Diffusion Rates and Transport Pathways of Fluorescein Isothiocyanate (FITC)-Labeled Model Compounds Through Buccal Epithelium

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The aim of this study was to characterize transport of FITC-labeled dextrans of different molecular weights as model compounds for peptides and proteins through buccal mucosa. The penetration of these dextrans through porcine buccal mucosa (a nonkeratinized epithelium, comparable to human buccal mucosa) was investigated by measuring transbuccal fluxes and by analyzing the distribution of the fluorescent probe in the epithelium, using confocal laser scanning microscopy for visualizing permeation pathways. The results revealed that passage of porcine buccal epithelium by hydrophilic compounds such as the FITC-dextrans is restricted to permeants with a molecular weight lower than 20 kDa. The permeabilities of buccal mucosa for the 4- and 10-kDa FITC-dextran (of the order of 10^{-8} cm/sec) were not significantly different from each other or from the much smaller compound FITC. The confocal images of the distribution pattern of FITC-dextrans showed that the paracellular route is the major pathway through buccal epithelium.

KEY WORDS: buccal drug delivery; buccal mucosa; fluorescein isothiocyanate-labeled dextrans; confocal laser scanning microscopy; drug transport pathways.

INTRODUCTION

Peroral administration of peptide and protein with the purpose of systemic drug delivery encounters serious obstacles. These potent compounds are often unstable in the gastrointestinal tract and absorb poorly. If partial absorption does take place, their blood concentration will also be reduced by hepatic first-pass elimination. Chronic parenteral administration is undesirable, and alternative delivery routes are being evaluated (1,2). The buccal mucosa has been investigated as a potential site for controlled delivery of macromolecular therapeutic agents, such as peptides, proteins, and polysaccharides (3–5), because of its accessibility and low enzymatic activity. Another interesting advantage is its tolerance (relative to the nasal mucosa and skin) to potential sensitizers (4). Because of the development of peptides and proteins as therapeutic agents, the possibility of utilizing the buccal mucosa for controlled delivery of these drugs is receiving more attention.

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A lack of knowledge about (a) the nature and location of the transport barrier(s) in the buccal epithelium and (b) the transport pathway(s) of macromolecules through this tissue is a major drawback in the assessment of the buccal epithelium as a site for systemic drug delivery (4,6,7). The aim of this study was to investigate the barrier properties of the buccal epithelium and to determine the transport pathways of model compounds across the buccal mucosa. Since it was assumed that the flux rates and transport pathways of permeants would depend strongly on their molecular size (8,9), a series of very hydrophilic FITC-labeled dextrans varying in molecular weight (4, 10, 20, and 40 kDa) was used as model permeants. Dextrans are neutral unbranched polyglucans to which FITC can be covalently attached. The FITC label allows fluorometric detection of the labeled dextrans in permeation studies and is an excellent fluorochrome for confocal laser scanning microscopy (CLSM). FITC was included in the permeation studies to compare the permeation characteristics of a low molecular weight compound (without any glucose units) with those of high molecular weight sugars.

The barrier properties of the epithelium were investigated by measuring transbuccal fluxes of a molecular weight series of FITC-dextrans. The penetration pathways of the fluorescent sugars were studied by visualizing the distribution of the fluorescent probes in the epithelium, using CLSM. Porcine buccal mucosa, which is a nonkeratinized epithelium (like human buccal mucosa but unlike rat or rabbit buccal mucosa), was used as a model tissue because its morphology and permeability are similar to those of human buccal epithelium (10). A schematic representation of the different cells in the buccal epithelium is given in Fig. 1 (11).

MATERIALS AND METHODS

Materials

FITC-dextrans of 4, 10, 20, and 40 kDa, average molecular weights of 4.4, 9.4, 19.6, and 35.6 kDa respectively, were purchased from Sigma (St. Louis, MO). The FITC content ranged from 0.008 to 0.010 mol FITC/mol glucose, determined by Sigma. The FITC-dextrans were used without any further purification steps. FITC was purchased from Sigma.

