Biochem. J. (1989) 261, 887–896 (Printed in Great Britain)

Oral mucosal pellicle

Adsorption and transpeptidation of salivary components to buccal epithelial cells

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The present investigation was carried out to examine the mechanism(s) whereby salivary molecules interact with human buccal epithelial cells. By utilizing antiserum against human parotid saliva, selected salivary components were detected by electrophoretic-transfer analysis of 1.5% SDS extracts of epithelial cells. Incubation of the cells and their aqueous cell-free extracts with ^{125}I-labelled parotid saliva resulted in the formation of an iodinated high-molecular-mass complex which was not present in ^{125}I-labelled saline alone. Formation of this complex was time-dependent and was inhibited by treating the buccal epithelial cells or their cell-free extracts with EGTA, iodoacetamide, *N*-ethylmaleimide or by heating at 100 °C for 15 min. The epithelial cells also promoted incorporation of [^{14}C]putrescine into high-molecular-mass complexes whose formation was inhibited by iodoacetamide, unlabelled putrescine and EGTA. Cell extracts mediated cross-linking of monodansylcadaverine into α-casein, and this interaction was inhibited by iodoacetamide. Significant amounts of radioactivity were recovered with the epithelial-cell envelopes after exhaustive extraction of ^{125}I-saliva- or [^{14}C]putrescine-treated epithelial cells with 4% (w/v) SDS/10% (v/v) β-mercaptoethanol. The incorporation of radioactivity into epithelial-cell envelopes was inhibited by pretreatment of the cells with putrescine, EGTA, iodoacetamide or heating at 100 °C for 15 min. These data suggest that: (1) oral mucosal pellicle is formed by the selective adsorption of saliva to the epithelial-cell plasma membrane and its associated cytoskeleton; and (2) the adsorbed salivary components may be cross-linked to each other or the epithelial cytoskeleton by epithelial transglutaminases.

INTRODUCTION

Salivary pellicle is a film which coats oral surfaces and functions as a moisture retainer, a protective barrier, a lubricant and a determinant for microbial colonization (Gibbons & Qureshi, 1976, 1978; Edwards, 1978; Moreno & Zahradnick, 1979; Gibbons & van Houte, 1980; Gibbons *et al.*, 1982; Stinson *et al.*, 1982; Tabak *et al.*, 1982; Hatton *et al.*, 1985; Levine *et al.*, 1985, 1987*a*,*b*; Bergey *et al.*, 1986). Previous studies suggest that pellicle is a multilayered film which is formed initially by the selective adsorption of salivary molecules to oral surfaces (Mayhall, 1970; Sonju & Rolla, 1973; Juriaanse *et al.*, 1981; Al-Hashimi & Levine, 1989) followed by homo- or hetero-typic complexing of these molecules with other molecules in the ambient saliva (Juriaanse *et al.*, 1981; Tabak *et al.*, 1982; Levine *et al.*, 1985, 1987*a*,*b*).

Salivary components which adsorb to oral mucosal epithelial cells (Bradway *et al.*, 1985; Erickson, 1985) comprise the mucosal pellicle. The forces which mediate the interactions between salivary molecules and the epithelial-cell surface most likely include non-covalent interactions involving electrostatic and hydrophobic forces. Preliminary data from our laboratory, however, suggest that these interactions may also be mediated by covalent bonds (Bradway *et al.*, 1986). Previous workers suggested that the surface of mucosal squames results from a metamorphosis in which the plasma membrane is simultaneously lined by the synthesis of an inner protein matrix and partially hydrolysed by phospholipases (Goldsmith, 1983; Gerson & Harris, 1984). The matrix, termed the 'epithelial-cell envelope' (Matoltsy & Matoltsy, 1966; Goldsmith, 1983), is synthesized by epidermal transglutaminase (protein-glutamine γ-glutamyltransferase, EC 2.3.2.13). Transglutaminases function through a double-displacement mechanism in which the γ-carboxamide group of an endoglutamine reacts with the enzyme thiol active site to yield a thioester bond and free NH_3. Subsequently the enzyme binds a primary amine which effects aminolysis of the thioester intermediate and participates in an amide bond with the glutamine residue (Williams-Ashman & Canellakis, 1980). Amide-bond (cross-link) formation is primarily between endoglutamine and lysine residues of cytosolic proteins; however, epidermal transglutaminase is capable of utilizing non-epithelial proteins and polyamines such as putrescine for substrates (Ogawa & Goldsmith, 1976). This suggests that the surface of oral epithelial squames may be characterized by a partially denuded protein matrix replete with an associated transpeptidase which may cross-link salivary proteins during mucosal-pellicle formation.

The purpose of the present study was to determine if salivary molecules can be covalently coupled to epithelial surfaces and to explore the mechanisms of these interactions. We demonstrate the apparent covalent incorporation of ^{125}I-parotid saliva and [^{14}C]putrescine into high-molecular mass complex(es) in the presence of

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; simulated salivary buffer, 0.021 M-Na_2HPO_4/NaH_2PO_4, pH 7.3, containing 36 mM-NaCl and 0.96 mM-$CaCl_2$; solubilizing buffer, 0.125 M-Tris/HCl, pH 6.8, containing 4% (w/v) SDS, 20% (v/v) glycerol and 10% (v/v) β-mercaptoethanol; PAGE, polyacrylamide-gel electrophoresis; DTT, dithiothreitol.

buccal epithelial cells. This reaction was inhibited by EGTA, iodoacetamide, heat inactivation and putrescine, thereby suggesting that salivary–epithelial-cell interactions may be catalysed by epithelial transglutaminases.

EXPERIMENTAL

Materials

The following reagents were purchased from the indicated sources: [^{14}C]putrescine (104.6 mCi/mmol) and En^{3}Hance were obtained from New England Corp. (Boston, MA, U.S.A.). Liquiscint scintillation cocktail was obtained from National Diagnostics (Somerville, NJ, U.S.A.). Glucose-oxidase-conjugated affinity-purified goat anti-rabbit IgG was supplied by Cooper Biomedical (Malvern, PA, U.S.A.). Chloramine-T, monodansylcadaverine, DTT, guinea-pig liver transglutaminase (2.3 units/mg of protein), α-casein, *N*-ethylmaleimide, EDTA trisodium salts, PMSF, SDS, phenazine methosulphate, Coomassie Brilliant Blue R250, and Nitro Blue Tetrazolium were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Intensifying screens for autoradiography (Cronex Lightning-Plus EB) were obtained from Du Pont (Wilmington, DE, U.S.A.) and X-Omat radiographic film, EGTA, 2-iodoacetamide, and β-mercaptoethanol were supplied by Eastman Kodak Co. (Rochester, NY, U.S.A.). Nitrocellulose blotting membranes and the Transblot transfer cell were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Sephadex G-25, Sepharose CL-2B and electrophoresis calibration kits containing phosphorylase-*b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), α-lactalbumin (14.4 kDa) were purchased from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A). *N*-Succinimidyl-3,4-hydroxyphenyl propionate was from Pierce Chemical Co. (Rockford, IL, U.S.A.) and Na^{125}I (13–17 mCi/μg) was purchased from Amersham International (Arlington Heights, IL, U.S.A.). All other reagents were obtained from either J.T. Baker (Philipsburg, NJ, U.S.A.) or Fisher Scientific Co. (Rochester, NY, U.S.A.).

General chemical and analytical methods

Total protein was determined by the Lowry *et al.* (1951) procedure, with bovine serum albumin as a standard. Polyacrylamide-gel electrophoresis was performed by the method of Laemmli (1970) in a Mini-Gel apparatus (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). Molecular masses were calculated from plots of the log(molecular mass) versus relative mobility of standard proteins.

