EXPERIMENTAL ORAL CARCINOGENESIS

With Particular Reference to Premalignant Lesions

VOLUME 1 OF TWO VOLUMES

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Thesis

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PREFACE

The work described in this thesis was undertaken in the University of Glasgow during 1969 and from September 1970 - 1973 while the author was a Lecturer in Oral Medicine and Pathology in Glasgow Dental Hospital and School. During the academic year 1969 - 70 the author was a Visiting Associate Professor in Oral Pathology in the University of Illinois, Chicago, U.S.A. and some of the work recorded in this thesis was undertaken during that time.

A part of the preliminary study recorded in Chapter 2 was repetition of previously published work, but the remainder of the work of this thesis is original and was either undertaken individually by the author or by technical staff directly supervised by the author.

Parts of the work of this thesis have been presented at scientific meetings:-

1. "Premalignant and malignant lesions in hamster cheek pouch" International Association for Dental Research Meeting, Chicago Division. January, 1970.

2. "A Comparative Study of Oral Premalignancy and Malignancy in Hamster Cheek Pouch and Human Oral Mucosa" (with Waterhouse, J.P.). The American Academy of Oral Pathology, San Diego, California, U.S.A. April, 1970.

3. "Experimental Carcinogenesis in the Hamster" Oral Pathology Club, Liverpool, September, 1970.

4. "The Application of Morphometric and Stereologic Methods to Oral Epithelium" International Association for Dental Research. British Division, Glasgow, April, 1971.

5. "Cellular Atypia in Experimental Hamster Oral Carcinogenesis" Oral Pathology Club, Newcastle, September, 1972.

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ORAL CARCINOGENESIS - HUMAN AND EXPERIMENTAL

1.1 INTRODUCTION

The practicing diagnostic oral pathologist finds many of his most difficult cases are lesions of oral epithelium in which malignant or premalignant changes are suspected. On a personal level this is a problem which has confronted the author in routine diagnostic work in about 20 per cent of biopsies submitted for diagnosis in Glasgow Dental Hospital. In the year 1971, for example, out of a total of 952 specimens submitted 8 were squamous cell carcinomas of oral epithelium, 8 lesions were considered to show evidence of premalignancy and in 25 cases lesions in which there were sufficient clinical grounds to justify biopsy were reported as showing no evidence of pre-malignancy. Histological evidence of possible malignant change was also examined for in cases of lichen planus, papillary hyperplasias of palate and squamous cell papillomas.

The criteria upon which a judgement about possible premalignancy is based, will be discussed in more detail in later parts of this and subsequent chapters. These criteria, however, are largely subjective and not infrequently the prognostic implications of the particular combination of histological changes in an individual specimen are based upon what might almost be described as an

emotional judgement by the observer. Clearly this is an unsatisfactory position, and it prompted this present investigation into oral carcinogenesis.

1.2 HUMAN ORAL MALIGNANCY AND PREMALIGNANCY

1.2.1 Incidence of Carcinoma of the Mouth

Pindborg (1965) discussed various means of expressing frequency of oral carcinoma in a given population and suggested that the ideal procedure was to give morbidity and mortality rates per 100,000 of the population. The study of Binnie et al (1972), for the years 1962 to 1964, quoted an average registration of 4.2 intraoral malignant neoplasms per 100,000 of the population for men and 1.9 per 100,000 for women in Scotland. The corresponding figures for lip cancer were 5.8 per 100,000 and 0.3 per 100,000. The mortality from intraoral malignancy was higher than that for lip malignancy being 3.6 per 100,000 in males for intraoral neoplasms and 0.4 per 100,000 in males for lip neoplasms.

The overall incidence of oral malignancy appears to be higher in Scotland than in England and Wales; 12.2 per 100,000 in Scotland (1962 to 1964 mean figures) as opposed to 9.7 per 100,000 in England and Wales (1960 to 1962 mean figures) and the mortality figures are also higher in Scotland (Binnie et al, 1972).

On a worldwide basis, the frequency of oral cancer is highest in south east Asia. Pindborg et al (1965) reported a frequency of 240 per 100,000 in a group of 10,000 Indians. Pindborg (1965) further indicated that in Bombay, oral cancers accounted for 47 per cent of all cancer.

The majority (over 90 per cent) of malignant neoplasms in the mouth are squamous cell carcinomas arising from the oral epithelium (Waldron, 1970a). The incidence of squamous cell carcinomas varies widely in different parts of the world and it is only in the last ten to fifteen years that the magnitude of the problem of oral cancer has really been apparent. However, it is not the purpose of this discussion to consider in detail the epidemiology of oral malignancy. The figures above are quoted to highlight the fact that squamous cell carcinoma of the oral cavity is a cause of significant morbidity and mortality in many parts of the world and is clearly a problem which requires further study.

Attempts to overcome the problem of oral cancer require to be made from several approaches. The first of these is essentially epidemiological and concerns the identification of aetiological factors in order that a rational basis for prevention of the disease may be determined. Secondly, new and improved

methods of treatment must be sought. Thirdly, it would be advantageous if improved diagnostic techniques could be developed. Improved techniques are required at several stages in the diagnostic process, but it would be especially useful if these techniques enabled lesions to be recognised at an early stage of malignancy or preferably in a premalignant phase prior to the development of overt malignancy. It is known that squamous cell carcinomas in the mouth may be preceded by a premalignant phase and that diagnosis of this stage requires microscopic examination of the epithelium. The aim of this work is to advance knowledge of some aspects of the premalignant phase in the development of oral cancer.

1.2.2 Premalignant Lesions of Oral Epithelium

The clinical observations of a number of investigators have established that squamous cell carcinomas in the mouth may be preceded by, or may co-exist with, a number of other clinically distinctive lesions. Histological study of these other lesions often reveals evidence of some of the changes characteristic of malignancy and the classification of precancer or premalignant lesion has been accorded to these lesions. Several types of lesion have been thought to be premalignant and the most important of these will be discussed briefly.

Leukoplakia is the principal premalignant lesion in the mouth (Renstrup, 1958). Leukoplakia is the name given to abnormal white patches or plaques on the oral mucosa. The term has been used in a number of ways and a survey by Sprague (1963) showed just how diverse even a group of diagnostic Oral Pathologists could be in their interpretations of this word. Waldron and Shafer (1960) used the term leukoplakia in a simple descriptive clinical sense of a white plaque which was not diagnosable as any other distinct entity such as lichen planus, syphilitic mucous patch, lupus erythematosus and several other specific forms of stomatitis. Pindborg and his co-workers further refined the definition for leukoplakia to "a well demarcated elevated white patch of 5 mm or more in diameter that could not be scraped off and could not be attributed to the presence of other disease" (Meyer et al, 1967). The definition of leukoplakia is that of a clinical condition and carries no histological connotation.

The incidence of malignant change in oral leukoplakias has been the subject of a number of studies. Cawson (1969) summarised these as indicating that the incidence of cancer in leukoplakia was probably 50 to 100 times greater than in the normal mouth, but that 90 - 95 per cent of leukoplakias will not develop cancer in a five year period. Hobaek (1946) reported

that 16 per cent of 246 patients with oral leukoplakia developed cancer. There was a difference in the frequency of malignant change at different intraoral sites; the frequency being greatest in cheek lesions. Studies, such as those of Renstrup (1958) and Shafer and Waldron (1961), found the incidence of malignant change in leukoplakia to be 9 per cent and 17.7 per cent respectively. However, more recent studies such as those of Einhorn and Wersall (1967), involving up to 44 years of follow-up on 782 patients, or of Pindborg et al (1968) on 248 patients indicated an incidence of malignant transformation of about 4 per cent.

In 1963, Pindborg et al, identified as particularly dangerous a group of leukoplakias which presented as white nodular patches intermingled with erythematous areas. To these the designation speckled leukoplakia was given. This finding, of a greater potential for malignant change in speckled as opposed to homogeneous leukoplakia, was confirmed in the series reported in 1968 by Pindborg et al.

The leukoplakia which is found in association with syphilis is also thought to be specially prone to undergo change to a squamous cell carcinoma (Cawson, 1969). For example, Weisberger (1957) noted that 14 patients with co-existing leukoplakia and [redacted]

evidence of syphilis all developed oral carcinomas. It is possible that this type of leukoplakia is now less frequent because of the decline in incidence of tertiary syphilis.

The association of chronic candidal infection and leukoplakia was discussed by Cawson (1966) who noted 15 cases of chronic candidosis in 138 biopsy specimens of leukoplakia. Candidal infection is most frequent in cases of speckled leukoplakia. Cawson and Lehner (1968) however found that it was impossible to point to any clinical feature which reliably differentiated those lesions in which candida was present. Renstrup (1970) found a 23 per cent incidence of candida in lesions from a series of 235 patients with leukoplakia. The majority of cases with candida were speckled leukoplakias although a 3 per cent incidence of candida in homogeneous leukoplakias was noted. Jepsen and Winther (1965) expressed doubts about the aetiologic significance of candidal invasion, but the work of Cawson (1966) and Renstrup (1970) does implicate candida directly in the aetiology of some leukoplakias.

Well defined red patches of oral mucosa have also been identified as premalignant lesions. Similar red patches on the glans penis were given the name "erythroplasia" by Queyrat (1911). The oral lesions

have been designated as "erythroplasia of Queyrat" by Williamson (1964) and "Bowen's disease" by Howarth (1935) and Gorlin (1950). In a recent review Shear (1972) pointed out that erythroplakia is a more appropriate term. Mehta et al (1971) diagnosed erythroplakia "when the oral mucosa was the seat of a well demarcated red, often fiery red, patch which could not be attributed to other causes." Shear (1972) distinguished three types of erythoplakia and indicated that a speckled variety was the one most likely to undergo malignant change. He further commented that this is probably the same type of lesion as Pindborg and his colleagues would diagnose as speckled leukoplakia.

Lichen planus is a condition which is usually not considered as predisposing to cancer (Lucas 1972a). However, Andraesen and Pindborg (1963) were able to find 46 cases reported between 1910 and 1961 in which carcinomas had developed in patients with oral lichen planus. Andraesen (1968) noted a further 18 cases reported since 1963 and indicated that the incidence of malignant transformation of lichen planus was quoted as between 1 per cent and 10 per cent by different authors. Cawson (1969) felt that the incidence was less than 1 per cent.

Chronic ulcers and fissures of oral epithelium are often cited as potentially malignant lesions (Lucas, 1972b). However, no detailed study of this is available and the position is made difficult by the fact that oral cancers often ulcerate making it difficult to know whether the ulcer or the tumour was present first.

Oral submucous fibrosis is a condition in which there are dense areas of fibrosis of the hard and soft palate, the fauces and sometimes the cheek. The condition is most frequent in India, and was first mentioned as being possibly premalignant by Paymaster (1956). Pindborg et al (1967), reported that of a series of 100 patients with oral cancers in South India, 40 suffered simultaneously from submucous fibrosis. In a later paper, Pindborg (1972) summarised a large volume of work on submucous fibrosis and concluded that it is undoubtedly a premalignant condition.

The oral mucosal atrophy seen in the Kelly-Paterson syndrome (Plummer-Vinson syndrome) predisposes to malignancy (Ahlbom, 1936). Hobaek (1946) showed that malignant change in leukoplakias was much more frequent in females than in males in his series of Norwegian cases. Wynder et al (1957) demonstrated that the high incidence of upper alimentary tract and

oral cancer in women in Sweden was correlated with the incidence of Plummer-Vinson syndrome. The mucosal atrophy seen in vitamin B deficiency has been thought to predispose to squamous cell carcinoma but evaluation of nutritional deficiencies in man is difficult and conclusive evidence is lacking. The mucosal atrophy in Plummer-Vinson syndrome and in vitamin B deficiency should probably not be regarded as premalignant per se, but merely as conditions in which carcinomas are more likely to arise than in normal mouths.

The squamous cell papilloma is a common benign neoplasm of oral mucosa. Opinion varies as to whether or not it should be considered premalignant. For example, McCarthy and Shklar (1964) and Shklar (1965) group this lesion with others which they consider premalignant, whereas Waldron (1970b) disagrees with this and does not regard the papilloma as premalignant. Of the other larger Oral Pathology texts Lucas (1972c) and Shafer et al (1963) do not regard the squamous cell papilloma as a premalignant lesion.

The clinical diagnosis of the premalignant lesions discussed above is unreliable because carcinomas, premalignant lesions and totally benign lesions may all show similar clinical appearances (Silverman and

Ware, 1960). Histological examination is necessary to accurately diagnose each individual lesion.

1.2.3 Histological Features of Oral Epithelial Premalignancy

The single most important histological criterion for diagnosing squamous cell carcinomas of the mouth is invasion of the underlying tissues by the tumour. The tumours can also exhibit a wide range of other abnormalities in cell appearances, keratinization and staining characteristics. Similar changes can be seen in lesions which lack the critical feature of invasion and it is these lesions which are recognised as premalignant on histological grounds.

The histological abnormalities thought to characterise epithelial premalignancy have been discussed by many authors under a number of headings. In the older literature and in America the term dyskeratosis is most commonly used. Strictly speaking this refers to an abnormality in keratinization, but by common usage the term has been extended to include all the features suggestive of precancer. In the gynaecologic literature the term dysplasia is used and this term is also sometimes applied to oral mucosa. The derivation of the word is from the Greek δυς meaning ill or abnormal and πλασσειν the verb 'to form'. Dysplasia therefore means abnormally formed and is thus probably a more appropriate term.

In the Scandinavian literature the terms epithelial atypia or cellular atypia have been used for many years to describe the same types of changes as other authors describe for dyskeratosis or dysplasia. Atypia is the term which has been adopted by the World Health Organisation and it is the term which will be used in the remainder of this dissertation.

The precise designation of the histological changes which constitute cellular atypia show minor variations in different authors' writings. However, the 13 changes suggested by Smith and Pindborg (1969) would probably satisfy the majority of investigators. These authors describe five abnormalities relating to the proportions of different cell compartments and the arrangement of cells. These features are basal cell hyperplasia, drop shaped rete ridges, irregular epithelial stratification, loss of polarity and loss of intercellular adherence. Of these features basal cell hyperplasia describes an increase in the size of the progenitor cell compartment and drop shaped rete ridges is also related to an increase in the number of progenitor cells, which form downgrowths into the lamina propria. Irregular stratification of cells probably relates to altered migration kinetics and loss of polarity, describing irregular orientation of cells especially in the progenitor cell compartment is probably a manifestation of altered cell production

and migration kinetics. Loss of intercellular adherence is probably also related to these changes.

The keratinization pattern may alter in atypia. Apart from changes in the surface characteristics which are not usually included among the features of atypia, keratinization below the normal keratinized layer can occur. This probably represents a disturbance in the maturation of cells.

Three features of atypia are descriptive of cytological abnormalities. These are hyperchromatic nuclei, anisocytosis and anisonucleosis, and pleomorphic cells and nuclei. The hyperchromatic nuclei may represent an increase in chromosomal material either in normal cells in the G_2 phase of the cell cycle (the premitotic postsynthetic gap) or they may be abnormal nuclei. The abnormal variation in sizes and shapes of cells and nuclei probably relates to disturbances in both the cell production and the maturation of epithelial cells.

Three histological changes described under cellular atypia refer to cell division. These are increased mitotic activity; mitoses at an abnormally superficial site in the epithelium, this being related to an increase in the size of the progenitor cell compartment, and the presence of bizarre mitoses.

Pindborg and his co-workers have used as their criterion for atypia, the presence of two or more of these 13 histological changes.

The importance of cellular atypia as an indicator of premalignancy has been debated. Cawson (1969) stated that "it should not be assumed that leukoplakia, dyskeratosis and carcinoma in situ are the usual or inevitable preliminary stages of invasive carcinoma". Waldron (1970c) also took up this point by casting doubt on the validity of the assumption that leukoplakic lesions showing atypia on biopsy were more sinister. In support of this he quoted the fact that Pindborg et al (1968) failed to demonstrate atypia in the biopsies of 4 of 11 patients who subsequently developed carcinomas. Waldron (1970c) does not however comment upon the possibility that the biopsies might not, in fact, have been representative of the lesions.

As noted previously in 1.2.2 speckled leukoplakias have a poorer prognosis than homogeneous leukoplakias. Pindborg et al (1963) found that 51 per cent of their cases of speckled leukoplakia showed atypia. In a larger series of 723 cases Mehta et al (1969) found atypia in 8.4 per cent of homogeneous leukoplakias and 59.1 per cent of speckled leukoplakias. Thus the incidence of atypia is substantially greater in the type of leukoplakia which is known to have a poorer

prognosis. This fact alone however, does not necessarily prove that these two observations are related.

Pindborg et al (1970) in a study of atypia in 51 cases of oral submucous fibrosis observed that 22.6 per cent of these exhibited atypia. In cases developing carcinomas, atypia was noted in 71.4 per cent of histological sections from areas adjacent to the carcinomas, whereas it was recorded in only 11.5 per cent of areas of oral submucous fibrosis remote from the carcinomas.

Mincer et al (1972) presented a follow-up study of 56 patients with what the authors classified as moderate or marked atypicality. Lesions with minimal atypicality were felt to be reactive in nature and were not included in the study. The results showed that lesions with atypia did not invariably proceed to malignancy, but that indeed just over 10 per cent reduced in size or disappeared without surgery. However, 35 per cent of cases recurred after surgery, 11.1 per cent showed increased severity of atypia on follow-up and 11.1 per cent developed into squamous cell carcinomas. The authors concluded that the presence of epithelia atypia in a biopsy indicated a significant likelihood that frank malignancy would develop.

Although this is an area which is still being debated the consensus of opinion is probably that, while carcinomas may develop without preceding lesions which show epithelial atypia on biopsy, there is a greater chance of malignancy developing in lesions which do show atypia. The individual histological changes which together give rise to the diagnosis of epithelial atypia are not exclusively found in the types of lesions considered to be premalignant. Rennie and MacDonald (1973) indicated the frequent occurrence of these histological changes in denture induced hyperplasias. As Mincer et al (1972) pointed out the difficulty in diagnosis lies in the failure of the present techniques to distinguish reversible atypia from that which will proceed to neoplasia.

1.2.4 Techniques for the Evaluation of Cellular Atypia

Most diagnostic pathologists assess cellular atypia subjectively and there is considerable individual variation in the importance different pathologists attach to particular features of atypia.

Kramer (1969) and Kramer et al (1970a and b) discussed the problems of subjective histological interpretation and presented details of an objective computer assisted system for evaluating some oral epithelial lesions. Leukoplakic lesions which subsequently underwent malignant change were compared

with leukoplakias not progressing to malignancy. Discriminant analysis gave the heaviest weightings to eight features of which six would be commonly included among the features of atypia. The two additional important changes noted were the presence of Russell bodies in the lamina propria and enlarged nucleoli in epithelial cells.

The use of computer assisted diagnosis must, at present, be limited to a few centres and used mainly as a research tool. The more immediate requirement is for a simple objective system of histological grading of atypia.

In 1969, Smith and Pindborg published a monograph describing an objective system for the histological grading of cellular atypia based on comparison of the specimen being studied with a standard series of photographs. The investigator using this system is instructed to examine the histological section for each of 13 features of atypia in turn. If an individual feature is absent it is graded as "none". If the feature is present it is graded into one of two categories of severity, essentially "slight" and "marked", by the use of the photographic standards. Weighted scores are assigned to the "slight" and "marked" categories. The actual weightings are based on the authors' subjective evaluation of the importance

of the individual changes in the interpretation of atypia. The sum of the scores of the individual features gives an overall atypia score or index for the lesion.

The diagnostic potential of the Smith and Pindborg (1969) technique of atypia evaluation is currently being assessed by Smith, Pindborg and others in relation to the World Health Organisation Study in oral precancerous lesions. The evaluation of a system such as this, using human material, presents some difficulties. There is an initial problem of obtaining a large enough series of suitable cases because, as discussed in 1.2.2, the incidence of malignant transformation is not high and a long follow-up period for individual lesions is required. For retrospective studies this presents difficulties in obtaining reliable previous records and for prospective studies the problem of keeping in touch with patients over many years can be considerable. Also, there are limitations on experimental design for studies on human material because of ethical considerations. For example, if at the time of biopsy a lesion is felt to show severe atypia this lesion cannot be left untreated to see if, in fact, overt malignancy supervenes. Because of the difficulties of obtaining large enough numbers of suitable cases, the findings on lesions from

different sites and of different duration and aetiology often need to be combined to allow statistical analysis to be undertaken.

The use of an experimental animal tumour system could overcome these problems. As many animals as are required can be used and standardisation of features such as age, sex and site of lesions can be achieved. The limitations on experimental design caused by ethical considerations need not necessarily apply.

1.3 EXPERIMENTAL CARCINOGENESIS IN ANIMALS

1.3.1 Historical Introduction to Experimental Carcinogenesis in Animals

The possible association of environmental factors and carcinogenesis was first recorded by Sir Percival Pott (1775) when he observed the relationship between carcinoma of the scrotum and exposure to soot. Subsequently many other environmental factors have been suspected as being associated with both human and animal neoplasia at many sites. The reports of clinical observations of the possible association between specific environmental factors and particular tumours stimulated laboratory workers to attempt to induce experimental tumours in animals.

The early workers in experimental cancer research used mainly crude coal tar and many early studies such as those of Hanau (1889) and Cazin (1894), on the rat

and the dog respectively, were unsuccessful because of unfortunate choices of animals and individual carcinogens (Woglom, 1926). The first successful induction of experimental tumours in 1915 is credited to Yamagiwa and Ichikawa (1918). These workers applied coal tar regularly to the skin of the rabbit ear and produced the first experimental tumours.

The next phase in the history of chemical carcinogenesis concerned mainly the extraction and isolation of pure carcinogens. In 1933, Cook et al identified the chemical structure of the potent carcinogenic constituent of coal-tar and this was followed by a period of study involving refined pure chemical carcinogens applied to various sites in a number of experimental animals.

1.3.2 Experimental Oral Carcinogenesis in Animals

The majority of early investigations on experimental carcinogenesis used skin as the site of tumour induction. Bonne (1927), however, described squamous cell carcinomas which developed in the palates of three out of 50 mice following applications of tar for 50 weeks. Oral leukoplakia was produced in rabbits by Roffo (1930) after prolonged exposure to tobacco smoke. These early attempts at oral carcinogenesis were discouraging because of the low tumour yield and long latent period as compared to skin.

Oral mucosa appears generally more resistant to chemical carcinogens than skin, especially in the rat and mouse. Levy (1948) failed to produce carcinomas of oral mucosa in mice despite the application of the powerful carcinogen 20 methylcholanthrene for 16 weeks. Levy (1958) did manage to induce carcinomas of the tongue following injection of methylcholanthrene. However, it was not until investigators turned to the hamster as an experimental animal that a reliable model oral tumour system was evolved (Salley, 1954).

The hamster was introduced as a laboratory animal rather later than the other small rodents. Adler is credited with the introduction of hamsters for laboratory studies in 1931 (Yerganian, 1972). The potential of the hamster in dental research was first suggested by Arnold (1942) who undertook studies on caries in molar teeth. Keyes and Dale (1944) described the anatomical and physiological features of the hamster cheek pouches. Ten years later Salley (1954) used this site in the first successful attempt to produce a reliable and consistent oral tumour system in which squamous cell carcinomas could be induced by the application of chemical carcinogens. Since that time, a considerable volume of research has been conducted on chemical carcinogenesis in the hamster and this will be discussed in 1.3.3 and 1.3.4.

Squamous cell carcinomas have been induced in the rat oral mucosa. Wallenius (1965) succeeded in producing carcinomas in one third of a series of otherwise normal rats but only after the powerful carcinogen dimethylbenzanthracene had been applied three times per week for 16 weeks. Wallenius (1965) believed that saliva exerted a protective effect upon the oral epithelium. Wallenius (1966) later demonstrated the protective effect of salivary mucin in experiments with desalivated rats in which tumours developed under circumstances in which no tumours arose in untreated control animals. Giunta and Shklar (1972) used the technique in which pure dimethylbenzanthracene is retained in contact with the mucosa by cyanoacrylates in an attempt to induce lingual carcinomas in rats. This technique is effective on hamster palate (Mesrobian and Shklar, 1969) but failed to produce tumours on rat tongue. It appears that whilst it is possible with appropriate manipulations to produce oral carcinomas in rats the time required is much greater than in the hamster.

No model system for experimental oral carcinogenesis in primates is available. However, Cohen et al (1971) described experimental epithelial atypia in response to exposure to chewing tobacco in Macaca Iris monkeys. Hamner (1972) also produced epithelial atypia in buccal epithelium of baboons using betel

quids with lime and tobacco similar to those used in India.

1.3.3 Experimental Carcinogenesis in Hamster Cheek Pouch

In 1954, Salley investigated the effects of application of three potent carcinogens to the hamster cheek pouch epithelium. The carcinogens were 9,10 dimethyl,1,2benzanthracene (D.M.B.A., synonym 7,12 dimethylbenz(a)anthracene), 20 methylcholanthrene and 3,4 benzpyrene. These were applied as 0.5 per cent solutions in either acetone or benzene and the pouches were painted with the carcinogen solution three times per week for 16 weeks and observed for a further nine weeks. Control animals were painted with either acetone or benzene alone. The animals treated with benzene solutions showed marked inflammatory changes and a high morbidity. After the initial inflammatory phase, Salley (1954) described four stages in the progression of pouch lesions. These were hyperplasia, papilloma formation, carcinoma in situ (preinvasive) and squamous cell carcinoma including metastases to lymph nodes. Salley concluded that the most effective carcinogen for the hamster cheek pouch was an acetone solution of D.M.B.A. This solution produced tumours in six to seven weeks. In 1955, Salley demonstrated that the use of mineral oil as the solvent for D.M.B.A. reduced the latent period for tumour development to about four and a half weeks.

Salley (1957a) investigated in more detail the early histologic changes in response to D.M.B.A. applications to the cheek pouch and in particular attempted to describe the sequence of changes occurring in the epithelium. He described the initial changes as inflammation followed by a period of degeneration and necrosis. This, in turn, was followed by regeneration with progressive hyperplasia developing cellular atypia and proceeding to neoplasia. He also noted the presence of whitish lesions similar to human oral leukoplakia after eight or nine carcinogen applications.

In 1961, Morris recorded the results of experiments to test the effects of age, differing D.M.B.A. concentrations and the frequency of carcinogen applications upon carcinogenesis in the hamster cheek pouch. The optimal age with regard to tumour production and ease of manipulation was about five weeks. The optimal carcinogen concentration was 0.5 per cent. Higher concentrations, for example 1.5 per cent, resulted in high morbidity of experimental animals and although tumours were produced by a 0.1 per cent D.M.B.A. solution, the latent period was extended. The latent period before tumour development was also extended by applying the carcinogen twice weekly instead of three times per week. Morris (1961) carefully described his carcinogen application

technique and recommended the "wiped brush" method. He further observed that the conditions of caging did not appear to affect the experimental results.

Morris and Reiskin (1966) investigated the early stages of carcinogenesis in the pouch in an attempt to decide at which stage the changes became irreversible with the inevitable development of neoplasia. They found that all animals painted for four weeks developed tumours after a short latent period, but that all animals painted for three weeks developed tumours after a more prolonged latent period. Animals painted for less than three weeks failed to develop tumours by 21 weeks. Thus the critical changes relating to malignant transformation in animals painted with 0.5 per cent D.M.B.A. in mineral oil had already occurred by three or four weeks before any clinical or microscopic indication of impending tumour development was evident.

In addition to studies simply designed to produce tumours in cheek pouch this model system has been used to evaluate the importance of a number of possible carcinogenic or co-carcinogenic agents. Renstrup et al (1962) showed that chronic mechanical irritation alone did not produce tumours, but that it hastened the development of carcinomas induced by D.M.B.A. Renstrup et al (1962) applied the carcinogen in an

adhesive vehicle (Orabase). Local applications of alcohol were shown to cause quicker tumour induction with D.M.B.A. and also to produce more aggressive tumours (Elzay, 1966). Salley (1963) indicated that cigarette smoke acted as a promoting agent by chronic irritation in hamster pouch carcinogenesis. Elzay (1969) studying the combined and individual effects of cigarette smoke and locally applied alcohol found that whole cigarette smoke was a more potent promoting agent than alcohol.

Shklar (1966a and 1967) found that animals injected with cortisone and painted with D.M.B.A. developed tumours more quickly than those animals merely painted with D.M.B.A. He also found that the tumours in cortisone treated group, although not more anaplastic, grew faster and were more invasive. Sabes et al (1963) recorded that cortisone applied topically prior to the application of D.M.B.A. enhanced the incidence of tumour production. The effect of cortisone might have been due to an altered immunologic response to the neoplasms. Woods (1969) and Giunta and Shklar (1971) found that treatment of hamsters with antilymphocyte serum produced more rapid development of more widespread neoplasms in the pouch. This occurred in spite of the fact that the hamster cheek pouch is an immunologically privileged site, (Frenkel, 1972) probably because of its unusual muco-

substances (Walker et al, 1970).

Rowe and Gorlin (1959) found that vitamin A deficiency promoted chemical carcinogenesis with D.M.B.A. in the cheek pouch and also that a restricted calorie diet inhibited tumour induction. Vitamin A palmitate applied topically has been shown to act as a co-carcinogen in D.M.B.A. carcinogenesis in the hamster pouch (Polliach and Levij, 1969, Levij et al, 1969). These authors felt that the co-carcinogenic effect might have been due to the labilising effect of the vitamin A on lysosomal membranes.

The effect of systemically administered anti-metabolite drugs on D.M.B.A. carcinogenesis has been studied. Shklar et al, (1966) found that methotrexate administration produced anaplastic carcinomas of cheek pouch and later Shklar (1972) obtained similar results with 5-Fluoruracil.

Several histochemical studies of pouch carcinogenesis have been reported. Studies of hydrolytic enzymes and dehydrogenases were undertaken by Mori et al (1962). Shklar's (1965b) results indicated a shift from aerobic respiration to anaerobic glycolysis. Santis et al (1964) observed alterations in hydrolytic enzymes in squamous cell carcinomas of pouch and noted that leukoplakic lesions showed enzyme activities

intermediate between those of normal mucosa and carcinomas.

Studies in the cell kinetics of pouch tumours were made by Reiskin and Mendelsohn (1964) and Nadarajathilagaratnam (1969). These indicated a constant rate of cell division in experimental tumours and a considerable reduction in the cell cycle time due mainly to a large reduction in the G_1 phase (post-mitotic pre-synthetic gap).

This review of previous work on cheek pouch carcinogenesis is not intended to be exhaustive, but to indicate the extent and nature of the investigations made on this model system. Many authors have made the assumption that the cheek pouch tumours are closely similar to tumours in man, but this is an assumption which has not always been properly considered. Kolas (1955) argued that the pouch was not a true intraoral site and was not subjected to the same environmental influences as the rest of the oral mucosa. Stormby and Wallenius (1964) felt that the unusual histology of the cheek pouch and the peculiar anatomical arrangement of the hamster oral cavity rendered it unsuitable as a model for oral carcinogenesis. Salley (1957b) however, pointed out that the pouch is derived embryologically from the primitive buccal cavity. He also indicated that the

pouch is susceptible to chemical carcinogenesis over its entire surface including the part adjacent to the oral opening where it is subjected to the same influences as the rest of the oral cavity. The important point would appear to be that before the pouch is used for a specific study in which the results are to be applied to the human situation, its comparability, with regard to that feature studied, must be investigated. For example, Smith (1968) studying the histochemistry of premalignant lesions in pouch found these to be closely similar to pre-malignant lesions of human oral mucosa and felt that as far as his experiments were concerned "the doubts that have sometimes been raised with regard to the truly oral nature of the cheek pouch mucosa are relatively unimportant."

1.3.4 Experimental Carcinogenesis at Other Intraoral Sites in the Hamster

Despite the large number of studies on the hamster cheek pouch, relatively few investigations on chemical carcinogenesis at other oral sites in hamster have been undertaken. Salley and Kreshover (1959) succeeded in producing palatal carcinomas in 54 per cent of hamsters treated with D.M.B.A. for 16 weeks. This was a lower and slower tumour yield than for cheek pouch or the skin of the ears. Stormby and Wallenius (1964) used a D.M.B.A. in acetone solution applied to the hamster palate in a study of the effect

of reduced salivation on experimental carcinogenesis. The normal control and the reduced salivation groups both developed tumours in four to six months. An increased incidence of malignancy was seen in the reduced salivation group.

In 1966, Al-Ani and Shklar studied the effects of applications of D.M.B.A. in mineral oil to the hamster gingiva. These authors found the gingiva to be less susceptible to carcinogenesis than the pouch in that after four months only areas of dyskeratosis and papillomas were noted. No carcinomas developed although the authors concluded that carcinomas would probably have appeared after a longer period. The washing effects of saliva and the effects of mastication were felt to have caused dispersion of the carcinogen. In order to overcome this difficulty and localise the carcinogen, Mesrobian and Shklar (1969) applied D.M.B.A. in powder form to the gingiva and then covered it immediately with cyanoacrylate. Carcinomas were detected clinically after 10 weeks and confirmed histologically at 16 weeks indicating that the modified application technique resulted in tumour induction in gingiva which took only approximately twice as long as that recorded in the cheek pouch.

Carcinomas of the hamster tongue were induced by Dachi in 1967. The author did not state which area of tongue was used in the study in which D.M.B.A. in dimethyl sulphoxide was applied for 30 weeks. 4 out of 15 hamsters developed carcinomas of tongue in that period, but larger and more anaplastic carcinomas were found in the same animals at other intraoral sites such as the lip, buccal mucosa and cheek pouches and also on the skin.

1.4 SOME ASPECTS OF THE ULTRASTRUCTURE OF ORAL MUCOSAL NEOPLASIA

The increased resolution afforded by the electron microscope as opposed to the light microscope has yet to be fully exploited in relation to oral mucosal lesions. However, several studies of leukoplakias, premalignant lesions and carcinomas are recorded.

In 1963, Frithiof et al examined human leukoplakic lesions showing hyperkeratosis but no atypia. They commented in detail on the altered ultrastructural patterns of keratinization observed. Silverman (1967) also compared the ultrastructure of normal and hyperplastic human buccal epithelium from 10 patients. In addition to describing changes in the superficial cell layers he observed a number of alterations in the basement membrane area in leukoplakic lesions. Prior to discussing these, mention of the normal structure of this area is required.

The ultrastructure of the normal basement membrane area of oral epithelium was described in detail by Susi et al (1967) and Susi (1969). A good summary of his previous work was published by Susi in 1971. This describes the basement membrane area as a highly ordered structure with a dense continuous layer known as the basal lamina or lamina densa into which a complex arrangement of fibrils is inserted from the connective tissue side. Between the lamina densa and the basal plasma membrane of the epithelial cells a clear zone is seen on electron micrographs and this is known as the lamina lucida. Specialised contact areas of the basal plasma membrane appear as discrete densely staining zones known as hemidesmosomes.

