made that intestinal protein may be synthesized preferentially from luminal rather than blood-borne amino acids (6). Thus, we studied the effect of the route of administration of precursor on intestinal protein synthesis. We have found that cells at every level of the villus synthesize protein equally well, and the intraluminal amino acids are utilized preferentially for protein synthesis. We conclude that the route of precursor administration must be carefully considered in the design and interpretation of studies involving intestinal protein synthesis.

METHODS

L-Leucine-^{14}C (uniformly labeled > 250 μCi/μmole) and L-leucine-^{3}H (4, 5) (30–50 μCi/μmole) were obtained from New England Nuclear Corp., Boston, Mass. O.C.T.™ was obtained from Fisher Scientific Co., Pittsburgh, Pa. Male Wistar rats (150–175 g) were purchased from National Laboratory Animal Co., St. Louis, Mo., and were fasted overnight before all experiments.

Intestine was sectioned transversely by the method of Nordstrom, Dahlqvist, and Josefsson (7) using an International Cryostat, model GTI (International Equipment Co., Needham Heights, Mass.). Pieces of intestine 1.0 × 1.0 cm were placed on a precut block of O.C.T., and the edges were trimmed. Section of 10 μ each were then cut; each fifth piece was examined histologically after staining with hematoxylin and eosin. The other four samples (10 μ each) were used for biochemical analysis.

^{3}H- or ^{14}C-labeled leucine was administered to animals by injecting 10 μCi in 2.0 ml of phosphate-buffered saline (pH 7.0) into the upper jejunum of animals anesthetized
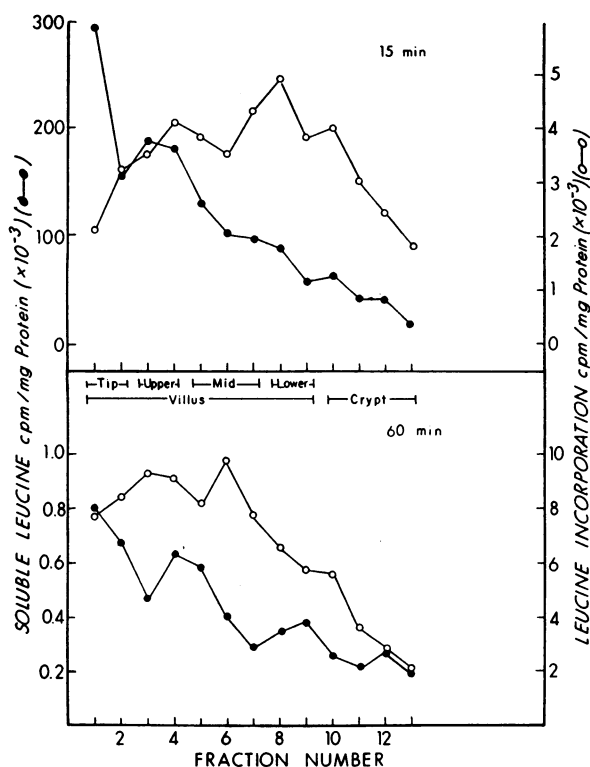
FIGURE 1 Incorporation of intraluminal leucine-¹⁴C into various levels of intestinal villi. Leucine-¹⁴C was administered intraluminally to three animals and intestine sliced as described in the Methods section. Each fraction corresponds to a 50 μ thickness of intestinal villus from each of two pieces of intestine from each animal. Thus, each point is the combined average of six samples.

with ether. The fluid filled a segment of about 10 cm in length, which was marked off by loose ligatures through the mesentery. The needle hole was sealed by application of Eastman 910 adhesive (Eastman Organic Chemicals, Rochester, N. Y.). After sacrifice, tissue was removed from the segment previously filled with fluid containing radioactive precursor. Absorption from this segment was usually complete in about 45 min.

