Penetration of the oral mucosa by radiolabelled chlorhexidine in guinea pigs

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The aim of the present study was to assess the penetration of chlorhexidine through the oral mucosa. Ten albino guinea pigs had the oral cavity exposed to 0.2 % ^{14}C-labelled chlorhexidine for 1 min after initial rinsing with water. Five animals were sacrificed after 30 min and 5 after 60 min. Biopsies from the tongue, palate, gingiva and the buccal mucosa were frozen in liquid nitrogen, cut, fixed and prepared for autoradiography. The surface layers of the epithelium contained the greater part of the labelled material in sections from the tongue, palate and gingiva. Below these heavily labelled surface layers a scattered distribution was noticed throughout the epithelium. Sections from the buccal mucosa showed less surface labelling, but a slight accumulation of label close to the basement membrane was seen. Though labelling was more abundant in the epithelium than in the connective tissue, chlorhexidine had also penetrated into the connective tissue in sections from the buccal mucosa, the gingiva and the ventral side of the tongue.

Key-words: Chlorhexidine; penetration; guinea pigs

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Chlorhexidine digluconate and diacetate have been shown to be retained in the mouth for hours after mouthrinses or after brushing the teeth with a chlorhexidine containing dentifrice (*Bonesvoll et al.*, 1974; *Rølla, Löe & Rindom Schiött*, 1971). The fact that chlorhexidine is retained in the oral cavity and released slowly, may be the cause of its plaque inhibiting capacity (*Jensen & Christensen*, 1971). Approximately 30 % of a 0.2 % chlorhexidine solution has been retained in the mouth (*Bonesvoll et al.*, 1974). As only small amounts could be recovered in after-rinses with water, the main part was considered to be firmly bound to oral surfaces. It has been hypothesized that little, if any part of the chlorhexidine could penetrate the oral mucosa and that it would accumulate in protein-chlorhexidine complexes on the epithelial surfaces (*Magnusson & Heyden*, 1973). However, even macro-molecules such as albumin have been shown to penetrate the gingival pocket epithelium (*Tolo*, 1971). As the binding of chlorhexidine to proteins in vitro has been shown to involve albumin in serum and saliva (*Hjeljord, Rølla & Bonesvoll*, 1973) the possibility exists that penetration of chlorhexidine even bound to macro-molecules as large as albumin may take place. The purpose of the

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present study was to investigate the penetration of chlorhexidine through the oral mucosa of guinea pigs.

MATERIAL AND METHODS

Eleven albino guinea pigs were used in the study. Their mean body weight was 450 g. The oral cavity of the animals was rinsed with water to remove food debris. A test solution of 0.2 % chlorhexidine digluconate containing ^{14}C-ring-labelled chlorhexidine (2 μCi/ml) was prepared*. The purity of the isotope labelled compound used was 96—97 %. The test solution was administered to the animals with a syringe containing 0.5 ml. The mucous membranes of the mouth were kept moist with the solution for 1 min before the animals were returned to the cage. Five animals were sacrificed after 30 min and five after 60 min by a lethal dose of pentobarbitalum (Nembutal®) intraperiotoneally. The oral cavity was rinsed with running water before biopsies of the palate, gingiva, tongue and buccal mucosa were placed on specimen-holders and frozen in liquid nitrogen.

The last animal served as control and was sacrificed without having been exposed to labelled chlorhexidine. Sections of 7 μm were cut in a cryostate, dried in the air for ½ hour and placed in paraformaldehyde vapor at 60°C for two hours. The sections were then coated with Kodak Emulsion NTB 2 for autoradiography. The autoradiographic procedure was performed in total darkness to reduce the background to a minimum. After three weeks exposure at 4°C the autoradiographs were developed, fixed and stained with hematoxylin and eosin.

As a control of the effect of fixation a few sections were treated according to a modification of Appleton's technique (*Appleton*, 1964). Microscope glass slides were coated with emulsion NTB 2 using the dipping technique, dried and stored in the cryostate. Sections were cut at 7 μm in the dark room under safelight. The cryostate sections were removed from the knife blade by the coated microscope slide. The slight warming-up that occurred when the slide was handled, made the sections adhere to the emulsion. The slides were transferred to a deep-freezer for exposure at a temperature of —20 to —30°C. Three weeks later the sections were allowed to thaw at room temperature, dried and fixed in 4 % solution of formaldehyde in phosphate buffer pH 7.4 and rinsed in distilled water in complete darkness. After development and fixation the sections were stained and mounted for microscopy.