The changes which Silverman (1967) noted in the lamina densa in leukoplakic lesions were reduplication of this lamina and also the presence of gaps in the lamina densa. Subjectively the number of hemidesmosomes was felt to be increased.

The basement membrane zone was much more thoroughly investigated by Frithiof (1969) in cases of hyperplastic oral epithelium and in what he called pre-invasive and invasive carcinoma. In severely inflamed epithelium gaps in the lamina densa and reduplication of the lamina densa were found. Cytoplasmic processes of epithelial cells, lacking a basement membrane

covering, and extending into connective tissue were thought to be related to lytic processes in the connective tissue (Frithiof, 1972). Similar changes have been recorded in skin lesions of psoriasis (Cox, 1969). In the preinvasive and invasive carcinomas Frithiof (1969) observed areas of lamina densa which were thin and defective and were associated with reduced numbers of hemidesmosomes. Uncovered cytoplasmic processes projected into the connective tissues. In some cases overproduction of abnormal basal lamina material was noted.

In 1972, Frithiof discussed the changes in pre-invasive and invasive carcinomas in more detail. He observed that two types of cytoplasmic projection through the lamina densa occurred. Long slender projections were seen only in preinvasive and invasive carcinomas and they were present in areas where the basal lamina was deficient. Frithiof (1972) considered these might be a useful diagnostic feature. The second type of cytoplasmic projection seen was a shorter more bulbous structure. Woods and Smith (1969a and 1970) described these as epithelial cell pseudopodia and observed them in experimental carcinomas of hamster cheek pouch and in human oral hyperkeratotic lesions. Woods and Smith (1969b) described similar pseudopodia developing in cheek pouches treated with 4-hydroxyanisole. This change

was reversible in contradistinction to the pseudopodia observed after treatment with carcinogen.

It would appear from this brief review that a number of changes are observed in the basement membrane zone of premalignant oral lesions. Of these, some changes may be essentially reactive whereas others, such as the presence of long slender cytoplasmic processes or a thin lamina densa with few hemidesmosomes may be of diagnostic value. Frithiof (1969) made some measurements of the thickness of the lamina densa, but most authors have relied upon subjective quantitation of ultrastructural changes. Better techniques of quantitation would appear desirable. As Weibel (1972) observed "Cancer cells don't look much different from normal cells, but it may well be that significant quantitative differences in their composition could be discovered if stereological studies were systematically performed."

1.5 QUANTITATION WITH PARTICULAR REFERENCE TO STEREOLOGY

Morphometry is the process of using quantitative data to describe structural features. In relation to the light and electron microscopic structural features of biological material it is a largely neglected field. This is partly because the obvious methods of simple measurement are tedious to apply on a large scale although some studies of this type have been recorded.

For example, Renstrup (1963) used a device with fine nylon thread to measure the length of basement membrane of epithelium. Meyer and Gerson (1964) measured the actual lengths and heights of epithelial cell profiles on photographic prints of histological sections. Barrington and Meyer (1969) and Meyer et al (1970) in addition, used a planimeter to measure surface areas on photographic prints of epithelium. Planimetric methods have also been applied to electron micrographs to measure the percentage of plasma membranes occupied by desmosomes (Chen, 1970) and desmosomes and hemidesmosomes in human crevicular epithelia (Geisenheimer and Han, 1971).

The types of quantitation described in the preceding paragraph in addition to being time consuming are also subject to considerable inaccuracies and a simpler and more accurate method of quantitation is available. This is stereology which is the name for a system of geometric analysis of structures and includes methods that allow direct estimation of metric properties of structures from two dimensional sections on the basis of geometrical statistical reasoning.

Weibel (1969) published an extensive review of stereological principals and nomenclature and traced some of the development of this speciality. The fundamental relationships of stereology were first

enunciated in 1847 by a French geologist Delesse, who indicated that the volume density V_v i.e. a volume of one constituent within a larger volume of material is, on average, equal to the areal density A_a of that constituent as seen on sections of the material. This relationship $A_a = V_v$ can be proven mathematically and holds provided that the sections studied are a representative random sample of the material. The volume density can thus be estimated from sections by measuring the areas on sections. Delesse (1847) accomplished this by tracing the profiles on heavy paper, cutting these out and then weighing them. Alternatively the areas could be measured by a planimeter.

Rosiwal (1898) however, showed that A_a could be obtained by linear integration. If a test line of length L were superimposed upon the section a measure of the length of that part of the line overlying the required areas was proportional to these areas and thus the volume. Thus $V_v = L_l$. A further advance in the method of calculation was proposed by Glagoleff (1933) who indicated that A_a could be obtained by superimposing a regular point lattice upon the section and counting the points falling on the required areas. Thus $V_v = P_p$. The first biological use of this approach was that of Chalkley (1943) who used a point counting device in the eyepiece of a microscope to

calculate the nucleo-cytoplasmic ratios of cells.

In addition to methods allowing calculation of volume densities, stereology provides methods for calculating profile lengths on sections and surface areas. These methods use counts of intersect points of grids of parallel lines with the structures being measured and are discussed well by Weibel et al (1966).

One problem in applying stereologic methods to biological systems is that sections of biological material often exhibit anisotropy. That is, the constituent elements of the tissues show preferential orientations which may mean that they do not fulfil the requirements of randomness mentioned previously. It is sometimes possible to overcome this problem from a stereologic standpoint by the methods of section preparation, but with regard to some tissues such as surface epithelia it is often better to conduct studies on precisely orientated sections containing the axis of anisotropy (Weibel, 1969). In oral epithelia this requires sections cut at right angles to the surface. This point will be discussed further in Chapter 6.

Stereologic methods have only recently been applied to oral tissues. Schroeder and Munzell-Pedrazzolli (1970) and Schroeder (1970) used point counting methods to study human gingivitis.

Angelopoulos (1972) devised an ingenious linear integrating device which he also used to study gingiva. Squier et al (1970) quantitated the effect of mild thermal injury of rat palatal epithelium and Liu et al (1973) studied the changes in rat lip following irradiation. Alvares et al (1973) reported preliminary stereologic studies on homogeneous oral leukoplakias in man.

1.6 AIM AND DESIGN OF THE PRESENT STUDY

The aim of the present study was to investigate the possibilities of using experimental oral epithelial lesions in hamsters to study the tissue changes characteristic of premalignancy. In particular it was intended to look at the possible diagnostic value of assessing these changes in predicting future malignant potential. The study is reported in five chapters (Chapters 2 - 6) which indicate progressive refinement of the research questions being asked.

The requirements of an experimental model for such a study of premalignancy and malignancy are several. Firstly, it should be possible to restrict the pathological changes to a prescribed area in which the induced changes should be reasonably uniform. This is desirable in that ideally a standard biopsy of the treated area should be employed and after a suitable follow-up period a standard excision of this

area should be performed. These requirements are basically those of standardisation of techniques in different animals. In Chapter 2 an evaluation of the technique proposed by Salley (1954) and Morris (1961) is reported and in Chapter 3 the development of a more refined technique to meet the above requirements for standardisation is described. Chapter 3 also records detailed histological studies of the features of cellular atypia in premalignant lesions and an evaluation of their diagnostic potential in predicting the development of malignancy. Some subjective observations about the similarities and differences between cheek pouch lesions and human oral lesions are also noted in Chapter 3.

Chapter 4 records a more thorough investigation of the diagnostic implications of cellular atypia and also a comparison of these features in human and hamster lesions. While the comparisons of human and hamster material were in progress it was observed that much of the literature dealing with histological changes associated with premalignancy had concentrated solely on the changes observable in such lesions. The question was asked "are the changes reported as indicative of premalignancy and malignancy observable in other oral mucosal lesions?" A simple computer study of the correlation of histological changes with five types or oral mucosa lesion is reported in Chapter 4.

The studies reported in Chapters 3 and 4 showed a close similarity in the features of human and hamster cellular atypia, but some differences were also apparent. In view of these differences and of the criticisms sometimes levelled at the use of the cheek pouch because of its unusual anatomy, a preliminary study was undertaken to assess the responses of several other intraoral sites to carcinogen applications. This study, reported in Chapter 5, suggested that the ventral surface of the hamster tongue was a site which merited further study.

The techniques of quantitation used in the studies recorded in Chapters 3, 4 and 5 were somewhat crude. It appeared highly desirable to use a more accurate method of quantitation and one which was objective. Chapter 6 records an evaluation of stereological techniques and their application to the assessment of some light and electron microscopic features of experimental lesions on the ventral aspect of hamster tongue induced by carcinogen applications.

A complex terminology is used to refer to clinical histological and ultrastructural features of oral mucosa and often different authors use terms in different ways. Appendix 1 records the definitions of terms as they are used in this present study.

HAMSTER CHEEK POUCH PRELIMINARY STUDIES

2.1 INTRODUCTION

As discussed in the preceding chapter, the hamster cheek pouch has been the most extensively used site for investigations on experimental oral carcinogenesis. Because of this, it was decided to examine the suitability of this site for assessing the histological changes associated with premalignancy and malignancy of oral epithelium.

In 1961, Morris attempted to standardise the technique used in the induction of experimental carcinomas of hamster cheek pouches in order that the results of different workers could be more readily compared. He advocated the use of young animals, ideally five weeks of age, and indicated an optimal concentration of carcinogen for the rapid production of malignant neoplasms. He also indicated that in his experiments no sex variation in response to carcinogen applications was observed and the conditions of caging had no apparent effect on the experimental results. Because of the fact that this standardised method was available it seemed appropriate to evaluate the potential of this technique.

Morris (1961), Salley (1954) and several other workers illustrate their papers with photomicrographs

of normal and treated hamster cheek pouches which show faults in the histological technique which have resulted in tearing and compression of sections. These faults are demonstrated in this present study in Fig. 2.13 and Fig. 2.19. Although previous authors do not discuss any such difficulties, observation of the photomicrographs suggests that these faults could have been due to difficulty in section preparation and a likely cause of this could have been poor fixation. The fixatives used by other workers were usually either a 10 per cent formalin solution or Bouin's fluid. It was decided in the preliminary experiment to test both of these fixatives and also to try other solutions to find the most satisfactory fixative for this tissue.

2.2 MATERIALS AND METHODS

2.2.1 Animals

Following the recommendation of Morris (1961) young animals were used. Five week old Syrian golden hamsters were purchased from Coombenhurst Preparatory School, Basingstoke, Kent. In order to standardise the animals and reduce any possible genetic or sex-related variations in response to the carcinogen, a line bred strain of hamsters was used and all animals were male. In spite of this, the weights of the animals ranged from 65 - 125 g at the time the experiment commenced. This was after a period in quarantine when the animals were eight weeks old.

Twenty-four male hamsters were randomly divided into three groups; Groups 1 and 2, each of ten animals and Group 3 of four animals. The animals were then marked on the ears to allow identification of each individually. The animals were housed individually in standard hamster boxes and were fed standard laboratory chow and water ad libitum.

2.2.2 Techniques for Examination of Cheek Pouch and Anaesthesia

In order to examine and experiment upon the epithelium of the hamster cheek pouch it is necessary that the animal be immobilised with the mouth open and the cheeks retracted. Morris (1961) advocated exposing the pouch for painting with carcinogen while holding the animal by hand and retracting the cheek with an instrument mounted on a stand. In the paper describing the technique he did not discuss the use of anaesthesia during this procedure. In preliminary tests it was found that carcinogen application was easier if the animal was anaesthetised and because of the frequency of carcinogen applications, inhalation anaesthesia appeared preferable to other forms of anaesthesia, such as intraperitoneal Nembutal injections. If individual animals were placed in a small chamber with ether until they were adequately anaesthetised, it was found that on removal from the chamber satisfactory anaesthesia persisted for about 1 minute. This period was insufficient to allow exposure and carcinogen painting

of both pouches using the technique of Morris (1961) in which each pouch is examined separately. Accordingly a more satisfactory technique of pouch exposure was sought.

Johansen (1952) described a device for the intra-oral examination of rodents and an example of this is shown in Fig. 2.1. Morris (1961) used such a device for detailed examination of the pouch in his experiments, but in the author's hands, this device appeared awkward to use and was too time consuming. The device illustrated in Fig. 2.2 is based on that described by Moss et al (1965). This device was found to fulfil the requirements for speed and ease of use. Animals in this device were restricted in their movements without the use of undue force and after a short period of experience most animals rested peacefully in the restrainer even after the effects of the ether anaesthesia had worn off.

The restraining device was used as follows. The animal was anaesthetised with ether and placed quickly in the detached chamber of the restrainer with its head through the hole in the top of the chamber (Fig. 2.3). This chamber was mounted on the base and the hamster's mouth was opened and held by elastic bands over the incisor teeth (Fig. 2.4). Subsequently it was found more convenient to use wire loops attached to the rubber bands and placed over the incisor teeth. The tension

of the rubber bands was checked carefully to avoid damage due to excessive opening of the jaws. Retractors were inserted into the cheek pouches and these provided excellent exposure of the medial wall of the pouch (Fig. 2.4). When the retractors were placed in the pouches they were held by an assistant while carcinogen applications were made to each cheek pouch. This entire procedure could be accomplished in about 30 - 40 seconds while the hamster was still anaesthetised.

2.2.3 Carcinogen

Dimethylbenzanthracene (DMBA) has been shown to be the most effective chemical carcinogen for use on hamster cheek pouch (Salley, 1954). In this preliminary study 7, 12 dimethylbenz (a) anthracene was used in a concentration of 0.5 per cent in acetone. (Salley, 1954). Acetone was chosen as the solvent in preference to benzene which caused much more tissue necrosis and high mortality of experimental animals. (Salley, 1954). Mineral oil has been used by several investigators as the solvent for the carcinogen, but it was felt that it was more difficult to control the spread of this solvent within the pouch and also that there was no advantage to be gained by accelerating the process of carcinogenesis as occurs with the use of mineral oil.

A fresh solution of the DMBA in acetone was

prepared weekly and any residue was discarded at the conclusion of carcinogen applications for that week. The freshly prepared carcinogen solution was stored in a dark bottle and a small volume, approximately 1-2ml, was transferred to a smaller bottle at the time of carcinogen applications. In this way it was hoped to avoid excessive concentration of the carcinogen due to evaporation of the acetone during the course of applications to several animals.

2.2.4 Carcinogen Applications

Following the recommendation of Morris (1961) the DMBA solution was applied with a camel-hair brush used in a circular motion to paint the carcinogen on the anterior medial wall of each cheek pouch. A No. 3 camel-hair brush was dipped in the carcinogen solution and wiped against the side of the bottle to remove excess solution before application to the cheek pouch.

Carcinogen applications were made three times per week, on Monday, Wednesday and Friday mornings. Each series of applications was commenced between 10.00a.m. and 11.00a.m. and took approximately one hour. The precaution of applying the carcinogen at approximately the same times on each occasion was taken because Sabes et al (1963) suggested that the time during the day at which carcinogen was applied could alter the tumour yield. These authors found that the yield of salivary gland tumours varied with the time of carcinogen

application but in their series this effect was not demonstrable in the case of hamster cheek pouch carcinomas. Nevertheless, it was felt that this was a possible variable which could be reduced by standardising the time of carcinogen application.

2.2.5 Experimental Procedure

The experimental design was that Group 1 consisting of ten animals should receive carcinogen applications three times per week for four weeks and Group 2, similar applications for six weeks. It was found that several animals showed moderately severe ulceration of the pouch epithelium at the end of the first week and the beginning of the second week of the experiment. One animal at this time showed a very severe reaction and was immediately sacrificed. Painting of ulcerated pouches was discontinued until the lesions healed. This usually occurred within a few days and no animal missed more than three carcinogen applications. Notes were made of the appearance of the pouch at the examination which preceded each carcinogen application.

A chart was kept recording the weights of individual animals at the beginning of each week. (This is shown in Table 2.3). Many animals lost weight during the first and second weeks, but then gained weight thereafter. The weight loss corresponded to the period of initial inflammatory reaction in the pouch. All animals were examined three times per week

by the author and were observed several times per day by trained animal house technicians. The majority of animals did not appear obviously distressed and were still taking food and water. Animals number 5 and 13 were sacrificed immediately on showing signs of distress.

All animals were killed with ether inhalation. Group 1 hamsters were killed at five weeks, this being one week after their last carcinogen application. Group 2 hamsters were killed at seven weeks. Consideration was given as to the best way of obtaining specimens of the pouch for histological examination. It was realised that future experiments would include examinations of small biopsies from living animals. Such biopsies would be in a state in which the muscle was contracted when the specimen was obtained. It was felt that this contracted state would be more reproducible than any state of stretching which could be produced and therefore all specimens were fixed in an unstretched condition. Examination of the pouches of freshly killed unfixed animals was most easily undertaken if the pouches were exposed by cutting through the lateral wall of the pouch and the skin with scissors and then laying back the edges as shown in Fig. 2.5. The cut margins of the pouch were then pinned to the adjacent skin without stretching and the excised head was fixed in a neutral buffered 10 per cent formalin solution. After fixation the pouches were dissected

out and representative samples were obtained for microscopic examination.

In addition to the detailed examination of the cheek pouch, a routine autopsy examination of all animals was undertaken on sacrifice. Samples for microscopic examination were taken from lungs, liver, kidney and spleen and any other organ which showed any unusual feature.

2.2.6 Safety Precautions

Precautions were taken during carcinogen applications to prevent exposure of the operator and the assistant to the DMBA. Great care was taken to avoid spillage of carcinogen solution and dripping from the loaded brush. Surgical rubber gloves, face masks and gowns were worn. All instruments in possible contact with carcinogen solutions were washed in acetone or benzene after each series of applications.

Darlow et al (1969) used Bacillus globigii spores in a study of the possible dissemination of substances applied to the clipped dorsal skin of mice. These authors suggested that their results indicated a level of dissemination into the atmosphere and into bedding which would constitute an important hazard if chemical carcinogens were being used. In the present study it was felt that the fact that the carcinogen was applied intraorally, greatly reduced this hazard. Peat and

straw were used in the standard solid based hamster boxes to try to confine any contamination which might have occurred and precautions were taken with the disposal of this bedding material before thorough weekly washing of the boxes.

2.2.7 Evaluation of Fixation and Histological Technique

The four untreated control animals in Group 3 were kept under the same standard animal house conditions as the experimental Groups 1 and 2. Two of these animals were sacrificed after five weeks and the remaining two after six weeks. These animals were used to provide tissue for comparison with experimental material and also for evaluation of different tissue fixatives. The fixatives tested were 10 per cent buffered formol saline, Bouin's fluid, Susa's fixative and formol-acetic-alcohol solutions which were prepared with either ethanol or with methanol. Details of these fixatives are noted in Appendix 2.

The tissue blocks were trimmed so that, after embedding, the plane of section to be cut was at right angles to the keratinized surface. All tissues were processed on an automatic tissue processor using the cycle detailed in Appendix 2. All blocks from control and treated pouches were sectioned with the keratinized surface being the first to meet the microtome knife and this surface was arranged as far as possible parallel to the knife edge. All sections were prepared on a

Leitz Minot rotary microtome.

2.3 RESULTS

2.3.1 Normal Hamster Cheek Pouch

The normal pouch is a uniform pink colour on gross examination and is dry due to the absence of salivary gland secretion into the pouch (Salley 1954). Histologically the epithelial lining is thin and orthokeratinised (Fig. 2.6). Apart from the few large rete pegs described by Gilette (1957) the epithelium is of uniform thickness. The progenitor cell compartment is restricted to the basal cell layer, defined as the layer of cells actually in contact with the basement membrane. This means that in normal pouches, mitoses are confined to the basal cell layer. A thin connective tissue layer is present between the epithelium and the muscle layers of the pouch.

2.3.2 Prematurely Sacrificed Animals

An noted previously two animals in the experimental groups were sacrificed immediately on showing signs of distress. Hamster number 13, after three applications of carcinogen, showed severe ulceration of both cheek pouches. Histological examination confirmed the presence of extensive ulceration (Fig. 2.7). The pouch epithelium showed obvious degenerative changes and much of the surface was replaced by a slough of necrotic epithelium, fibrin and inflammatory cells. Other areas of epithelium showed hyperplasia and foci of cellular

atypia were present (Fig. 2.8). A marked inflammatory reaction was present in the underlying connective tissue and muscle. Routine gross and histological examination of the organs as detailed in 2.2.5 showed no obvious abnormality.

Hamster number 5 showed a rapid weight loss during the second week of the experiment. A purulent exudate was present in both cheek pouches and bacteriological examination of smears from the pouches yielded a heavy growth of a Proteus species. The pouches which had previously been ulcerated appeared to have healed. Autopsy examination showed congestion of the lungs. The liver, spleen, kidney and heart showed no obvious abnormality. Histological examination of the cheek pouches revealed an altered epithelium, showing areas of acanthosis and atrophy, overlying areas of hyalinised scar tissue. Small foci of ulceration were present, but in general the pouch appeared to have healed well. A purulent exudate was noted on the surface of the pouch. In view of these findings it was felt that the most likely cause of the animal's weight loss was a Proteus septicaemia originating from the cheek pouches. Routine examination of other organs as detailed in 2.2.5 showed no obvious abnormality.

2.3.3 Experimental Group 1

This group of hamsters received carcinogen applications for a period of four weeks and the animals were sacrificed one week after the last application. At this time the group consisted of 9 animals. Of the 18 pouches in the group 9 showed no significant gross abnormality, 4 showed slight scarring at the distal end of the pouch and 3 showed moderate scarring. Severe scarring with considerable contraction and distortion of the pouch was noted in both pouches of one animal. An irregular surface perhaps best described as a cobblestone effect was noted in parts of two pouches. Distinct white patches were noted in two pouches and in one pouch a small papilloma was present near the anterior end.

The histological findings are summarized in Table 2.1. No carcinomas were seen, but histology did confirm the presence of the papilloma noted grossly (Fig. 2.9). This lesion showed cellular atypia (Fig. 2.10). Cellular atypia was present in all pouches (Fig. 2.11). Areas of hyperkeratosis, acanthosis and atrophy were also present in most pouches. Dense scar tissue, hyalinised in areas, was present in 15 of the 18 pouches and in parts this formed a distinct dense layer into which prolongations of the overlying epithelium extended (Fig. 2.12). 3 of the 18 pouches showed small areas of ulceration which had not been

detected during the gross examination. The routine examination of the other organs showed no detectable abnormality.

2.3.4 Experimental Group 2.

This group also consisted of 9 animals which had received carcinogen applications for two weeks longer than the animals in group 1. In this group gross examination showed abnormalities in all 18 pouches. 7 pouches showed mild scarring and 4 moderate scarring towards the distal end of the pouch. A cobblestone surface effect was noted in one pouch and white "leukoplakic" lesions were seen in 10 pouches. Papillomas were noted grossly in two pouches.

The findings of the histological examination of the group 2 pouches are noted in Table 2.2. In one pouch from hamster 20 an early squamous cell carcinoma was seen (Fig. 2.13). This lesion was an elevated papillomatous tumour and the appearances suggested that the lesion could have been due to malignant change in a papilloma. In this same pouch another papilloma was present. This lesion showed cellular atypia but there was no evidence of invasion (Fig. 2.14). Two other papillomas were seen histologically in other pouches. One of these had been noted grossly, but the other was small and had not been observed on gross examination. The other suspected papilloma observed grossly was an elevation due to scarring in the underlying connective

tissue. This had caused a lesion which was readily misinterpreted with the naked eye as an epithelial neoplasm.

Areas of cellular atypia, hyperkeratosis and acanthosis were seen in all 18 pouches. Atrophy and dense scar tissue were often present, but ulceration was noted in only one pouch.

Section artefacts such as those discussed later in 2.3.7 were present in sections from all 36 of the pouches of group 1 and group 2 animals.

The study of other organs as detailed in 2.2.5 showed no obvious abnormality.

2.3.5 Cellular Atypia

Cellular atypia was found in both pouches of all animals in groups 1 and 2. The appearance of individual areas was very variable and Fig. 2.15(A,B,C) shows some of the more frequent appearances. It was noted that the changes were more extensive and severe in group 2 animals than those in group 1 which had received fewer carcinogen applications over a shorter period. The appearances of some areas of cellular atypia were very similar to those seen in human oral mucosa. In one area marked epithelial changes were seen which placed the lesion in the borderland between severe cellular atypia and early invasive squamous cell carcinoma (Fig. 2.16). One change was observed which

had not been seen in human oral mucosa and this is illustrated in Figs. 2.17 and 2.18. This abnormality consisted of an area of acantholysis usually associated with an area of premature individual cell keratinisation.

2.3.6 Animal Weights

The weekly weights of individual animals in both the control and experimental groups are shown in Table 2.3. At week 1, the starting point of the experiment, the mean weight of the control group was 110.25 g (S.D. 10.66 g) and of the combined experimental groups was 96.11 g (S.D. 12.44 g). Comparison of these two groups by Student's "t" test yielded a "t" value of 2.599 and $P < 0.05$ indicating a significant difference in the starting weights of the control and experimental groups. The two experimental groups were each considered individually. The mean for group 1 at week 1 was 92.56 g (S.D. 12.70 g) and group 2 was 99.67 g (S.D. 11.80 g). Comparison of these values with the control group indicated a significant difference between group 1 and the controls ($P < 0.05$) but experimental group 2 was not significantly different from the control group ($P > 0.2$).

It was desirable to determine if the experimental procedure had produced a significant systemic effect on the animals and it was felt that comparison of the weight gained by the controls in a given period with that gained by the experimental group would help to

indicate the magnitude of the systemic effect. Since it could be assumed that the control group and the experimental group 2 were drawn from the same universe comparison between the differences in weight of individual animals between week 1 and week 5 in these two groups was undertaken. These differences are shown in Table 2.4. The mean weight gain in the control group of 31.25 g is much greater than that of experimental group 2 (3.11 g). The difference in these values is highly significant ($P < 0.001$) indicating that the experimental procedure produced significant systemic effects.

2.3.7 Control Animals - Tissue Fixatives

The 10 per cent buffered formol saline solution used was a standard histological fixative. The tissue fixed in this solution gave blocks which frequently proved difficult to cut. Examination of the histological sections revealed frequent artefacts, similar to those seen in the experimental groups. These artefacts were particularly seen in the region of the epithelial connective tissue interface. Also the nuclear detail observed after formalin fixation was poor compared to that seen with the other fixatives (Fig. 2.19).

Bouin's solution gave inconsistent results. On occasions excellent results were seen but artefacts similar to those noted after formalin fixation were

present in some sections and the blocks were sometimes difficult to cut. In addition the tissue tended to stain heavily with eosin after use of this fixative. Susa's fixative similarly gave excellent results on occasions but there was also a tendency for the block to be very hard and difficult to cut.

The best results were obtained with formol-acetic-alcohol. This fixative gave good nuclear detail and produced a very attractive aesthetic result allowing a delicate eosin staining. Nuclear detail was excellent and mitotic figures were particularly clearly seen (Fig. 2.20). Some blocks were very difficult to cut. It was felt that this could have been due to evaporation of the alcohol during trimming of the specimen. Such evaporation could have hardened the tissue by itself or it may have allowed the acetic acid to cause tissue damage. This problem was overcome by processing fairly large pieces of cheek pouches, about one centimetre square which were dissected out quickly to prevent excessive alcohol evaporation. These large blocks were accurately trimmed just prior to the stage of blocking out in paraffin.

The paraffin wax used was Paramat manufactured by Gurr and this proved satisfactory for these specimens.

2.4 DISCUSSION

In premalignant oral epithelial lesions in man the histological changes which are thought to be indicative of potential malignancy are those grouped together under the heading of cellular atypia. If the proposed animal model were to be useful in studying the prognostic value of individual histological features then corresponding changes should be seen in the animal lesions and in human oral mucosal lesions. The results of this preliminary study indicated that the model system did satisfy these criteria in that changes similar to those seen in human premalignant oral lesions were present in all pouches.

The model system was unsatisfactory in some other respects, however. The principal defect appeared to be lack of standardisation of the response between pouches and when different areas of the same pouch were studied. It was not possible with this technique to predict where areas of pathological change would be seen although the general observation was made that the changes were more severe towards the distal end of the pouch. There are several possible reasons for such a variation. There may be differing degrees of reactivity to carcinogen in different parts of the pouch or the local environment may vary with respect to contact with food or the cleaning action of the tongue.

It was also felt possible that gravity might be a factor in the differing response of parts of the pouch in that while the animal was in the restrainer there would be a tendency for the carcinogen solution to drain into the distal end of the pouch probably resulting in a higher carcinogen dosage in this region. This technique, therefore, did not fulfill the criteria detailed in 1.6 for the induction of a standard lesion which was reasonably reproducible. The scarring and dense hyalinised connective tissue which were seen in many pouches greatly complicated the histological interpretation and it was not possible to distinguish clearly the abnormalities related to regeneration and repair from the changes which were primarily associated with malignant change.

The detailed histological examination also gave rise to some doubt about the suitability of the model system. The only carcinoma observed appeared to arise in a papilloma and the four papillomas observed all showed evidence of obvious cellular atypia. Human oral papillomas are not generally considered as premalignant lesions.

The study of the weekly weights of the individual animals confirmed that the carcinogen applications had a systemic effect on the animals and suggested the need for a better controlled and less toxic application of

the carcinogen. The significant difference in the

It had been thought that the allocation of animals to different groups had been random, but the subsequent evaluation revealed that this was not so in relation to the mean starting weights of animals in different groups. This indicated a requirement to check that the procedures for random allocation to different groups had in fact produced a random distribution in terms of the parameteres to be investigated later.

2.5 CONCLUSIONS

This model system based on the technique proposed by Salley (1954) and standardised by Morris (1961) does produce lesions in the hamster cheek pouch with the histological features of cellular atypia. The localisation of these changes is not sufficiently predictable to allow a standard biopsy and excision technique to be employed, but if this problem could be overcome, then the system warrants further detailed study.

HAMSTER CHEEK POUCH - SECOND EXPERIMENT

3.1 INTRODUCTION

The experiment reported in Chapter 2 indicated that the application of DMBA in acetone to the hamster cheek pouch produced changes similar to those seen in human premalignant oral epithelial lesions. The main defect to be overcome, with regard to the experimental technique, was the poor localisation and standardisation of the response to the carcinogen.

The aim of the experiment reported in this Chapter was to attempt to localise the epithelial changes to a pre-determined area of the cheek pouch and to biopsy this area after a period of carcinogen application. It was then planned to observe this treated area over a period and later excise the whole treated area. The aim was then to analyse the histological changes seen in the biopsy and to try and relate these to the subsequent changes in the treated area.

3.2 MATERIALS AND METHODS

3.2.1 Animals

The preliminary experiment proved the suitability of the line bred Syrian golden hamsters and further animals were obtained from the same supplier. Forty male hamsters, seven weeks of age at the commencement of the experiment were divided randomly into a control group of ten animals, experimental group 1 of twenty

animals and experimental group 2 of ten animals. These animals were then marked on the ears to allow individual identification. Because of the number of animals in this experiment individual caging was considered impracticable in the space available. The animals were housed in standard hamster boxes, two or three hamsters per box. It was felt that this would not seriously affect the experimental technique as Morris (1961) had shown no difference in his study with animals caged under differing conditions. The animals were fed standard laboratory chow and given water ad libitum. The safety precautions detailed in 2.2.6 were again followed.

3.2.2 Immobilisation and Anaesthesia

The techniques described in 2.2.2 for anaesthesia and immobilisation of animals during the carcinogen applications were found very satisfactory in use and were repeated in this experiment. In order to obtain biopsies of the cheek pouch, more prolonged anaesthesia was required. This was achieved by intraperitoneal injections of veterinary Nembutal. The anaesthetic solution used was that described by Cruikshank (1965). This consisted of a stock solution of 60 mg/ml of pentobarbitone sodium which was diluted 1 in 10 with 10 per cent ethyl alcohol. 1ml per 100 g body weight of this final solution, on intraperitoneal injection produced deep anaesthesia for 30 minutes to one hour.

3.2.3 Carcinogen Application

As in the preliminary experiment, the carcinogen solution was 7,12 dimethylbenz (a) anthracene dissolved in acetone. The 20 animals in group 1 received applications of a 0.5 per cent DMBA solution and the 10 animals in group 2 were treated with a more dilute solution containing 0.25 g DMBA per 100 ml of acetone. Morris (1961) demonstrated that a 0.25 per cent DMBA solution produced a slower tissue response although it ultimately gave a good tumour yield. It was therefore felt that painting with the more dilute carcinogen might extend the time scale of the appearance and progression of the early changes in the process of carcinogenesis. The solutions were prepared as detailed in 2.2.3 and the same precautions, as previously, were taken to minimise concentration of the solution due to evaporation of acetone.

The experimental design was that the 30 animals in the experimental groups should receive applications of carcinogen three times per week for a period of six weeks. A standard biopsy was obtained two weeks after the last carcinogen application. In 11 of the 60 biopsies, the histological sections were unsatisfactory due to the tissues being hard and difficult to cut. These eleven pouches were rebiopsied at a site adjacent to the original biopsy, two weeks after the first biopsy was obtained. The animals were observed

for a further seven weeks before sacrifice. During the period of carcinogen application if it was considered that ulceration of the treated area of the pouch was likely to occur carcinogen painting was suspended until the pouch appeared more healthy. In this way the extensive ulceration and subsequent scarring of the pouches noted in some animals during the preliminary experiment were avoided. All animals received between 15 and 17 carcinogen applications.

As an additional aid to evaluating the systemic effects of the carcinogen applications, a chart was kept recording the weights of individual animals at the beginning of each week. This chart is shown in Table 3.2

3.2.4 Localisation of Experimental Changes

The distribution of the pathological changes seen in the pouches of the animals in the preliminary experiment suggested that carcinogen solution had flowed into the depths of the pouches and not remained localised to the actual area of application. The problem of ensuring that carcinogen applications were localised to a specific area during the course of the experiment clearly had two distinct facets. Firstly, a system allowing accurate recognition of the test area was needed and secondly it was necessary to be able to visualise where the carcinogen was present upon application and then restrict it to the desired

area. It was decided that the experimental area should be a 1 cm square portion of mucosa on the medial wall of each pouch near the opening of the pouch anteriorly. This was the most accessible area of the pouch when it was exposed using the retractors as in the preliminary experiment. Some authors have routinely everted the pouch prior to each carcinogen application, but it was felt that this was unnecessarily traumatic and also too time consuming. It was felt that the first problem of recognition of the treated area might be solved by tattooing the periphery of the required area.