Leucine-³H (3 μCi/μmole), at a concentration of 0.12 μmole/ml, with a mixture of the other 19 amino acids (0.1 μmole/ml) in Krebs-Ringer bicarbonate buffer pH 7.0 was infused through a 20 cm segment of jejunum beginning at the ligament of Treitz, according to Jacobs and Luper (8). During leucine perfusion through intestinal segments, doses of leucine-¹⁴C were injected simultaneously in the saphenous vein to give an estimated specific activity of 3 μCi/μmole without significantly expanding the extracellular leucine pool. Intravenous infusion was contained for 1 hr with leucine-¹⁴C in normal saline at a concentration of 0.12 μmole/ml (3 μCi/μmole) to approximate blood leucine concentration. The rate of infusion was 0.04 ml/min. The perfusion rate was 0.04 ml/min. After 1 hr, the 20 cm intestinal segment was removed, rinsed with normal saline containing nonradioactive leucine, and placed on the cryostat for slicing.

Polyribosomes were isolated from intestine as previously described (9). Differential scraping of intestinal mucosa

was performed as reported by Dietschy and Siperstein (10). This technique removed villi at the junction between crypts and villi. The homogenizing medium contained 0.25 M sucrose, 0.025 M Tris (pH 7.4), and 0.005 M MgCl. The homogenate was centrifuged at 10,000 g for 15 min. The postmitochondrial supernate was layered over sucrose zones of 0.5 and 2.0 M (11) and centrifuged at 105,000 g for 8 hr to ensure full recovery of ribosomes. The total RNA of the postmitochondrial fraction differed by less than 10% from the total RNA of the homogenate. Thus, the RNA on the gradients represented most of the cellular RNA. RNA was isolated from the ribosomes according to the technique of Fleck and Munro (12) and measured by the orcinol reaction (12). pH 5 fraction was prepared from scrapings of both villi and crypts (13). L-Leucine incorporation by isolated polyribosomes was measured as previously described (9).

The slices from the transverse sectioning of the intestine on the cryostat saved for biochemical studies were analyzed for leucine incorporation into protein and for protein, or applied to polyacrylamide gels for electrophoresis. For most experiments, 0.5 ml of 5% trichloroacetic acid (TCA)[1] was added and the protein extracted according to the method of Siekevitz (14). In some experiments the first supernatant fraction (containing soluble radioactive leucine) was saved. The final precipitate was solubilized in 0.5 ml of NCS (Nuclear-Chicago Corp., Des Plaines, Ill.) and added to 10 ml of toluene containing 0.4% p-bis-2,5-diphenyloxazole and 0.005% p-bis-2'-(5'phenyloxazolyl)benzene. The samples were counted in a Packard model 3320 liquid spectrometer (Packard Instrument Co., Downers Grove, Ill.). Protein was assayed by the technique of Lowry, Rosebrough, Farr, and Randall (15).

For electrophoretic studies, the slices from the cryostat were sonicated in 0.5 ml of 0.05 M sodium phosphate buffer, pH 7.4, and centrifuged at 105,000 g for 60 min. The particulate fraction was resuspended by sonicating in 0.5 ml of phosphate buffer. The protein was then solubilized in 3% sodium dodecyl sulfate (SDS) and 1% β-mercaptoethanol (BME). 300 μg of protein were applied to 0.6 × 12 cm polyacrylamide gels made in 0.1% SDS (16) in 0.1 M sodium phosphate buffer at pH 7.2. After electrophoresis gels were stained with 0.05% Coomassie blue, and the pattern of protein was recorded. Then the gels were extruded in a Savant auto gel divided model AGD-30A (Savant Instruments, Inc., Hicksville, N. Y.), and the eluate was collected directly into counting vials and counted in 10 ml of Bray's solution (17) as described above.

RESULTS

Metabolism of intraluminal leucine. After leucine-¹⁴C was injected in the lumen of the jejunum, tissue was removed, sliced on the cryostat, and analyzed to determine how much amino acid was soluble and how much was incorporated into protein. Fig. 1 shows that at 15 min the cells along the villus contained a large number of counts which did not precipitate with TCA. At 15 min the fluid removed from the intestinal lumen showed that the leucine-¹⁴C was not completely absorbed. Over 95% of the TCA-soluble counts chromatographed with leucine in butanol:acetic acid: H₂O, 4:1:2. The cells at

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[1] *Abbreviations used in this paper:* BME, β-mercaptoethanol; DOC, deoxycholate; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

the tip of the villus contained the largest concentration of soluble leucine in agreement with earlier radioautographic studies (18). However, all cells along the villus and in the crypt incorporated leucine into protein. By 60 min, when all of the leucine had been absorbed from the lumen, soluble counts in leucine were now much lower, but more leucine was incorporated into protein. In contrast to the pattern at 15 min, the cells in the upper and mid villus incorporated more leucine per milligram protein than did cells in the lower villus and crypt. This finding may be explained by observation that the cells in the upper villus had been exposed to larger amounts of leucine-¹⁴C. Radioautographic studies showed that after 1 hr almost all grain counts were over mucosal epithelial cells and not lamina propria.