Grain counting was performed by using a square ocular grid at × 150. The grid was orientated with one of the peripheral lines at and parallel to the basement membrane with the grid extending into the connective tissue. The grains in the connective tissue of the ten squares most distant to the basement membrane were counted. The mean number of grains was calculated for each section. The background was similarly assessed by placing the grid with one peripheral line paralell to the epithelial surface and the grains in the emulsion of the most distant 10 squares were counted. Both background and connective tissue grain counting were made in the same localisation of the sections to minimize errors. Student's »t» test was used to test the differences between the grains in the emulsion outside the specimen and in the connective tissue.

* Kindly supplied by I.C.I., Ltd. Macclesfield, U.K.

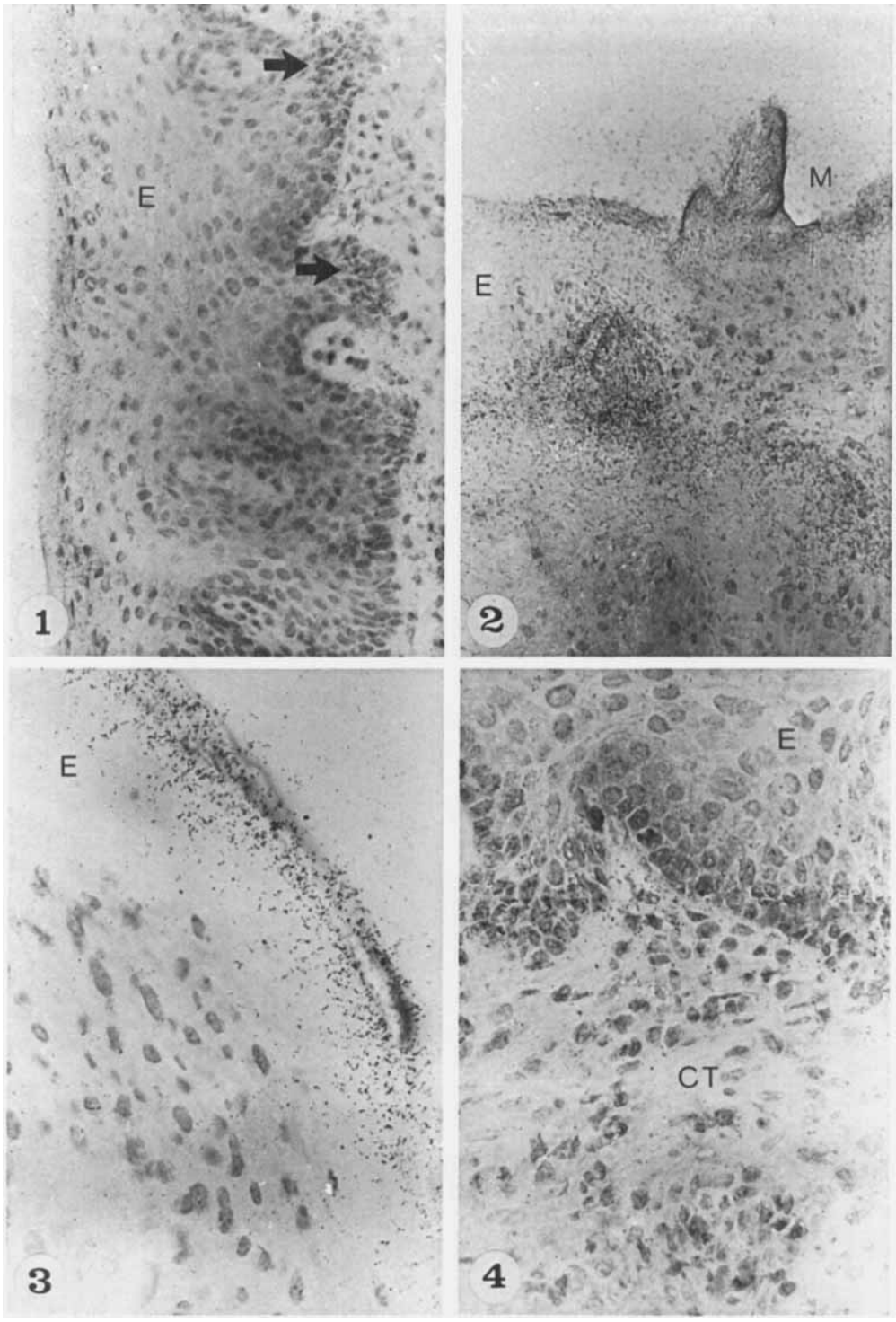

Fig. 1. Distribution of labelling in a section from the ventral side of the tongue. Heavy labelling in the outer 1/5 of the epithelium (E), a scattered labelling in the deeper layers and an accumulation at the basement membrane (arrows). × 300.

Fig. 2. Penetration into the middle part of the dorsal epithelium (E) of the tongue. The mucin layer (M) on the tissue also contains labelled material. × 300.

Fig. 3. The main part of the labelled material is bound to the outer cells and a more scarce labelling is seen in the deeper layers of the gingival epithelium (E). × 480.

Fig. 4. Accumulation of labelling at the basement membrane and penetration through the epithelium (E) into the connective tissue (CT). Tongue. × 480.

