J. Membrane Biol. 19, 55–78 (1974)
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Galactose Fluxes Across Brush Border of Hamster Jejunal Epithelium: Effects of Mucosal Anaerobiosis

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Received 19 March 1974; revised 27 June 1974

Summary. We have determined unidirectional influxes by measuring rate of uptake between 0.5 and 1.0 min of incubation in 5 mM galactose. ^{3}H-inulin was used to correct for galactose in residual mucosal solution. Influx was greatly depressed by removing Na^+ from or adding 10^{-4} M phlorizin to the incubation medium. Influx was also greatly depressed by making the mucosal solution anaerobic during a 10-min preincubation period. The same severe inhibition of galactose influx by mucosal anaerobiosis also occurred under conditions in which the epithelial cells did not gain Na^+. After taking into account the effect of the mucosal unstirred water layer, influxes could be normalized to a concentration of 1.0 mM at the membrane surface. It was estimated that at 1.0 mM the phlorizin-sensitive component of galactose influx was reduced 93% by 10 min of mucosal anaerobiosis. Unidirectional effluxes were estimated by subtracting steady-state downhill mucosal-to-serosal flux from influx. The ease of efflux was apparently augmented 2.8-fold by mucosal anaerobiosis. After 10 min of mucosal anaerobiosis, there was no longer any directional preference for movement of galactose across the brush border and there was no uphill transport, in spite of the persistence of a large Na^+ gradient. These results provide strong evidence against the theory that ion gradients provide the major source of energy for directional preference and uphill transport.

It is well known that uphill transport of hexoses into intestinal absorptive cells across their brush borders can be stopped by interfering with oxidative metabolism. With *in vitro* preparations of hamster jejunum, removal of oxygen from the mucosal solution is an effective way to stop uphill transport of sugars; oxygen in the serosal solution is not necessary, nor is it sufficient by itself [3]. However, oxygen in the serosal solution is necessary

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and sufficient to support the basal transmural electrical potential and short-circuit current across hamster and rat jejunum [3]. Thus, there seem to be two pools of oxidative metabolic activity, one which can be supplied with oxygen from the mucosal side and another which can be supplied from the serosal side. Sugar transport can be abolished by arresting only that portion of total oxidative metabolism which is accessible to oxygen in the mucosal solution.

Mucosal anaerobiosis could have this effect if it resulted in (1) depression of influx into the absorptive cells across the brush borders, (2) enhancement of efflux from the cells across the brush borders, or (3) both depression of influx and enhancement of efflux. The main purpose of the present study was to test these alternatives.

The results have importance in evaluating the Na^+-gradient hypothesis according to which the energy used for uphill pumping of sugars across brush borders is derived from the Na^+ and K^+ concentration gradients across the plasma membrane of microvilli, rather than directly from the hydrolysis of ATP [21]. The Na^+-gradient hypothesis predicts that (1) sugar influx should not be greatly depressed by arrest of oxidative metabolism as long as the Na^+ concentration in the mucosal solution is unchanged [13], and (2) sugar efflux should be augmented by arrest of oxidative metabolism if, and only if, intracellular Na^+ activity increases.

Materials and Methods

Male golden hamsters, weighing between 95 and 145 g, were used. They were allowed food and water *ad libitum*. Each animal was anesthetized with ether, and a segment of upper jejunum was cut from the mesentery and immediately placed in oxygenated Krebs-Henseleit solution [17] at 37 °C (pH 7.4; 287 milliosmolar), in which it remained for nearly all of the subsequent preparations. The segment was well rinsed inside and out and everted with a stainless steel rod. The segment was then tied to the glass apparatus illustrated in Fig. 1, which is somewhat similar to apparatus described by Oh and Beck [18] and by Dietschy *et al.* [10]. Each segment was 8 cm long, and its upper end was originally about 2 cm from the duodenal-jejunal junction. The apparatus and jejunal segment were immersed to the top of the segment in a graduated cylinder or plastic test tube containing Krebs-Henseleit solution, and then filled with 3.4 ml of the same medium. The outside medium is called the mucosal solution and the inside medium is the serosal solution. Both solutions were continuously and vigorously gassed during subsequent incubations with humidified 5% CO_2 in either O_2 or N_2. The inside solution circulated because of the bubble lift effect. The interval between removal of segment from animal and insertion into outside incubation medium was about 2 to 3 min. The entire preparation and subsequent incubations were done in an environmental room, maintaining the gut at 37 °C. Three types of experiments were performed: (1) transmural transport studies, (2) uptake studies, and (3) measurements of streaming potentials.

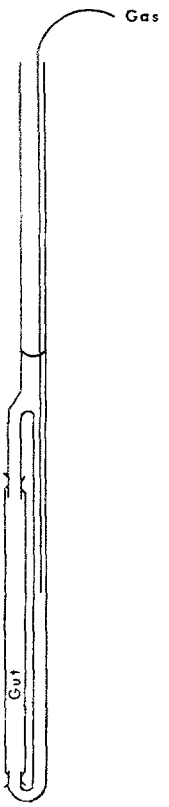

Fig. 1. Glass apparatus with everted jejunal segment attached. Distance between cannula tips is 8.0 cm. The level of the inside solution fluctuates widely with intestinal contractions. Gas is bubbled in through a polyethylene tube

Transmural Transport Studies

The everted jejunum was incubated for 60 min, while gassing both sides with 5% CO_2 in either O_2 or N_2. At zero time the mucosal solution contained 5.0 mM D-galactose with a trace of D-galactose-1-^{14}C (New England Nuclear), and its volume was 12.0 ml for uphill transport studies and 30.0 ml for downhill transport studies. Sometimes the serosal solution was initially identical to the mucosal solution, but sometimes it was galactose-free. The mucosal and serosal solutions were sampled (20 μliters) every 15 min and the samples dissolved in Aquasol (New England Nuclear) and counted in a liquid scintillation counter. Counts were converted to amounts of galactose by dividing by the specific activity. The final volumes were determined gravimetrically. Any change in volume was assumed to have occurred uniformly throughout the incubation period.

At the end of each experiment the segment was cut from the apparatus, slit open, and quickly rinsed in ice-cold 5.07% mannitol solution. It was then blotted on filter paper and laid on a glass plate resting on ice. The mucosa was scraped off with a glass microscope slide and placed in a tared centrifuge tube. After weighing, 3.0 ml of 70% ethanol were added to the tube, and extraction was allowed to proceed for at least 18 hr with occasional vigorous agitation. The tube was centrifuged, and 50 μliter samples of

the supernatant were added to vials containing Aquasol and counted. Tissue dry weight/wet weight ratios were determined in preliminary experiments by drying mucosa to constant weight at 110 °C. Thin-layer chromatograms of samples of extracts and final serosal and mucosal solutions revealed no evidence for radioactivity in anything but galactose.

