HISTOLOGICAL CHANGES IN THE ORAL MUCOSA OF THE WISTAR RAT TREATED WITH COMMERCIAL LIME (CALCIUM HYDROXIDE)—AN OPTICAL AND SUBMICROSCOPIC STUDY

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THE high incidence of oral cancer in different population groups in many south-east Asian nations has been stressed repeatedly in early and recent reports (Orr, 1933; Balendra, 1949; Cooray, 1944; Khanolkar, 1959; Muir, 1962; Pindborg, 1965). In India, and elsewhere, epidemiological and biological investigations have been done, or are in progress, on any possible correlation between the habitual use of the betel chew or " pan " and oral cancer. The involvement of the main constituents of " pan "—betel leaf (*Piper betel*), betel nut (*Areca catechu*), tobacco (*Nicotiana tabacum*), slaked shell or stone lime (slaked calcium hydroxide) and catechu (Acacia catechu) have also been considered individually or in combination (Mody and Ranadive, 1959; Muir and Kirk, 1960; Dunham, Muir and Hamner, 1966). The role of lime appears rather controversial, though some reports do suggest its correlation with the occurrence of oral cancer. It was therefore considered worthwhile to study the effects of slaked commercial lime on the oral mucosa of laboratory animals. The main aims of the study were to evaluate the part played by lime in the causation of oral malignancy or of oral conditions considered to be precancerous and the trauma it caused to epithelial and subepithelial tissue. This paper reports the histological changes produced on short term and prolonged application of lime to the oral mucosa of normal, diet conditioned and hormone treated Wistar rats.

MATERIAL AND METHODS

One hundred and thirty-nine weanling Wistar rats (66 male and 73 female) were divided into four experimental groups as follows:

Group I—36 animals on standard laboratory diet (wheat 66%; cracked lentils 15%; pea nut 5%; fish meal 7%, shark liver oil 1%, sesame oil 1%).

Group II—35 animals on a protein deficient diet (milled rice 1000 g.; NaCl 10 g.; vegetable oil 50 ml.; cod liver oil 20 ml.; B-complex tablets (100 mg.) 3 tablets).

Group III—36 animals on a vitamin B deficient diet (Sucrose 34·5%; corn starch 34·0%; vegetable oil (vitamin A + D — 700 Units) 6·7%; vitamin free casein 20%; NaCl 1·0%; $CaCO_3$ 0·5%).

Group IV—32 animals on standard laboratory diet, pretreated locally with deoxycorticosterone acetate (DOCA). The hormone (Decortone—Cipla Ltd., Bombay) was injected on the palate biweekly in a dose of 1 mg. per injection.

The experiment was started after pretreatment with 5 mg. in each animal. The total hormone dosage was as follows:

Period of time (months)	Total animals	Total dosage (mg.)
1	6	12
2	6	21
3	5	29
4	6	38
5	5	47
6	4	56

A week after the introduction of solid diet, and in Group IV, after 5 mg. of DOCA had been injected locally, the animals in each experiment were divided into six groups, each consisting of one littermate control and five experimental animals. The palate and buccal mucosa of all the experimental animals were painted daily, for five days a week, with the test substance. The control animals were stroked identically with a camel hair brush, simulating the mechanical irritation produced during the oral painting. Animals in Group I were killed in batches of 6 (1 control and 5 experimental) at 2, 4, 6, 8, 10, and 12 months after start of painting. Animals in the diet deficient groups were killed in batches of 6 (1 control and 5 experimental) at 2, 3, 4, 5, 6 and 7 months after start of painting, as it was difficult to keep them alive for longer time periods. The hormone treated animals were killed in 6 batches (1 control and 5 experimental) at 1, 2, 3, 4, 5 and 6 months after start of painting.

The palate, tongue and buccal mucosa were fixed in Lillie's 10% neutral formalin and divided in two portions for optical and electron microscopy.

6 μ thick serial paraffin sections were stained with haematoxylin and eosin, Mallory's trichrome, phosphotungstic acid haematoxylin (PTAH), Weigert's resorcin fuchsin for elastica, aqueous 0·5% toluidine blue at pH 4·5.

For electron microscopy small pieces of palatal and buccal mucosal sub-epithelial connective tissue were isolated under microscopic control from the formalin fixed tissue, and washed for two hours in distilled water to remove traces of the fixative. This material was then teased into a fine suspension of isolated fibrils. Micro drops of this suspension were placed on coated 200 mesh copper specimen grids. The samples were air dried, metal shadowed lightly at an angle of 15–20° with chromium, and scanned in an RCA-EMU 2D electron microscope with an objective aperture of 0·001″ internal diameter.

The design of experiments in all four groups is shown in Table I.

The test substance used was slaked lime (calcium hydroxide) sold commercially as a paste for use in the betel chew. Lime is prepared either from the calcareous coating of marine invertebrates, harvested along the coast line, or from stone quarried in three Indian provinces, Marwar, Uttar Pradesh or Bihar. The chemical reaction involved in the commercial manufacture of this substance is $CaCO_3 \xrightarrow{heat} CaO + CO_2$; $CaO + H_2O = Ca(OH)_2$.

RESULTS

In all four groups gross and microscopic observations on male and female animals will be described together as no significant sex difference was observed in the tissue response.