Fresh buccal tissue from swine (weighing approx. 70–100 kg) was obtained from the Experimental Surgery Department of the University Hospital (Leiden, The Netherlands), from the Institute for Veterinary Research (Zeist, The Netherlands), or from local slaughterhouses. The tissue was removed after the animals were killed by exsanguination or electroshock and immediately transported in Krebs buffer. The period between isolation and experiment was approximately 2 hr.

An isotonic Krebs buffer (pH 7.5) was used for both the *in vitro* penetration and the microscopy studies. All chemicals used were of analytical grade and were dissolved in freshly prepared twice-distilled water.

In Vitro Flux Measurements

The fresh buccal tissue was dermatomed at a thickness

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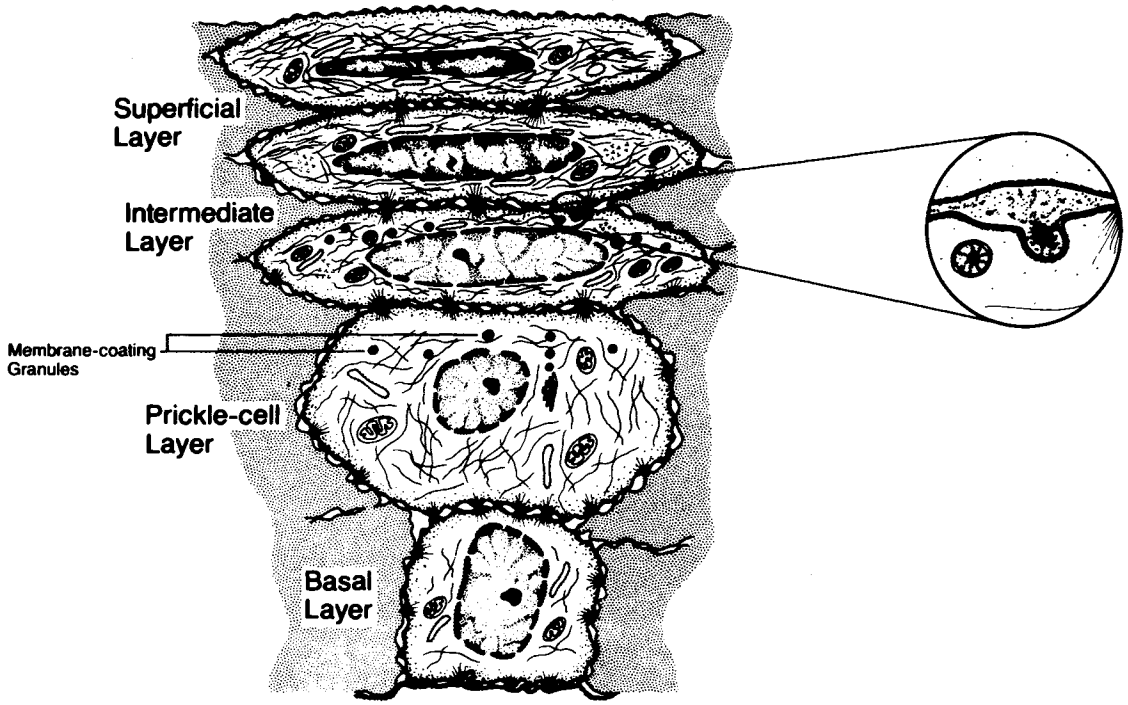

Fig. 1. Schematic representation of buccal epithelium, showing cells in different layers [taken with permission from Wertz and Squier (11)].