Uptake Studies

Each segment was everted and incubated for 10 min with Krebs-Henseleit solution on both sides. The volume of the mucosal solution was 15.0 ml. The serosal solution was always gassed with 5% CO_2 in O_2, but N_2 sometimes replaced O_2 for gassing the mucosal solution. Following this 10-min "preincubation," the apparatus and intestinal segment were quickly transferred to a second mucosal solution (15.0 ml) containing 5.0 mM D-galactose with traces of D-galactose-1-^{14}C, and inulin-methoxy-^{3}H (both obtained from New England Nuclear). Mucosal and serosal solutions were gassed with the same gases used during preincubation. Incubation continued for various periods up to 5 min, after which the apparatus and intestinal segment were quickly plunged into two changes of ice-cold 5.07% mannitol solution to remove adhering mucosal solution. The segment was cut from the apparatus, immediately blotted on filter paper, and placed in a tared centrifuge tube. Extraction and sampling were as described above. The counts attributable to ^{14}C and to ^{3}H were determined in a liquid scintillation counter. The ^{3}H counts were used to correct the total ^{14}C counts for that portion present in residual mucosal solution, assuming that inulin did not enter the epithelium. Thus, the amount of galactose which had entered the epithelium across the brush border was estimated. This quantity was divided by the wet weight of the tissue to yield a value referred to as "uptake." In a number of experiments the final serosal solution was collected and sampled; no ^{14}C-labeled galactose was detected in it after uptake periods of 2 min or less. On many occasions the tissue was extracted for a few days instead of the usual 18 hr with no change in results. Thus, an 18-hr extraction was sufficient.

Thin-layer chromatograms of samples of the extracts were prepared in a few experiments performed without inulin; these were scanned for radioactivity. Only one spot was found with an R_f corresponding to that found with the original sample of ^{14}C-labeled galactose.

Measurement of Streaming Potentials

Each segment was incubated for 10 min with oxygenated Krebs-Henseleit solution on both sides. Then the segment was quickly transferred to a new mucosal solution (15.0 ml) which contained 100 mM-D-mannitol in addition to the usual inorganic constituents. Transmural electrical potential difference (p.d.) was continuously measured essentially as described in a previous publication [4]. After the streaming potential was fully developed and transmural p.d. was stable, the segment was transferred back to Krebs-Henseleit solution and the decay of the streaming potential was followed. The object of these experiments was to estimate the effective thickness of the unstirred layer covering the mucosal surface, using the approach described by Dainty and House [8] and Diamond [9].

In this paper, in the text and in the tables, the measure of variability presented is the standard error of the mean.

Results

Effect of Mucosal or Serosal Anaerobiosis on Transmural Transport of Galactose

The ability of this preparation of hamster jejunum to transport galactose uphill is illustrated in Fig. 2. In this experiment galactose was initially present at approximately equal concentrations (5.0 mM) on both sides. At the end of 1 hr with mucosal and serosal solutions both oxygenated, 21.6 μmoles of galactose had been transported into the serosal solution and a serosal/mucosal concentration ratio of 8.7 developed. Table 1 shows the effect of mucosal or serosal anaerobiosis on uphill transport of galactose. Mucosal anaerobiosis completely blocked uphill transport, but serosal anaerobiosis had no effect.

Mucosal-to-serosal (M→ S) movement of galactose into an initially galactose-free serosal solution is shown in Fig. 3. A steady rate of transport was reached within 15 min and remained essentially unchanged until at least 45 min. No effect of serosal anaerobiosis on M→ S flux was detected, but mucosal anaerobiosis, within about 20 min, reduced M→ S flux to a level which was only 7.9% of that obtained with mucosal O_2. The average

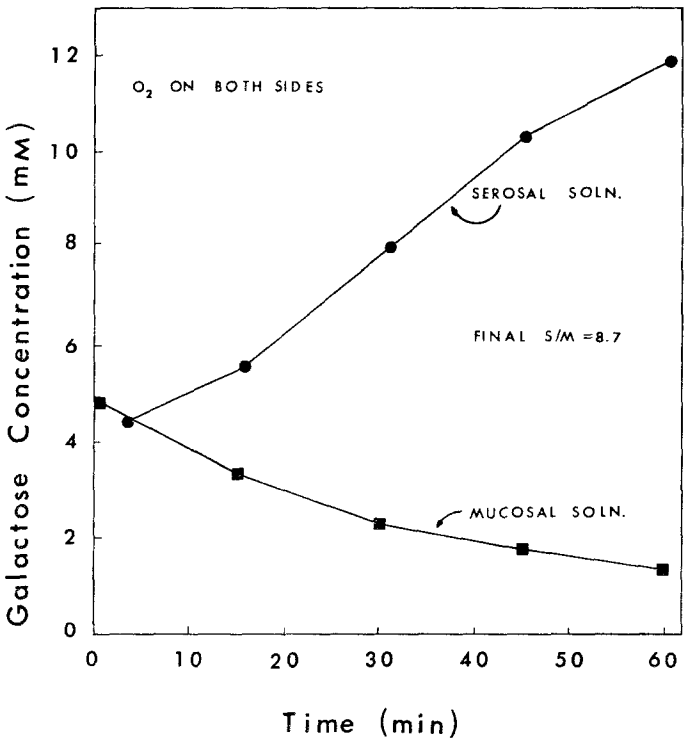

Fig. 2. A sample experiment showing the ability of this preparation of hamster jejunum to perform uphill transport of galactose

Table 1. Effect of mucosal or serosal anaerobiosis on uphill transport of galactose across everted hamster jejunum

Anaerobiosis	Galactose transported[a] (μmoles/hr)
None	21.9±4.02 (3)
Serosal	20.8±2.45 (3)
Mucosal	− 0.6±0.51 (3)

[a] This is the amount of galactose found in the serosal solution at the end of 1 hr, minus the amount originally present. Results are expressed as mean ± standard error of mean. Number of animals is in parentheses.