Macroscopic observations.—All control and experimental animals were weighed weekly. Those on stock diet in Groups I and IV recorded a normal weight curve, whereas animals maintained on a protein and vitamin B deficient diet in Groups II and III showed gradual inanition and appeared generally unhealthy, with dull hair and soreness of skin.

At the time of killing the majority of the animals in all four groups showed a marked mauve discolouration of the oral mucosa.

Microscopic observations.—Table II gives a summary of the epithelial response in control and experimental animals in all four groups.

A mild to moderate palatal hyperplasia occurred in 21 out of 24, and buccal mucosal hyperplasia in 17 out of 24 control animals (Fig. 1 and 2). Other more abnormal palatal epithelial changes were cell vacuolation in 7 animals, a prominent stratum granulosum in 9 animals, hyperkeratosis in 5 animals and parakeratosis in 1 animal. In the buccal mucosa these changes occurred as follows: cell vacuolation in 6 animals, a prominent stratum granulosum in 9 animals, hyperkeratosis in 5 animals and parakeratosis in 1 animal. No control animal in any four groups showed cell atypia or acanthosis of the epithelium.

In a total of 115 palates painted with the lime the epithelial changes were mild to moderate to massive hyperplasia and a prominent stratum granulosum in all animals (Fig. 3 and 4), hyperkeratosis in 96 animals, marked cytoplasmic vacuolation with a large nucleus and single or multiple nucleoli in 50 animals (Fig. 5 and 6). Attempts at invagination of rete pegs into the papillary layer were seen in 59 animals (Fig. 3 and 4), whilst frank acanthosis was found in 26 animals (Fig. 5 and 6). Similarly, in a total of 115 buccal mucosa painted with the lime the changes were mild to moderate to massive hyperplasia and a prominent stratum granulosum in all animals (Fig. 7 and 8) marked cytoplasmic vacuolation with large nuclei in 71 animals, mild hyperkeratosis in 82 animals (Fig. 9, 10 and 11), invagination of rete pegs into the papillary layer in 59 animals and frank acanthosis with cell atypia in 28 animals (Fig. 9, 10 and 11).

Table III summarises the connective tissue response in all the four groups. All control and test animals in Groups II and III showed a scarcity of fibrous connective tissue. In the 24 control animals, submucosal hyalinisation was seen in 12 animals, while fibroblastic proliferation was seen in animals kept on a B-complex deficient diet and in all animals given the hormone. A mild inflammatory exudate was seen in 4 animals, 3 of whom belonged to Group IV where injection injury had occurred. There was no dilatation and congestion of blood vessels in any control animal. A progressively enhanced submucosal connective tissue reaction was found in all test animals. In all four groups there was considerable increase in fibroblastic proliferation (Fig. 6 and 11), oedema (Fig. 11), connective tissue hyalinisation (Fig. 4 and 5), and a chronic inflammatory exudate. Blood vessels were dilated and congested in 104 lime-exposed animals.

Fibroblasts in the protein and vitamin B deficient group had a foamy vacuolated sparse cytoplasm, while those in the group of rats treated with DOCA were large with abundant cytoplasm.

Three differential stains for connective tissue were used on serial sections in this study. Table IV summarises the reaction of oral mucosal collagen to Mallory's trichrome and phosphotungstic acid haematoxylin, (PTAH) and Weigert's resorcin fuchsin for elastica. In the 24 control animals, the palatal collagen of only one and the buccal mucosal collagen of only 2 animals showed tinctorial

abnormality in occasional areas, identical coarse collagen staining pink with the trichrome stain and purple with the PTAH. There was no increase in the resorcin positive element in any tissue. In the 115 experimental animals, palatal collagen of 49 animals and buccal mucosal collagen of 99 animals showed this tinctorial alteration, in an occasional fibre bundle (Fig. 12 and 13).

The mast cell response, seen in Tables V and VI differed markedly in the palate and the buccal mucosa. Mast cells were very few in the palate and few to moderate in the buccal mucosa of all untreated control animals in the four groups.

EXPLANATION OF PLATES

FIG. 1 to 15 illustrate the tissue reaction in the oral mucosa of Wistar rats, irritated with a dry camel hair brush or painted with commercial lime.

FIG. 1.—Section of the palate of diet control animal kept on a protein deficiency for 3 months. A very mild epithelial hyperplasia and sparse connective tissue are seen. H. & E. ×65.

FIG. 2.—Section of the buccal mucosa of a control animal irritated with a dry camel hair brush for 6 months. There is very mild epithelial hyperplasia and a prominent stratum granulosum. No abnormal keratinisation or hyalinisation of the subepithelial connective tissue is seen. H. & E. ×65.

FIG. 3.—Section of the palate from an animal painted for 4 months. There is moderate to marked epithelial hyperplasia and a prominent stratum granulosum. Epithelial cords invaginate into the corium. H. & E. ×78.

FIG. 4.—Section of the palate painted for 5 months. The epithelium is moderately to markedly hyperplastic and has a very prominent stratum granulosum. Acanthosis of the epithelium into the corium is seen. Subepithelium connective tissue is hyalinised. H. & E. ×65.

FIG. 5.—Section of the palate painted for 8 months showing marked hyperplasia, a prominent stratum granulosum, and much acanthotic invagination into the corium. The subepithelial connective tissue is sparse and hyalinised. H. & E. ×78.