Collection of parotid saliva and preparation of rabbit anti-(parotid saliva) antiserum

Human parotid saliva was collected from a 37-year-old healthy male donor using a Carlsen–Crittenden apparatus (Curby, 1953) and 2% (w/v) citric acid as a gustatory stimulus. The saliva contained ≈ 2.5 mg protein/ml and was either used directly (fresh) or after resuspension from a freeze-dried form. When saliva was to be freeze-dried, approx. 30 ml was collected into a chilled tube containing a 'cocktail' which inhibits proteinase activity [2.5 ml of cold 0.1 M-Tris/HCl, pH 7.5, with 2% (w/v) Na_2EDTA, 10% (v/v) propanol and 0.2 mM-PMSF]. This mixture was then filtered through glass wool, dialysed against 10 vol. of cold 2% Na_2EDTA, dialysed extensively against cold distilled water, then freeze-dried.

Rabbit anti-(parotid saliva) antiserum was prepared by immunizing a single New Zealand White rabbit as previously described by Levine & Ellison (1973) and Shomers *et al.* (1982). Briefly, parotid saliva was emulsified at a concentration of 1 mg/ml in a solution containing 0.5 ml of phosphate-buffered saline (0.01 M-Na_2HPO_4/NaH_2PO_4, pH 7.5, containing 0.154 M-NaCl) and 0.5 ml of Freund's complete adjuvant and injected subcutaneously in the flank three times at 2-week intervals. The antibody production was determined by immunoelectrophoresis, and the immune serum was obtained from whole blood after clotting initially for 1 h at room temperature and then at 4 °C overnight. Potential blood-group activity was removed by three sequential adsorptions (4 °C for 1 h) of antiserum with equal volumes of freshly washed and packed red blood cells from the parotid-saliva donor (Issitt, 1972). The completeness of adsorption (absence of agglutination) was determined by microscopic examination after 1 h incubation at room temperature. After adsorption, the serum was divided into portions and frozen at −20 °C until further use. In the following text this preparation will be designated 'anti-salivary antiserum'.

Collection of buccal epithelial cells and detection of salivary-derived mucosal pellicle

All experiments were performed in the morning without prior eating or routine oral hygiene for at least 8 h. Initially, the buccal mucosa of the parotid-saliva donor was rinsed three times with distilled water and approx. 1×10^6 cells were collected by scraping with a dull metal spatula in 1.5 ml of ice-cold 'simulated salivary buffer' (0.021 M-Na_2HPO_4/NaH_2PO_4, pH 7.3, containing 36 mM-NaCl and 0.96 mM-$CaCl_2$; Bennick & Cannon, 1978). The cell suspension was divided into two equal aliquots and one aliquot was then vigorously washed three times by vortex-mixing and centrifugation (13000 ***g*** for 4 min at room temperature) in 1.0 ml of simulated salivary buffer. The total time for these procedures was less than 20 min. Both epithelial-cell pellets (washed and unwashed) were then extracted for 30 s at room temperature in 40 μl of 1.5% SDS in distilled water. The extracts were then diluted 1:1 in 'solubilizing buffer' (0.125 M-Tris/HCl, pH 6.8, containing 4% SDS, 20% glycerol, and 10% β-mercaptoethanol), heated for 2 min at 100 °C and examined by SDS/10%-PAGE. Electrophoretic transfer of antigens from SDS/PAGE gels was carried out by the methods of Burnette (1981) and Towbin *et al.* (1979) in a transblot transfer cell. The nitrocellulose blots were incubated in blocking buffer (0.01 M-Na_2HPO_4/NaH_2PO_4, pH 7.2, containing 0.154 M-NaCl, and 0.05% Tween-20) for 1 h and incubated with a 1:750 dilution of rabbit anti-saliva antiserum in blocking buffer overnight. Antigen–antibody complexes were then allowed to react with a 1:1000 dilution of glucose-oxidase-conjugated affinity-purified goat anti-rabbit IgG (in blocking buffer containing 0.1% merthiolate) for 4 h. The nitrocellulose was washed in three changes of blocking buffer (100 ml) at room temperature and antigens revealed by utilizing 0.326 mM-phenazine methosulphate, 2.45 M-Nitro Blue Tetrazolium and 41.7 mM-dextrose in 0.1 M-Na_2HPO_4/NaH_2PO_4

buffer, pH 6.9. Non-immune rabbit serum (diluted 1:750) and glucose-oxidase-conjugated affinity-purified goat anti-rabbit IgG (diluted 1:1000), alone, were used as negative controls. In additional experiments, human foot callus (4 mg) was pulverized under liquid nitrogen and then extracted in 40 μl of 1.5% SDS in distilled water for 30 s at room temperature for use as a source of epithelial-cell extracts which had not been exposed to saliva. The results of these experiments are shown in Fig. 1 (below). Portions of electrophoretic gels containing molecular-mass standards were removed and stained with Coomassie Brilliant Blue before electrophoretic transfer.

Preparation of ^{125}I-parotid saliva

Initially, parotid saliva was iodinated by the method of Greenwood *et al.* (1963) for use as a tracer during subsequent preparative procedures. Briefly, 100 μg of saliva was resuspended in 100 μl of 0.1 M-K_2HPO_4/KH_2PO_4, pH 7.4, and iodinated at room temperature by the addition of 1 mCi of $Na^{125}I$ (in 10 μl of 0.01 M-NaOH) and 5 μl of chloramine-T (0.8 mg/ml in distilled water). The reaction was stopped after 1 min by the addition of 5 μl of sodium metabisulphite and 5 μl of KI (both at a concentration of 2 mg/ml in distilled water) and the ^{125}I-saliva was desalted on a column (1 cm × 10 cm) of Sephadex G-25 (fine grade) equilibrated and eluted (0.5 ml fractions) with distilled water. Elution of the column was monitored by γ-radiation counting, and fractions containing ^{125}I-parotid saliva (specific radioactivity 0.072 μCi/μg of protein) were stored at 4 °C for later use.

For preparative procedures, the following method of iodination was used to label both lysine and tyrosine residues. Saliva (1 mg) was resuspended in 200 μl of 0.2 M-borate buffer, pH 8.6, and transferred to 1.5 ml polypropylene tubes coated with 100 μl of *N*-succinimidyl-3,4-hydroxyphenyl propionate (1 mg/ml in benzene). The reaction mixture was incubated for 30 min on ice and the reaction was repeated twice more by transferring the saliva preparation to tubes prepared in the same manner. After the addition of 1 μg of previously iodinated saliva (0.072 μCi/5 μl) as a tracer, the mixture was desalted at room temperature by repeated (twice) passage through columns (1 cm × 10 cm) of Sephadex G-25 (fine) equilibrated with distilled water. Fractions (0.5 ml) were monitored for radioactivity with a Beckman model 5500 γ-radiation counter. The *N*-succinimidyl-3,4-hydroxyphenyl propionate-derivatized saliva (815 μg of protein) was freeze-dried and dissolved in 0.1 M-K_2HPO_4/KH_2PO_4, pH 7.4. This material was iodinated with 1 mCi of $Na^{125}I$ by the method of Greenwood *et al.* (1963) and desalted by one passage through Sephadex G-25 as described above. Fractions containing the iodinated saliva were pooled and stored at 4 °C. The ^{125}I-parotid saliva (which will hereafter be designated '^{125}I-saliva') had a specific radioactivity of 0.380 μCi/μg of protein.

