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THE RENEWAL OF GINGIVAL EPITHELIUM IN MARMOSETS (CALLITHRIX JACCHUS) AS DETERMINED THROUGH AUTORADIOGRAPHY WITH THYMIDINE-H^3*

by

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Stratified squamous epithelium as a tissue is constantly being renewed through the formation of new cells in the basal epithelial layers. The presence of mitoses in the developing gingival epithelium of monkeys is reported by *McHugh* (1960) while the rate of mitoses in the gingivae of two human age groups is discussed by *Meyer et al.* (1956). *Greulich* (1961) has shown that a variation in the mitotic activity exists between the epithelial attachment and the rest of the oral gingiva. This finding is supported by the results of *Beagrie & Skougaard* (1962) which suggested that in mice the epithelial cells against the enamel are renewed every 3—5 days, while in the oral epithelium there appears to be a renewal time of 8—10 days. *Löe* (1961) found in an experi-

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ment carried out on dogs a continuous renewal of the epithelium of the cuff.

It was felt necessary to repeat on primates the experiments already reported on mice, and for this purpose the marmoset (Callithrix Jacchus) was chosen.

The marmoset has a dentition and dental histology similar to that of other primates. For autoradiographic purposes this animal has the added advantage of a size and weight (200—350 g) which from an economical point of view makes it suitable for experiments with expensive radioactive isotopes.

The aims of the present experiment were:

to observe the renewal time and manner of cell migration in the gingival epithelium of marmosets (Callithrix Jacchus), and to make comparison between the rate of turnover in the attached gingiva and the epithelial cuff,

to determine the type of cell population by means of the radioactive index and through this index and the DNA synthesis time,

to calculate the renewal time of gingival epithelia.

MATERIAL AND METHOD

Twenty-four marmosets (17 male and 7 female) were used in the experiment. Prior to the experiment the gingivae of the animals were examined under the stereomicroscope in order to exclude from the material animals with clinical manifestations of periodontal disease. As the animals were not born and reared at the animal house of the Royal Dental College, Århus, it was impossible to determine their age, but with the exception of one, they were all adult. The weights of these ranged from 187 grams to 333 grams.

20 marmosets were used to repeat the experiment on mice (*Beagrie & Skougaard*, 1962). Injections of tritiated thymidine were given intraperitoneally at a dosage of approximately 1 micro curie per gram body weight. The intervals between injection and sacrifice were from ½ hour to 6 days. These intervals as well as the sex and body weights of the animals are shown in Table 1.

Table 1.

Animal	Weight	Sex	Date of injection	Hour of injection	Interval between injection and sacrifice
M. 40	265 g	♀	13- 1-62	9^{00}a.m.	½ h
M. 18	187 g	♂	23- 9-61	4^{00}a.m.	1 h
M. 22	230 g	♂	19-11-61	8^{30}p.m.	1 h
M. 41	273 g	♂	13- 1-62	9^{00}a.m.	1 h
M. 45	230 g	♀	23- 2-62	1^{30}p.m.	1 h
M. 46	200 g	♂	23- 2-62	1^{30}p.m.	1 h
M. 47	200 g	♀	23- 2-62	11^{30}p.m.	1 h
M. 50	273 g	♂	11- 4-62	11^{30}p.m.	1 h
M. 23	268 g	♀	19-11-61	8^{30}p.m.	3 h
M. 42	272 g	♂	13- 1-62	9^{00}a.m.	4 h
M. 49	290 g	♂	23- 2-62	1^{30}p.m.	8 h
M. 24	293 g	♂	19-11-61	8^{30}p.m.	12 h
M. 20	294 g	♂	23- 9-61	4^{00}a.m.	24 h
M. 81	318 g	♂	26- 5-62	10^{30}a.m.	24 h
M. 84	333 g	♂	26- 5-62	10^{30}a.m.	24 h
M. 48	240 g	♂	23- 2-62	1^{30}p.m.	24 h
M. 21	250 g	♀	23- 9-62	4^{00}a.m.	48 h
M. 60	127 g	♂	13- 1-62	9^{30}a.m.	72 h
M. 19	273 g	♂	23- 9-61	4^{00}a.m.	6 d
M. 79	250 g	♀	21- 6-62	9^{30}a.m.	6 d

From the remaining four animals similarly injected, biopsies were taken of oral mucosa at intervals from ½ hour to 12 hours (Table 2). During this time the animals were kept anaesthetized by an intramuscular injection of Nembutal. Dosage of Nembutal was in the order of 0.018 mg/g body weight.

