HIGH-RESOLUTION RADIOAUTOGRAPHY OF PHLORIZIN-^{3}H IN RINGS OF HAMSTER INTESTINE

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ABSTRACT

Quantitative light microscope radioautographs of galactose-^{3}H and phlorizin-^{3}H were prepared from freeze-dried plastic-embedded hamster small intestine incubated in vitro. The usual uphill epithelial cell accumulation of galactose accompanied by a somewhat smaller lamina propria accumulation was observed in control tissue incubated 3 min in 1 mM galactose-^{3}H. The addition of 5×10^{-4} M phlorizin to the medium blocked uphill accumulation, but did not prevent galactose equilibration with the epithelial cells. The galactose content of the lamina propria was considerably less than the galactose content of the epithelial cell. Varying the phlorizin-^{3}H content of the medium from 0.6 to 60 μM revealed a brush border binding of phlorizin which followed a Langmuir adsorption isotherm with a half-saturation constant of 13 μM and a maximum binding of 84 μmoles of phlorizin/liter of microvilli or 2.6×10^6 sites/epithelial cell. The phlorizin content of the epithelial cell compartment, excluding microvilli, never exceeded 10% that of the medium after 20 min of incubation. These findings directly support the view that phlorizin is a nontransported inhibitor which binds glucose-galactose carriers at the surface of epithelial cell microvilli.

INTRODUCTION

The plant glycoside phlorizin is widely recognized as a selective inhibitor of active sugar transport in the intestine (21, 28) and renal tubule (16, 19). In the intestine, micromolar doses are effective in vitro (12) while slightly larger doses are required in vivo (20). It is also an inhibitor of energy metabolism, but this effect requires millimolar doses; hence, phlorizin is generally thought to interfere directly with the glucose-galactose transport mechanism. The luminal or brush border surface of the intestinal epithelium was first proposed (24) as the site of phlorizin action on the basis of the rapid reversibility of glucose transport inhibition in vivo, and this concept is now generally accepted (13, 23) on the basis of recent quantitative studies of in vitro sugar absorption. It has been suggested, from the saturative (18) and competitive (2) nature of this inhibition, that a tight association between phlorizin and membrane carrier sites of the microvilli limits the availability of these sites for glucose-galactose transfer. Whether phlorizin itself is actively transferred is not known.

These views, being based almost exclusively on indirect inhibitory evidence, clearly point to the need of direct observations on the effects and behavior of phlorizin in inhibitory situations. This

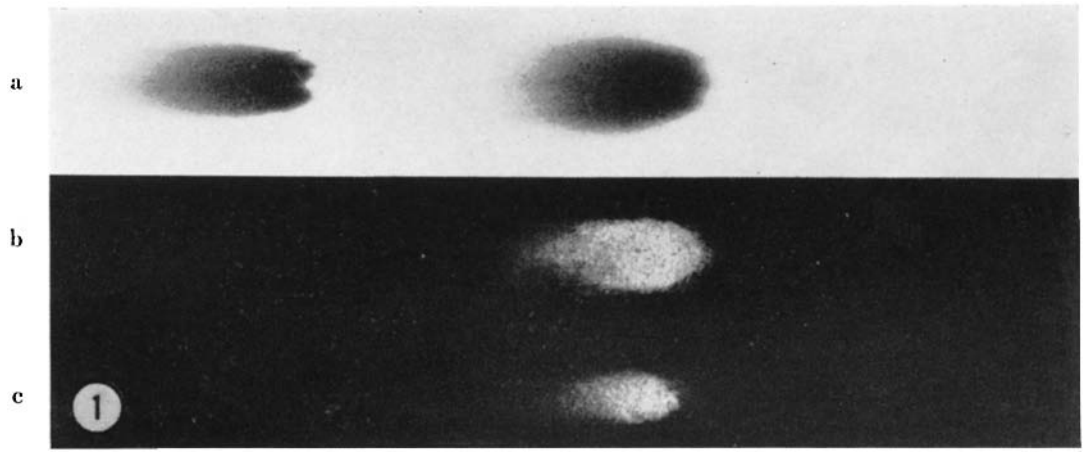

FIGURE 1 Chromatography of phlorizin: (*a*) chalcone precursor (left) and phlorizin-^{3}H (right), spotted with diazotized benzedine; (*b*) radiochromatogram of phlorizin-^{3}H; and (*c*) radiochromatogram of final incubation medium (PB-2) containing 11 μM phlorizin-^{3}H. Development was ascending in 15% acetic acid and water. Radiochromatograms were made by sandwiching paper chromatogram between glass plates coated with Kodak NTB 2 emulsion and exposing them for 8 wk. × 0.6.

study continues and extends the method of high-resolution water-soluble radioautography (26) by applying the method to the problem of phlorizin inhibition. I present quantitative evidence from both phlorizin-^{3}H and galactose-^{3}H radioautographs showing that phlorizin is a nontransported inhibitor which binds to epithelial cell microvilli.

METHODS AND MATERIALS

Details of the methods and analysis of radioautographic precision are given in a previous publication (26). Briefly, narrow rings of hamster small intestine were incubated for variable periods in a bicarbonate buffer containing 15% albumin and appropriate amounts of radioactive test compounds. After incubation, they were removed, dissected into 1–2 mg bits, and quenched in propane cooled to −184°C. The bits of tissue were then freeze-dried, fixed in osmium tetroxide vapor, and embedded in siliconized plastic. Light microscope radioautographs were prepared by coating 1-μ sections, which had been collected over water, with liquid Kodak emulsion. The absolute concentration of a radioactive test compound in a 2-μ band (e.g. brush border) of radioautographs prepared according to this procedure could be measured with over-all accuracy of ±20% by comparing the grain density (number of silver grains/μ^2) of the band and that of the medium whose concentration had been measured by scintillation counting. Grain densities were determined from photomicrographs (results) and a given measurement was based on 100 or more grains.

Test compounds included galactose-^{3}H (New England Nuclear Corp., Boston), natural phlorizin (Nutritional Biochemical Corp., Cleveland), and tritium-labeled phlorizin. The phlorizin-^{3}H was prepared by tritium reduction (performed by the New England Nuclear Corp., Boston) of its chalcone precursor, synthesized according to the method of Zemplen and Bognar (29). Pure phlorizin-^{3}H (2.86 c/millimole) was obtained by paper chromatography of the crude reduction product (Fig. 1). A physiological assay (Fig. 2) showed the synthetic compound to be equally as potent as natural phlorizin. Radioautographic exposures varied from 4 to 10 days for galactose-^{3}H and 3 to 60 days for phlorizin-^{3}H.