Characterization of buccal-epithelial-cell–salivary interactions

In these experiments, a whole-cell preparation of buccal epithelial cells or an aqueous cell-free extract of these cells, were treated with ^{125}I-saliva. For experiments utilizing whole cells, four samples containing approx. 5×10^5 buccal epithelial cells were collected in simulated salivary buffer, transferred to 1.5 ml polypropylene tubes and centrifuged at 13000 *g* for 4 min at room temperature. The first sample, representing a zero-time incubation, was resuspended in 40 μl of reaction buffer containing final concentrations of 0.05 M-Tris/HCl, pH 7.5, with 20 mM-$CaCl_2$ and 10 μl (0.760 μCi) of ^{125}I-saliva. These cells were washed immediately in 1 ml of simulated salivary buffer, centrifuged for 4 min at 13000 *g* and then frozen. A second sample was treated as above, but incubated for 4 h at 37 °C (4 h incubation). The remaining two samples were pretreated for 15 min in the reaction buffer used above either by heating at 100 °C or by incubation with a final concentration of 20 mM-iodoacetamide at 37 °C before the addition of ^{125}I-saliva. ^{125}I-saliva (10 μl) was combined with 40 μl of 0.05 M-Tris/HCl, pH 7.5, containing 20 mM-$CaCl_2$ and incubated for 4 h at 37 °C to act as a negative control. After 4 h incubation, the cells were pelleted at 13 000 *g* for 4 min, washed once in 1 ml of simulated salivary buffer and then extracted (including the zero-time incubation) in 30 μl of solubilizing buffer at room temperature for 30 s. Solubilized samples were recovered by centrifugation at 13000 *g* for 4 min and heated at 100 °C for 2 min. Equal amounts of radioactivity either from the extracts or negative controls were examined by SDS/10%-PAGE, and the gels were dried for autoradiography. Autoradiography was performed at −70 °C utilizing Kodak X-Omat AR radiographic film and a high-speed calcium tungstate intensifying screen. The results are shown in Fig. 2 (below).

For the preparation of epithelial-cell extracts, approx. 3×10^6 epithelial cells were incubated, with gentle stirring (50 rev./min with a Teflon stir bar in a V-bottomed reaction vial), in 1 ml of 0.05 M-Tris/HCl, pH 7.5, containing 0.2 mM-PMSF for 18 h at 4 °C. After recovery by centrifugation (room temperature, 13000 *g*), the supernatant (extracts) (0.35 mg of protein/ml as determined by the Lowry method) was transferred, in five 200 μl aliquots, to 1.5 ml polypropylene tubes. The first two samples were each pretreated by the addition of 20 mM-$CaCl_2$, and one of these samples was heated at 100 °C for 15 min. The remaining samples were pretreated by 20 mM additions of either EGTA, iodoacetamide or *N*-ethylmaleimide. ^{125}I-saliva (0.760 μCi/10 μl) was then added to all of the samples, and these were incubated at 37 °C for 18 h. After incubation, aliquots of each of the extract samples were combined (1:1) with 15 μl of solubilizing buffer, heated at 100 °C for 15 min, and then examined by SDS/10%-PAGE, followed by autoradiography (24–48 h exposures). The results are presented in Fig. 3 (below).

Isolation of high-molecular-mass salivary complexes

Gel-filtration chromatography was utilized to isolate the high-molecular-mass salivary complexes formed from the interaction between buccal epithelial cells and ^{125}I-saliva constituents. Epithelial cells (approx. 3×10^6) were harvested with a metal spatula and collected in 3 ml of ice-cold simulated salivary buffer, followed by centrifugation at 4 °C at 13000 *g*. The cell pellet was resuspended in 500 μl of 0.05 M-Tris/HCl, pH 7.5, containing 20 mM-$CaCl_2$ and 17 μCi of ^{125}I-saliva. The cells were incubated for 4 h at 37 °C, recovered by centrifugation at 13000 *g* and extracted twice for 5 min at 100 °C with 500 μl of solubilizing buffer. The extracts (containing 3.74 μCi of ^{125}I-saliva) were recovered by

centrifugation for 4 min at 13000 *g* at room temperature and applied to a column (1.5 cm × 120 cm) of Sepharose CL-2B equilibrated in 0.1 M-NH_4HCO_3, pH 7.8, containing 0.1% SDS and 0.05% NaN_3. Fractions (1.5 ml) were eluted in equilibrating buffer at a flow rate of 10 ml/h and monitored by γ-radiation counting of 50 μl aliquots. ^{125}I-saliva alone (3.73 μCi) was heated in 1 ml of solubilizing buffer at 100 °C for 5 min and eluted from the same column under the same conditions. Fractions containing ^{125}I-labelled components were pooled, and aliquots from each pool ($\approx$ 10000 c.p.m.) were heated at 100 °C for 5 min in solubilizing buffer and then analysed by SDS/10% PAGE–autoradiography (Fig. 4 below).

Detection of primary-amine incorporation into proteins of buccal-epithelial-cell preparations

Primary-amine incorporation into proteins of whole buccal epithelial cells was detected by using SDS/PAGE–fluorography to monitor [^{14}C]putrescine incorporation in epithelial-associated proteins. Epithelial cells were collected as described above and transferred in six samples of approx. 5×10^5 cells at room temperature to 1.5 ml polypropylene tubes. The cells were pelleted by centrifugation at 13000 *g* for 4 min at room temperature, and three samples were resuspended to 30 μl of a reaction mixture containing (final concns.) 0.05 M-Tris/HCl, pH 7.5, 1 mM-EDTA, 1 mM-DTT, 20 mM-$CaCl_2$, and 10 μl of [^{14}C]putrescine (104.6 nmol; 104.6 mCi/mmol) was added at various times. [^{14}C]Putrescine was immediately added to one sample which was incubated for 8 h at 37 °C (the experimental sample). The other two samples were either heated to 100 °C for 15 min before the addition of [^{14}C]putrescine (a heat-inhibited sample) or quenched with 40 μl of 1.0 M-putrescine immediately after the addition of [^{14}C]putrescine and stored at 4 °C (a zero-time incubation). The remaining three samples were treated (before the addition of [^{14}C]putrescine) for 15 min at 37 °C with reaction buffer in which final concentrations of either 20 mM-EGTA, 20 mM-iodoacetamide or 100 mM unlabelled putrescine were substituted for $CaCl_2$. Freeze-dried saliva (prepared as previously described) was reconstituted to 1.87 mg of protein/ml in 40 μl of 0.05 M-Tris/HCl (pH 7.5)/1 mM-EDTA/1 mM-DTT, 20 mM-$CaCl_2$ and 104.6 nmol of [^{14}C]-putrescine (104.6 mCi/mmol per 10 μl) and incubated at 37 °C for 8 h to serve as a negative control. After incubation the samples were all extracted as described for buccal-epithelial-cells–^{125}I-saliva experiments, and equal volumes of extracts or negative-control reaction mixture were subjected to SDS/10%-PAGE. The gels were then treated with En^{3}Hance, according to manufacturer's instructions, dried, and fluorographs were exposed at −70 °C for 48–72 h (Fig. 5, below).

