Quantitative Radioautography of Sugar Transport in Intestinal Biopsies from Normal Humans and a Patient with Glucose-Galactose Malabsorption

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ABSTRACT Both galactose accumulation and phlorizin binding by columnar epithelial cells have been investigated in vitro with a recently developed technique for high-resolution, plastic-section radioautography which is particularly suited to small quantities of biopsy tissue. Grain density analysis of the radioautographs provides definitive support for the view that the cellular mechanisms underlying glucose-galactose absorption in laboratory animals are fully applicable to the small intestine of man. Even the number of sugar carriers at the microvillar membrane appears similar and the major quantitative difference, lower affinity for phlorizin in man, correlates with the finding that phlorizin is also a less potent inhibitor of uphill, galactose transport at the microvilli. In addition, radioautographs of biopsies taken 2 yr apart from a patient with glucose-galactose malabsorption provide evidence that the cellular defect in this inborn error of transport is a persistent reduction in the number of functioning sugar carriers at the microvillar membrane.

INTRODUCTION

According to the generally accepted model for intestinal absorption (Fig. 1), sugars of the glucose-galactose class are transported uphill into the columnar epithelial cells of the small intestine by a sodium-coupled carrier which also possesses an inhibitory binding site for phlorizin. Within the cells both sugar and sodium diffuse to the lateral membranes. Sugars move downhill across these membranes, presumably by facilitated diffusion, and enter the intercellular spaces. Sodium is actively pumped across the lateral membranes, creating a hyperosmolality within the intercellular spaces. The resulting osmotic movement of water into these spaces generates hydrostatic pressure which both dilates the spaces and causes a bulk flow of absorbed fluid across the basement membrane to the circulation. Some sugar may also diffuse directly from the basal ends of the cells. The accumulated evidence upon which this model is based was derived mainly from in vitro animal studies and is the subject of several extensive reviews (1–6).

The above model, or some variation thereof, is thought to apply to man also; yet there is limited direct evidence, even with regard to sugars, to support this view (2, 7–9). The recent discovery of familial glucose-galactose malabsorption (10, 11) emphasizes the need for such data. Although the precise defect is yet to be explained, studies of several patients with this disorder indicate that it is a specific inborn error of sugar transport. Patients exhibit a life-threatening impairment of glucose-galactose absorption, while absorption of fructose (12–14), amino acids (15, 16), sodium (16), and apparently other metabolites is normal. Their pedigrees indicate that the disorder is inherited in an autosomal recessive manner (9, 17). The simultaneous presence of a mild defect in renal glucose transport in most patients thus far examined (18) is reminiscent of the inborn errors of amino acid transport which affect the renal tubule and to a lesser extent the small intestine (19). Lastly, the apparently normal intestinal morphology (13, 16) is consistent with a specific defect in the absorptive mechanism.

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The above characteristics coupled with the radioautographic observation that columnar cells from a patient with glucose-galactose malabsorption failed to accumulate galactose-^{14}C led us (13) to postulate a defect in the brush border, glucose-galactose carrier. To further test this hypothesis, we have used recently developed, high-resolution radioautography (20) to measure the intracellular distribution of galactose-^{3}H and phlorizin ^{3}H in cells from the same patient. We reasoned that if this patient possesses a defective carrier mechanism her columnar cells would neither establish a steep uphill galactose gradient nor bind phlorizin at the brush border. First, however, it was necessary to demonstrate the existence of these phenomena in normal human intestine. We found steep uphill galactose gradients and microvillar binding of phlorizin in normal intestinal cells, but not in the patient's cells.

METHODS

Human material. Our results are based on in vitro radioautographic studies of mucosal biopsy specimens taken perorally from near the duodenal-jejunal junction of three adults, a child of 1 yr, and a patient with glucose-galactose malabsorption at ages 1 and 3 yr. Permission for each biopsy was obtained after a detailed description of the procedure and the potential complications. No complications occurred. Protocols have been approved by the Human Experimentation Committee of the Upstate Medical Center.

The adult subjects were three males, 30–40 yr of age, all in good health, and specifically exhibiting no evidence of gastrointestinal disease. The 1 yr old boy was subjected to peroral biopsy for diagnostic purposes and was found to be normal both clinically and histologically. He had previously been on a gluten-free diet without confirmation of disease. The female patient with glucose-galactose malabsorption was first biopsied in 1965 (13) and then rebiopsied in 1967 when she was 3 yr old. Her clinical status in 1967 was basically unaltered. Fructose remained the major source of dietary carbohydrate; from dietary records kept by her mother, 30–40% of the patient's caloric intake derived from carbohydrate, approximately 70% of which consisted of fructose. Thus, only about 10% of her caloric intake was derived from sugars other than fructose. Dietary indiscretions led to prompt increases in watery stools. A preliminary study of the patient's renal tubular transport maximum for glucose (Tm), employing endogenous creatinine clearance as a measure of glomerular filtration rate (GFR), gave a mean Tm/GFR in milligrams per milliliter of 1.35 ±0.10 SD. This value, which is approximately 50% of normal (9), was derived from seven clearance periods at elevated plasma glucose levels ranging from 292 to 410 mg/100 ml. Mild glucosuria was observed at the patient's control glucose level of 88–90 mg/100 ml.

In vitro radioautography. Immediately after removal, mucosal biopsy specimens ranging between 50 and 150 mg were washed 10 min in iced saline and divided into small bits about 5–10 mg each. The tissue bits were preincubated 10 min at 37°C in Krebs-Henseleit medium equilibrated with 5% CO_2 in O_2 and transferred to individual test incubation flasks containing 1–2 ml of medium, appropriate amounts of test compounds, and 15% bovine albumin, added to enhance medium identification in the radioautographs

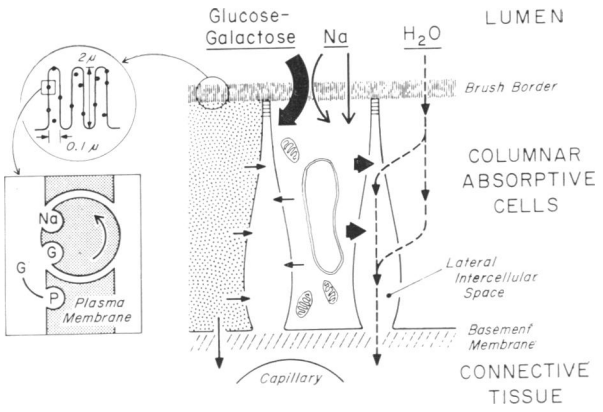


FIGURE 1 Model of cellular mechanisms for sugar absorption in small intestine. Broad arrows indicate uphill glucose-galactose transport across the brush border and uphill sodium transport across the lateral membrane; small arrows indicate downhill movement, which for sugar at lateral and/or basal membranes is presumed to involve carrier-mediation (5). Dilated lateral spaces are associated with sodium-dependent water absorption (6). Circular inset shows individual microvilli; dots represent glucose-galactose carriers located in the cell membrane (Discussion). An expanded view of a carrier is given in the square inset. Both free carrier and the ternary complex of carrier plus sodium (Na) and sugar (G) are thought to translocate across the membrane (5). Binding of the phloretin moiety of phlorizin (G-P) to a membrane site near the carrier is postulated to produce both allosteric, i.e. indirect, inhibition plus placement of the glucose moiety in a propitious position to compete directly with sugar molecules for the transport site on the carrier (4).

(20). After a selected period of incubation, the tissue was removed, placed on aluminum foil, and quickly frozen in liquid propane at −185°C. The frozen tissue was then processed for high-resolution radioautography. An extensive description of these procedures has been reported (20). Briefly, the tissue was freeze dried, fixed in osmium tetroxide vapor and embedded in a partially waterproof, silicone and epoxy resin mixture. Plastic sections 1 to 2 μ thick, cut with glass knives and collected over water, were placed on microscope slides and coated with liquid nuclear track emulsion. Exposures ranged from 1 to 10 wk, depending upon medium radioactivity. After development of the overlying radioautographic image, tissue sections were lightly stained with basic fuchsin in acetone to enhance contrast with phase microscopy. In the case of the earliest biopsies studied (Fig. 8, legend), galactose-^{14}C, frozen-section radioautographs instead of galactose-^{3}H, plastic-section radioautographs were prepared by a method then in use in our laboratory (21). Tissue incubation and freezing procedures were essentially identical, but resolution was satisfactory only to the cellular and not to the subcellular level (13). However, the close correspondence of quantitative results at the cellular level (Fig. 8) helps to validate the high-resolution, plastic-section technique.