of approximately 500 μm to remove the majority of the connective tissue from the epithelium. Buccal epithelium was mounted in Ussing chambers with a diffusion area of 0.75 cm^2 and a compartment volume of 7 mL. Prior to the experiment, the diffusion chambers (with the buccal epithelia mounted between them) were filled with the Krebs buffer (pH 7.5) and placed in a thermostat of 34°C. After an equilibration period of about 0.5 hr, the buffer was replaced with an FITC-dextran or FITC solution (approximately 0.5 m*M* in the Krebs buffer) on the donor (mucosal) side and with fresh buffer on the acceptor (serosal) side. The temperature was maintained at 34°C during the experiment with a water jacket, which also protected the diffusion cells from light, and carbogen (a mixture of 95% O_2 and 5% CO_2) was circulated through both compartments to maintain tissue viability and to provide stirring. Samples were collected from the acceptor compartment at hourly intervals from 0 to 8 hr (1 mL in the case of FITC and 500 μL in the case of FITC-dextran experiments) and from the donor compartment at the beginning and the end of the experiment (50 μL). Evidence is available that porcine buccal mucosa remains viable during this experimental time period (10). The samples were wrapped in foil and stored at 4°C until analysis.

CLSM

Confocal microscopy can be used to obtain a high-quality image from a focal plane below the surface of a specimen, without interference from fluorescence outside of the focal plane. An aperture in front of the detector prevents out-of-focus light from being detected, so that only signals from in-focus points contribute to the image build up by a computer from the points of emitted light. The resulting image is an "optical section." The optical sectioning can be performed with unfixed specimens, so that tissue preparation, embedding, and microtoming procedures, which can result in redistribution of the diffusing agent and tissue damage, are not required (12,13).

The plane of focus can be moved along the *Z* axis by focusing the microscope with a stepper motor. The stack of optical slices obtained in this way forms a three-dimensional picture. An important consideration in CLSM investigations of a thick sample is that the quality of deeper images may be affected by absorption and/or scattering of the excitation and emission light, as well as tissue autofluorescence at those wavelengths. Furthermore, the depth of optical sectioning is ultimately limited by the working distance of the lens used, which can be quite small for high NA lenses, useful in confocal microscopy. In our studies, we used a lens with a working distance of 0.13 mm (see dashed line in Fig. 2A). Buccal epithelium does absorb and scatter light to some degree. In addition, the structures of interest in deeper sections (parallel to the outer surface of the tissue) were less bright and not as well resolved as the superficial ones, making them difficult to interpret. To overcome these limitations and to corroborate results obtained from optical sectioning in intact tissue, we combined confocal optical sectioning with mechanical cross-sectioning.

Samples were prepared by cutting with a clean single-edged razor blade as shown in Fig. 2A. Subsequently, this face of the cut was positioned against the coverslip; in other words, the cross section was rotated 90° (see Fig. 2B). An *X–Y* optical section of the tissue was made below the plane of cut, as indicated by the dashed line in Fig. 2B to avoid interference by fluorescence from damaged cells. This en-

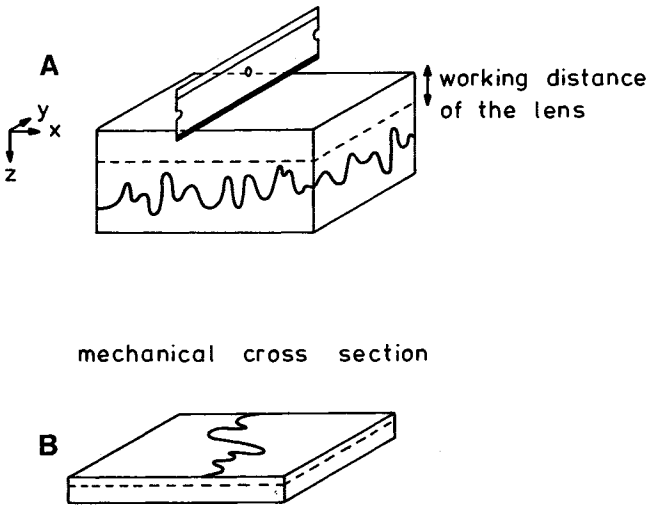

Fig. 2. Mechanical cross section of buccal epithelium. (A) The dashed line displays the working depth of the lens in buccal mucosa. The cross section was made using a razor blade. (B) An image taken below the cutting plane, on the dashed line.

abled us to obtain a true estimation of the distribution pattern of the fluorescent probe, without the attenuation that was evident in the optical sections made parallel to the surface in an intact specimen.