Ability of isolated polyribosomes to synthesize protein. To corroborate the finding that cells in the villus have the necessary machinery for protein synthesis, polyribosomes were isolated from villi and crypts and tested for their ability to synthesize protein in vitro. Table I shows that the recovery of ribosomal RNA from crypts was nearly the same whether or not sodium deoxycholate (DOC) was present to dissolve microsomal membranes. On the other hand, recovery from the villi without DOC was only about one-half of that when DOC was added. Since the yield of RNA from the crypt was not improved by the use of DOC, it seemed likely that the ribosomes in this fraction were mostly unattached to membrane, whereas the reverse was tried in the villi. These results confirm the impression gained earlier by electron microscopy (19). However, when polyribosomes from villi or crypts were tested for their ability to sustain protein synthesis in vitro, no difference was noted per ribosome (milligram protein). Moreover, ribosomes from the villi were just as active whether attached to membrane (without DOC), or separated from it (with DOC).

TABLE I
Protein Synthesis by Intestinal Polyribosomes

Tissue	RNA content of polyribosomes		Leucine-¹⁴C incorporation	
	Without DOC	With DOC	Without DOC	With DOC
	mg RNA/g tissue		*cpm/mg ribosomal protein*	
Villi	0.68	1.33	1380	1500
Crypts	1.52	1.49	1440	1490

Villi and crypts were scraped from the entire small intestine of each of three rats and combined. Polyribosomes were isolated and used for in vitro protein synthesis as described in the Methods section. Deoxycholate concentration was 0.25%. The values are the mean of three separate determinations.

TABLE II
Diurnal Variation of Intestinal Protein Synthesis

Time	Intraluminal leucine-¹⁴C incorporation		Intravenous leucine-³H incorporation	
	Homogenate	Brush border	Homogenate	Brush border
	cpm/µg protein		*cpm/µg protein*	
10 a.m.	6.37 ±2.37	7.96 ±2.94	9.14 ±1.35	8.85 ±1.63
1 p.m.	5.98 ±0.57	6.43 ±0.78	8.63 ±1.03	9.62 ±0.58
5 p.m.	7.57 ±1.66	7.37 ±1.19	9.14 ±1.74	10.01 ±1.22
10 p.m.	5.18 ±0.75	5.82 ±0.93	7.79 ±0.92	8.99 ±0.57
2 a.m.	6.25 ±1.38	6.93 ±1.15	8.43 ±1.08	9.51 ±0.82

Four rats were used for each time point. Animals had no food during the experiment and were exposed to light from 8 a.m. to 6 p.m. 20 µCi of leucine was given intravenously and 10 µCi of leucine-¹⁴C was given intraluminally at the time noted in the table, and the animals were sacrificed 2 hr later. Mucosa was removed by scraping, homogenized in 5 mM EDTA, and brush borders isolated by the method of Forstner, Sabesing, and Isselbacher (21). Trichloroacetic acid was added (5% final concentration) to samples of homogenate and brush borders, and the protein extracted as described in the Methods section. After solubilization in 1 N NaOH, samples were taken for radioactivity and for protein analysis. Values reported are the mean ±1 SD. None of the differences within each group was statistically significant by Student's *t* test.

Diurnal variation of protein synthesis. Diurnal variation of protein synthesis has been reported in other organs such as liver (20) where the precursor arrives via the blood stream. Since much of intestinal protein synthesis may utilize intraluminal precursors, we examined the effect of the route of precursor administration on diurnal variation in protein synthesis. Table II demonstrates that whether leucine was given intravenously or intraluminally, there was no significant diurnal variation noted either in leucine incorporation into total intestinal proteins or into brush border proteins. Inconstant differences were noted when animals were allowed access to food (Purina Rat Chow; Ralston Purina, Checkerboard Square, St. Louis, Mo.) during the duration of the experiment.

