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Enzyme characterization studies on the rate-limiting barrier in rabbit buccal mucosa

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Summary

In the present study, hyaluronidase, neuraminidase, chondroitinase ABC, heparinase, heparitinase, and trypsin were used to help characterize components of the rate-limiting barrier, i e., upper epithelial cell layers, of rabbit buccal mucosa Visualization of horseradish peroxidase permeability and quantitative measurements of glycerol, choline, and salicylic acid permeability, in the presence and absence of enzymes, were employed to evaluate the enzyme's effect on the tissue. The results indicate that the ability of an enzyme to alter the permeability of a diffusing solute through buccal mucosa appears to depend on the chemical properties of the solute itself Moreover, it appears that protein as well as chondroitin sulfate and dermatan sulfate are important constituents, i.e., large amounts and/or most accessible, of the rate-limiting barrier of rabbit buccal mucosa.

Introduction

From much of the published literature on drug absorption, in particular, for peptides in nonparenteral routes, including the buccal mucosa, the tissues are usually quite restrictive in their permeability characteristics, often resulting in a limited and/or subtherapeutic response. Consequently, penetration enhancement may play an important role in the successful delivery of these substances via the noninvasive routes. In general, penetration facilitation of a solute through a mucous membrane involves the use of an agent, such as a bile salt, surfactant, or organic solvent, which is often indiscriminate in its effects on the epithelial membrane. However, methods of modulating tissue permeability may be better controlled with information related to the physicochemical properties of conducting pathways for compounds traversing multiple cell layers.

Thyrotropin releasing hormone, a tripeptide, appears to migrate predominately through the paracellular pathway in the rate-limiting barrier, i.e., upper 50 μm or so of the epithelium, of rabbit buccal mucosa (Dowty et al., 1992). The major constituents of the extracellular space usually consist of a maze of polysaccharides and

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TABLE 1

Various enzymes and their substrates and products

Enzyme	Substrates	Products
Chondroitinase ABC	chondroitin sulfate, dermatan sulfate, hyaluronic acid (slow)	disaccharides
Hyaluronidase	chondroitin sulfate, dermatan sulfate, hyaluronic acid	tetra- and octasaccharides
Heparinase	heparin, heparan sulfate, chondroitin sulfate, hyaluronic acid	high molecular mass products
Heparitinase	heparan sulfate	di- and tetrasaccharides
Neuraminidase	α-D-configuration *N*-acylneuraminic acid	sialic acid removal
Trypsin	protein	cuts carboxyl side of Lys of Arg

proteins (Hay, 1981; Alberts et al., 1989; Darnell et al., 1990). To this end, a series of enzymes have been employed to help identify that portion of the extracellular material that contributes to the permeability barrier of rabbit buccal mucosa. These enzymes include hyaluronidase, neuraminidase, chondroitinase ABC, heparinase, heparitinase, and trypsin (Suzuki et al., 1968; Yamagata et al., 1968; Hovingh and Linker, 1970; Boyer, 1971; Cuatrecasas, 1972; Linker and Hovingh, 1972, 1977; Suzuki, 1972; Horowitz and Pigman, 1978; Kanwar et al., 1980; Simionescu et al., 1981; Squier, 1984; Sunnergren, 1987; Groggel et al., 1988; Kazama et al., 1989; Koob, 1989), which are able to metabolize various sugar residues and protein linkages of polysaccharides, proteoglycans, glycoproteins, and glycolipids potentially present in the matrix between cells (see Table 1). The methods utilized to study the enzyme's effect on the tissue include: (a) visualization of horseradish peroxidase permeability, an intercellular probe which is rate-limited by the upper 100 μm or so of the epithelium (Squier, 1973, 1984); and (b) quantitative measurements of the permeability of several hydrophilic and similar molecular weight solutes: glycerol (neutral charge), choline (positively charged), and salicylic acid (negatively charged).