FIG. 6.—Section of the palate from protein deficient animal painted for 6 months. There is marked hyperplasia, a prominent stratum granulosum, nuclear vacuolation and acanthotic invagination. Subepithelial areas show marked fibroblastic proliferation and inflammation. H. & E. ×107.

FIG. 7.—Section of the palate from a protein deficient animal painted for 6 months. Epithelial hyperplasia and a very prominent stratum granulosum are seen. Cell dissociation and atypical staining of nucleoplasm are evident. H. & E. ×214.

FIG. 8.—Section of the palate from a protein deficient animal painted for 6 months. Picture shows an area of marked hyperplasia, with a prominent stratum granulosum and parakeratosis. Cell dissociation and vacuolation were also seen. Juxta-epithelial areas were highly cellular, showing fibroblasts with a foamy nucleoplasm and inflammatory cells. H. & E. ×214.

FIG. 9.—Section of the buccal mucosa painted for 8 months showing cell dissociation, atypical nuclear staining and invagination of epithelial cells into the corium. There is a chronic inflammatory exudate and much fibroblastic proliferation in the corium. H. & E. ×107.

FIG. 10.—Section from the buccal mucosa of DOCA treated animal painted for 1 month. Picture shows an area of marked hyperplasia, and a prominent stratum granulosum. There are marked acanthotic epithelial cords projecting into the corium, individual cells showing dissociation dyskeratosis and hyperchromatic nuclei. H. & E. ×214.

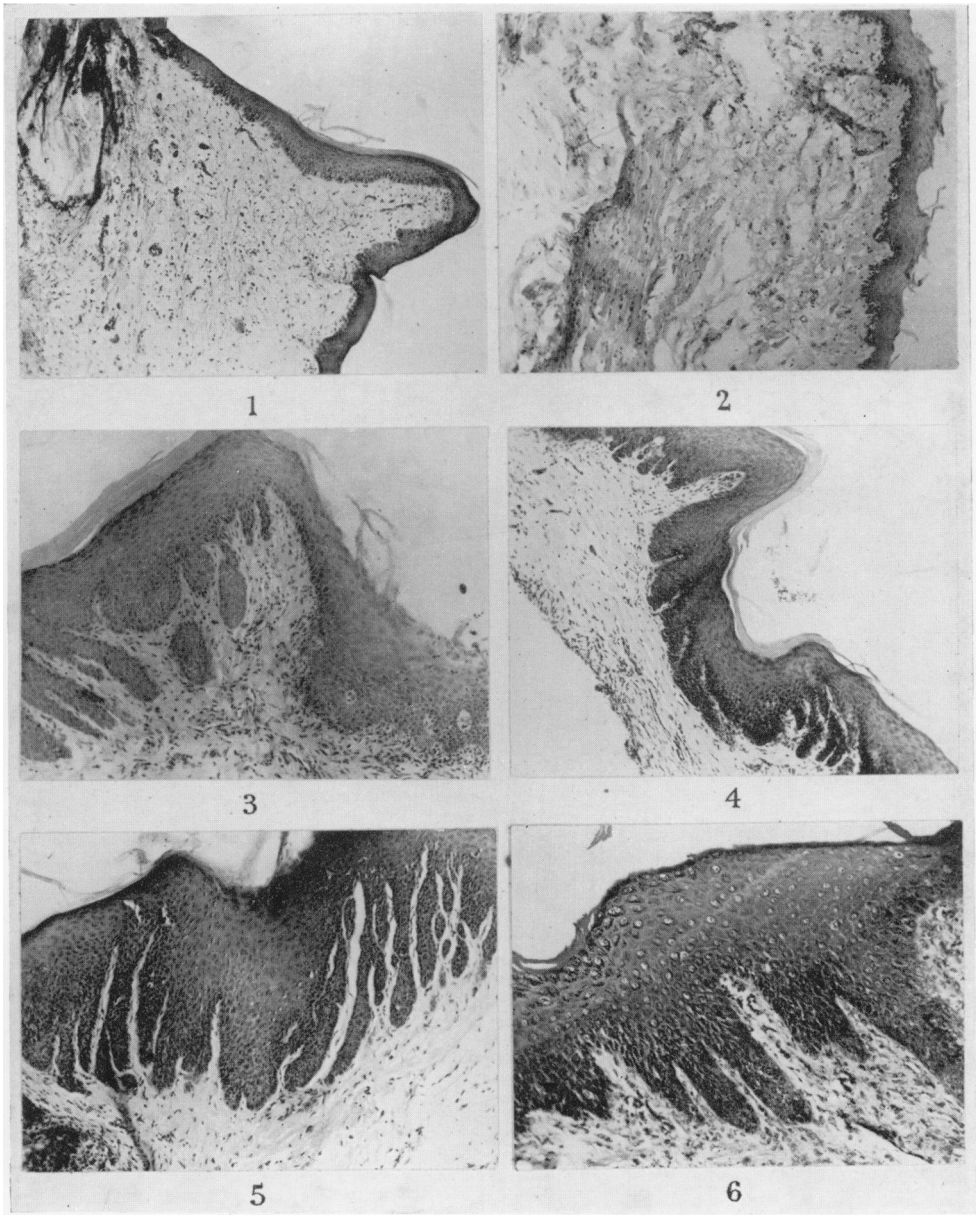
FIG. 11.—Section of the buccal mucosa of DOCA pretreated animal for 5 months. There is hyperplasia and a very prominent stratum granulosum. Invagination of the rete pegs into the corium is marked. The subepithelial mucosa shows oedema and much fibroblastic proliferation. H. & E. ×65.