Several techniques of tattooing were tried including the use of a tattooing machine obtained from a local professional tattoo artist. None of the techniques employed was entirely satisfactory. The main difficulty encountered was in producing adequate tensioning and immobilisation of the area to be tattooed, even when the animal was deeply anaethetised with Nembutal. This was due to the fact that the pouch is not firmly attached to the underlying tissues and also has a muscular coat. The pouch is distensible but elastic and it proved impossible to maintain even stretching in situ while attempting to tattoo the experimental area. The subepithelial tissues in the experimental area are very lax and even the insertion of a thin strip of cardboard through an incision

within the pouch into the space between the pouch mucosa and the Masseter muscle did not sufficiently stabilise the area. It was felt that an alternative technique for delineation of the experimental area in the pouch was required.

Observation of the medial wall of the pouch indicated that this is bounded anteriorly by a prominent raphe and vein (Fig. 3.1). It was felt that this vein could serve as a reference point if carcinogen applications could be made with a standard implement bearing a consistent relationship to this vein on successive applications.

An applicator 1 square centimetre in size was required. Experiments with 1 cm square pieces of foam rubber were conducted, but these proved unsatisfactory because of difficulties in constructing the applicator and of applying it evenly to the required area. Also it was difficult to standardise the amount of carcinogen carried on the applicator on different occasions. The alternative solution which was adopted was to use an artist's oil painting brush which was 1 cm in width and which was trimmed to 1 cm in length. This brush was dipped in the carcinogen solution and wiped against the edge of the container to remove excess carcinogen before being applied in such a manner that the brush was placed on the experimental

area with a single movement with the long axis of the brush parallel to the epithelial surface. The brush was placed such that the junction of the bristles and the metal part of the handle lay on the prominent vein described previously and the brush was applied centrally in the opening of the medial wall of the pouch. The brush was then lifted off the epithelium perpendicularly to the surface and withdrawn from the pouch. It was felt that this technique would allow a uniform application to the whole experimental area.

The problem of visualising where applied carcinogen solution was actually present does not seem to have been tackled by previous investigators. This problem arose because the acetone solution was clear and could not be seen on the mucosa. The obvious solution was to incorporate a dye in the solution. After experimenting with several standard histological dye solutions it was found that the most suitable dye was Sudan Black. When a concentration of this dye of between 0.05 mg and 0.1 mg per 100 ml of carcinogen solution was used the black colour indicated the extent of the carcinogen application. It was felt that this very weak dye solution would not materially alter the tissue responses and subsequent comparison of the changes in this experiment with those observed in the first experiment substantiated this view.

Before the carcinogen was applied, the experimental area was gently dried with a small piece of cotton wool and any food debris present was removed. This reduced the immediate dispersion of the carcinogen solution upon application. It was felt that if the solvent could be evaporated quickly after application, the carcinogen would be deposited only on the epithelium of the desired experimental area. The rapid evaporation of the acetone was achieved by blowing into the pouch a gentle current of air from a rubber chip syringe immediately after carcinogen application. The acetone evaporated within 5 to 10 seconds and gross inspection indicated that this technique successfully restricted the spread of the carcinogen. It was recognised that the possibility of later spread of carcinogen still existed, but it was felt that this constituted a much less serious problem than the initial uncontrolled spread of the carcinogen solution in acetone. Attempts to restrict this possible later spread of carcinogen were not made.

3.2.5 Biopsy Technique

It was desirable that a standardised technique of biopsying the experimental area was used. The animals were anaesthetised with intraperitoneal Nembutal in order to allow adequate time for the operation to be undertaken without undue haste.

Attempts were made to devise as atraumatic a technique as possible. A device similar to that suggested by Davenport (1969) was constructed. This consisted of a modified bur for use in a conventional dental handpiece. Fig. 3.2 shows this biopsy drill consisting of the shank of a contra-angle bur to which was soldered a metal cup having a sharpened cutting edge. It was planned to apply the rotating cup to the mucosa and cut through the epithelium and lamina propria down to the underlying muscle. It was hoped that the biopsy could then be dissected free with scissors by cutting across the base of the specimen. In practice it was found that this technique was unsuitable in that it failed to cut the pouch cleanly and tended to twist part of the pouch around the rotating cup. Variations of the cutting speeds and tensioning of the pouch mucosa failed to solve the problems of using this biopsy technique.

It was then decided that the standard biopsy should consist of a 4 mm square area in the antero-cranial corner of the experimental area. It proved difficult to excise this area neatly and without undue trauma simply by use of a scalpel. A combination of cuts made with a scalpel and fine pointed sharp scissors was found to give the best results. Two techniques were used. At first two parallel incisions 4 mm apart were made in an antero-posterior direction and then two

scissor cuts at right angles to these incisions completed the outlining of the biopsy which could be held carefully at one corner by fine forceps and readily freed from the underlying connective tissue by a scissor cut parallel to the epithelial surface. This technique had the disadvantage that the original scalpel cuts ran across the line of the muscle bundles of the Masseter muscle. The alternative technique of making the first incision with a scalpel close to and parallel to the prominent vein at the opening of the pouch and completing the outlining of the biopsy with scissors, proved more satisfactory. The surface of the pouch was not touched before the biopsy was taken in order to reduce the risk of removing the superficial cells in the stratum corneum. Sterilized instruments were used and between biopsies the scalpel blade was wiped with 70 per cent alcohol and the scissors and forceps were stored in 70 per cent alcohol. These precautions were taken to reduce the risk of bacterial cross-infection. Before use, the instruments were wiped to remove excess alcohol.

After the biopsy was removed, the pouch was observed to contract, greatly reducing the size of the wound. Bleeding was minimal and the wound was left untreated. Hamster number 14 was observed to be bleeding from the biopsy site several hours after the operation and was sacrificed. Healing in other

animals was rapid and uneventful and after one week it was difficult to detect the site of the biopsy in many animals.

3.2.6 Laboratory Techniques

The 4 mm square biopsy specimen was placed flat upon a small piece of filter paper with the connective tissue surface towards the paper. This ensured that the specimen remained flat during the fixation for one hour in the formol-acetic-alcohol solution described in 2.3.7. After fixation the specimen was halved to provide a face cut accurately at right angles to the keratinized layer thus allowing a good surface upon which to block out the tissue. The filter paper was removed from the back of the specimen and the tissues were then placed in 70 per cent alcohol before routine paraffin processing. Care was required to prevent tissue damage due to evaporation of the alcohol during the trimming of the specimens and removal of the filter paper and it was thought to be damage due to this cause which necessitated the taking of repeat biopsies of some pouches.

All animals were sacrificed and the pouches exposed as described in 2.2.5. All pouches were photographed and then fixed in formol-acetic-alcohol. After fixation the experimental area of 1 square centimetre was measured out and excised. The experimental area was kept moist with fixative solution

during this procedure. The experimental area was processed as a single piece and before embedding in paraffin it was cut cranio-caudally into four equal sized strips which were then blocked out to allow the sections to be cut at right angles to the keratinized surface. Areas of the pouch outwith the treated area which showed obvious pathological changes were also excised and processed for light microscopic study.

3.2.7 Histological Evaluation

6 μm thick sections of the paraffin embedded biopsy specimens were prepared. From each biopsy, two ribbons each of three sections were mounted, such that the first to the third and approximately the seventh to the ninth sections were retained. This gave a choice of sections on each slide if an individual section was damaged and allowed an interval of at least 40 μm between the first and the last sections available from an individual block. A ribbon of three sections was also prepared from each of the four blocks of the excised experimental area. All sections were stained with haematoxylin and eosin.

A preliminary histological assessment was made by noting the presence or absence of a number of pathological changes in the biopsy and final excision sections. These changes were atrophy of the epithelium, acanthosis, hyperorthokeratosis, hyperparakeratosis, disordered stratification, premature individual cell

keratinization and acantholysis. The presence of papillomas and carcinomas was also noted.

A more detailed evaluation of possible premalignancy was required which would consider all the features of cellular atypia. The interpretation of these individual changes is open to considerable variation between observers and in order to attempt to standardise such interpretation and render it more objective, the atypia scoring technique of Smith and Pindborg (1969) was used. A form based on that of Smith and Pindborg (1969) is shown in Table 3.1. This form was used in conjunction with the Smith and Pindborg (1969) standard photographs to assess cellular atypia in one section from each of the pouch biopsies.

The first section in the ribbon of sections from each biopsy was marked on the glass coverslip and then the sections from the 58 pouches were renumbered by a technician using random sampling numbers. During the subsequent evaluation by the author, only these numbers were used to identify the slides. This evaluation was thus "blind" and avoided any bias due to a knowledge of the animals in which carcinomas subsequently developed.

Prior to undertaking the full analysis of the 58 sections, it was felt necessary to ensure that the assessment by the use of the photographic comparisons was being made consistently. Five sections were scored

on three occasions a few days apart. Differences of up to 20 per cent in the scores between the first and second occasions were recorded. These differences were due to difficulty in assigning individual cases to a slight or marked grade in features such as basal cell hyperplasia and increased nucleo-cytoplasmic ratio and in the recognition of bizarre mitoses. Little difference was noted between the second and third assessments and study of these differences allowed decisions to be made about accurate grading such that the grading of the larger series would be adequately standardised.

3.2.8 Statistical Methods

The comparison of weekly weights of groups of animals in experiment 2 and the changes in weight during the experiment involved comparison of actual weights which were thus measured on a ratio scale. The same is true of weight comparisons between groups in experiments 1 and 2. It was assumed that the weights in individual groups were normally distributed and that the data was amenable to analysis by parametric methods. Comparison of the weights of different groups of animals was thus made using the Student's 't' test.

The initial histological characterisation of the response to carcinogen applications at the time of biopsy and after sacrifice of the animals involved the

recording of the presence or absence of particular features in these two groups. The result obtained gave a proportion of cases showing each particular feature. This proportion was converted to a percentage value and the 95 per cent confidence limits of that percentage were calculated. The lack of overlap of corresponding confidence limits indicated a significant difference at the 5 per cent level.

The more detailed evaluation of cellular atypia produced a numerical score for each biopsy section. The scale of measurement involved had at least the strength of an ordinal scale but it was doubted if it had the strength of an interval scale (Siegel, 1956). It was also felt undesirable to assume that the scores were normally distributed. These two factors required that the data be analysed by nonparametric methods. The research question asked was "are the cellular atypia scores of the biopsies in those cases in which tumours subsequently developed, different from those of the group which did not develop tumours?" If the cellular atypia score was to have prognostic value, then the research hypothesis had to be that the scores of the cases developing tumours were significantly greater than those in cases not developing tumours. The appropriate nonparametric test for such a study is the Mann-Whitney U test (Siegel, 1956) and this was the test employed.

3.3 RESULTS

3.3.1 Animal Weights

The weekly weights of individual animals in the experimental groups are recorded in Table 3.2. Comparison of the week 1 weight of animals in group 1 with those in group 2 showed no significant difference. In order to determine if the different carcinogen dosages employed in the two experimental groups caused a difference in the weight gained by the animals during the experiment, the differences in weights at week 11 and week 1 in the two groups was calculated. Animal 14 was omitted from this and subsequent parts of the study. The mean weight gain in this period was greater in group 2 than in group 1 but this difference was not statistically significant (Table 3.2). This indicated that the animals painted with the weaker carcinogen solution did not show significantly less systemic effect.

The weights of the control animals were not recorded as these animals were kept for a variable period and used to evaluate several technical and histological procedures.

The weight gain in experimental animals in the second experiment was compared with that of experimental and control animals in the first experiment as recorded in 2.3.6 (Table 3.3). Animals in the second

experiment were started at 7 weeks of age; one week earlier than in the first experiment. The week 2 weights in the second experiment were thus studied and these did not differ significantly from the week 1 weights of either the experimental or control groups in the first experiment. The weight difference of week 2 to week 6 in experiment 2 was compared to the week 1 to week 5 differences in the first experiment. During these corresponding periods the animals in experiment 2 gained significantly less weight than the controls ($P < 0.001$) but significantly more weight than the experimental animals in the first experiment ($P < 0.001$). It was therefore concluded that the experimental procedure in the second experiment had caused a systemic effect resulting in a lowered weight gain in experimental animals as opposed to controls, but that this effect was less severe than that noted in the experimental animals in the first experiment.

3.3.2 Macroscopic Evaluation

The pouches were examined carefully three times per week during the period of carcinogen applications. All pouches remained normal until the end of the second week when a few pouches appeared rather erythematous and atrophic. During the third and fourth weeks approximately one third of the pouches showed what appeared to be superficial erosions, most of which were

within the experimental area. By the beginning of the fifth week of the experiment all pouches had a normal healthy appearance grossly and in no instance was there the scarring and contraction which had been evident in the first experiment. The first papilloma observed was seen in an animal in group 1 at the end of the fifth week of the experiment.

At the time of biopsy which was two weeks after the last application of carcinogen, small papillomas were observed in 13 pouches in 11 animals. In 8 pouches the tumours were within the experimental area, but in 5 cases the papillomas were elsewhere in the pouch. At this time also, many pouches were observed to have a rough surface with white hyperkeratotic areas.

At sacrifice, exophytic tumours were observed in 29 pouches in 19 animals. In 21 pouches these lesions were in the experimental area. It was not possible to state with certainty whether an individual lesion was benign or malignant until the histological examination was undertaken later and showed that 5 of the tumours within the experimental area and 3 outwith the area were squamous cell carcinomas.

The findings in individual pouches are summarised in Table 3.4. The observations at the time of the first biopsy were not significantly different from those two weeks later when 11 pouches were re-biopsied.

The results at the time of first and second biopsies were therefore combined and compared with the findings at sacrifice.

Comparison of the groups painted with the differing concentrations of carcinogen revealed no significant differences at the time of biopsy. At sacrifice, no difference was noted either in the overall incidence of tumours or in the incidence of carcinomas within the experimental area. In group 2, which received applications of the weaker carcinogen solution, only 2 pouches developed tumours outwith the experimental area. This contrasted with 14 pouches in group 1 in which tumours developed outwith the experimental area and this difference was significant ($P < 0.05$).

Comparison of the photographs taken at the time of biopsy with those at the time of sacrifice revealed a variable picture. In some cases a small tumour at the time of biopsy had obviously enlarged by the time of sacrifice (Fig. 3.3) while in other cases a large tumour was present at sacrifice in an area which seven weeks previously had shown little evidence of pathological change (Fig. 3.4). At the time of sacrifice, multiple papillomas were present in some pouches. Hyperkeratotic areas were frequently seen (Fig. 3.5) but in 17 pouches no obvious pathological

change was evident on naked eye examination. In one instance a skin tumour was evident on the muco-cutaneous junction of the lip (Fig. 3.6).

3.3.3 Microscopic Evaluation

The wide range of histological changes observed in the first experiment which was reported in Chapter 2, was also seen in this experiment. The small exophytic tumours designated as papillomas showed epithelial hyperplasia and often obvious and sometimes severe cellular atypia was noted (Fig. 3.7). All the carcinomas appeared to be exophytic growths and early invasion was seen in the bases of papillomatous tumours (Fig. 3.8 and Fig. 3.9) suggesting that this was the mode of development of all the carcinomas observed in this experiment.

The skin tumour observed grossly, on histological examination appeared to be a carcinoma which was probably derived from skin appendages (Fig. 3.10).

A preliminary microscopic examination was undertaken to discover the frequency of occurrence of certain histological changes in the biopsy and sacrifice specimens. The findings of this examination are summarised in Tables 3.5 and 3.6.

The features examined for were focal atrophy, acanthosis, hyperorthokeratosis and hyperparakeratosis. Prior to the fuller study of cellular atypia undertaken

later it was felt that a preliminary indication of premalignancy might be obtained by recording the presence or absence of disordered epithelial stratification. This feature was evaluated subjectively without the aid of photographic standards for comparison as were used in the later study (3.3.4). While this study was in progress it was noted that premature keratinization and acantholysis were frequently seen and these characteristics were also included in the preliminary evaluation.

From the biopsy material, the two ribbons of three sections each were examined and a short ribbon of sections from each of the four blocks of the experimental area at sacrifice was studied. The features noted in the previous paragraph were recorded as present or not present if they were observed in any of the sections of a particular specimen. No attempt was made to quantitate the extent to which a particular feature was present in an individual specimen.

The results of the first and second biopsy specimens were assessed as a single group and are shown in Table 3.5. Corresponding observations at the time of sacrifice are shown in Table 3.6. It is evident that all the changes assessed were seen more frequently at the time of sacrifice than in the biopsy specimens.

Table 3.7 shows the 95 per cent confidence limits of the percentages of pouches showing each feature studied. No overlap was present in these limits when the biopsy specimens were compared to the final specimens. In all cases therefore these differences were statistically significant at the 5 per cent level. The histogram (Fig. 3.11) emphasises the striking increases in the frequency of focal atrophy, hyperparakeratosis and disordered stratification between the biopsy and final specimens.

Comparison of group 1 with group 2 both at the time of biopsy and at sacrifice revealed no significant difference in the frequencies of any of the histological changes studied in the groups painted with different carcinogen concentrations.

3.3.4 Cellular atypia

The atypia scores of one section from each pouch biopsy are shown in Table 3.8. These scores range from 2 to 54 out of a possible total of 75. As discussed in 3.2.8 analysis was undertaken by non parametric methods and the scores were thus ranked from 1 to 58. Tied values were assigned the average of the tied ranks.

The first question asked was whether the cellular atypia scores of the biopsies from the 5 pouches in which carcinomas subsequently developed in the

experimental area were significantly greater than the scores in the remaining 53 biopsies. The results of this analysis are shown in Table 3.8. The mean atypia score of the pouches developing carcinomas was 35.0 whereas that of the remaining pouches was 22.68. The ranks of three of the cases developing tumours were 54, 55 and 56, but carcinomas also developed in the specimens ranked 14.5 and 25.5. Using the Mann-Whitney U test the one tailed probability of obtaining this difference in ranks between the carcinoma group and the non carcinoma group was P=0.0559. (Application of the correction for ties (Siegel, 1956) did not alter this probability). This difference, although nearly so, was not significant at the 5 per cent level. Thus the ranks of the atypia scores do not justify the rejection of the null hypothesis in favour of the research hypothesis that the atypia scores of cases developing carcinomas were significantly greater than in the remaining biopsies.

The number of cases developing squamous cell carcinomas was small and all these tumours presented as exophytic growths which appeared to have arisen from papillomatous tumours. Many of the papillomas noted showed evidence of cellular atypia and it was felt that, had the period of observation before sacrifice been longer these lesions would probably have become malignant. Accordingly a comparison was made of the

atypia scores of biopsies from pouches subsequently developing any neoplasm in the experimental area with those in which no neoplasm was found by the time of sacrifice. The mean atypia score of the tumour group of 21 pouches was 28.67, while that of the non tumour group of 37 pouches was 20.95. A Mann-Whitney U test comparing these groups gave a value of P=0.0951. Thus the atypia scores at the time of biopsy of the group developing tumours were not significantly greater than those in the pouches not developing tumours.

Mann-Whitney U tests also failed to reveal any significant difference between the atypia scores of the groups painted with different carcinogen concentrations (P=0.0853) and analyses of carcinoma and tumour cases within the two groups separately also revealed no significant differences in atypia scores.

3.4 DISCUSSION

3.4.1 Localisation and Standardisation of Response

The initial aim of the experiment described in this chapter was to devise a technique to localise the tissue exposure and response to carcinogen to a defined area. The microscopic observations in 3.3.2 indicated that at the time of biopsy 57 per cent of the pouches with tumours contained these lesions in the experimental area of 1 square centimetre. At the time of

sacrifice, tumours were found in more than twice as many pouches and in 72 per cent of these, the lesions were within the experimental area. The technique in this experiment was clearly providing a much better localisation of response than was found in the first experiment recorded in Chapter 2.

The comparison between the groups treated with different dosages of carcinogen showed a marked difference between these groups at the time of sacrifice in that the group painted with the 0.25 per cent DMBA solution showed significantly fewer tumours outwith the experimental area. There was no significant difference in the tumour yield within the experimental area in the groups painted with the two different concentrations of carcinogen solution and the use of the more dilute solution was therefore felt to provide the better experimental model.

The technique in the second experiment achieved a satisfactory tumour yield without causing the marked tissue distortion due to ulceration and subsequent healing with scar tissue as was seen in the first experiment. This greatly simplified the histological assessment. The comparison of the weight changes during the course of experiments 1 and 2 also indicated that the technique in the second experiment produced much less severe systemic effects although the animals

did gain significantly less weight than untreated controls.

3.4.2 Histological Analysis

The preliminary histological evaluation (3.3.3) showed a significant increase in the frequency of occurrence of the features studied, between the biopsy and sacrifice specimens. These features were focal atrophy, acanthosis, hyperorthokeratosis, hyperparakeratosis, disordered stratification, premature keratinization and acantholysis. It could be argued that this difference was due to the fact that the amount of tissue examined in the sacrifice specimens was five times as great as that studied in the biopsy specimens. However, the features recorded were often present as multiple foci in both biopsy and sacrifice specimens and the scoring system merely recorded whether or not the abnormality was present. It was thus felt that, while the difference in specimen size could have affected the results obtained, it was probable that this effect was small and that the differences observed represented a real qualitative difference between the specimens.

The high incidence of acanthosis and hyperorthokeratosis in the biopsy specimens suggested that these were part of the initial reactive phase and might not be indicative of a premalignant change. The striking increases in the incidence of focal atrophy,

hyperparakeratosis and disordered stratification between the biopsy and sacrifice specimens were interpreted as indicating progressive alternations in cell turnover and cell interactions in the epithelium and were felt to be similar to changes noted in human oral premalignancy.

The premature keratinization observed was of the same type as that noted in the first experiment (Fig. 2.18). This was present in 36 per cent of the biopsies and almost 90 per cent of the sacrifice specimens. The acantholysis which had a frequency of occurrence very similar to the premature keratinization in both the biopsy and sacrifice specimens was usually found in association with the premature keratinization. This acantholysis was of the type illustrated in Fig. 2.17 and was characterised by rounding up of cells in the stratum spinosum with loss of intercellular attachment and increase in the intercellular space. The premature keratinization and acantholysis did increase significantly between the biopsy and sacrifice specimens, but subjectively it was felt that this change did not show an obvious relationship to the development of tumours. Even in those cases in which papillomas and carcinomas developed the premature keratinization and acantholysis did not show any clear relationship to the tumours. The foci showing these changes were usually seen as discrete

localised areas occuring in otherwise relatively normal parts of the epithelium.

3.4.3 Cellular atypia

The analysis of the cellular atypia scores in the biopsy specimens showed that a higher mean atypia score was obtained in those cases in which carcinomas subsequently developed than in the remainder of pouches. The differences in atypia scores were not significant at the 5 per cent level but it was felt that this finding might have been related to poor sampling. Only one section from each biopsy was scored. Further study involving better sampling of individual biopsies was thus required and this is discussed in Chapter 4.

A further possible cause for the lack of significant difference in the atypia scores of cases developing carcinomas might be related to the actual scoring technique. Observations on the use of the Smith and Pindborg (1969) technique are noted in 3.4.4.

3.4.4 Subjective Assessment of Cellular Atypia Evaluation Technique

The assessment of cellular atypia using the Smith and Pindborg (1969) technique presented some difficulties. Some practice to produce reasonable consistency in interpretation was first required before the full analysis was undertaken.

The instructions on page 8 of Smith and Pindborg's (1969) monograph state "By comparison of the photographs and the section it will become apparent which grade most closely resembles the most marked features seen down the microscope". In practice the majority of sections in the present study fell between the slight and marked cases illustrated and it was often found difficult to say exactly where an individual case lay. In these circumstances a quantitative element of the extent of a given change was used to arrive at the designation of slight or marked. It was felt that this subjective element in the analysis could have been reduced by utilising slightly different grades for the standard photographs; such that the photographs indicated the extremes of the ranges of the none, slight and marked categories. This would then have allowed the evaluation "slight" change to be awarded to cases showing a change equal in severity to the "slight" illustration but less than the "marked" illustration. Cases with the particular characteristic absent or less severe than the "slight"

illustration could be graded as none.

The assessment of characteristics relating to cell division carries considerable weight in the evaluation of cellular atypia. In fact, a possible 25 points out of the total of 75 in the atypia score relate the features 11, 12 and 13 which are those concerned with mitosis. The evaluation of these characteristics is simplified in the case of the hamster cheek pouch by the fact that the progenitor cell compartment is restricted to the basal cell layer, defined as comprising those cells in contact with the basement membrane. The amount of mitotic activity is assessed purely subjectively and it is difficult to conceive of producing a more accurate system of assessment without greatly complicating the measuring technique. This is because the number of mitoses seen in a given section is a function of a number of variables such as mitotic duration and the rate of entry of cells into mitosis both of which show diurnal variation. Also the circulating adrenaline levels affect mitosis and these are influenced by stress. Some of these factors have been discussed by the author elsewhere. (MacDonald 1971).

The level in the epithelium at which mitotic figures are seen is a good objective measurement in hamster cheek pouch, but the assessment of the presence of bizarre mitosis is more subjective as it involves

the decision as to whether or not individual mitotic figures "conform to any normal stage of the mitotic cycle". (Smith and Pindborg, 1969). This is dependent upon the investigator's concept of the normal appearances and is complicated by the plane of section of individual mitoses. Factors such as total volume of chromatin per dividing cell, thickness and irregularity of chromatin threads and pattern of chromatid division at anaphase and telophase are all involved in this assessment. In the present study 18 biopsies showed single bizarre mitoses and 15 biopsies showed multiple bizarre mitoses. This seems a rather high frequency of bizarre mitoses and it may be that more detailed criteria are required for the definition of an abnormal mitosis which has prognostic significance as regards premalignancy.

3.4.5 Comparison of Experimental Hamster Carcinogenesis and Human Oral Mucosal Lesions

The stimulus to research into experimental oral carcinogenesis in the hamster has come largely from a desire to elucidate some of the problems encountered in studying human oral malignancy. It is therefore important to decide whether or not it is reasonable to apply the results obtained in the experimental animal to the human situation.

Subjective comparison of the hamster response to carcinogen with the author's concept of the changes seen in human oral mucosa suggested that the acanthosis, hyperkeratosis and plaques of hyperparakeratosis were very similar to the findings in human oral leukoplakia. The plaques showing cellular atypia were also very similar to human lesions.

The histological appearances of cheek pouch papillomas were felt to be different from human oral papillomas. The hamster lesions rarely showed the multiple epithelial fronds typical of the human lesion and the acanthosis and atypia in the hamster were much greater than is usual in human lesions.

In the present experiment all the carcinomas observed appeared to have arisen by invasion in the base of papillomatous neoplasms. A similar observation was made by Woods and Smith (1969a) who noted in their gross observations that all tumours, if allowed to develop, eventually became malignant neoplasms.

McCarthy and Shklar (1964) state that dyskeratotic changes are often seen in human oral papillomas and may signify the possibility of malignant transformation. However, this is not a common way for squamous cell carcinomas to arise in human oral mucosa.

The frequent finding of foci of premature keratinization and acantholysis in the experiments recorded in this chapter and in Chapter 2 was a distinctive feature of the hamster response to carcinogen which is not found in human oral lesions. This change has not been recorded by previous authors and personal discussion with other workers who have used this model system suggests that it may be peculiar to the experimental circumstances of the author's studies, perhaps related to the strain of animals used or to the particular mode of carcinogen application.

The observations therefore suggested that while there were similarities in the hamster response to carcinogen applications to the cheek pouch and human oral mucosal lesions, there were important differences in the response which required further analysis before a statement about the applicability of the experimental results to the human situation could be made.

3.5 CONCLUSIONS

The study reported in this chapter produced a localisation of response to carcinogen applications to a defined area of the cheek pouch. The assessment of cellular atypia by the Smith and Pindborg (1969) technique gave results which suggested that those pouches subsequently developing carcinomas showed

higher atypia scores at biopsy but as indicated in 3.4.3 a repeat analysis with better tissue sampling was required.

The comparison of the hamster and human lesions which was discussed in 3.4.5 was highly subjective and more accurate information about the features characterising the human oral lesions appears to be required before conclusions can be drawn about the wider application of the animal results.

FURTHER STUDIES ON CELLULAR ATYPIA AND COMPARISON OF HAMSTER AND HUMAN LESIONS

4.1 INTRODUCTION

In Chapter 3 it was noted that the failure to observe significantly greater atypia scoring in the biopsies from pouches subsequently developing carcinomas as compared to the remainder of the pouches might have been due to inadequate sampling. It was desirable, therefore, to repeat the analysis using a technique to provide more representative sampling of the biopsies.

As a progression from the subjective comparisons of human and hamster lesions noted in 3.4.5 a more objective examination of the features of cellular atypia as evidenced by hamster and human lesions appeared desirable. This required the assessment of a number of human oral premalignant lesions by the Smith and Pindborg (1969) method and comparison of the findings with the previously completed analysis in hamsters.

It was suggested in 3.4.2 that some of the pathological changes observed in sections of the biopsies of the hamster cheek pouches might have been basically reactive or hyperplastic and not indicative of premalignancy. It was felt that the same might be true of human premalignant oral mucosal lesions.

It is common for pathological textbooks to describe in detail the histological features of oral premalignant lesions and squamous cell carcinomas as individual entities, while failing to discuss these histological changes in the wider perspective of a range of oral mucosal lesions. It was therefore felt pertinent to examine which pathological changes were significantly correlated with neoplasia and which changes were seen also in non neoplastic oral mucosal lesions.

4.2 MATERIALS AND METHODS

4.2.1 Cellular Atypia - Further Analysis of Experiment 2

The results of the analysis of atypia scores in experiment 2, reported in 3.3.4, were considerably influenced by the low scores of two of the cases in which carcinomas subsequently developed. These were in pouches 18A and 28A (see Table 3.8). The obvious preliminary to a further analysis was to see if the sections of these biopsies which were scored were in fact the most severely involved areas of the biopsies. Serial sections of these two biopsies were prepared and every 5th section was examined. Subjectively it was readily apparent that there were areas showing more severe atypia than those scored in the original sections.

The analysis of cellular atypia depends on the evaluation of the most severely involved areas observed.

The actual scoring procedure is time consuming when applied to a large number of sections and it was felt appropriate to select subjectively the section showing the most severe cellular atypia and use the score obtained from that section for further analysis. If the procedure of selecting the most severely involved section was to be applied to the carcinoma specimens then clearly further sampling of all the other biopsy specimens was also required. The blocks from the remaining 56 pouches were therefore recut and sampled at three levels in the block such that three short ribbons of sections were retained with approximately ten 5 um sections between each ribbon being discarded. By subjective evaluation of the cellular atypia the most severely affected section from each biopsy was selected and the overlying coverslip was marked. It proved impossible to obtain further sections from the blocks of the biopsies of 3 of the 58 pouches. The most severely affected of the previously available sections was used to obtain the atypia score in these cases. As indicated in 3.2.7 it was desirable that analyses be made blind and the individual sections were thus numbered and scored randomly such that the author was unaware of which pouch each slide came from until after all sections were scored for cellular atypia using the Smith and Pindborg (1969) technique.

4.2.2 Cellular Atypia - Human Material

Biopsies of human oral mucosal lesions were obtained from the files of the routine diagnostic pathology service in Glasgow Dental Hospital and School. All cases received after August, 1971 and reported as showing cellular atypia were collected until twenty such cases were available. All available sections of these cases were examined and subjectively the sections showing the most severe cellular atypia in each biopsy were selected for detailed scoring. In some cases it was difficult to decide subjectively which section showed the most severe involvement and in these cases more than one section was scored and the section in which the atypia score was highest was the one used in further studies.

4.2.3 Comparison of Cellular Atypia in Human and Hamster Material

Before a detailed comparison of human and hamster material was possible it was essential to obtain a group of hamster lesions with comparable atypia scores to the human cases scored. Twenty hamster sections were selected which had the same, or as closely similar as possible, atypia scores to the twenty human lesions. The comparability of the hamster and human groups was checked using a Mann-Whitney U test.

When comparable groups of hamster and human cases of cellular atypia were obtained the distribution of cases in the thirteen variables used in the cellular atypia analysis was examined. The human and hamster series were studied separately and for each of the thirteen variables the number of cases in the "none" "slight" and "marked" categories was recorded. It was felt that comparison of the breakdown of human and hamster material into these categories would allow of a more detailed comparison than that provided by the atypia scores alone.

4.2.4 Histological Features of Some Human Oral Mucosal Lesions

As indicated in the introduction, it is common for the features of lesions found in particular diseases to be described without indicating which histological characteristics are closely associated with these lesions specifically and which are shared with the lesions of other diseases. Accordingly, it was felt desirable to sample a group including several different types of oral mucosal diseases and to evaluate the histological features of the lesions. This part of the study was conducted in the University of Illinois while the author was a Visiting Associate Professor in the Department of Oral Pathology.

The sections studied were from the routine diagnostic pathology service and were selected by

starting with the specimens from 1st January, 1967, and taking all specimens received after that date on which a diagnosis was made of squamous cell carcinoma, premalignant oral mucosal lesion, papilloma, papillary hyperplasia of palate or acanthosis with or without focal keratosis (noted in Table 4.6 as hyperplasia). This latter group was restricted to cases of clinical leukoplakia in which the pathologist was asked to rule out malignancy or premalignancy. As expected the selection was biased in favour of the hyperplasia group and after the first 39 cases had been selected only squamous cell carcinomas and premalignant lesions were added to the group for analysis.

All available sections of each case were examined and the original diagnosis was confirmed. Cases were retained in the study only if the sections were felt to show enough of the lesion to be representative and to show adjacent normal or relatively normal epithelium. A judgement was also made as to whether or not the section had been cut at right angles to the mucosal surface. Obviously oblique sections were not used as it was desirable to evaluate features such as atrophy and acanthosis which could be considerably influenced by oblique sectioning. The totals of each type of lesion finally included in the study were 12 squamous cell carcinomas, 4 premalignant lesions, 5 papillomas, 3 papillary hyperplasias of palate and 24

hyperplasias.

The histological features studied related to keratinization, cellular organisation, mitotic activity, connective tissue changes and the morphology of the lesion. In all, 26 features were assessed. (Table 4.5 lists these features) The individual features were noted as present or not in each case. This information was transferred to 80 column punch cards along with additional information about the sites of the lesions. In all, 39 variables for the 48 cases were recorded. A computer programme for the correlation of the 39 variables was used and this provided r values for simple correlations between each of the 39 variables. The 8 variables relating to the sites of the lesions have been discarded from this present study because of the small numbers of lesions at each site.