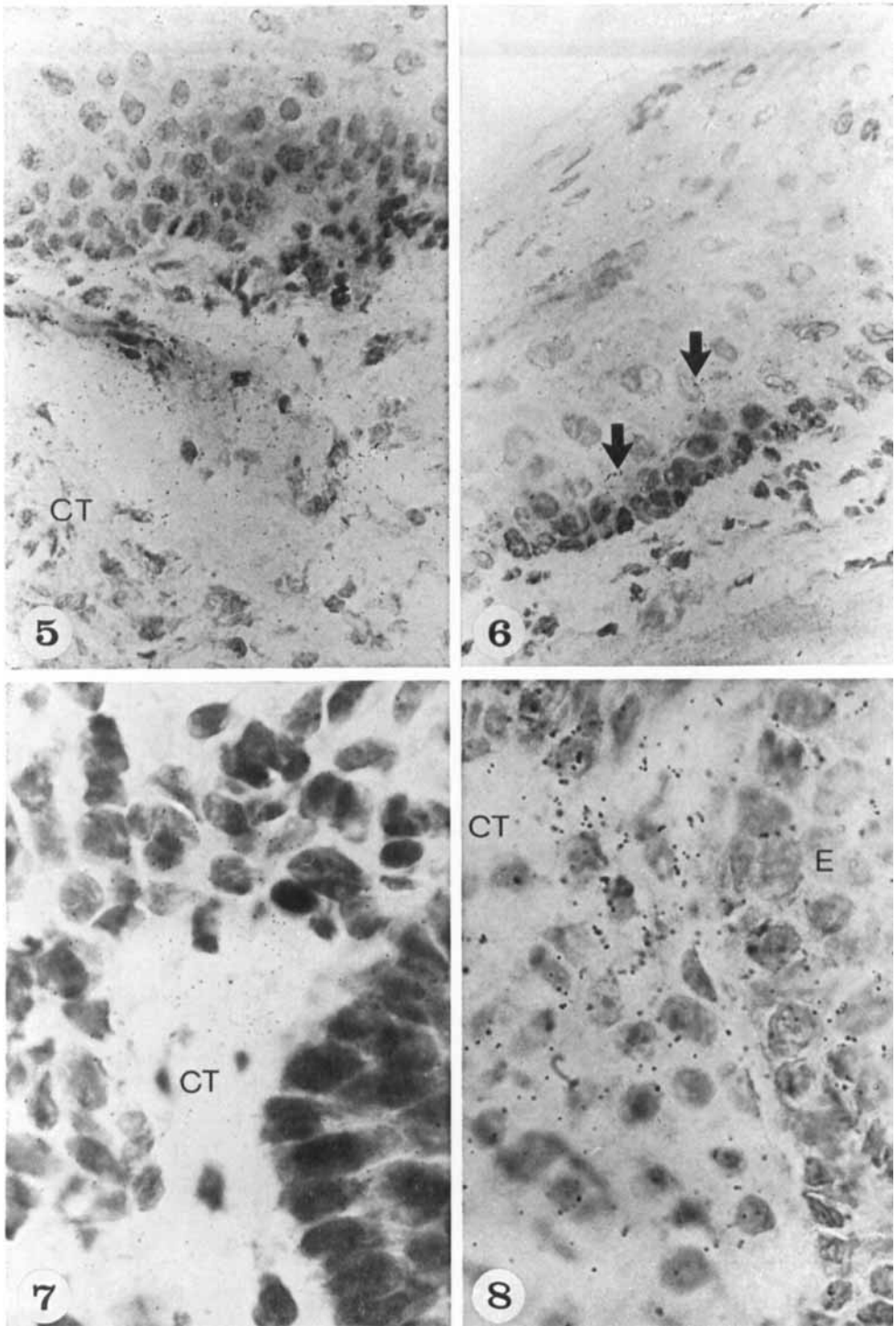

Fig. 5. Uneven distribution of labelled material in connective tissue from the ventral side of the tongue (CT). × 480.

Fig. 6. Note lack of binding of labelled material to the surface layer of buccal mucosa. A slight accumulation of the labelled material in the deeper layers of the epithelium is present (arrows). × 480.

Fig. 7. Distribution of ^{14}C-activity in a section from the ventral side of the tongue. Note distinct labelling in the connective tissue (CT). Autoradiograph made according to Appleton's technique. × 1200.

Fig. 8. Labelling in the deeper part of the epithelium (E) and connective tissue (CT), from the same biopsy as Fig. 7, showing a similar distribution of ^{14}C-labelled chlorhexidine. Autoradiographs was prepared with a conventional technique. × 1200.

RESULTS

The surface layers of the epithelium were heavily labelled in the sections from the tongue, the palate and the gingiva. The greater part of the radiolabelled material was found in the outer 1/3—1/5 of the epithelium (Figs. 1, 2). Below this heavily labelled zone a more scattered distribution of labelled material was found throughout the epithelium (Fig. 3). Labelling was also found in the connective tissue, though more scarce than in the epithelium (Fig. 4). Most parts of the connective tissue showed a scattered distribution, but areas with heavier grain concentration were also found (Fig. 5). Background labelling of the emulsion was relatively low. A heavy labelling was seen on the surface of some of the specimens, especially from the tongue and the palate. The sections from the buccal mucosa lacked