Quantitation. Radioautographic grain densities over particular tissue structures (silver grains/μ^2) were determined either with an ocular grid in the phase-contrast microscope or from measured areas in photomicrographs. With high-

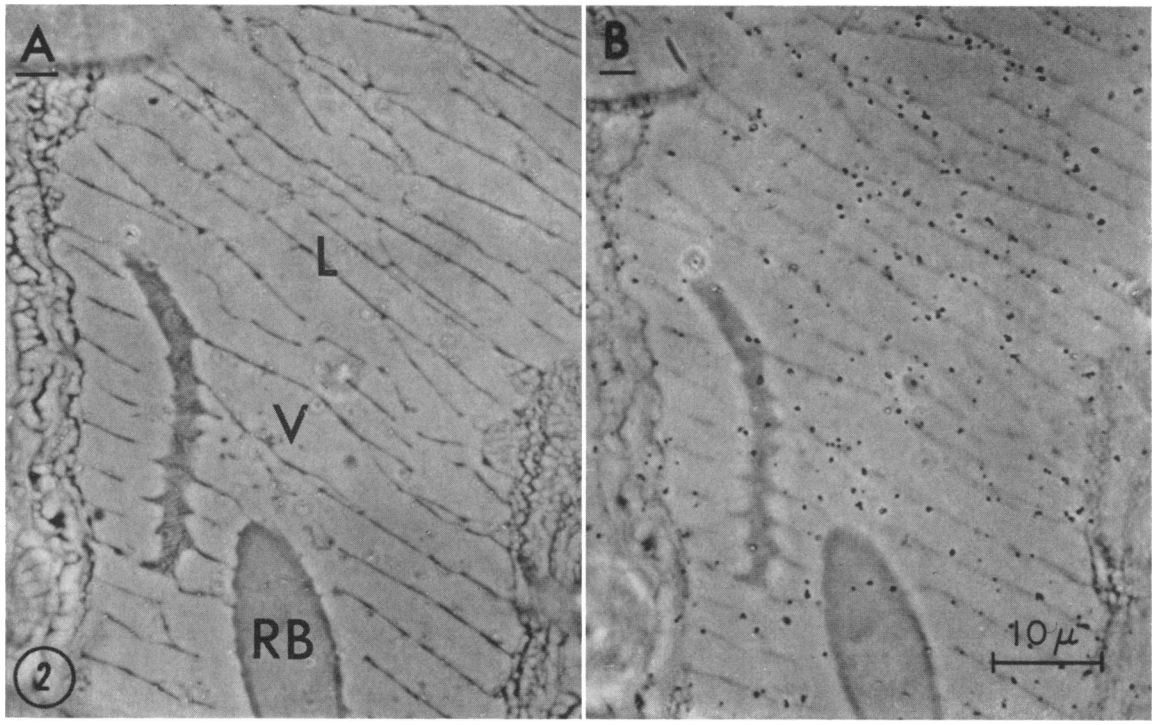


FIGURE 2 Evaluation of radioautographic resolution with a water-soluble compound. A. Phase-contrast photomicrograph of a section of freeze-dried, plastic-embedded, amphibian plasma which contained 2 mM glucose-^{3}H. The plasma was slowly frozen (in blood vessel of intact *Necturus*) so as to produce large, solute-free ice crystals which appear as voids (V) bounded by "eutectic lines" (L). These lines should contain all former solutes including plasma proteins and the labeled sugar (text). Note two shrunken, red blood cells (RB), the result of intracellular dehydration during slow freezing (24). B. Microscope focused on developed silver grains (black dots) in the overlying radioautographic emulsion. The grain distribution indicates that the ^{3}H label was successfully localized to the eutectic lines, i.e., only about 15% of the grains lie outside of imaginary 1 μ-wide bands centered on the lines. The thicknesses of the plastic section and the emulsion layer were 1 and 1–3 μ, respectively. × 1400.

resolution, plastic-section radioautographs, the concentration of ^{3}H label in a 2 μ-wide band, e.g. brush border, can be related to that in medium with an accuracy of ±20% when each grain density measurement is based on 100 or more grains (20). With the earlier frozen-section radioautographs, the concentration of ^{14}C label in a 30 μ-wide band, e.g. columnar epithelium, can be related to that in medium with an accuracy of about ±20% when appropriate corrections are made for source geometry (21). Relative grain density values were converted to absolute content or concentration by scintillation spectrometry of label in the medium. The terms *content* and *concentration* are used interchangeably with galactose, the view being that most galactose is dissolved in tissue water and not bound (Discussion).

Further evaluation of leaching and resolution with plastic-section radioautographs. An important advantage offered by the present freeze-dried, plastic-section technique as compared to other methods, for example frozen-section radioautography, is the ability to collect tissue sections over water and coat them with wet radioautographic emulsions. These steps insure flat sections and intimate contact with a uniformly thin (1–3 μ) emulsion film, two essential requirements for high-resolution radioautography (22). In addition, the presence of the embedding plastic facilitates quantitation by imparting to the various tissue elements a uniform density and, thus, uniform self-absorption of the weak beta emission from tritium (23).

However, contact with aqueous media may result in gross leaching of water-soluble compounds from plastic sections. In the initial report with the present technique (20), loss of galactose-^{3}H and phlorizin-^{3}H during water collection was conservatively estimated at 25% of the total in the sections. This value represented the upper limit of a small number of measurements. A more extensive study of leaching into the collecting water during sectioning (D. D. S. Mackenzie and W. B. Kinter, unpublished observations) revealed that losses from 0.5, 1.0, and 1.5 μ-thick sections were generally less than 10% and averaged 7 and 6% for galactose-^{3}H and phlorizin-^{3}H, respectively. Likewise, for galactose-^{14}C and inulin-^{14}C in plastic sections with the same three thicknesses, losses averaged 8 and 6%, respectively.

Previous estimates of radioautographic resolution (20) showed that 85 ±4% SD of the silver grains produced by a 1 μ-wide band of succinic-^{3}H epoxy resin embedded in

methacrylate were located over the band. This evaluation is open to the criticism that, since the tritium label (succinic acid-^{3}H) was incorporated into the insoluble epoxy resin, the capability of resolving water-soluble compounds was not properly tested. Thus, we devised a second, more rigorous resolution test. Slow freezing of extracellular fluids or incubation media produces large ice crystals whose interstices or "eutectic lines" contain all the original solutes in precipitated form (24). When a labeled solute such as glucose-^{3}H is present, the widely spaced eutectic lines in sections of slowly frozen fluid provide a satisfactory model for testing resolution. Grain counts from radioautographs such as that in Fig. 2 revealed that 85 ±4%SD of the silver grains were localized to 1 μ-wide bands centered on the eutectic lines, thus substantiating our prior evaluation of resolution. Furthermore, the agreement between the two tests shows that, subsequent to freezing, local displacement of water-soluble compounds is negligible with the present plastic-section technique.

Test compounds. D-galactose-1-^{3}H, 72–83 mCi/mmole, and D-galactose-1-^{14}C, 2–8 mCi/mmole (New England Nuclear Corp., Boston, Mass.), were used to investigate active sugar absorption and D-glucose-1-^{3}H, 0.53 Ci/mmole, from the same supplier was used to test for resolution. In inhibition and binding studies, we used unlabeled phlorizin (Nutritional Biochemicals Corp., Cleveland, Ohio) and tritium-labeled phlorizin, 2.86 Ci/mmole (25), in which the label was located between the phenolic groups. Thus, any removal of the glucose moiety by hydrolysis would yield phloretin-^{3}H from the parent 2′,4′,6′-trihydroxy-3-(*p*-hydroxyphenyl)-propiophenone-2′-β-D-glucoside.