4.3 RESULTS

4.3.1 Cellular Atypia - Further Analysis of Experiment 2

As noted in 4.2.1 the serial sections of the biopsies of pouches 18A and 28A revealed more severely affected areas than those scored in the analysis reported in 3.3.4. Fig. 4.1 shows the most severely affected area of the original sections of 18A. Fig. 4.2 shows much more severe atypia revealed by the subsequent serial sectioning of the same tissue block.

The atypia scores of the most severely affected areas of 18A and 28A respectively were 59 and 53. This contrasted with scores of 15 and 20 obtained in the earlier study.

The scores of the second analysis are shown in Table 4.1 which also gives the ranks of the scores and indicates the pouches with carcinomas or any tumour observed grossly in the experimental area. The mean atypia score of the 58 pouches was 30.02 and this was higher than the mean score of 23.74 obtained in the first analysis. Further comparison of the scores obtained in the first and second analyses is noted in 4.3.2.

The ranks of the cellular atypia scores of the cases in which squamous cell carcinomas subsequently developed were compared with the ranks of the remainder of the specimens by a Mann-Whitney U test, (Siegel 1956) and the result is shown in Table 4.1. The P value of 0.0014 indicates that the atypia scores of the cases developing carcinomas were significantly greater than in those cases which did not develop carcinomas.

In the comparison of the first and second analyses of cellular atypia it was clear that in some individual cases the scores in the first analysis were higher than those in the second study. As the cellular atypia

scoring relates to the most severely involved area of each specimen the comparison of carcinoma and non-carcinoma groups was repeated using as the score for each individual biopsy, the higher of the scores from the two analyses. A Mann-Whitney U test in this case gave a one tailed probability of P=0.0018 indicating that the atypia scores of the carcinoma group remained significantly higher than those of the group in which carcinomas did not develop.

The atypia scores of the biopsies from the 21 pouches in which any tumour developed in the experimental area were compared with the scores from biopsies of pouches not developing tumours. A Mann-Whitney U test yielded a value P=0.0036 indicating that the atypia scores of the tumour group were significantly greater than those of the non tumour group.

The atypia scores of the pouches developing carcinomas were not the five highest ranked scores. The lowest ranked score of a case developing a carcinoma was in pouch 13A with a score ranked 47 out of 56. 8 other cases, excluding those developing carcinomas, had higher atypia scores. In these 8 cases the histology at sacrifice showed 2 cases of carcinoma in situ; 5 of severe atypia with a query about the possible presence of early invasion in 2 instances; and 1 case of moderate cellular atypia.

4.3.2 Comparison of the Two Analyses of Cellular Atypia in Hamster Cheek Pouch

It was noted in the previous section that the mean atypia score in the second analysis was higher than that in the first analysis. It was felt desirable to compare the results of the two analyses more fully. Since the sections studied represented two samples drawn from the same population a Wilcoxon matched pairs signed-ranks test (Siegel 1956) was used. This test examines the direction and magnitude of the differences observed between pairs of values. The analysis is shown in Table 4.2. This yielded a value of P=0.002 indicating that the scores in the second analysis were significantly greater than those in the first analysis. This finding was in agreement with the subjective impression that the more extensive sampling had revealed in a number of cases areas of more marked cellular atypia in the second analysis.

4.3.3 Comparison of Cellular Atypia in Human and Hamster Material

The atypia scores of the 20 cases from human oral mucosa and the 20 hamster lesions with equivalent or nearly equivalent cellular atypia scores were compared. The mean atypia score of the human lesions was 34.85 and that of the hamster lesions was 35.1. A Mann-Whitney U test was performed to show whether or not the atypia scores of the hamster were significantly higher than the human cases. This test yielded a

value of P=0.4522 indicating that there was no significant difference in the cellular atypia scores of the hamster and human lesions.

Having obtained comparable groups of human and hamster lesions showing cellular atypia the individual scoring sheets were examined to show the numbers of cases in the "none" "slight" and "marked" categories of each of the 13 variables examined in the calculation of the cellular atypia score. The human and hamster groups were examined separately and a summary of the findings is shown in Table 4.3.

The best statistical test to use for comparing data of the type shown in Table 4.3 is the X^2 test. However, there are limitations on the use of this test related to the magnitude of the expected frequency in any cell (Siegel, 1956). Cochran (1954) recommends that for X^2 tests with more than one degree of freedom fewer than 20 per cent of cells should have an expected frequency of less than 1. If these requirements are not met the established procedure is to combine adjacent categories to fulfil the requirements. When this was done with the data relating to the comparison of the 13 separate histological features in human and hamster atypia 2 x 2 contingency tables were obtained. A X^2 test was then performed on this data unless the expected frequency in any cell was

less than 5, in which case a Fisher exact probability test was used (Siegel, 1956). For the analysis of the data in Table 4.3 in the case of feature 13, the presence of bizarre mitoses, the data can properly be analysed by a 3 x 2 X^2 test. In the case of the remaining 12 features adjacent categories had to be combined to fulfil the requirements detailed in the preceding paragraph. In each case two 2 x 2 contingency tables were prepared. The "none" and "slight" categories were first combined for both the hamster and human material and then the "slight" and "marked" categories were combined and the analysis was repeated.

No difference was observed between the hamster and human lesions with regard to 8 of the 13 histological features. These features were irregular epithelial stratification, keratinization of cells below the keratinized layer, basal cell hyperplasia, loss of polarity, hyperchromatic nuclei, anisocytosis and anisonucleosis, pleomorphism of cells and nuclei and the presence of bizarre mitosis. The five features in which a significant difference between hamster and human atypia was observed are detailed in Table 4.4. The features which were more frequent or more marked in human lesions were drop shaped rete ridges, loss of intercellular adherence and increased mitotic activity. There was a greater increase in

the nucleo-cytoplasmic ratio in the hamster and the animal lesions also showed more mitotic figures above the normal progenitor cell compartment.

4.3.4 Histological Features of Some Human Oral Mucosal Lesions

Table 4.5 lists the histological features recorded and Table 4.6 shows the findings in the 48 individual cases. Table 4.7 shows the positive and negative correlations noted and their levels of significance. Only those instances in which a significant correlation was found are noted.

Of the features relating to keratinization, hyperorthokeratosis and hyperparakeratosis were not particularly related to any diagnostic group. Hyperorthokeratosis was absent from the cases of papillary hyperplasia of palate and a significant negative correlation of hyperorthokeratosis and this diagnosis was found.

Keratin pearls and premature individual cell keratinization showed a significant positive correlation with squamous cell carcinoma, but not with the other diagnostic groups, although keratin pearls were seen in some premalignant lesions, papillomas and papillary hyperplasias of palate. No cases of hyperplasia showed keratin pearls. Occasional premature keratinized cells were seen in all diagnostic categories.

Of the features relating to the cellular organisation, acanthosis was the most frequently found, being noted in 93.8 per cent of the cases studied. This characteristic was not particularly correlated with any of the diagnostic categories.

Focal atrophy was noted in all 12 cases of carcinoma; there being a highly significant correlation between the presence of focal atrophy and carcinomas. Ulceration was also noted in 9 of the 12 cases of carcinoma. All of the remainder of the characteristics of altered cellular organisation studied were significantly correlated to carcinomas with the exception of intracellular oedema in the basal cell layer. Of these characteristics, the only ones which showed a significant correlation with premalignant lesions were those of abnormal variation in cell sizes and nuclear sizes in the basal cell layer and above the basal cell layer. There was a significant negative correlation between the diagnostic group of hyperplasias and the features positively correlated with the carcinomas.

As might be anticipated elevated mitotic activity and abnormal mitoses were significantly related to carcinomas and showed a significant negative correlation with the hyperplasias although both of these features were observed in all diagnostic groups.

The high correlation of acute inflammation with the carcinoma group is probably related to the high frequency of ulceration observed in the carcinomas. Plasma cells were also prominent in the infiltrate related to carcinomas in contrast to the hyperplasia group in which a significant positive correlation was noted to the presence of a chronic inflammatory cell infiltrate from which plasma cells were absent.

4.4 DISCUSSION

4.4.1 Cellular Atypia Scoring in Hamster Cheek Pouch

The results reported in 4.3.1 clearly showed that the sampling technique employed in the previous study recorded in 3.3.5 was inadequate. The extensive sampling of the biopsies to select the most severely involved area of epithelium for atypia scoring provided areas of atypia which gave high atypia scores in all cases in which carcinomas subsequently developed. This is good evidence for the reliability of the system of atypia scoring as a predictor of the prognosis of the lesions in the hamster cheek pouch.

The atypia scores of the biopsies of all pouches in which tumours developed subsequently were also significantly higher than the scores of the biopsies from the pouches not developing tumours. This finding supports the suggestion made in 3.3.4 that papillomas developing in this experimental model system are probably potentially malignant.

4.4.2 Comparison of Cellular Atypia in Human and Hamster Lesions

The values in Table 4.3 indicated that the atypias in human and hamster lesions were closely similar. However, it was important to consider the five categories in which differences were observed. It was felt that these were largely related to differences in the basic histology of human and hamster mucosae rather than in the pattern of cellular atypia. Fig. 4.3 compares normal hamster cheek pouch epithelium, normal human buccal mucosa and normal human hard palate.

The difference in the frequency with which drop shaped rete ridges were seen in hamster and human lesions may be related to the fact that the normal hamster cheek pouch epithelium does not show rete ridges. The different frequency of mitoses above the normal progenitor cell compartment may be explicable by consideration of the normal histology of the hamster pouch in which the progenitor cell compartment is restricted to the basal cell layer - defined as consisting of cells in contact with the basement membrane. It is thus easy to decide if a cell is in mitosis superficial to the normal progenitor cell compartment in the hamster. In human oral mucosa the progenitor cell compartment consists of several layers of cells and it is less easy to recognise whether or not an individual mitoses is at an abnormally superficial level in the epithelium.

The proportions of human and hamster cases showing increased mitotic activity were very similar, but a larger number of the human lesions were noted as showing a marked increase in activity and this difference was statistically significant. The assessment of mitotic activity was made subjectively without an actual count of mitoses being made. Also the decision as to whether or not an increase in mitotic activity was present was dependent on the observer's subjective concept of the normal frequency with which mitoses should be seen at the site being examined. These factors made the measurement of mitotic activity rather imprecise and it is doubtful if the difference shown by the values in Table 4.5 represents a real difference of the statistical significance shown.

There is a considerable difference in the size of human and hamster oral epithelial cells. The hamster cells are much smaller and have a higher nucleo-cytoplasmic ratio. This latter fact is probably at least part of the explanation for the greater increase recorded in the nucleo-cytoplasmic ratio in hamster atypia.

The greater degree of loss of intercellular adherence noted in human lesions was felt to be probably a real difference unrelated to any defect

in the technique of atypia scoring. It is interesting that this difference was still apparent in spite of the subjective impression of the frequency of the focal acantholysis with premature keratinization seen in the hamster lesions.

The similarity demonstrated between atypia in the hamster cheek pouch and human oral lesions is good evidence in support of the applicability of the results obtained in the hamster to man. This suggests that cellular atypia scoring should be a reliable prognostic indicator in human oral cellular atypia.

4.4.3 Histological Features of Some Human Oral Epithelial Lesions

The study reported in 4.3.4 was of limited extent and only a few cases were present in some of the diagnostic categories. The interpretations of the results must be made cautiously, although the larger number of cases in the carcinoma and hyperplasia groups allows more confidence to be placed in the validity of the findings relating to these groups.

The histological features which showed a significant positive correlation with the diagnostic group of carcinomas were those which most textbooks describe as features of oral squamous cell carcinomas. The high correlation of focal atrophy with carcinomas was interesting as this association was also suggested by

the sharp increase in the presence of this feature between the hamster biopsy and sacrifice specimens in the experiment recorded in 3.3.3.

The only features showing a significant positive correlation with the premalignant group of lesions were those relating to abnormal variations in cell size and nuclear size of the epithelial cells. This may represent the earliest indication of malignant potential.

4.5 CONCLUSIONS

The results discussed in this chapter confirm the importance of adequate sampling of biopsies being scored for cellular atypia and suggests that the subjective selection for scoring of the most severely affected areas is a valid procedure.

Despite the differences in experimental carcinogenesis in hamster cheek pouch and human oral lesions, as noted in 3.4.5 the analysis recorded in this chapter indicates the close similarity of the patterns of atypia in hamster and human lesions. This fact supports the use of this model system in the evaluation of premalignant oral lesions and the application of the results to the human situation.

EARLY REACTIONS TO CARCINOGEN APPLICATION TO THE INTRAORAL DRAINAGE AREA IN THE HAMSTER

5.1 INTRODUCTION

In man, approximately 70 per cent of cases of squamous cell carcinoma in the mouth occur within an area comprising only 20 per cent of the area of the oral mucosa (Moore and Catlin, 1967). These authors called this area the drainage area and it consists of the mucosa of, and immediately adjoining, the lingual sulcus and the floor of the mouth. Carcinogens present in the mouth which are carried in saliva are most likely to drain to that area and then proceed backwards to the pharynx. It seemed likely that this drainage area might also be at greater risk in the hamster and it was decided to investigate the early stages of the response of this area of epithelium to carcinogen applications.

As noted in 1.3.2 Wallenius, (1968) stressed the importance of the protective action of saliva during his studies of chemical carcinogenesis in the rat. It was therefore felt that further studies were required on areas of epithelium in the hamster mouth which were constantly moistened by saliva as opposed to the cheek pouch epithelium which is usually dry.

The experiment described in this chapter was also designed to discover the most suitable part of

the "drainage area" in the hamster mouth for further studies.

5.2 MATERIALS AND METHODS

5.2.1 Animals

The same strain of hamsters as was used in the studies recorded in Chapters 2 and 3 was used in this study. Ten male hamsters, eight weeks of age at the commencement of the experiment were used. These animals were housed two or three to a standard hamster cage and given standard laboratory chow and water ad libitum.

5.2.2 Experimental Techniques and Design

The carcinogen used was a 0.5 per cent solution of 7,12 dimethylbenz(a)anthracene (DMBA) in acetone and the solution was prepared as noted in 2.2.3. Applications of carcinogen solution were made after the animals had been lightly anaesthetised with ether and placed in the restrainer described in 2.2.2. The carcinogen was applied with a No. 3 camel's hair brush which had been dipped in the DMBA solution and wiped against the edge of the container before being introduced into the lower lingual sulcus and rotated against the lingual gingiva and lateral surface of the tongue. This procedure was carried out once on each side of the tongue.

The experimental design was that the hamsters should each receive three carcinogen applications per week for six weeks. At this time, it was planned that two animals would be sacrificed while the remainder of the animals received further carcinogen applications. It was planned, thereafter, to continue carcinogen applications and to sacrifice two animals each week until ten weeks from the start of the experiment. Unfortunately, two hamsters escaped during the course of the experiment and at weeks eight and ten, only one animal was sacrificed.

5.2.3 Laboratory Methods

After the period of carcinogen application, the hamsters were killed by ether inhalation and the heads removed and placed in 10 per cent buffered formol saline. After 24 to 48 hours fixation, the heads were removed from the fixative solution and the lower jaw was dissected from the remainder of the head by cutting through the vertical ramus of the mandible. The anterior portion of the tongue was removed by a coronal cut to the base of the lingual frenum. This part of tongue, about 6 to 8 mm in length was trimmed into two blocks and routinely processed for paraffin embedding.

The remainder of the lower jaw was then bisected anteroposteriorly through the symphysis and the two

halves of the jaw were decalcified. The decalcifying fluid employed was 4N formic acid (Brain, 1968) and the decalcification process was checked by X-raying the specimen. Decalcification was complete in four to five days.

After decalcification the anterior portions of the jaws with the incisor teeth were discarded and the remainder of the tissue including the whole lingual sulcus was divided by coronal sections into four or five tissue blocks for each side. After washing for 18 hours in running water, the tissue blocks were processed for paraffin embedding.

Two blocks of tissue were available from the anterior part of each hamster tongue. These blocks were sampled at four levels to provide representative sections of the whole area. A preliminary histological study was undertaken and the ventral mucosa was examined for the presence of five histological features which were recorded for each animal. These features were focal atrophy, acanthosis, hyperkeratosis, cellular atypia and inflammation in the epithelium and lamina propria. From each animal the section showing the most marked cellular atypia was selected for further study. In some cases more than one section was selected as subjectively it was difficult to decide which one showed the most marked degree of atypia.

The sections selected were then scored for cellular atypia using the Smith and Pindborg (1969) technique.

The decalcified blocks of lower jaw were cut in order to provide cross sections of the lingual sulcus at several points. These sections were examined by the same procedures as were described above for the ventral surface of the tongue. Separate observations were made of the lateral aspect of the tongue and of the lower lingual attached gingiva.

Histological sections of normal tissues for comparison were prepared from the retained heads of the control animals of the experiment recorded in Chapter 3.

5.3 RESULTS

5.3.1 Normal Control Animals

The distinction between the ventral epithelium and the lateral margins and dorsum of the tongue is quite clear cut (Fig. 5.1). Histologically, the ventral aspect of the tongue is distinguished by the lack of papillae and by a much more regular epithelium than is seen on the dorsum of the tongue. Fig. 5.2 reveals that the normal ventral surface epithelium of the tongue is orthokeratinized and the epithelium shows short rete ridges. The progenitor cell compartment involves the deepest two or three cell layers.

The epithelia of the lower lingual attached gingiva and lateral aspect of tongue in about the middle third of the tongue tend to be slightly thicker than the ventral surface of the tongue as is shown in Fig. 5.3, but the histological appearances are otherwise very similar.

5.3.2 Response to Carcinogen Exposure - Ventral Surface of Tongue

The results of the preliminary histological study on the ventral epithelium of the tongue are shown in Table 5.1. This study indicated that focal atrophy was not observed at this site, but that acanthosis was frequent although it was not until after nine weeks of carcinogen exposure that hyperkeratosis was seen.

The feature recorded as inflammation and which was present in all specimens was an infiltration of mononuclear cells in the lamina propria and deeper layers of the epithelium (Fig. 5.4). This was sometimes associated with the appearances of hydropic degeneration of the basal cells.

In the initial subjective assessment, cellular atypia was thought to be present in all specimens. The section showing the most severe cellular atypia was selected and scored for atypia using the Smith and Pindborg (1969) technique. The atypia scores are shown in Table 5.1. It is interesting that foci of

atypia were already present by six weeks. These foci showed irregular epithelial stratification, loss of intercellular adhesion and premature keratinization similar to that recorded in the cheek pouch (Fig. 5.5). Fig. 5.6 illustrates the atypia seen after nine and ten weeks of carcinogen application and it is clear that this closely resembles the findings in the hamster cheek pouch.

The atypia scores did not show any obvious correlation with the duration of carcinogen application. This was confirmed by the Spearman rank correlation coefficient (Siegel 1956) which is shown in Table 5.1. This yielded a value of $r = 0.4345$ and $P > 0.05$.

5.3.3 Response to Carcinogen Application on Lower Lingual Gingival Epithelium and Lateral Surface of Tongue

An assessment similar to that recorded in 5.3.2 for the ventral surface of the tongue was undertaken and the results are summarised in Table 5.2. It is clear from this table that the finding of acanthosis was slightly less frequent on the ventral surface of the tongue and hyperkeratosis was more frequent. The hyperkeratosis recorded was mainly seen as orthokeratosis, only one small focus of parakeratosis being observed. Inflammation in the form of a mononuclear cell infiltrate similar to that observed in the ventral surface of the tongue was present in most cases and was more marked on the gingival than on lingual

epithelium (Fig. 5.7).

An early papilloma was observed on the gingival epithelium of one animal after 10 weeks of carcinogen applications (Fig. 5.8).

Cellular atypia was less frequently observed in the gingiva and lateral surface of tongue than on the ventral surface of the tongue. 7 of the 16 specimens of gingiva and 9 of the 16 specimens of lateral surface of tongue showed cellular atypia. The most severe cellular atypia observed was noted on the lingual epithelium of animal 7 (Fig. 5.9).

5.3.4 Comparison of Atypia at Three Sites in the Drainage Area

The frequency of occurrence of cellular atypia at the three sites was first examined (Table 5.3). The gingiva and lateral surface of tongue were compared by a X^2 test of a 2 x 2 contingency table and the frequency of occurrence of atypia at these two sites was not significantly different.

Because of the fact that all the specimens of the ventral surface of tongue showed cellular atypia, one of the cells in a 2 x 2 contingency table showed a value of 0. In this instance a X^2 test was therefore inappropriate and a Fisher exact probability test was undertaken (Table 5.3). When the atypia frequency on the ventral surface of the tongue was compared to

that seen on the lingual gingiva and then to that seen on the lateral surface of the tongue values of $P < 0.01$ were found in each case. This indicated that the frequency of occurrence of cellular atypia in this study was significantly greater on the ventral surface of the tongue than at the other two sites.

The cellular atypia scores on the ventral aspect of the tongue appeared to be higher than at the other two sites. The atypia scores at the three sites were examined by Mann-Whitney U tests and these showed that the atypia scores of the specimens of the ventral surface of tongue were significantly higher than those of gingiva ($P=0.04$). No significant difference was noted between the atypia scores of ventral surface of tongue and lateral surface of tongue or between the lateral surface of the tongue and the lower lingual gingiva (Table 5.3).

5.4 DISCUSSION

The experiment reported in this chapter indicated that cellular atypia developed in the epithelium of the "drainage area" of the hamster mouth in response to the application of chemical carcinogen solution. Of the three areas studied the ventral surface of the tongue showed the highest incidence of cellular atypia and also the most severe degree of atypia was noted at this site. The only tumour observed in this

group of animals developed on the lower lingual gingiva.

Of the three sites studied, the ventral surface of the tongue was the most easily visualised and was also the easiest site upon which to conduct experimental work. It had the further advantage that it was not closely related to bone and therefore sections of tissue, in their natural relationships, could be prepared without the requirement for decalcification.

5.5 CONCLUSIONS

It was concluded that the "drainage area" of the hamster mouth is susceptible to the action of chemical carcinogens. The most favourable area of the "drainage area" for further investigations on cellular atypia would appear to be the ventral aspect of the tongue.

STEREOLOGICAL STUDIES OF SOME ASPECTS OF THE RESPONSE OF HAMSTER TONGUE EPITHELIUM TO CARCINOGEN

6.1 INTRODUCTION

6.1.1 General Considerations

The histological studies of the oral epithelial response to carcinogen application reported in the preceding four chapters have all used very crude systems of measurement. A better understanding of the tissue changes involved requires more sensitive and accurate measuring techniques and as indicated in Chapter 1 morphometry and in particular stereology can provide such methods.

It was decided to examine the possibility of applying stereological methods to study some of the early changes of chemical carcinogenesis. The derivation of reliable and accurate stereological data is dependent on precise technique and upon fulfilling the several assumptions which the stereological methods require. Much of this chapter will be concerned with the development of techniques to satisfy the requirements of these assumptions.

Several of the changes studied previously in the scoring of cellular atypia related to the basal cells of the epithelium and it was decided that the stereological analysis should be mainly concerned with these cells. It was decided to investigate the

following features; basal cell hyperplasia (here defined more accurately as increase in the volume of the progenitor cell compartment), nucleo-cytoplasmic ratio of basal cells and the junctions between basal cells and the basement membrane. The first two of these features form part of the group of changes constituting cellular atypia and the inter-relationship of epithelium and basement membrane is clearly important in connection with invasion of malignant neoplasms. Tarin (1968) reported that the numbers of hemidesmosomes appeared decreased in experimental carcinogenesis of mouse skin and Frithiof (1972) reported that human oral squamous cell carcinomas showed a marked reduction in the number of hemidesmosomes.

As with the experiments recorded in Chapter 3 it was desirable to use a specified site which could be uniformly exposed to carcinogen, then biopsied and the remainder of the treated area followed for a period to allow examination of the further development of the response to carcinogen.

6.1.2 Stereological Methods: Assumptions and Requirements

The requirements for obtaining reliable stereologic data can be considered in three categories. These are sampling, identification of structures being measured and assumptions relating to specific measuring techniques.

Sampling is important because stereological methods make the assumption that the areas used for the counting procedures are a random sample of the tissue being studied. The requirements of the random sampling must be considered at all levels of the investigation from the selection of animals to the precise area of an electron microscope section to be photographed for further counting. The sampling technique must also be considered in relation to the particular structure of the tissue under study. The important point in this regard is the type of distribution of components within the tissue. Oral epithelium is an anisotropic tissue. That is, the components of the tissue, the cells, have a particular structured inter-relationship which, in oral epithelium, results in the formation of strata of cells. Stereological analysis of a tissue such as liver is usually undertaken on randomly orientated sections. With anisotropic tissues it is generally preferable to use precisely orientated sections which are prepared to include the axis of anisotrophy in the plane of the section (Weibel, 1969). With oral epithelium this entails the preparation of sections cut perpendicular to the keratinized surface. A further point about section orientation is that if the study relates to changes in individual cell layers, then clearly accurate identification of the

cell layers is mandatory and in oral epithelium it is frequently impossible to distinguish between basal cells and stratum spinosum cells in randomly orientated sections.

Two important factors of experimental design relate to the identification of structures. The first is the establishing of criteria for the accurate delineation of structures to be quantitated. The second factor is that the sample size in terms of the volume of tissue studied should be large enough to be representative of the whole tissue. For reasons of economy of time and effort it is thus important to use the lowest magnification possible, consistent with a high enough resolution, to allow accurate identification of structures being measured.

Just as consideration must be given to anisotropy in the tissue, so it is important to consider possible anisotropy in the measuring system used. Clearly a system of parallel lines such as is used for intercept point counting is highly anisotropic and the technique of using such a system must take account of this fact.

6.2 MATERIALS AND METHODS

6.2.1 Animals

As the initial experimental procedures described in this chapter were undertaken while the author was

in America it was not feasible to use the same animals as in the previous experiments. An alternative line-bred strain of Syrian golden hamsters was obtained and 30 male hamsters, 7 weeks of age, were purchased. Each hamster was individually marked on the ears for identification and the animals were housed two or three to a standard hamster cage. The animals were given standard laboratory chow and water ad libitum and this was supplemented once or twice a week with fresh carrots.

6.2.2 Experimental Site

It was decided to use a truly intra-oral site in this study and as indicated in the experiments recorded in Chapter 5, the best site to use was felt to be the ventral surface of the tongue.

Fig. 6.1 demonstrates the sharp demarcation between the lateral and anterior margins of tongue bearing papillae and the thinner lining epithelium of the ventral surface. It was desirable that a standard area of the ventral epithelium be used for this study and an area 4 mm square, equally distributed across the midline, was selected. This area was delineated by five tattooed points located with the aid of the modified cover slip forceps shown in Fig. 6.2. Tattooing was performed by dipping a 30 gauge needle in an Indian ink solution and pricking this needle

through the epithelium into the connective tissue and muscle at the required sites. The presence of the tattooed points (Fig. 6.1) ensured that carcinogen applications and subsequent surgical procedures were always applied to the same site.

6.2.3 Carcinogen Application

The application of a DMBA in acetone solution to the ventral surface of the tongue was found to be less easy than to the cheek pouch because of the greater mobility of the tongue. Also it was felt that carcinogen spread would be much less easily controlled on the tongue which is a site constantly washed with saliva.

Mesrobian and Shklar (1969) used butylcyanoacrylate and DMBA in the induction of palatal tumours in hamsters. Their technique was to apply carcinogen powder and immediately cover this with the butylcyanoacrylate which set to give a protective covering over the applied carcinogen. At the time the experiment reported in this chapter was being undertaken, supplies of cyanoacrylate tissue adhesives were difficult to obtain. Commercially available intraoral bandage was, however, readily obtained. This was Squibb intraoral adhesive bandage (Orahesive) and it was decided to apply the carcinogen powder and retain it in position with this bandage. This technique

offered the additional advantage that if the carcinogen were to be actually applied on the bandage, then this bandage could be cut to a desired size and the carcinogen then restricted to a precise area.

The actual technique used was that suitably anaethetised animals were placed in the restrainer described in 3.2.3. A 4 mm square piece of intra-oral bandage was prepared and this was dusted lightly on the sticky surface with the carcinogen 9,10 dimethyl 1,2 benzanthracene (DMBA) in pure powder form. The ventral surface of the tongue was then dried and the bandage with the DMBA was applied to the experimental area, demarcated by the tattoo marks.

6.2.4 Anaesthesia

It was found that the hamsters were able to remove the intraoral bandage from the tongue fairly readily on recovery from the anaesthetic. It was therefore decided that in order to give the carcinogen a reasonable period of contact with the epithelium, it would be necessary to keep the treated animals anaesthetised for that period. Anaesthesia for a minimum of one hour was considered desirable.

Anaesthesia was obtained by intraperitoneal injection of veterinary Nembutal prepared as described in 3.3.3. This solution of pentobarbitone sodium in alcohol was given at the recommended dosage of 1 ml

per 100 g body weight and produced anaesthesia for periods in excess of 30 minutes. Attempts to prolong the anaesthesia by increased initial dosage resulted in the death of some animals under the anaesthetic during the course of the experiment. This occurred with eight animals in the experimental group. It was found much safer to observe the animals after anaesthetising with a small dosage and to give repeated small doses of anaesthetic intraperitoneally if the animal was recovering within a period of 1 hour after the carcinogen application. Precautions were taken to keep the animals warm and in a draught free area until recovery from the anaesthetic.

6.2.5 Experimental Design

The animals were divided into experimental and control groups. The experimental group consisted initially of 20 animals. Unfortunately the eight anaesthetic deaths occurred in this group and a further three animals died from unexplained causes during the course of the experiment. The experimental group was therefore reduced to nine animals.

The control group consisted of ten animals. Three of these animals were used in studies of the tattooing technique and biopsy techniques. Three animals were studied as untreated controls and the remaining four animals were sham treated controls.

These sham treated controls were operated upon in a manner identical to that of the experimental animals except that no carcinogen powder was applied to the intraoral adhesive bandage.

It was desirable to make carcinogen applications over a period of several weeks, then biopsy the treated area and later sacrifice the animals after a further period of observation. Because of the difficulties experienced with the anaesthetic technique it was decided to restrict carcinogen applications to once per week. Carcinogen applications were thus made in the manner described in 6.2.3, once per week for ten weeks. The animals were then observed for periods of between eight and nine weeks after which a biopsy of the treated area was obtained. The animals were observed for a further six weeks after which time they were sacrificed. It would have been preferable to have prolonged the period of observation following the biopsy before sacrificing the animals. The early sacrifice was necessitated by the fact that the author was nearing the termination of his visiting appointment in the University of Illinois.

6.2.6 Biopsy Technique

The obtaining of a suitable biopsy from the ventral surface of the tongue proved to be more difficult than was the case with the cheek pouch. A

major cause of this difficulty was in immobilising the tongue. The solution adopted finally was to anaesthetise the hamster with Nembutal using 0.6-0.7 mg per 100 ml body weight of the anaesthetic solution described in 3.3.3. The animal was then placed in the restrainer and two sutures were placed through the tip of the tongue and secured to a retort stand. The sutures were adjusted so that the tongue was held vertically under slight tension. With the tongue thus supported, the operator had both hands free to undertake the biopsy.

The biopsy consisted of half of the experimental area of 4 sq. mm and was a strip 4 x 2 mm on the left half of the tongue. This was obtained by steadying the tip of the tongue with forceps and making three incisions with a scalpel in the midline, the tip and the lateral margin of the tongue through the appropriate tattooed points. These incisions were joined to each other and by the use of a fine pair of scissors a mucosal flap was freed from the tongue. One corner of the biopsy towards the tip of the tongue was held in fine toothed forceps and the scissor cuts were made parallel to the epithelial surface to give a flap 1 to 1.5 mm thick. When the flap was 4 mm long a scissor cut at the base of the tongue freed the biopsy.

This technique had several advantages. The even tensioning of the tongue by the sutures greatly facilitated the initial incisions and the scissor cuts. After the biopsy was freed by the scissor cuts it reduced in length and it was felt that provided the initial stretching of the tongue by the sutures was not excessive, then the biopsy specimens would probably all contract a consistent amount.

Because the tongue was under tension, relatively little haemorrhage occurred initially. After the biopsy was completed and the sutures were removed, haemorrhage did occur. It was found that such haemorrhage was impossible to control if the biopsy was too deep and had involved the larger vessels shown in Fig. 6.3. One of the control animals had to be sacrificed because of profuse haemorrhage after the biopsy. An important point regarding this haemorrhage was that it appeared less severe if the animal recovered quickly from the anaesthetic. Accordingly the anaesthetic dosage was reduced as far as practicable. The postoperative healing in all animals except the one sacrificed because of haemorrhage, was rapid and uneventful.

6.2.7 Specimens Obtained at Sacrifice

In order that the specimens of the experimental area obtained at sacrifice should be comparable with those obtained at biopsy the procedure detailed in 6.2.6 was repeated. Firstly a 4 x 2 mm strip of mucosa was taken from the right side of the tongue. This represented the remaining half of the original experimental area. A similar strip was then removed from the left side of the tongue and this was the healed site of the biopsy. **Examination of this tissue will form part of a future study not reported in this thesis.**

After these specimens were obtained, the animals were not allowed to recover but were sacrificed with an overdose of Nembutal. All animals were given a post-mortem examination, but no pathological changes of note were found in any organs other than the tongue.

6.2.8 Laboratory Procedures - Fixation and Embedding of Tissues

It was decided that both light and electron microscopic studies should be conducted on plastic embedded material. The detailed procedure adopted for the biopsy and final specimens is shown diagrammatically in Fig. 6.4. The fresh biopsy specimen was laid on dental wax and cut into four equal sized strips 2 x 1 mm in surface area. The anterior two strips were fixed in gluteraldehyde and the posterior two strips were fixed in osmium. After primary fixation, when the aldehyde fixed tissues were in

buffer further trimming of each strip of mucosa was undertaken to obtain from each, four blocks measuring 1 x 0.5 mm in epithelial surface area. The blocks were trimmed such that the long axis of each block lay either in the long axis or the short axis of the tongue. These blocks were then post fixed in an osmium tetroxide solution. The tissues fixed primarily in the osmium tetroxide solution were processed to the stage of 95 per cent alcohol at which stage they were trimmed in a manner similar to that indicated above for the aldehyde fixed tissue. The details of the fixative solutions and processing cycle are shown in Appendix 2.

All tissue blocks were processed in araldite and flat embedded in latex moulds of the type illustrated in Fig. 6.5. The previous careful trimming of the blocks enabled the epithelial surface to be accurately located in the centre of the block face with the plane of the epithelial surface at or nearly at right angles to the block face. The tissues were embedded such that the block face represented a section either directly along or across the tongue.

6.2.9 Section Preparation

The stages in the preparation of light microscopic and electron microscopic sections from the araldite blocks are shown in Fig. 6.6.

