Influence of the enteric surface coat on the unidirectional flux of acetamide across the wall of rat small intestine[1]

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Summary. In vivo treatment of the jejunal mucosa with glycosidic enzymes seems to remove the enteric surface coat of the enterocyte. As a consequence, the mucosa-to-serosa unidirectional flux of acetamide increases remarkably. The glycocalyx probably represents a barrier to the diffusion of small hydrosoluble solutes.

A molecule that passively permeates the intestinal wall transcellularly moves across several barriers[2]. The first one is the unstirred water layer (UWL) lining the apical pole of the enterocyte[3], which can be reduced by rapidly mixing and/or recirculating the bulk luminal fluid. Such an UWL could represent up to 36% of the total transport resistance to several solutes[4]. Another barrier is the enteric surface coat (glycocalyx), which forms an additional UWL that cannot be reduced by rapidly mixing the bulk luminal fluid. The microvillus membrane, the basolateral membrane and the subepithelial tissues with their UWLs represent the final barriers. The involvement of the glycocalyx as a hindrance to diffusion during disaccharide absorption was suggested long ago[5,6]. Recently it has been reported that the membrane surface mucous coat represents an important diffusion barrier for nutrients[7]. Since the glycocalyx could also be a considerable hindrance to the passive movement of small water-soluble molecules (such as acetamide, urea, etc.), we tried to eliminate this layer. To this purpose we studied the action of several enzymes on the surface coat glycoconjugates. It has been reported that the removal of the glycocalyx by mucolytic or proteolytic agents was mostly unsuccessful because this integral part of the cell is remarkedly stable[8]. However, some successful results have been obtained with the agar replica technique used to study the activity of the enzymes adsorbed on the mucus coat[9]. By assuming that the glycocalyx contains sialic acid, neuraminidase treatment was often employed but the results thus obtained were inconsistent. We know, however, that the sialic acid content of the glycocalyx depends on the species of animal, since it is present in certain animals such as the rat[10] but at very low levels in others such as the rabbit[11] and man[10]; moreover, there is also a different regional distribution of sialic acid in various animal species[12]. The sugar sequence of glycoconjugates of the glycocalyx is still unknown; therefore, by taking into consideration the carbohydrate sequence of the nonreducing termini of the etheropolysaccharide chain of membrane glycoproteins, we used several glycosidases in order to remove the surface coat (under in vivo conditions). Then the intestine thus treated was used in vitro to study the unidirectional flux of acetamide. We employed this molecule because it is a non-metabolizable water-soluble substance of low molecular weight (59.07 M_r), nontoxic and, as we have previously demonstrated, it diffuses passively across the intestinal barrier[2,13]. Other authors have used this molecule, as well as other hydrosoluble molecules, for the study of passive movement of water-soluble nonelectrolytes[14].

Materials and methods. The experiments were carried out as follows: albino male rats (Wistar strain, 240–270 b.wt) were starved overnight (with free access to water). Under barbiturate anesthesia the abdomen was opened and the jejunum (with its intact vascular supply) was washed with 20 ml of Krebs-Ringer bicarbonate solution at 37 °C and emptied. Then it was connected with a syringe at the caudal end while an L-shaped glass cannula (its vertical branch functioning as a reservoir) was inserted into the upper part of the jejunum. A known volume (4 ml) of a 12 mM citrate buffer at pH 4.5 (which is the optimum value for the enzymes used) was introduced into the lumen. Neuraminidase (0.5 U/ml, Sigma Chem. Co.) was placed in the reservoir, mixed with the syringe and allowed to act for the 1st 10 min. Then the following enzymes (Sigma Chem. Co.) were added sequentially: β-galactosidase (0.2 U/ml), β-N-acetyl-glucosaminidase (0.25 U/ml), sulfatase (74 U/ml), α-mannosidase (0.5 U/ml), β-glucosidase (1 U/ml) and β-glucuronidase (2377 U/ml). The enzymes (final specific activities in parentheses) were allowed to act for 60 min; the solution was mixed every 5–10 min. At the end of the incubation the luminal fluid was collected and analyzed for the determination of sialic acid[15] and sugars[16] released. The jejunum was rapidly washed, isolated and incubated at