For the detection of transglutaminase activity in cell-free extracts, fluorescence spectrophotometry with monodansylcadaverine as a substrate was carried out as described by Lorand *et al.* (1971). The extract was prepared as described above in 1 ml of 0.05 M-Tris/HCl, pH 7.5, containing 0.2 mM-PMSF, recovered by centrifugation at 13000 *g*, freeze-dried and reconstituted with 200 μl of 0.05 M-Tris/HCl, pH 7.5, containing 20 mM-$CaCl_2$, 2 mM-DTT and 6 mM-NaCl. One half of this preparation (100 μl) was then diluted with 600 μl of the same buffer, resulting in a reaction mixture containing 0.085 M-Tris/HCl, pH 7.5, with 20 mM-$CaCl_2$, 2 mM-DTT, 6 mM-NaCl, 40 mM-α-casein and 2×10^{-5} M-monodansylcadaverine. The reaction was carried out at 37 °C, and increases in relative fluorescence intensity accompanying monodansylcadaverine incorporation into α-casein were monitored by utilizing a Perkin–Elmer 650-40 fluorescence spectrophotometer equipped with a Kipp and Zonen (strip graph) recorder. The reaction mixture was excited at 360 nm and fluorescent emission was monitored between 400 and 650 nm at 1 h intervals. Both excitation and emission slits were set at 10 mm. The remaining aliquot of cell extract was incubated under the same conditions in a reaction mixture containing 20 mM-iodoacetamide. Liver transglutaminase (0.005 unit) was used as a positive control in both sets of experiments. All reaction spectra were compensated for background and/or solvent emission utilizing reaction mixtures containing only monodansylcadaverine or α-casein. The results are shown in Fig. 6.

Interactions of ^{125}I-saliva and [^{14}C]putrescine with epithelial-cell envelopes

These experiments were performed to determine whether ^{125}I-saliva or [^{14}C]putrescine interact with the epithelial-cell envelope and whether such interactions were epidermal-transglutaminase-mediated. Six triplicate sets of cells ($\approx 1.7 \times 10^5$ cells) were treated with ^{125}I-saliva or [^{14}C]putrescine using the experimental conditions described previously. After incubation at 37 °C for 8 h, the cells were pelleted at 13000 *g* for 4 min. Cell envelopes were recovered by repeated extraction with 1 ml of solubilizing buffer at 65 °C over a 72 h time period. The extractions were considered complete when the radioactivity in the extraction supernatant was decreased to background. Radioactivity in extraction supernatants and cell envelopes from ^{125}I-saliva and [^{14}C]putrescine-treated cells was determined by γ-radiation counting and liquid-scintillation counting in Liquiscint (Fig. 7 below).

RESULTS

Detection of salivary components in extracts of buccal epithelial cells

Initial experiments using electrophoretic-transfer analysis with rabbit anti-saliva antisera demonstrated the selective nature of saliva's interaction with buccal epithelial cells. Buccal-epithelial-cell extracts (unwashed or washed) from the saliva donor showed a different electrophoretic profile of components (Fig. 1, lanes b and c) than that seen with saliva alone (Fig. 1, lane a). Control electrophoretic transfers of epithelial-cell extracts which had not been exposed to saliva (foot callus) revealed only three components which were reactive with rabbit anti-saliva antiserum (Fig. 1, lane d). These components did not appear to correspond to those seen on electrophoretic transfers of saliva alone. Non-immune rabbit serum reacted slightly with one component in unwashed cell extracts (Fig. 1, lane e). Glucose-oxidase-conjugated goat anti-rabbit IgG alone did not react with components of buccal-epithelial-cell or foot-callus extracts (results not shown). Collectively these observations indicate that the reactivity of anti-saliva antiserum with buccal epithelial extracts is specific for saliva constituents.

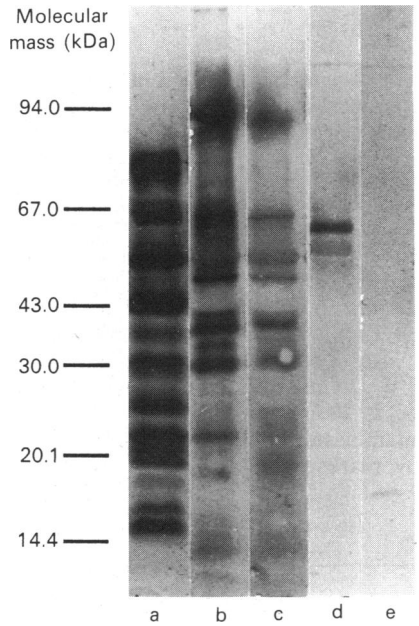

Fig. 1. Immunoblot patterns of parotid saliva and buccal-epithelial-cell extracts allowed to react with rabbit anti-human parotid saliva

Comparison of the immunoblot patterns produced by freshly collected parotid saliva (lane a) with extracts of unwashed (lane b) and washed (lane c) buccal-epithelial-cell extracts shows that only selected components of human parotid saliva were extracted from buccal epithelial cells. Rabbit anti-(parotid saliva) antiserum reacted with three components of non-oral epithelial extracts (foot callus) that did not correspond to bands in parotid saliva or buccal-epithelial-cell extracts (lane d). Non-immune rabbit serum reacted slightly with one component in unwashed buccal-epithelial-cell extracts (lane e).

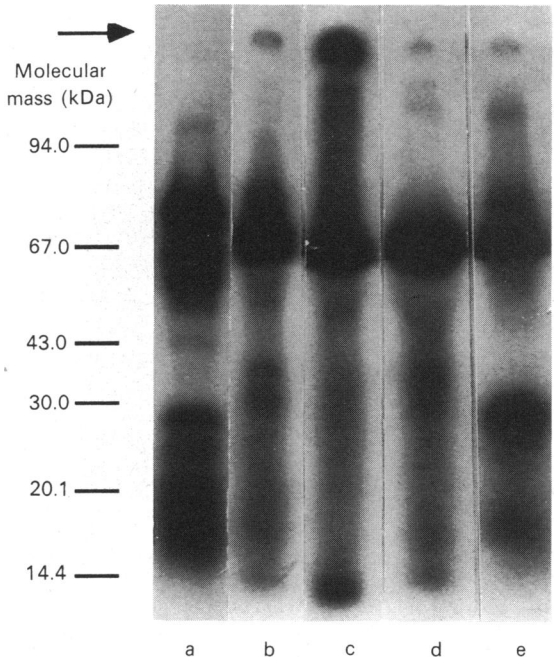

Fig. 2. Presence of ^{125}I-parotid saliva in a high-molecular-mass complex after incubation with buccal epithelial cells

A high-molecular-mass complex is obtained from ^{125}I-parotid-saliva-treated buccal epithelial cells after a 4 h incubation at 37 °C. This complex, as shown by SDS/10%-PAGE–autoradiography (lane c), is not present in ^{125}I-parotid saliva alone when incubated in the same reaction buffer at 37 °C (lane a). A comparison of a zero-time incubation (lane b) and a 4 h incubation (lane c) shows that the production of the high-molecular-mass complex increases with time. The formation of the high-molecular-mass complex is inhibited by heating the buccal epithelial cells at 100 °C for 15 min (lane d) and by 15 min pretreatment with an alkylating agent (20 mM-iodoacetamide, lane e) known to inhibit transglutaminases. The unlabelled arrow marks the top of the 3% stacking gel.

Characterization of saliva–epithelial-cell interactions

To examine further the interaction between saliva and buccal epithelial cells, ^{125}I-saliva was used. SDS/PAGE–autoradiographs of buccal-epithelial-cell extracts after incubation with ^{125}I-saliva under various conditions are shown in Fig. 2. Cell extracts obtained by incubation with ^{125}I-saliva resulted in a protein profile markedly different from that obtained with ^{125}I-saliva alone (Fig. 2, lane c versus lane a). The presence of a high-molecular-mass complex which just entered the 3% stacking gel (see arrow in Fig. 2, lane c) was noted in the cell extracts obtained after 4 h incubation. The formation of this complex could be inhibited by either freezing the epithelial-cell reaction mixture immediately after or by heating the cells at 100 °C before the addition of ^{125}I-saliva (Fig. 2, lanes b and d respectively), suggesting that its formation may be mediated by an enzymic reaction. Pretreatment of the cells with 20 mM-iodoacetamide (Fig. 2, lane e) inhibited the formation of the high-molecular-mass complex. This observation in combination with previous literature suggested that the putative enzyme may be a transglutaminase.