TABLE 2

Preparation, source, and incubation temperatures of enzymes used

Enzyme [a]	Source	Temperature (°C)	Preparation
Hyaluronidase (type VI-S)	bovine testes	37	500 U/ml in 0.1 M phosphate buffer and 0.15 M NaCl at pH 6.0
Neuraminidase (type VI)	*Clostridium perfringens*	37	0.1 mg/ml in 0.1 M phosphate buffer at pH 6.0
Chondroitinase-ABC	*Proteus vulgaris*	37	5 U/ml in 0.1 M Tris-HCl at pH 7 4
Heparitinase	*Flavobacterium heparinum*	37	0.4 U/ml in 0.1 M sodium acetate at pH 7 0
Heparinase	*F heparinum*	30	5 U/ml in 0.1 M sodium acetate and 0.01 M $CaCl_2$ at pH 7.0
Trypsin	porcine pancreas	37	1% in 25 mM Hepes isotonic with NaCl at pH 7.4

[a] All from Sigma, St. Louis, MO

Materials and Methods

Horseradish peroxidase permeability studies

Rabbit buccal mucosa was excised and prepared as described previously (Dowty et al., 1992). Small tissue pieces from the same rabbit were placed in a glass vial with an enzyme solution, i.e., test tissue, listed in Table 2 or its respective buffer without enzyme, i.e., control tissue, for either 2 or 5 h. The solutions were gassed with