Effect of route administration of precursor on site of protein synthesis. In contrast to the intraluminal administration of leucine-¹⁴C (Fig. 1), after a single intravenous injection of leucine-¹⁴C, cells in the crypt were most active in protein synthesis (Fig. 2, upper half). However, when leucine-³H was administered into the jejunum simultaneously to animals which had received the leucine-¹⁴C intravenously, the pattern obtained for ³H incorporation was the reverse of that for ¹⁴C—i.e., cells near the villus tip had the highest specific activity of protein synthesis. When the isotopes were reversed, the same patterns were seen. Thus, all cells in the intestinal mucosa synthesized protein, if the precursor was presented to the cells. The pattern of acid-soluble leucine-¹⁴C from the intravenous injection (not shown) followed the pattern of leucine incorporation, demonstrating that after intravenous injection the cells in the crypt are most heavily exposed to radioactive precursor. A constant infusion of leucine in the blood and jejunum exaggerated the findings seen after a single intravenous
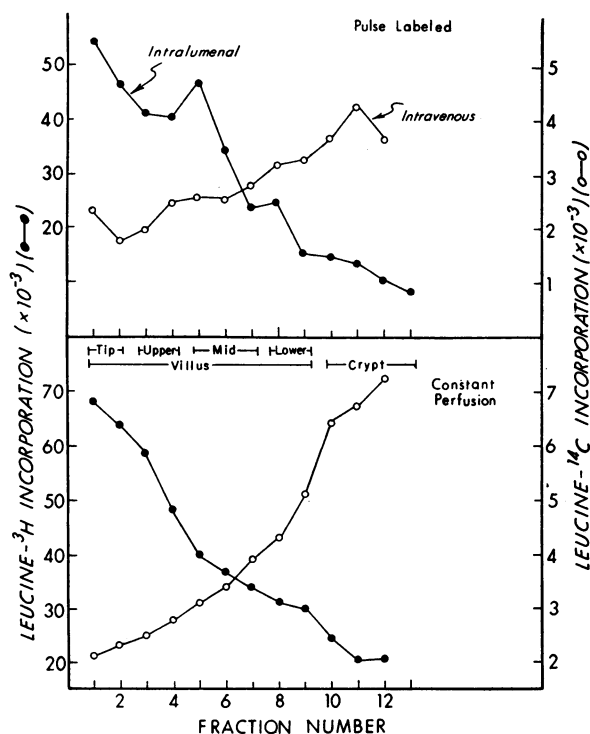
FIGURE 2 Effect of route of administration on the incorporation of leucine into intestinal proteins. Leucine was administered as described in the Methods section. Samples were removed from animals after 1 hr and sliced as described in Fig. 1. Incorporation of leucine is measured as cpm/mg protein. Pulse labeled refers to a single intravenous and jejunal injection of amino acid.

and jejunal injection (Fig. 2 [lower half]). When precursor was perfused through the lumen, there was a linear gradient of incorporation decreasing from villus tip to crypt. After intravenous injection, the reciprocal gradient was found. Thus, the route of administration markedly affected cellular incorporation of the radioactive amino acid precursor.

Fig. 2 also demonstrates that, even under conditions when leucine of comparable specific activity was perfused in the blood and in the lumen, the specific activity of incorporated leucine from the lumen was about 10 times that from the blood. This is consistent with the concept that intraluminal amino acids may be most important in the synthesis of the proteins of the intestinal cell.

Products of protein synthesis in crypts and villi. To determine whether the proteins made in crypts and villi were similar, rats were injected intraluminally with leucine-14C and incorporation allowed to proceed for 1 hr. After slicing the intestine, the proteins from these fractions were separated by polyacrylamide gel electrophoresis. The patterns of labeled protein obtained are demonstrated in Fig. 3. Fig. 3 shows that for particulate proteins there was a shift in labeling such that the larger

proteins closer to the origin were labeled most heavily in the upper villus, whereas in the crypt the smaller and rapidly migrating proteins were labeled most heavily. The pattern with soluble proteins (not shown) demonstrated that the larger proteins closer to the origin were more heavily labeled in the crypt. Using intravenous leucine similar results were obtained.

