

The Permeability of Skin and Oral Mucosa to Water and Horseradish Peroxidase as Related to the Thickness of the Permeability Barrier

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The permeability of porcine skin and keratinized and nonkeratinized oral mucosa to tritium-labeled water and horseradish peroxidase (HRPO) was determined using perfusion chambers. Small blocks from each tissue were also incubated with HRPO and the extent of penetration visualized microscopically; this enabled measurements to be made of the thickness of the permeability barrier to this water-soluble tracer. Results obtained after inverting the oral mucosa in the chambers or adding metabolic inhibitors indicated that both compounds diffuse across the tissue. The permeability constants derived directly in the study showed that skin was less permeable than oral mucosa and that the floor of the mouth was significantly more permeable than all other regions. When these constants were normalized in terms of a standard permeability barrier thickness and the different tissues compared, the values obtained for skin were again less than those of the oral regions but, of these, the buccal mucosa was significantly higher. The difference in permeability between epidermis and keratinized oral epithelium may be due to differences in the volume density of membrane-coating granules known to exist between the tissues; differences between the oral mucosal regions may reflect differences in the nature of the intercellular barrier material.

The relative impermeability of mammalian skin first became apparent from measurements of percutaneous water loss which showed that diffusion of water through skin is slower than through other tissues [1]. The barrier was shown to be located in the stratum corneum and more recently it has been suggested that the intercellular region of this layer may represent the principle barrier of the epidermis to the penetration of lipid- and water-soluble compounds [2].

The oral mucosa, like the skin, serves as a protective covering for the deeper tissues and is also used to deliver substances locally and systemically for therapeutic purposes [3,4]. In previous work we have demonstrated the existence of an intercellular barrier to ultrastructural tracers in the stratum corneum of keratinized regions and in the superficial cell layers of nonkeratinized oral epithelium [5,6]. The discovery of an intercellular permeability barrier in nonkeratinized oral epithelium, a tissue often assumed to be permeable [7,8], was surprising. However, the finding may be explained by differences in permeability between therapeutic agents, which are predomi-

nantly lipid-soluble [3,4] and the water-soluble substances used as tracers. Alternatively, it may reflect the limitations of using a microscopically localized tracer to study permeability.

Although there have been numerous studies of percutaneous permeability using radioisotopes, few workers have applied these techniques to the oral mucosa [9-12]. There have been no comparisons of keratinized oral epithelium with epidermis or with the nonkeratinized oral regions, nor have most comparisons allowed for the considerable differences in thickness between different regions. Galey et al [11] used total thickness of the sample to standardize their permeability data, but this is not very meaningful if the permeability barrier does not extend through the full thickness of the mucosa. The use of horseradish peroxidase (HRPO) as a microscopic tracer provides a means of visualizing, and thus obtaining measurements of, the epithelial barrier to water-soluble substances. This, in turn, enables permeability data to be related to barrier thickness. The study to be described here used isotopically labeled HRPO and water to determine permeability constants for various regions of porcine oral mucosa and skin; these values were then normalized in terms of barrier thickness and differences among regions compared.

MATERIALS AND METHODS

All tissues were taken from pigs at slaughter and transported in phosphate-buffered saline (PBS, 0.01 M, pH 7.5); they were generally used within 3 h of removal. Skin was taken from the glabrous region of the belly, keratinized oral mucosa from the attached gingiva between incisor and premolar teeth, nonkeratinized buccal mucosa from the region posterior to the angle of the mouth, and nonkeratinized floor of mouth mucosa from the region close to the base of the tongue. Submucosal and subcutaneous tissue was trimmed from the biopsies and specimens were used as follows.

Histologic Assessment

Small portions of each region were fixed in formalin, embedded in paraffin wax, the sections stained with hematoxylin and eosin, and examined with the light microscope. All sections were cut normally to the surface as determined by the profiles of the narrow elongate connective tissue papillae.

Visualization of the Permeability Barrier

Blocks not exceeding 1 mm³ from each region were incubated in 1% HRPO (Sigma type VI, Sigma Chemical Co., St. Louis, Missouri) for 1 h, then fixed immediately in buffered 2% formaldehyde-2.5 glutaraldehyde for 40 min, rinsed and snap-frozen in isopentane cooled in liquid nitrogen. Sections were cut at 6 μ m in a cryostat and incubated with hydrogen peroxide and diaminobenzidine to demonstrate the localization of the HRPO [13].