The araldite blocks were initially trimmed by hand with razor blades. The blocks were then mounted in the chuck of either a Sorvall MT2B or a Cambridge Huxley ultromicrotome with the epithelial surface vertically orientated. In this position the angulation of the keratinized surface to the block face could be readily seen. The glass knife in the ultromicrotome was then set at right angles to the keratinized surface of the epithelium and the block face trimmed to give the required plane of section (as discussed in 6.1.2) perpendicular to the keratinized surface.

The block face was then reduced in size to include full thickness of the epithelium and a small thickness of lamina propria and muscle. The long sides of this block were trimmed back at an angle of 30 degrees such that the edges of the block face were exactly parallel. The block was then turned through 90^{o} in order that the keratinized surface was parallel to the knife edge and would also be the first part of the tissue to meet the knife. The angulation of the block was carefully adjusted to ensure that the previously trimmed plane of the block face remained the plane of section.

Sections were cut with glass knives, at 0.5 µm to 0.6 µm for light microscopy. These sections were transferred from the water bath to a clean glass slide with the aid of a wire loop. The sections, on a drop of water on the slide, were flattened by holding a brush which had been dipped in chloroform close to the water surface. Flattening was completed by placing the glass slides on a hotplate at 70°C. When the water had fully evaporated the sections adhered to the glass. The sections were then stained by the technique of Aparicio and Marsden (1969). This method uses a methylene blue and borax stain with a basic fuchsin counterstain. In many instances the counterstain was omitted as the detailed structure of the epithelium could be seen better with the blue stain alone.

For electron microscopy the block face was reduced by cutting back about half of the length of the epithelial surface by 40 µm. Thin sections in the silver to lemon yellow interference range were then cut with glass knives from the remaining part of the block face. Short ribbons of sections were mounted upon copper grids with rectangular slots. (LKB pattern 4829A-8). Fig. 6.7 illustrates the manner in which the section was laid on the grid such that the rectangular slot ran through the thickness of the epithelium at right angles to the surface. Initially

large blocks such as illustrated in Fig. 6.7 were used, but subsequently blocks which provided sections covering only three grid spaces were found to give superior results. Where possible, uncoated grids were utilised, but some specimens proved rather fragile and in these instances formvar coated grids stabilised with carbon were utilised.

Sections for electron microscopy were stained for 15 to 20 minutes in a saturated aqueous solution of uranyl acetate followed by 4 minutes in lead citrate (Reynolds, 1963). Sections were examined in an A.E.I. EM6B electron microscope at 60 Kv.

6.2.10 Sampling Procedures for Stereologic Study

The stereologic studies were undertaken on four control and two experimental groups. The four control groups were the osmium fixed untreated specimens in the long axis of the tongue and in the short axis of the tongue and corresponding sham treated specimens. The two experimental groups were the osmium fixed biopsy and sacrifice specimens in the short axis of the tongue. The sacrifice specimens were those from the remaining half of the original treated area (6.2.7). The osmium fixed tissues were chosen because of the greater clarity with which membranes were seen in osmium as opposed to gluteraldehyde fixed material.

The numbers of specimens were as follows:-

Untreated controls - Long axis 3

Four blocks of tissue from each specimen were processed and arbitrarily numbered from 1 to 4. Block number 1 from each specimen was then examined and, if the orientation and tissue preservation were satisfactory, the sections for detailed study were prepared from this block. If the first block was found to be unsuitable the further blocks were examined in numerical sequence until one showing satisfactory orientation and tissue preservation was obtained.

The selection of the area to be photographed was made by examining the slide on the microscope with a x 10 objective, orientating the slide such that the epithelial surface was parallel to the long axis of the photographic negative and centering the slide on the ground glass viewing screen of the photomicrograph attachment. The objective was then changed to a x 40 and the photomicrograph was obtained without further adjusting the position of the slide.

Photographs of the entire area of basal cells between two adjacent sets of grid bars were prepared. This required two sets of four negatives to cover the required areas. Fig. 6.9 illustrates the sampling

procedures. Prints from negatives at 5,000 times were enlarged to 12,500 times and these allowed nuclear and cytoplasmic boundaries to be adequately distinguished (Fig. 6.10).

The higher power ultrastructural study of the basement membrane area was made on four prints from each specimen. The same areas sampled for the low power electron microscopic study were used and the four areas of basement membrane adjacent to the grid bars were photographed at a magnification of 20,000 times (Fig. 6.9). Prints were prepared at a final magnification of 50,000 times (Fig. 6.11).

In preliminary studies all prints were prepared using a Kodak Autoprocessor in order to avoid possible print distortion due to prolonged wetting of the paper during processing and washing. There was some loss of clarity and resolution in these prints however, and dish developed prints were preferable. Detailed measurements indicated that the distortion in these prints was very small and it was concluded that it would have little effect on the present studies.

6.2.11 Stereologic Techniques for Light Microscopy (Level 1)

Quantitation of possible basal cell hyperplasia in carcinogen treated tissues was required. This involved calculation of the volume densities of progenitor cells. In addition, the volume densities

of the stratum spinosum and granulosum cells and the stratum corneum were assessed. The progenitor cells were distinguished by their relatively small volume of cytoplasm and the cell morphology. The progenitor cells were further divided into two groups. These were basal cells, defined as those actually seen to be in contact with the basement membrane in the sections studied and those not in contact with basement membrane.

The distinction between progenitor cells and stratum spinosum cells was in most cases clear cut, but sometimes intermediate cells were present. Cells which on section had a length, as measured in the axis parallel to the epithelial surface, of more than twice the vertical dimension of the cell, the measurement at right angles to the epithelial surface, were designated as stratum spinosum cells.

The counting procedures were undertaken on photographic prints of the thick sections described in 6.2.10. Point counting to establish volume densities was made with a grid of parallel lines forming 1 cm squares. This grid was scratched on a clear acrylic sheet which was laid over the print, with the scratched lines in contact with the print, and lit obliquely with a lamp in order that the internal reflection made the scratches show as fine bright lines.

A grid of this nature may have the same periodicity as some tissue constituents and it is also anisotropic. The problems of anisotropy and periodicity were overcome by sellotaping the print lightly to a board on which three lines at 60° angles were drawn. Three point counts were then made with the grid orientated along each of the lines at 60° angles.

6.2.12 Stereologic Procedures for Ultrastructural Study (Levels 2 & 3)

The nucleo-cytoplasmic ratio was obtained by a point counting method using a grid of parallel lines forming 3 cm squares. This grid was prepared and used in the same manner as that described in 6.2.11. This provided a differential point count and therefore volume densities of nuclei and cytoplasm of basal cells.

It was felt that a measure of the irregularity of the basement membrane zone would also be useful. Accordingly an intercept point count of the lamina densa was made with a grid of parallel lines at 1 cm intervals prepared and used in a manner similar to that described in 6.2.11. The intercepts counted were where the grid lines crossed the connective tissue side of the lamina densa.

The actual length of basement membrane studied in each section was obtained using the formula derived from the solution of the Buffon Needle Problem

(Buffon, 1777, quoted by Weibel, 1968). This formula allows the calculation of the boundary length of a curve B by intercept point counts using a grid of parallel lines equally spaced apart. The length of the curve B is a function of the number of intercept points and the distance between the lines. The equation used is $B = \frac{\pi}{2} \times I \times d$, where I is the intercept point count and d is the distance between the parallel lines. This equation was modified to $L_{ld} = \frac{\pi}{2} \times \frac{I_{ld} \times 10,000}{3 \times 12,500}$ where L_{ld} equals length of the lamina densa and I_{ld} is the count of intercept points with the connective tissue side of the lamina densa. The distance between the grid lines was 1 cm or 10,000 µm. The result was divided by 3 because three intercept point counts for each print were made to allow for anisotropy and by 12,500, this being the print magnification. This formula reduced in this instance to $L_{ld} = 0.419 \times I_{ld}$. The result was expressed in µm of lamina densa.

The area of basal cells in each set of prints was calculated. Since the grid consisted of 3 cm squares each point represented 9 cm^2. This represented an area of 5.76 μm^2 of the tissue sample. The area of basal cells in each print was calculated as $A_{bc} = P_{bc} \times \frac{5.76}{3} \mu m^2$. The division by 3 was required because 3 intercept point counts were made

to allow for anisotropy. It was then possible to relate the basement membrane length in each series of prints to the area of basal cells in these prints.

The technique of intercept point counting was also applied to the higher magnification prints (level 3). Intercept point counts were made of hemidesmosomes and free basal plasma membrane to allow the calculation of the proportion of the basal plasma membrane involved in hemidesmosomes.

Most hemidesmosomes were readily distinguished, but it was felt important to establish criteria for the delineation of some structures which were less clearly defined. In order for an area of basal plasma membrane to be counted as a hemidesmosome the following conditions had to be satisfied:-

1. A distinct lamina densa, lamina lucida and basal plasma membrane had to be seen.
2. A discrete thickening of the basal plasma membrane had to be present.
3. This discrete thickening had to be at least 3 times as thick as the adjacent basal plasma membrane.

Structures fulfilling these conditions were designated as hemidesmosomes and the entire remainder of the basal plasma membrane was noted as "free basal plasma membrane". This procedure ensured that the designation of these structures was consistent throughout the study, but it is probable that it resulted in a slight underestimate of the proportion of basal plasma membrane involved in hemidesmosomes.

The intercept point count of the basal plasma membrane of the basal cells was used to calculate the length of basal plasma membrane in individual prints using the previously detailed equation $L_{pm} = \frac{\pi}{2} \times I_{pm} \times \frac{10,000}{3 \times 50,000}$. In this case L_{pm} was the length of the basal plasma membrane and I_{pm} the intercept point count. The magnification of the prints in this case was 50,000.

From the previous calculation of the proportion of basal plasma membrane occupied by hemidesmosomes it was then possible to calculate the actual length of hemidesmosomes present in each print. This was a measure of the sum of the lengths of the individual hemidesmosomes.

In each print studied a count of the total number of hemidesmosomes, N_{hd}, was made. The mean profile length of hemidesmosomes was then calculated

as $\frac{L_{hd}}{N_{hd}}$, the result being in μm. The hemidesmosome frequency was calculated as $\frac{N_{hd}}{L_{pm}}$ and this expressed the number of hemidesmosomes per μm of basal plasma membrane.

6.3 RESULTS

6.3.1 General Considerations

The study described in this chapter concerns the accurate evaluation of specific features of normal and carcinogen treated epithelium. No attempt was made to undertake a full subjective assessment of the changes in the carcinogen treated tissues. However, a few general observations were made during the course of the sampling for the stereologic study and it is pertinent to note these before examining the results of the detailed study.

At the light microscopic level (level 1) the subjective impression was gained that the epithelia in the carcinogen treated groups were thinner than in the controls. Cellular atypia was not observed in any of the carcinogen treated specimens and in particular basal cell hyperplasia was not noted.

At the low power ultrastructural level (level 2) the main difference noted between the normal and carcinogen treated specimens was that the intercellular spaces between basal cells were greater in the

carcinogen treated tissue. Occasional structures suggestive of the pseudopodia described by Woods and Smith (1969) were observed but these were very infrequent (Fig. 6.12). Woods and Smith (1969) felt that such pseudopodia were an indication of premalignancy.

The higher power ultrastructural investigation revealed changes in the lamina densa of the basement membrane in the carcinogen treated specimens. These showed a greater than normal variation in the thickness of the lamina densa and gaps and areas of reduplication were also noted (Fig. 6.13). Prior to the stereologic study no overall difference in hemidesmosomes was noted between the carcinogen and the normal specimens.

6.3.2 Normal and Sham Treated Controls - Level 1

The results of the light microscopic stereologic study (level 1) on the normal and sham treated controls at the time of biopsy are summarised in Table 6.1. The values shown in this table are the point counts of the various cell layers. These are proportional to the area densities and the volume densities of the different cell layers in the two different axes of the tongue studied. In addition, the ratios of the progenitor cell compartment and the basal cells to the total thickness of the epithelium and the proportion

of the progenitor cell compartment formed by basal cells are shown.

Comparisons of the means and standard deviations were made by "t" tests. The normal control sections in the short axis of the tongue did not differ significantly from the normal control sections in the long axis of the tongue with respect to the volume densities of any of the cell layers or in the proportions of the epithelium formed by different cell compartments.

Similarly no significant differences were noted between the sham treated control specimens cut in the long axis of the tongue and those cut in the short axis. When the normal short axis sections were compared to the sham treated short axis specimens, no significant differences were demonstrable. However, the normal long axis sections showed a significantly greater volume density of basal cells ($P < 0.01$) than the sham treated controls in the long axis. No significant differences were noted between these specimens in the other parameters studied.

The reasons for the greater volume density of the basal cell compartment recorded in the preceding paragraph were not clear, but as this was the only difference between the sham treated and normal controls

and the total numbers in the groups were small it was felt reasonable to combine the results of the normal controls and the sham treated controls. The combined values of the control groups cut on the short axis of the tongue were compared with the combined values of the controls in the long axis and proved not to be significantly different.

6.3.3 Carcinogen Treated Specimens - Level 1

Since the values of the normal and sham treated control sections in the short axis of the tongue were not significantly different, they were combined to give a group of sections from 7 animals for comparison with the 9 animals from which biopsies and later sacrifice specimens of the carcinogen treated area were available (Table 6.2).

When the control sections were compared with the carcinogen treated tissue obtained at biopsy, no significant differences were noted in the overall thickness of the epithelium or in the sizes of the keratinized, maturation or progenitor cell compartments. The progenitor cell compartment was larger in the carcinogen treated material, but this difference was not regarded as significant. The volume density of basal cells was significantly greater in the carcinogen biopsy specimens ($P = 0.032$) and this was reflected in the larger proportion of the whole

epithelium formed by basal cells (P = 0.01) and the greater proportion of the progenitor cell compartment formed by basal cells (P = 0.008). Since the volume densities of the progenitor cell compartments in the control and carcinogen biopsy specimens did not differ significantly, it was felt that the explanation for the greater proportion of basal cells was possibly that the length and irregularity of the basement membrane had increased causing a greater proportion of the progenitor cell compartment to be counted as basal cells.

When the control sections were compared to the carcinogen treated material obtained at sacrifice the carcinogen treated material was found to show a thinner epithelium (P = 0.001). This was due to a reduction in the volume density of the maturation compartment (P = 0.002) and the progenitor cell compartment (P = 0.0005). The volume densities of the keratinized compartment and the basal cells did not differ significantly. Although the carcinogen treated material showed a thinner epithelium the proportions of this epithelium formed by the progenitor cell compartment and by basal cells did not differ from the corresponding proportions found in the control material. The proportion of the progenitor cell compartment formed by basal cells was significantly greater in the

carcinogen treated tissue (P = 0.005) and as in the case of the biopsy material this was interpreted as suggestive of an increase in basement membrane length and complexity in the carcinogen treated specimens.

The carcinogen treated biopsy specimens were compared with the tissue obtained at sacrifice of the animals six weeks after the biopsy specimens were taken. The sacrifice specimens showed significantly thinner epithelium (P = 0.011). The volume densities of the keratinized compartments did not differ, but the volume densities of the maturation and progenitor cell compartments and basal cells were all significantly less in the sacrifice specimens (P values of 0.034, 0.001 and 0.006 respectively). The proportion of the epithelium formed by progenitor cells was less in the sacrifice material (P = 0.048) but the ratios of the basal cells to total epithelium and to the progenitor cell compartment did not differ in the biopsy and sacrifice material.

6.3.4 Normal and Sham Treated Controls - Level 2

Table 6.3 shows the values of the point counts of nuclei and cytoplasm and the intercept point counts of the lamina densa in the normal control sections cut in the short axis of the tongue. The point count for each individual animal is the sum of the counts obtained on the two areas of basal cells studied. From

these basic point counts the values for the nucleo-cytoplasmic ratio and the area of basal cells per unit length of basement membrane were obtained as detailed in 6.2.12.

Similar calculations to those described in the preceding paragraph were performed for the other groups of animals and the means and standard deviations for the normal and sham treated control groups are shown in Table 6.4.

The nucleo-cytoplasmic ratio of basal cells in the normal controls in the short axis of the tongue did not differ significantly from that of the sections of normal controls in the long axis of the tongue or sections of the sham treated controls in the short axis of the tongue. The standard deviation of the normal control group in the short axis of the tongue was large and the sample size was small. These factors may account for the lack of significant difference between the groups. The normal control sections in the long axis of the tongue had a significantly lower nucleo-cytoplasmic ratio than corresponding sham treated controls. When the combined control group of the normal and sham treated specimens in the short axis of the tongue was compared to the corresponding combined groups in the long axis no difference in the nucleo-cytoplasmic ratios was noted.

Comparisons of the area of basal cells per unit length of basement membrane in the prints of normal and sham treated controls revealed no significant difference. However, when the combined normal and sham treated groups in the short axis of the tongue were compared with combined normal and sham treated groups in the long axis a significant difference was apparent. The sections in the long axis showed a larger area of basal cells per unit length of basement membrane. Comparison of the values for A_{bc} and L_{ld} suggested that this difference was due to a larger value in the area of basal cells rather than a change in the length of the basement membrane.

6.3.5 Carcinogen Treated Specimens in the Short Axis of the Tongue - Level 2

The findings at the time of biopsy and of sacrifice are shown in Table 6.5 and are compared with the findings of the combined normal and sham treated controls. No difference in nucleo-cytoplasmic ratio was found between the control and carcinogen treated epithelia.

When the area of basal cells per unit length of basement membrane was studied an increased value was found in the biopsy group and a decrease was noted in the sacrifice group. These values did not, however, differ significantly from those of the control group. Comparison of the biopsy and sacrifice groups showed

that the decrease in the area of basal cells per unit length of basement membrane was significant (P = 0.001) and evaluation of the A_{bc} and L_{ld} values suggested that the result was due to a combination of a reduced area of basal cells and an increased length of basement membrane in the sacrifice group as opposed to the biopsy group.

6.3.6 Normal and Sham Treated Controls - Level 3

The results of the high power stereologic analysis are summarised in Table 6.6. The value N indicates the number of electron micrograph prints studied in each group. The values in the column HD% indicate the proportion of the basal plasma membrane of basal cells involved in hemidesmosomes, expressed as a percentage. No significant difference in this percentage was found between the normal and sham treated controls in the short axis of the tongue or between the normal and sham treated controls in the long axis of the tongue. Similarly no significant difference was noticed between the combined normal and sham treated specimens in the short axis and the corresponding combined group in the long axis of the tongue.

The results of the analysis of mean lengths of hemidesmosomes revealed no differences between the normal and corresponding sham treated controls. A difference was observed when sections in the short

axis of the tongue were compared with those in the long axis. The mean hemidesmosome profile lengths as visualised in sections cut in the long axis of the tongue were greater than those in the short axis. With the combined normal and sham treated controls the difference was significant with a P value of 0.017.

The frequency of hemidesmosomes per unit length of basal cell basal plasma membrane did not differ between the normal controls and corresponding sham treated controls. The sections in the short axis showed a higher frequency of hemidesmosomes than sections in the longer axis. In the comparison of the combined normal and sham treated controls in the two axes of the tongue this difference was significant ($P = 0.008$).

6.3.7 Carcinogen Treated Epithelium at Biopsy and Sacrifice - Level 3

The results of the quantitation of hemidesmosomes in the carcinogen treated epithelium are summarised in Table 6.7. The proportions of basal plasma membrane involved in hemidesmosomes did not differ between the combined control group and the carcinogen treated groups.

The mean length of hemidesmosome profiles and the hemidesmosome frequency were found not to differ in control and carcinogen biopsy material cut in the short axis of the tongue.

When the carcinogen sacrifice sections in the short axis of the tongue were compared to the corresponding controls, the sacrifice specimens showed significantly longer hemidesmosome profiles ($P = 0.001$), and a lower hemidesmosome frequency ($P = 0.00002$).

Comparison was made between the carcinogen biopsy specimens and the carcinogen sacrifice specimens. The sacrifice specimens showed significantly longer hemidesmosome profiles ($P = 0.032$) with a greatly reduced frequency ($P = 0.000002$).

6.4 DISCUSSION

6.4.1 General Considerations

The stereologic studies were conducted in three parts. Firstly a comparison was made of the normal and sham treated control sections. Secondly, the control sections in the two axes of the tongue at right angles to each other were compared and thirdly, the control and experimental treated sections in one axis of the tongue were compared.

6.4.2 Comparison of Normal and Sham Treated Controls

At the light microscopic level no overall difference in the normal and sham treated controls was observed. It therefore was reasonable to combine the values of these two separate groups to form a larger group for comparison with the carcinogen treated material. The importance of the finding of

a significant difference in the relative volumes of the basal cells between the normal controls and the sham treated controls in the long axis of the tongue is not clear. This was an unexpected result and may have been caused by deficiencies in the measuring technique due possibly to the relatively poor resolution of such high power light microscopic pictures, rather than being representative of a real difference between the specimens.

In the low power ultrastructural study (level 2) the nucleo-cytoplasmic ratio of basal cells in the normal short axis sections did not differ significantly from that in the corresponding sham treated controls. It was therefore felt to be a valid procedure to combine the results of these two groups for comparison with the carcinogen treated groups in the short axis of the tongue.

The difference in the nucleo-cytoplasmic ratios of the basal cells in the normal sections cut in the long axis of the tongue and the corresponding sham treated controls although it was of a low level significance does require explanation. In contrast to the normal and sham treated controls in the short axis, the values for the nucleo-cytoplasmic ratios in the long axis showed smaller standard deviations. While the observed difference may be a real one

between the normal and sham treated specimens the large variability of the measurements suggests that the basal cells studied may not have been derived from a uniform population. The progenitor cell compartment of an epithelium does not form a homogeneous compartment, being comprised of cells destined to migrate to the surface and also of cells which will remain in the progenitor cell compartment (MacDonald, 1971 a). MacKenzie (1970) indicated that basal cells in epidermis form functional units with cells at the periphery of the units remaining for further division and those in the centre of the unit migrating to the more superficial layers. It seems probable that this organisation of functional units also applies to the basal cells of the epithelium in this study and if this is so it is reasonable to expect that these cells with different functions will show morphological differences. Further study on a larger sample is required to evaluate this possibility.

The comparison of the area of basal cells supported by a unit length of basement membrane showed no difference between the normal and the sham treated controls. This suggests that there was no difference in the degree of folding or complexity of the basement membrane between these two groups of controls.

In the high powered ultrastructural study no differences in hemidesmosomes were noted between the normal and sham treated controls cut in the short axis of the tongue. Similarly no differences in hemidesmosomes were noted between the normal and sham treated controls cut in the long axis of the tongue.

In summary, no differences were observed in any of the main parameters studied at the three levels of investigation between the normal and sham treated control sections prepared in the short axis of the tongue. Minor differences were observed between the sections of normal and sham treated controls cut in the long axis of the tongue, but it was not clear whether these were due to faults in technique or to lack of homogeneity of the material studied. Comparisons of the control and carcinogen treated materials were therefore better made using the sections prepared in the short axis of the tongue.

6.4.3 Comparison of Control Sections Cut in the Short Axis and the Long Axis of the Tongue

At the light microscopic level no significant difference was observed between sections cut in the two axes of the tongue other than the single difference discussed in 6.4.2. It would be surprising if the results had been otherwise.

At the low power electron microscopic level (level 2) the nucleo-cytoplasmic ratio did not differ in the two axes studied. The difference in the area of basal cells per unit length of basement membrane in the combined controls in the short axis as opposed to those in the long axis (Table 6.4) was difficult to explain. Had this difference been due to variation in the degree of folding of the basement membrane it would have been readily conceived of, but as the difference appears to have been due to increased areas of basal cells in the prints of sections in the long axis of the tongue, the most likely explanation is that this result was due to the lack of homogeneity of the basal cells as discussed in 6.4.2.

In the level 3 ultrastructural study the proportion of the basal plasma membrane involved in hemidesmosomes did not differ in sections in the two axes of the tongue. This finding fails to confirm the author's previous observations on a smaller sample of the same material (MacDonald, 1971 p).

The differences in the mean lengths of hemidesmosome profiles and the frequency of hemidesmosomes observed in sections in the two axes of the tongue were a clear indication of "horizontal" anisotropy in this tissue and represent an important variable which will require to be considered in future quantitative

studies. It confirmed the need for careful specimen orientation in stereologic studies. As the proportions of basal cell plasma membrane involved in hemidesmosome attachments did not differ in the two axes of the tongue it follows that the lower frequency of longer hemidesmosome profiles in the long axis balanced the higher frequency of shorter hemidesmosomes in the short axis. This concept is illustrated diagrammatically in Fig. 6.14. The hemidesmosomes are possibly oval with their long axis in the long axis of the tongue.

6.4.4 Comparison of Control and Carcinogen Biopsy Specimens

Stereologic studies of the carcinogen biopsy specimens did not show basal cell hyperplasia evidenced either as an absolute increase in the progenitor cell compartment or an increase in the proportion of the epithelial thickness consisting of progenitor cells. The volume density of actual basal cells was thought to be larger and this was felt to be due to an increase in the length and complexity of the basement membrane. The low power ultrastructural study failed to confirm this idea despite the ten weeks of carcinogen application and a further eight weeks of observation period. It is striking that no difference was observed in the carcinogen biopsy group in the nucleo-cytoplasmic ratio of basal cells or in the features of the hemidesmosomes studied.

6.4.5 Comparison of Control and Carcinogen Sacrifice Specimens

The time interval between the taking of the biopsies and the sacrifice of the animals was six weeks. The sacrifice specimens were the other halves of the original carcinogen treated areas left after biopsy. It was felt that six weeks was a sufficient interval to allow the immediate affects of the biopsy technique to have subsided but it is not possible to state to what extent the surgical procedure of biopsy influenced the progress of the remaining half of the carcinogen treated area.

Differences between the control and carcinogen sacrifice specimens were observed at the three levels of magnification studied. At the light microscopic level the essential change was of epithelial atrophy due to a reduction in the size of the maturation and progenitor cell compartments. Basal cell hyperplasia was not present. The proportion of the progenitor cell compartment formed by basal cells was greater in the carcinogen sacrifice specimens. The suggestion that this result could be explained by an increase in the length and complexity of the basement membrane was supported by the findings of the low power ultrastructural study. In addition, this study suggested reduction in the height of the basal cells possibly from a low columnar type of cell to a

cuboidal cell. The nucleo-cytoplasmic ratio did not alter however.

The most striking change in the carcinogen sacrifice specimens was in the increased length of hemidesmosome profiles and reduced hemidesmosome frequency in the sections in the short axis of the tongue. Despite these marked changes however, the proportion of the basal plasma membrane involved in hemidesmosome attachments did not alter significantly.

6.4.6 Comparison of Carcinogen Biopsy and Carcinogen Sacrifice Specimens

Comparison of the biopsy and sacrifice specimens confirmed that the changes in the sacrifice specimens discussed in 6.4.5 had almost all occurred in the six week interval between the taking of the biopsy and the time of sacrifice. It is conceivable that these changes were not related to the biopsy procedures and the study of Shklar (1968) would support this concept. However, the changes did occur in a short period of time and clearly there are important considerations with respect to clinical practice which require that the effects of the biopsy procedures be carefully evaluated.

6.5 CONCLUSIONS

The studies reported in this chapter confirm the feasibility of applying stereologic methods to the evaluation of the features of cellular atypia. They further indicate the sensitivity and the comparative simplicity of these methods.

These studies do indicate a requirement for further investigation of normal control tissues especially at the lower power ultrastructural level where more information about possible morphological variation in basal cells would be desirable.

It should be possible to apply stereologic methods to the evaluation of all of the features of cellular atypia and this will be required for the full assessment of the diagnostic potential of the cellular atypia scoring technique of Smith and Pindborg (1969).

SUMMARY

Squamous cell carcinoma of the oral cavity is a cause of significant morbidity and mortality in Scotland and in many other parts of the world. From a pathologist's viewpoint one of the important problems relating to this disease is in the diagnosis of early lesions particularly those in the premalignant phase which can precede tumour development. The aim of the work described in this thesis was to advance knowledge of the premalignant phase in the development of squamous cell carcinoma of the mouth.

It was desirable to use an experimental animal oral cancer model in this study and a preliminary evaluation of the standard technique for inducing hamster cheek pouch carcinomas was made. This technique gave poor localisation of the response to carcinogen application and also had the disadvantage of producing severe ulceration. A new technique of tumour induction in hamster cheek pouch was devised to overcome these problems. This technique was then used to produce premalignant lesions in the hamster cheek pouch which were biopsied and then followed to relate the subsequent progress to the histological changes seen at the time of biopsy. A detailed objective quantitative analysis of the changes of cellular atypia which characterise the premalignant lesions was made according

to the technique of Smith and Pindborg (1969). It was shown that this technique was a good prognostic indicator in hamster cheek pouch lesions.

In order to examine the validity of applying the results obtained in the hamster cheek pouch to human oral lesions comparison of human and hamster premalignant lesions was undertaken. Although a few obvious differences were noted, detailed comparisons of the cellular atypia as evaluated by the Smith and Pindborg (1969) technique indicated that the human and hamster premalignant lesions were closely similar. It was concluded that the Smith and Pindborg (1969) technique should be a reliable prognostic indicator in human oral lesions.

A study of the histological features present in human oral lesions indicated that although the features usually described as characterising squamous cell carcinomas were most frequent in these lesions they were also demonstrable in some non-neoplastic oral mucosal lesions.

Some authors have criticised the hamster cheek pouch tumour model on the grounds that the cheek pouch is not exposed to the same environment as the mouth proper. Evaluation was therefore made of the early responses to carcinogen application at three truly

intraoral sites in the hamster. The areas studied were in the "drainage area" of the lingual sulcus and the specific sites examined were the ventral surface of tongue, the lateral surface of tongue and the lingual gingivae. Premalignant changes were observed at all three sites although these developed less rapidly than in the case of cheek pouch lesions. The site showing the most consistent and marked response was the ventral surface of the tongue and it was felt that this was the site which most merited further study.

The quantitative techniques used in the earlier analysis of premalignant changes were fairly crude and it was felt desirable to derive more accurate quantitative data. The problems of applying stereologic methods to oral mucosa were studied and these methods were applied to normal and carcinogen treated ventral tongue epithelium in the hamster. The investigation was concerned mainly with basal cells and the basement membrane zone. At a light microscopic level, quantitation of the cell compartments of the epithelium was undertaken and this gave a measure of the atrophy in carcinogen treated epithelium. The nucleo-cytoplasmic ratios of basal cells were studied at a low power electron microscope level and at a higher power the basement membrane zone and in particular hemides-

mosomes were studied. Control sections demonstrated a previously unrecorded "horizontal" anisotropy in tongue epithelium in that hemidesmosomes were shown to be less frequent, but of longer profiles on average in sections in the long axis as opposed to the short axis of the tongue. In carcinogen treated epithelium a further reduction in numbers of hemidesmosomes was observed, but these had longer profiles in section than hemidesmosomes in control tissues.

The derivation of valid quantitative data by stereologic techniques can only be achieved after a number of requirements concerning such factors as random selection, orientation and the limitations of particular measuring techniques have been satisfied. This study has shown how this may be achieved. It is suggested that these methods could form a basis for future quantitative studies of other cell changes found in premalignancy with a view to evaluating their diagnostic potential in human lesions.

APPENDIX 1.

DEFINITIONS OF WORDS AND ABBREVIATIONS

A - area of section.

Acantholysis - loss of coherence between epithelial cells, with rounding up of individual cells.

Acanthosis - abnormal increase in thickness of stratum spinosum.

Atrophy - as applied to a stratified squamous epithelium; abnormal decrease in thickness of the progenitor cell and maturation compartments.

Atypia - either epithelial atypia or cellular atypia. Atypia of oral epithelium is present when the tissue exhibits two or more of the following histological alterations; drop shaped rete ridges, irregular epithelial stratification, keratinization of cells below the keratinized layer, basal cell hyperplasia, loss of intercellular adherence, loss of polarity, hyperchromatic nuclei; increased nucleo-cytoplasmic ratio in basal cell and prickle cell layers, anisocytosis and anisonucleosis, pleomorphic cells and nuclei, increased mitotic activity, mitoses at abnormally superficial level or presence of bizarre mitoses.

B - boundary length of profile.

Basal cell - a keratinocyte seen to be in contact with the light microscopic basement membrane or the electron microscopic lamina densa in the particular section studied.

Compartment - as applied to tissue, a collection of cells with a specific function. Oral epithelia consist of a progenitor cell compartment, a maturation compartment and a cornified cell compartment (MacDonald, 1971).

Dyskeratosis - synonym for epithelial or cellular atypia.

Dysplasia - synonym for epithelial or cellular atypia.

Hyperkeratosis - the presence of an abnormally thick stratum corneum at a site which is normally keratinized. A more precise description may indicate hyperorthokeratosis or hyperparakeratosis.

I - number of intersection points.

L - length of structure.

Lamina densa (synonym basal lamina) - the electron opaque layer at the epithelial connective tissue interface into which connective tissue fibrils are inserted.

Lamina lucida - the electron lucent zone between the lamina densa and the basal plasma membrane of basal epithelial cells.

Leukoplakia - a clinical descriptive term for a white patch on the oral mucosa which cannot be scraped off and which is not attributable to any specific mucosal lesion such as lichen planus or lupus erythematosus. Leukoplakias may be further characterised as homogeneous if of a uniform white appearance or speckled if present as discrete white foci upon an erythematous area of epithelium.

Morphometry - the use of quantitative data in the description of structural features.

N - number of structures.

Orthokeratosis - the presence of a stratum corneum consisting of anucleate squames.

P - number of (test) points.

Parakeratosis - the presence of a stratum corneum consisting of nucleated squames.

S - surface of structure.

S.D. - standard deviation.

Semikeratinization - a description applied by Meyer et al (1967) to epithelia in leukoplakias showing a mixture of histological traits of keratinized and non-keratinized areas of oral mucosa.

Stereology - a system of geometric analysis of structures and textures, including methods that allow direct derivation of metric properties of structures from two-dimensional sections on the basis of geometrico-statistical reasoning.

V - volume of structure.

V_v - volume density of structure in tissue i.e. the volume of one constituent within a larger volume.

$\bar{x}$ - arithmetical mean.

APPENDIX 2.