RESULTS

Galactose-^{3}H Radioautographs

That phlorizin blocks uphill sugar accumulation by intestinal epithelial cells incubated in vitro was postulated on indirect grounds (23) and recently demonstrated at the villar level with ^{14}C-radioautography (13). The radioautographs of Figs. 3 and 4 demonstrate the effects of phlorizin on the villar distribution of galactose-^{3}H after 3 min of incubation. The large uphill accumulation typical of control tissue (Fig. 3) was almost completely blocked by the addition of 5×10^{-4} M phlorizin to the bathing medium (Fig. 4). The high magnification radioautographs of Fig 5 show the galactose-^{3}H distributions at the subcellular level. By

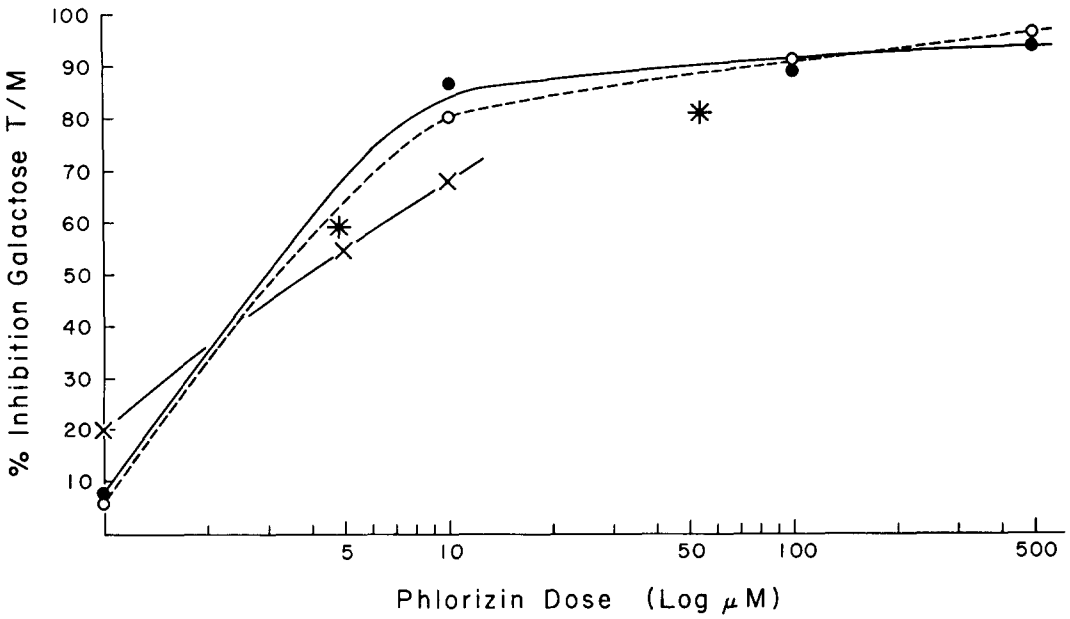

FIGURE 2 Phlorizin dose response curves. Ordinates are single measurements of per cent inhibition of tissue-to-medium galactose concentration ratios (T/M) for rings of hamster intestine incubated 20 min at 37°C in 5 mM galactose and indicated concentrations of phlorizin. Solid and open circles were responses to phlorizin-^{3}H and natural phlorizin, respectively, with tissue from the same animal. Asterisks were responses to phlorizin-^{3}H from a second animal. Crosses are Diedrich's measurements (6) for natural phlorizin inhibition of glucose T/M in rings of hamster intestine. Analyses were performed on ≈150 mg of tissue in duplicate with the Nelson-Somogyi method (22).

eye, the grain density of the inhibited epithelial cells (Fig. 5 *a*) was indistinguishable from that of the medium but definitely higher than that of the lamina propria. This impression was verified by grain density measurements; the relative values presented in Table I suggest a slight accumulation, no more than 5% that of control cells. Except for the absence of an uphill brush border step, galactose distributions of control and inhibited tissue were quite similar. Both control and inhibited tissue exhibited an almost uniform content of cytoplasmic galactose and galactose-poor intercellular spaces and nuclei. The persistence of the downhill step across the basal lamina of inhibited tissue suggests no increased permeability of the basal exit barrier. A possible decrease in permeability is less certain, but previous studies (13, 23) suggest this effect to be unlikely. Thus, it would seem that the only effect of phlorizin was to depress uniformly the absolute galactose content of the tissue by blocking an uphill transfer of sugar into the microvilli. This finding further emphasizes the importance of examining the distribution of phlorizin.

Phlorizin-^{3}H Radioautographs

One would expect binding of phlorizin to glucose-galactose transport sites to be most evident as a preferential accumulation of inhibitor in the brush border at concentrations below saturation. Accordingly, phlorizin-^{3}H tissue distributions were examined at medium concentrations ranging from threshold (0.6 μM) to near maximum (60 μM) inhibition. The most striking feature of the radioautographs prepared from intestine incubated at the lower medium concentrations was a large accumulation of phlorizin-^{3}H in the brush border; the radioautographs in Figs. 6 and 7 are representative of this finding. In the lower magnification dark-field view (Fig. 6), this accumulation was evident as a bright band of silver grains at the free surface of the villi. The continuity of this band over the entire free surface of the villi revealed a uniformity, among small bowel epithelial cells, resembling that of galactose accumulation (Figs. 3 and 5 *b*). The sharpness of these grain density boundaries may be appreciated from the high magnification view in Fig. 7; clearly, the accumu