Permeability Measurements

Discs of tissue 7 mm in diameter were punched out and a soft rubber gasket with an internal diameter of 5 mm sealed to the epithelial surface with cyanoacrylate adhesive. The tissue was then clamped between the two halves of a Plexiglass perfusion chamber, each of which was filled with 6 ml of PBS and air was bubbled through the chamber to provide aeration and mixing. Either tritiated water (0.6 μ Ci) or tritiated HRPO (5 μ Ci; New England Nuclear, Boston, Massachusetts) was added to the epithelial (hot) side of the chamber. When HRPO was used, sufficient cold HRPO (Sigma Type VI) was added to give a total concentration of 1%, which is similar to that used for the

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Abbreviations:

ANOVA: an analysis of variance

HRPO: horseradish peroxidase

K_p : permeability constant

PBS: phosphate-buffered saline

PMSF: phenyl methyl-sulfonylfluoride

microscopic tracer studies. Samples of 0.1 ml were taken from the hot side and the total volume (6 ml) removed from the cold side, the original chamber volumes being restored by adding PBS. The samples were mixed with scintillation fluid and counted until counts reached a 2σ value of 1%; quenching was corrected automatically in the counter. Initially, samples were removed after 15, 30, 60, 120, 180, 240, 400, and 440 min and the amount of isotope traversing the tissue (expressed as counts/min) plotted against time to determine the lag time for each tissue and each compound.

All tissue regions showed a lag phase of less than 1 h, by which time the rate of transfer had reached a linear phase that continued as long as readings were taken (approximately 8 h) (Fig 1). Values used for computing a permeability constant were taken at 1 or 2 h after reaching a steady state.

A permeability constant (K_p) was calculated from values obtained after a steady state was reached using the relationship [14]:

$$K_p = \frac{Q}{A \cdot T \cdot (C_o - C_i)}$$

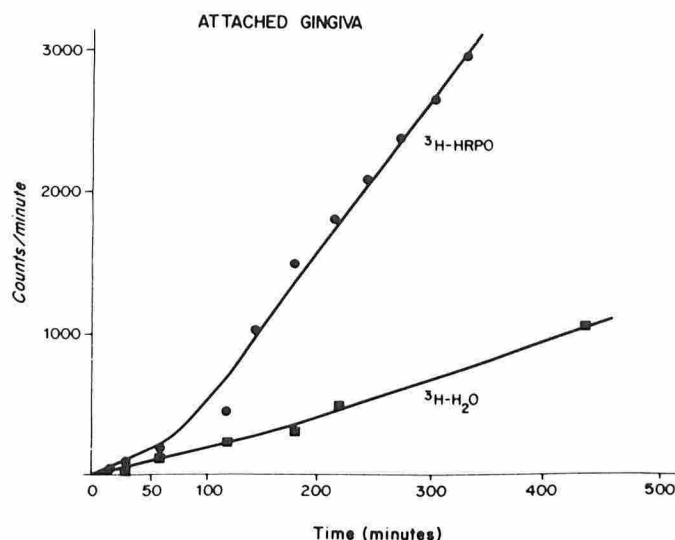


FIG 1. Representative plots of total transfer of isotope with time for both isotopes and one of the oral tissues (attached gingiva).

where Q is the quantity of compound transversing the mucosa; C_o and C_i , the concentration of the compound on the outer (or epithelial) and inner (or connective tissue side) of the specimen, respectively; A is the area of exposed tissue in cm^2 and T is time of exposure in minutes. The units of K_p are thus cm/min .

Some of the tissues were removed from the chambers after 60- to 180-min exposure and incubated with HRPO, as described above, so as to be able to examine the integrity of the permeability barrier microscopically.

To determine whether penetration might represent an active, energy-requiring process, measurements were also made for all tissue regions and compounds in the presence of 0.002% potassium cyanide (KCN) or with the specimen inverted so as to expose the connective tissue to the isotope.

HRPO is a protein that might be degraded if there were proteolytic activity at the mucosal surface. To reduce this possibility, phenyl methyl-sulfonylfluoride (PMSF), a protease inhibitor [15], was added to the solution in the chambers at a concentration of 0.002% in some of the experiments using oral mucosa. To further determine whether the isotope counted in the HRPO experiments remained attached to the protein molecule or had dissociated or become metabolically separated, samples from both sides of the chamber were counted before, and after, passing through a Millipore immiscible molecular separator (Millipore Corp., Bedford, Massachusetts). This retains molecules with a nominal molecular weight greater than 10,000.