FIG. 12.—Section of the palate of DOCA treated animal painted for 5 months. Picture shows pale to intense uneven staining collagen with chromophobic areas. An occasional fibre is abnormally stained orange/blue or orange (black in the picture). Mallory's Trichrome. ×90.

FIG. 13.—Section of the buccal mucosa painted for 6 months showing areas of coarse collagen fibres, stained partially orange blue. Mallory's Trichrome. ×180.

FIG. 14.—Electron micrograph of isolated collagen from palate of DOCA treated 4 months control animal. Long stranded collagen with a normal 640 Å periodicity is seen. There is mild lateral swelling of the fibrils and increased amorphous material. Cr shadowed. ×27,500.

FIG. 15.—Electron micrograph of isolated collagen from the palate painted for 2 months. Long stranded normal collagen with slight lateral swelling and mild increase in amorphous material are seen. Cr shadowed. ×21,600.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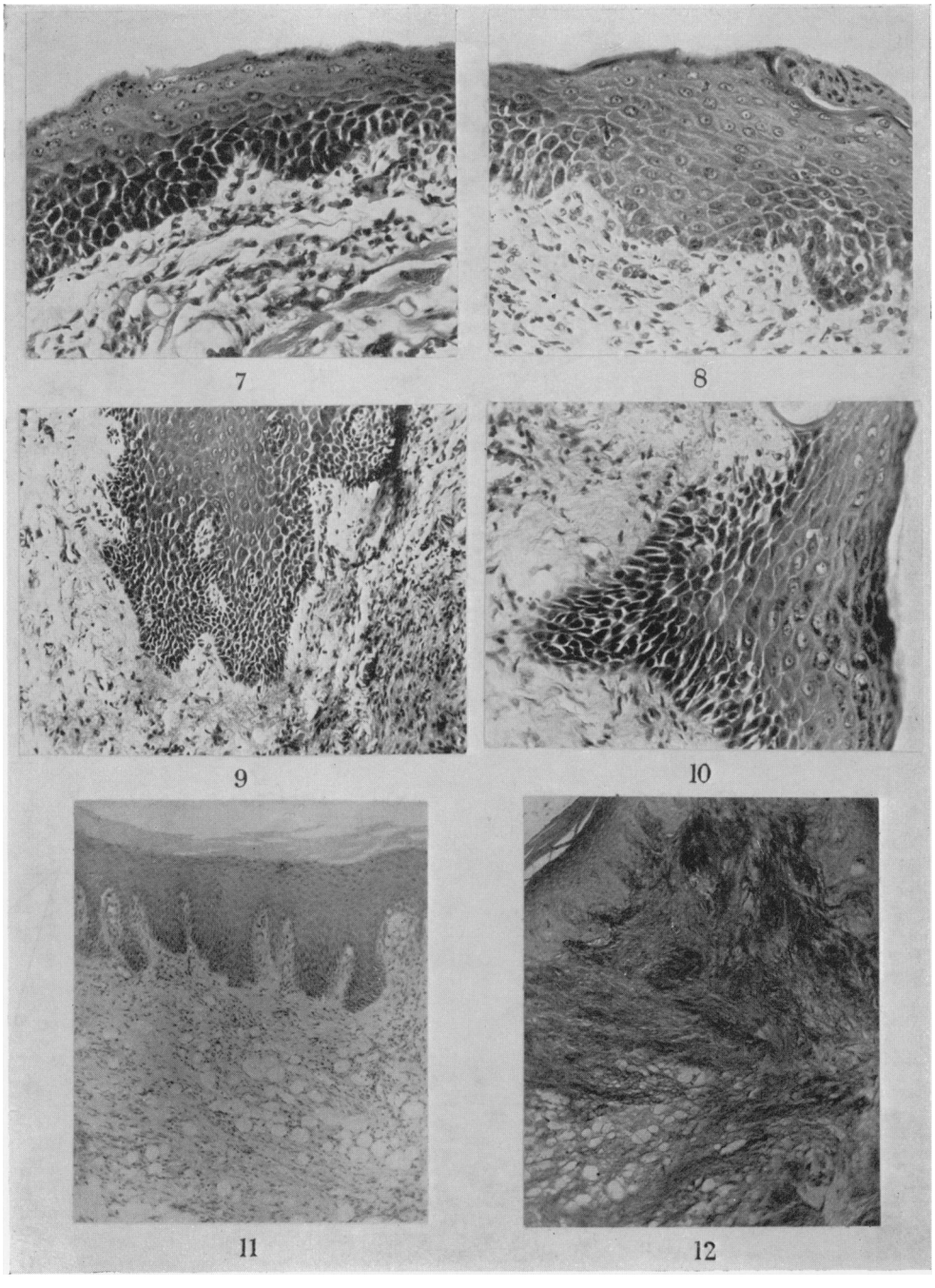