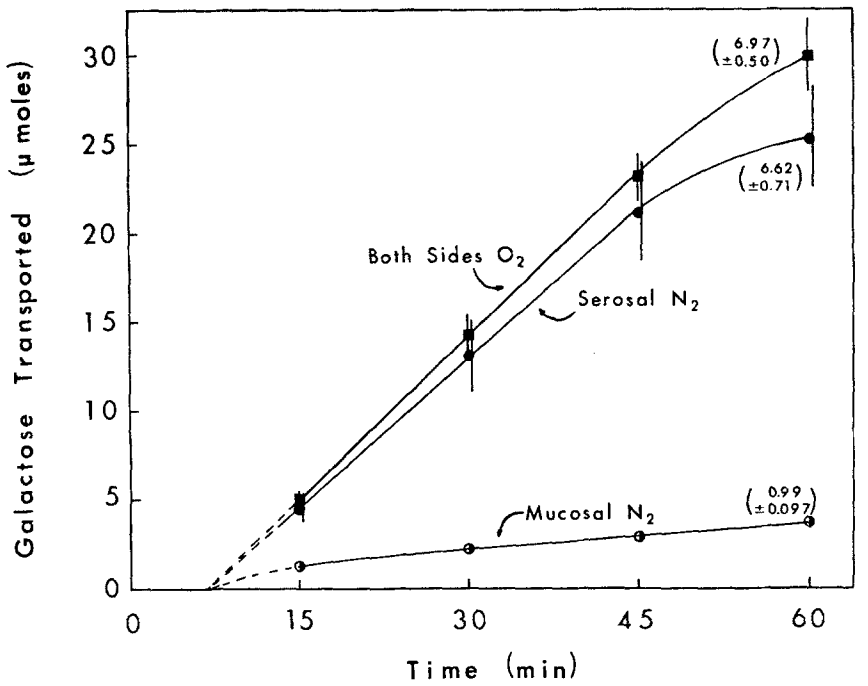

Fig. 3. Galactose transported into the serosal solution as a function of time. At zero time there was no galactose in serosal solution and a concentration of 5.0 mM in mucosal solution. Mucosal or serosal anaerobiosis were produced by gassing with 5% CO_2 in N_2 instead of O_2. The figures in parentheses are the mean final galactose concentration ratios between mucosal tissue water and mucosal solution. The vertical lines represent the standard error of the mean; for the data with mucosal N_2 these lines are not shown because they were smaller than the symbols used to represent the means. The number of animals was seven with O_2 on both sides, five with mucosal N_2, and four with serosal N_2. The dashed lines represent extrapolation to a lag-time of 6.7 min

concentration ratio between the mucosal tissue water at the end of 60 min and the final mucosal solution is also indicated in Fig. 3. Mucosal anaerobiosis prevented uphill accumulation of galactose in the tissue.

We tried to determine if mucosal anaerobiosis has the above effects by inhibiting galactose influx across the apical surface of absorptive cells; or by accelerating efflux from the cells back into the mucosal solution, thus nullifying the effectiveness of influx; or by both of the above.

Galactose Uptake

The Correction for Galactose in Residual Mucosal Solution. In many previous investigations of unidirectional influx of sugars, amino acids, etc., an attempt was made to correct total uptake for the amount present in residual mucosal solution, the volume of which was estimated from the content of labeled inulin in the tissue extracts (e.g., *see* Refs. [13, 14, 22]). We have taken the same approach. However, to validate this correction, it would be necessary to demonstrate that during the time periods used for uptake the inulin space remained constant. The data on residual mucosal solution are presented in Table 2. It is evident that uptake of inulin was not complete even by 2.0 min. This result was expected because of the presence of a mucosal unstirred water layer. Sallee *et al.* [20] have discussed this problem. They found it took about 3 min for ^{14}C-inulin to equilibrate with residual mucosal solution. Consequently, the volume of residual mucosal solution is underestimated unless the uptake period is at least 3 min. We have found that at 1.0 min the estimated volume is only about 55% of the true volume (assuming the value found at 5.0 min to be the true volume). Unfortunately, as shown below, unidirectional influx cannot be measured with uptake periods much beyond 1.0 min (except when uptake is inhibited in some way) because of gradually increasing efflux. Thus, there is a perplexing situation. With uptake periods of a minute or less there is a large error in the correction for extracellular galactose; with uptake periods exceeding 1 min, uptake no longer measures unidirectional influx. The correction terms cannot simply be adjusted by using theoretical values for

Table 2. Volume of residual mucosal solution remaining after rinsing and blotting mucosal surface: Estimated from the amount of ^{3}H-labeled inulin in jejunal extracts

Uptake period (min)	Residual mucosal solution (μliter/g wet weight)
0.5	21.9±2.8 (6)
1.0	24.8±1.8 (35)
2.0	32.1±3.0 (23)
5.0	44.8±4.8 (12)

percentage inulin equilibration because the variation in residual mucosal solution among individual experiments is quite large. Fortunately, however, the problem is not too serious. During uninhibited galactose uptake the correction for galactose in the residual mucosal solution was a very small fraction of total galactose in the extract. For example, with an uptake period of 1.0 min the estimated amount of galactose in residual mucosal solution averaged only about 3.5% of the total. The average correction should really have been about 6.4% of the total; thus the error is unimportant and has been neglected in this paper. The correction for galactose in residual mucosal solution becomes a much larger fraction of total galactose in the extract when uptake is inhibited in some way; but, fortunately, when uptake is inhibited, longer uptake periods can be used, giving ^{3}H-inulin a chance more nearly to equilibrate with the unstirred layer. For example, with mucosal anaerobiosis and an uptake period of 2 min, estimated galactose in residual mucosal solution averaged about 9% of the total. At 2 min ^{3}H-inulin was about 72% equilibrated with residual mucosal solution; so the proper correction should have averaged about 12.5% of the total. Again, no large error is incurred even though ^{3}H-inulin has not completely equilibrated.

Effect of Mucosal Anaerobiosis on Unidirectional Influx. The results of uptake experiments are shown in Fig. 4. These data have all been corrected for galactose in residual mucosal solution using the actual values for ^{3}H-inulin uptake, accepting the small errors discussed in the previous paragraph. With mucosal O_2, galactose uptake into the epithelium was not a linear function of time over the entire 2-min period examined. We believe that unidirectional influx into the epithelium is best given by the maximum slope of the curve. Several seconds were required for maximum slope to be attained; this period probably reflects the time required for galactose to diffuse through the unstirred layer outside the absorptive cells (*see* discussion of this point in Ref. [20]). After 1 min the slope decreased. We think this decrease was caused mainly by the development of appreciable efflux from the epithelium back into the mucosal solution; movement into the serosal solution could also contribute, as suggested by Sallee *et al.* [20], but was never detected in these experiments within the 2-min time period. After 2 min of uptake the concentration of galactose in tissue water averaged 4.1 mM.

It would appear from Fig. 4 that uptake from 0.5 min to 1.0 min can be used to estimate the maximum slope and, therefore, unidirectional influx. Over this period, influx was 2.24 μmoles/min/g when both mucosal