After sacrifice the tissues were fixed in 10 % neutral buffered formalin, and following decalcification double-embedded in celloidin and paraffin wax. Serial sections were cut at 4—5 microns and covered with autoradiographic stripping film (Kodak A.R. 10) according to the method described by *Pelc* (1947).

The covered sections were exposed in light-tight boxes at 5°C for 40 days. Kodak D 19 was used for developing the exposed films, and after fixation the sections were stained with Ehrlich's haematoxylin and eosin as outlined by *Pelc* (1956). For inter-

Table 2.

The percentage of labelled epithelial cell mitoses from oral mucous membrane biopsies at various time intervals.

Hours after injection	% labelled mitosis
½	none
1	4.8
1½	9.1
2	81.8
3	84.0
3½	80.0
4	85.7
5	90.9
5½	81.0
6	83.4
7	91.1
7½	72.7
8	81.1
8½	28.0
9	20.0
10	18.7
12	21.4

pretation of results the oil immersion lens was used. The Zeiss 40×oil immersion (apo. 1.0) proved to have the advantage of high resolution and depth of dimension.

RESULTS

On examination, some sections of the material were discarded because of ulceration of the crevicular epithelium or downgrowth of epithelium on to the cementum.

In all the 1 hour experiments, the sections showed labelled cells in the basal layer of all parts of the gingival epithelium. The labelling of cells seemed to be lighter than that seen in the mouse experiment.

Grain counts carried out on 250 labelled cells showed that the average number of exposed silver grains on the labelled cells in the one hour sections was of the order of 18 (Figs. 1 & 2 b).