Determination of Epithelial and Barrier Thickness

Tracings were made of sections from blocks incubated with HRPO and the epithelial-connective tissue interface, the epithelial surface and the boundary at which HRPO penetration ceased were delineated (Fig 2g). Three nonserial sections from every tissue region of at least 3 animals were traced. The areas of epithelium and of the barrier region that had been traced were measured on a digitizer and from a knowledge of the length of epithelium traced, the mean thickness of epithelium and of the barrier layer was calculated.

Analysis of Data

All comparisons were made using an analysis of variance (ANOVA). Groups showing significant differences at the 5% level were identified using Duncan's multiple range test.

RESULTS

Microscopic Preparations

Fig 2a-d shows routine histologic preparations of the 4 major tissue regions used for the permeability studies; these differ in

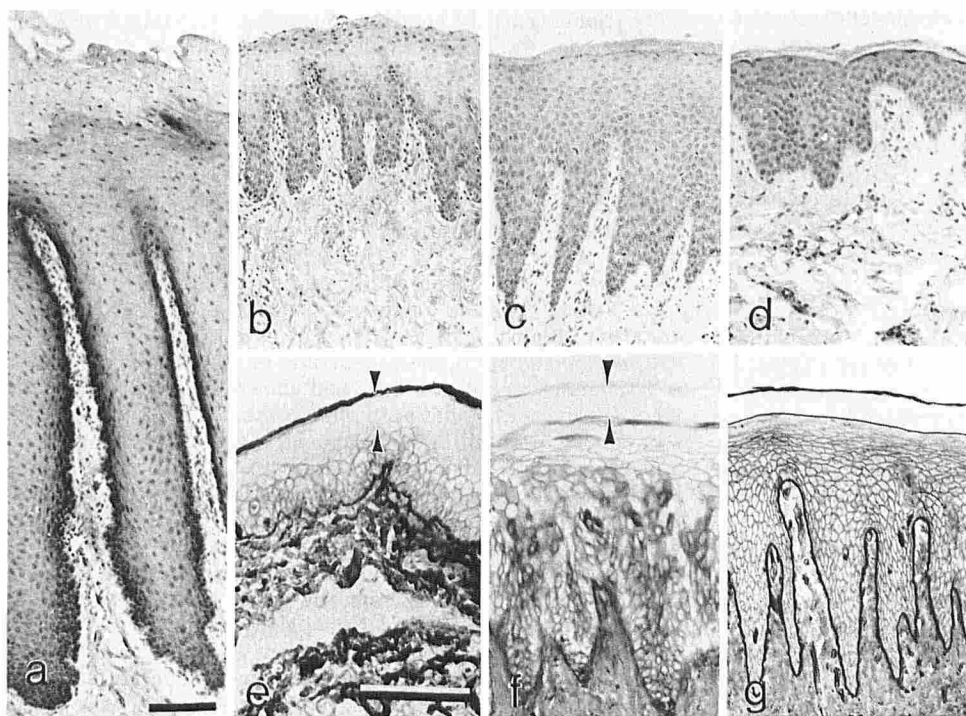


FIG 2. a-d, Histologic preparations of the 4 porcine tissue regions used in the study stained with H & E and reproduced at the same magnification (bar = 50 μm). Note differences in epithelial thickness and keratinization between (a) buccal mucosa, (b) floor of mouth, (c) gingiva, and (d) skin. e-g, Sections prepared from blocks of porcine tissue incubated with HRPO and reacted so as to show the penetration of tracer. All are reproduced at that same magnification (bar = 50 μm). Floor of mouth (e) and gingiva (f) are shown after being mounted in the perfusion chamber. Although HRPO has leaked into many cells, the superficial epithelial barrier (arrows) is intact. (g) shows the method of determining barrier and epithelial thickness in gingiva by tracing the outlines of these regions.