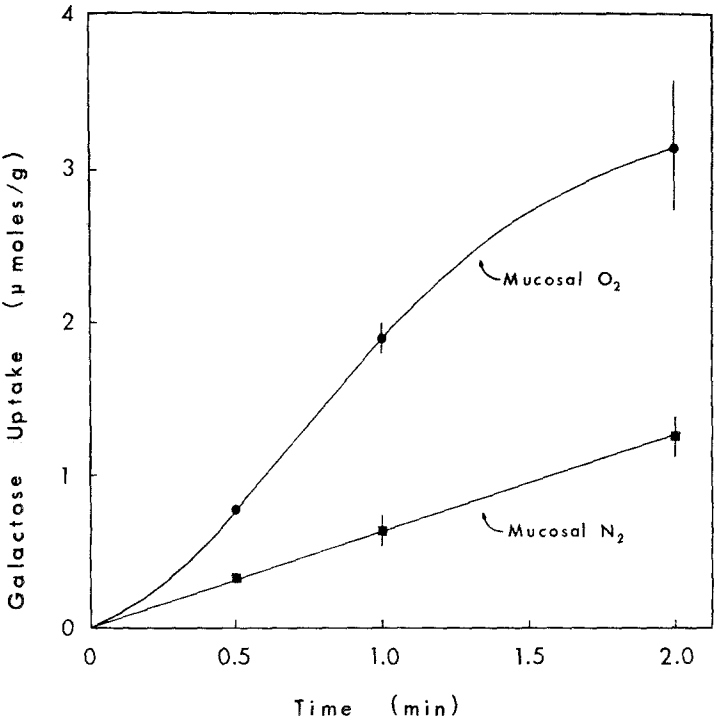

Fig. 4. Uptake of galactose by everted jejunum as a function of time. Uptake values have been corrected for galactose in the mucosal ^{3}H-inulin space (*see text*) and are expressed as μmoles per g wet weight of intestine. Each point represents the mean from 4 experiments (except the 1-min data with mucosal O_2 for which there were 6 experiments). Standard errors are indicated by vertical lines except when obscured by the symbols

and serosal solutions were oxygenated. When the mucosal solution was gassed with N_2 instead of O_2 (during the 10-min preincubation period and the uptake period), uptake was a linear function of time for at least 2 min, and influx was reduced to 0.63 μmoles/min/g, a 72% reduction. Thus, most of the influx measured in these experiments was dependent upon oxygen in the mucosal solution.

Table 3 shows uptake values for experiments in which the usual 10-min preincubation was extended to 20 min in order to test for reversibility of the inhibition and to determine if a greater inhibition occurs when anaerobiosis is prolonged. For comparison, uptake values following the usual 10-min preincubation are also shown. If the mucosal solution was gassed with N_2 for 10 min and then with O_2 for an additional 10 min, about 50% of the oxygen-sensitive uptake was restored. Mucosal anaerobiosis for 20 min

Table 3. Galactose uptake—effects of various conditions

Na^+ Concentration in mucosal solution (mEquiv/liter)		Uptake period (min)	Phlorizin in mucosal solution	Mucosal gassing during preincubation and uptake periods	Galactose uptake (μmoles/min/g)
Pre-incubation period	Uptake period				
143	143	1	—	O_2	1.90 ±0.106 (6)
143	143	2	—	N_2	0.63 ±0.065 (4)
143	143	1	—	O_2[a]	1.72 ±0.139 (4)
143	143	1	—	N_2[a]	0.51 ±0.066 (6)
143	143	1	—	$N_2 \rightarrow O_2$[b]	1.19 ±0.076 (4)
25	143	1	—	O_2	1.49 ±0.118 (6)
25	143	1	—	N_2	0.52 ±0.039 (6)
143	0	2	—	O_2	0.29 ±0.016 (6)
143	0	2	—	N_2	0.14 ±0.022 (6)
143	143	5	10^{-4} M	O_2	0.044±0.006 (6)
143	143	5	10^{-4} M	N_2	0.067±0.010 (6)

[a] 20-min preincubation period instead of usual 10 min.
[b] 10-min preincubation with mucosal N_2 followed by another 10 min with mucosal O_2; uptake period with O_2.

resulted in a somewhat greater inhibition of unidirectional influx than did anaerobiosis for 10 min, but the difference was not statistically significant.

Is the Effect of Mucosal Anaerobiosis Mediated by a Rise in Intracellular Na^+ Concentration? In the above experiments, preincubation and uptake both took place in a medium containing 143 mEquiv/liter Na^+, and it is likely that during the 10-min preincubation period intracellular Na^+ concentration rises more when the mucosal solution is gassed with N_2 than when it is gassed with O_2. As pointed out by Goldner *et al.* [13], inhibiting metabolism might influence sugar influx indirectly via an elevated intracellular Na^+ concentration, rather than by directly affecting the influx mechanism.

Consequently, we carried out another series of uptake experiments in which preincubation was done in a mucosal solution containing only 25 mEquiv/liter Na^+. Choline chloride was used to replace all the NaCl; the osmolarity was adjusted to 287 milliosmolar (measured by freezing-point depression) by adding choline chloride. The serosal solution was normal Krebs-Henseleit solution. For the uptake period the regular incubation medium was used; this contained 143 mEquiv/liter Na^+. The uptake period was 1 min for all these experiments. During mucosal oxygenation the 1-min uptake value alone should be an underestimate of true influx (*see* Fig. 4).

The results are shown in Table 3. When the mucosal solution was oxygenated, preincubation in the low Na^+ medium resulted in a statistically significant depression of galactose uptake. Some inhibition should have been expected because of the fact that following preincubation in a medium containing only 25 mEquiv/liter Na^+, the Na^+ concentration at the outer surface of the epithelium would rise only gradually during the early portion of the uptake period because of the presence of an unstirred layer of mucosal solution covering the epithelium. We do not believe that this inhibition provides evidence for the hypothesis that intracellular Na^+ influences sugar transport [15]. This effect of preincubating in a choline medium was also found by Goldner *et al.* [14]. They found a 19% reduction in 3-O-methylglucose influx into rabbit epithelium; we found a 22% reduction in galactose uptake. (In a later paper [13] they reported no effect on 3-O-methylglucose influx). The important point is that mucosal anaerobiosis still drastically reduced influx even after preincubating in a medium with a low Na^+ concentration. Mucosal anaerobiosis for 10 min caused a 65% reduction in 1-min uptake following preincubation in the low-Na^+ medium, and a 67% reduction following preincubation in the regular medium.

A separate set of experiments was done to determine the amounts of Na^+ and K^+ in the mucosa following incubation for 10 min with either 25 or 143 mEquiv/liter Na^+ in the mucosal solution. The serosal solution always contained 143 mEquiv/liter Na^+ and was always oxygenated. In these experiments, following incubation, the segment was slit open, rinsed, blotted, and the mucosa scraped off as described above for the studies on transmural transport. Extraction was in 0.1 N HNO_3 instead of 70% ethanol. Concentrations of Na^+ and K^+ in the extract were determined by atomic absorption spectrophotometry. The extracted segment plus the extract were then dried to constant weight so that the water content of the segment could be determined. To estimate intracellular Na^+ and K^+, total tissue content must be corrected for that in the residual mucosal solution and in the serosal extracellular space. The volume of the residual mucosal solution was taken to be 4.5% of the wet weight of the entire segment (mucosal and muscle layers combined), a value derived from the ^3H-inulin data of Table 2. The concentrations in residual mucosal solution were assumed to equal those in the mucosal solution. The volume of the serosal extracellular space could not be measured with any accuracy in 10-min incubations, but we can be reasonably sure from various reports in the literature (e.g., Ref [11]) that it is not more than 20% or less than 10% of the total mucosal water; a value of 15% was selected in order to obtain the estimates shown in Table 4. The concentrations of Na^+ and K^+ in serosal extracellular space were assumed