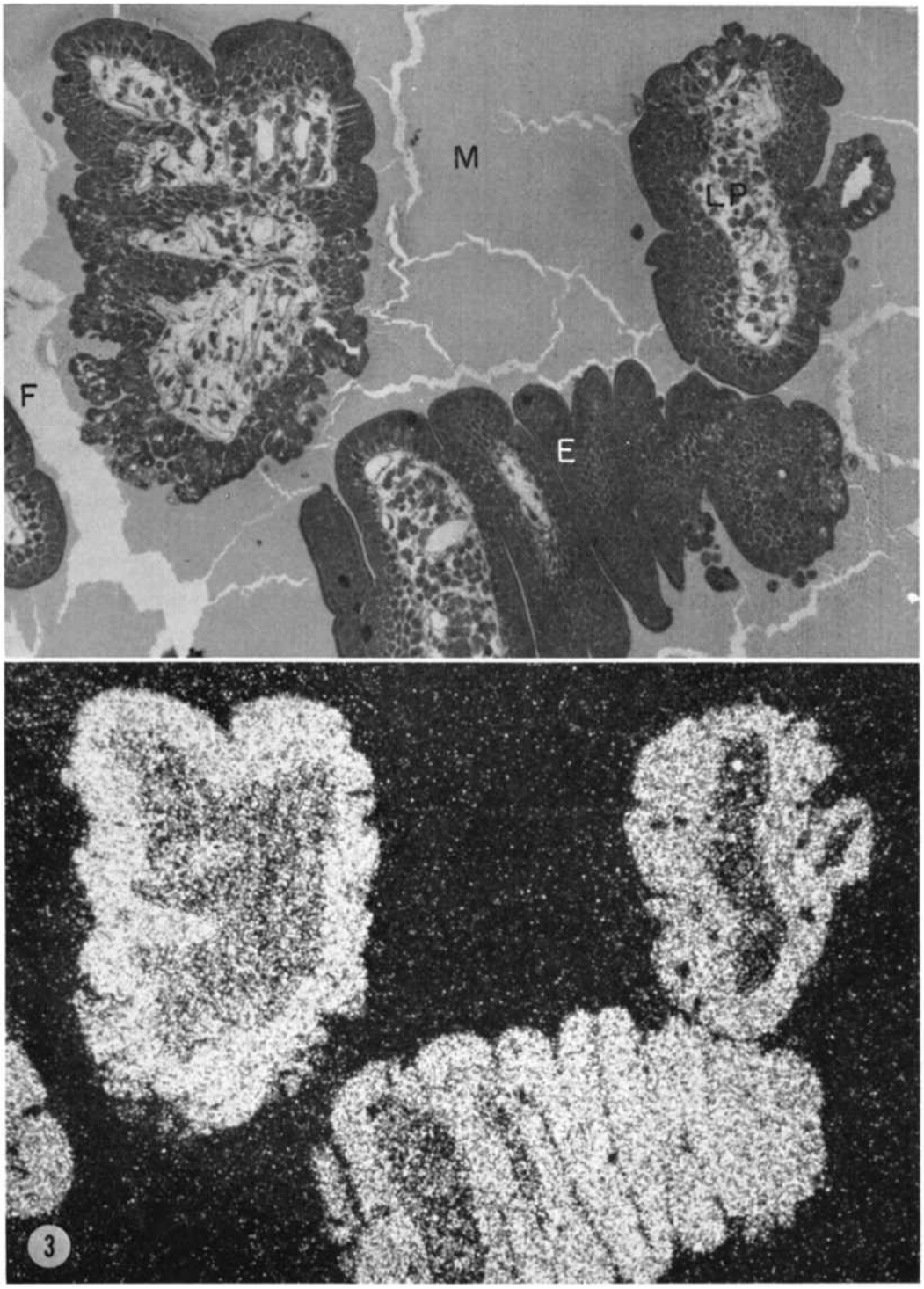

FIGURE 3 Bright-field (top) and dark-field micrographs of nearby stained section (basic fuchsin) and radioautograph from control hamster intestine (HG-2) incubated 3 min in 1 mM galactose-^{3}H. Incubation medium (*M*) interspersed with freezing fractures (*F*) surrounds villi cut longitudinally and in cross-section. The bright dots of the dark-field image (bottom) are silver grains of the photographic emulsion and correspond to disintegrations of tritium label. They show a large galactose-^{3}H accumulation by the epithelium (*E*) and a somewhat smaller accumulation in the lamina propria (*LP*). × 250.