To determine if the putative enzyme was soluble, the previous ^{125}I-saliva experiments were repeated utilizing cell-free extracts and ^{125}I-saliva as well as known transglutaminase inhibitors. Results obtained from autoradiographs of these preparations confirmed the presence of a high-molecular-mass complex in the experimental sample which was not present in ^{125}I-saliva alone (Fig. 3, lanes a and b). This complex-formation was inhibited by pretreatment of the extracts at 100 °C for 15 min (Fig. 3, lane c) or by the incorporation of EGTA, iodoacetamide or *N*-ethylmaleimide (Fig. 3, lanes d, e and f) into the reaction mixture. Some components of ^{125}I-saliva also appeared to sustain a decrease in molecular mass. Comparison of saliva alone (Fig. 3, lane a) with the experimental reaction mixtures (Fig. 3, lane b) show a decrease in salivary components in the 67–94 kDa molecular-mass range. It is possible that these components may have been depleted by cross-linking; however, the same components also appear to be missing in the inhibited reaction mixtures (Figs. 3, lanes c–f).

The formation of an epithelial-cell-mediated high-molecular-mass ^{125}I-saliva-containing complex was confirmed by gel-filtration chromatography of epithelial-cell extracts on Sepharose CL-2B in the presence of 0.1% SDS. When cell extracts were chromatographed on Sepharose CL-2B, three peaks (I, II and III) were detected (Fig. 4, top). Autoradiographs of pooled fractions from peak I, subjected to SDS/10%-PAGE under reducing conditions, showed a high-molecular-mass band which

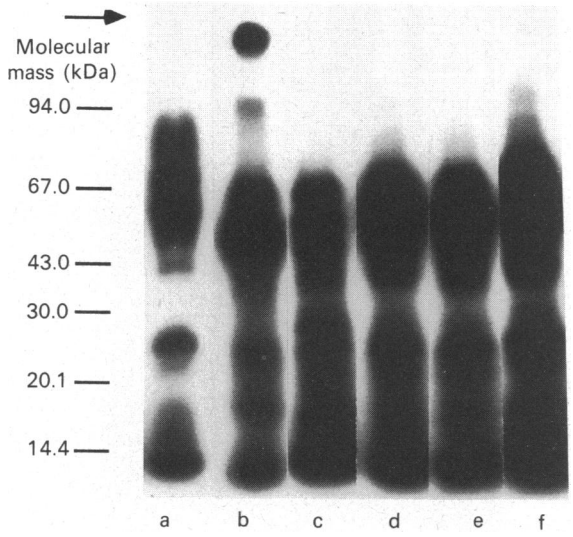

Fig. 3. Presence of ^{125}I-parotid saliva in a high-molecular-mass complex after incubation with cell-free extracts of buccal epithelial cells

A high-molecular-mass complex is produced in ^{125}I-parotid-saliva-treated cell-free epithelial extract after a 4 h incubation at 37 °C. This substance, shown by SDS/10% PAGE–autoradiography (unlabelled arrow, lane b), is not present in ^{125}I-parotid saliva alone (lane a) or in a cell-free epithelial extract which had been heated at 100 °C for 15 min (lane c). Formation of the high-molecular-mass complex was also attenuated by pretreatment with reagents (lane d, 20 mM-EGTA; lane e, 20 mM-iodoacetamide; lane f, 20 mM-*N*-ethylmaleimide) known to inhibit transglutaminases, before the 4 h incubation. The unlabelled arrow marks the top of the 3% stacking gel.

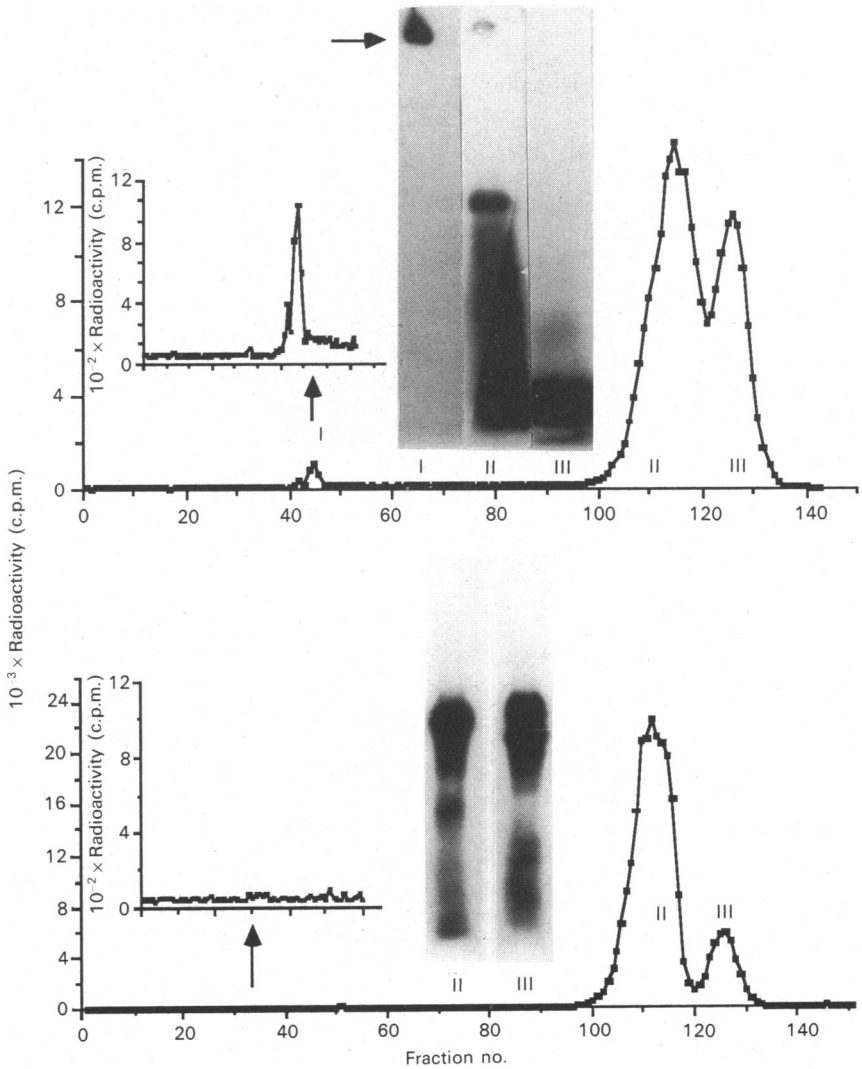

Fig. 4. Gel filtration on Sepharose CL-2B of extracts from buccal epithelial cells incubated with ^{125}I-parotid saliva

The insets represent scaled-up chromatographs of fractions 1–60 from each column. Top: extracts after incubation of buccal epithelial cells with ^{125}I-parotid saliva resulted in three peaks. The high-molecular-mass complex from peak I is indicated by the arrow (at the top of 3% stack) on SDS/PAGE–autoradiography in lane I. Peaks II and III are shown in lanes II and III respectively. Bottom: the fractionation of ^{125}I-parotid saliva alone resulted in two peaks with no high-molecular-mass component. Lanes II and III represent peaks II and III examined by SDS/PAGE–autoradiography on 10% gels.

barely penetrated the 3% stacking gel (Fig. 4, lane I, top). In contrast, ^{125}I-saliva alone revealed only two included peaks (II and III) (Fig. 4, bottom). Further comparison of the banding pattern in Fig. 4, lane III, top, with lane III, bottom, shows an apparent decrease in the molecular mass of salivary proteins incubated with epithelial cells. Comparison of the relative areas of peak III (top and bottom) suggests that there is also a relative increase in the amount of lower-molecular-mass salivary components recovered from cell extracts. These data verify that the interaction between the epithelial cells and saliva resulted in the formation of a high-molecular-mass salivary complex and also suggest that salivary components may be modified by proteolysis.