Table 4. Estimated intracellular Na^+ and K^+ concentrations and water content in jejunal mucosa following incubation for 10 minutes

Na^+ concentration in mucosal solution during incubation (mEquiv/liter)	Gassing	Water content (% of wet weight)	Estimated intracellular concentration after incubation (mEquiv/liter)	
			Na^+	K^+
143	O_2	84.7±0.5 (n=5)	45.2±3.0 (n=5)	109.7±2.9 (n=5)
143	N_2	85.6±0.3 (n=6)	56.4±5.6 (n=5)	90.8±3.4 (n=6)
25	N_2	85.1±1.1 (n=6)	40.6±2.3 (n=6)	94.8±7.1 (n=6)

to equal those in the serosal solution. As anticipated, the data in Table 4 show that intracellular Na^+ concentration was somewhat higher after the mucosal solution was gassed with N_2 than after it was gassed with O_2; but the difference was not very great and would seem to be an unlikely cause of the very marked difference in galactose influx. Reducing the Na^+ concentration in the mucosal solution to 25 mEquiv/liter prevented the rise in intracellular Na^+ concentration brought about by mucosal anaerobiosis.

We realize that the concentrations shown in Table 4 probably do not accurately represent the concentrations at the inner surface of the brush border membrane because of unequal distribution in various cell types and compartmentalization within cells. Furthermore, the chemical activities are certainly much lower than the concentrations. These data merely show the relative overall concentrations. It seems likely, but remains unproved, that if the overall Na^+ concentration does not rise (during mucosal anaerobiosis in 25 mEquiv/liter Na^+), then neither does the pertinent Na^+ activity in the apical cytoplasm.

Effect of Phlorizin and of a Na^+-free Mucosal Solution. We needed to make sure that the influx being measured was via the well known Na^+-dependent phlorizin-sensitive transport pathway into the absorptive cells. To this end, uptake was measured from a Na^+-free mucosal solution and from a mucosal solution containing 10^{-4} M phlorizin. In both types of experiments preincubation was in regular Krebs-Henseleit solution. The Na^+-free medium was prepared by substituting choline chloride for NaCl and $KHCO_3$ for $NaHCO_3$. A quick rinse of the mucosal surface in Na^+-free medium was performed between the preincubation and uptake periods

whenever uptake was to occur in the Na^+-free medium. In the phlorizin experiments the mucosal solution contained the usual concentration of Na^+ (143 mEquiv/liter). The serosal solution was always regular Krebs-Henseleit solution and was always oxygenated. As shown in Table 3, uptake was severely depressed by the absence of Na^+ or the presence of phlorizin in the mucosal solution; phlorizin was somewhat more effective than the Na^+-free condition. When influx was measured from the Na^+-free medium, mucosal anaerobiosis still had an inhibitory effect. Also note that there was still a substantial Na^+-dependence during mucosal anaerobiosis; a finding which confirms that of Bihler *et al.* [6]. In the presence of 10^{-4} M phlorizin, mucosal anaerobiosis did not inhibit influx. In fact, there was a small increase ($p = 0.077$ by two-tailed t test) in galactose influx brought about by mucosal N_2 in the presence of phlorizin. We can conclude that the effect of mucosal anaerobiosis is on the phlorizin-sensitive transport pathway. Most of the uptake was still blocked by phlorizin even with mucosal anaerobiosis; this finding is important in interpreting subsequent data on efflux because it shows that anaerobiosis did not simply destroy the membranes.

It is hard to tell if the observed effects of phlorizin, of the Na^+-free medium, and of mucosal anaerobiosis were maximal. A higher concentration of phlorizin or a longer period of mucosal anaerobiosis might have resulted in even more inhibition than shown in Table 3. It is very likely that when the bulk mucosal medium was initially Na^+-free, uptake was not actually occurring from a Na^+-free solution, since the unstirred layer outside the absorptive cells probably contained some Na^+ that had not been rinsed off or that had leaked from the cells. This consideration may explain why inhibition was not as complete with the Na^+-free medium as it was with phlorizin, and why some inhibition by mucosal N_2 took place even in the Na^+-free medium. In spite of these uncertainties it is clear that mucosal anaerobiosis blocked a very substantial portion of the Na^+-sensitive and phlorizin-sensitive component of influx.

Effect of Preloading the Epithelium with Galactose. We wanted to know if preloading the epithelium with galactose would influence galactose influx. A series of experiments was done in which unlabeled galactose (5 mM) was present in the mucosal Krebs-Henseleit solution during the 10-min preincubation period. Preliminary experiments demonstrated that under these conditions an accumulation of galactose in mucosal tissue water occurred to a concentration of about 20 mM by 10 min, and that longer incubation (up to 1 hr) resulted in little or no further increase in tissue accumulation. Uptake of labeled galactose into preloaded epithelium averaged 1.62 ± 0.144

μmoles/min/g in six experiments. This value is slightly lower than the 1.90 ± 0.106 μmoles/min/g obtained in six experiments without preloading; however, the probability of the null hypothesis is 0.15 (by two-tailed t test). Thus, we cannot conclude that there was an effect of preloading. Furthermore, a small apparent inhibition of influx might be anticipated because of dilution of labeled galactose in the unstirred layer by unlabeled galactose leaking from the cells. (In fact, this latter problem would severely complicate the interpretation of results even if a large effect had been observed.)

Influence of the Mucosal Unstirred Layer on Galactose Influx. Influx of galactose into the absorptive cells involves movement across two barriers in series: the unstirred layer of fluid covering the mucosal surface, and the plasma membrane of the brush border. The more easily a substance penetrates the plasma membrane, the more important is the contribution of the unstirred layer to toal resistance against influx [23]. Therefore, it is possible that in the presence of mucosal O_2 the unstirred layer has a relatively more important contribution than it does during mucosal anaerobiosis. If so, the effect of mucosal anaerobiosis on the ease with which galactose can penetrate the plasma membrane might be underestimated by considering only the data in the previous section.

To evaluate the role of the unstirred layer, we attempted to measure its thickness. The approach described by Dainty and House [8] and by Diamond [9] was employed. They showed that

$$l = \sqrt{\frac{T_{\frac{1}{2}} D}{0.38}} \tag{1}$$

where l is the thickness of the unstirred layer, D is the diffusion coefficient of a test substance within the unstirred layer, and $T_{\frac{1}{2}}$ is the time required for development of one-half the streaming potential that occurs when one surface of the tissue is suddenly exposed to the test substance. It is assumed that the test substance cannot penetrate the membrane and that the magnitude of the streaming potential that it induces across the membrane is directly proportional to its concentration at the outer surface of the membrane. If these assumptions are correct, then l can be estimated equally well from the $T_{\frac{1}{2}}$ for build-up of a streaming potential and from $T_{\frac{1}{2}}$ for decay of a streaming potential after the tissue is again exposed to the same osmolarity on both sides. Derivation of the above equation assumes the membrane is a plane surface; this, of course, is not true with intestine. The villi and microvilli present anything but a plane surface to the mucosal unstirred layer. The values calculated, therefore, must be regarded as "effective"

Table 5. Transmural streaming potentials induced by mucosal mannitol (100 mM), and effective thickness of the mucosal unstirred layer

Mucosal gassing	Δp.d. (mV)		$T_{\frac{1}{2}}$ (sec)		l (μm)		
	Build-up	Decay	Build-up	Decay	Build-up	Decay	Mean
O_2 (n= 7)	3.8 ±0.28	3.9 ±0.38	17.9 ± 3.31	17.6 ± 2,52	208	206	207
N_2 (n= 6)	4.0 ±0.31	4.0 ±0.37	14.1 ± 1.50	22.8 ± 2.54	184	234	211

thicknesses. The value used for D was 9.15×10^{-6} cm^2/sec. This value was obtained by correcting to 37 °C the Handbook value of D for mannitol at 25 °C.