the heavy labelling in the surface layer (Fig. 6). A scattered distribution of grains was found, and in the deeper layers of the epithelium close to the basement membrane, a slight accumulation was noticed. The subjacent connective tissue was also labelled (Fig. 6). Essentially the same pattern of distribution was found at the two time intervals.

Table I. *Labelling of connective tissue and background emulsion in terms of mean number and range*

		$\bar{x}$	R
Tongue ventral side	Connective tissue	199**	259—167
	Background	64	83—54
Buccal mucosa	Connective tissue	89*	95—86
	Background	45	50—45
Gingiva	Connective tissue	99*	114—89
	Background	53	60—48
Palate	Connective tissue	59N.S.	67—52
	Background	50	53—48

*$0.05 > p > 0.02$, **$p < 0.01$, N.S. Not significant

When comparing the autoradiographs made by the two fixation techniques no clear differences in the distribution of labelled material were evident (Figs. 7, 8).

The results from the grain counting are seen in Table I. The differences between the number of grains outside the specimen (background) and in the connective tissue were significant for all tissues except for the palatal mucosa. This indicated that the test material had penetrated into the connective tissue in 3 of the 4 tissues examined.

DISCUSSION

Penetration of the epithelium may be defined as an entering of the test material from the surface into the epithelium. The finding of test material in the connective tissue may be considered a penetration from the epithelium into the connective tissue. With ^{14}C-labelled chlorhexidine a penetration of both the epithelium and the connective tissue was found.