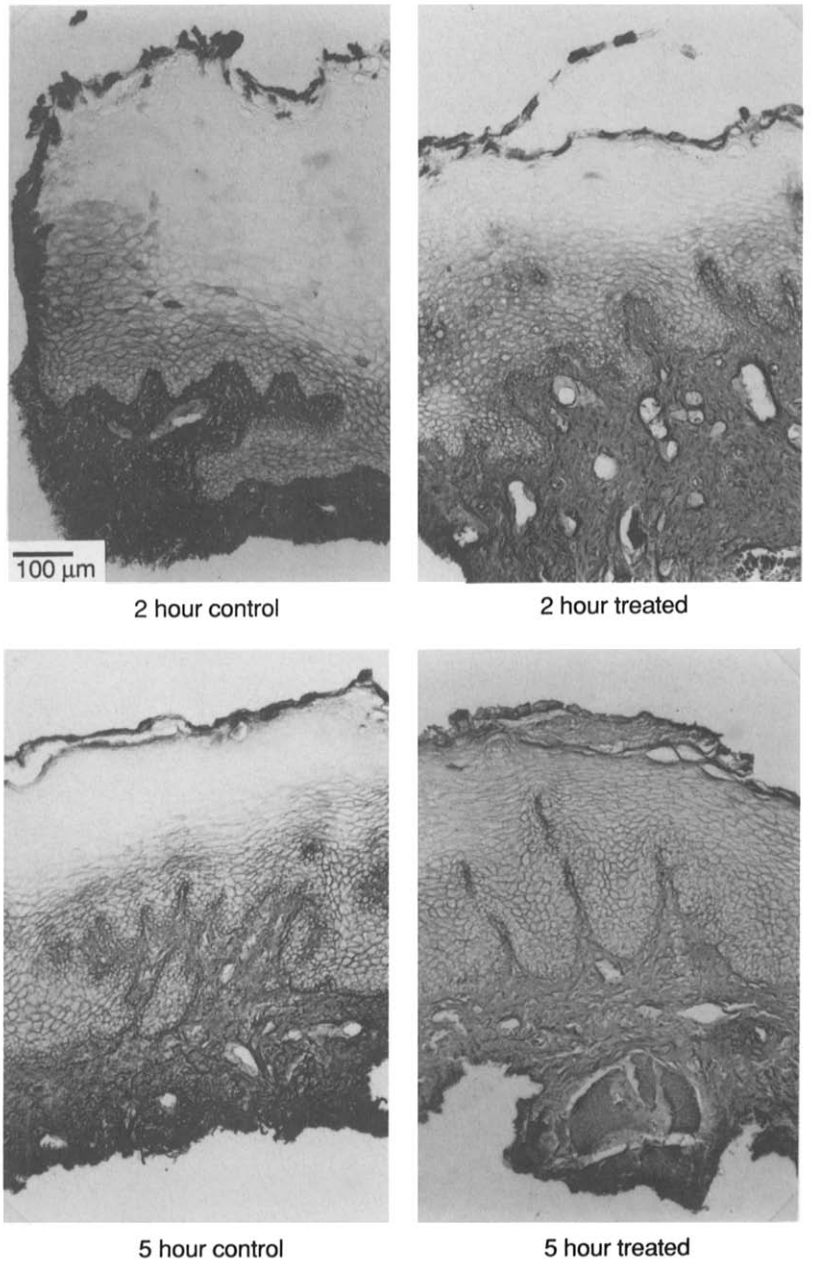

Fig. 1. Effect of chondroitinase ABC digestion on the permeability of horseradish peroxidase in rabbit buccal mucosa in vitro. HRP is present in the intercellular spaces of the rate-limiting barrier, i.e , upper 100 μm or so of the epithelium, after 5 h, but not after 2 h of enzyme exposure. All photos are at the same magnification.

humidified 95% O_2/5% CO_2 which induced mixing and were maintained in a shallow water bath at the appropriate temperature listed in Table 2. After 2–5 h incubation time, tissues were removed and washed in 0.1 M Tris-HCl at pH 7.4, followed by the addition of 30 mg/ml horseradish peroxidase (Type II, Sigma) for 1 h at 37°C. Tissues were fixed overnight at 4°C in 2%