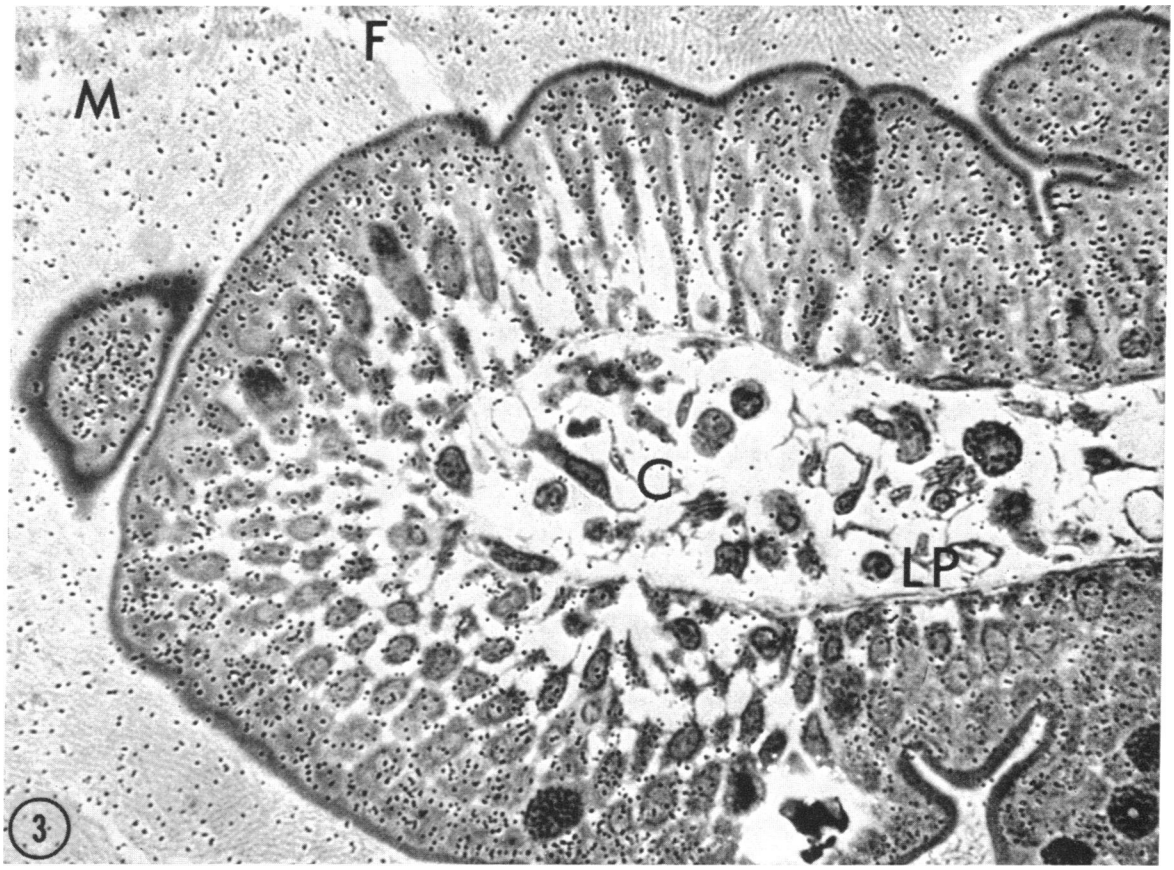
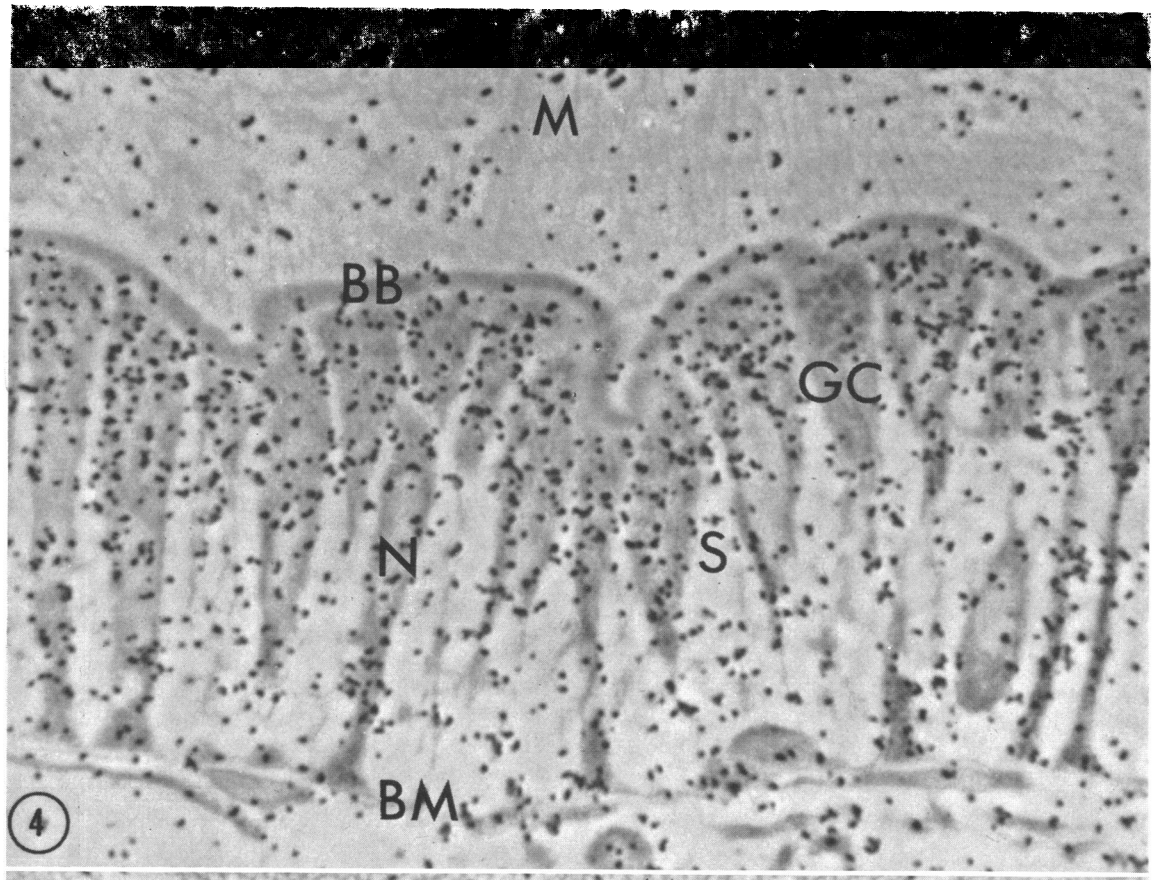


FIGURE 3 Radioautograph of a villus tip illustrating the general pattern of galactose-^{3}H distribution in biopsy tissue from a normal subject (J. S., 1967). The focus in this phase-contrast photomicrograph was adjusted to show both the lightly stained tissue section and the developed silver grains of the overlying emulsion (black dots) with a fair degree of sharpness. The surrounding incubation medium (M) exhibits an occasional fracture (F) produced during freezing, drying, or embedding. The central connective tissue core of the villus, i.e. lamina propria (LP), possesses numerous capillary loops (C), one of which contains a trapped red cell. The lamina propria is separated from the medium by a layer of columnar epithelium in which a few cells were cut longitudinally and the rest tangentially. The region with the longitudinally sectioned columnar cells (top) is shown at higher magnification in Fig. 5. Incubation conditions for this particular bit of the biopsy specimen are given in the legend to that figure. × 950.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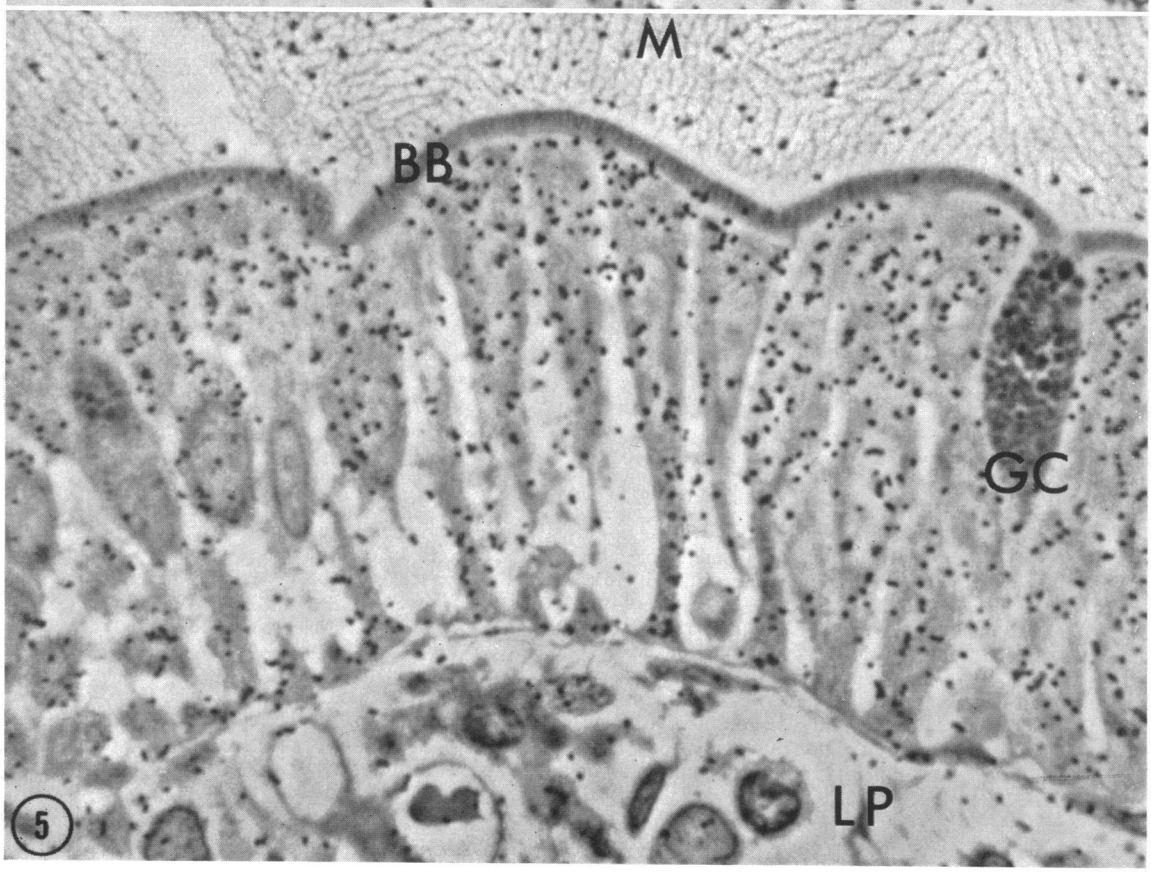
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RESULTS