Porcine buccal tissue was incubated in the same way as for the flux experiments, in Ussing chambers with 0.5 m*M* solutions of the various FITC-dextrans, and after a certain period (2, 4, 6, and 8 hr) the tissue pieces were examined in the microscope.

The confocal microscope system used was a BioRad MRC600 confocal unit equipped with an argon-ion laser (excitation lines are 488 and 514 nm) and mounted on a Zeiss IM-35 inverted microscope. The FITC label was detected using the Bio-Rad blue high-sensitivity filter block, which selects the 488-nm laser line to illuminate the specimen and passes emitted light with a wavelength longer than 515 nm. Confocal images were obtained using a Zeiss Plan-Neofluar 25×/0.8 oil/water/glycerin lens (Zeiss, Oberkochen, Germany).

Analytical Procedure for Flux Measurements

FITC-dextrans concentrations were determined with a size exclusion chromatograph (Separon HEMA BioPeek SEC column, Tessex, Aarhus, Denmark) equipped with a fluorescence detector (Shimatzu RF530, Den Bosch, The Netherlands; excitation wavelength, 498 nm; emission wavelength, 520 nm). The eluant used consisted of 20 m*M* NaH_2PO_4 adjusted to pH 9 with 1 *M* NaOH. The system was calibrated using standard solutions of the FITC-dextrans in the Krebs buffer. In the FITC permeability experiments, the sample concentrations were measured using a fluorometer only (Perkin Elmer 3000; same wavelengths as mentioned above), because the fluorescence intensity of FITC is approximately 10 times higher than the fluorescence intensity of the same concentration of the 4- and 10-kDa FITC-dextrans. The fluorometer was calibrated with standard solutions of FITC in the Krebs buffer.

Analysis of Flux Data

Flux data were plotted as the cumulative amount of FITC-dextran that diffused from the mucosal to the serosal side of the epithelium versus time. The permeability coefficient, P, was calculated from the formula

$$P = (dQ/dt)/(\Delta C * A) \quad (1)$$

where dQ/dt is the steady-state slope of the cumulative flux curve, ΔC is the concentration difference across the buccal mucosa, and A (0.75 cm^2) is the effective cross-sectional area available for diffusion. A one-way analysis of variance was used to test for statistical significance of differences.

RESULTS

In Vitro Flux Studies

The detection limit of the assay for all FITC-dextrans used was approximately 10 n*M*. Degradation of the FITC-dextrans (which would result in fractions with a lower molecular weight, or the cleavage of the FITC label) was examined by scanning the chromatogram over an elution time ranging from 0 to 20 min. The retention times of the intact FITC-dextrans ranged between 4 and 5 min. No additional peaks were seen. Furthermore, comparison of the elution profile of the donor solution prior to and at the end of the diffusion experiment revealed no differences. Hence, it was concluded that there are no enzymes acting on dextrans in excised porcine buccal mucosa.

Individual flux curves of the various FITC-dextrans are depicted in Fig. 3. Only FITC and the 4- and 10-kDa FITC-dextrans were able to penetrate the epithelium at a detectable rate. The permeability coefficients were calculated from the steady-state part of the flux curves of all experiments performed. These results are listed in Table I. The permeability of porcine buccal mucosa was $1.12 \pm 0.69 * 10^{-8}$ cm/sec for the 4-kDa FITC-dextran and $2.96 \pm 2.07 * 10^{-8}$ cm/sec for the 10-kDa FITC-dextran. The permeabilities for the 20- and the 40-kDa FITC-dextran were measured but could not be detected. The permeabilities of the 4- and 10-