With Krebs-Henseleit solution on both sides, rather stable transmural p.d.'s were obtained of about 3 to 4 mV (serosal side positive). Upon transfer to a mucosal solution containing 100 mM mannitol, the p.d. gradually decreased and sometimes reversed. A new stable value was usually reached in about 1 min. The change in p.d. (Δp.d.) is assumed to be a streaming potential. Upon transfer to a mannitol-free mucosal solution again, p.d. gradually returned to about its previous value. The results are shown in Table 5. First note that Δp.d. was the same during mucosal anaerobiosis as it was during mucosal oxygenation. It is comforting to conclude from this observation that mucosal anaerobiosis does not destroy the integrity of the membrane; otherwise, the reflection coefficient toward mannitol and, consequently, Δp.d. would probably be decreased.

Also note that with mucosal O_2 the $T_{\frac{1}{2}}$ for build-up was about the same as that for decay, as it should be if Δp.d. is a linear function of mannitol concentration at the surface of the membrane. However, with mucosal anaerobiosis, $T_{\frac{1}{2}}$ was greater during decay than during build-up. We offer no explanation for this discrepancy. If the $T_{\frac{1}{2}}$ values for build-up and decay are averaged and effective thickness (l) calculated from this average, l is about the same with either O_2 or N_2, about 209 μm.

An independent estimation of apparent unstirred layer thickness can be obtained from the data on galactose uptake shown in Fig. 4. With mucosal O_2 there was a delay in development of steady influx, presumably owing to diffusion of galactose through the unstirred layer. In 1953 Barrer introduced the "time-lag" method to study diffusion [5], by which it is possible to estimate the path length (in this case the thickness of the unstirred layer) from the time-lag. The time-lag is defined as the intercept on the time axis made by the straight line relating amount diffused to time during steady

flux. Barrer showed that

$$l = \sqrt{6DL} \tag{2}$$

where L is the time-lag. In the present experiments L was 9.2 sec, and consequently $l = 223$ μm (assuming 9.01×10^{-6} cm^2/sec for D). This value for l is quite close to that estimated from streaming potentials.

A value of around 200 μm for l seems reasonable and is within the range found by Westergaard and Dietschy [25] in rabbit jejunum. Diamond [9] and Smulders and Wright [24] found unstirred layer thicknesses of about 100 μm for the mucosal surface of rabbit gallbladder, which is a smoother surface and consequently should have a thinner unstirred layer than intestinal mucosa.

Net flux through the unstirred layer (J_L) would be expected to obey the following relationship

$$J_L = \frac{AD}{l}(C_1 - C_2) \tag{3}$$

where A is the surface area of the unstirred layer in cm^2/g wet weight of intestine, C_1 is the concentration of galactose in the bulk mucosal solution, and C_2 is the average concentration at the outer surface of the plasma membrane. We assumed l to be 210 μm and D to be 9.01×10^{-6} cm^2/sec. We determined A in eight segments (still mounted to the apparatus and immersed in mucosal solution) by measuring diameter from villous tip to villous tip with a micrometer ($A = 26.8$ cm^2/g). In the presence of mucosal O_2 galactose influx was 2.24 μmoles/min/g, and assuming a quasi-steady state, this equals J_L. From the above information it can be estimated that during galactose influx with mucosal O_2 and a concentration of 5.0 mM in the bulk mucosal solution, C_2 was only 1.75 mM. A similar calculation shows that during mucosal anaerobiosis $C_2 = 4.09$ mM. Assuming that during mucosal anaerobiosis influx across the plasma membrane is a linear function of C_2 (up to 4.09 mM), influx would have been only 0.27 μmoles/min/g if C_2 had been 1.75 mM. Thus, if C_2 had been 1.75 mM, mucosal anaerobiosis would have reduced influx by 88%. If only the phlorizin-sensitive component of influx were considered, a slightly greater effect of N_2 would be noted.

Effect of Mucosal Anaerobiosis on Galactose Efflux

The next major question concerns efflux: Is efflux (back out of the cells across the brush border) influenced by oxidative metabolism? This question

is more difficult than the one about influx. Probably the best way to estimate efflux is to subtract steady-state, downhill, transmural, M→ S transport from unidirectional influx. In the steady-state the remainder is a good estimate of efflux as the following argument shows. The notation used by Schultz *et al.* [22] will be employed. During steady-state transmural, M→ S transport

$$J_{ms}-J_{sm}=J_{mc}-J_{cm}=J_{cs}-J_{sc} \tag{4}$$

where J signifies a unidirectional flux and the subscripts represent the mucosal solution (m), epithelial cells (c) and serosal solution (s); the donor and recipient compartments are represented, respectively, from left to right in the subscript. We can assume that in our experiments on downhill M → S transport (*see* previous section), J_{sm} was negligible in comparison to J_{ms}, at least during the earliest period of steady flux, because initially there was no galactose on the serosal side; and even when the serosal solution contains 5 mM galactose we have found (*unpublished*) that steady-state S→ M flux is only 5.0% of the rate of M→ S transport. Therefore,

$$J_{cm}=J_{mc}-J_{ms}. \tag{5}$$

Table 6 shows the best estimates of these fluxes that we can make with the present data. The values for J_{ms} were calculated from the 15 to 45 min data in the same experiments depicted in Fig. 3. We have no direct measure-

Table 6. Effect of mucosal anaerobiosis on unidirectional galactose fluxes during steady M→ S transport

		Mucosal gassing		
		O_2	N_2	N_2/O_2
Fluxes (μmoles/min/g)	J_{mc}	2.43[a]	0.73	0.30
	J_{ms}	1.18	0.10	0.08
	J_{cm}	1.25	0.63	0.50
Estimated galactose concentration at donor surface of membrane (mM)	Outside	2.69	4.76	1.77
	Inside	24.00	4.42	0.18
Estimated Flux from a concentration of 1.0 mM (μmoles/min/g)	$J_{mc}^{1.0}$	1.92[a]	0.153	0.08
	$J_{cm}^{1.0}$	0.052	0.143	2.8
Directional preference at 1.0 mM	$\frac{J_{mc}^{1.0}}{J_{cm}^{1.0}}$	36.9	1.07	

[a] Calculated assuming $J=\frac{2.88\,C_2}{0.5+C_2}$.

ment of J_{mc} during steady-state M→ S transport; in fact, it is not possible to make this measurement because the specific activity in the unstirred layer would be unknown. Since apparent unidirectional influx of galactose was not influenced much by preloading the tissue with galactose (*see above*), it might be assumed that J_{mc} during steady-state M→ S transport equals J_{mc} during maximal uptake. Thus, the first inclination is to use 2.24 and 0.63 μmoles/min/g for J_{mc} with mucosal O_2 and N_2, respectively. However, this assumption would be wrong; J_{mc} should be greater during steady-state M→ S transport than during maximal uptake because the concentration of galactose at the outer surface of the membrane should be greater. Efflux from the loaded cells into the unstirred layer would be expected to elevate the concentration there above that present when the cells are unloaded.