DISCUSSION

The data presented here demonstrate the importance of the route of amino acid administration in studying intestinal protein synthesis. Contrary to previous interpretations (2–5), cells all along the intestinal villus make protein (Figs. 1 and 2). The incorporation of leucine into various levels of the villus cannot be attributed to cells in the lamina propria, since these cells incorporated negligible amounts of radioactivity by radioautography, at least during the short interval of these experiments. Moreover, the polyribosomes from both crypt and villi incorporated leucine at equal rates. The method of preparing isolated villi includes the villus-crypt junctional area in the villus fraction. If the crypts and junctional region were the most active in synthesizing protein, as previously claimed (2–5), one would not expect polyribosomes from the entire villus fraction to incorporate leucine into protein at a rate equal to that of the crypt alone. The fact that they did (Table I) again shows that polyribosomes from cells along the entire villus make protein at a rate equal to that of crypt cell polyribosomes.

Actually, cells on the villus in vivo made protein at a more rapid rate than those in the crypt (Fig. 2), possibly due to the greater availability of amino acids from the lumen. The mucosal surface actively transports amino acids, and intraluminal concentrations of amino acids are often higher than those in blood (22). On the other hand, amino acid precursor accumulation in the lower villus after intravenous administration may be due to the fact that crypt cells utilize amino acids from the blood stream. Since they are more proximally situated to the blood supply than the villus cells, the distribution of protein labeling seen after intravenous administration may be explained by the paucity of substrate reaching the more distal cells. Thus, although there is some overlap, intraluminal precursors were incorporated into cells in the upper half of the villus in high concentrations and intravenous precursors were incorporated into the crypt and lower villus in lower concentrations. A final difference noted was that the pattern of proteins most heavily labeled by the precursor was different in the villus and crypt. Therefore, depending upon the proteins one is interested in studying, different results might be obtained by the use of one method of labeling as compared with the other. For

example, thymidine kinase is an enzyme found almost exclusively in the crypt cells (23). Thus, intravenous injection of precursor would maximally label this protein. However, disaccharidases are not present at all in the crypt cells, but only in the mature villus cells (7). Jejunal injection of precursor would be most appropriate in this case.

Most previous studies have utilized parenteral amino acids as a precursor for intestinal protein synthesis (2–5). However, as seen from the present data, these authors studied mostly proteins made by the cells in the lower half of the intestinal mucosa (crypt and lower villus). Hirshfield and Kern used both parenteral and oral amino acids, but assumed that a homogenous population of cells was being studied (6). Finally, in studies where rates of protein synthesis were measured, no consideration has been given to the fact that parenteral amino acid administration, by measuring primarily protein synthesis in the crypt and lower villus at a suboptimal rate may measure only a fraction of total intestinal protein synthesis. It is possible, for example, that villus protein synthesis is the most affected by starvation or other experimental manipulations.

The data from previous studies becomes more clear when the foregoing facts are considered. Lipkin and Quastler (3) noted maximal leucine incorporation in the crypt cells after intraperitoneal injection, but also noted a slow loss of grain counts as cells moved up the villus. Since protein content does not change along the villus(24),[a] these data are consistent with protein turnover and new synthesis of protein in the villus. Shorter and Creamer (4) noted some grain counts at the tips of villi after intraperitoneal methionine, but could not adequately explain this finding. Hirschfield and Kern (6) showed that at 3 and 6 hr, after giving leucine, oral leucine was incorporated better into jejunal mucosa than was intraperitoneal leucine. These differences were not apparent in the ileum or at a later time, in either tissue. These data may be explained by the greater availability of oral leucine or other precursor amino acids to the villus epithelial cells of the jejunum.