Galactose accumulation by normal mucosa. The pathway for glucose-galactose absorption across the intestinal epithelium was investigated with bits of human biopsy tissue incubated for periods up to 10 min in media containing labeled galactose, usually at 1 mmole/liter, plus in some instances partially inhibitory levels of unlabeled phlorizin. Galactose is generally metabolized more slowly than glucose and short incubation periods were used so as to ensure that most of the tissue radioactivity would represent labeled sugar molecules and not metabolic products. The representative high-resolution radioautographs in Figs. 3–5 depict the distribution of galactose-^{3}H in normal mucosal tissue after 5 min of incubation. Since each small bit of mucosa tended to contract or "ball up" with villi projecting outward, only the outer portions of individual villi were in direct contact with the incubation medium. One such villus tip with its connective tissue core or lamina propria is shown at intermediate magnification (Fig. 3) and examples of columnar epithelial cells extending from medium to lamina propria are shown at maximal light microscope magnification (Figs. 4 and 5). Passing from medium to lamina propria there is an abrupt increase in the grain density or galactose concentration at the brush border, followed by an abrupt decrease at the basement membrane to roughly half the concentration in medium. The galactose accumulated within the columnar absorption cells appears to be distributed rather uniformly except for the nuclei, which contain lower levels. Representative grain density measurements of brush border, corrected for medium between microvilli, and of apical and basal regions exclusive of cell nuclei, are given in Table I. The latter two measurements provide a quantitative assessment of cytoplasmic galactose distribution and give no evidence of preferential accumulation at either end of the cells. However, variations of less than $\pm 20\%$ would not be detected, due to the limitations of the method. The enlarged intercellular spaces, characteristic of intestinal epithelium absorbing sodium and water (6), also contain less galactose than the cytoplasm (Figs. 3–5). Measurement of the actual grain density of these spaces was precluded by their small size and the presence of fine cytoplasmic trabeculae which are only partially visible in present light microscope radioautographs (Fig. 4, legend). Qualitatively, galactose distribution was similar in tissue incubated for times other than 5 min; quantitatively, cell to medium concentration ratios increased with incubation time (Fig. 8).

The glucoside, phlorizin, affected only the rate of galactose uptake and not the general pattern of distribution in normal mucosal tissue. When 0.5 mM phlorizin was included in the incubation medium, the 5 min accumulation of galactose-^{3}H by human columnar cells was impaired (Figs. 3 and 5) but not nearly the degree expected from previous animal work (25). Grain density measurements (Table I) demonstrate uniform reductions in the galactose content of various intracellular regions which amounted to 41–47% and 66–71% of the control values with 1.0 and 0.25 mM galactose-^{3}H in media, respectively. Graphical analysis of cell to medium concentration ratios (Fig. 8) indicates that phlorizin inhibition was rapid in onset and did not diminish with increased incubation time. These findings are consistent with the view that phlorizin acts as a competitive inhibitor of uphill galactose transport at the brush border.

Galactose radioautographs of patient's mucosa. The galactose distribution in the patient's columnar epithelium was examined with tissue bits from two separate biopsies. The radioautograph in Fig. 6 is representative of the distribution in the first biopsy after 3 min exposure to 1 mM galactose-^{3}H. The most salient finding was the presence of a downhill concentration gradient across the brush border. Grain density measurements (Fig. 8)

FIGURE 4 Galactose-^{3}H radioautograph of columnar epithelium from a normal mucosal biopsy (J. S., 1967). Tissue bit was incubated 5 min in medium containing 1 mM labeled galactose. The grain density (black dots) over medium (M) represents the 1 mM concentration. Absorptive cell nuclei (N) divide the cytoplasm roughly into a thick apical portion contiguous with the distinct brush border (BB) and a tenuous basal portion portion terminating on the indistinct basement membrane (BM). Complex extensions of cytoplasm frequently protrude as fine trabeculae (would be clearer with electron microscope) into the expanded lateral intercellular spaces (S). An occasional goblet cell (GC) interrupts the layer of columnar absorptive cells. Only the latter accumulate galactose under present conditions. × 1700.

FIGURE 5 Galactose-^{3}H radioautograph of normal columnar epithelium (J. S., 1967) illustrating partial phlorizin inhibition. Tissue bit was incubated 5 min in medium containing 1 mM labeled galactose plus 0.5 mM unlabeled phlorizin. Under these conditions the galactose accumulation within columnar absorptive cells was reduced about 50%, as seen both by visual comparison with a control radioautograph from the same biopsy (Fig. 4) and by grain density measurements (Table I). Major features of this particular tissue section, such as the lamina propria (LP), are pointed out in Fig. 3. Also note the prominent goblet cell (GC), the well defined, 2 μ-wide band of brush border (BB), and the fine ice crystal pattern of the rapidly frozen medium (M), as contrasted with the coarse pattern of the slowly frozen plasma in Fig. 2. × 1700.

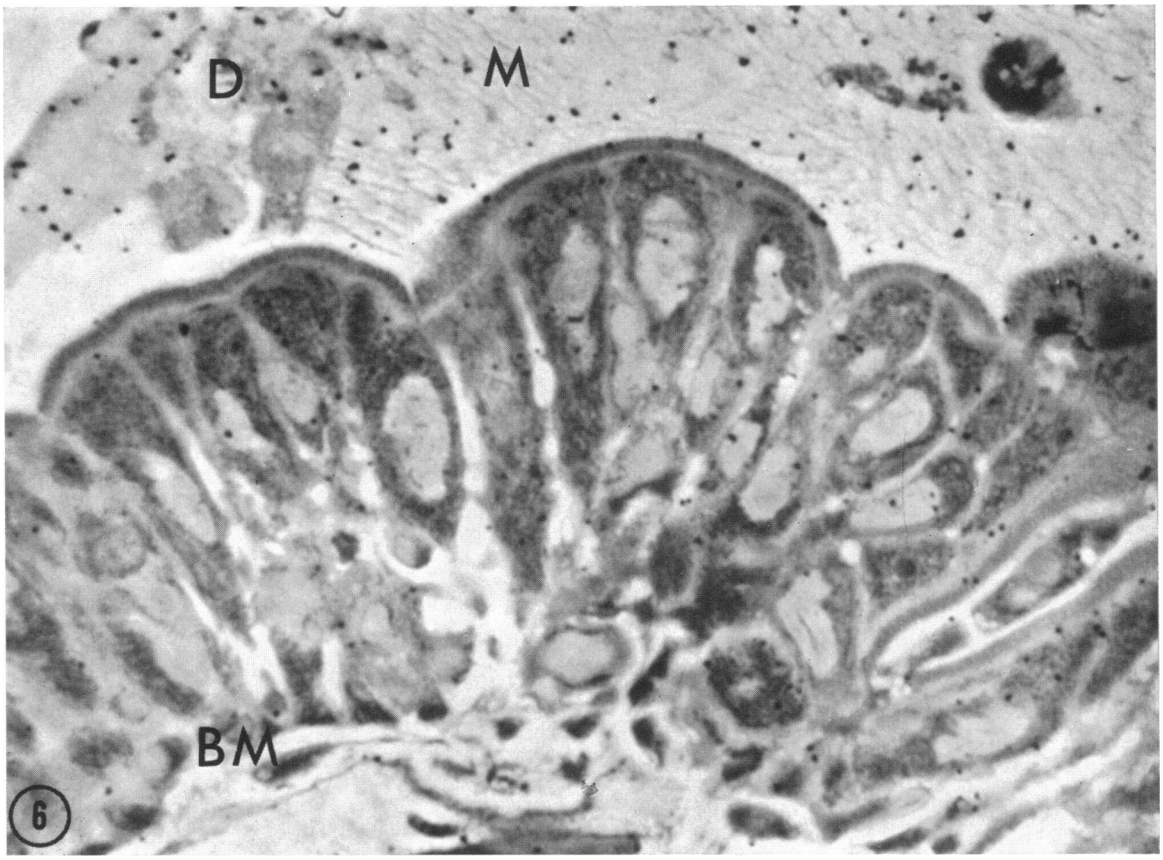


FIGURE 6 Galactose-^{3}H radioautograph of columnar epithelium from a patient with glucose-galactose malabsorption (1965 biopsy). Tissue bit was incubated 3 min in medium containing 1 mM labeled galactose. Cell debris (D) is scattered throughout the medium (M). The basement membrane (BM) demarcates the lower boundary of the well stained, absorptive cells whose galactose content was only about 50% that of medium. × 1800.