In the epithelial cuff, the labelled cells were unevenly distri-

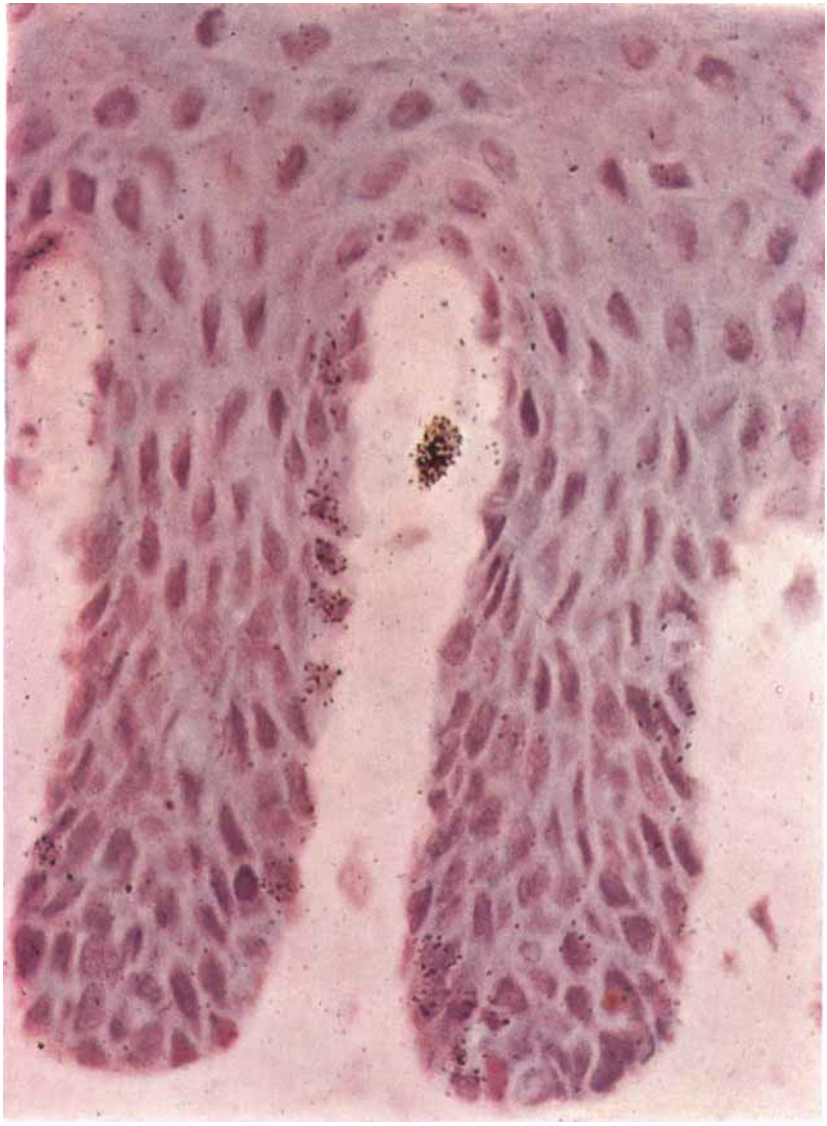

Fig. 1

Epithelial ridges (rete pegs) from attached gingiva of marmosets showing labelling in the basal layer 1 hour after injection with thymidine-H^3. Note the labelling intensity as compared to the heavily labelled connective tissue cell.

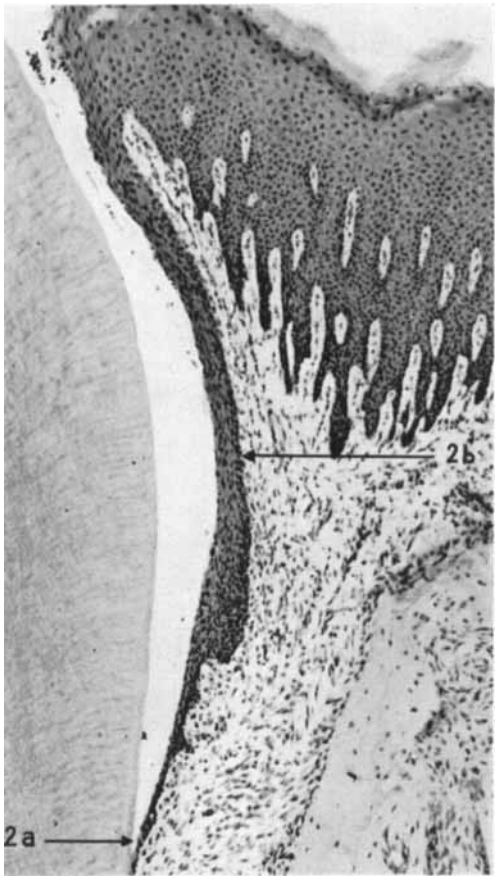

Fig. 2. The epithelial cuff of marmoset 1 hour after injection with thymidine-H^3.

buted. A higher number of labelled cells were present in the marginal half of the cuff than in the part nearer the cemento-enamel-junction, but labelled cells were frequently seen directly in contact with or near to the C.E.J. (Fig. 2 a). Where epithelial ridges (rete pegs) were present in the attached gingiva these tended to show a high number of labelled cells (Fig. 3).

The 3, 4, 8, and 12 hour specimens showed an increase in the number of labelled cells in both parts of the gingival epithelium when compared to the 1 hour sections. The labelled cells were

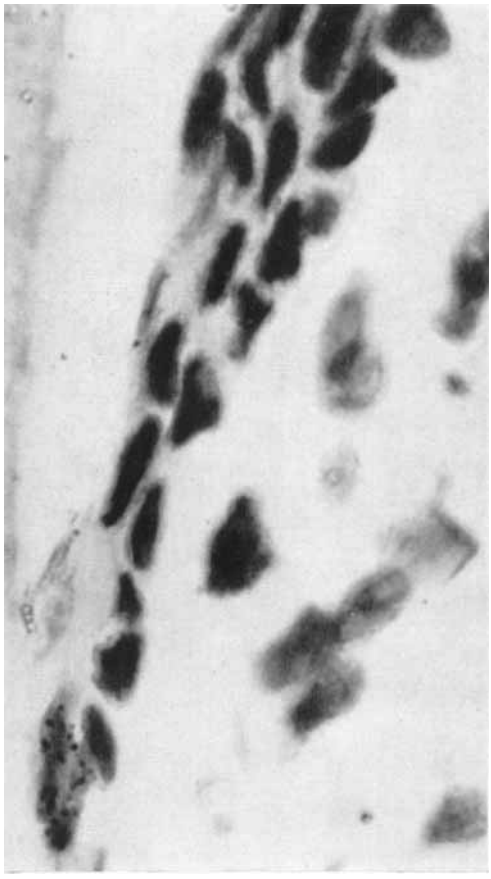

Fig. 2 a. The base of the cuff where two labelled cells are seen against the tooth with a desquamated cell above them.

still located in the area of the basal layer with the exception of the 12 hour specimen in which some labelled cells were seen one or two cells peripheral to the basal layer.