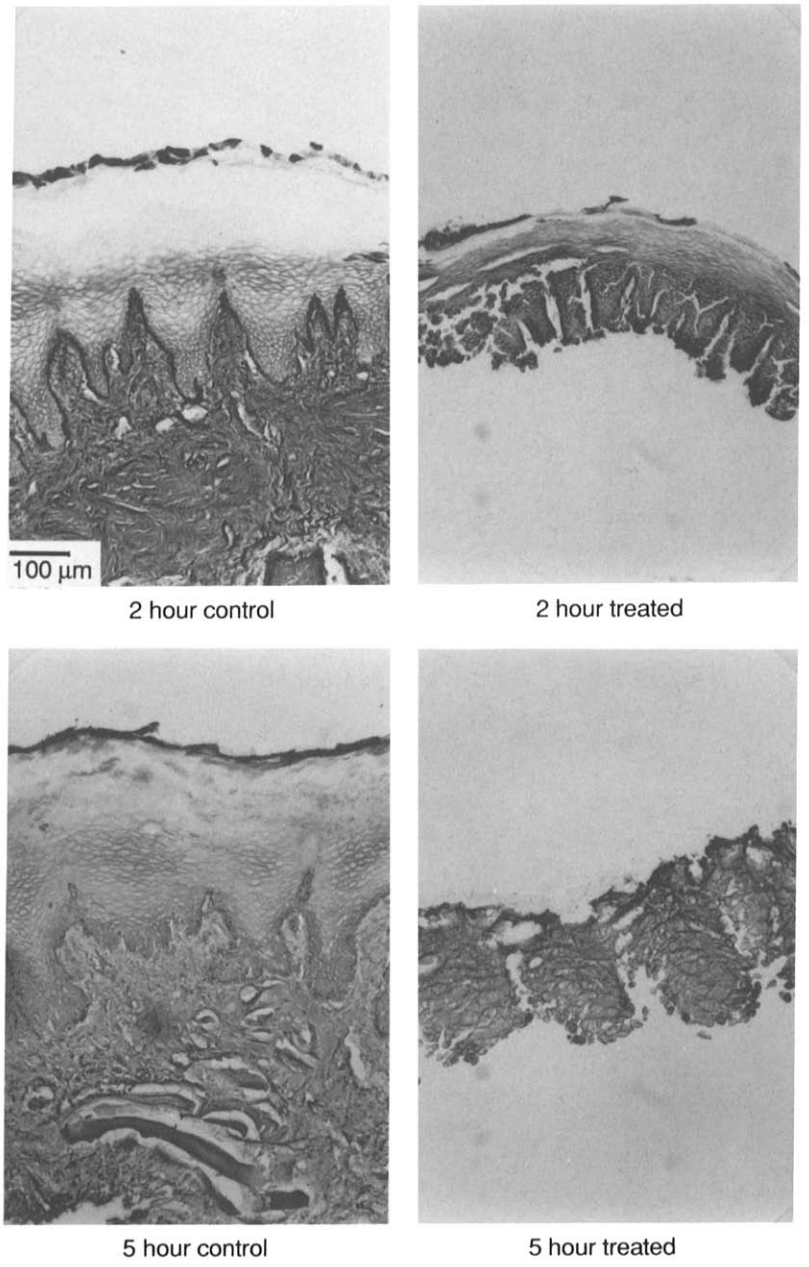

Fig 2. Effect of trypsin digestion on the permeability of horseradish peroxidase in rabbit buccal mucosa in vitro. The connective tissue has been removed after 2 h of exposure to the enzyme and HRP is seen throughout the epithelial layers Cellular damage is apparent after 5 h of exposure to trypsin All photos are at the same magnification

formaldehyde and 2.5% glutaraldehyde in 0.08 M cacodylate buffer at pH 7.4. Specimens were then rinsed three times in 0.08 M cacodylate buffer. For light microscopy, tissue blocks were mounted on cork disks (Mackenzie, 1974) and snap-frozen in isopentane cooled in liquid nitrogen. Serial 15 μm sections were made on a 5030 microtome (Bright Instruments Co. Ltd, Huntington, U.K.) at -20°C. Sections were then stained with 3,3′-diaminobenzidine (DAB); working solution: 0.3% DAB, 0.01% H_2O_2, and 0.26% $Ni(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in 0.1 M Tris buffer. Specimens were visualized with a Leitz Orthoplan universal large field microscope.

Glycerol, choline, and salicylic acid permeability studies

Solutes were as follows: (1) [1,3-^{14}C]glycerol (ICN Radiochemicals, Irvine, CA); Mol. Wt, 92; specific activity, 100 mCi/mmol; and purity, 99.6%; (2) [1,2-^{14}C]choline chloride (NEN Research Products, DuPont, Wilmington, DE); Mol. Wt, 104; specific activity, 7.2 mCi/mmol; and purity, 99%; and (3) [7-^{14}C]salicylic acid (NEN Research Products); Mol. Wt, 138; specific activity, 56.0 mCi/mmol; and purity, 98.5%.

Rabbit buccal mucosa was mounted in a side-by-side diffusion cell (Grass and Sweetana, 1988; Precision Instrument Design, Los Altos, CA). One