TABLE I

Distribution of Galactose-^{3}H in Human Absorptive Cells after 5-min Incubation

Biopsy	Medium composition		Autoradiographic grain density ratios*		
	Galactose-^{3}H	Phlorizin	Brush border/ medium	Apical cytoplasm/ medium	Basal cytoplasm/ medium
	mmoles/liter				
J. S., '67	1.0	—	3.7 ±0.8 (6)‡	4.5 ±0.8 (10)	4.2 ±0.6 (6)
	1.0	0.5	2.0 ±0.2 (4)	2.4 ±0.6 (6)	2.5 ±0.3 (4)
Per cent phlorizin inhibition:			46%	47%	41%
T. M., '68	1.0	—	2.6 (2.1, 3.0)§	4.3 (4.1, 4.5)	3.5 (3.6, 3.5)
	0.25	—	6.5 (6.7, 7.8, 5.0)	7.4 (7.8, 8.5, 6.0)	6.8 (7.7, 7.0, 5.7)
	0.25	0.5	1.9 (2.6, 1.1)	2.4 (3.3, 1.4)	2.3 (3.0, 1.6)
Per cent phlorizin inhibition:			71%	68%	66%

* For each tissue section, the individual counts for medium, brush border, etc., represent at a minimum 100 and on the average 300 silver grains apiece. Brush border values corrected for 20% extracellular space (20).
‡ Mean ±SD (number of sections counted). Fractional SD values ranged from 10 to 25%.
§ Average (individual ratios for sections).

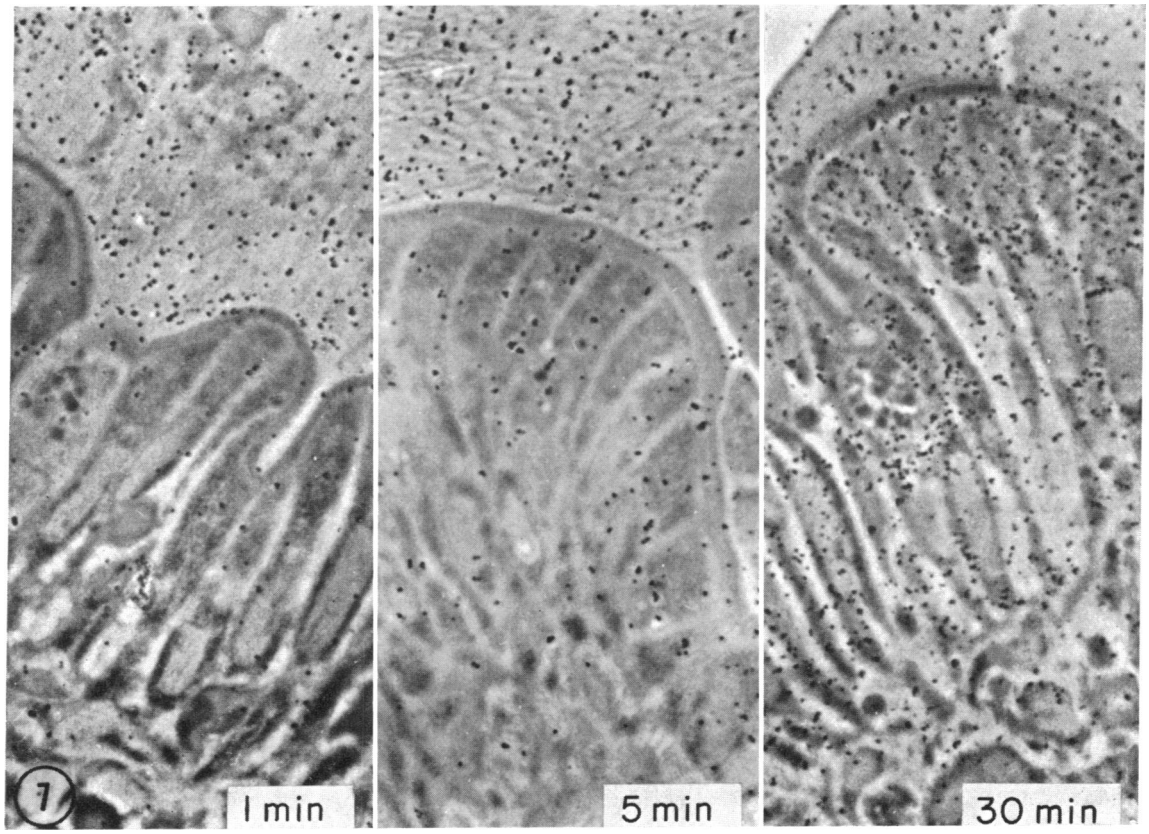


FIGURE 7 Galactose-^{3}H radioautographs of columnar epithelium from patient at age 3 yr (1967 biopsy). Tissue bits were incubated for 1, 5, and 30 min in medium containing 1 mM labeled galactose. Limited uphill accumulation of galactose within absorptive cells had occurred by 30 min. × 1300.

indicate that the galactose content of the patient's columnar cells was not quite half that of the medium, whereas after the same period of incubation the galactose content of normal cells was three to four times that of the medium. The galactose distribution in cells from the second biopsy after 1, 5, and 30 min of incubation may be compared in Fig. 7. Cellular entry was just detectable after the 1 min exposure and, even after 30 min, the intracellular concentration barely exceeded that of the medium. The slowed rate of entry is shown graphically in Fig. 8. The galactose that did enter, however, appeared to distribute as in normal cells. For example, the 1 min corrected brush border (Table I, footnote) and apical cytoplasm to medium ratios averaged 0.23 and 0.21, the 5 min 0.45 and 0.41, and the 30 min 1.2 and 1.5. It is also of interest that the single data point for the patient's 1965 biopsy falls on the galactose accumulation curve obtained with the biopsy taken 2 yr later (Fig. 8). This finding, in agreement with the continued dietary intolerance toward glucose and galactose (Methods), indicates no abatement of the patient's absorptive lesion. With the 1965 biopsy, no morphological anomalies were detected at either light or electron microscope levels (13). Similarly, with the 1967 biopsy no anomalies are evident, at least at the light level. Moreover, present micrographs of both biopsies (Figs. 6 and 7) reveal numerous open intercellular spaces, the presence of which is consistent with normal salt and water absorption.

Phlorizin binding. Although phlorizin proved to be a less effective inhibitor of sugar accumulation in human (Fig. 8) than in hamster (25) cells, the inhibition was of sufficient magnitude to suggest that normal human microvilli might also bind phlorizin. Thus, we examined the cellular distribution of tritium-labeled phlorizin at medium concentrations ranging from 5 to 500 μmoles/liter. Normal epithelium exposed for just 1.5 min to 5 μM phlorizin-^{3}H (Fig. 9) exhibited a significant brush border accumulation which did not increase further with 15 min incubation. At high medium concentrations, the microvillar capacity to accumulate phlorizin was not apparent. For example, after 15 min exposure to 100 μM phlorizin-^{3}H (Fig. 10) the brush border grain density,

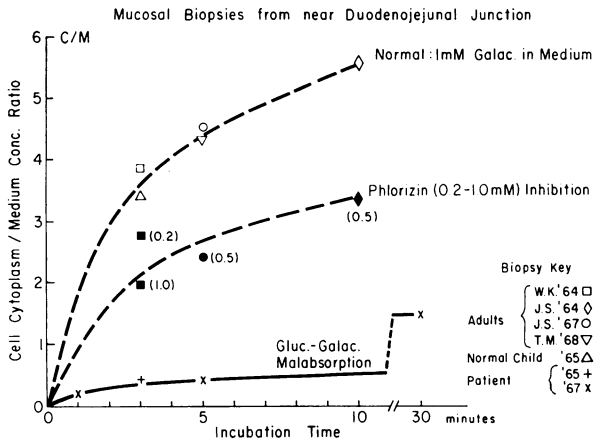


FIGURE 8 Quantitative comparison of galactose accumulation by normal, phlorizin-inhibited, and patient's absorptive cells. All incubation media contained 1 mM labeled (usually ^3H) galactose. Each data point represents the average cell (apical cytoplasm with galactose-^3H) to medium grain density ratio for an individual tissue bit (grain counts from two or more sections); symbol indicates biopsy specimen. Curves were fitted by eye. Data points for the earliest biopsies (W. K., 1964 and J. S., 1964) were obtained from galactose-^{14}C, frozen-section radioautographs (13) by applying source geometry corrections to grain densities over bands of columnar epithelium (Methods). The data points with 0.2 and 1.0 mM phlorizin (W. K., 1964) suggest that inhibition increased with phlorizin concentration. With a repeat biopsy on subject J. S. (J. S., 1967) galactose-^3H radioautographs were prepared (Figs. 3–5). Both galactose-^{14}C frozen-section (13) and galactose-^3H plastic-section (Fig. 6) radioautographs were prepared from bits of the patient's 1965 biopsy incubated for 3 min; the columnar epithelium to medium ratio averaged 0.41 in the former and the apical cytoplasm to medium ratio averaged 0.43 in the latter.