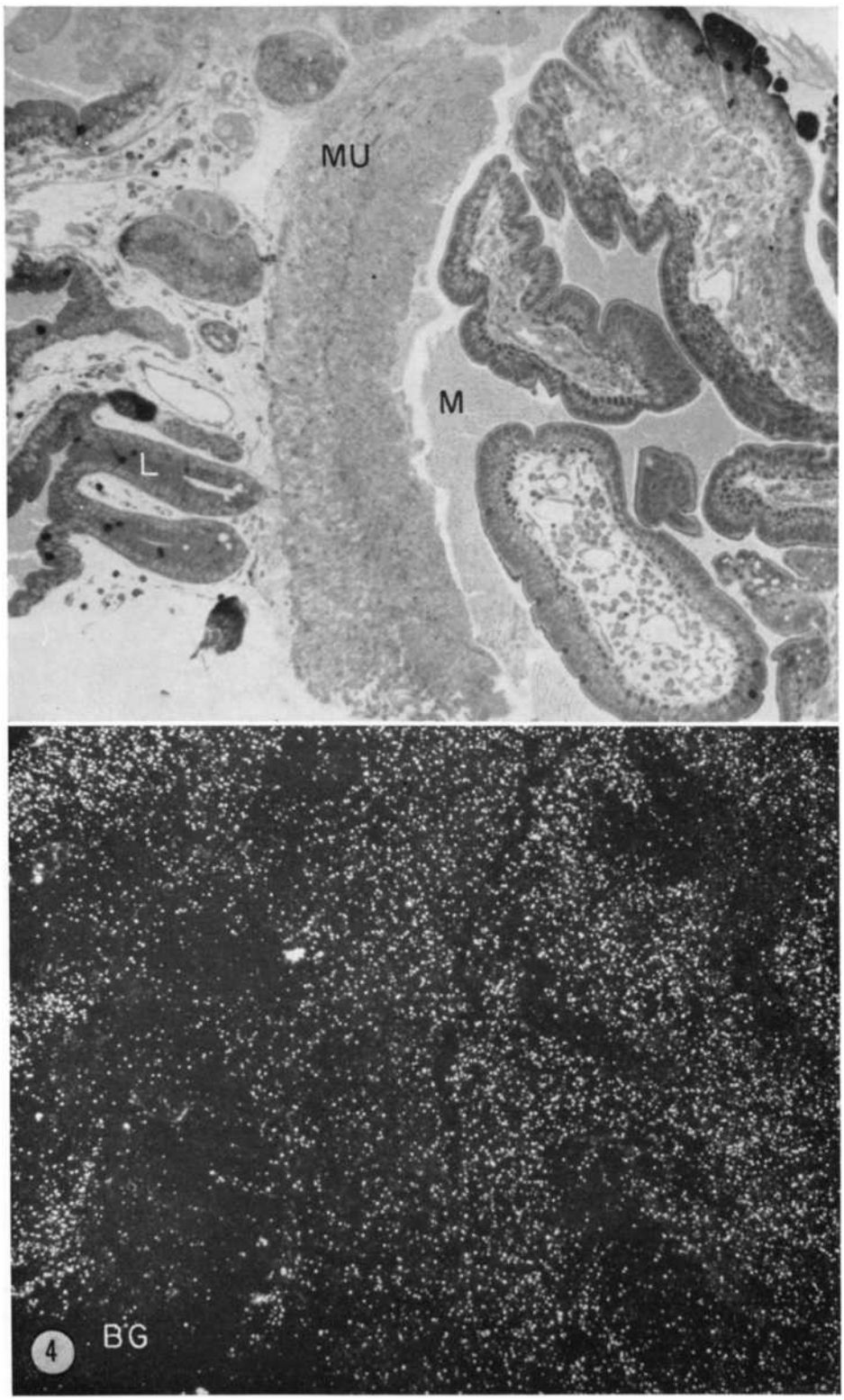

FIGURE 4 Bright-field (top) and dark-field micrographs showing distribution of galactose-^{3}H in same intestine as in Fig. 3, when 5×10^{-4} M phlorizin was added to the test incubation medium. The pre-incubation medium did not contain phlorizin. Epithelium and medium (*M*) are indistinguishable in the dark-field image. However, lamina propria and crypts of Lieberkühn (*L*) are indicated by grain densities intermediate to those of background (*BG*) and medium. The muscularis (*MU*), usually stripped away during preparation, was left intact in this animal (HG-2). × 250.

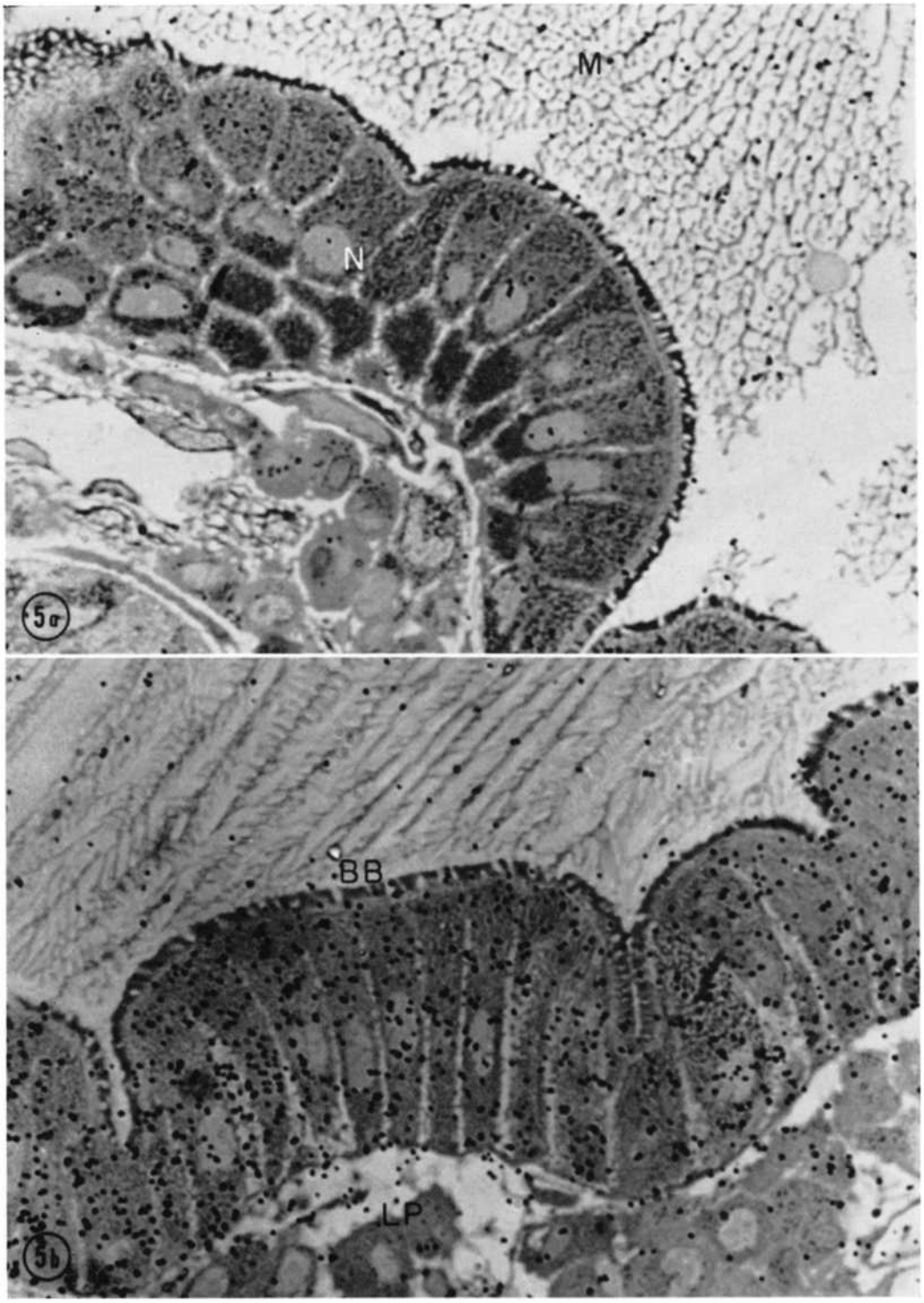

FIGURE 5 High magnification bright views of radioautographs from same tissue as in Figs. 3 and 4 showing subcellular distribution of galactose-^{3}H with (*5 a*) and without (*5 b*) phlorizin in the incubation medium. The silver grains appear as black dots overlying a slightly out-of-focus image of the tissue. The incubation medium (*M*) is reticulated and the brush border (*BB*) slightly distorted. Nuclei (*N*) of epithelial and lamina proprial cells (*LP*) are lightly stained. Uphill accumulation (*5 b*) was completely blocked by phlorizin (*5 a*), but grain pattern in the tissue appears to be unaltered (see Table I for measurements). × 1500.

TABLE I

*Effect of Phlorizin on In Vitro Galactose Absorption**

Animal	Relative galactose-^{3}H content‡					
	Medium	Corrected§ brush border	Apical band	Nuclear band	Basal band	Lamina propria
HG-2 (Figs. 3–5)						
Control	1.0	10.8	11.6	12.1	11.6	3.2
Phlorizin	1.0	1.1	1.3	1.4	1.2	0.3
HG-4 (not shown)						
Control	1.0	5.7	9.2	10.9	6.4	2.5
Phlorizin	1.0	1.0	1.4	1.4	1.1	0.4

* Hamster rings incubated 3 min in 1 mM galactose-^{3}H (Control) plus 5×10^{-4} M phlorizin (Phlorizin).

‡ Grain density ratios based on single photographic measurements of 100 or more grains each after correction for background. Micrographs in Figs. 3–5 illustrate part of measured area from HG-2. Bands indicate divisions of epithelial cell.