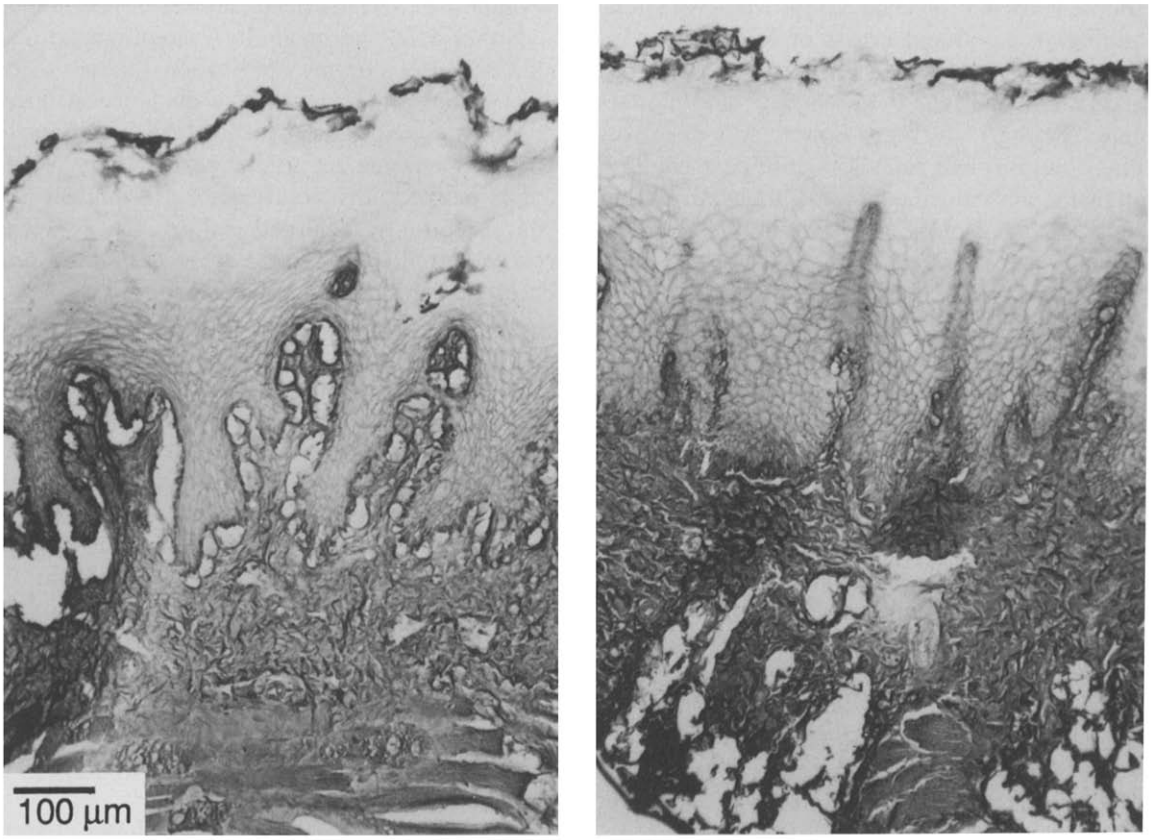

Fig. 3. Effect of hyaluronidase digestion on the permeability of horseradish peroxidase in rabbit buccal mucosa in vitro at 5 h. HRP is unable to penetrate into the upper 100 μm or so of the epithelium, i.e., rate-limiting barrier, in either control or enzyme-treated tissue. The same results were obtained at 2 h with this enzyme and at 2 and 5 h with heparitinase, neuraminidase, and heparinase. All photos are at the same magnification.

of the two rabbit cheeks was incubated on both sides with an enzyme solution as indicated in Table 2, while the other cheek was placed in the corresponding enzyme buffer without the enzyme. In addition, the donor solution of each cell contained approx. 1 μCi/ml of a radioisotope indicated above. A sample of the donor compartment was taken at the start of the experiment to establish initial concentration of the diffusing solute. Samples were taken at various times over a 6 h period and placed in a scintillation vial. 10 ml of scintillation cocktail (Bio-Safe II) was added and total dpm were counted on a Tri-Carb 460CD using the external standard channels ratio method of quench correction to determine counting efficiency.

Simple diffusion of each solute was evaluated by adding an equal and excess of cold solute (50 mM glycerol (J.T. Baker Chem. Co., Phillipsburg, NJ); 0.15 mM salicylic acid (Sigma); and 1 mM choline (Sigma)) to both donor and receiver bathing solutions and calculating the permeability through the buccal mucosa of 1 μCi/ml of the respective radioisotope placed in the donor chamber.