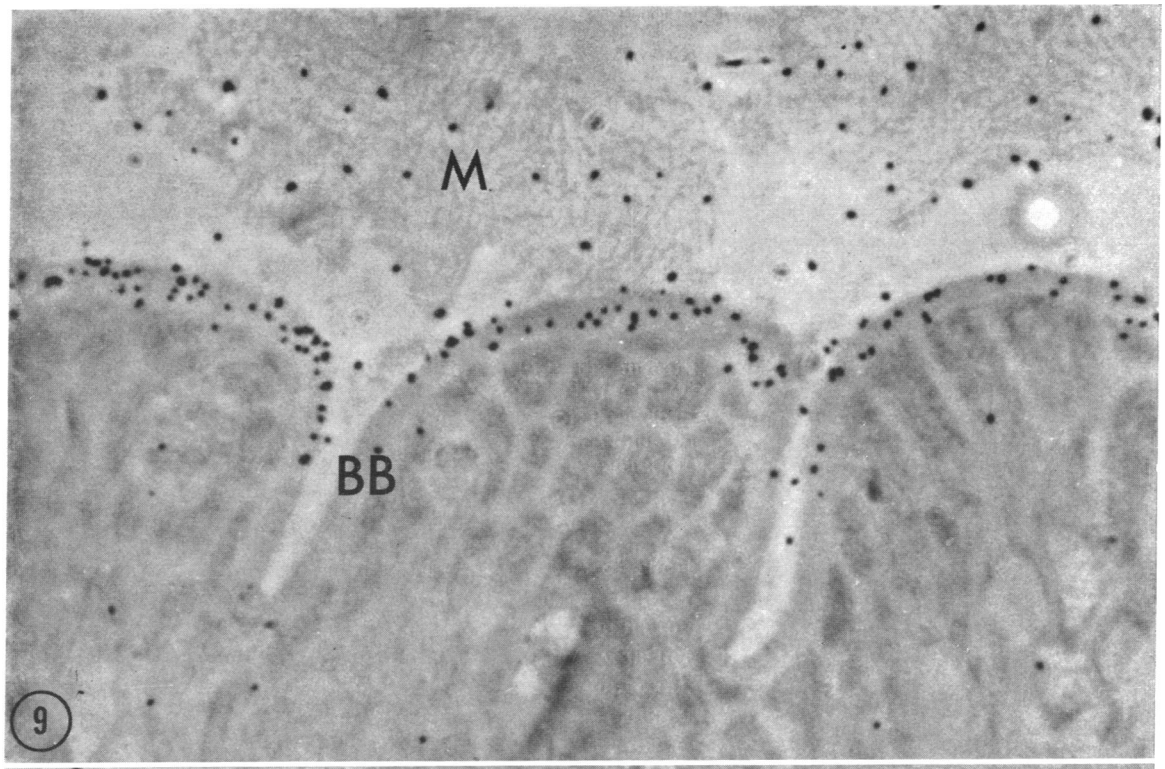
corrected for extracellular space, was only half that of medium. Entry of phlorizin into absorptive cells, on the other hand, was not appreciable under any of the conditions employed. Irrespective of medium concentration and incubation time, both the cell cytoplasm to medium and the lamina propria to medium grain density ratios averaged 0.1 or less.

Limited cellular entry of phlorizin and lack of brush border accumulation at high phlorizin concentrations had previously been observed with hamster intestine (25). These observations suggest that the free surface of the intestinal microvilli possesses a finite number of phlorizin-binding sites which become filled at high concentrations. This view is further supported by the grain density measurements from phlorizin-^3H radioautographs representing two normal biopsies. As shown in Fig. 12, the actual data points are closely fitted by Langmuir's adsorption equation (26) arranged in the form, $B = B_{max}\, m/m + K$, where B is the amount of phlorizin bound, m is the medium concentration, and K is the half-saturation constant. The phlorizin-binding curve for hamster (25) is included for comparison. The difference between the maximum binding, B_{max}, for man, 91 μmoles/liter, and for hamster, 84 μmoles/liter is considerably smaller than the experimental error of these measurements, ±20%, and, therefore, negligible. The difference in K values for man, 72 μmoles/liter, and hamster, 13 μmoles/liter, is well beyond the limits of experimental error and clearly indicates a difference in affinity which correlates with the inhibitory action of phlorizin, i.e., lower affinity and less inhibition in man.

The observed phlorizin binding by normal human microvilli raises a question central to the explanation of glucose-galactose malabsorption. Namely, do the patient's microvilli also bind phlorizin? Thus tissue bits from the 1967 biopsy were incubated 15 min in media containing 1, 25, and 100 μM phlorizin-^3H. Even at the 1 μM level, where binding should be most evident, radioautographs showed no brush border accumulation of phlorizin (Fig. 11). However, quantitative analysis of grain distributions (Fig. 12) did indicate a limited amount of binding. The K value of 55 μmoles/liter cannot be considered different from that for normal man, due to the experimental error involved, but the B_{max} of 8 μmoles/liter is only about 10% of normal and does correlate with the limited galactose uptake observed after 30 min with the patient's 1967 biopsy.

FIGURE 9 Phlorizin-^3H radioautograph of columnar epithelium from a normal mucosal biopsy (T. M., 1968). Tissue bit was incubated 1.5 min in medium containing 5 μM labeled phlorizin. Radioautograph was selected to show areas of maximal brush border (BB) content relative to medium (M). With this biopsy specimen, brush border accumulation of phlorizin did not increase with longer incubation, i.e., for bits incubated 1.5 and 15 min (not shown) the corrected brush border (Fig. 12, legend) to medium grain density ratios averaged 1.7 and 1.5, respectively. Absorptive cell cytoplasm to medium ratios were less than 0.1, irrespective of incubation period. × 1800.