TABLE I. Regional comparisons of permeability

Tissue region	H ₂ O			HRPO		
	Number of animals	Number of experiments ^a	Mean K_p ^b	Number of animals	Number of experiments	Mean K_p ^b
Skin	4	6	131.8 ± 27.1	8	12	60.8 ± 11.8
Gingiva	3	5	279.4 ± 36.1	10	19	222.2 ± 30.1
Buccal mucosa	4	8	450.8 ± 72	6	13	117.6 ± 18.4
Floor of mouth	3	7	753.2 ± 106.8	6	12	430 ± 46

^a Each experiment consisted of 2–5 permeability determinations.

^b ± SEM × 10⁻⁷ cm/min.

^c Values not different from another; all others significantly different ($p < 0.05$).

TABLE II. Regional differences in permeability expressed in terms of a uniform permeability barrier

Tissue region	Thickness (μm ± SEM)		Mean K_p expressed in terms of a uniform barrier 100 μm thick (± SEM × 10 ⁻⁷)			
	Total epithelium	Permeability barrier	H ₂ O		HRPO	
Skin	69 ± 4 ^a	16 ± 1	21.1 ± 4.3	9.4 ± 1.8		
Gingiva	208 ± 9	35 ± 4	98.3 ± 16.0	79.5 ± 11.4		
Buccal mucosa	772 ± 20 ^a	282 ± 17 ^a	173.2 ± 24.6	99.1 ± 10.6		
Floor of mouth	192 ± 7	23 ± 1	1271.3 ± 203.1	331.6 ± 51.9		

^a Thickness significantly different from other tissues ($p > 0.05$).

keratinization and in overall epithelial thickness. Specimens incubated with HRPO after being mounted in perfusion chambers showed some damage in terms of cell leakage in the nucleated layers, but the superficial barrier always retained its integrity (Fig 2e,f).

Effect of Inhibitors, Tissue Inversion, and HRPO Filtration

Values for the permeability constant for each tissue and each compound were compared to those obtained: (a) when the tissue was inverted, (b) in the presence of KCN, and (c) for HRPO, when PMSF was added. An ANOVA showed that, for any tissue and any compound, none of these treatments had a significant effect on the permeability constant.

From the ratio of the counts obtained for labeled HRPO before, and after, passage through the molecular separator it was determined that approximately 60% of the label remained attached to the peroxidase. This proportion differed only slightly between the hot and cold sides of the chambers (mean % of labeled HRPO from 5 determinations was 68.7 and 55.3, respectively) which suggests that there was little, if any, metabolic breakdown of the penetrating HRPO. A similar value was obtained using fresh isotope in the absence of tissue, suggesting that labeled HRPO may not be entirely stable; unfortunately, the manufacturers are unable to specify the stability of this compound.

Regional Differences

Values obtained were compared using an ANOVA according to the tissue region for each labeled compound (Table I). Regardless of the compound, the sublingual mucosa always showed a significantly ($p < 0.05$) greater permeability than any other region and skin showed the lowest permeability. However, for HRPO, buccal mucosa was not significantly more permeable than skin whereas, with water, gingiva did not differ significantly from skin in its permeability.

The tissues used show considerable differences in epithelial thickness and keratinization (Fig 2); measurements of epithelial and barrier thickness made from tracings of sections of tissue exposed to HRPO tracer are shown in Table II. When the total epithelial thickness was compared, epidermis was found to be significantly thinner, and the buccal epithelium significantly thicker, than all other regions. The permeability

barrier of the buccal mucosa was significantly thicker than all other regions.

The permeability constants obtained for the different regions (Table I) were normalized in terms of the thickness of the barrier measured in these regions. This was done by multiplying the values of the permeability constants by the thickness (permeability is inversely related to thickness) and arbitrarily dividing by 100 so as to provide a constant reflecting the permeability of a uniform barrier, 100 μm thick, in each region (Table II). When these values were examined, some interesting differences emerged; the values for skin were still lowest in terms of both water and HRPO, and the difference between skin and the oral mucosa was accentuated. Among the oral regions the values for gingiva, which is keratinized, remained the lowest, but the nonkeratinized floor of mouth epithelium now showed a much lower value than that of the buccal mucosa.

DISCUSSION

It is generally accepted that the movement of substances across the permeability barrier of the skin occurs by diffusion and is not an active process [16]. The results obtained in this study after inverting the oral mucosa or adding metabolic inhibitors suggest that water and HRPO also diffuse across keratinized and nonkeratinized oral tissues. This is in agreement with studies by Siegel on dog and rabbit, although it has been suggested recently that not all substances cross oral mucosa by simple diffusion and there is some limited evidence to support the concept of carrier-mediated transport (facilitated diffusion) [17]. As nonkeratinized tissues tend generally to be more friable than are keratinized tissues, there might be some concern that the former would break down while mounted in perfusion chambers. However examination of nonkeratinized tissue with HRPO after removal from the chamber revealed an intact superficial barrier (see Fig 2b).