§ Corrected for extracellular space of 21% (26).

lation of phlorizin was limited to the brush border band of the epithelium.

Unlike galactose, phlorizin in the brush border did not rapidly equilibrate with the cellular compartment; the association with microvilli was evidently tight. Indeed, the content of the cellular compartment appeared to be well below that of the medium. One was also impressed by a low cellular content at higher doses (Fig. 8). Grain density measurements of tissue incubated at various phlorizin-^{3}H concentrations are compared in Table II. These relative values show that, regardless of medium concentration, the epithelial cell content was no more than 10% of the medium concentration. Some phlorizin may have entered via the lamina propria; however, one would predict that the brush border was the primary route of entry since the phlorizin concentration of the lamina propria was low compared to that of the medium.

Phlorizin bound to the microvilli of the brush border was not apparent at high medium concentrations. The radioautographs of Fig. 8 illustrate the effect of increased medium concentration on phlorizin binding. The large concentration difference between brush border and medium found in tissue incubated at 3 μM (Fig. 6 *a*) was absent in in the 60-μM tissue (Fig. 6 *b*). The brush border-to medium grain density ratio was about one (Table II), suggesting a saturation of binding sites characteristic of adsorption phenomena. The dependence of brush border binding on medium concentration was examined quantitatively in Fig. 9. A plot of phlorizin content of the microvilli against medium concentration gave a curvilinear relationship which closely fitted a Langmuir adsorption isotherm (11), with a half-saturation constant of 13 μM and a maximum binding capacity of 84 μmoles of phlorizin/liter of microvilli (see Fig. 9 for calculations).

This half-saturation constant is consistent with phlorizin inhibitory constants ((K_i) determined from kinetics of sugar-transport inhibition. The values reported in the literature depend upon the particular sugar and vary from 0.45 μM for 1, 5 anhydro-D-glucitol (2) to 600 μM for xylose (1) in hamster intestine. The above finding and the observation that saturation doses also cause maximum inhibition suggest that phlorizin binding is specific (see Discussion).

DISCUSSION

The present results offer convincing support for the view that low doses of phlorizin inhibit active sugar absorption withour interfering with energy metabolism. Of the various methods used to study its metabolic effects, cell-free preparations are probably the most sensitive, requiring doses of about 10^{-4} M to elicit interference (17). The radioautographic measurements in Table II show that intestine exposed 20 min to phlorizin concentrations which drastically reduced galactose absorp-

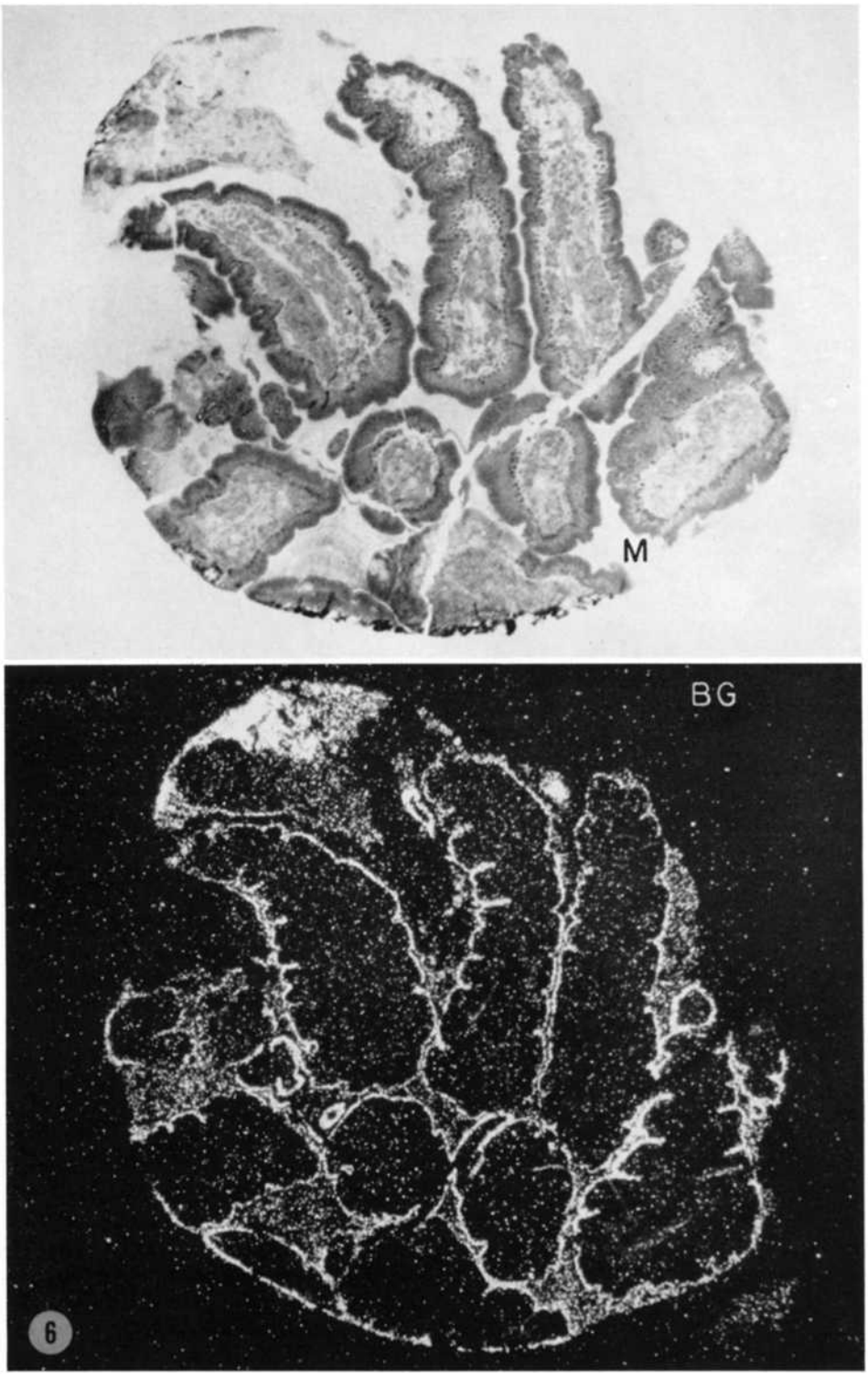

FIGURE 6 Bright-field (top) and dark-field micrographs of a stained section and radioautographs showing distribution of phlorizin-^{3}H at the villar level (PB-1); incubation was 20 min in a 0.6 μM medium containing no albumin. The bright lines outlining the villi (bottom) demonstrate a heavy accumulation of phlorizin-^{3}H at the free surface of the columnar epithelium. The grain densities of epithelium and lamina propria are larger than that of background (*BG*) which is somewhat high owing to a 60-day exposure. In the absence of albumin, patches of medium (*M*) are sparse. × 145.