FIGURE 10 Phlorizin-^3H radioautograph of normal columnar epithelium. (T. M., 1968) illustrating saturation of brush border accumulation at high medium concentration. Tissue bit was incubated 15 min in medium containing 100 μM labeled phlorizin (specific activity 20-fold lower than in Fig. 9). For this bit, the corrected brush border to medium and absorptive cell cytoplasm to medium grain density ratios averaged 0.5 and 0.1, respectively. × 1800.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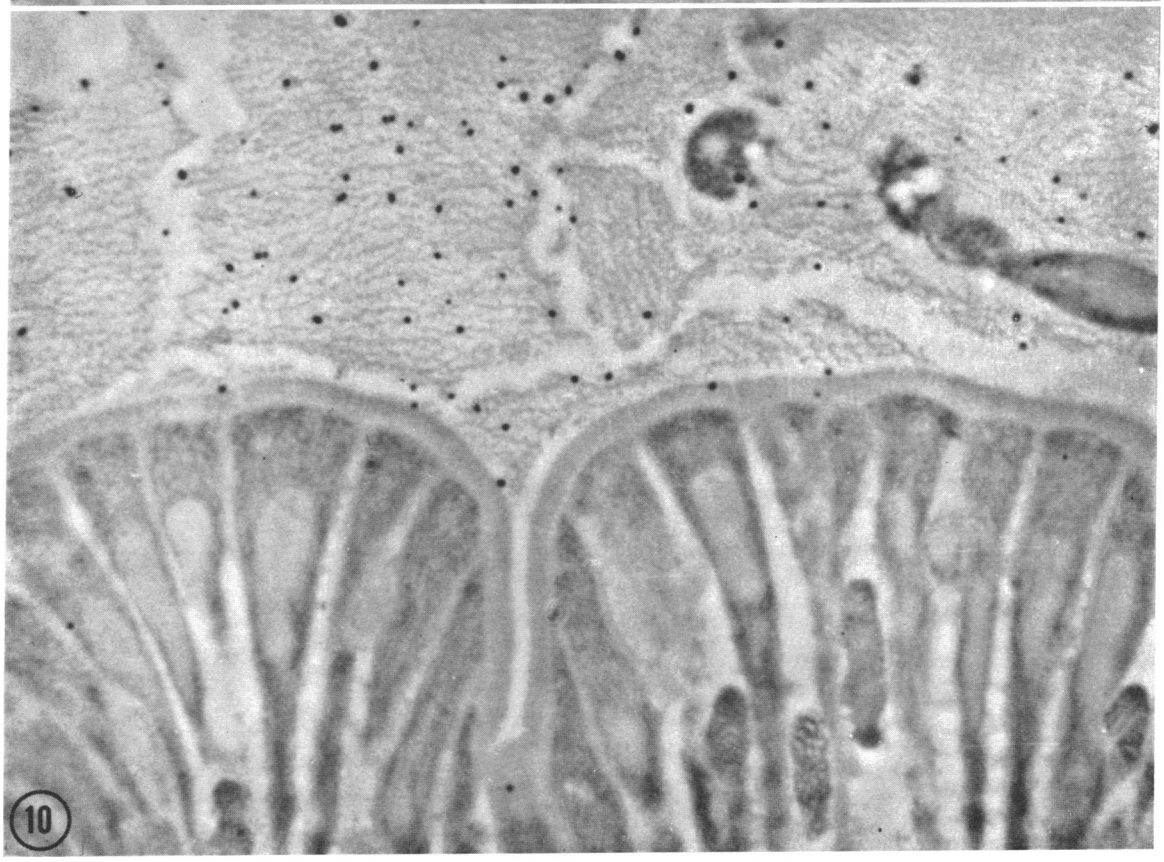
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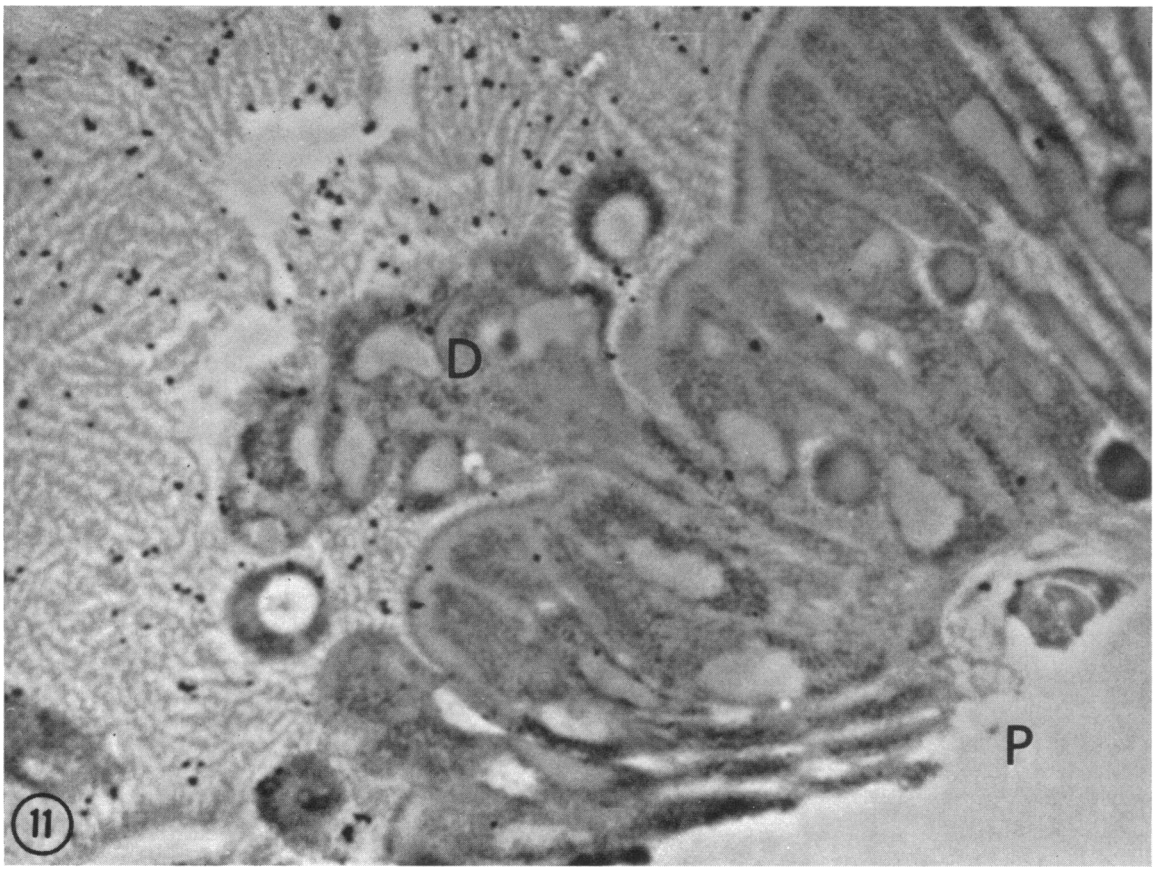

FIGURE 11 Phlorizin-^{3}H radioautograph of columnar epithelium from patient with glucose-galactose malabsorption (1967 biopsy). Tissue bit was incubated 15 min in medium containing 1 μM labeled phlorizin and the corrected brush border to medium and absorptive cell cytoplasm to medium grain density ratios averaged 0.2 and < 0.1, respectively. The low background grain density of the radioautograph can be appreciated from the tissue-free area of plastic (P). Cell debris (D) was in the process of being extruded from the epithelium. $\times$ 1700.

DISCUSSION

The similarity of sugar absorption in man to that in laboratory animals was initially suggested by in vivo studies which, due to the restricted methodology necessary with human subjects, are difficult to interpret (2, 7). A direct in vitro comparison was provided by the radioautographic demonstration that columnar epithelium from normal man (13), like that from hamster (21), rapidly accumulates galactose. More recently, Elsas, Hillman, Patterson, and Rosenberg (9) have shown that the kinetics of glucose accumulation in biopsied human mucosa are quantitatively similar to those in intestinal tissue from small animals; the accumulation also requires the presence of sodium (9, 15). Comparison of the present high-resolution radioautographs with those of hamster cells (20, 25) offers further direct evidence that the basic cellular mechanisms outlined in the Introduction (Fig. 1) are equally applicable to glucose and galactose absorption in man.

The abrupt increase in sugar content seen at the brush border in galactose radioautographs leaves little doubt that the microvilli of human absorptive cells possess an uphill glucose-galactose pump. Whatever doubt remains hinges on the physical state of cytoplasmic sugar which is generally thought to be unbound and, thus, free to diffuse laterally and basally. Consequently, transcellular movement should be accompanied by a diffusion gradient. The apparent absence of any apical to basal gradient in galactose radioautographs is not, however, sufficient evidence to exclude the presence of free sugar. The calculations of Stirling and Kinter (20) show that, if the diffusion coefficient in cytoplasm is but 5% of that in water, a gradient too small to be detected by present radioautography would suffice to transport galactose from apical to basal cytoplasm at a rate commensurate

with the initial rate of entry into hamster cells. Since the radioautographic analysis of galactose entry in Fig. 8 closely agrees with that for hamster (20), the same arguments are applicable to human cells. In addition, Csáky and Esposito (27) have recently provided a direct assessment of the state of cytoplasmic sugar in epithelial cells of frog intestine; mucosal accumulation of the nonmetabolized competitor of glucose-galactose transport, 3-*O*-methylglucose, was accompanied by an increase in intracellular water which indicates that the sugar analog was probably in a free, osmotically active form. Thus, assuming that accumulated galactose is likewise unbound within the cytoplasm of human cells, our radioautographs confirm both uphill entry from medium at the brush border and downhill exit to the lamina propria. The most probable route of sugar exit is across the lateral membranes; certainly the interdigitations and trabeculae which project into the intercellular spaces present a much greater surface area than the limited amounts of cell membrane adjacent to the basement membrane, e.g., Fig. 3 and electronmicrographs of hamster cells (20). Our observation that intercellular spaces contain less galactose than cytoplasm is readily explained by the proposed bulk flow of fluid down these channels (Fig. 1), particularly as the present incubation media always contained sodium. This explanation is supported by the abundance of dilated intercellular spaces especially at villus tips (Fig. 3), a morphological pattern which Tomasini and Dobbins (6) have correlated with high rates of sodium-dependent water absorption in rat ileum in vivo. In addition, exit of sugar into intercellular spaces is an integral part of the specific model proposed by Fordtran, Rector, and Carter (8) to explain the stimulatory effect of glucose and galactose on the absorption of sodium and water by human jejunum in vivo. Finally, it should be noted that exit along the lateral membrane would mean less sugar diffusing all the way from the brush border to the basal end of the cell, less of a concentration gradient between apical and basal cytoplasm, and less possibility of detecting that gradient by radioautography.