Results and Discussion

Light microscope images of horseradish peroxidase (HRP) permeability from 2 and 5 h enzyme studies are shown in Figs 1–3. Hyaluronidase results at 5 h, depicted in Fig. 3, were identical with 2 h control and treated tissue and, moreover, are representative of what was seen with heparitinase, neuraminidase, and heparinase. There was a change in HRP permeability in the rate-determining barrier, the upper 100 μm or so of the epithelium, in the presence of chondroitinase (Fig. 1; 5 h) and trypsin (Fig. 2; 2 and 5 h). Cell damage by chondroitinase has been shown by Squier (1984) to be minimal with electron microscopy. On the other hand, there appears to be extensive damage to cells with trypsin after 5 h. However, 1% trypsin has been used in this laboratory (Dowty, 1991) on rabbit buccal mucosa under the same conditions for 4 h to collect cells for culture. This procedure results in greater than 90% viable cells using trypan blue exclusion. The poor preservation of the tissue may then be due, in part, to the extreme fragility of trypsinized tissue and to the processing techniques to allow for microscopic examination.

Buccal mucosa exposed to heparitinase, hyaluronidase, neuraminidase, and heparinase all showed no apparent change in HRP permeability in the rate-limiting barrier (represented by Fig. 3). Moreover, at the light microscope level, the tissues appear to be intact. However, cell damage may be apparent at the electron microscope level. Electron micrographs of hyaluronidase and neuraminidase treated tissue have been reported by Squier (1984).

Overall, such data are qualitatively useful in evaluating enzyme effects. However, it is not clear whether HRP permeability was enhanced or inhibited after enzyme application relative to control. Therefore, quantitative evidence is needed to draw conclusions regarding the specific action of each enzyme on solute permeability. To this end, permeability coefficients of similarly sized but differently charged solutes, i.e., glycerol, choline, and salicylic acid, were calculated in the presence and absence of each enzyme.

For each glycerol, choline, and salicylic acid permeability experiment, the total amount of radioactivity being transported from donor to receiver solutions was determined as a percent of the total donor radioactivity. Data from the linear portion of the curve (i.e., steady-state kinetics) were fitted with the best least-squares line and each slope (% min^{-1}) was subsequently recorded. The permeability coefficient (cm s^{-1}) was then determined with the following equation from Fick's laws of diffusion (see Table 3):

$$P = [(\text{slope})(1.2\ \text{ml})] \Big/ [(0.785\ \text{cm}^2)(100\%)(60\ \text{s min}^{-1})]$$

where 1.2 ml is the volume of the donor chamber, 0.785 cm^2 corresponds to the exposed surface area of tissue to transport, 100% is the initial amount in the donor cell, and 60 is the conversion factor for minutes to seconds. The permeability coefficients of each solute were indepen-

dent of concentration in the range of 0.0083–50 mM for glycerol, 0.12–1 mM for choline, and 0.015–0.15 mM for salicylic acid, indicating all three solutes appear to traverse buccal mucosa by simple diffusion. Moreover, the permeability coefficient of each solute was dependent on the buffer and temperature used (refer to Table 2). However, these particular conditions were used to optimize the activity of each enzyme.

From Table 3 and Fig. 4, it is apparent that the permeability coefficient can vary greatly between different rabbits. However, the ratios of the solute permeability in the presence of the enzyme over that in its absence are much closer for different rabbits. These ratios or enhancement factors for glycerol, choline, and salicylic acid are summarized in Table 4. The permeability of each of the solutes statistically increased in the presence of trypsin and remained relatively the same with addition of hyaluronidase and neuraminidase. These results appear to be consistent with the HRP data, in that an increase in solute permeability corresponded to the presence of HRP in the rate-limiting layer, whereas no change in solute permeability was associated with the absence of HRP in the barrier region. On the other hand, the other three enzymes affected the permeability of the solutes differently. Choline (positively charged) permeability in the presence of chondroitinase, heparitinase, and heparinase was the same as that for control tissue while glycerol (neutral) diffusion decreased in each case indicating a possible increase in membrane binding. Moreover, the permeability of salicylic acid (negatively charged) was enhanced with chondroitinase but remained essentially equal in the presence of heparitinase and heparinase. These results suggest that the chemical properties of the solute play a role in determining its permeability enhancement or inhibition in the presence of an enzyme-modified environment. The size of the probe appeared to be less important, for exam-