Detection of primary-amine incorporation into components of buccal-epithelial-cell preparations by the use of [^{14}C]putrescine

The incorporation of primary amines into buccal-epithelial-cell components was detected by utilizing [^{14}C]putrescine as a substrate. Incubation of cells with [^{14}C]putrescine for 8 h resulted in the incorporation of radioactivity into a high-molecular-mass complex (arrow) as well as multiple lower-molecular-mass components of the epithelial cells (Fig. 5, lane c). This labelling was completely inhibited by the addition of 0.5 M unlabelled putrescine immediately after the addition of [^{14}C]putrescine (Fig. 5, lane b). Pretreatment of the cells at 100 °C for 15 min inhibited the incorporation of [^{14}C]putrescine (Fig. 5, lane d), as did iodoacetamide and 100 mM unlabelled putrescine (Fig. 5, lanes f and g respectively). Only partial inhibition was seen in cell preparations treated with 20 mM-EGTA (lane e). EGTA (20 mM) appeared to inhibit completely high-molecular-mass formation in ^{125}I-saliva incubated with cell extracts (Fig. 3, lane e); however, [^{14}C]putrescine and ^{125}I-saliva cross-linking into epithelial-cell envelopes from whole-cell preparations was also only partially inhibited by 20 mM-EGTA [Figs. 7*a* and 7*b* (below) lanes 3)]. This suggests that partial inhibition of putative transglutaminase activity by EGTA in whole-cell preparations may be due to incomplete chelation of cellular calcium stores. Parotid saliva, in the absence of cells, was not labelled when treated with [^{14}C]putrescine (Fig. 5, lane a).

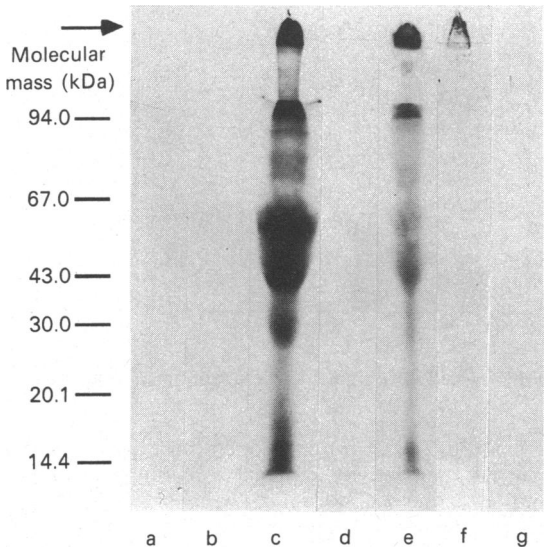

Fig. 5. Detection of transglutaminase activity by SDS/10% PAGE–fluorography after incubation of buccal epithelial cells with [^{14}C]putrescine

Radiolabelling of molecules in extracts obtained from buccal epithelial cells incubated for 8 h at 37 °C with [^{14}C]putrescine is shown by SDS/10%-PAGE–fluorography (lane c). No radiolabelling was obtained when [^{14}C]putrescine was incubated with parotid saliva alone (lane a). Radiolabelling was inhibited by the immediate addition of 0.5 M unlabelled putrescine (lane b) or by heating the epithelial cells preparation at 100 °C for 15 min (lane d). Labelling was also inhibited by pretreatment of the epithelial cells for 15 min with transglutaminase inhibitors (lane e, 20 mM-EGTA; lane f, 20 mM-iodoacetamide; lane g, 100 mM-putrescine) before the 8 h incubation. The unlabelled arrow marks the top of the 3% stacking gel.

The results of fluorescence spectrophotometric assays to detect the presence of transglutaminase activity showed an increase in the incorporation of monodansylcadaverine, with time, into α-casein in the presence of cell extracts (Fig. 6*a*). Monodansylcadaverine incorporation was completely inhibited by iodoacetamide, and none could be detected in the absence of extract. Similar results were obtained in control experiments utilizing 0.005 unit of guinea-pig liver transglutaminase as a source of transglutaminase activity (Fig. 6*b*). Combined data from these experiments suggest that transglutaminase activity is present in buccal epithelial cells and their extracts utilized in this study.

Saliva and [^{14}C]putrescine interactions with epithelial-cell envelopes

In our previous experiments, cell-associated radioactivity could only be partially removed from [^{14}C]putrescine- or ^{125}I-saliva-treated epithelial cells by single extractions in 4% SDS/10% β-mercaptoethanol. Repeated extractions of the cells over 72 h with 4% SDS/10% β-mercaptoethanol resulted in the recovery of insoluble epithelial-cell envelopes (as revealed by electron microscopy; results not shown) that were identical in appearance with those described by Matoltsy & Matoltsy (1966). The radioactivity associated with cell envelopes prepared from cells incubated with [^{14}C]putrescine or ^{125}I-saliva are shown in Figs. 7(*a*) and 7(*b*) respectively. Incorporation of ^{125}I-saliva or [^{14}C]putrescine was almost completely inhibited in zero-time incubations (Figs. 7*a* and 7*b*, lanes 1) and by treatment with iodoacetamide or by preincubation of the cells at 100 °C for 15 min (Figs. 7*a* and 7*b*, lanes 5, respectively). Treatment of the cells with EGTA only partially inhibited ^{125}I-saliva and [^{14}C]putrescine incorporation (lanes 3, Figs. 7*b* and 7*a* respectively), whereas treatment with unlabelled putrescine almost completely inhibited [^{14}C]putrescine incorporation (Fig. 7*a*, lane 6). All forms of inhibition or increased incorporation were statistically significant (by one-way analysis of variance) at the $P < 0.01$ level.

The combined data from the ^{125}I-saliva and [^{14}C]putrescine experiments suggest that transglutaminase activity is present on the surface of buccal epithelial cells. Furthermore, this enzyme may cross-link salivary substrates to other proteins on the cell surface, including those in the buccal-epithelial-cell envelope.

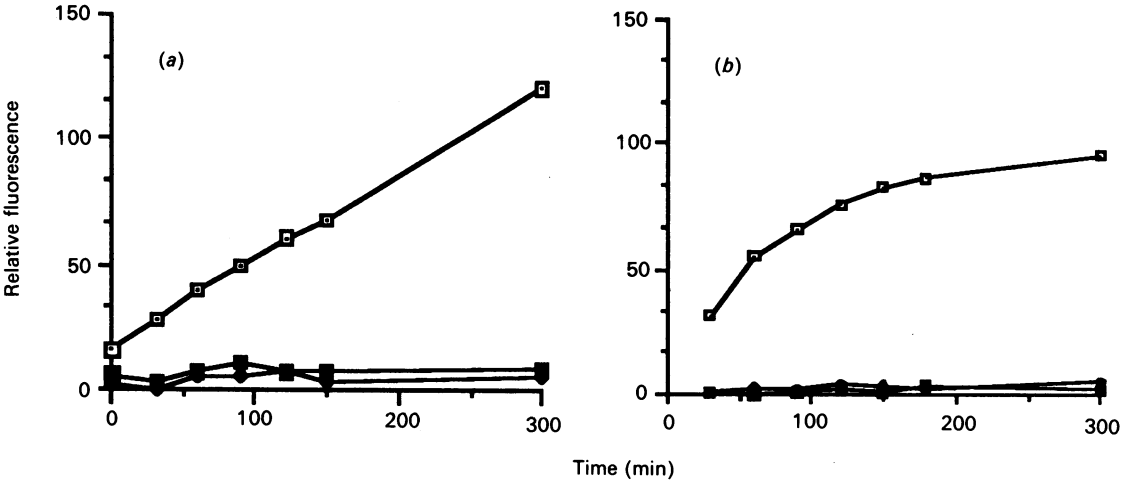

Fig. 6. Detection of transglutaminase activity in cell-free epithelial extract utilizing monodansylcadaverine in a fluorescence spectrophotometric assay

Transglutaminase activity in cell-free epithelial extract was detected by monitoring a characteristic increase in the fluorescent intensity of monodansylcadaverine after its incorporation into α-casein. An increase in the fluorescent intensity was detected in experimental reaction mixtures containing cell-free epithelial extract (*a*) or liver transglutaminase (*b*) using 40 mM-α-casein and 2×10^{-5} M-monodansylcadaverine (⊡). This increase was abolished by pretreating the same reaction mixtures with 20 mM-iodoacetamide (■) or heating them at 100 °C for 15 min (◆).