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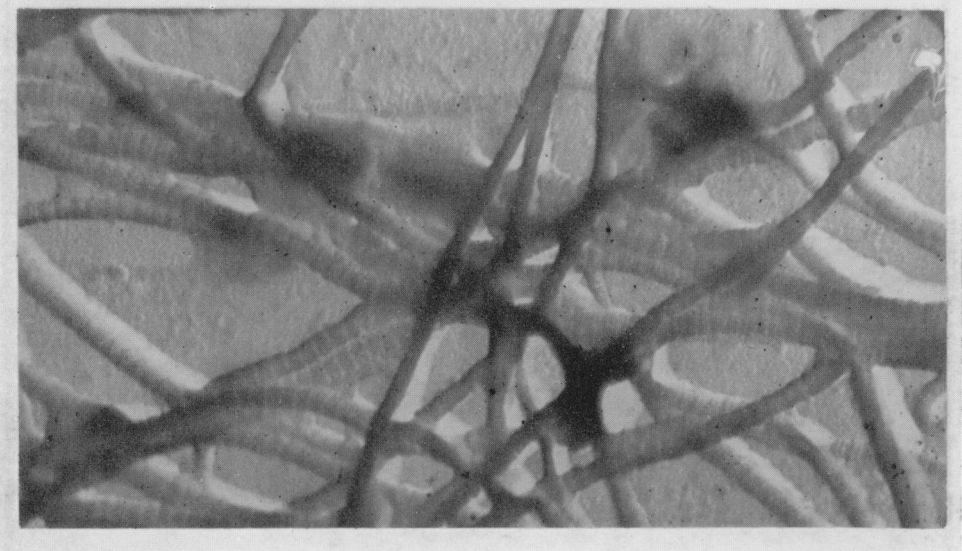

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TABLE I.—*Local Painting of Oral Mucosa of Wistar Rats with Commercial Lime = Design of Experiment*

Group	Duration of experiment (months)																								Total animals	
	1		2		3		4		5		6		7		8		9		10		11		12			
	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E
I Stock diet	–	–	1	5	–	–	1	5	–	–	1	5	–	–	1	5	–	–	1	5	–	–	1	5	6	30
II Protein deficient	–	–	1	5	1	4	1	5	1	5	1	5	1	5	–	–	–	–	–	–	–	–	–	–	6	29
III Vitamin B deficient	–	–	1	5	1	5	1	5	1	5	1	5	1	5	–	–	–	–	–	–	–	–	–	–	6	30
IV Stock diet DOCA treated	1	5	1	5	1	4	1	5	1	4	1	3	–	–	–	–	–	–	–	–	–	–	–	–	6	26

C = Control
E = Experimenta

TABLE II.—*Local Painting of Oral Mucosa of Wistar Rats with Commercial Lime—Summary of Epithelial Response in Four Groups*

Group	Total animals	Epithelium													
		Hyperplasia		Vacuolation		Prominent stratum granulosum		Hyperkeratosis		Parakeratosis		Invagination		Acanthosis	
		Palate	Buccal mucosa	Palate	Buccal mucosa	Palate	Buccal mucosa	Palate	Buccal mucosa	Palate	Buccal mucosa	Palate	Buccal mucosa	Palate	Buccal mucosa
Stock diet															
I Control	6	5	4	1	1	1	1	1	1	—	—	—	—	—	—
Experimental	30	30	30	11	17	30	30	21	16	1	3	13	13	5	6
II *Protein Deficient diet*															
Control	6	6	3	2	3	2	1	2	—	—	—	—	—	—	—
Experimental	29	29	29	18	25	29	29	25	19	11	17	12	11	7	7
III *B-Complex Deficient diet*															
Control	6	5	5	3	1	4	4	1	1	1	1	—	—	—	—
Experimental	30	30	30	19	21	30	30	24	23	13	14	14	15	8	8
Stock diet															
DOCA—Control	6	5	5	1	1	2	3	1	—	—	—	—	—	—	—
Experimental	26	26	26	8	8	26	26	26	24	11	8	20	20	6	7

TABLE III.—*Local Painting of Oral Mucosa of Wistar Rats with Commercial Lime—Summary of Connective Tissue Response in Four Groups*

Group	Total animals	Connective tissue response: Fibroblast proliferation — Palate	Fibroblast proliferation — Buccal mucosa	Hyalinisation — Palate	Hyalinisation — Buccal mucosa	Inflammation — Palate	Inflammation — Buccal mucosa	Dilatation of blood vessels — Palate	Dilatation of blood vessels — Buccal mucosa
I *Stock diet*									
(i) Control	6	—	—	1	1	—	—	—	—
(ii) Experimental	30	30	30	29	30	28	30	13	30
II *Protein deficient*									
(i) Control	6	—	—	3	3	—	—	—	—
(ii) Experimental	29	23	23	29	29	29	29	10	27
III *Vitamin B-Complex deficient*									
(i) Control	6	6	6	4	4	1	1	—	—
(ii) Experimental	30	30 (Vacuolated)	30 (Vacuolated)	30	30	30	30	13	30
IV *DOCA treated*									
(i) Control	6	6	6	4	4	3	3	—	—
(ii) Experimental	26	26	26	26	26	26	26	20	26

TABLE IV.—*Local Painting of Oral Mucosa of Wistar Rats with Commercial Lime—Summary of Response of Fibrous Connective tissue to Differential Stains*