Phlorizin is well known as an inhibitor of active glucose-galactose transport in renal and intestinal tissue of many vertebrates. This general finding has been extended to both galactose and glucose accumulation by human mucosa in vitro (13, 16). Action from the luminal or brush border side of the intestine, rapid onset and reversibility of inhibition, and fully competitive kinetics for phlorizin but not its aglycone, phloretin, have been the principal evidence marshaled (1, 2, 4) in formulating the view that phlorizin blocks sugar absorption by binding near and interacting with glucose-galactose carriers located in the cell membrane at the surface of microvilli (Fig. 1). Radioautographs of human cells in

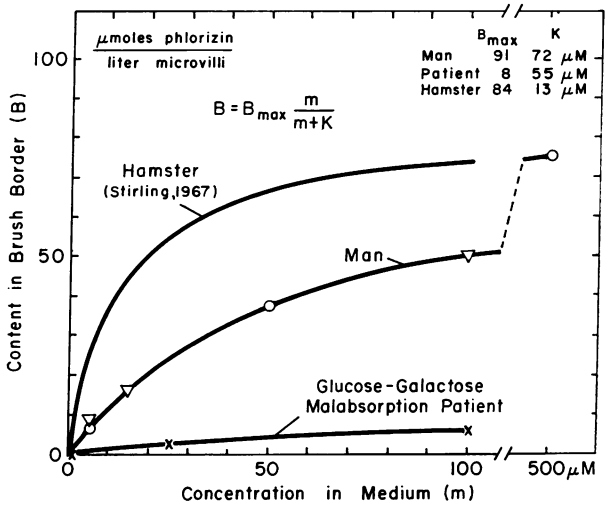

FIGURE 12 Phlorizin-^{3}H binding curves. Human tissue bits were incubated 15 min to ensure equilibrium. Brush border-bound phlorizin, B, was calculated from the relationship, B = m (microvilli grain density/medium grain density), where m is medium concentration and microvilli grain density is brush border grain density corrected for 20% extracellular space between microvilli (20). Data points represent average grain density ratios from two or more sections of individual bits; symbols indicate biopsies and correspond to key in Fig. 8. The curves were calculated from the adsorption equation (text) after the maximum binding, B_{max}, and half-saturation, K, constants were obtained from intercepts of straight lines fitted by eye to reciprocal plots of the data points. The hamster curve is from a previous study (25) and was obtained with tissue incubated 20 min.

conjunction with those of hamster (20, 25) provide the following critical support for this view. First, location of the uphill, phlorizin-sensitive carriers at the microvillar surface is consistent both with the high galactose content of microvilli, i.e., corrected brush border, which approximates the galactose content of cell cytoplasm and with the inhibitory effect of phlorizin on the rate of sugar entry which is manifested by equal reductions in the galactose content of microvilli and cytoplasm (Table I). Secondly, binding of phlorizin at specific sites near the carriers is consistent both with the rapid phlorizin binding in microvilli which exhibits saturation (Fig. 12) and with the slow entry into cytoplasm that indicates a lack of phlorizin transport via the carriers. It may, of course, be argued that the observed microvillar binding of phlorizin is largely nonspecific and unrelated to the inhibition of sugar transport; however, the quantitative relationships between binding and inhibition suggest otherwise. Both binding (Fig. 9) and inhibition (4) take place very rapidly. The low microvillar affinity for phlorizin in man as compared to hamster (Fig. 12) is accompanied by less potency in inhibiting the rate of sugar entry. For example, the phlorizin concentration

TABLE II

Estimates of Sugar Carrier Density Based on Phlorizin Molecules Bound per Cell

Epithelial cells	Method	K*	Carriers/cell
		μmoles/liter	
Human intestine:			
Normal	Radioautography	72	2.8×10^6
Patient	Radioautography	55	2.4×10^5
Hamster intestine:	Radioautography (25)	13	2.6×10^6
	Isolated brush borders (28)	77	3.9×10^8
Dog kidney:	Whole kidney (31)	—	1.0×10^7
Rat kidney:	Isolated brush borders (30)	3.4	6.0×10^6

* Half-saturation concentration for phlorizin binding.

required to halve the accumulation of galactose was about 500 μmoles/liter for human cells with 1 mM galactose in the medium (Fig. 8) as compared to about 5 μmoles/liter for hamster tissue with 5 mM galactose (25). Also for human mucosa with 5 mM glucose, 100 μM phlorizin only about halved the 10 min uptake of this more rapidly metabolized sugar, as evidenced by the accumulation of ^{14}C label (16).

If one accepts specific phlorizin sites near the sugar carriers, then measurements of microvillar binding afford a means of estimating the number of carriers. A potential source error is the possibility than human brush border may contain a phlorizin hydrolase similar to that reported in hamster intestine (28, 29). Since, however, only phlorizin-^3H with the label in the phloretin moiety (Methods) was used for human and hamster (25) radioautographs, any hydrolysis would yield phloretin-^3H. Thus, assuming that all microvillar ^3H label represents either phlorizin or phloretin bound specifically in 1:1 association with glucose-galactose carriers, the number of carriers per cell can be calculated (25) from the maximum binding, B_{max} (Fig. 12). In Table II, carrier densities so obtained are compared with estimates from the literature also based on maximal phlorizin binding. As estimated by radioautography, values for normal human and hamster intestine are very similar. The much larger value that Diedrich obtained with isolated brush border fragments from hamster intestine (28) may reflect exposure of nonspecific phlorizin-binding sites due to disruption of microvilli during the isolation procedure. On the other hand, Bode, Baumann, Frasch, and Kinne obtained a value with isolated brush border particles from rat kidney (30) which is close to the values estimated for normal intestinal cells by radioautography. Finally, it should be emphasized that on the basis of simple calculations (25) density values of even 10^7 carriers per cell (Table II) are too small for the binding of low affinity, $1/K$, substances to be detected in radioautographs. In fact, with human intestinal cells the affinity for phlorizin, $K = 72$ μmoles/liter, was just high enough to permit binding measurements (Fig. 12). Consequently, still lower affinities for transported sugars, e.g. $K = 4200$ μmoles/liter for glucose in man (9), would preclude detection of molecules bound to transport sites on carriers in the presence of the necessarily much larger numbers of molecules in incubation media between microvilli (25). Thus, the microvillar accumulation of sugar observed in present galactose radioautographs (Table I) almost certainly represents molecules which have undergone uphill transport across the cell membrane at the surface of the microvilli (Fig. 1).

Turning to the nature of the transport defect in glucose-galactose malabsorption, previous in vitro studies with biopsy tissue have suggested that the defect is confined to the uphill, phlorizin-sensitive entry of sugar into columnar cells (9, 13, 15, 16). Present high-resolution radioautographs provide direct evidence of a markedly reduced rate of galactose entry across the patient's microvillar membrane. As with control cells, the galactose content of the microvilli approximated that of cytoplasm, but almost 30 min, instead of about 1 min, of incubation were needed for these intracellular levels to exceed the 1 mM concentration in medium, i.e., to become uphill (Figs. 7, 8). Slow entry capable of proceeding uphill suggests limited carrier function in this particular patient and is consistent with reports from two other patients that glucose absorption in vivo proceeded at about 10% of the normal rate (12, 16). Furthermore, evaluation of glucose-galactose carriers by phlorizin binding (Table II) indicates that the carrier density in the patient's microvillar membrane was about 10% of normal, while, at least with respect to phlorizin affinity or $1/K$, the function of the existing carriers was unchanged. Thus, the molecular basis of familial glucose-galactose malabsorption appears to be a reduced number of functioning sugar carriers which, presumably, represent protein molecules under specific genetic control. This conclusion is supported independently by the elegant kinetic study of Elsas et al. (9) with tissue from presumed heterozygotes, i.e. close relatives of a patient, which showed that the maximum rate of glucose accumulation in vitro was about 75% of normal while the glucose affinity was unchanged. Failure of Elsas et al. and others to detect uphill accumulation of glucose in patients' mucosa by whole tissue analysis (9, 15, 16) is not surprising in view of the limited uphill accumulation of galactose seen in the epithelium alone (Fig. 7) and the more rapid metabolism of glucose. Likewise, failure to detect any effect of phlorizin on the already impaired entry of glucose into a patient's mucosal tissue (16), probably reflects the fact that radioautography, which

did detect phlorizin binding in such tissue, is a more sensitive technique than whole tissue analysis.

In concluding, it should be noted that past studies utilizing peroral biopsies have been limited to only a few experimental conditions because of the small quantities of tissue available. As our studies show, radioautography circumvents this limitation, i.e., from a single biopsy (patient, 1967) weighing less than 100 mg, six distinct experimental conditions were examined. Thus, high-resolution, plastic-section radioautography not only yields quantitative data at the subcellular level, but is also particularly suited to studies on small amounts of rare tissue. For example, the exciting possibility that renal glucose-galactose transport involves two systems under separate genetic control, the "nonintestinal" system being unaffected in present malabsorption patients (18), could be investigated if renal tissue from such a patient should become available.

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