The permeability constants derived directly in this study confirm the generally held view that skin is less permeable than oral mucosa, although the keratinized gingival epithelium was not significantly different from epidermis in its permeability to water. Of the oral regions, the floor of the mouth was always significantly more permeable than the other areas. The actual values obtained for the permeability of pig skin to water are lower than those quoted by Galey et al [11] but close to the values reported for human epidermis by Scheuplein [16]; this is reasonable, as pig skin has been shown by other workers to have a permeability similar to human skin [18]. Although there was up to a 5-fold difference between the permeability of skin and oral mucosa, this is less than other published ratios which have ranged from 10- to 100-fold [9–11]. However, no other comparisons have been made on a variety of tissues from the same species under the same conditions.

There are no published values for the permeability of skin or oral mucosa to HRPO although the molecule has been used on numerous occasions as a microscopically demonstrable tracer. In assessing the permeability constants obtained with labeled HRPO it must be remembered that some of the tritium may become separated so that only about 60% of the isotope counted

is still attached to the larger peroxidase molecule. This will tend to overestimate the permeability constants obtained for HRPO, but will not affect the comparisons made between tissues. Nevertheless it is surprising to find HRPO crossing the tissue when histochemical procedures indicate a barrier to this compound. One explanation is the difference in sensitivity between a histochemical reaction utilizing a single section and measurements of isotopic flux across an area of membrane. Furthermore, the histochemical incubations are carried out for 1 h, which corresponds to the lag time determined in our isotope experiments. As might be expected for a large water-soluble substance, the differences in the permeability constants obtained for the various tissues with HRPO tended to parallel those for water but the actual values were less. It is interesting that skin was not significantly different from buccal mucosa in its permeability and this may reflect the restraining effect of an extremely thick, if inefficient (see below), barrier layer on a relatively large, charged molecule.

Comparisons of the permeability constants of various tissues provide an estimate of real differences but offer little insight into the nature of the barrier if there is morphologic variation between them, such as in thickness. Galey et al [11] attempted to standardize their measurements in terms of thickness of the overall mucosa but the use of HRPO provides a means to measure the extent of the barrier to water-soluble substances (and possibly to other compounds [2]). In this way an estimate of permeability per unit of barrier thickness or of the "quality" of the barrier can be obtained. When this was done a very real difference emerged between skin and oral mucosa, including the keratinized gingival region. In attempting to explain this, several possibilities might be considered. There are likely to be variations in partition coefficients and diffusivity between the tissue regions for any given compound that will account for some of the differences in permeability [16,17]. The oral tissues are all bathed in fluid and regions such as buccal mucosa show a greater degree of hydration than does skin, as indicated by dry weight measurements [19]. The permeability of certain oral regions might thus be likened to that of occluded, hydrated skin, which shows a slightly higher permeability than does dry skin [16,20]. On the other hand, orthokeratinized oral regions (such as gingiva and hard palate) show no greater degree of hydration than does epidermis, despite their aqueous environment [19]. However, some clarification is provided by the stereologic data of Schroeder [21] who, using human tissue, has compared the relative volume density of membrane-coating granules in all the regions used in this study. Schroeder showed that not only are membrane-coating granules more abundant in epidermis than in oral epithelia, but that they are more completely discharged and none remains in the superficial cells, as occurs in nonkeratinized oral epithelium.

Finally, the differences in permeability that were revealed between various regions of oral epithelium may reflect regional differences in the type of membrane-coating granule, despite their similar concentrations in oral tissues. The membrane-coating granules of keratinized oral epithelia are identical to those of epidermis [22] whereas those of nonkeratinized tissues have a different morphology, possessing an amorphous core enclosed by a membrane rather than a series of parallel lamellae [23,24]. This difference might be reflected in the nature of the intercellular barrier material produced in these tissues, and it is of interest that our initial results (unpublished) of lipid

analysis of pig buccal epithelium reveal a pattern of glycolipids differing from that present in epidermis and the gingival epithelium.

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