This finding was more evident after 24 hours. A considerable increase in the number of labelled cells had then taken place. The average grain count from 250 labelled cells now was of the order of 8 grains per cell (Fig. 3) which is consistent with what

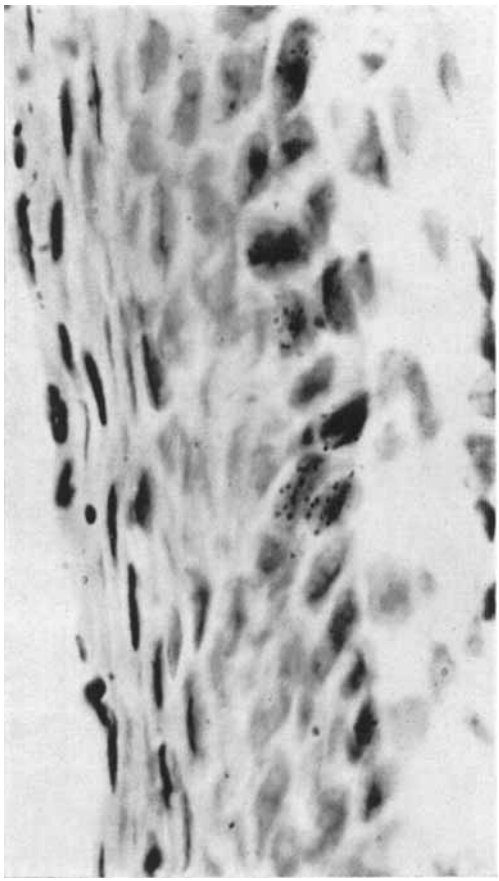

Fig. 2 b. The middle of the cuff showing labelling of the basal cells.

would be expected following division of the cells and proportional dilution of the radioactivity.

In the 48 and 72 hour specimens the position of the labelled cells varied according to the thickness of the cuff. Where a thin cuff consisting of 3—6 layers was present the labelled cells were found throughout the epithelium (Fig. 4). Where a thicker cuff was present the position of the labelled cells were still some cell layers away from the surface. The labelled cells at the surface

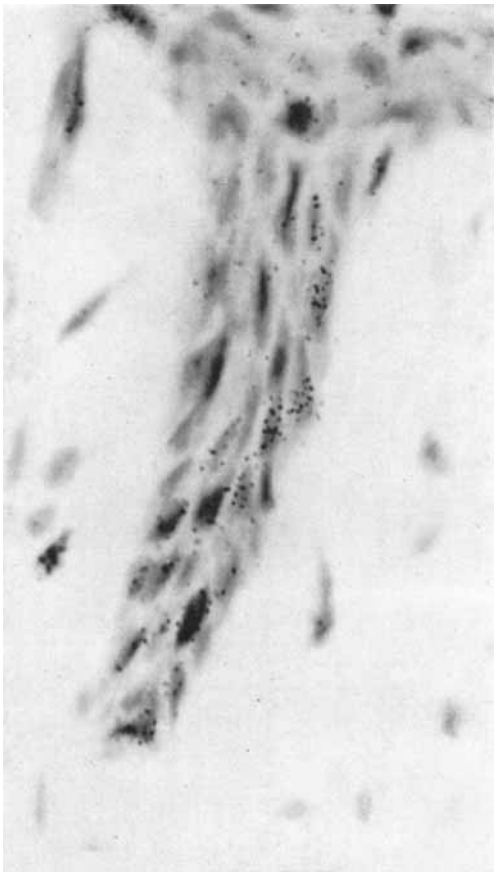

Fig. 3. Epithelial ridge (rete peg) 24 hours after injection with thymidine-H^3 showing a high number of labelled cells and a proportional decrease in the labelling intensity as compared to the labelled cells in Fig. 1.

seemed to be lighter labelled than other cells belonging to the same cell generation (Figs. 4 and 5). It was difficult to make accurate observations on the 6 days specimens from the cuff due to the small number of exposed silver grains found over the divided cells, but labelled cells carrying a small number of grains were found on the surface in all parts of the cuff.