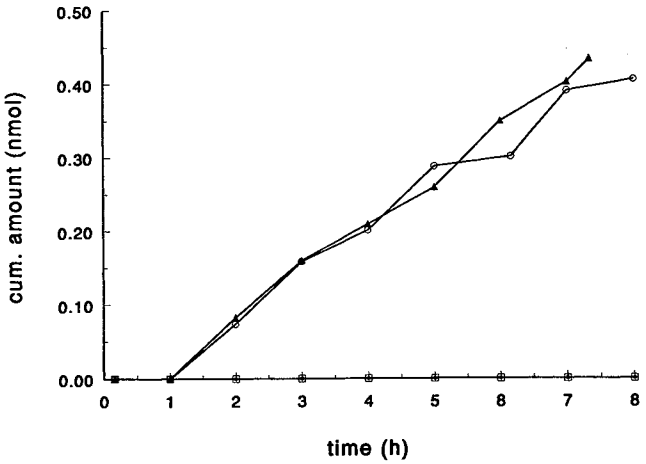

Fig. 3. Typical examples of flux curves of FITC-dextrans: cumulative amount that penetrated porcine buccal mucosa *in vitro* versus time for 4-kDa (▲), 10-kDa (○), 20-kDa (□), and 40-kDa (■) FITC-dextran.

Table I. Permeabilities of Porcine Buccal Mucosa

Compound	M_w (Da)	M_w/M_n[a]	Permeability ($*10^{-8}$ cm/sec)	n[b]
FITC	389	1	3.05 ± 2.77	9
FITC-dextran 4000	4,400	<1.5	1.12 ± 0.69	10
FITC-dextran 10,000	9,400	<1.5	2.96 ± 2.07	7
FITC-dextran 20,000	19,600	<1.25	≤0.05[c]	6
FITC-dextran 40,000	35,600	<1.25	≤0.05[c]	6

[a] The ratio between weight-average molecular weight and number-average molecular weight.
[b] Number of experiments.
[c] The detection limit is $0.05 * 10^{-8}$.

kDa FITC-dextrans were not significantly different from each other or from that of FITC (Fig. 4). However, the scatter in the *in vitro* permeabilities was rather large.

CLSM Studies

Typical examples of two to six CLSM experiments per FITC-dextran per time point are shown in Figs. 5A–E. Each micrograph depicts an optical slice ±25 μm below the surface of a mechanical cross section through buccal epithelium (i.e., an *XY* plane, parallel to the cut edge). The confocal images show the distribution of the labeled dextrans after diffusion of 4-kDa FITC-dextran for 4 hr (A), 4-kDa FITC-dextran for 6 hr (B), 10-kDa FITC-dextran for 4 hr (C), 20-kDa FITC-dextran for 4 hr (D), and FITC for 4 hr (E). All images were averages of 10 scans and were obtained with the same laser intensity, filter block, lens, black level, and scan speed; the images were not enhanced after acquisition.

To investigate the autofluorescence properties of the epithelium, porcine buccal mucosa was incubated in Ussing chambers with Krebs under the same conditions as mentioned above but in the absence of FITC-dextrans. Buccal tissue in combination with Krebs buffer did not produce any fluorescence of its own (see right part of Fig. 5D), when excited either with the 488-nm line of the laser or with the 514-nm line.