FIXATIVES AND TISSUE PROCESSING PROCEDURES

FIXATIVES

10% neutral buffered formol-saline

Sodium dihydrogen orthophosphate	8.4 g
Disodium hydrogen orthophosphate	15.6 g
Sodium chloride	21.6 g
Neutral formaldehyde	240 ml
Distilled water	2160 ml

Bouin's fluid

Picric acid, saturated aqueous solution	75 ml
Formalin (40% formaldehyde)	25 ml

Heidenhain's "Susa"

Mercuric chloride	4.5 g
Sodium chloride	0.5 g
Trichloracetic acid	2 g
Acetic acid	4 ml
Formalin	20 ml
Distilled water	100 ml

Formol-acetic-alcohol

Formaldehyde	100 ml
Acetic acid	50 ml
Alcohol (ethanol or methanol)	850 ml

This formol-acetic-alcohol solution was tried with either ethanol or methanol. Methanol should theoretically cause less tissue shrinkage and subjectively it was felt that the blocks from tissues fixed in the methanol containing solution were easier to cut.

Electron Microscopy

Gluteraldehyde 2% solution in 0.2M sodium cacodylate buffer.
pH 7.2
osmotic pressure approximately 350 mOsm

Osmium tetroxide 1% solution in 0.2M sodium cacodylate buffer, with 5.5% sucrose.
pH 7.2
osmotic pressure approximately 330 mOsm

TISSUE PROCESSING CYCLES

Light Microscopy

Automatic tissue processor with following cycle:-

1.	50% methylated spirits	30 minutes
2.	80% methylated spirits	1 hour
3.	8% phenol in methylated spirits	2 hours
4.	8% phenol in methylated spirits	1 hour
5.	8% phenol in methylated spirits	2 hours
6.	Absolute alcohol	2 hours
7.	Absolute alcohol	3 hours
8.	50% absolute alcohol, 50% chloroform	30 minutes
9.	Chloroform	3 hours
10.	Chloroform	4 hours
11.	Paraffin wax	2 hours
12.	Paraffin wax	2 hours

Electron Microscopy

1.	Gluteraldehyde	2 hours
2.	Sodium cacodylate buffer	1 hour
3.	Osmium tetroxide	30 minutes
4.	Distilled water	rinse
5.	50% alcohol	5 minutes
6.	75% alcohol	5 minutes
7.	95% alcohol	5 minutes
8.	Absolute alcohol	3 changes of 10, 10 and 30 minutes.
9.	Propylene oxide	2 changes of 30 minutes each.
10.	50% Propylene oxide, 50% Araldite	30 minutes
11.	33% Propylene oxide, 67% araldite	1-4 hours
12.	Araldite (room temperature)	5-8 hours
13.	Embed and incubate at 60°C	48 hours

If the primary fixation was in osmium tetroxide, steps 1 and 2 were omitted and step 3 increased to 1 hour.

The araldite used was Fluka (Durkapan) using the proportions:- Solution A/M 10 ml; B 10 ml, C 0.5 ml and D 0.75 ml.

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EXPERIMENTAL ORAL CARCINOGENESIS

With Particular Reference to Premalignant Lesions

VOLUME 2 OF TWO VOLUMES

DONALD GORDON MacDONALD

B.D.S.(Glas). M.R.C.Path.

Thesis

Submitted for the Degree of Doctor of Philosophy

University of Glasgow, May 1973.

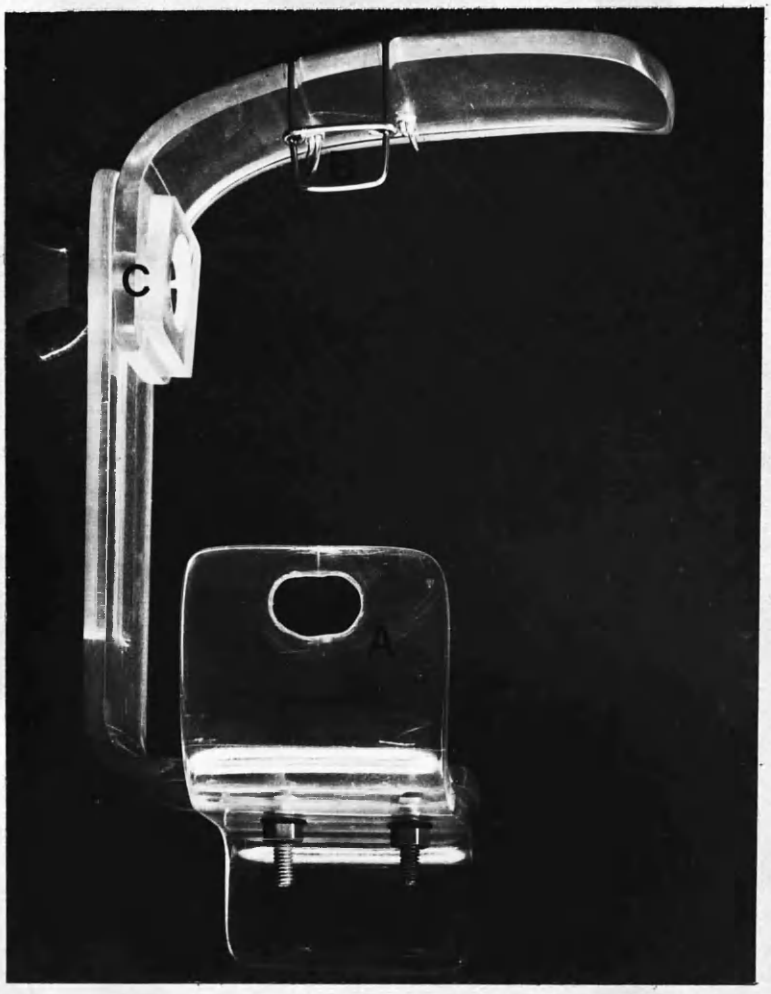


Fig. 2.1 Device for the intraoral examination of rodents. The lower incisor teeth are placed through the hole A and the upper incisors and snout are held in B. The mouth is opened by adjustment of part C.

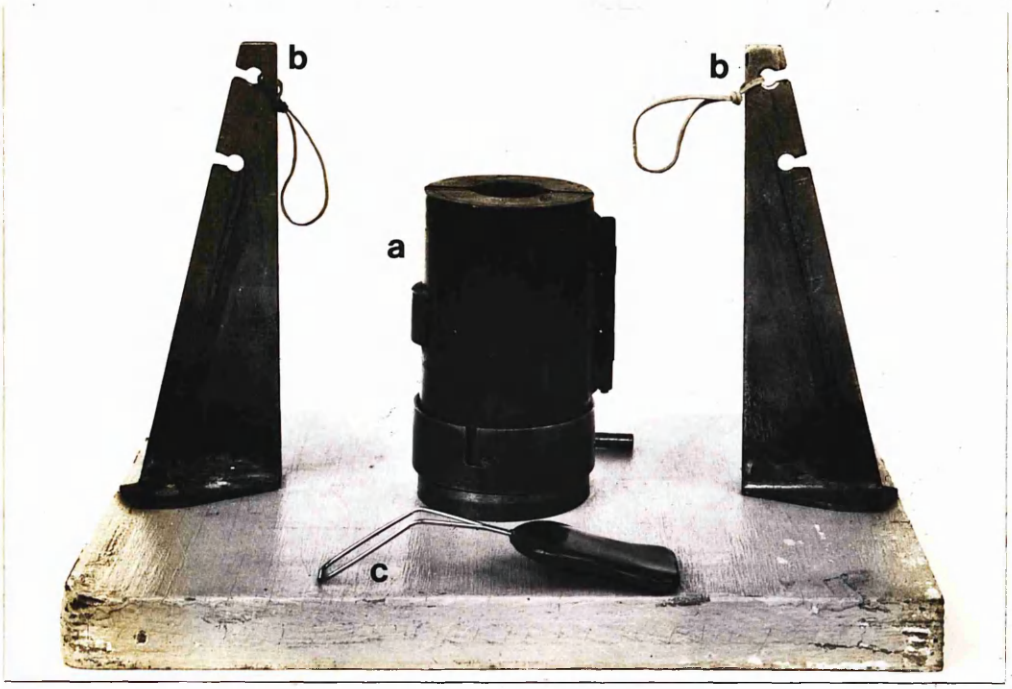


Fig. 2.2.A Restraining device for intraoral examination of hamsters.
a. Cylinder for holding body of animal.
b. Stands with elastic bands to hold incisor teeth.
c. Cheek retractor.

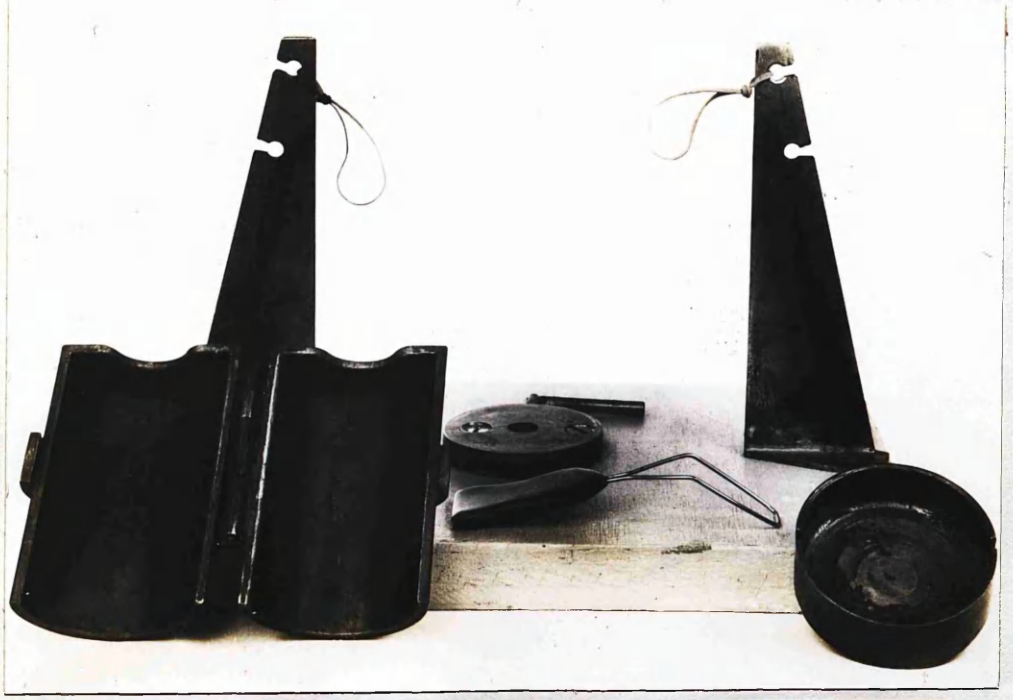


Fig. 2.2.B Restraining device for intraoral examination of hamsters.
View with parts separated.

Fig. 2.3 Hamster in restraining device for intraoral examination.

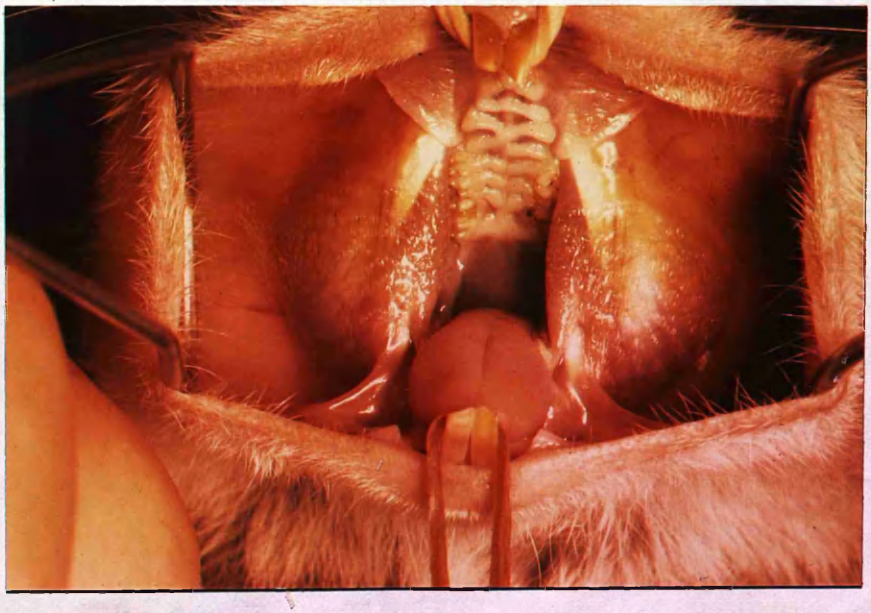


Fig. 2.4 Exposure of cheek pouches with hamster in restraining device. Untreated normal control.

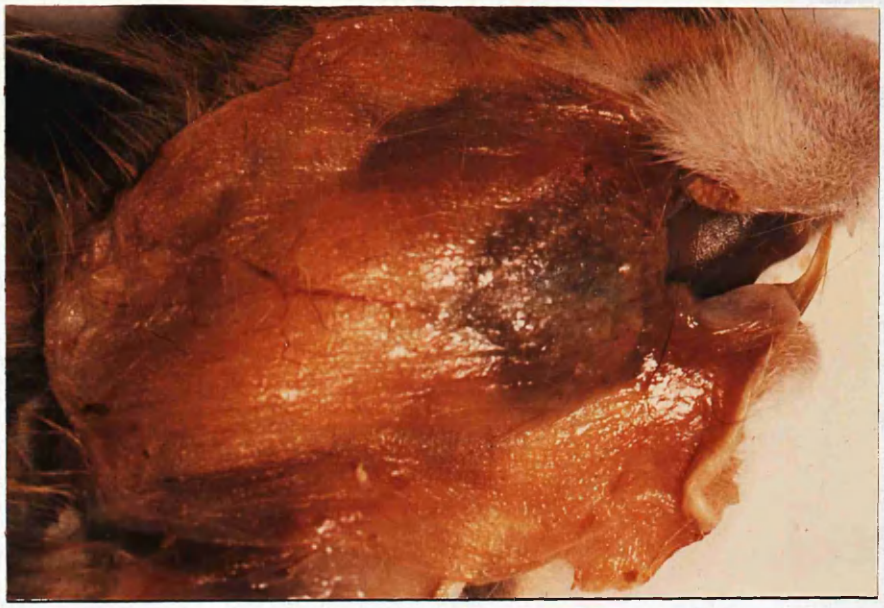


Fig. 2.5 Pouch exposure after sacrifice. This is a carcinogen treated animal and the pouch shows early pathological changes.

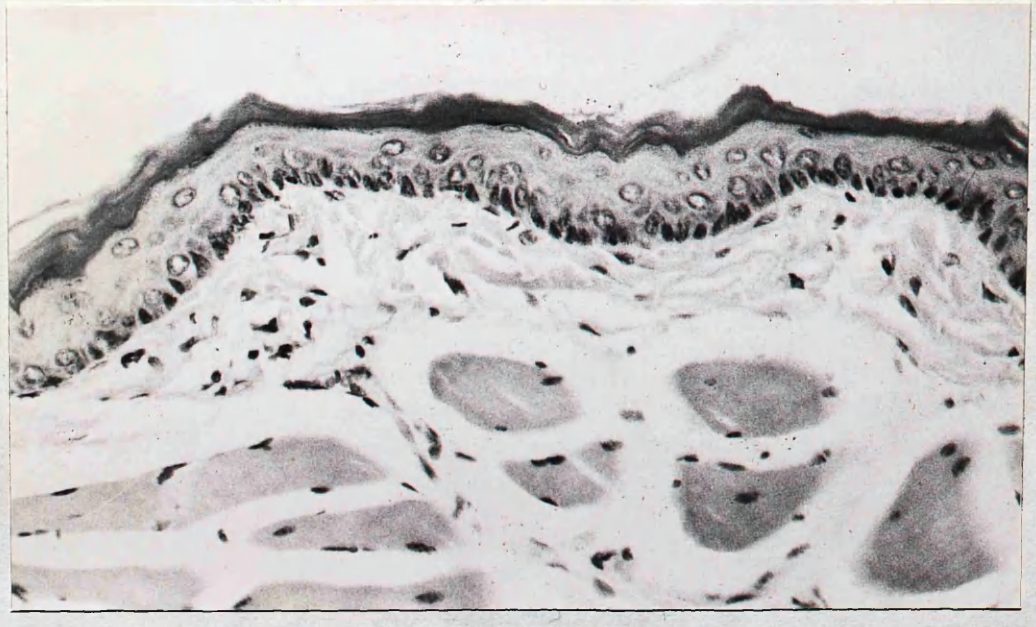


Fig. 2.6 Normal hamster cheek pouch mucosa.
H. and E. x 310.

Fig. 2.7 Hamster number 13. Extensive ulceration of the pouch and a thick slough(s) of necrotic tissue are present.
x 115.

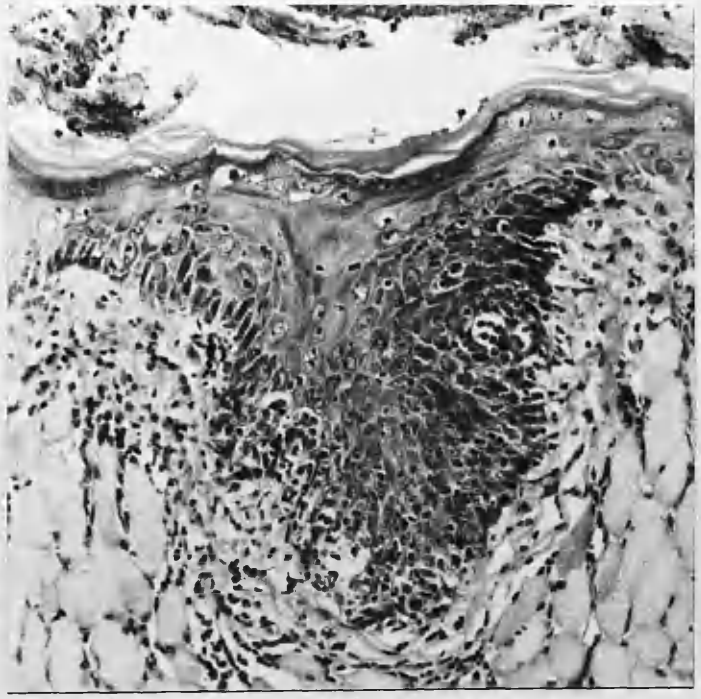


Fig. 2.8 Hamster number 13. Area of hyperplastic pouch epithelium showing cellular atypia.
x 210.

POUCH		CARCINOMA	PAPILLOMA	CELLULAR ATYPIA	HYPER-KERATOSIS	ACANTHOSIS	ATROPHY	DENSE SCAR	ULCERATION
1	A			+	+	+	+	+	
	B			+	+		+	+	
2	A			+	+	+	+	+	
	B			+	+	+	+		
3	A			+	+	+	+		+
	B			+		+	+	+	+
4	A			+	+	+	+	+	+
	B			+	+	+	+	+	
6	A			+	+	+	+	+	
	B			+	+	+	+	+	
7	A			+	+	+	+	+	
	B			+	+	+		+	
8	A			+	+	+	+	+	
	B		+	+	+	+	+	+	
9	A			+	+	+	+	+	
	B			+	+	+	+	+	
10	A			+	+	+		+	
	B			+	+	+	+		
TOTAL	18	0	1	18	17	17	16	15	3

Table 2.1 Histological changes observed in the cheek pouches after four weeks of DMBA applications.
(Experimental group 1)

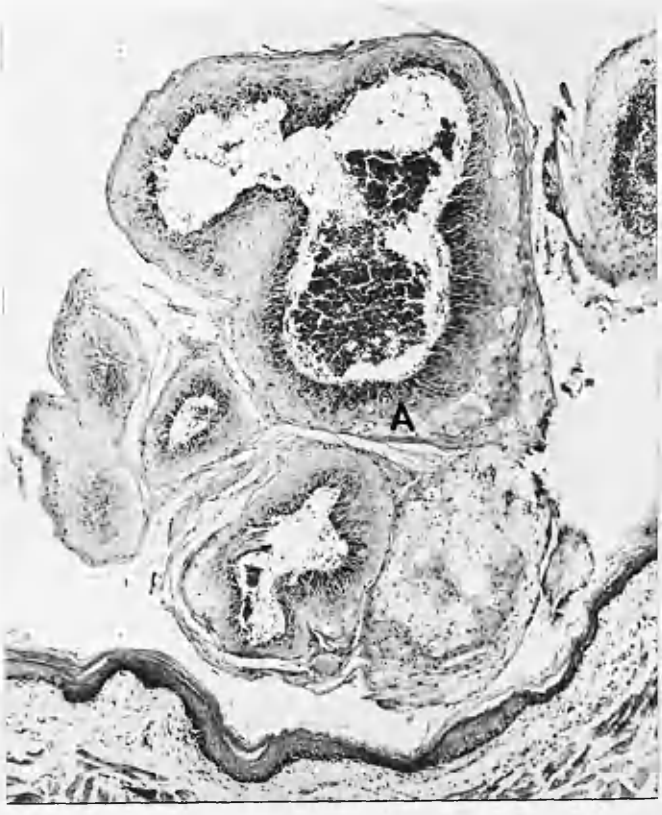


Fig. 2.9 Papilloma seen in pouch 8B after four weeks of DMBA application. Note cellular atypia at A.
x 50.

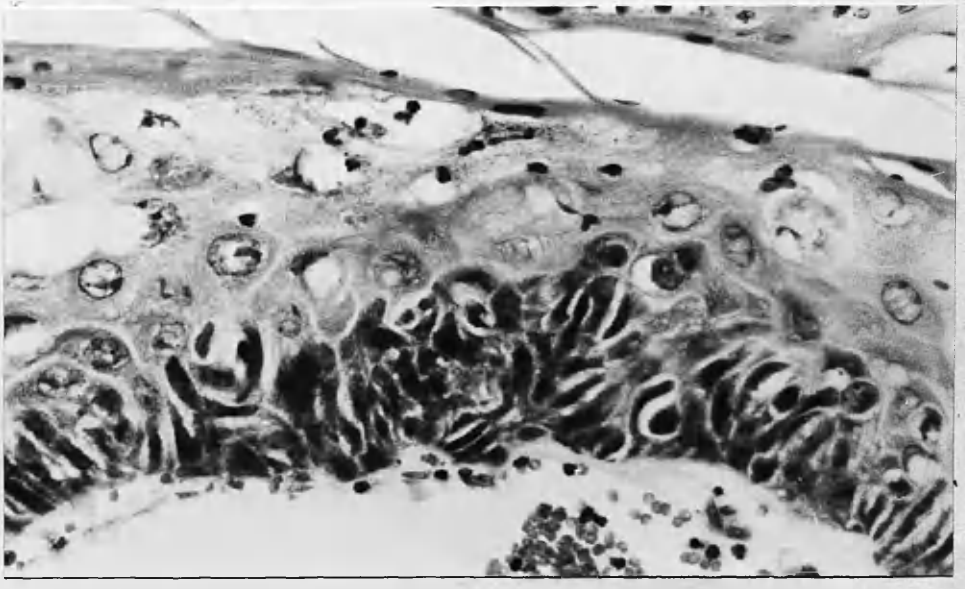


Fig. 2.10 Higher magnification of area A in Fig. 2.9 showing cellular atypia.
x 390.

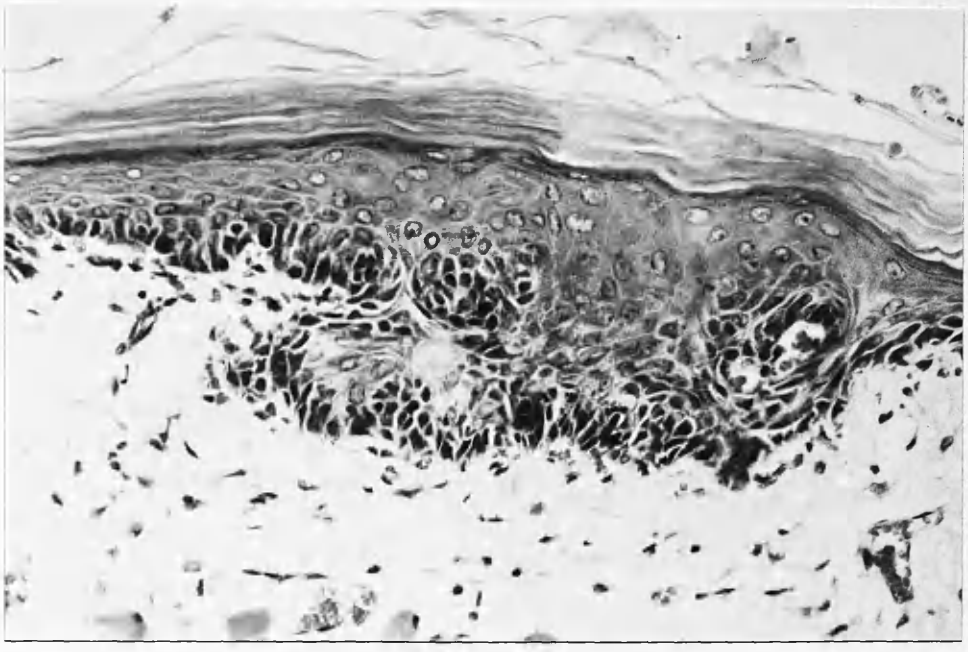


Fig. 2.11 Pouch in group 1 animal showing cellular atypia. Basal cell hyperplasia, loss of polarity, disordered stratification and hyperchromatism are among the changes seen. x 250.

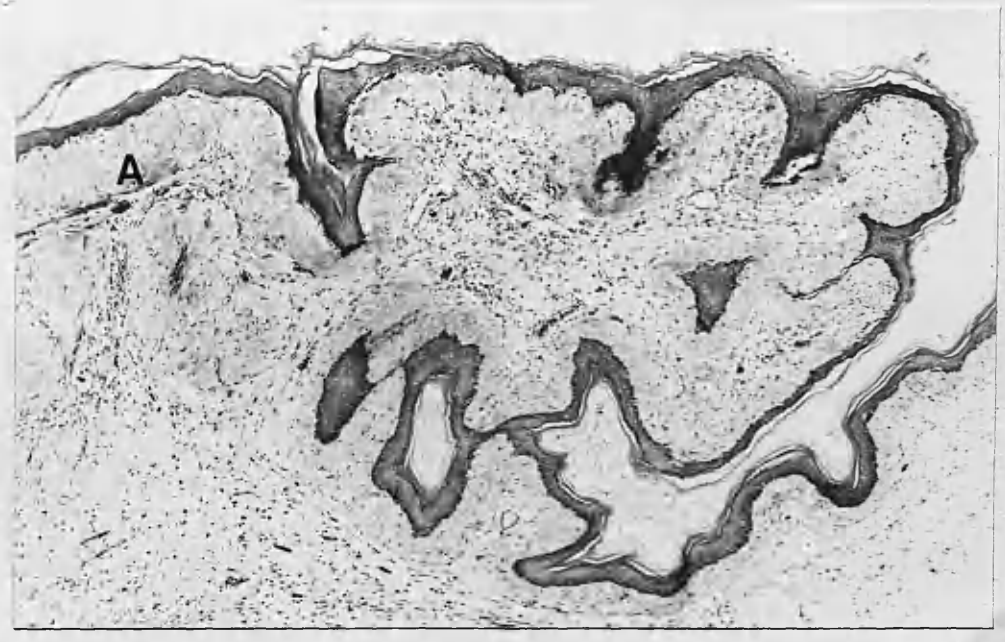


Fig. 2.12 Healed area of ulceration showing dense scar tissue (A) and irregular regenerated epithelial lining.
x 130.

POUCH		CARCINOMA	PAPILLOMA	CELLULAR ATYPIA	HYPER-KERATOSIS	ACANTHOSIS	ATROPHY	DENSE SCAR	ULCERATION
11	A			+	+	+	+	+	
	B			+	+	+	+		
12	A			+	+	+	+	+	
	B			+	+	+	+		
14	A			+	+	+	+	+	
	B			+	+	+			
15	A			+	+	+	+		
	B			+	+	+			
16	A			+	+	+	+	+	
	B		+	+	+	+	+	+	
17	A			+	+	+	+	+	
	B			+	+	+	+	+	
18	A		+	+	+	+			
	B			+	+	+	+	+	+
19	A			+	+	+	+	+	
	B			+	+	+	+		
20	A	+	+	+	+	+	+	+	
	B			+	+	+	+	+	
TOTAL	18	1	3	18	18	18	15	11	1

Table 2.2 Histological changes observed in the cheek pouches after six weeks of DMBA applications.
(Experimental group 2)

Fig. 2.13 A. Squamous cell carcinoma - exophytic tumour exhibiting invasion at a. (Hamster number 20). Note also the artefact at b due to a fault in section preparation. x 60.
B. Higher power of invasion at a. x 410.

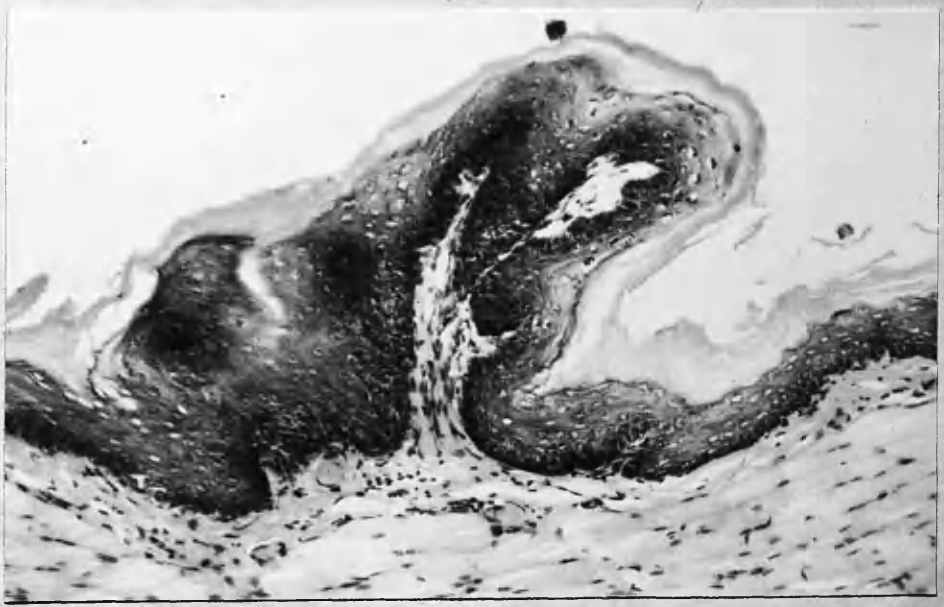


Fig. 2.14 Hamster number 20. Early papillary neoplasm.
x 150.

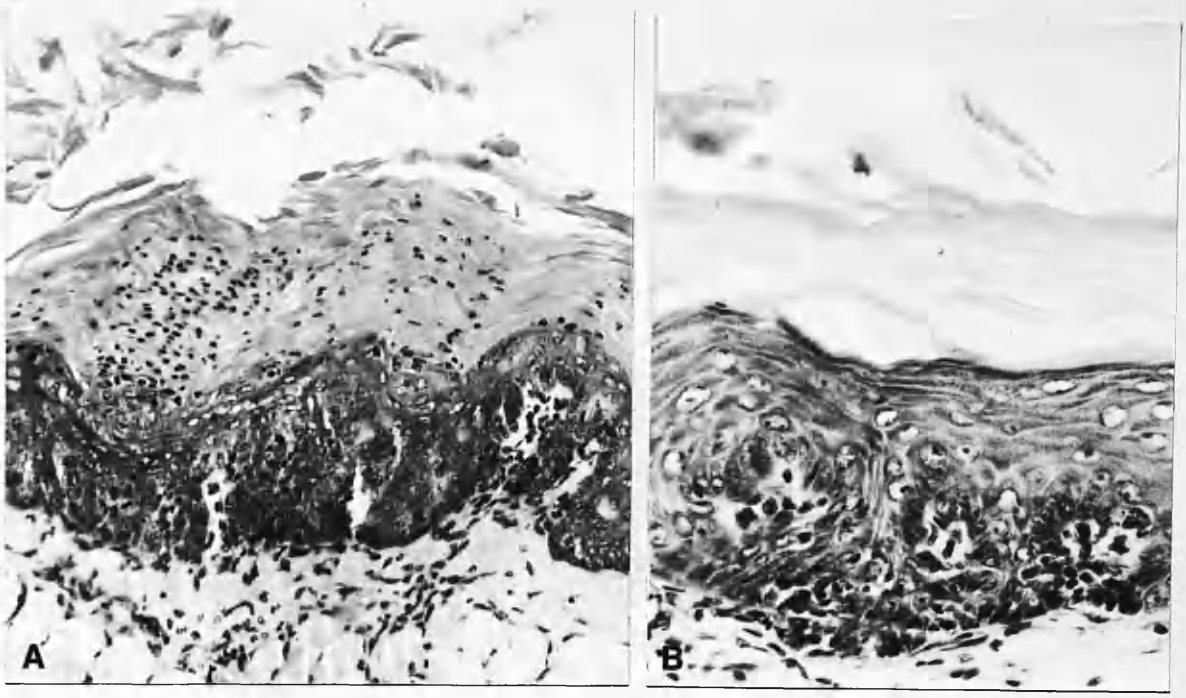


Fig. 2.15 A. Pouch exhibiting hyperparakeratosis and moderately severe cellular atypia. x 210.
B. Pouch with basal cell hyperplasia and moderately severe loss of polarity and disordered stratification. x 335.

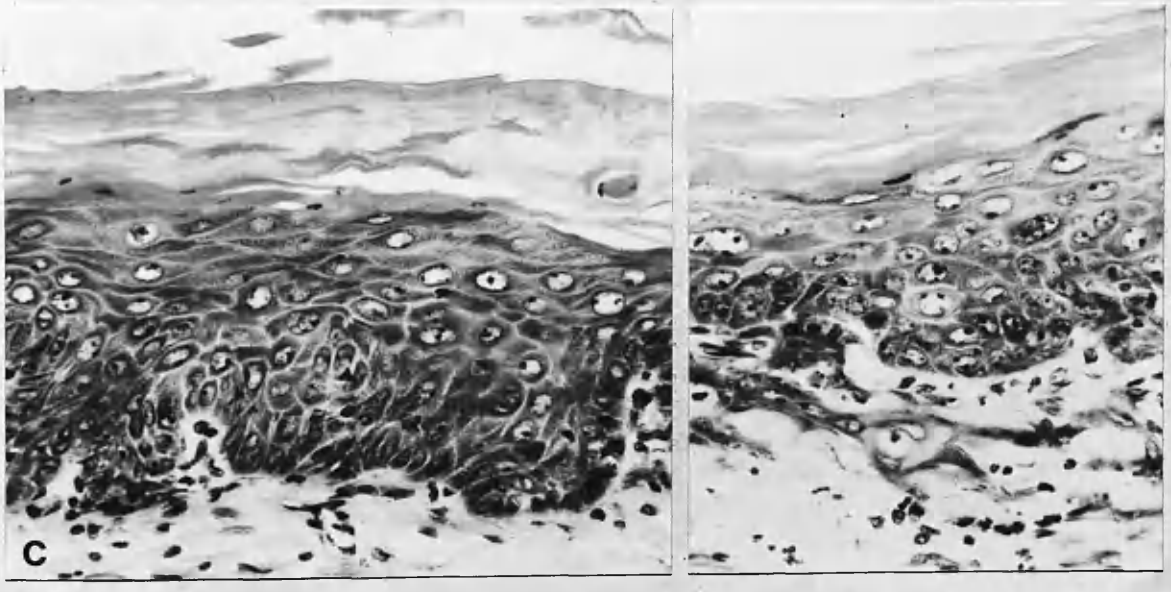


Fig. 2.15 C. Pouch with mildly disordered epithelial stratification and loss of polarity. x 325.