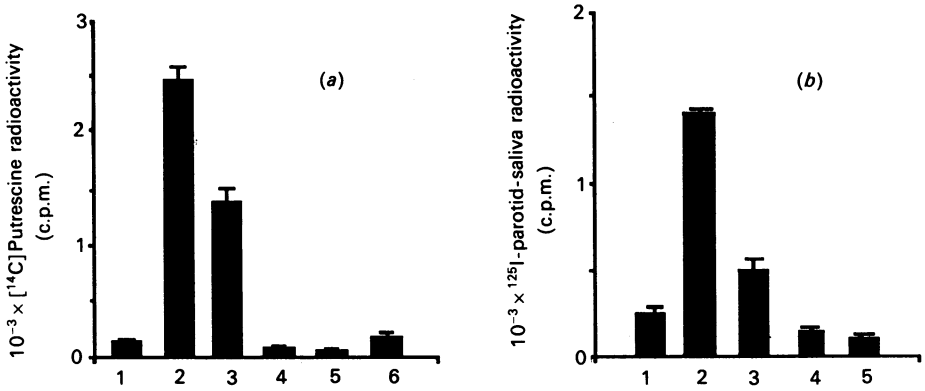

Fig. 7. [^{14}C]Putrescine (*a*) and ^{125}I-parotid saliva (*b*) recovered with buccal epithelial-cell envelopes after extraction of buccal epithelial cells

Buccal epithelial cells were incubated with either [^{14}C]putrescine (*a*) or ^{125}I-parotid saliva (*b*) and the epithelial-cell envelopes were recovered by extraction with SDS/β-mercaptoethanol. The amount of radioactivity remaining in the epithelial-cell envelope was then determined. Zero-time incubation (*a* and *b*, lane 1) and pretreatment of buccal epithelial cells at 100 °C for 15 min (*a* and *b*, lane 5) significantly decreased the amount of radioactivity recovered with buccal-epithelial-cell envelopes as compared with the experimental sample (*a* and *b*, lane 2).Transglutaminase inhibitors iodoacetamide (20 mM, *a* and *b*, lane 4), EGTA (20 mM, *a* and *b*, lane 3) and putrescine (100 mM, *a*, lane 6) also significantly decreased the amount of radioactivity recovered with buccal-epithelial-cell envelopes.

DISCUSSION

Oral pellicle has traditionally been thought to form by the selective adsorption of salivary molecules on to relatively inert surfaces. The synthesis of the epithelial-cell envelope, however, may endow desquamating buccal epithelial keratinocytes with a surface which is unique from other oral surfaces as regards pellicle formation. Epithelial-cell envelopes maintain the peripheral integrity of keratinocytes and protect them from physical, chemical and osmotic damage. Indeed the surface of dermal epithelial keratinocytes is almost completely composed of epithelial-cell-envelope protein (Goldsmith, 1983). The epithelial envelope, however, is composed of cross-linked proteins and therefore may be porous enough to allow the diffusion of cytosolic or membrane-associated proteins out of the cell. During the terminal stages of differentiation, the epithelial plasma membrane is initially lined by the epithelial-cell envelope and then partially hydrolysed (Gray *et al.*, 1978; Gerson & Harris, 1984). Consequently, the epithelial-cell envelope may be partially exposed to the oral environment, allowing transglutaminase or possibly lysosomal enzymes to be released into the immediate oral environment to mediate

the cross-linking or proteolysis of extracellular molecules. Thus it may be possible to propose a model of epithelial cell–saliva interaction based on adsorption, transpeptidation and proteolysis. In this model, selected salivary components would be adsorbed to either plasma membrane or partially denuded epithelial-cell envelopes, and transglutaminase substrates within this group could then be cross-linked to other transglutaminase substrate molecules. Depending on the surface to which it is adsorbed, salivary components may be cross-linked either to other salivary proteins, structural components of epithelial-cell envelopes, plasma-membrane proteins or possibly proteins on microbial surfaces. Our data support this model and also suggest that the adsorption of salivary components from human parotid saliva is selective.

Recovery of ^{125}I-saliva and [^{14}C]putrescine with extracted epithelial-cell envelopes suggests that epithelial-cell envelopes are not only exposed to the oral environment, but may act as acceptors for salivary cross-linking. However, the amount of saliva these cells can adsorb or cross-link is difficult to determine in these experiments. Buccal epithelial cells have previously been exposed to saliva, and their capacity for both adsorption and transpeptidation may be nearly saturated. Thus the apparently small amount of ^{125}I-saliva cross-linked in our experiments may only represent a portion of the buccal-epithelial-cell's total salivary cross-linking capacity.

Release of transglutaminase activity into cell-free extracts suggests that transglutaminase may diffuse from the exposed epithelial-cell envelope to the extracellular environment. It is possible, however, that the cells are damaged during the preparation of extract, allowing the release of cytosolic contents. Previous characterization of keratinocytes suggest, however, that the epithelial-cell envelope and other components of associated cytoskeleton are capable of protecting the keratinocyte from extreme physical and chemical insults. Methodologies in this study used for the preparation of cell-free extracts included centrifugation at 13000 *g* and stirring at 50 rev./min for 18 h; however, buccal epithelial cells should withstand this treatment. Counting of cells performed before and after extract preparation showed complete recovery of cells that were microscopically intact (results not shown), suggesting that the cells were not grossly damaged during this procedure.

The present data suggest that epithelial cells may also be a source of lysosomal proteinases, which escape into the extracellular environment and hydrolyse salivary components. This is not surprising, considering the number and diversity of proteolytic enzymes produced by stratified squamous epithelial cells. In experiments using extracts, some components in the 67–94 kDa molecular-mass range were lost from ^{125}I-saliva. It is possible that some of these components were depleted from ^{125}I-saliva by cross-linking into high-molecular-mass complexes and/or hydrolysed into smaller constituents. Evidence for this latter possibility is also provided in the results shown in Fig. 3, which shows an apparent decrease in the molecular masses of salivary components after incubation with buccal epithelial cells.