	Group	Total No. of animals	Reaction to differential connective tissue stains: Palate — Mallory's trichrome — Normal	Palate — Mallory's trichrome — Abnormal	Palate — PTAH — Normal	Palate — PTAH — Abnormal	Palate — Weigert's R.F. — Normal	Palate — Weigert's R.F. — Abnormal	Buccal mucosa — Mallory's trichrome — Normal	Buccal mucosa — Mallory's trichrome — Abnormal	Buccal mucosa — PTAH — Normal	Buccal mucosa — PTAH — Abnormal	Buccal mucosa — Weigert's R.F — Normal	Buccal mucosa — Weigert's R.F — Abnormal
I *Stock diet* 1 to 12 mths.	Control	6	6	—	6	—	6	—	6	—	6	—	6	—
	Expt.	30	14	16	18	12	14	16	3	27	3	27	3	27
II *Protein deficient diet* 1 to 7 mths.	Control	6	6	—	6	—	6	—	6	—	6	—	6	—
	Expt.	29	20	9	20	9	19	10	3	26	3	26	2	27
III *B-Complex deficient diet* 1 to 7 mths.	Control	6	6	—	6	—	6	—	6	—	6	—	6	—
	Expt.	30	11	19	11	19	12	18	7	23	7	23	6	24
IV *Stock diet* 1 to 7 mths.	Control	6	6	—	5	1	6	—	4	2	4	2	6	—
	Expt.	26	11	15	11	15	12	14	3	23	3	23	3	23

PTAH = Mallory's Phosphotungstic Acid, Haematoxylin
Weigert's R.F. = Weigert's Resorcin Fuchsin
Expt. = Experimental animals.

TABLE V.—*Local Painting of Oral Mucosa of Wistar Rats with Commercial Lime—Summary of Mast Cell Response in Four Groups*

Group	Total number of animals		Mast cells (palate)									
			Very few		Few		Moderate		Increased		Considerably increased	
	Control	Expt.	Control	Expt.	Control	Expt.	Control	Expt.	Control	Expt.	Control	Expt.
I *Stock diet* 1 to 12 mths.	6	30	3	20	3	8	—	—	—	—	—	—
II *Protein deficient diet* 1 to 7 mths.	6	29	4	23	—	2	2	4	—	—	—	—
III *B-Complex deficient diet* 1 to 7 mths.	6	30	5	24	1	5	—	—	—	—	—	—
IV *Stock diet* DOCA pretreated 1 to 6 mths.	6	26	6	26	—	—	—	—	—	—	—	—

Expt. = Experimental animals.

TABLE VI.—*Local Painting of Oral Mucosa of Wistar Rats with Commercial Lime—Summary of Mast Cell Response in the Buccal Mucosa in Four Groups*

Group	Total number of animals		Mast cells (buccal mucosa)									
			Very few		Few		Moderate		Increased		Considerably increased	
	Control	Expt.	Control	Expt.	Control	Expt.	Control	Expt.	Control	Expt.	Control	Expt.
I *Stock diet* 1 to 12 mths.	6	30	—	—	—	7	6	15	—	3	—	5
II *Protein deficient diet* 1 to 7 mths.	6	29	—	7	—	2	4	13	1	—	1	7
III *B-Complex deficient diet* 1 to 7 mths.	6	30	4	5	—	—	1	20	—	—	1	5
IV *Stock diet DOCA pretreated* 1 to 6 mths.	6	26	—	2	1	8	5	13	—	—	—	3

Expt. = Experimental animals.

In the experimental animals, the distribution of mast cell in the palate was very sparse, while in the buccal mucosa they were very few to moderate to considerably increased in number in the four groups.

Submicroscopically, untreated control animals in all four groups showed normal long-stranded collagen with a 640 Å periodicity (Fig. 14). The only submicroscopic alteration seen in the deficient and DOCA-treated experimental animals was a mild lateral swelling. The DOCA-treated group also showed some increase in amorphous material. There was no loss of the 640 Å periodicity (Fig. 15) which was not often obscured by a coating of increased amorphous material.

DISCUSSION

This study should optimally have been carried out on a number of species simultaneously. However, designs of experiment are often limited by technical difficulties. It was possible therefore to test only one rodent species, the Wistar rat, which has been maintained in a controlled inbred state since its import in 1942. Varied experimental conditions have been obtained by inducing dietary or hormonal stress in parallel with exposure to the irritant.

The role of diet in the causation of changes in the oral cavity has been mentioned (Sharp, 1956; Wolbach and Bessey, 1942; Forbus, 1952; Martin and Koop, 1942; Khanolkar, 1944; Shanta and Krishnamurthi, 1963). The lack of protein in the diet produces many tissue changes in the oral cavity such as thinning of the mucosal epithelium (Stahl, Sandler and Cahn, 1955; Unakar, 1960) and retarded wound healing (Udupa, Woessner and Dunphy, 1956). Vitamin B has been correlated with oral mucosal changes by a number of investigators. Martin and Koop (1942) have implicated vitamin B deficiency in the etiology of degenerative changes in the human oral mucosa usually antedating malignant transformation. In this investigation therefore two groups of Wistar rats consisting of 35 and 36 animals were deprived of protein and vitamin B-complex respectively. The animals kept on a B-complex deficient diet were not given an additional dose of any sulpha compound as the aim was to induce only a mild deficiency such as that found in malnourished Indian people and not a total lack of the vitamin.