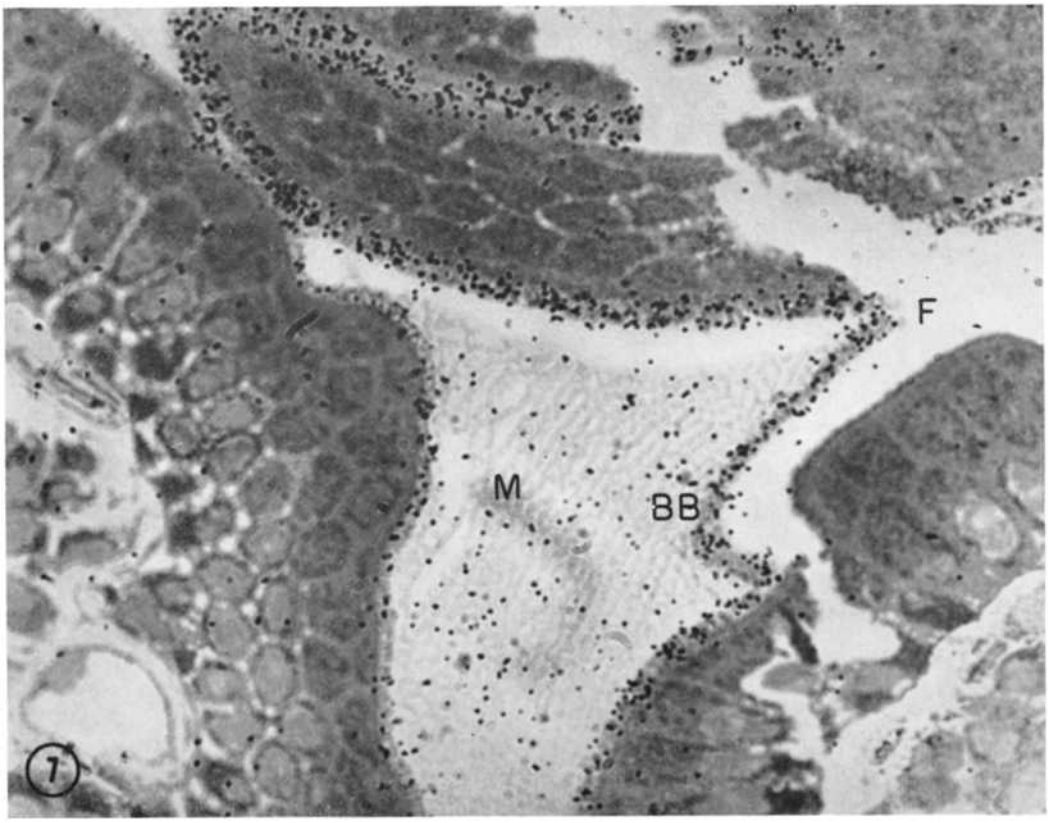

FIGURE 7 High magnification bright-field view of radioautograph in Fig. 6 showing phlorizin-^{3}H distribution at the cellular level. The bright bands seen in the dark-field image (Fig. 6) represent phlorizin-^{3}H accumulation in the brush borders of the columnar epithelium. This localization is made quite clear by a freezing fracture (*F*) separating brush border (*BB*) from epithelium. The patch of medium (*M*) emphasizes the low phlorizin content of the columnar cells (see Table II). × 1350.

TABLE II

*Phlorizin-^{3}H Distributions at Different Medium Concentrations**

Animal	PB-1	PB-2	PB-1	PB-1	PB-2	PB-3	PB-1
Fig.‡	6, 7		8				8
Medium§ conc. (μM)	0.6	1.0	3.0	6.0	11	60	60
Relative grain density‖							
Medium	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Brush border	4.5	6.6	5.8	3.7	3.2	1.0	1.1
Cell	0.1	0.1	0.1	0.0	0.1	0.1	0.1
Lamina propria	0.1	0.1	0.2	0.2	0.1	0.3	0.1

* Rings of hamster intestine incubated 20 min.

‡ Figure illustrates part of measured area.

§ Measured by scintillation-counting.

‖ Brush border values (uncorrected for extracellular space) are average ratios of either ten measurements (PB-1) or four measurements (PB-2 and PB-3); cell and lamina propria ratios represent single measurements. All were made from photomicrographs, based on 100 or more grains/measurement and corrected for background.