Transglutaminase-mediated cross-linking may be instrumental in stabilizing homo- or hetero-typic complexes between molecules of salivary and/or epithelial origin which may contribute to the functional characteristics of the mucosal pellicle. Indeed, it has been suggested that homo- and hetero-typic interactions between salivary molecules during pellicle formation endow these films with enhanced moisture retention, lubricity and/or barrier properties (Tabak *et al.*, 1982; Levine *et al.*, 1985, 1987*a*). This argument has been supported by reports of decreased mucosal permeability (Tolo *et al.*, 1977) and increased lubrication (Hatton *et al.*, 1985), which are mediated by heterotypic complexing of salivary molecules on oral surfaces. Transglutaminase-mediated salivary cross-linking with epithelium has not been previously described. However, transglutaminase-mediated cross-linking of exogenous molecules to the surface of spermatozoa and blastocysts has been shown in the female reproductive tract. Mukherjee *et al.* (1982, 1983) have hypothesized that uretoglobin, a protein present in mucosal secretions of the uterus, is cross-linked to the β_2-microglobin subunit of the transplantation antigen on the surface of spermatozoa and blastocysts. This reaction is presumed to inhibit maternal lymphocyte response to these reproductive cells in a manner which prevents transplantation-antigen–lymphocyte interactions. Similar extracellular cross-linking by a membrane-bound transglutaminase isoenzyme has been reported in hepatocytes (Barsigian *et al.*, 1988). By analogy, cross-linked salivary molecules on buccal epithelial cells may block epithelial receptors which mediate host–microbial interactions and, at the same time, may provide new receptors for other microbes. This 'conditioning' of the mucosal surface may promote specific microbial adherence which has been proposed previously for non-mucosal surfaces (Gibbons & van Houte, 1980; Stinson *et al.*, 1982; Tabak *et al.*, 1982; Levine *et al.*, 1985, 1987*a*).

Clinically, the presence of an epithelial-derived transglutaminase may have implications for the design of artificial salivas for individuals with decreased salivary function or xerostomia. A prominent symptom of xerostomic individuals is the sensation of a dry mucosa which is only transiently abated by present topical-replacement therapies. On the basis of the observations of the present study, it may be possible to enhance the substantive effect of artificial salivas by designing preparations which contain substrates for covalent cross-linking to mucosal epithelium.

This work was supported in part by Physician Scientist Award DE00185 and grants DE07034, DE08240, and DE07585 from the U.S. Public Health Service.

REFERENCES

Al-Hashimi, I. & Levine, M. J. (1989) Arch. Oral Biol. **34**, 289–295
Barsigian, C., Fellin, F. M., Jain, A. & Martinez, J. (1988) J. Biol. Chem. **263**, 14015–14022
Bergey, E. J., Levine, M. J., Reddy, M. S., Bradway, S. D. & Al-Hashimi, I. (1986) Biochem. J. **234**, 43–48
Bradway, S. D., Jones, P. C. & Levine, M. J. (1985) J. Dent. Res. **64** (spec. issue), Abstr. 719
Bradway, S. D., Bergey, E. J., Jones, P. C. & Levine, M. J. (1986) J. Dent. Res. **65** (spec. issue), Abstr. 854
Bennick, A. & Cannon, M. (1978) Caries Res. **12**, 159–169
Burnette, N. W. (1981) Anal. Biochem. **112**, 195–203
Curby, W. A. (1953) J. Lab. Clin. Med. **41**, 493–496
Edwards, P. A. W. (1978) Br. Med. Bull. **34**, 55–56
Erickson, D. (1985) Scand. J. Dent. Res. **93**, 320–328

Gerson, S. J. & Harris, R. R. (1984) in The Structure and Function of Oral Mucosa (Meyer, J., Squire, C. A. & Gerson, S. J., eds.), pp. 36–37, Pergamon Press, Elmsford, NY

Gibbons, R. J. & Qureshi, J. V. (1976) in Microbial Aspects of Dental Caries (Stiles, H. M., Loesche, W. J. & O'Brien, T. C., eds.), pp. 163–181, Information Retrieval, Washington

Gibbons, R. J. & Qureshi, J. V. (1978) Infect. Immun. **22**, 665–671

Gibbons, R. J., Etherden, I. & Moreno, E. C. (1982) Infect. Immun. **42**, 1006–1012

Gibbons, R. J. & van Houte, J. (1980) in Bacterial Adherence (Beachy, E. H., ed.), pp. 61–104, Chapman and Hall, New York

Goldsmith, L. A. (1983) in Biochemistry and Physiology of the Skin (Goldsmith, L. A., ed.), pp. 184–196, Oxford University Press, New York

Gray, G. M., King, I. A., Yardly, H. J. (1978) J. Invest. Dermatol. **71**, 131–135

Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963) Biochem. J. **89**, 114–119

Hatton, M. N., Loomis, R. E., Levine, M. J. & Tabak, L. A. (1985) Biochem. J. **230**, 817–820

Issitt, P. D. (1972) Applied Blood Group Serology, pp. 16–17, Spectra Biologicals, Oxnard, CA

Juriaanse, A. C., Booij, M., Arends, J. & Ten Bosch, J. J. (1981) Arch. Oral. Biol. **26**, 91–96

Laemmli, U. K. (1970) Nature (London) **227**, 680–685

Levine, M. J. & Ellison, S. A. (1973) Arch. Oral Biol. **18**, 839–853

Levine, M. J., Tabak, L. A., Reddy, M. S. & Mandel, I. D. (1985) in Molecular Basis of Oral Microbial Adhesion (Mergenhagen, S. E. & Rosan, B., eds.), pp. 125–130, American Society of Microbiology, Washington

Levine, M. J., Reddy, M. S., Tabak, L. A., Loomis, R. E., Bergey, E. J., Jones, P. C., Cohen, R. E., Stinson, M. W. & Al-Hashimi, I. (1987*a*) J. Dent. Res. **66**, 436–441

Levine, M. J., Jones, P. C., Loomis, R. E., Reddy, M. S., Al-Hashimi, I., Bergey, E. J., Bradway, S. D., Cohen, R. E. & Tabak, L. A. (1987*b*) in Oral Mucosal Diseases: Biology, Etiology and Therapy (Proceedings of the Second Dows Symposium) (Mackenzie, I. C., Squier, C. A. & Dabelsteen, E., eds.), pp. 24–27, Laegeforeningens Forlag, Denmark

Lorand, L., Lockridge, O. M., Campbell, L. K., Myhrman, R. & Bruner-Lorand, J. (1971) Anal. Biochem. **44**, 221–231

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275

Matoltsy, A. G. & Matoltsy, M. N. (1966) J. Invest. Dermatol. **46**, 127–129

Mayhall, C. W. (1970) Arch. Oral Biol. **15**, 1327–1341

Moreno, E. C. & Zahradnick, R. T. (1979) J. Dent. Res. **58**, 896–903

Mukherjee, A. B., Ulane, R. E. & Agrawal, A. L. (1982) Am. J. Reprod. Immunol. **2**, 135–141

Mukherjee, D. C., Agrawal, A. K., Manjunath, R. & Mukherjee, A. B. (1983) Science **219**, 989–991

Ogawa, H. & Goldsmith, L. A. (1976) J. Biol. Chem. **251**, 7281–7288

Shomers, J. P., Tabak, L. A., Levine, M. J., Mandel, I. D. & Ellison, S. A. (1982) J. Dent. Res. **61**, 397–399

Sonju, T. & Rolla, G. (1973) Caries Res. **7**, 30–38

Stinson, M. W., Levine, M. J., Cavese, J. M., Prakobphol, A., Murray, P. A., Tabak, L. A. & Reddy, M. S. (1982) J. Dent. Res. **61**, 1390–1393

Tabak, L. A., Levine, M. J., Mandel, I. D. & Ellison, S. A. (1982) J. Oral Pathol. **11**, 1–17

Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354

Tolo, K., Brandtzaeg, P. & Jonsen, J. (1977) Immunology **33**, 733–743

Williams-Ashman, H. G. & Canellakis, Z. N. (1980) Physiol. Chem. Phys. **12**, 457–472

Received 14 December 1988/17 February 1989; accepted 28 February 1989