The use of DOCA seems to need some explanation. Reports describe an enhanced tissue reaction with the use of certain anabolic hormones (Bauer and Clark, 1953; Pirani *et al.*, 1950; 1951; Sirsat and Khanolkar, 1960; Ketkar and Sirsat, 1966). There was originally some confusion as to whether DOCA was responsible for the production of inflammation *per se*. A detailed study was carried out by Ketkar and Sirsat (1966) on the exact morphological and histochemical changes in connective tissue treated with DOCA. They concluded that DOCA itself did not induce inflammation. When the trauma of injection accompanied DOCA administration the inflammation was more marked than that in which injection injury was inflicted without parallel hormone administration. In a study of capsaicin treatment in DOCA pretreated oral mucosa in the Wistar rat, Sirsat and Khanolkar (1960) reported a total increase in sensitivity to capsaicin, observed optically and submicroscopically, in the hormone treated animals. It was in the light of these observations that this anabolic mineralocorticoid was used in this study.

The histology of the untreated control palate, carried out in these studies, also notes the marked divergence in the number of epithelial cell layers which normally

occur in different parts of the palate (Kutuzov and Sicher, 1952). It was therefore extremely necessary, that in a study which takes into account cell multiplication, care should be taken and uniformity maintained in the palatal area being described. This study compares mainly the palatal epithelium of the rugal ridges, in the three groups—normal untreated, solvent control and palates painted with the lime. This however did not rule out description of marked change occurring in other parts of the palate.

It is seen throughout that the buccal mucosa presents a greater degree of tissue change than that observed in the palate. Observations on control and treated oral mucosa described in this paper lead one to conclude that in the Wistar rat, hyperplasia alone should not be considered a specific pathological change provoked by the irritant. It appears to be more a biological disturbance seen routinely in the rat palate as a constant reaction to mastication of solid food. Invagination of rete cones into the papillary layer was found in 59 lime-treated animals, while frank acanthosis occurred in only 28. Similar acanthosis has been noted by Dunham, Muir and Hamner (1966) in 26 hamsters treated with calcium hydroxide. Two valid conclusions are possible from this study of the oral epithelium— (1) In animals not exposed to an extraneous traumatic agent, continuous injury due to solid diet, coupled with enhanced sensitivity of the mucosa due to dietary or hormonal stress can occasionally produce an epithelial change which can be called abnormal, (2) Acanthosis of the oral epithelium is found only in animals exposed to lime, and reflects probably a deeper injury to the cellular growth pattern and also a chronically altered epithelium connective tissue interrelationship.

Table III shows the inflammatory response in the control and experimental animals. The palate and buccal mucosa of all experimental animals react to the lime-inflicted trauma with marked dilatation of blood vessels and inflammatory exudation. Inflammation in response to the whole betel chew has been mentioned by Muir and Kirk (1960). These authors painted the ears of 53 Swiss mice with an aqueous extract of a typical Singapore tobacco, betel nut, shell lime " quid " for 2 years and produced a chronic inflammatory exudate with a rather oedematous dermis after 5 months of painting. In this study, the presence of protein and vitamin B deficiency and the exposure to DOCA no doubt were factors that augmented the oral tissue response to this highly traumatic ingredient of the betel chew.

Like the epithelial reaction, the mast cell response appears to be considerably more marked in the buccal mucosa than the palate. This could really be so, for the same reason suggested for the greater epithelial hyperplasia in the buccal mucosa than in the palate—a more intimate exposure to the irritant. A point has however to be kept in mind that even in untreated controls, under normal conditions, the palatal mast cell population is much lower than in the buccal mucosa. Even so, proportionately speaking, the mast cell response is very marked in the buccal mucosa in all four groups and suggests again the highly irritant nature of lime and the great susceptibility of the subepithelial tissue to this substance.

Subepithelial hyalinisation was a marked feature in the oral mucosa of all animals exposed to lime. The biological significance of this phenomenon, which is a non-specific limited connective tissue response to injury, and the epithelial reaction provoked by altered connective tissues have often been mentioned

(Gillman *et al.*, 1955; Sirsat and Pindborg, 1967). Suffice it here to emphasise that the epithelium overlying a hyalinised submucosa is an uneasy one, mainly due to a defective nutritional status resulting from the replacement of an active vascular tissue by an inert hyalinised substance. The abnormal staining reaction seen in the palates of 70 animals and in the buccal mucosa of 99 out of 115 animals exposed to lime fall within the definition given by Gillman *et al.* (1954, 1955) for a pseudoelastic transformation of the collagen fibres. Submicroscopically, however, the only change seen in the collagen fibrils isolated from the experimental animals is a lateral swelling. No frank alteration to degraded material similar to that seen in human oral submucous fibrosis (Sirsat and Khanolkar, 1957) and rat mucosa treated with capsaicin (Sirsat and Khanolkar, 1960) is found. Therefore the tinctorial changes do not reflect an outright transformation to frankly pathological degradation of an elastotic nature. Recent work on atypical staining of collagen suggests another possibility. Scheuner and Gabler (1965) carried out a comparative histological and histochemical study of collagen and elastic fibres in sections from 28 human organs and tissues from newborn to 28 years old, taken 0–7 days post-mortem, and in dogs and rabbits. Staining was done with a wide spectrum of 13 compounds. The factors involved in the aberrant staining of collagen were not ageing, post-mortem or pathological effects. These authors admit a similarity between the atypical stained collagen and fibrinoid in degraded connective tissue, but conclude that this phenomenon occurs not uncommonly in non-pathological collagen also. Craik and McNeil (1966) studied the differential staining of human collagen from the abdominal wall, after subjecting it to stress by artificial stretching. The fibrous reaction to stress was tinctorial alteration to red in Mallory's trichrome. They felt that the stretching probably reorientated the collagen structure, removed the aniline blue positive mucopolysaccharide and exposed fuchsinophilic protocollagen molecules. Melcher (1963) also described such altered staining of collagen in gingival inflammation.