In the attached gingiva it was not possible to see when the labelled cells reached the surface as the labelling was lost in the granular layer. This seemed to be the case after 3—6 days.

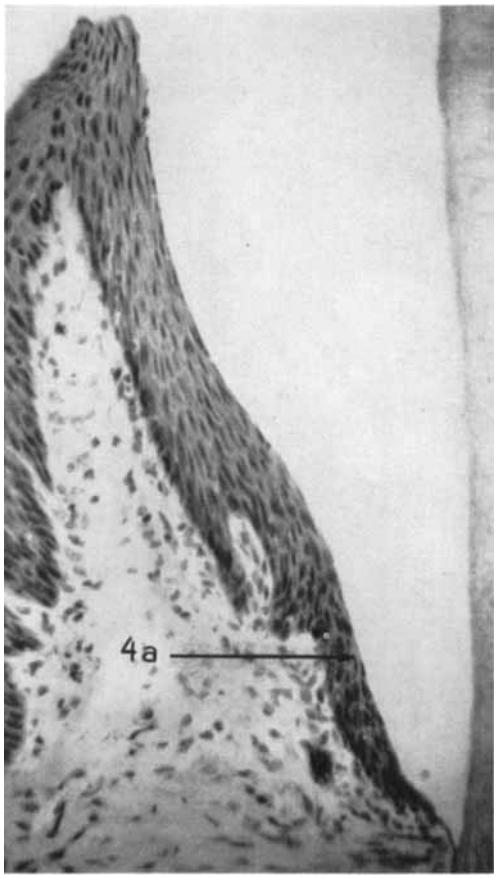

Fig. 4. Epithelial cuff 48 hours after injection with thymidine-H^3.

RADIOACTIVE INDEX

Table 3 shows the radioactive index (% labelled cells) of the epithelium of the attached gingiva and the epithelial cuff. These figures were tabulated after counting 10,000 cells in the 1 hour, 24 hour and 3 day specimens.

No figure is included for 3 day specimen of the attached gingiva because of the difficulty in making an accurate calculation. The results, however, comply with the criteria for a tissue with a renewing cell population as outlined by *Messier & Leblond* (1960).

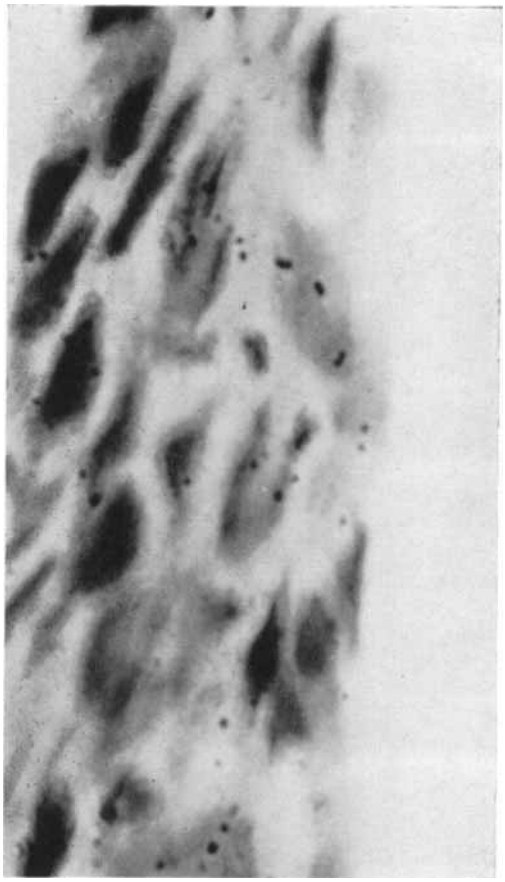

Fig. 4 a. Higher magnification at area indicated by arrow in Fig. 4 showing labelled cells throughout the epithelium and at the surface next to the enamel space.

Table 3.

Radioactive Index (% labelled cells) at intervals of 1 hour, 24 hours, and 3 days after injection of thymidine-H^3.