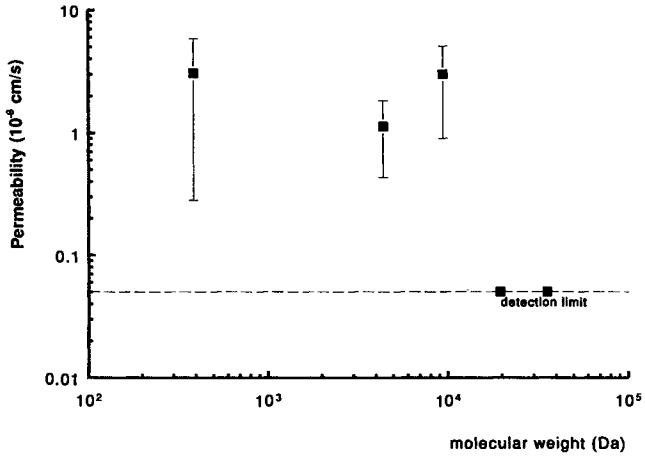

Fig. 4. Mean permeability coefficients of porcine buccal mucosa *in vitro* for FITC and FITC-dextrans versus molecular weights of the permeants. Error bars represent the SD. The dashed line ($0.05 * 10^{-8}$ cm/sec) displays the minimum permeability that could be detected.

The confocal images of the distribution pattern of FITC-dextrans showed that the 4-kDa dextran penetrated the epithelium through the extracellular space (Fig. 5A). After 6 hr a large part of the fluorescent probe was taken up in the basal and prickle cells (Fig. 5B; and for the histology see Fig. 1), i.e., increased fluorescence in the cytoplasm. The 10-kDa dextran, however, was still confined to the extracellular space (Fig. 5C) after the completion of the 8-hr diffusion experiment. The 20-kDa dextran was not able to penetrate the buccal mucosa (Fig. 5D).

As far as free FITC was concerned, the micrograph taken after 4 hr (Fig. 5E) revealed the presence of the dye both inside and outside the cells in almost all strata of the epithelium. FITC was able to pass the cell membranes of all cells in the epithelium. FITC seemed to be distributed equally over the cytoplasm of the cell, while the intensity in the cell nuclei is very low.

DISCUSSION

FITC-dextrans over a range of molecular weights were chosen as model compounds for high molecular weight hydrophilic drugs. As apparent from both the kinetic and the microscopic studies, the labeled dextrans with a molecular weight of 4 and 10 kDa were able to penetrate the buccal epithelium, whereas the larger, 20- and 40-kDa FITC-dextrans were not. The limiting molecular weight for these compounds was not established but appears to lie between 10 and 20 kDa. This indicates that hydrophilic macromolecules with molecular weights up to 10 kDa may be able to pass the epithelial barrier of the buccal mucosa. The permeabilities measured in the flux experiments for the 4- and the 10-kDa dextrans were not significantly different from each other and were in the same range as that observed for the low molecular weight fluorescent dye FITC.

The permeability coefficients found in the present study (in the range of 10^{-8} cm/sec) are of the same order of magnitude as reported for buccal transport of hydrophilic compounds such as 4-kDa FITC-dextran (14), the tripeptide thyrotropin releasing hormone (15), and an ACE inhibitor (16). Although the total flux across buccal epithelium is rather low, buccal penetration of such macromolecules would probably be enhanced by coadministration of absorption enhancers such as bile salts (17).

The results obtained with the CLSM showed that during the first 4 hr, the 4- and 10-kDa FITC-dextran both penetrated the buccal epithelium via the extracellular space. This observation corresponds with the 2-hr treatment of rabbit buccal mucosa with 4-kDa FITC-dextran by Harris and Robinson (14). In our study, after 6 hr of application, the smallest dextran, M_w = 4 kDa, was taken up by the cells in the basal layer and in the prickle cell layer; this was not found for the 10-kDa FITC-dextran. It can be excluded that the intracellular uptake of the 4-kDa FITC-dextran is a consequence of cell membrane damage, because in that case the 10-kDa FITC-dextran would also pass the cell membrane. An active uptake mechanism, specifically for the 4-kDa and excluding the 10-kDa fraction, is even more unlikely. However, noting that FITC (the smallest compound) is taken up in all epithe-