The characteristics of intraluminal (or combined with parenteral) administration of amino acid precursors are: (a) greater rate of incorporation, and (b) labeling of villus epithelial cells, resulting in a more complete evaluation of total intestinal protein synthesis. One further characteristic of intestinal protein synthesis is the lack of diurnal variation in the nonfed animal (Table II). Such a variation has been noted in other tissues (20), and may be consistent with the observation that, in nocturnal animals such as the rat, blood amino acid levels are lowest in the late afternoon (25). Other data from our laboratory shows that the intracellular
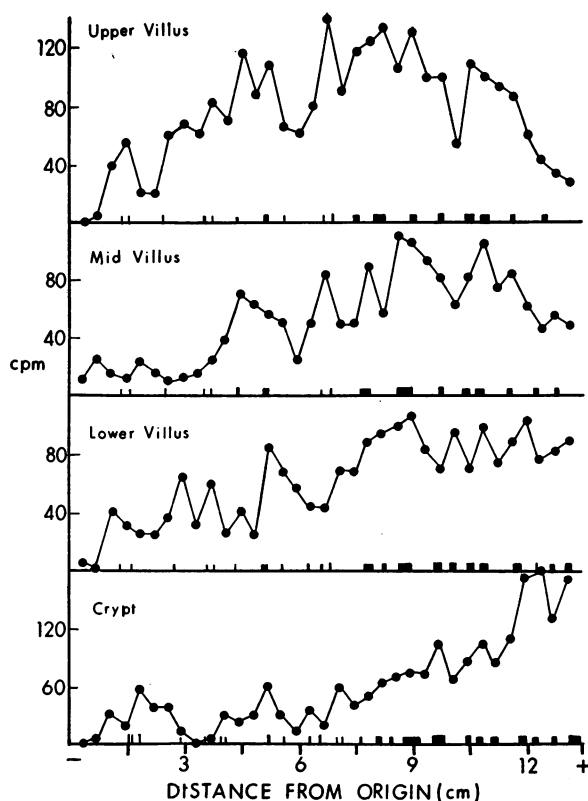

FIGURE 3 Synthesis of particulate proteins in the intestinal villus. 10 μCi of leucine-14C was given intraluminally into each of three rats sacrificed 1 hr later. Duplicate fractions obtained from the cryostat from each animal were combined into groups correpsonding to the villus fractions depicted in Figs. 1 and 2. Counts in the "tip" fraction were inadequate for analysis. Samples were homogenized in 0.05 M sodium phosphate buffer pH 6.0, centrifuged at 105,000 g for 1 hr, and the supernatant fraction was removed. The pellet was solubilized in 3% SDS and 1% BME. 300 μg of protein from this fraction was applied in duplicate to 5% polyacrylamide gels made 0.1% in SDS and electrophoresed at 8 ma/tube. One gel was stained with Coomassie blue. The marks at the bottom of each graph correspond to the protein stain. The width of the mark is proportional to the intensity of the stain. The other gel was extruded in a Savant autogel divider, model AGD-30A, with an extrusion rate of 0.5 cm/min. Thus, the extruded fraction could be correlated with the stained protein bands. The extruded gel was collected directly into counting vials for analysis as described in the Methods section.

pool concentration of amino acids is directly related to the rate of protein synthesis.[a] The lack of diurnal variation noted may be explained by the fact that many amino acids available to the epithelial cells probably are derived from the lumen rather than the blood stream (Fig. 2). Large variations in intraluminal amino acid content may be caused by ingestion of food, and feeding

[a] Alpers, D. H. Unpublished observations.

[a] Alpers, D. H., and S. O. Thier. Unpublished observations.

of standard Purina Rat Chow led to much variation in our results at different times of day. Thus, if intraluminal administration is to be used reproducibly, animals should be without food or should be injected at the same time after a standard tube feeding.

The villus epithelial cells synthesize protein rapidly. However, the protein content per cell does not increase, and the cells are not known to secrete large amounts of proteins. These data are consistent with several interpretations. First, cells or parts of cells may be lost from the villus during migration. Protein secretions are well documented for Paneth cells (26), but these constitute no more than a small fraction of the cells in the intestinal mucosa. There is at present no evidence for loss of whole cells or parts of other cells from the side of the villus. Sucrase (27) and other disaccharidases (28) turn over at a rapid rate during cell migration along the villus, and this turnover could be due to loss of membrane attached proteins from the cells. However, these enzymes account for only a small fraction of the leucine incorporated into the entire mucosa (28). A second interpretation of the data presented above is that many intestinal proteins are rapidly broken down and replaced. This seems a likely possibility and is consistent with the data of Rosenweig, Herman, and Stifel (29) that levels of enzyme activity in the mucosa can change within hours in response to a variety of stimuli. Recent data from our laboratory demonstrate that there is a spectrum of rates of turnover of brush border proteins (30). Thus, the intestinal mucosa does not seem to make protein primarily in the crypt and lower villus with little subsequent modification of that protein during cell migration. Our data suggest a much more dynamic process continuing along the entire length of the villus.

ACKNOWLEDGMENTS

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