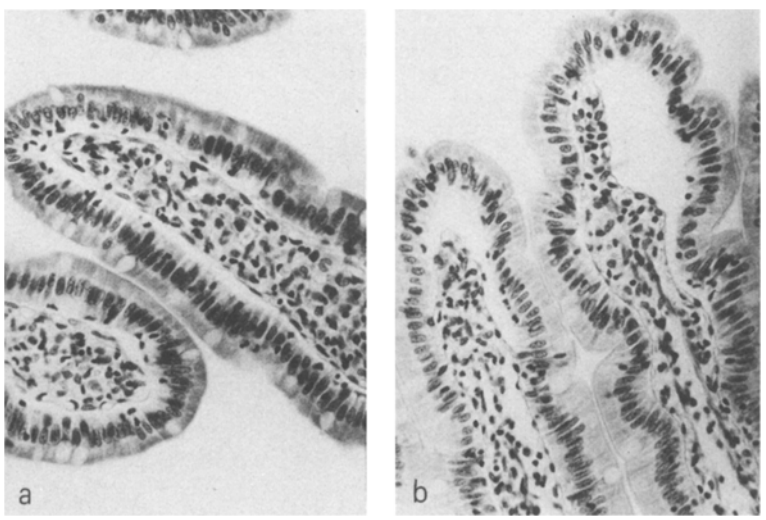

Figure 1. Light micrograph of a section of rat jejunum (magnification × 375) after 1 h incubation in vivo with citrate buffer solution (a) and with the same solution added with glycosidic enzymes (b). The tissue was fixed in Bouin's solution and stained with haematoxylin and eosin.

28 °C in 50 ml of Krebs-Ringer bicarbonate solution added with 5.56 mM glucose (serosal solution). The lumen was perfused (2.5 ml/min) by means of a peristaltic pump with 5 ml of the same solution (mucosal solution) except that it contained ^{14}C-acetamide (0.6 μCi/ml, New England Nuclear Co. corresponding to 115 nmoles/ml) and ^{3}H-polyethyleneglycol 900 M_r (1 μCi/ml, New England Nuclear Co.) for the determination of water movement and intestinal leakiness, if any. Both the mucosal and serosal solutions were gassed with 95% O_2 and 5% CO_2. After a pre-perfusion period of 5 min the experiment started and serosal samples were taken every 10 min for 1 h. The radioactivity was measured by liquid scintillation spectrometry and the mucosa-to-serosa flux of acetamide was thus determined. Cell Na^+ and K^+ concentrations were determined by flame photometry both in control and enzyme-treated intestines in order to check the viability of the epithelium. In fact, any initial alteration of the function of the absorbing cells affects cell electrolyte concentration as well as net Na^+ (and water) transepithelial transport.

Table 1. Release of sialic acid and total neutral sugars by the intestine after treatment with glycosidases

	Sialic acid ($mg\ g^{-1}\ h^{-1}$)	Total neutral sugars ($mg\ g^{-1}\ h^{-1}$)
Controls	0.21 ± 0.04 (3)	4.63 ± 2.64 (4)
Enzyme-treated	0.42 ± 0.10 (3)	27.49 ± 4.02 (4)
R	2.01	5.94

Sialic acid and total neutral sugars released after 60 min of in vivo treatment of the intestine with several glycosidases, expressed as $mg\ g^{-1}$ of dry tissue weight and per h. Values ± SEM. and number of experiments (in parentheses) are reported. R represents the ratio between treated and control intestines.

Table 2. Some parameters used to assess the viability of the small intestinal epithelium under control and enzyme-treated experiments after 1 h of in vivo incubation

	Cell Na^+ (mEq)	Cell K^+ (mEq)	Net fluid transport ($ml \cdot g^{-1} \cdot h^{-1}$)
Controls (4)	60 ± 9	124 ± 10	4.9 ± 2.7
Enzyme-treated (4)	51 ± 6	108 ± 2	5.5 ± 1.5

Cell electrolyte concentration is given in mEq/l of cell water. Net fluid transport is expressed in ml/g dry tissue weight of total intestine per h. Values ± SEM and number of experiments (in parentheses) are reported.