We can estimate this concentration in the same way that it was estimated for the influx experiments, simply by equating J_L in Eq. (3) with J_{ms}. By this calculation, at 15 min with mucosal O_2, $C_2 = 2.69$ mM; and at 15 min with mucosal N_2, $C_2 = 4.76$ mM. We have preliminary data on the kinetic constants for galactose uptake from the unstirred layer. These data are not being presented in detail at this time, but they have been used, along with the above values for C_2, to calculate the J_{mc} values shown in Table 6; these should be regarded only as approximations, but are at least better than the J_{mc} values measured during maximal uptake.

The right-hand column of Table 6 shows that J_{cm} was depressed 50% by mucosal N_2. However, in the M→ S transport experiments the final concentration of galactose in mucosal tissue water was 24.0 ± 1.41 mM with mucosal O_2 and only 4.42 ± 0.33 mM with mucosal N_2, and J_{cm} is expected to be some function of tissue concentration. If J_{cm} is divided by tissue concentration, we arrive at a quantity which represents the ease of efflux provided that efflux is a linear function of tissue concentration. This calculation shows that efflux appears to be 2.8 times easier with mucosal N_2 than with mucosal O_2.

Normalized Fluxes and The Directional Preference for Galactose Movement Across the Brush Border Membrane

To compare the ease of membrane penetration in the two directions, the four unidirectional transmembrane fluxes presented in Table 6 must be normalized to account for the fact that the concentrations at the donor surfaces were different in all four cases. To this end, a quantity called $J^{1.0}$ is presented in Table 6. $J^{1.0}$ is the estimated flux that would have occurred from a concentration of 1.0 mM. With mucosal O_2, $J_{mc}^{1.0}$ was calculated

using the hyperbolic relationship shown in the footnote to Table 6. (At the present time the constants shown have not been accurately determined but are close enough for this purpose.) The other three $J^{1.0}$ values were calculated merely by dividing J by the appropriate concentration shown in Table 6. The inside concentration was assumed to equal the concentration in total mucosal tissue water. This may be a slight underestimation of the concentration at the inner surface of the membrane but cannot be far off. The assumption of linearity between J_{cm} and inside concentration may not be valid, but there is no better information available.

In any event, we estimate that if the concentration were 1.0 mM on both sides of the brush border membrane, galactose would move in about 37 times faster than it would move out (when mucosal O_2 is present). Mucosal anaerobiosis essentially abolishes this directional preference by decreasing the ease of influx and increasing the ease of efflux. It should also be noted that at 1.0 mM about 92% of the influx is dependent upon mucosal O_2, according to the approximations given in Table 6.

The $J^{1.0}$ data in Table 6 can easily be corrected to exclude the phlorizin-insensitive component if we assume that $J_{mc}^{1.0} = J_{cm}^{1.0}$ for the phlorizin-insensitive pathway (i.e., assume no directional preference for this pathway). We estimate that phlorizin-sensitive $J_{mc}^{1.0}$ was reduced 93% and $J_{cm}^{1.0}$ was increased 3.0-fold by mucosal anaerobiosis. The directional preference at 1.0 mM for the phlorizin-sensitive component was 44.3-fold with mucosal O_2 and 1.08-fold with mucosal N_2.

Discussion

In the *in vitro* preparation of hamster jejunum used in this study, making the mucosal solution anaerobic completely abolished transmural uphill transport and greatly diminished downhill mucosal-to-serosal transport of galactose, apparently because uphill movement from the mucosal solution into the absorptive cells was prevented. Making the serosal solution anaerobic had no effect on galactose transport. The inability of the apical ends of absorptive cells to transport galactose uphill during mucosal anaerobiosis might be caused by an inhibition of influx across the apical plasma membrane, an acceleration of efflux, or both. The main purpose of this investigation was to test these alternatives.

The results clearly show that mucosal anaerobiosis markedly reduces unidirectional influx of galactose into the jejunal epithelium from the mucosal solution. This reduction was entirely at the expense of the phlorizin-

sensitive component of influx. In fact, the phlorizin-insensitive component of influx was slightly increased by mucosal anaerobiosis. By taking into account the effects of the unstirred layer at the mucosal surface, it was estimated that if the concentration of galactose were 1.0 mM at the membrane surface, about 93% of phlorizin-sensitive unidirectional influx would be dependent upon mucosal O_2. The serosal solution was always oxygenated in these uptake studies; furthermore, no attempt was made to block anaerobic metabolism. Thus, it is possible that the residual phlorizin-sensitive influx is at least partly dependent upon metabolism.

Goldner *et al.* [13] measured unidirectional influx of 3-O-methylglucose into rabbit ileal epithelium and found substantial inhibition by cyanide and dinitrophenol. However, even after these agents were allowed to act for 30 min, the degree of inhibition (about 55%) was less than observed in the present study. Furthermore, Goldner *et al.* found that when their preincubation was done under conditions in which the absorptive cells did not gain Na^+, the degree of inhibition was less (roughly 25%). They attributed most of the inhibitory effect of metabolic poisons to an elevated intracellular Na^+ concentration (in spite of the fact that when they lowered intracellular Na^+ concentration by preincubating in a Na^+-free medium, they found no acceleration of influx). On the other hand, we found essentially the same severe inhibition of influx after preincubating under conditions in which the intracellular Na^+ concentration did not rise. We cannot attribute the effect observed to changes in Na^+ concentration, and must favor the idea of a more direct coupling between metabolism and influx.

If we express our data in the same units used by Goldner *et al.* [14], galactose influx under control conditions was about 5.0 μmoles/hr/cm^2, which is more than twice that obtained by Goldner *et al.* [14] in rabbit ileum in spite of the fact that they had 20 mM galactose in the mucosal solution and we had only 5 mM. If the difference in influx between the two preparations is at the expense of the metabolically dependent component, less metabolic dependence would be expected in rabbit ileum than in hamster jejunum. We have done a few experiments with everted rabbit terminal ileum using the technique that we used for hamster jejunum. These experiments cannot be presented in detail here, but we can report that we find values for galactose influx that are entirely consistent with those reported by Goldner *et al.* [14]. Moreover, 10 min of mucosal anaerobiosis (in a medium containing 143 mEquiv/liter Na^+) produced roughly 35 to 40% inhibition of galactose influx, a value which seems consistent with the findings of Goldner *et al.* [13]. Rabbit ileum may simply function differently than hamster jejunum.