Tissue	1 hour	24 hours	3 days
Epithelial cuff	5.1 %	11.5 %	3.1 %
Attached gingiva	2.8 %	8.7 %	*)

*) labelling intensity too weak to give an accurate figure.

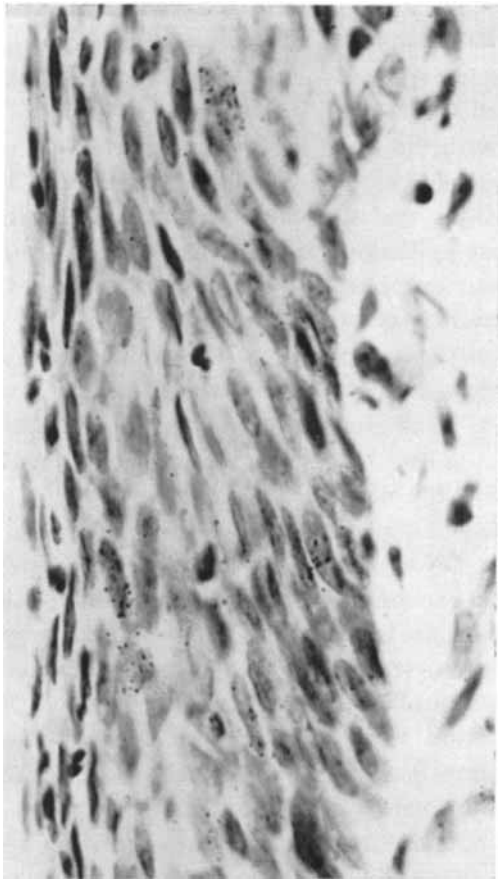

Fig. 5. Epithelial cuff 72 hours after injection with thymidine-H^3. In this thicker area labelled cells are seen some layers away from the surface.

Observations on the biopsy material were made with a view to determining when the mitoses were first labelled and further at what time interval after injection the mitoses were unlabelled.

The results presented in Table 2 were compiled from observations on biopsies from oral mucous membrane of the four animals reserved for this purpose.

DISCUSSION

The manner of cell migration observed in the gingiva of marmosets was exemplified in the 24 hour sections of the cuff where

the labelled cells were found 1–2 cells from the basal layer. Their migration seemed to be towards the enamel. Oral epithelium at this stage showed a similar pattern of migration towards the prickle cell layer.

Compared with the same experiment carried out on mice (*Beagrie & Skougaard,* 1962) it was more difficult in the marmoset material to follow the movement of the half labelled cells after division. Mouse epithelial cells had an average grain count of 65 on the one hour specimens reduced to 22 grains after 24 hours, whereas the marmoset sections had an average of 18 grains and 8 grains, respectively. Such a variation in grain count was interesting in view of the fact that the same body weight dosage of thymidine-H^3 was given to both sets of animals, and that the exposure time of the marmoset sections (40 days) was double that of the mice (21 days).

Such a difference in labelling intensity may be a reflection of a variation in DNA metabolism between the two animals. Alternatively, the exchange system for thymidine to the epithelium from the blood vessels in the connective tissue may be less efficient in marmosets than in mice. The possibility exists that there is reduced uptake of thymidine into the blood of the marmoset following injection. However, the presence of very heavily labelled cells in the connective tissue immediately beneath the basal layer of the epithelium was a constant finding (Fig. 1). The labelling of these cells was so intense that grain counting was impossible and indicates that the tracer was available to them in sufficient quantities. Even when a test intravenous injection was given to one animal not listed in the tables the phenomenon remained the same.

It was previously suggested that the tissue renewal time could be expressed as the time taken for the last fully labelled cell to pass from the basal to the surface layer. This definition is somewhat inaccurate since there is great variation in the life time of cells in epithelial tissue on account of the variation in the distance that individual cells have to migrate to the surface for desquamation. Thus, if the turnover time of the tissue is expressed as the time taken for the last fully labelled cell to be desquamated, many of the cells in the tissue will have been replaced more than once. Furthermore, it is impossible to de-

termine when the last fully labelled cell reaches the surface, because at this time, an assortment of labelling is present in the epithelium. The fully labelled cells through division will be closely followed by half labelled cells and these in turn by cells carrying a still lessened amount of tracer.