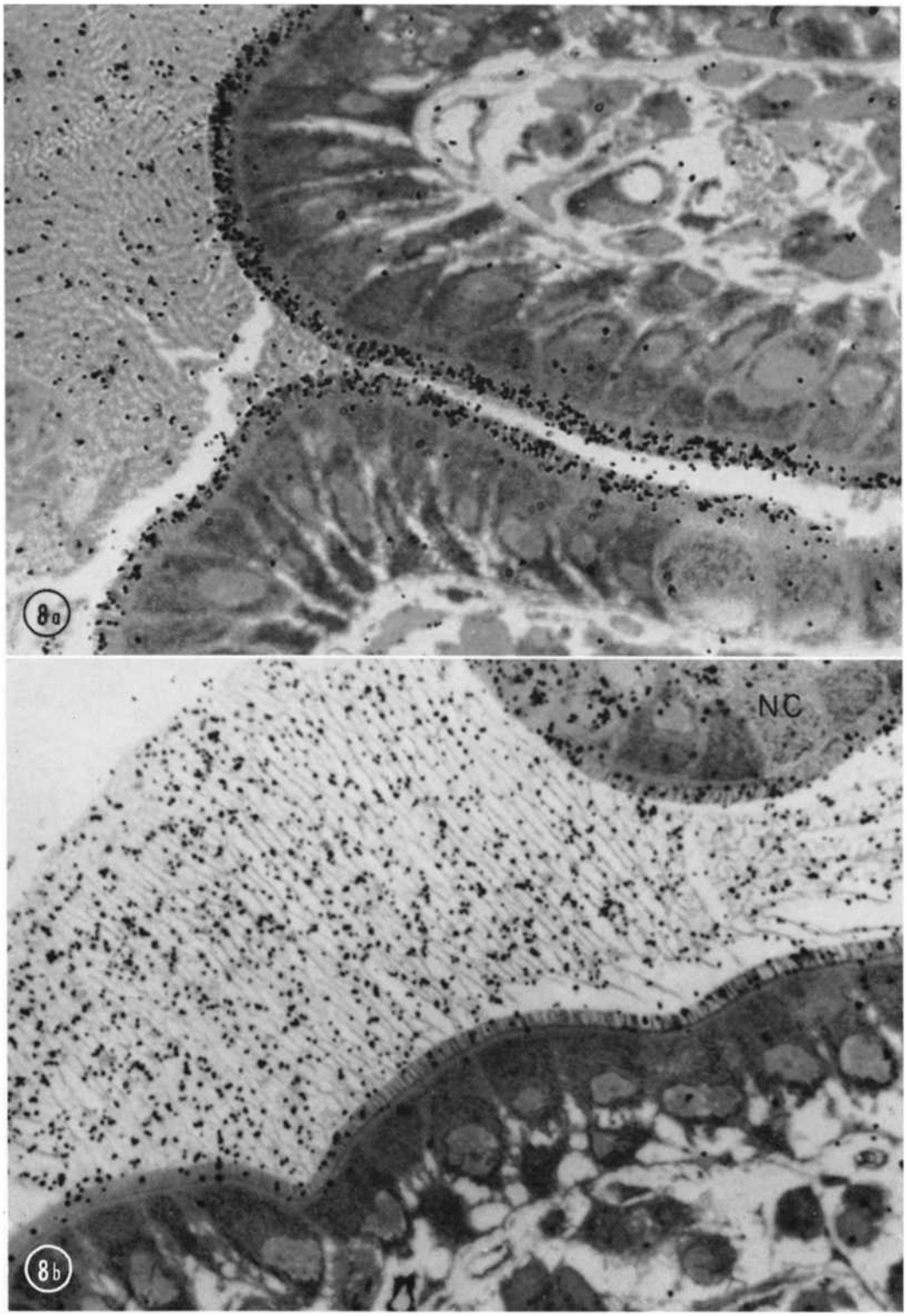

FIGURE 8 Radioautographs contrasting phlorizin binding by microvilli of intestine (PB-1) incubated 20 min in 3 μM (Fig. 8 *a*) and 60 μM (Fig. 8 *b*) media. At the lower concentration, the phlorizin content of the brush border is large relative to that of the medium, but they are nearly equal at the higher concentration, suggesting a saturation of phlorizin-binding sites. Some light-staining cells (*NC*) show an increased permeability to phlorizin and are probably necrotic. This type of cell also shows poor galactose accumulation and increased permeability to mannitol (26). × 1650.

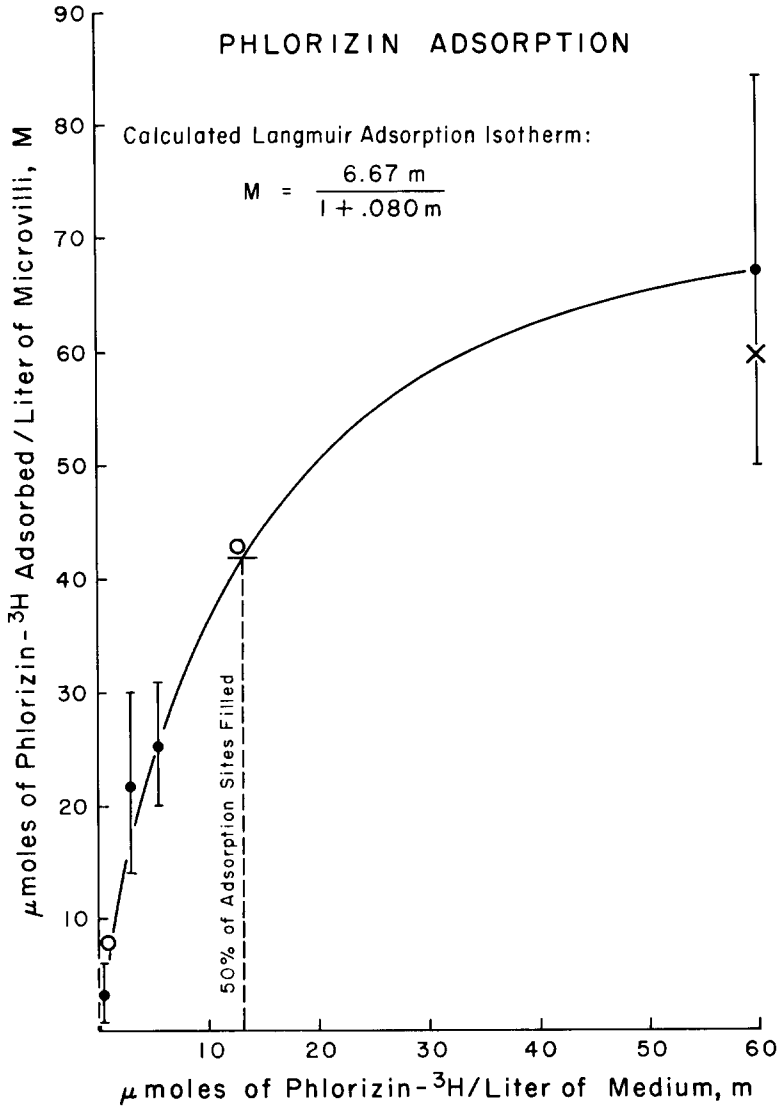

FIGURE 9 Dependence of phlorizin binding on concentration. Microvilli concentration (M) of phlorizin was determined from the relationship: $M = m \frac{(\text{microvilli grain density})}{(\text{medium grain density})}$, where m is medium-concentration (scintillation-counting measurement) and microvilli grain density is brush border grain density corrected for an extracellular space of 21% (26). Filled circles (PB-1) are averages ± SD of ten to fifteen sample measurements at each concentration; open circles (PB-2) and cross (PB-3) are averages of three to four measurements. The theoretical curve was determined by a least squares fit of the open circles to the linear form of the Langmuir adsorption equation (11). Extrapolation to infinite medium concentration gave a maximum binding capacity of 84 μmoles of phlorizin/liter of microvilli; the half-saturation constant is 13 μM. Albumin was omitted from the incubation media of animal PB-2 and from the 0.5 μM medium of animal PB-1; the close agreement among measurements from all animals indicates that albumin has a negligible effect on phlorizin binding at these concentrations.

tion (Fig. 2) does not attain cellular levels close to 10^{-4} M. In addition, a 3-min incubation was sufficient to effect almost complete inhibition of uphill accumulation (Figs. 3–5); one may assume that during this short exposure the cellular levels were negligible.