In this investigation, too, much tissue damage is inflicted with the presence of oedema and continuous inflammation and a number of stressor agents come into play. The change in the collagen is seen only at the optical levels, tinctorially so, there being no submicroscopic degradation in fibril structure. The altered staining in this study could therefore also be due to a reorientation in the physical properties of the fibrils due to stress and not to any pathological reorientation at molecular levels in the basic structure of the scleroprotein fibril. Lateral swelling in the fibrils found submicroscopically confirms the swelling seen optically in all lime treated collagen. This swelling is brought about by an overall tissue hydration due to the pathological oedema and is a non-specific phenomenon. At the level of the fibril structure it reflects a change in the inter-molecular bonds with increased width of the chain.

The indirect aim in animal studies such as this one is always to evaluate the test substance as a possible causative agent of human oral disturbance. The correlation is attempted by a comparison of tissue changes in the human dyscrasia in question and the experimental study. Three main oral disorders come to mind in connection with the oral mucosa in the Indian people—submucous fibrosis, leukoplakia and frank oral cancer. In view of the tissue changes produced by commercial lime in the rat oral mucosa, it is worth discussing its probable role in the etiology of morbid conditions of the human mouth. Submucous fibrosis of the oral mucosa has been recognised as a not uncommon entity found

mainly in the Indian population (Joshi, 1953; Pindborg, 1965; Sirsat and Khanolkar, 1957, 1960) and occasionally in other south-east Asia countries (Pindborg and Sirsat, 1966). The main tissue reaction in this condition is epithelial atrophy in most cases (Pindborg *et al.* 1965) hyperplastic epithelium being seen in only a few advanced cases. There is dense hyalinisation of connective tissue, coexistent with a chronic inflammation (Sirsat and Khanolkar, 1957). Acute inflammatory changes followed by hyalinisation are found in this study in the lime treated mucosa also. The epithelial reaction is always a hyperplasia, very different from that usually seen in submucous fibrosis. It is very unlikely that the acute injury inflicted by lime could lead to the chronic productive response seen in submucous fibrosis (Sirsat and Khanolkar, 1960). A clinical reason to support this contention is that submucous fibrosis can occur in young subjects who have never chewed pan, and so never been exposed to long-term irritation by lime.

It has already been mentioned that lime has been correlated many years ago to the causation of leukoplakia (Fells, 1908; Orr, 1933; Bentall, 1908; Balendra, 1949). More recently, detailed surveys have been reported by Atkinson *et al.* (1964) and Forlen *et al.* (1965). Atkinson *et al.* studying the oral mucosa of islanders in New Guinea mention their custom of trailing the mucosal surface with a stick dipped in shell lime, subsequent to chewing the betel nut. They felt that the presence of white leukoplakic patches traced the pathway along which the lime must be continually deposited. Forlen *et al.* (1965) have reported a correlation between the use of lime and a high incidence of leukoplakia in these same people. Leukoplakia in all these studies rightly denotes just the clinical presence of white patches. The histological aspect of this term postulates the presence of hyperplasia, hyper- or para-keratosis, acanthosis and dyskeratosis (Shafer, Hine and Levy, 1964). In the majority of animals exposed to lime in this study, these changes were seen in the epithelium. The mucosal epithelium of the rat is normally more keratinised than that of man (Provenza, 1964); it is also notoriously more resistant to change induced by external agents. It therefore appears logical that if lime can evoke a disturbed growth pattern in the rat mucosa, the human buccal mucosal epithelium would be even more traumatised. Lasting mucosal changes could then occur. The question that followed concerns the further involvement of commercial lime in frank oral cancer. In this study, painting for periods of time up to 12 months did not produce a single recognisable malignancy, although atypia was seen in some epithelia. This need not rule out the involvement of this high-grade irritant in the causation of human oral cancer. On the basis of the moderate to massive epithelial and subepithelial tissue damage, caused by this irritant, two surmises can be made on the mode by which a long-term use of the betel chew might lead to oral cancer. A continuous coexistence of an highly altered epithelium and underlying connective tissue and a chronic interference with normal metabolic functions could lead to a neoplastic alteration of the epithelium. The other hypothesis is based on the combined use of stone or shell lime and tobacco, a practice prevalent in all parts of India, though not so in New Guinea according to Atkinson *et al.* (1964). It could be that the tobacco would exert a surer carcinogenic effect on tissue damaged severely or already rendered hyperplastic by the lime. Lime could then, when used along with tobacco, be termed rather loosely a co-carcinogen. Muir and Kirk (1960) produced squamous carcinoma or papilloma in the ears of Swiss mice painted daily for two years with a crude aqueous extract of the whole betel quid. They attributed their

success in obtaining tumours where purer tobacco extracts failed to elicit them (Mody and Ranadive, 1959) to the possible presence of co-carcinogenic factors in the whole betel quid. It could well be that lime, which is invariably present in the betel quid or " pan ", acts thus by virtue of the tissue reaction it provokes, as the co-carcinogenic agent.

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