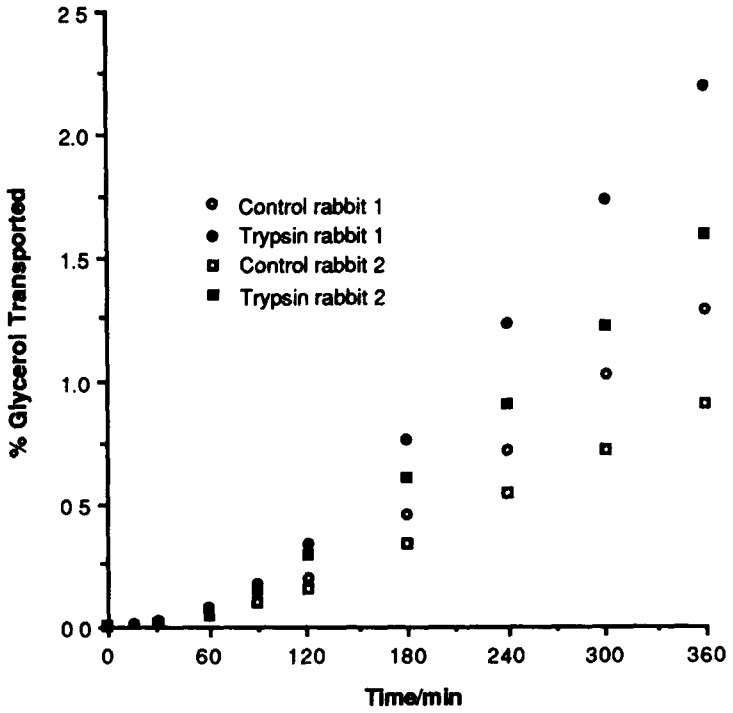

Fig 4. Permeability kinetics of glycerol through rabbit buccal mucosa in vitro in the presence and absence of trypsin Note that there is a large variation in permeability between the two rabbits (see also Table 3), however, the enhancement factors are similar (refer also to Table 4).

TABLE 3

Permeability coefficients of glycerol, choline, and salicylic acid in rabbit buccal mucosa in vitro in the absence (control buffer) and presence of the indicated enzyme

Enzyme	Permeability coefficient ($\times 10^7$) (cm s^{-1})		
	Glycerol	Choline	Salicylic acid
Control buffer	5.0(0.4)	16(2)	23(2)
+ chondroitinase ABC	3.7(0.5)	16(2)	23(2)
Control buffer	4.2(0.6)	5 3(0 5)	9.4(0.7)
+ heparitinase	3.6(0.5)	5 3(0 6)	9.4(0.4)
Control buffer	2.2(0 8)	2.9(0.5)	4 4(0 7)
+ heparinase	1.8(0.5)	2 9(0.4)	4.4(0 8)
Control buffer	10(2)	18(2)	12(2)
+ trypsin	17(3)	53(26)	36(5)
Control buffer	5.6(1.1)	17(2)	33(4)
+ hyaluronidase	5 6(1.0)	18(2)	34(2)
Control buffer	1 9(0.4)	11(2)	23(2)
+ neuraminidase	1.8(0.3)	11(1)	23(2)

Values are the averages of four experiments. Number in parentheses is the standard error of the mean

ple, in the presence of chondroitinase, the permeability of glycerol was inhibited and that of salicylic acid was enhanced. Hyaluronidase and heparinase, which are similar to chondroitinase in that they can digest chondroitin sulfate, did not enhance the permeability of any of the probes

TABLE 4

Summary of effects by various enzymes on the permeability of glycerol, choline, and salicylic acid in rabbit buccal mucosa in vitro