The finding that phlorizin blocks uphill transfer of galactose across the brush border without appreciably penetrating the epithelium leads to the inescapable conclusion that it interferes with an active sugar pump located in the microvilli. The rapid onset of inhibition, its competitive kinetics (2), and close parallelism to phlorizin adsorption further suggest that the interference is a result of adsorption to glucose-galactose carriers at the surface of the microvilli. Since cellular levels were less than 10% that of the medium after 20 min, reaction with the carrier must not confer any special permeability upon phlorizin as is generally proposed for sugars of the glucose-galactose class. Alvarado and Crane (3) have argued on theoretical grounds that carrier-mediated entry would be slow because of phlorizin's high affinity for the carrier (small K_t).[1] Under present conditions, this argument is untenable since transfer across the brush border barrier was slow at concentrations equal to or less than that which would half saturate transport (K_t). The observation that cellular content after 20 min was nearly proportional to medium concentration, which varied from 0.6 to 60 μM, suggests diffusion kinetics for phlorizin entry.

Either steric hindrance or secondary binding could prevent intracellular transfer. Diedrich (6) has advanced convincing arguments, based on the configurational specificity of phlorizin and inhibitory analogs, for secondary binding by the aglycone ring. This view is consistent with the observations that phloretin (aglycone phlorizin) is a noncompetitive inhibitor of intestinal glucose absorption (8) and is decidedly less effective than phlorizin (12). In light of present findings, this hypothesis becomes particularly attractive since it not only accounts for phlorizin's high affinity for the carrier but also explains the absence of cellular transfer. Active transport of several phenylglycosides has been reported (3, 14), but there is no information available on the transport of glycosides containing two phenyl groups. However, one might speculate that the addition of a second phenyl group would prove too formidable a bulk for intracellular transfer. It would be of interest, in regard to both steric hindrance and secondary binding, to examine the transport of certain phlorizin analogs which are less potent inhibitors (e.g. phlorizin 4′-glucoside, 6).

The phlorizin-binding measurements bear directly on the question of glucose-galactose carrier density. If it is assumed that binding is specific and that each carrier combines with only one phlorizin molecule, a density of 2.6×10^6 carriers/cell is calculated from the maximum binding capacity of the microvilli (Fig. 9) and the microvilli volume/cell. This volume was estimated as the brush border volume in light micrographs less 21% extracellular space (26). Since the total carrier/cell is known, the turnover number of sugar-carrier complex can be calculated from influx measurements. The initial influx of a single epithelial cell exposed to 1 mM galactose-^{3}H was estimated from radioautographs (26) as 3.0×10^{-17} moles/sec $\times$ cell. At concentrations which saturate the galactose accumulation process in rings of hamster intestine (5), the initial influx is about three times this value. Thus, the maximum turnover number (TN) is about twenty-one galactose molecules/carrier $\times$ sec. These values with estimates of total glucose carrier per cell and turnover numbers from the literature are presented in Table III. Estimates of cell membrane surface density, calculated from appropriate anatomical data, also have been included. If one considers the diverse tissues and methods of calculation, these values are remarkably consistent. More importantly, these estimates are consistent with the general concept of a membrane carrier; that is to say, neither turnover numbers nor surface densities are prohibitive. The maximum turnover number for a diffusible carrier depends on carrier size and membrane viscosity, neither of which is known; turnover number of a nondiffusing carrier is even a more open question. This problem has been discussed by Diedrich (7). If it is assumed that the carrier is a sphere with a maximum diameter of 100 A, the thickness of the plasma membrane, the

[1] According to current carrier concepts (27), substrate concentrations $\gg K_t$ saturate the carriers, producing a near maximum transport rate. If (phlorizin) medium $>$ (phlorizin) cell and both $\gg K_t$, both inward and outward transport would be saturated, yielding a net rate of entry close to zero in a system far from equilibrium. K_t is that sugar concentration which half-saturates the transport capacity of the system.

TABLE III

Estimates of Maximum Carrier Density and Turnover Number

Estimated as:	Tissue	Number of carriers per Cell	Number of carriers per μ^2 of cell surface	TN
Phlorizin-^{3}H content of microvilli at saturation (Fig. 7)	Hamster intestine	2.6×10^6	1,700*	21
Bound phlorizin at low blood levels (7)	Whole dog kidney	1×10^7	6,700*	25
p-Chloromercuribenzene sulfonate titratable $-$SH groups (25)	Human erythrocyte	1.4×10^6	11,400‡	180
Glucose-^{14}C retained by red cell ghosts (15)	Human erythrocyte	5×10^5	4,100‡	500

* Estimated from electron micrographs (10) as 2,000 microvilli/cell, each 2×0.12 μ^2.

‡ Approximated as surface of an oblate spheroid with 7.7- and 3-μ axes.

maximum fraction of cell surface occupied by the carrier would, according to the estimates of Table III, range from 13% for the intestine to 89% for the red cell.

Finally, it is of interest to ask whether or not one can expect to see radioautographically a membrane accumulation of sugar in the intact cell. According to the above estimates of carrier density and transport kinetics, the concentration of carrier-bound sugar would actually be less than that of the free sugar bathing the membrane because the half-saturation constants (K_t) are larger than the concentration of total carrier in the membrane.[2] Detection of membrane binding by intact cells is to be expected only with compounds whose half-saturation constants are considerably less than the carrier concentration, e.g. phlorizin in the intestine. The absence of brush border binding in galactose-^{3}H radioautographs (26) supports this conclusion. Recent suggestions of glucose binding to disrupted intestinal microvilli by Faust, Wu, and Faggard (9) and to fragments of red cell ghosts by Bobinski and Stein (4) are not inconsistent with this conclusion. In these studies, the addition of a large volume of cell membranes to solutions of glucose-^{3}H, sorbose-^{14}C (4) glucose-^{14}C, mannose-^{3}H (9) caused an alteration in the original distribution ratio, indicating binding of glucose. This procedure is a more sensitive index of binding than the present radioautographic method because the large membrane volume permits an accurate comparison between the free and bound sugar concentrations. A similar comparison in the intact cell would be insensitive because the cell membrane is a very small fraction of the total volume. When binding of a compound can be measured radioautographically, as demonstrated here with phlorizin-^{3}H, the results are more meaningful in that they represent the functioning cell.

[2] For galactose accumulation in rings of hamster intestine and glucose transport in the human erythrocyte, K_t are about 2 mM (5) and 9 mM (27), respectively. If the thickness of the plasma membrane is 100 A, the membrane concentration of carrier would range, according to the estimates of Table III, from 0.3 mM for intestinal microvilli to 1.8 mM for the red cell.

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