Fig. 2.16 Pouch with moderate cellular atypia and appearance suggestive of early invasion. x 320.

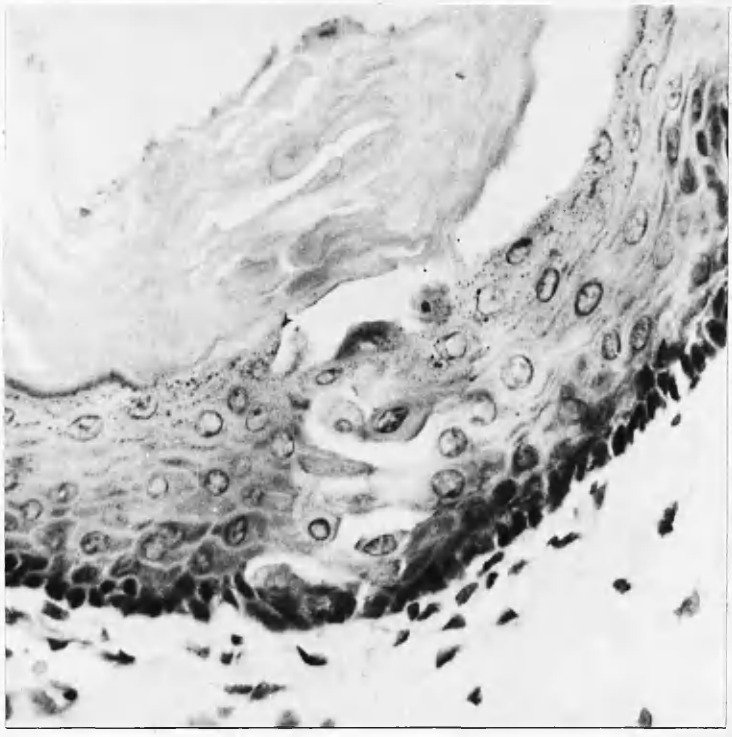


Fig. 2.17 Pouch epithelium showing columns of acantholytic cells. x 380.

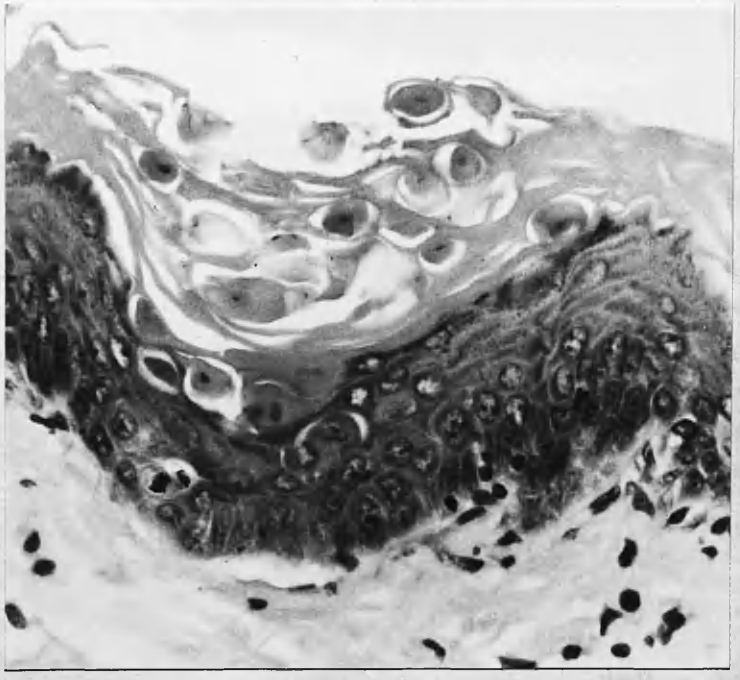


Fig. 2.18 Pouch showing marked premature cell keratinisation and acantholysis.
x 385

	WEEKS							
ANIMAL	1	2	3	4	5	6	7	
1	65	68	74	80	83			
2	100	98	95	100	115			
3	100	100	84	92	96			
4	85	86	79	85	95			
5	90	95	74	sacrificed				Experimental Group 1
6	95	93	96	105	113			
7	100	90	90	100	100			
8	105	105	108	115	116			
9	100	95	97	104	112			
10	83	80	83	86	87			
11	105	105	106	112	117	120	128	
12	100	105	105	105	110	112	114	
13	93	sacrificed						
14	103	98	83	97	98	100	95	
15	106	105	100	110	117	123	123	Experimental Group 2
16	70	63	70	75	76	79	78	
17	106	95	94	95	94	100	102	
18	110	105	103	108	113	113	110	
19	100	90	98	105	105	110	108	
20	97	85	88	92	95	98	102	
21	110	120	121	123	129	130		
22	106	120	126	134	144	145		Control Group
23	125	130	142	152	158			
24	100	105	113	122	135			

Table 2.3 Individual animal weights recorded at the beginning of each week of the experiment.

WEEK 1 WEIGHTS IN GRAMS

Experimental Groups (animals 1 - 20 excluding 5 & 13)	mean	96.11
	S.D.	12.44
Experimental Group 1 (1 - 10 excluding 5)	mean	92.56
	S.D.	12.70
Experimental Group 2 (11 - 20 excluding 13)	mean	99.67
	S.D.	11.80
Control Group (21 - 24)	mean	110.25
	S.D.	10.66

Experimentals compared to Controls	"t" = 2.324	$P < 0.05$
Experimental Group 1 compared to controls	"t" = 2.599	$P < 0.05$
Experimental Group 2 compared to controls	"t" = 1.597	$P > 0.1$

ANIMAL	WEEK 1	WEEK 5	DIFFERENCE WEEK 5 - WEEK 1	
11	105	117	+12	
12	100	110	+10	
14	103	98	-5	
15	106	117	+11	
16	70	76	+6	Experimental Group 2
17	106	94	-12	
18	110	113	+3	
19	100	105	+5	
20	97	95	-2	
21	110	129	+19	
22	106	144	+38	Control Group
23	125	158	+33	
24	100	135	+35	

Difference:- weight in week 5 - weight in week 1.

Experimental Group 2 mean 3.11
S.D. 8.07

Control Group mean 31.25
S.D. 8.42

Experimental Group 2 compared to Control Group

"t" = 5.63
$p < 0.001$

Table 2.4 Weight change in individual animals during the course of the experiment.

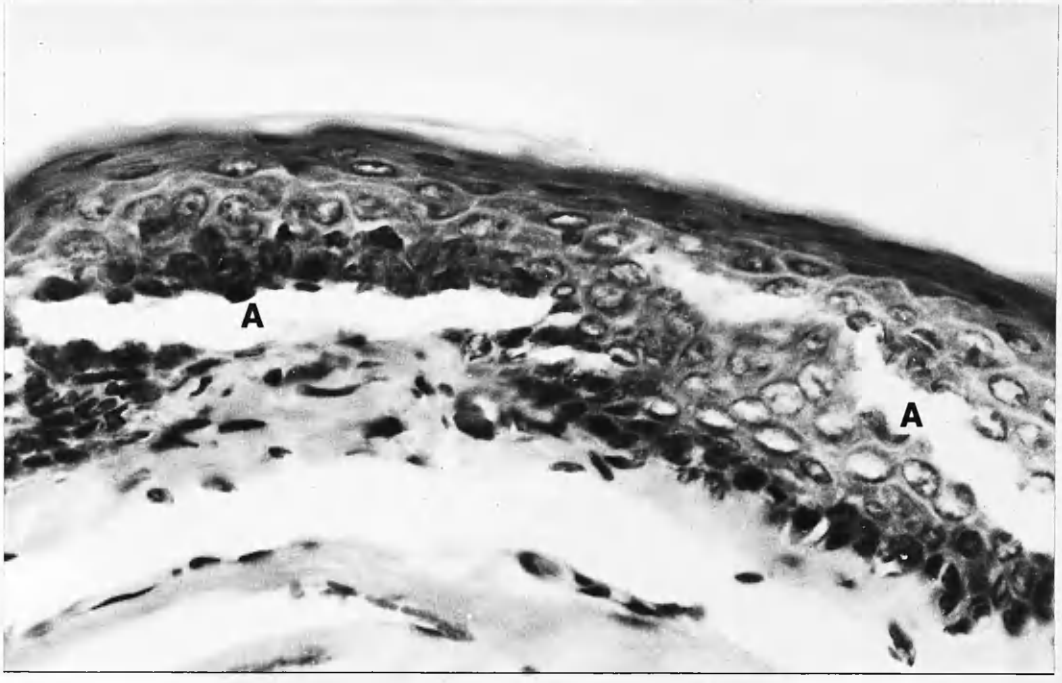


Fig. 2.19 Normal hamster cheek pouch after formalin fixation. Note sectioning artefacts at A and poor nuclear detail. H. & E. x 250.

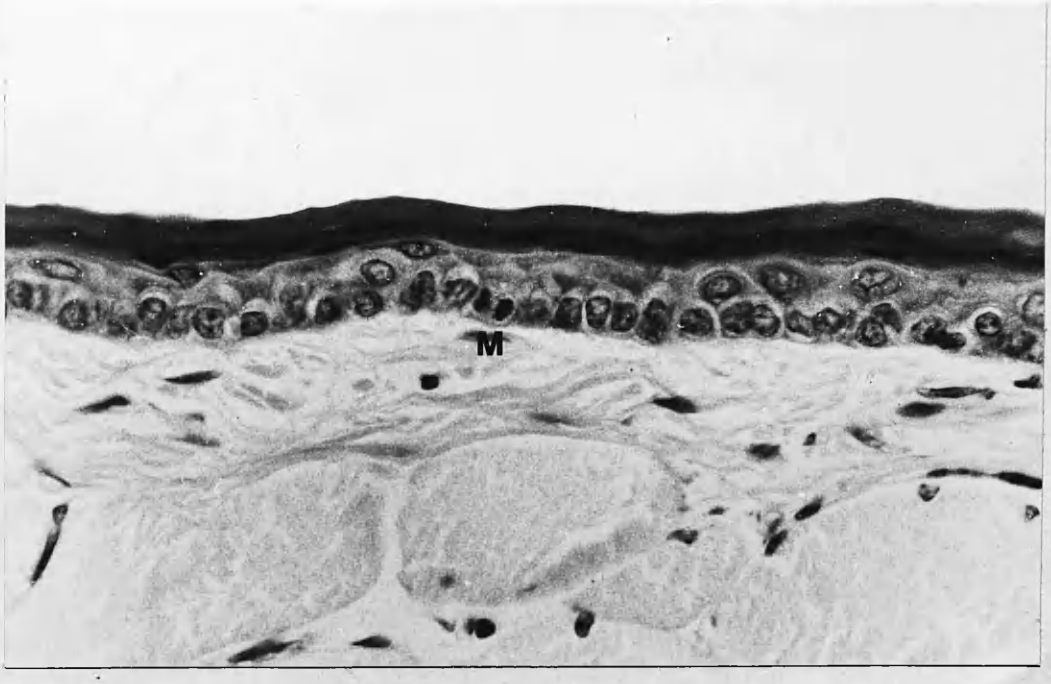


Fig. 2.20 Normal hamster cheek pouch. Fixation in formol-acetic-methanol. Note the crisp cytological picture and the mitosis at M. H. & E. x 200.

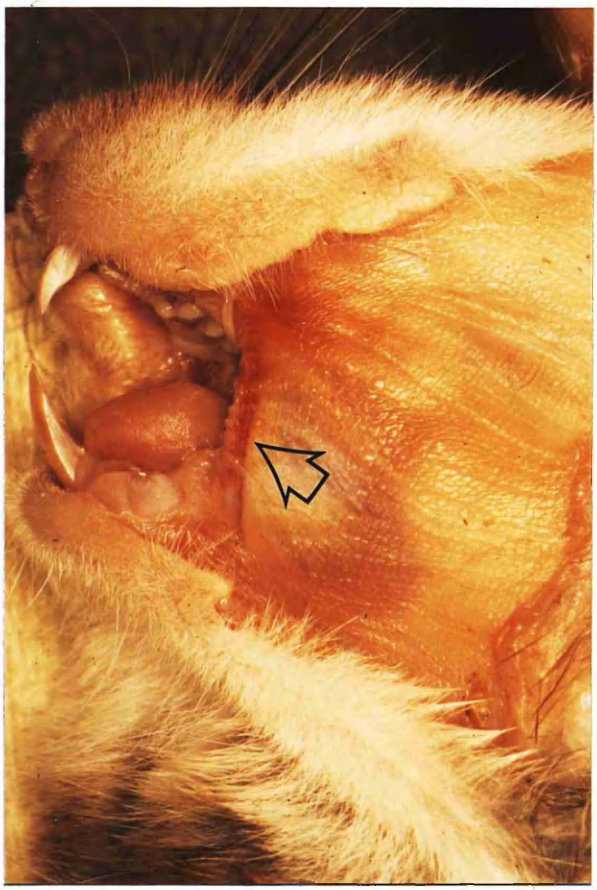


Fig. 3.1 View of the medial wall of the cheek pouch exposed to show the prominent blood vessels (arrowed) at the anterior border of the masseter. These vessels are also shown clearly in Fig. 2.4.

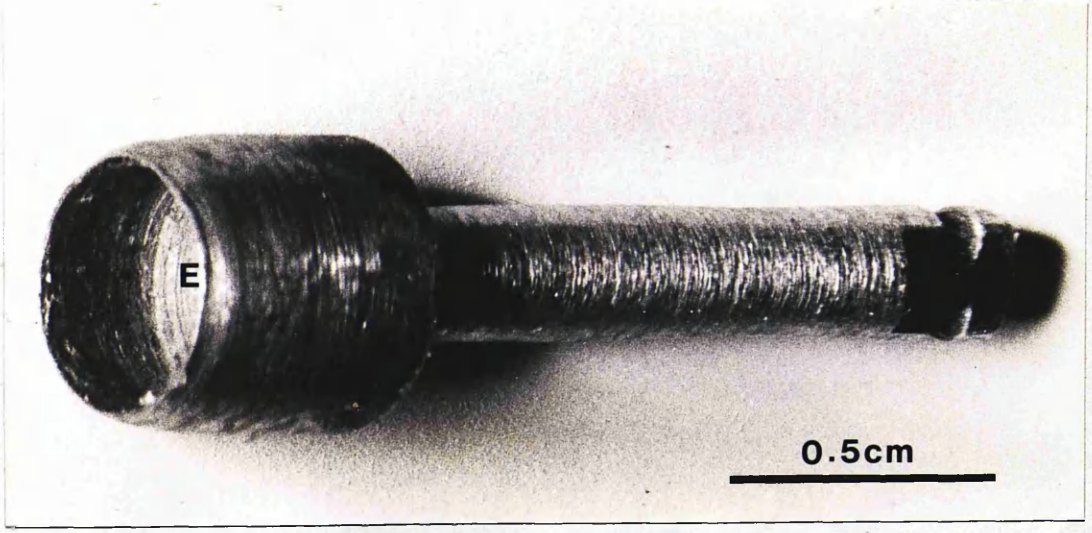


Fig. 3.2 Modification of dental bur for use as a biopsy device. A head with a cutting edge (E) has been soldered to the shaft of a contra-angled bur.

ORAL EPITHELIAL ATYPIA	ANIMAL NO.	SECTION NO.
Characteristic to be observed	Grades	Score
1. "DROP-SHAPED" RETE RIDGES	NONE (0) SLIGHT (2) MARKED (4)	
2. IRREGULAR EPITHELIAL STRATIFICATION	NONE (0) SLIGHT (2) MARKED (5)	
3. KERATINIZATION OF CELLS BELOW KERATINIZED LAYER	NONE (0) FEW/SHALLOW (1) MANY/DEEP (3)	
4. BASAL CELL HYPERPLASIA	NONE (0) SLIGHT (1) MARKED (4)	
5. LOSS OF INTERCELLULAR ADHERENCE	NONE (0) SLIGHT (1) MARKED (5)	
6. LOSS OF POLARITY	NONE (0) SLIGHT (2) MARKED (6)	
7. HYPERCHROMATIC NUCLEI	NONE (0) SLIGHT (2) MARKED (5)	
8. INCREASED NUCLEO-CYTOPLASMIC RATIO (INCREASED DENSITY) IN BASAL AND PRICKLE-CELL LAYERS	NO INCREASE (0) SLIGHT INCREASE (2) MARKED INCREASE (6)	
9. ANISOCYTOSIS AND ANISONUCLEOSIS	NONE (0) SLIGHT (2) MARKED (6)	
10. PLEOMORPHIC CELLS AND NUCLEI	NONE (0) SLIGHT (2) MARKED (6)	
11. MITOTIC ACTIVITY	NORMAL (0) SLIGHT INCREASE (1) MARKED INCREASE (5)	
12. LEVEL OF MITOTIC ACTIVITY	NORMAL (0) LOWER ½ ONLY (3) ALSO UPPER ½ (10)	
13. PRESENCE OF BIZARRE MITOSES	NONE (0) SINGLE (6) MULTIPLE (10)	

Table 3.1 Scoring system for analysis of cellular atypia - after Smith and Pindborg (1969).

	Week									Weight change	
Animal	1	2	3	4	5	6	7	11	15	Wk1-11	Wk2-6
1	90	100	63	110	112	112	118	120	121	30	12
2	84	94	98	102	105	108	114	112	114	28	14
3	98	107	114	120	120	124	130	135	137	37	17
4	86	93	100	105	106	110	114	120	125	34	17
5	78	80	82	80	82	85	90	95	100	17	5
6	77	80	86	94	96	100	105	102	90	25	20
7	94	96	100	105	106	110	110	118	113	24	15
8	75	83	88	95	98	100	104	108	111	33	17
9	78	80	85	85	88	92	95	105	103	27	12
10	93	98	100	103	108	112	116	125	127	32	14
11	75	83	86	90	93	93	98	102	105	27	10
12	77	90	84	90	96	98	100	110	114	33	8
13	85	92	94	98	100	104	111	118	120	33	12
14	72	80	86	87	92	96	100	sacrificed			16
15	82	90	96	100	106	112	120	127	132	45	22
16	77	82	84	84	85	85	90	95	91	18	3
17	96	104	110	117	119	120	126	132	131	36	16
18	92	98	98	100	103	110	112	117	116	25	12
19	77	86	88	92	98	103	111	121	122	44	17
20	98	106	105	108	114	121	125	135	135	37	15
21	82	85	88	92	98	104	113	120		38	19
22	80	86	94	100	104	110	110	117		37	24
23	86	96	103	107	113	118	122	129		43	22
24	79	86	93	97	103	108	108	117		38	22
25	80	87	90	96	98	102	102	105		25	15
26	95	102	105	107	110	110		120	125	25	8
27	82	92	100	105	106	107		114	118	32	15
28	87	92	98	102	103	105		110	115	23	13
29	85	90	97	105	105	106		115	120	30	16
30	77	86	94	102	100	102		115	118	38	16

Table 3.2 Weekly weight in grams of individual animals.

Group 1 hamsters, painted with 0.5% DMBA, consisted of 1-15 and 26-30.
Group 2, painted with 0.25% DMBA, included animals 16-25.

Comparison of Experiment 2 and Experiment 1.

1. Comparison of weights at 8 weeks of age (weights in grams)

Week 2	Experiment 2 (Nos.1-30)	mean	90.80
		S.D.	8.02
Week 1	Experiment 1 (Nos.1-20)	mean	96.11
		S.D.	12.44
	Experiment 1 (Controls)	mean	110.25
		S.D.	10.66

Comparison.

Experiment 2 (1-30) vs Experiment 1 (1-20)

"t" = 1.689 $P > 0.1$

Experiment 2 (1-30) vs Experiment 1 (Controls)

"t" = 3.519 $P < 0.01$

2. Weight change over 4 weeks (in grams)

Experiment 1.	(Controls) week 5 - week 1 (Omitting 5 & 13)	mean	31.25
		S.D.	8.42
Experiment 1.	(Nos. 1-20) week 5 - week 1	mean	6.22
		S.D.	8.36
Experiment 2.	(Nos. 1-30) week 6 - week 2	mean	14.43
		S.D.	5.49

Comparison Experiment 1 (Controls) v Experiment 2 (Nos.1-30)

"t" = 3.886 $P < 0.001$

Experiment 1 (1-20) v Experiment 2 (1-30)

"t" = 3.714 $P < 0.001$

Table 3.3 Comparison of animal weights and weight changes in experiments 1 and 2.

POUCH		AT BIOPSY WITHIN AREA	AT BIOPSY OUTWITH AREA	AT SACRIFICE WITHIN AREA	AT SACRIFICE OUTWITH AREA
1	A	+		+	+
	B	+		+	
2	A				+
	B			+	+
3	A				+
	B				+
4	A				
	B				
5	A				
	B				
6	A		+	+	+
	B			+	+
7	A			+	
	B		+		
8	A			+	
	B			+	+
9	A				
	B				
10	A				+
	B				
11	A				
	B				+
12	A				
	B				
13	A	+		+	+
	B	+		+	
15	A		+	+	
	B				
16	A				
	B	+		+	
17	A				+
	B			+	
18	A			+	
	B	+		+	
19	A		+	+	
	B				
20	A				
	B				
21	A			+	
	B				
22	A				
	B				
23	A	+		+	
	B			+	+
24	A				
	B				
25	A				
	B				
26	A				
	B				
27	A				
	B		+		
28	A			+	
	B				
29	A			+	+
	B	+			+
30	A				+
	B				
TOTAL	58	8	3	21	16

Tumours outwith experimental area at sacrifice.

Group 1 v Group 2

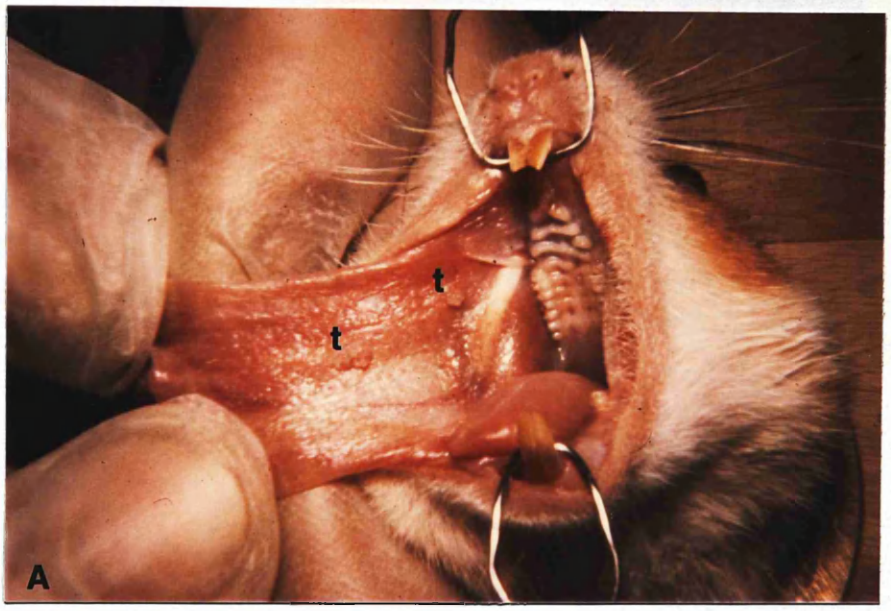
$x^2 = 4.726$

$P < 0.05$

Table 3.4 Tumours observed during gross examination of pouches at the times of biopsy and of sacrifice.

For each animal, A is the left cheek pouch and B the right cheek pouch. A + indicates the presence of one or more tumours which are specified as being within or outwith the experimental area of 1 sq. cm.

Group 1, animals 1 - 15 and 26 - 30, received applications of 0.5% DMBA.
Group 2, animals 16 - 25, received applications of 0.25% DMBA.



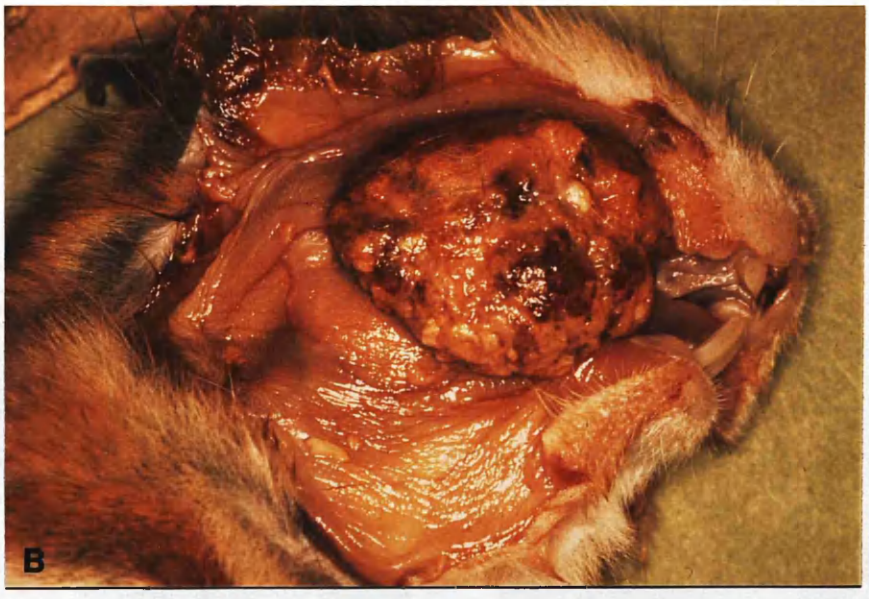


Fig. 3.3 A. Appearance of cheek pouch No. 16B after carcinogen applications and immediately prior to biopsy. Note two small tumours (t).

B. Pouch 16B. The same pouch as shown in 3.3A, at the time of sacrifice of the animal showing a large carcinoma. The time interval between A and B was 7 weeks and the animals received no treatment during this period.

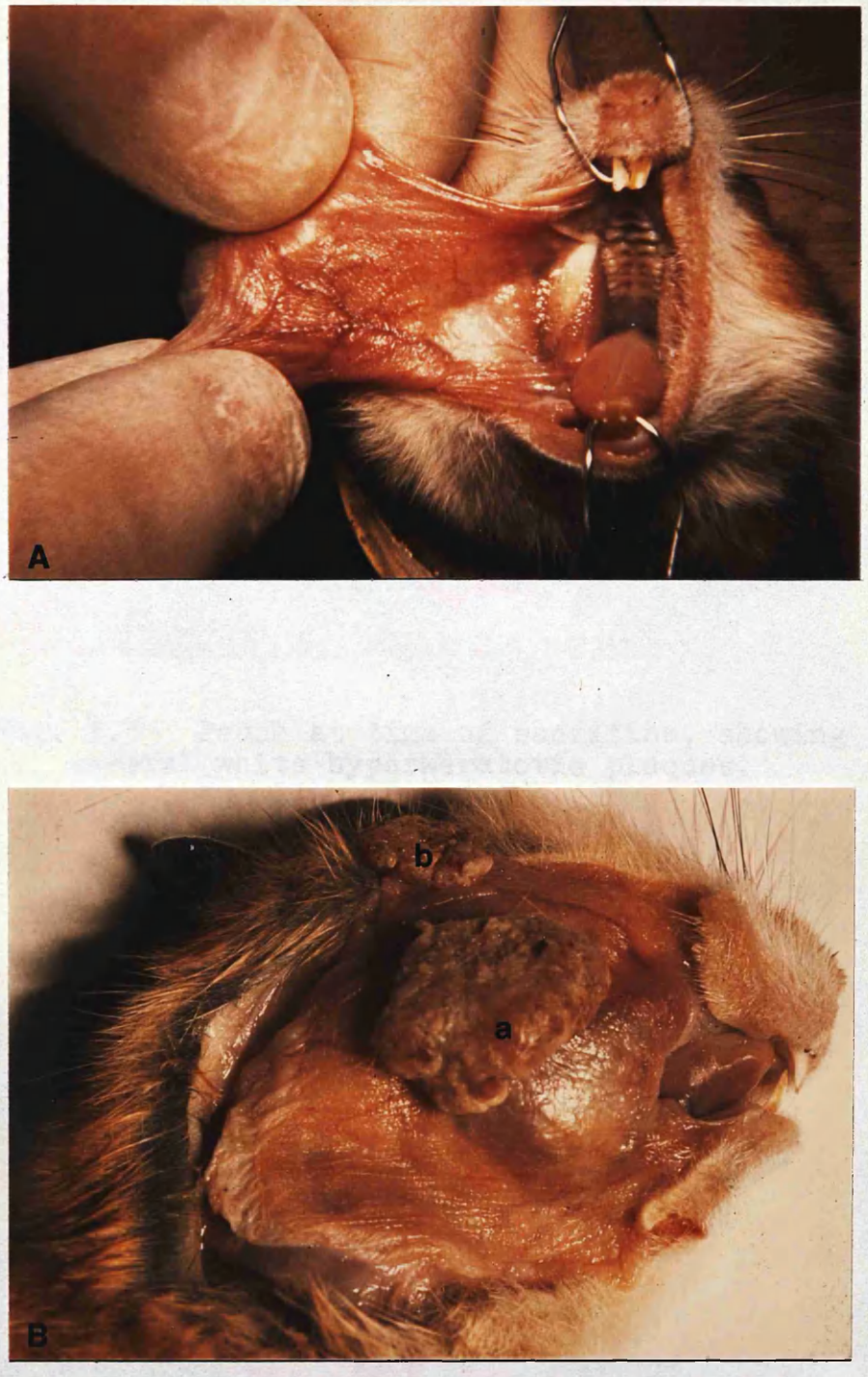


Fig. 3.4 A. Treated cheek pouch immediately prior to biopsy (pouch No. 2B). No obvious abnormality is evident.

B. Pouch 2B. 7 weeks after photograph in 3.4A was taken. The lesions (a) a squamous cell carcinoma and (b) a papilloma, have developed although the pouch has received no interference since the biopsy.

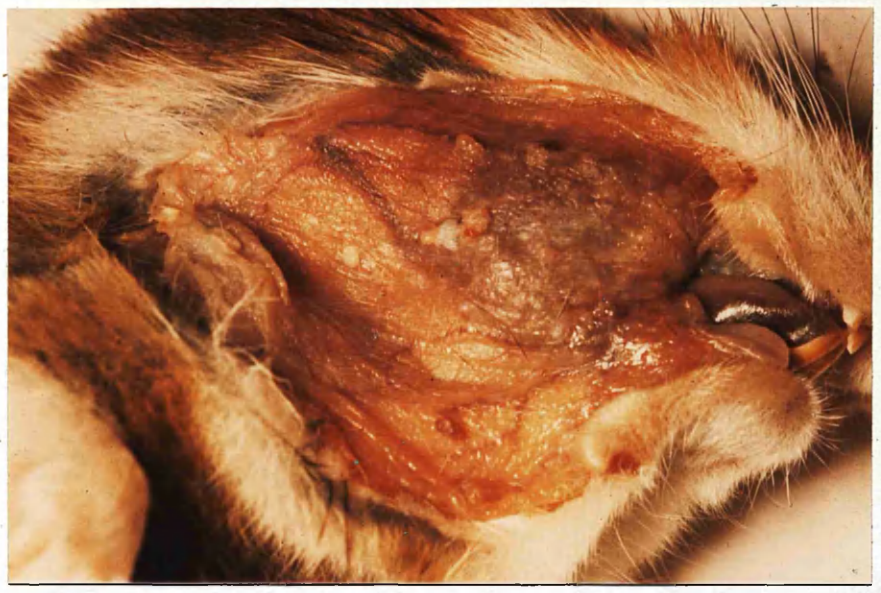


Fig. 3.5 Pouch at time of sacrifice, showing several white hyperkeratotic plaques. (Pouch No. 8B).

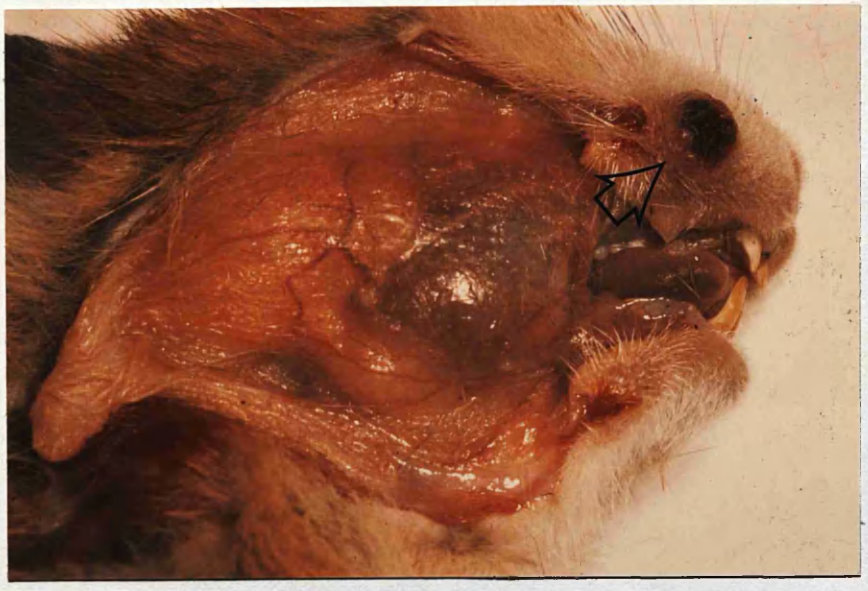


Fig. 3.6 Skin carcinoma (arrow) in animal No. 15

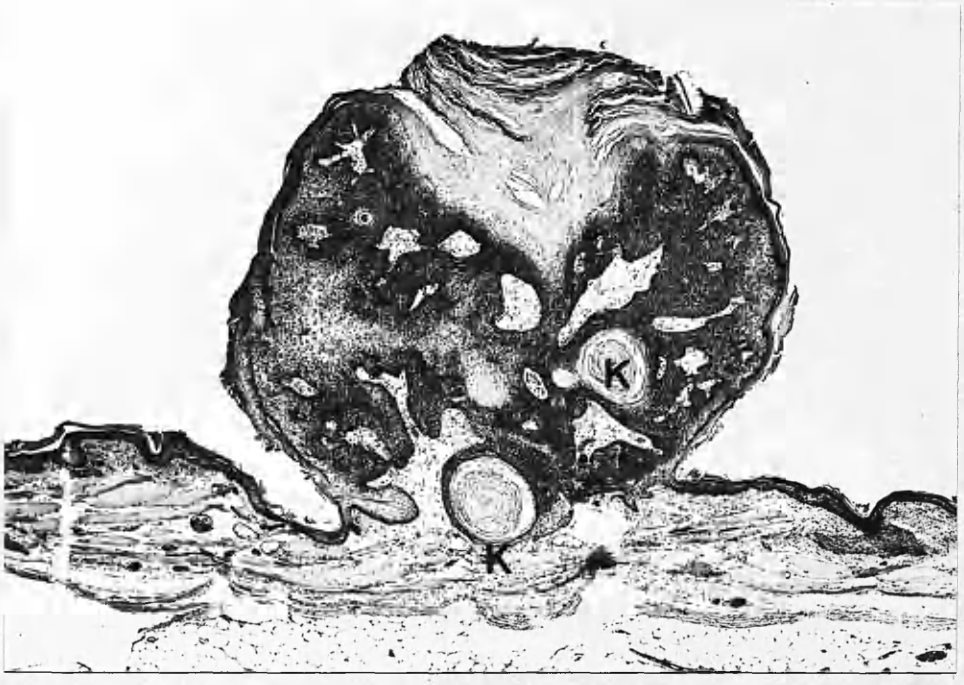


Fig. 3.7 Papilloma showing marked basal cell hyperplasia and keratin pearls (K). x 35.