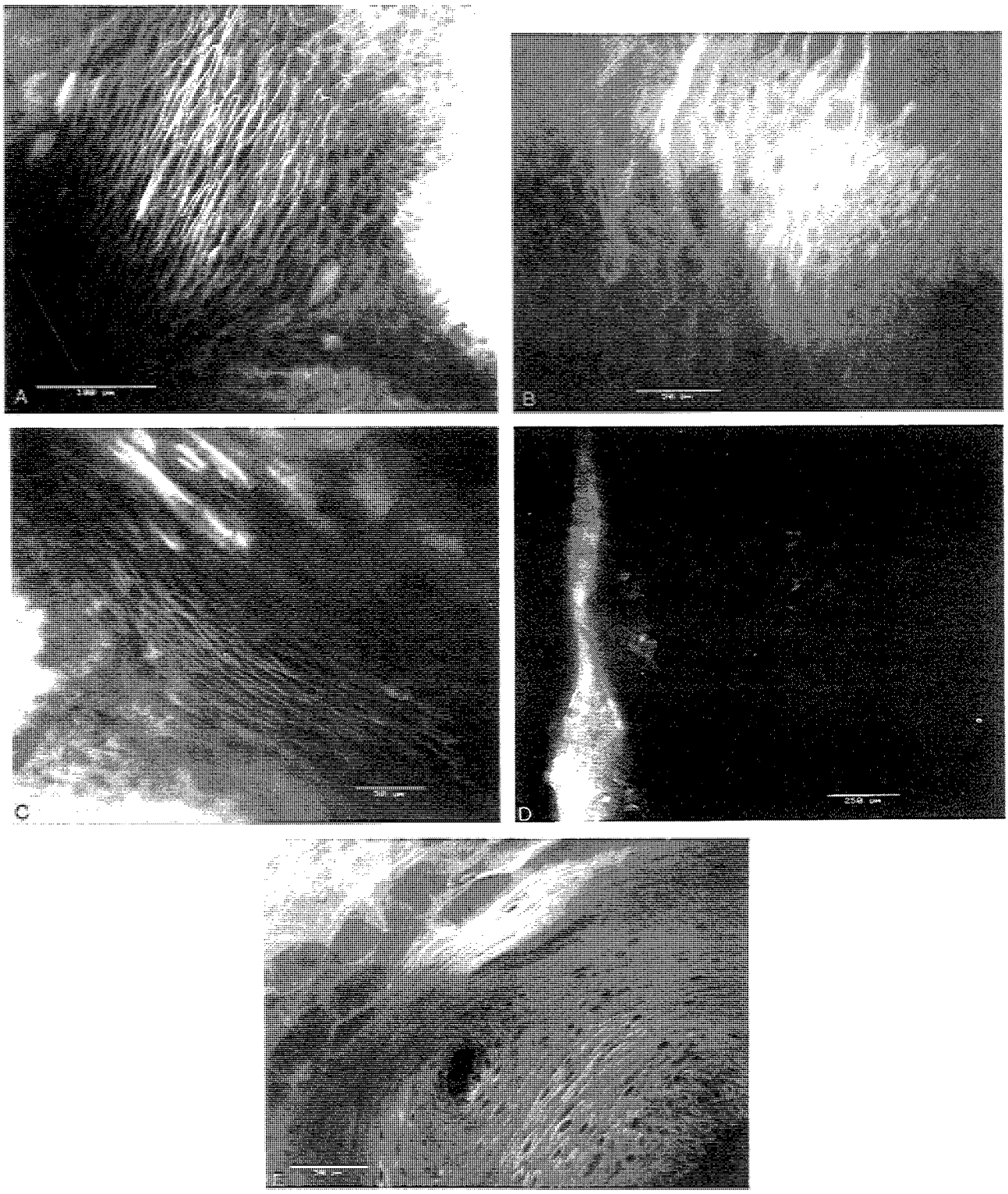

Fig. 5. Confocal micrographs of mechanical cross sections through porcine buccal epithelium illustrating the penetration of 4-kDa FITC-dextran for 4 hr (A), 4-kDa FITC-dextran for 6 hr (B), 10-kDa FITC-dextran for 4 hr (C), 20-kDa FITC-dextran for 4 hr (D), and FITC for 4 hr (E).