Enzyme	Enhancement factor		
	Glycerol	Choline	Salicylic acid
Chondroitinase ABC	0.77(0 02)	1 00(0.02)	1 54(0.06)
Heparitinase	0.85(0.03)	1.00(0.02)	0 99(0.02)
Heparinase	0.88(0.03)	1.00(0.02)	0 98(0.01)
Trypsin	1.71(0 20)	3.01(0.40)	2.78(0.05)
Hyaluronidase	1.02(0.02)	1.02(0 03)	1.00(0.01)
Neuraminidase	0.99(0.01)	0.98(0.02)	1.01(0.01)

Enhancement factor denotes the permeability change in the presence of enzyme relative to control without enzyme. Values are the averages of four experiments. Number in parentheses is the standard error of the mean

used. This may have had something to do with the size of the products formed in combination with the extent of proteoglycan digestion (see Table 1).

A question which arises from this work is whether or not the enzymes, which did not affect the permeability barrier, were able to gain access to this region. The molecular masses of the enzymes used are as follows: chondroitinase, 150 kDa (Yamagata et al., 1968); heparinase, 62 kDa (McLean et al., 1984); heparitinase, 45 kDa (Yang et al., 1985); neuraminidase, 56 kDa (Balke et al., 1974); trypsin, 23.2 kDa (Lehninger, 1982); and hyaluronidase, 50 kDa (Boyer, 1971). Chondroitinase, which is the largest enzyme listed, evidently gained access into the superficial barrier in that HRP permeability was altered. In addition, HRP, a 40 kDa protein, was seen to penetrate up to both borders of the barrier region in the presence of heparinase, heparitinase, neuraminidase, and hyaluronidase, which suggests that these enzymes of 50 kDa should not have had difficulty in reaching the tissue. Nevertheless, it is possible that the extracellular constituents are arranged in a manner which disallows substrate accessibility by the enzyme.

In discussing related work, Squier (1984) has shown that chondroitinase, hyaluronidase, and neuraminidase produced the same qualitative effect on HRP permeability as described here. His work also demonstrated that, while chondroitinase was the only enzyme of the three mentioned to increase HRP permeability through the barrier, it had minimal damaging effects on epithelial cells. Adams (1975) suggested that neuraminidase is effective in reducing the glycocalyx of the epithelium. However, it was ineffective in altering the permeability of a fluorescent dye which is similar to the results presented here. George et al. (1980) examined the binding of limulus polyphemus, which is a lectin specific for sialic acid residues, in freshly excised human buccal mucosa. Their results indicated the absence of lectin binding in the buccal region. To this end, neuraminidase, with no substrate to act upon, would not be able to modify the buccal mucosa, which is in agreement with the work of Squier (1984), Adams (1975), and this study. Aungst and

Rogers (1989) showed that the use of chondroitinase or hyaluronidase did not enhance the absorption of insulin through buccal mucosa. However, the experiments were performed with rat oral epithelium which is apparently devoid of glycosaminoglycans in the intercellular spaces (Pedlar, 1979). Consequently, an effect by these enzymes should not have been anticipated in the rat animal model.

Overall, trypsin was the most effective at enhancing solute permeability indicating, unsurprisingly, the prevalence of protein in the extracellular space. At the same time, chondroitinase, in most cases, appeared to be effective in altering solute permeability, increasing or decreasing it, which suggests the presence of proteoglycans containing chondroitin sulfate and/or dermatan sulfate. However, heparan sulfate and heparin may play a smaller role (i.e., smaller amounts and/or less accessible) in this regard. Moreover, hyaluronic acid as well as sialic acid residues appear to be absent, already modified, or inaccessible to the applied enzymes. To this end, the use of compounds which are specific for protein, chondroitin sulfate, or dermatan sulfate may be the most useful in altering the permeability characteristics of a drug which uses the paracellular route while traversing buccal mucosa dependent on the drug's chemical properties.

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