Unfortunately it is impossible to determine whether the individual cell is fully or half labelled, whether it started off from the basal layer towards the surface shortly after the injection or later after further division. Cells from the same division carrying the same amount of tracer do not necessarily show the same number of exposed silver grains on the autoradiograph. Due to the very short range of the tritium beta-rays, the labelling intensity is not only dependent on the amount of tracer in the nucleus, but also on the distance from the DNA containing parts of the cell to the film emulsion.

A cell saturated in thymidine-H^3 and sectioned at right angles to its long axis will give a reduced beta-ray emission when compared to one cut in the long axis and equally saturated in the isotope.

Taking the above factors into consideration it would thus seem desirable to have a more accurate method for determining the turnover rate of stratified epithelium. It is apparent that a definite relationship exists between the radioactive index and the tissue renewal time, i.e. the shorter the renewal time the greater the radioactive index. Knowledge of the radioactive index permits calculation of the renewal time but for this it is necessary to know the time required by cells for synthesis of DNA prior to mitoses.

A proliferating cell passes through various phases leading up to mitosis: a post mitotic gap (G_1) a DNA synthesis phase (S), a premitotic gap (G_2) in strict order, as illustrated below.

$$\text{MITOSIS} — G_1 — S — G_2 — \text{MITOSIS}.$$

If experimental animals are sacrified at different time intervals after injection with thymidine-H^3, it is possible to calculate the length of the synthetic phase (S) and the nonsynthetic phases (G_2, mitosis, and G_1) by observing how long after injection the mitoses are labelled. In animals sacrificed shortly after injection, cells showing mitotic figures will not be labelled as the cells at

the time of injection will have been in a non-synthetic phase, that is G_2 or early mitoses. On the other hand, labelling of the mitoses indicates that the cells were in the S phase at injection time and where at longer intervals the mitoses again fail to show labelling, these cells will have been in the non-synthetic post-mitotic gap (G_1) when the injection was given. The lengths of these phases have been calculated for intestinal epithelium of mice (*Quastler*, 1960) and appear to be 2 hours for G_2 plus mitoses, 7½ hours for S, and 9½ hours for G_1.

From Table 2 it can be seen that 90 % of the mitotic figures were unlabelled after 1½ hours, whereas at the 2 hour period 80 % of mitoses were labelled. This seems to indicate that in marmosets the G_2 phase is approximately of 2 hours duration. It also appears from Table 2 that a change in labelling of mitoses again takes place between 8 and 8½ hours suggesting that the S phase is of approximately 7 hours duration.

In order to recognize the mitoses through the silver grains the biopsy autoradiograph was exposed for only 14 days. Such a short exposure time reduced the number of exposed grains per labelled cell to 3—4. Because of this it was difficult sometimes to determine which mitoses were labelled since each half nucleus carried a maximum of 2 grains. Although with this short exposure time "background" was minimal, never-the-less the possibility of false labelling cannot be ruled out.

When the S time of the cell and the radioactive index of the tissues are both known, the tissue renewal time can be reasonably accurately calculated through the following formula:

$$t = \frac{100}{r} \times \frac{S}{24}$$

Where t = renewal time in days,
S = DNA synthesis time in hours,
r = the one hour radioactive index in %.

This calculation does not take account of the diurnal variation in cell division, but such an inaccuracy will be reduced considerably if the radioactive index used for the calculation represents an average number compiled from several experimental animals injected at different hours of the day. The accuracy would also

be dependent upon the time during which radioactive thymidine is available in the blood after injection. For rats this was found to be maximal 20 minutes after subcutaneous injection with a rapid decrease thereafter (*Messier & Leblond,* 1960). In man a rapid fall in radioactivity of the blood plasma was found one hour after intravenous injection (*Cronkite, Fliedner et al.,* 1959).

In the ½ hour specimens the degree of labelling was of a similar type to that of the one hour section, and it is therefore probable that the clearance time of thymidine from the blood of marmosets will be similar to that reported for rats. This is at present under further investigation through counting of blood plasma samples.

When the 1 hour radioactive index (Table 3) and the S phase figures are applied to the formula, the renewal time would appear to be

$$\text{for the epithelial cuff, } \frac{100\times 7}{5\times 24} = 5.8 \text{ days, and}$$

$$\text{for the attached gingiva, } \frac{100\times 7}{2.8\times 24} = 10.4 \text{ days.}$$

This renewal time for the epithelial cuff is in agreement with the times estimated by observation. For the keratinized attached gingival epithelium where it was impossible to make satisfactory late observations, the calculation method is considered to be more accurate.