According to the Na^+-gradient hypothesis [7, 21], the energy used for uphill pumping of sugars across brush borders is derived from the Na^+ and K^+ concentration gradients across the plasma membrane of microvilli, rather than directly from the hydrolysis of ATP. The Na^+-gradient hypothesis predicts that sugar influx should not be greatly depressed by arresting metabolism [13], but that efflux back out of the cells should be increased because of the rise in intracellular Na^+ concentration which would occur following shutdown of the Na^+ extrusion mechanism.

The quantitative importance of the contribution that the Na^+ gradient makes to directional preference and uphill transport in hamster jejunum must be questioned. It would seem from the present experiments that metabolically energized influx provides the major drive to directional preference and uphill transport across hamster brush borders. It is very important to note that directional preference was essentially abolished by 10 min of mucosal anaerobiosis even though at this time there was still a large Na^+ gradient. Other evidence against the Na^+-gradient hypothesis has recently been reviewed by Kimmich [15], especially his own important observation that sugars can still move uphill into isolated chick intestinal epithelial cells even after reversal of the normal gradients for monovalent cations. Moreover, Armstrong *et al.* [1] have recently demonstrated that the energy stored in the Na^+ chemical activity gradient across brush borders of bullfrog small intestine is not sufficient to account for observed galactose accumulation. However, if the energy stored in both the Na^+ and K^+ chemical activity gradients or the energy in the Na^+ electrochemical activity gradient could be utilized, then enough energy would theoretically be available, although rather high efficiencies of energy transfer (about 30 and 60%, respectively) would be required [1].

The model put forward by Kimmich [15] (*see also* Kimmich and Randles [16]) offers an attractive explanation for the apparent direct coupling between oxidative metabolism and sugar transport. He postulates that the same high-energy phosphorylated intermediate in the brush border membrane that is thought to energize Na^+ and K^+ transport can, alternatively, be diverted to energize sugar or amino acid transport. However, there is at least one observation which is not consistent with this model, and which might lead to reservations about its acceptance: most, if not all, of the Na^+, K^+-ATPase (and, therefore, the phosphorylated intermediate) is associated with the basal-lateral membranes rather than with the brush border membranes [12, 19]. We feel that the most that can be accepted confidently at the present time is a strong augmentation of sugar influx by some source of metabolic energy (perhaps ATP). This augmentation of

influx seems to require extracellular Na^+. It may involve a direct coupling with hydrolysis of ATP at the brush border, or may be mediated in some way other than by ion gradients. Kimmich has suggested that if coupling between hydrolysis of ATP and sugar transport is not direct, then perhaps it is mediated by the electrical potential difference across the brush border membrane [15].

Mucosal anaerobiosis also appeared to increase the ease of galactose efflux back out of the absorptive cells across the brush borders. Table 6 gives our best estimates of the fluxes expected when the galactose concentration is 1.0 mM at the donor surface of the membrane ($J^{1.0}$). At this concentration efflux ($J_{cm}^{1.0}$) is increased 2.8-fold by mucosal anaerobiosis. This is a substantial effect; nevertheless, it is relatively small compared to the 12.5-fold augmentation of $J_{mc}^{1.0}$ brought about by mucosal oxygenation. Furthermore, this effect may be overestimated. It was necessary to assume a linear relationship between J_{cm} and tissue concentration of galactose. If this relationship is actually hyperbolic over the pertinent concentration range (up to 24 mM), the true effect of mucosal anaerobiosis on efflux would be less. A portion of the apparent increase in ease of efflux caused by mucosal N_2 is probably due to an increase through the phlorizin-insensitive pathway. In the presence of phlorizin in the mucosal solution, observed J_{mc} was 0.044 μmoles/min/g with mucosal O_2 and 0.067 μmoles/min/g with mucosal N_2 (*see* Table 3). Using Eq. (3), we find the average galactose concentrations at the outer surface of the brush border membrane (C_2) during these influxes would have been about 4.94 and 4.90 mM, respectively. Consequently, phlorizin-insensitive $J_{mc}^{1.0}$ was 0.0089 μmoles/min/g with mucosal O_2 and 0.0137 μmoles/min/g with mucosal N_2. Assuming no directional preference through the phlorizin-insensitive pathway, these would also be the values for $J_{cm}^{1.0}$. Therefore, mucosal anaerobiosis increases that portion of $J_{cm}^{1.0}$ which takes place via the phlorizin-insensitive pathway by only 0.0048 μmoles/min/g; this is only 5.3% of the total increase in $J_{cm}^{1.0}$. Nearly all of the increased ease of efflux must be attributed to an effect on the phlorizin-sensitive pathway. We also showed that during 10 min of mucosal anaerobiosis, in regular Krebs-Henseleit solution, the intracellular Na^+ concentration rises somewhat more than it does during mucosal oxygenation. It is possible that the increased ease of efflux over the phlorizin-sensitive pathway is mediated by an elevated intracellular Na^+ concentration. We were unable to design experiments to test this possibility.

Thus, our main question is answered: The reason mucosal anaerobiosis prevents transmural uphill transport and tissue accumulation of galactose is that it eliminates the directional preference for transport across the

apical membrane by inhibiting unidirectional influx and probably also by augmenting unidirectional efflux via the phlorizin-sensitive pathway. The former effect is by far the most important, in contradiction to the Na^+-gradient hypothesis.

In unpublished experiments we have measured transmural flux of galactose from a serosal solution containing 5 mM galactose to an initially galactose-free mucosal solution and found it to be, with mucosal and serosal oxygenation, only 5.0% of the mucosal-to-serosal flux reported in this paper. In other words, for transmural fluxes in this preparation of everted hamster jejunum, there is a 20-fold directional preference when the donor solutions contain 5 mM galactose. The 37-fold directional preference across the brush border membrane, estimated above for the situation in which the donor compartments contain 1.0 mM galactose, is probably enough to explain the entire transmural directional preference. There would seem to be no need to postulate a contribution from the basal-lateral membrane, contrary to our previous preliminary suggestion [2]. However, evidence for an active role of the basal-lateral membrane *in vivo* has recently been presented by Esposito *et al.* [11]. Our previous experiments were preformed on noneverted hamster jejunum, and we are reluctant to abandon the idea that, at least under some conditions, the basal-lateral membrane can contribute to transepithelial directional preference.

The authors are indebted to Mrs. Jo Llu Long for her skilled and devoted technical assistance. This study was supported by NIH Grant AM-05778 and by U. S. Army Medical Research and Development Command, Contract No. DADA-17-68-C-8114.

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