Two major difficulties had to be considered in this study. A tritium labelled isotope could give a clear picture of the distribution as the resolution for tritium is between 0.5 and 1.0 μm (*Rogers*, 1967). A disadvantage in using tritium is that the labelling of the chlorhexidine molecule may not be stable. Tracing of radiolabelled molecules in the tissue is based on the assumption of a stable linkage of the isotope to the test material. ^{14}C-labelled chlorhexidine seems to fulfill this demand. Metabolic cleavage of chlorhexidine is minimal. Absorbed chlorhexidine is excreted via the kidney mainly unchanged (*Winrow*, 1973). The autoradiographic results presented with ^{14}C-labelled isotope should therefore be a reliable sign of presence of chlorhexidine in the tissues examined.

The other difficulty in this experiment was the danger of contaminating the biopsy with the isotope during removal of the tissue. Furthermore, there was a possibility of redistribution of labelled material within the biopsy. To eliminate unbound chlorhexidine the oral cavity was rinsed with water. As the biopsies were trimmed in the cryostate before collecting the sections, possible contamination from the biopsy procedure ought to be eliminated. The apex of the tongue was removed in toto and frozen with the cut surface against the specimen holder. Thereby any possible contamination from the technical procedure was eliminated from this biopsy since the sections obtained had a circumferent epithelial lining.

The standard techniques of histology and autoradiography presuppose that labelled material in the specimen is insoluble in a wide range of polar and non-polar solvents. Freezing the tissue in liquid nitrogen and sectioning in a cryostate should be a reliable way to eliminate any redistribution in the tissue during sectioning. It has been suggested that the knife-edge of the cryostate acts like an ice skate, thawing the block by pressure at the line of contact. However, this effect does not seem to be of major importance as a refreezing of the thawed zone takes place immediately (*Rogers*, 1967). It must be admitted however that no way of sectioning frozen specimens completely eliminates the danger of redistribution of a watersoluble test material. The coating of the sections with the warm emulsion introduces a danger of redistribution. Therefore, the freezing technique for autoradiography of soluble materials (*Appleton*, 1964; *Rutherford & Black*, 1970) was used for some sections and compared to sections from the same biopsy fixed in paraformaldehyde vapor. Appleton's technique is based on the assumption that there is little chance of loss or displacement of radiolabelled material when care is taken to keep the tissues frozen during the entire process from sectioning and throughout the autoradiographic procedures. In this study the glass slides were coated in fresh emulsion, dried and stored at —18°—20°C. The biopsies were cut in the darkroom, and the sections were collected on these prepared slides which were kept in the freezer during exposure. Thereby the tissues were kept frozen during the entire procedure. This method ensured that the sections would not come in contact with any fluid which might extract or redistribute the labelled material. As no differences in the distribution of labelled chlorhexidine could be found in the section treated by the two methods of fixation, it seems safe to recommend conventional paraformaldehyde vapor fixation for further studies of isotope labelled chlorhexidine.

It should be noted, however, that by the paraformaldehyde vapor fixation technique with subsequent dipping of the slides in the emulsion, the sections will be covered by the emulsion. With the Appleton technique the section will be on top of the emulsion. This resulted in better histologic details and stainability but in the disadvantage of finding the developed grains at a lower focal plane. This is evident when Figs. 7 and 8 are compared.

Tissue from one animal that was not exposed to chlorhexidine was included in this study. Non-radioactive tissue, fixed in the same way as the others will serve as a measure of the chemography present. Chemography depends on diffusion of reactive molecules into the emulsion from the specimen and their chemical interaction with the silver halide crystals

(*Rogers*, 1967). Sections from non-radioactive tissue give a more accurate estimation of background than using emulsion outside the sections. Background counts can then be taken from identical volumes of emulsion over similar areas of active and inactive specimens. This approach was used in the study. However the number of grains in the emulsion over and distant from the specimen was almost identical in the control animal. As chemography seemed to be negligible, grain counting over and distant from the specimen was made in the same autoradiograph since differences in the emulsion thickness might bias the results (*Leblond, Kopriwa & Messier*, 1963). Table I reveals significant differences in the mean number of grains in the emulsion over and more distant to the specimen for the tongue, the buccal mucosa and the gingiva, but not for the palate. This might be explained by the thickness of keratinized epithelium of the palate, rendering it less penetrable than the other tissues examined.