The results from this experiment as well as those from the experiment carried out on mice, show that the epithelial cuff in both animals is renewed every 3—6 days.

It is difficult to imagine how a permanent "epithelial attachment" can exist under these circumstances.

SUMMARY AND CONCLUSIONS

1. Twenty-four marmosets were injected intraperitoneally with tritiated thymidine and observations were made on the gingival epithelium by autoradiography.
2. The DNA synthesis phase of epithelial cells was calculated from biopsies of oral mucous membrane and found to be 7 hours.

3. The manner of cell migration of epithelial cells of the gingiva including the epithelial cuff appeared passive in type. Movement towards the surface seemed to occur as a result of new cell formation in the basal layers.
4. The cell populations of both epithelia conformed to the criteria of *Leblond & Messier* for renewing cell populations.
5. The renewal time for epithelium of the epithelial cuff was observed to be approximately 6 days. This observation was supported by calculation made from the radioactive index of animals sacrificed one hour after injection. For attached gingiva the figure of 10 days was obtained.
6. A permanent attachment of surface epithelial cells to the enamel of the tooth is unlikely in view of the continued loss and renewal of these cells.

RÉSUMÉ ET CONCLUSIONS

LE RENOUVELLEMENT DE L'ÉPITHÉLIUM GINGIVAL CHEZ LES MARMOUSETS (CALLITHRIX JACCHUS) D'APRÈS AUTORADIOGRAPHIES À L'AIDE DE LA THYMIDINE-H^3.

1. Vingt-quatre marmousets ont reçu de la thymidine au tritium en injection intra-péritonéale et leur épihélium gingival a été observé à l'aide d'autoradiographies.
2. On a calculé, en se basant sur des biopsies de la muqueuse buccale, la phase de la synthèse d'ADN, et on a trouvé qu'elle était de 7 heures.
3. Le mode de migration cellulaire des cellules épithéliales de la gencive comprenant l'attachement épithélial a paru être de type passif. Les mouvements vers la surface se produisant ont semblé être le résultat d'une nouvelle formation cellulaire dans les couches basales.
4. Les populations cellulaires des deux épithéliums étaient conformes aux critères de *Leblond et Messier* pour le renouvellement des populations cellulaires.
5. On a observé que le temps de renouvellement pour l'épithélium de l'attachement épithélial était de 6 jours environ. Cette observation a été confirmée par le calcul fait en se basant sur l'index radio-actif d'animaux sacrifiés une heure

après l'injection. Pour la gencive adhérente, on a obtenu la valeur de 10 jours.

6. Un attachement permanent des cellules épithéliales superficielles à l'émail de la dent est peu vraisemblable en considération de la perte et du renouvellement continus de ces cellules.

ZUSAMMENFASSUNG UND SCHLUSSFOLGERUNGEN

1. 24 Krallenaffen wurden mit H^3-Thymidin intraperitoneal injiziert, und Beobachtungen wurden an dem Gingivalepithel mit Autoradiographie gemacht.
2. Die DNA-Syntesenzeit der Epithelienzellen wurde von Biopsien der oralen Mukosa auf 7 Stunden bestimmt.
3. Die Art der Zellenwanderung der gingivalen Epithelienzellen einschliesslich des Epithelansatzes schien passiver Art zu sein. Bewegungen auf die Oberfläche zu stellten sich als Ergebnis einer Neubildung der Zellen in den basalen Schichten heraus.
4. Die Art der Zellen der beiden Epithelien stimmte mit den Kriterien von *Leblond und Messier* über "renewing cell population" überein.
5. Die Erneuerungszeit für Epithel des Epithelansatzes wurde auf ungefähr 6 Tage bestimmt.
   Diese Observation wurde von Berechnung von radioaktiven Indices in Tieren, die eine Stunde nach der Injektion getötet wurden, unterstützt.
   Für befestigte Gingiva war die Erneuerungszeit etwa 10 Tage.
6. Eine permanente Befestigung der oberflächlichen Epithelzellen des Epithelansatzes an dem Zahnschmelz ist in Anbetracht der dauernden Erneuerung dieser Zellen nicht wahrscheinlich.

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