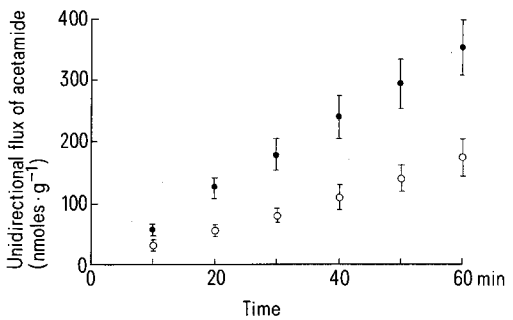

Figure 2. Mucosa-to-serosa unidirectional flux of acetamide in rat jejunum expressed in nmoles/g of dry tissue weight (ordinate) as a function of time (abscissa). Open circles (n = 4) represent controls while solid circles (n = 5) represent enzyme-treated intestines.

Cell electrolyte concentrations were determined in the scraped mucosa after correction for the extracellular space[17,18]. At the end of the experiment, samples of the intestinal mucosa were processed for morphological examinations in order to verify any tissue damage due to the enzymatic treatment.

Results and discussion. The relevant release of sialic acid and sugars (see table 1) into the luminal fluid during the in vivo incubation with enzymes, seems to indicate that the glycocalyx is, at least in part, removed. As shown in table 2 none of the parameters taken to ascertain the viability of the enterocyte was altered. As a matter of fact, cell Na^+ and K^+ concentrations are similar under both experimental conditions as well as net transepithelial water transport. In addition, diffusion of radioactivity due to ^{3}H-polyethyleneglycol 900 into the serosol compartment at the end of the experiment, under the different experimental conditions, was negligible. In other words, the final radioactivity in the serosal compartment was about 2% of that initially present in the mucosal side and no difference was found between control and enzyme-treated experiments. Figure 1 finally shows that the morphology of the epithelium was not altered by the enzymatic treatment. These results seem to demonstrate that the enzymes do not alter the function and the morphology of the intestinal epithelium. As figure 2 clearly shows, the unidirectional flux of acetamide from the mucosal to the serosal side is doubled when the enteric surface coat has been subjected to the action of enzymes. The flux difference begins to be statistically significant from the 10th min on. This seems to indicate that the glycocalyx represents a diffusion barrier to the movement of a passively diffusing hydrosoluble nonelectrolyte. This conclusion is in agreement with previous reports by other authors concerning the influence of the glycocalyx on the absorption of disaccharides and other nutrients[5-7]. As a matter of fact, the thickness of this layer is about 0.3–0.5 μm[5,8] depending on the animal species, and is 1 order of magnitude thicker when the mucus coat is considered[7]. Some authors did not succeed in removing the surface coat enzymatically; however, they used the everted intestinal sac, i.e. they operated under in vitro conditions, while our experiments were performed in vivo under more favorable conditions.

As a conclusion, the glycocalyx seems to represent an additional and significant diffusion barrier against the movement of small hydrosoluble molecules.

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Effect of carbon sources on the polar lipid fatty acids of *Microsporum gypseum* grown at different temperatures

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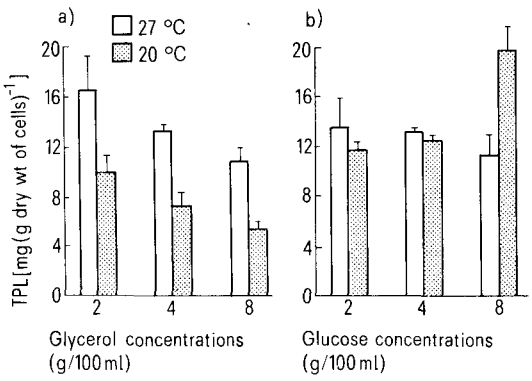
Summary. Total phospholipids and their constituent fatty acids exhibited significant alterations in presence of glucose or glycerol when *M. gypseum* was grown at 20 and 27 °C. Cells grown on a glucose medium showed a higher degree of unsaturation as compared to cells grown on a glycerol containing medium.