lial cells, we may come up with a much more likely explanation: The observed trend (no intracellular uptake of 10 kDa, uptake of 4 kDa only in basal and prickle cells, and uptake of FITC in all cells) might imply that the size constraint of cell membrane passage strongly depends on the level of cell differentiation, in the sense that closer to the basal layer of the epithelium, passage of the cell membrane apparently can be accomplished by larger molecules than

closer to the distal cell layers of the epithelium. The lack of intracellular uptake of the 4- and 10-kDa FITC-dextrans in the upper layers may be related to specific events occurring during cell differentiation. As cells migrate toward the upper prickle cell layer, an extensive network of filaments appears in the cytoplasm of the cells (18). This and the thickening of the cell membrane might obstruct the uptake of dextrans in the superficial and intermediate layers of the buccal epithelium.

The present observation that the permeability of 4-kDa FITC-dextran is not significantly different from the permeability of 10-kDa FITC-dextran, as measured by flux measurements, suggests that the uptake of 4-kDa FITC-dextran in basal and prickle cells, occurring long after steady state is reached, does not influence the steady-state transport rate through the epithelium. If there is a transcellular pathway for these compounds, pronounced differences in permeabilities are to be expected. This does not exclude the possibility that the time-dependent redistribution of material from the intercellular to the intracellular domain may play a role in the initial (non-steady-state) phase of the transport event. Hence, we conclude that the hydrophilic compounds investigated appear to penetrate the buccal epithelium via the paracellular pathway. Moreover, even the smaller permeant FITC does not penetrate significantly faster through the epithelium, although this compound is able to pass all epithelial cell membranes. Even when only the paracellular pathway is involved, differences in molecular weight are expected to give rise to changes in permeability. From reports studying the nasal and pulmonary epithelium, consisting of only one cell layer, and the blood–brain barrier, one layer of endothelial cells, it was concluded that an increase in the molecular weight of the FITC-dextrans causes a decrease in permeability (19–21). In addition, using the theoretical formulae for predicting epithelial permeability developed by Potts and Guy (9), the logarithm of P/K (P = permeability; K = partition coefficient) of permeants across an epithelium linearly decreases with increasing molecular volume. Comparing the 4-kDa FITC-dextran with the 10-kDa FITC-dextran, one would expect the highest molecular weight [i.e., molecular volume (21)] permeant to have the lowest permeability. However, the results from this study disagree with the proposed theory, possibly due to the complexity of the multilayered, highly differentiated buccal epithelium and the inhomogeneity of the intercellular material.

The size-restricted passage across buccal epithelium, observed both in the flux experiments and in the confocal examinations, could be explained by the size limitations of the permeation pathway, being the extracellular space. The molecular volume of the 20-kDa FITC-dextran presumably prohibits its diffusion between cells of the buccal epithelium.

The results of this study are completely in agreement with those of Squier and Lesh (22) and Dowty *et al.* (15) showing the intercellular compartment as the major pathway in buccal drug transport. The observation that the 20-kDa FITC-dextran does not permeate into the epithelium suggests that the superficial cells present a barrier layer. Direct evidence for the involvement of the basal lamina in the barrier properties (7) cannot be extracted from the present findings. To obtain more information on the location of the transport barrier inside the buccal epithelium, time-resolved visualization studies are currently being executed.

To increase the permeability for buccal drug transport, it is necessary to investigate the intercellular contents of the buccal epithelium in detail and the effects penetration enhancers may have on the intercellular material. These studies are now in progress in our laboratory.

In conclusion, passage of porcine buccal epithelium by hydrophilic compounds such as the FITC-dextrans is restricted to permeants with a molecular weight lower than 20 kDa. Below this size limit, there appears to be no relation between the degree of transport across porcine buccal mucosa and molecular weight of the FITC-dextrans. The major transport pathway of these high molecular weight, hydrophilic compounds through porcine buccal epithelium is the paracellular route.

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