The greater part of the ^{14}C-labelled material was found in the outer layers of the epithelium in the palate, the tongue and the gingiva, which agrees with observations in chlorhexidine fed mice by *Magnusson & Heyden* (1973). They found that radioactive chlorhexidine accumulated on the filiforme papillae of the tongue, but there was also a scattered distribution in the deeper layers of the connective tissue of the tracheal wall. This is in accordance with the present findings. Though the surface layers of the keratinized tissues contained the greater part of labelled material, the distribution of the label in the deeper layers should be noted. In sections from the ventral side of the tongue, areas of heavier labelling could also be found in the subjacent connective tissue, but a scattered distribution was the most common finding. Most likely the heavy labelling in the connective tissue was due to a transport of the test material by the vasculature. This explanation was supported by the finding of dense labelling in some areas of the connective tissue, which by serial sectioning proved to be small vessels (Fig. 5).

The buccal mucosa showed a different distribution. The heavy labelling in the surface layer was lacking, and labels were scarce in the outer layers of the epithelium. Chlorhexidine had been administered to the animals with a syringe, and care had been taken to bring the solution in contact with all parts of the mucosa where biopsies were to be taken. Evidently, the chlorhexidine had not been bound to the buccal mucosa in the same manner as in the keratinized epithelium of the mouth. In the deeper layers of the epithelium a slight accumulation of label was noticed. This finding is in accordance with that of *Tolo* (1971), who showed an accumulation of labelled albumin in the basal layers of the pocket epithelium in guinea pigs, whereas less labelled material was observed in the connective tissue. Seemingly, a transport of a protein with a molecular weight of 68.000 can occur through the gingival pocket epithelium.

The diameter of albumin is about 40A, and it was evident in *Tolo*'s study that the oral basement membrane exerted a restricting effect upon molecules of this size. Chlorhexidine has a molecular weight of 505.5 (free base). The accumulation found in the deeper layers of the epithelium may be explained by a restricting effect of the basement membrane upon molecules of this size or may indicate that chlorhexidine penetrated bound to carrier molecules.

Background was low in sections from the buccal mucosa. In sections from the

other tissues the background was low well away from the specimen. But close to the heavily labelled epithelial layers. the emulsion was considerably more labelled. Even though the oral cavity was rinsed with water to remove excessive chlorhexidine before the biopsies were taken, a mucin layer will still be left coating the mucosa. Probably this mucin layer accounts for some of the labelling found outside the sections. It cannot be outruled that in the washing procedure both the mucin layer and the outer layers of the epithelium have been eliminated from the buccal mucosa, giving an impression of no binding of chlorhexidine to the surface in this tissue.

When the autoradiographs from the four areas of the oral mucosa were compared, the keratinized tissues clearly contained much greater amounts of chlorhexidine than non-keratinized. In a study by *Davies & Hull* (1973) oral mucosa of dogs were exposed to radio-labelled chlorhexidine. The results were expressed as μg of chlorhexidine per cm^2 oral mucosa after combusting the samples to ^{14}C-labelled CO_2. Values from the tongue and the palate were 79 μg and 39 μg, whereas the cheek only contained 1.5 μg per cm^2. The present autoradiographic results support their observation of different binding capacity of the oral mucosa. But the possibility of penetration into the deeper layers of the epithelium of the buccal mucosa (Fig. 6) with a negligible surface binding might explain the differences in their results. Surface square measurements of the different parts of the oral mucosa will not reveal the true square surface because of anatomic differences as papillae etc.

No difference in labelling was seen between the exposure intervals of 30 and 60 minutes. This might be explained by a rapid penetration of the tissues requiring less than 30 minutes or that a depot of chlorhexidine is retained on the mucosal surfaces. From this depot both penetration and release to the oral cavity may take place. In this experiment a single application was used. Repeated applications as used in the clinic may be of importance in a possible immunization of the host. The rate of penetration may therefore be changed (*Tolo*, 1974). This suggestion should be elucidated further.

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