Manipulation of membrane lipid components by changing the growth medium composition is a valuable tool for studying the role of phospholipids in microorganisms[3]. Hence, the use of a defined growth medium is essential for bringing about controlled modification in microbial biomembranes[4]. In order to ascertain the role of apolar head groups of phospholipids in biological membranes, extensive studies have been undertaken using either fatty acid auxotrophs[5-7] or microorganisms grown in the presence of cerulenin, an antibiotic that may be used as a tool for introducing controlled alterations in cellular fatty acid composition[8]. However, similar studies on filamentous fungi are scanty[9]. Considerable effort has been made in our laboratory to study the effects of various environmental factors on the phospholipid composition of dermatophytes[10-15], and significant alterations were observed both in polar as well as apolar head groups of phospholipids. In view of the earlier observations on the influence of various carbon sources and temperature on the lipid composition of *Rhizopus arrhizus*[16], we have also compared the effects of glucose and glycerol on the phospholipid fatty acids of *Microsporum gypseum*, grown at different temperatures.

Materials and methods. The source of *Microsporum gypseum* used in this study is the same as has been reported earlier[17]. *M. gypseum* was grown as surface cultures in Sabouraud's media (pH 5.4–5.6) containing 2, 4 or 8% glucose and 1% peptone (Biological grade, Centron, Bombay, India) at 20 or 27 °C. In glycerol media, glucose was replaced with equal concentrations of glycerol. The growth pattern of *M. gypseum* was determined by plotting dry weight of the mycelium against the age of the culture. Various cultures grown at 27 °C were harvested after 15 days, while those grown at 20 °C were harvested after 21 days; at these times the cells were in the log phase of growth. The harvested cells were blotted dry, weighed and a known portion of the fungus kept for dry weight determination while the rest was used for extraction of lipids according to the method of Folch et al.[18]. Phospholipids were quantitated by estimating phospholipid phosphorus after perchloric acid digestion by the method of Bartlett[19] as modified by Marinetti[20]. Phospholipids were separated from total lipids either by acetone precipitation or by silicic acid column chromatography, and methyl esters of phospholipid fatty acids were prepared by transesterification with methanol in the presence of thionylchloride[11]. Fatty acids were identified by comparing their retention times with those of standards. The relative composition of fatty acid was calculated by the triangulation method. Unsaturated/saturated (U/S) fatty acids ratios were calculated by dividing the total unsaturated fatty acids ($C_{16:1}+C_{18:1}+C_{18:2}$) by the total saturated fatty acids ($C_{10:0}+C_{12:0}+C_{14:0}+C_{16:0}+C_{18:0}$).

Results and discussion. Glycerol may be comparable to glucose as the carbon substrate for biosynthesis of *M. gypseum* phospholipids, particularly at higher temperatures and lower concentrations. Total phospholipids showed a significant decrease ($p < 0.05$) with increased glycerol concentration, at both the growth temperatures tested (fig., a). However, no change in total phospholipid content was observed up to 4% glucose, at either temperature (fig., b). With 8% glucose, there was a slight decrease in total phospholipids at 27 °C while a significant increase ($p < 0.001$) was observed at 20 °C. The observed alterations in phospholipid content with these 2 carbon sources may be due to the differences in their utilization for phospholipid biosynthesis by *M. gypseum*. Similar observations have been made with various fungal species, which have been shown to differ in their ability to convert various carbon substrates to lipids[9].

Significant changes in the phospholipid fatty acid composition of *M. gypseum* were observed in the presence of either glycerol or glucose (tables 1 and 2). At 27 °C, there was a decrease in the U/S fatty acid ratio when glycerol concentration was increased from 2% to 4% (table 1). The decrease in unsaturation with 4% glycerol was due mainly to an



Effect of different concentrations of glycerol or glucose on the total phospholipids (TPL) of *M. gypseum* at 27 and 20 °C. Values are mean ± SD of 6 independent determinations.