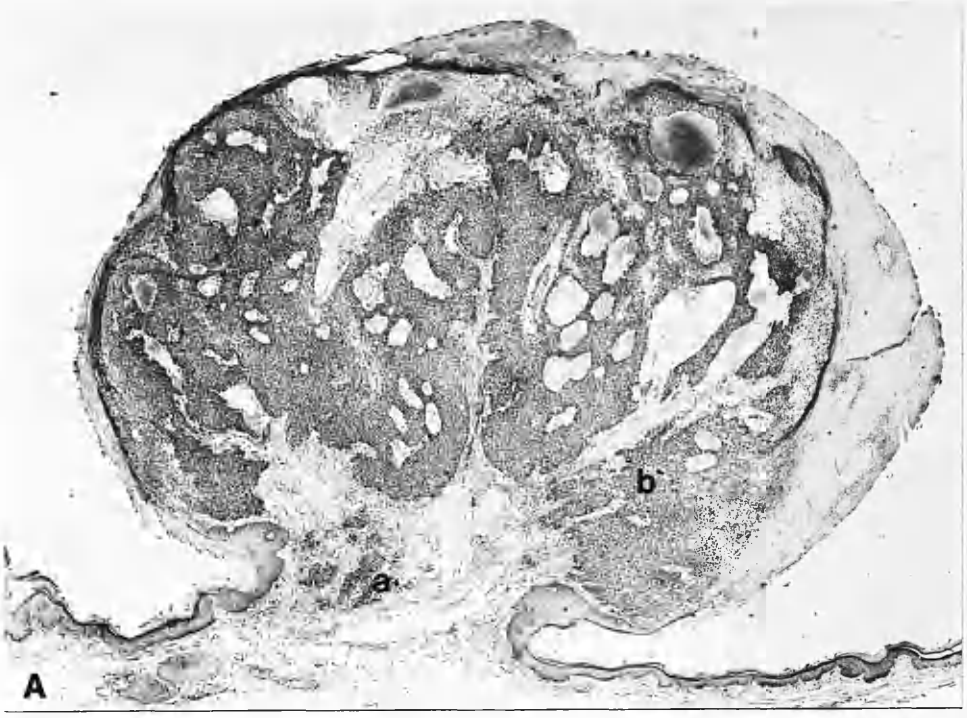
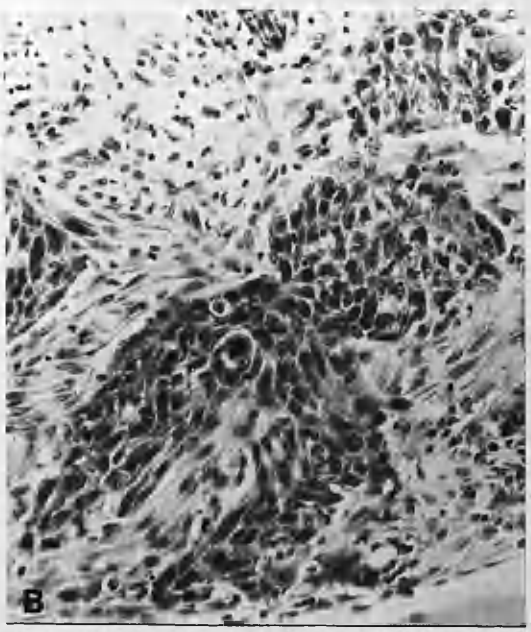
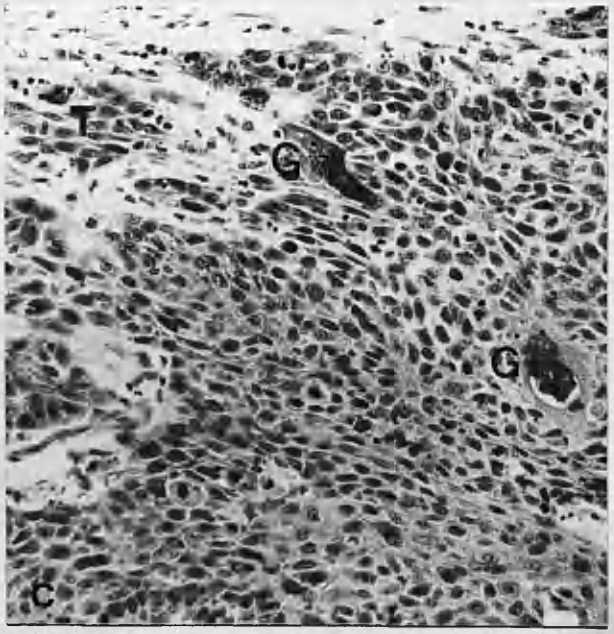


Fig. 3.8 A. Exophytic carcinoma in pouch. x 25.

B. Higher power of area a in 3.8A showing area of early invasion at the base of the tumour. x 210.

C. Higher power of area b in 3.8A. Note the marked cellular atypia with numerous mitotic figures including tripolar mitosis (T) and tumour giant cells (G). x 170.





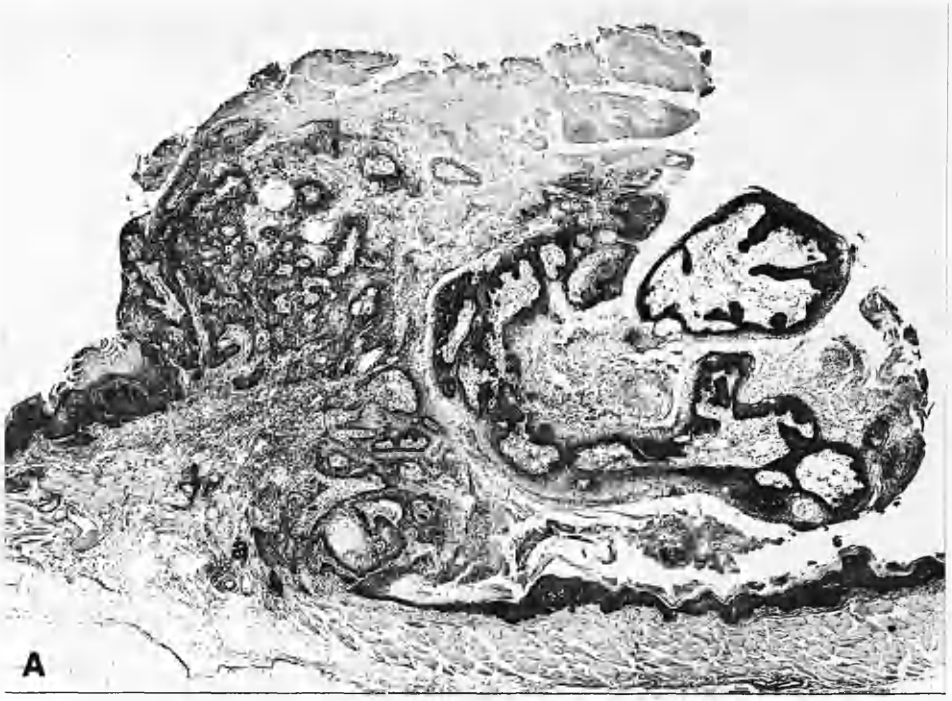
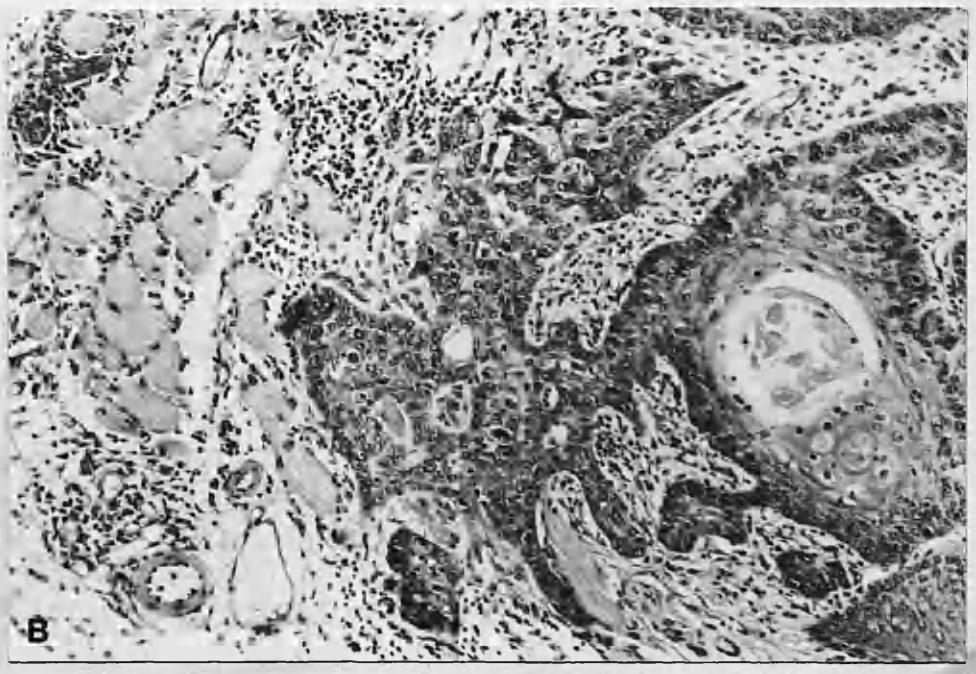


Fig. 3.9A Exophytic carcinoma in pouch. Early invasion of muscle is present in the base of the lesion at a. x 25.

B Higher power of tumour in area a of 3.9A, invading the underlying striped muscle of the pouch. x 130.



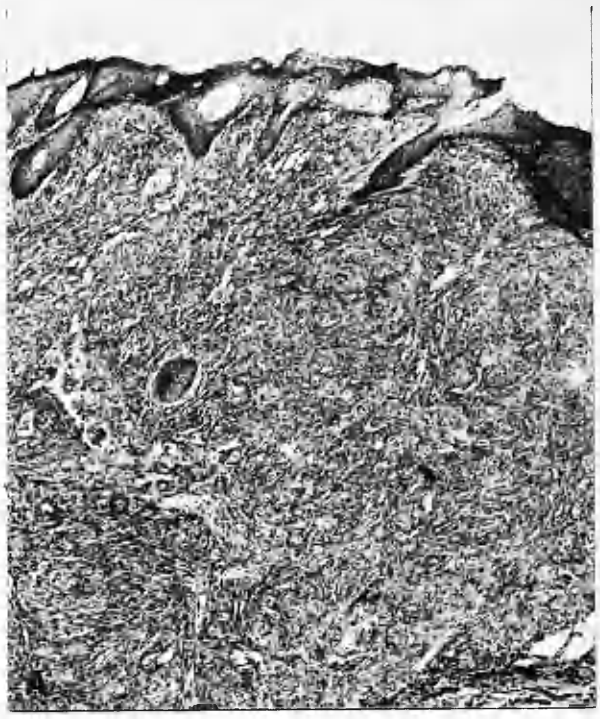
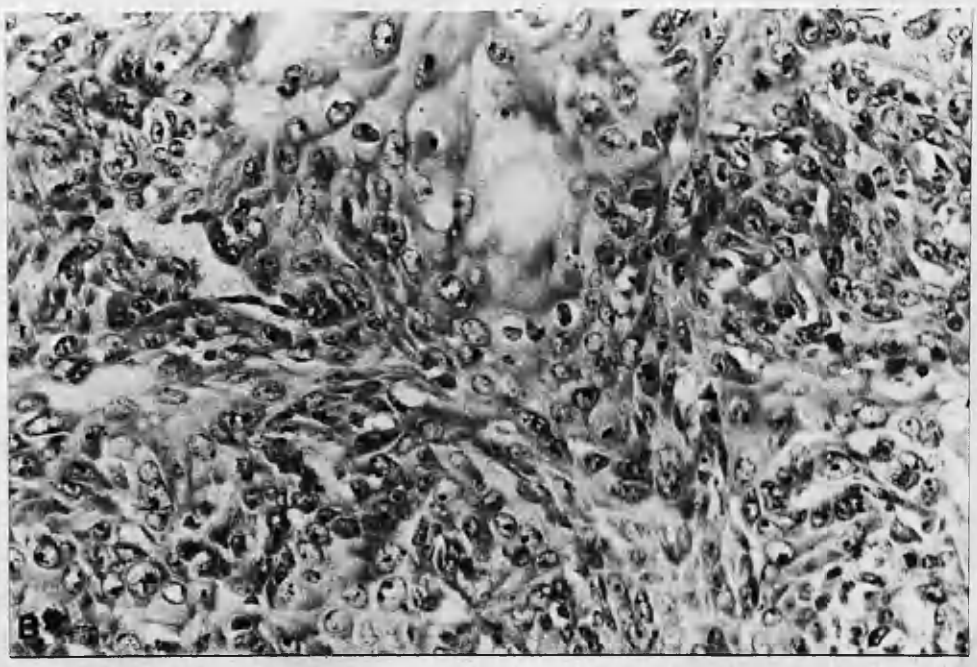


Fig. 3.10 Histological appearances of skin tumour shown in Fig. 3.6

A. Low power view of tumour in dermis. x 36.

B. Higher power showing poorly differentiated squamous cell carcinoma. x 230.



POUCH		ATROPHY	ACANTHOSIS	HYPERORTHO-KERATOSIS	HYPERPARA-KERATOSIS	DISORDERED STRATIFICATION	PREMATURE KERATINISATION	ACANTHOLYSIS
1	A		+	+	+	+	+	+
	B		+	+	+	+	+	+
2	A		+				+	+
	B		+	+		+		
3	A		+	+			+	
	B							
4	A							
	B		+	+			+	+
5	A		+	+				
	B		+	+	+			
6	A		+	+		+	+	+
	B		+	+			+	+
7	A		+					
	B		+	+				
8	A		+	+			+	+
	B							
9	A							
	B							
10	A		+	+				
	B		+	+				
11	A		+	+				
	B		+	+				
12	A	+					+	+
	B							
13	A		+	+				
	B		+	+			+	
15	A		+	+				
	B		+	+				
16	A		+	+				+
	B		+	+	+			
17	A		+	+				
	B		+	+			+	+
18	A		+	+				
	B		+	+				
19	A		+	+			+	+
	B		+	+			+	+
20	A		+	+				
	B		+	+				
21	A		+	+			+	+
	B		+	+				
22	A		+	+	+	+	+	+
	B		+	+				
23	A		+	+			+	+
	B							
24	A		+	+			+	+
	B		+	+				+
25	A							
	B		+	+				
26	A		+	+				
	B		+	+	+	+	+	+
27	A	+	+	+	+		+	+
	B							
28	A		+	+	+		+	+
	B		+	+			+	+
29	A		+	+			+	+
	B		+	+				
30	A		+					
	B		+					
TOTAL	58	2	49	45	7	7	21	22

Table 3.5 Summary of histological changes in biopsy specimens. A + indicates that the feature was present.

POUCH		ATROPHY	ACANTHOSIS	HYPERORTHO-KERATOSIS	HYPERPARA-KERATOSIS	DISORDERED STRATIFICATION	PREMATURE KERATINISATION	ACANTHOLYSIS
1	A	+	+	+	+	+	+	+
	B	+	+	+	+	+	+	+
2	A	+	+	+	+	+		+
	B	+	+	+		+	+	
3	A	+	+	+	+		+	+
	B	+	+	+	+	+	+	+
4	A	+	+	+	+		+	+
	B	+	+	+		+	+	+
5	A	+	+	+				+
	B	+	+	+	+	+	+	+
6	A		+	+	+	+	+	+
	B		+	+	+	+	+	+
7	A	+	+	+	+	+	+	+
	B		+	+	+	+	+	+
8	A	+	+	+	+	+	+	+
	B	+	+	+	+	+	+	+
9	A	+	+	+	+		+	+
	B	+	+	+			+	+
10	A		+	+			+	+
	B	+	+	+	+		+	+
11	A	+	+	+	+	+	+	+
	B	+	+	+	+	+	+	+
12	A	+	+	+	+		+	+
	B	+	+				+	+
13	A	+	+	+	+	+	+	+
	B	+	+	+	+	+	+	+
15	A	+	+	+			+	+
	B		+	+			+	+
16	A	+	+	+	+	+		
	B		+	+		+		
17	A	+	+	+	+	+	+	+
	B	+	+	+	+	+	+	+
18	A	+	+	+	+	+	+	+
	B	+	+	+	+		+	+
19	A	+	+	+	+	+	+	+
	B	+	+	+	+	+	+	+
20	A	+	+	+	+	+	+	+
	B	+	+	+			+	+
21	A		+	+	+	+	+	+
	B	+	+	+	+	+	+	+
22	A	+	+	+	+			
	B	+	+	+			+	+
23	A	+	+	+	+		+	+
	B	+	+	+	+	+	+	+
24	A	+	+	+	+	+	+	+
	B	+	+	+	+	+	+	+
25	A	+	+	+		+	+	+
	B	+	+	+		+	+	+
26	A	+	+	+				+
	B	+	+	+			+	+
27	A	+	+	+	+	+	+	+
	B	+	+	+		+	+	+
28	A	+	+	+	+	+	+	+
	B	+	+	+			+	+
29	A	+	+	+	+	+	+	+
	B	+	+	+	+	+	+	+
30	A	+	+	+	+	+	+	+
	B	+	+	+	+	+	+	+
TOTAL	58	51	58	57	41	39	52	54

Table 3.6 Summary of histological changes observed in the specimens of the experimental area at the time of sacrifice.

	BIOPSY			SACRIFICE		
	No. of pouches	% of pouches	95% confidence limits of %	No. of pouches	% of pouches	95% confidence limits of %
ATROPHY	2	3.4	0 - 8.2	51	87.59	79.4 - 96.5
ACANTHOSIS	49	84.5	75.0 - 94.0	58	100	
HYPERORTHO-KERATOSIS	45	77.59	66.6 - 88.5	57	98.3	94.9 - 100
HYPERPARA-KERATOSIS	7	12.1	3.5 - 20.6	41	70.7	58.7 - 82.6
DISORDERED STRATIFICATION	7	12.1	3.5 - 20.6	39	67.2	54.9 - 79.6
PREMATURE KERATINISATION	21	36.2	23.6 - 48.8	52	89.7	81.7 - 97.7
ACANTHOLYSIS	22	37.9	25.2 - 50.7	54	93.1	86.4 - 99.8

Table 3.7 Comparison of the frequency with which some histological changes were observed in biopsy and sacrifice specimens.

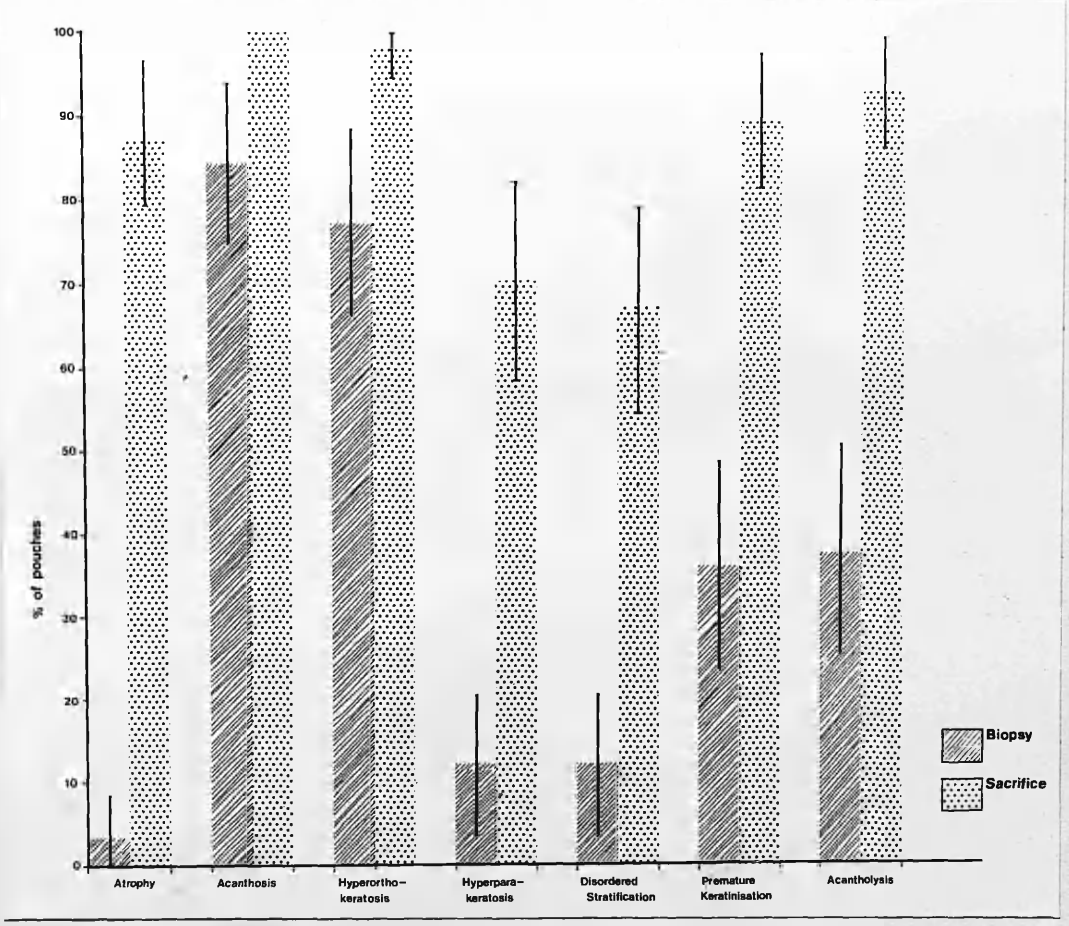


Fig. 3.11 Histogram showing the difference in percentage incidence of histological changes as detailed in Table 3.7. The range marks on each column represent the 95 per cent confidence limits of the percentages shown.

POUCH		ATYPIA SCORE	RANK	CARCINOMA	TUMOUR
1	A	44	53		+
	B	40	51		+
2	A	19	23		
	B	48	56	+	+
3	A	9	6.5		
	B	34	48		
4	A	11	9		
	B	25	37.5		
5	A	11	9		
	B	17	17.5		
6	A	33	46.5		+
	B	12	11		+
7	A	32	44		+
	B	50	57		
8	A	54	58		+
	B	30	42		+
9	A	17	17.5		
	B	15	14.5		
10	A	23	31		
	B	7	4		
11	A	25	37.5		
	B	32	44		
12	A	9	6.5		
	B	8	5		
13	A	47	55	+	+
	B	22	27.5		+
15	A	23	31		+
	B	29	41		
16	A	19	23		
	B	45	54	+	+
17	A	18	20.5		
	B	22	27.5		+
18	A	15	14.5	+	+
	B	13	12		+
19	A	11	9		+
	B	32	44		
20	A	24	34.5		
	B	23	31		
21	A	24	34.5		+
	B	25	37.5		
22	A	27	40		+
	B	35	49.5		
23	A	17	17.5		+
	B	3	1		
24	A	17	17.5		
	B	19	23		
25	A	14	13		
	B	5	2.5		
26	A	23	31		
	B	33	46.5		
27	A	20	25.5		
	B	18	20.5		
28	A	20	25.5	+	+
	B	35	49.5		
29	A	23	31		+
	B	25	37.5		
30	A	5	2.5		
	B	41	52		

Mann-Whitney U test

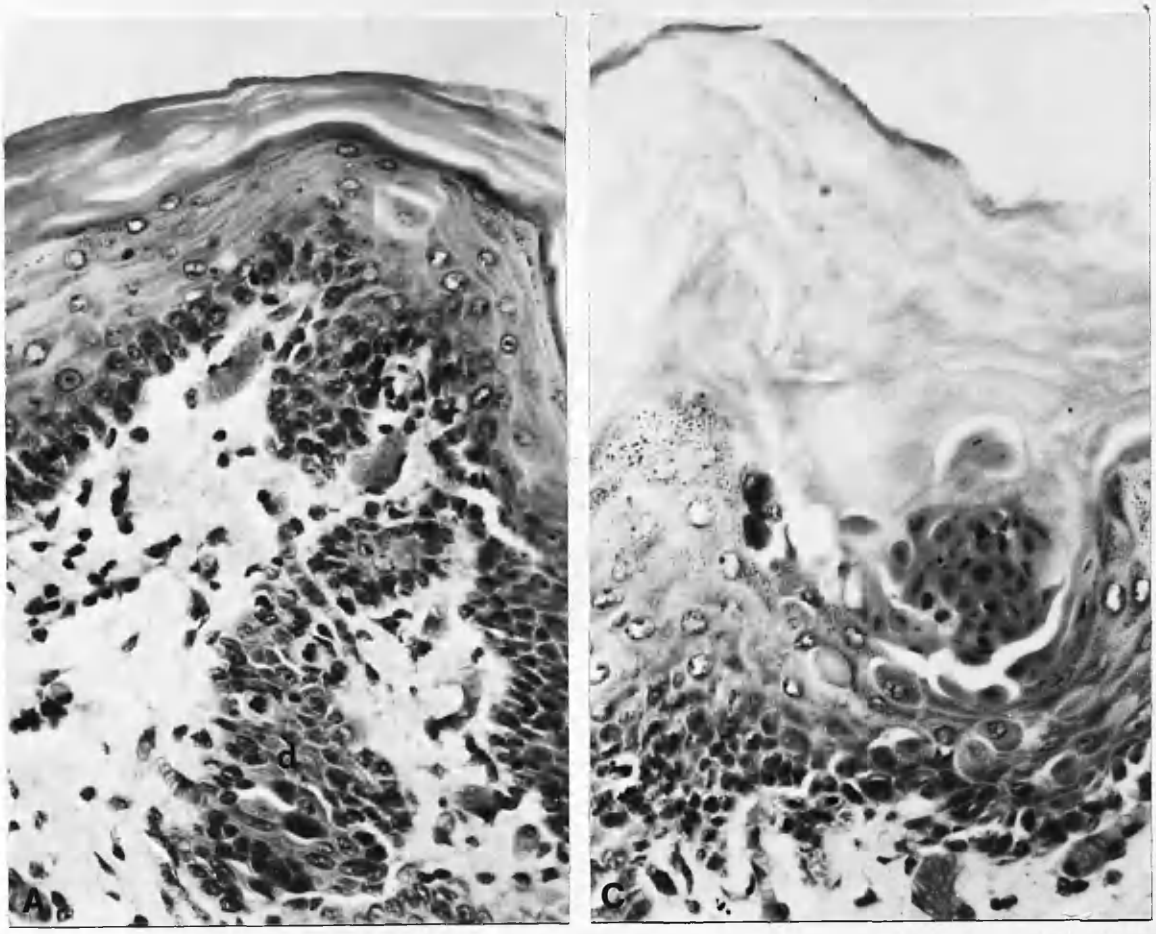
Carcinoma v remainder
P = 0.0559

Any tumour v no tumour
P = 0.0951

Group 1 (0.5% DMBA)
v group 2 (0.25% DMBA)
P = 0.0853

Table 3.8 Cellular atypia scores from one section of each biopsy. A + indicates that by the time of sacrifice a carcinoma or any tumour had developed in the experimental area.

Fig. 4.1 Mild cellular atypia in pouch 18A. There is slight basal cell hyperplasia and irregular stratification of cells. The atypia score of the section of which this is a part is 15. x 300.



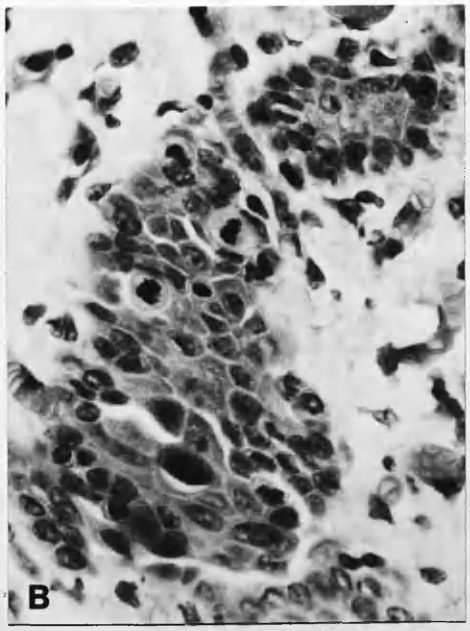


Fig. 4.2 Areas of cellular atypia from the most severely involved section of biopsy of pouch 18A. Atypia score was 59.

A. Note abnormalities in the basal layer and epithelial downgrowth at d. x 300.

B. Higher power of area d showing mitoses and nuclear pleomorphism. x 500.

C. Premature keratinization and acantholysis. x 300.

POUCH		ATYPIA SCORE	RANK	CARCINOMA	TUMOUR
1	A	53	54		+
	B	40	42.5		+
2	A	14	11		
	B	42	46	+	+
3	A	24	22.5		
	B	40	42.5		
4	A	28	25.5		
	B	38	39		
5	A	40	42.5		
	B	11	9.5		
6	A	60	58		+
	B	41	45		+
7	A	28	25.5		+
	B	57	56		
8	A	31	30		+
	B	36	37		+
9	A	20	18.5		
	B	19	16.5		
10	A	9	6.5		
	B	5	3		
11	A	24	22.5		
	B	31	30		
12	A	8	5		
	B	15	12		
13	A	43	47.5	+	+
	B	20	18.5		+
15	A	25	24		+
	B	18	14.5		
16	A	23	21		
	B	50	51	+	+
17	A	53	54		
	B	32	32.5		+
18	A	59	57	+	+
	B	51	52		+
19	A	48	49		+
	B	49	50		
20	A	32	32.5		
	B	18	14.5		
21	A	35	35		+
	B	37	38		
22	A	10	8		
	B	35	35		
23	A	19	16.5		+
	B	7	4		+
24	A	17	13		
	B	29	27.5		
25	A	31	30		
	B	3	2		
26	A	40	42.5		
	B	43	47.5		
27	A	11	9.5		
	B	2	1		
28	A	53	54	+	+
	B	39	40		
29	A	9	6.5		+
	B	29	27.5		
30	A	35	35		
	B	22	20		

By Mann-Whitney U test

Carcinoma v remainder

P = 0.0014

All tumour cases
v no tumour cases

P = 0.0036

Table 4.1 Second analysis of cellular atypia by Smith and Pindborg (1969) technique in hamster cheek pouch lesions.

POUCH		SCORE IN 2nd. ANALYSIS	SCORE IN 1st. ANALYSIS	DIFFERENCE IN ATYPIA SCORES (d)	RANK OF d	RANK WITH LESS FREQUENT SIGN
1	A	53	44	9	26.5	
	B	40	40	0	0	
2	A	14	19	-5	17	17
	B	42	48	-6	20.5	20.5
3	A	24	9	15	38	
	B	40	34	6	20.5	
4	A	28	11	17	42.5	
	B	38	25	13	34	
5	A	40	11	29	48.5	
	B	11	17	-6	20.5	20.5
6	A	60	33	27	47	
	B	41	12	29	48.5	
7	A	28	32	-4	12.5	12.5
	B	57	50	7	23.5	
8	A	31	54	-23	46	46
	B	36	30	6	20.5	
9	A	20	17	3	9	
	B	19	15	4	12.5	
10	A	9	23	-14	36	36
	B	5	7	-2	6	6
11	A	24	25	-1	2	2
	B	31	32	-1	2	2
12	A	8	9	-1	2	2
	B	15	8	7	23.5	
13	A	43	47	-4	12.5	12.5
	B	20	22	-2	6	6
15	A	25	23	2	6	
	B	18	29	-11	31.5	31.5
16	A	33	19	14	36	
	B	50	45	5	17	
17	A	53	18	35	52	
	B	32	22	10	29	
18	A	59	15	44	55	
	B	51	13	38	54	
19	A	48	11	37	53	
	B	49	32	17	42.5	
20	A	32	24	8	25	
	B	18	23	-5	17	17
21	A	35	24	11	31.5	
	B	37	25	12	33	
22	A	10	27	-17	42.5	42.5
	B	35	35	0	0	
23	A	19	17	2	6	
	B	7	3	4	12.5	
24	A	17	17	0	0	
	B	29	19	10	29	
25	A	31	14	17	42.5	
	B	3	5	-2	6	6
26	A	40	23	17	42.5	
	B	43	33	10	29	
27	A	11	20	-9	26.5	26.5
	B	2	18	-16	39	39
28	A	53	20	33	51	
	B	39	35	4	12.5	
29	A	9	23	-14	36	36
	B	29	25	4	12.5	
30	A	35	5	30	50	
	B	22	41	-19	45	45

T = 426.5

Atypia scoring

2nd analysis v 1st analysis

$$z = \frac{T - \frac{N(N+1)}{4}}{\sqrt{\frac{N(N+1)(2N+1)}{24}}}$$

$$= \frac{426.5 - \frac{55(55+1)}{4}}{\sqrt{\frac{55(55+1)(110+1)}{24}}}$$

$$= -2.88$$

$$P = 0.002$$

Table 4.2 Comparison of two analyses of [illegible] in hamster cheek pouch by Wilcoxon matched-pairs signed-rank [illegible]

	HUMAN			HAMSTER		
	None	Slight	Marked	None	Slight	Marked
1. Drop shaped rete ridges	6	8	6	14	5	1
2. Irregular epithelial stratification	0	11	9	0	10	10
3. Keratinization of cells below keratinized layer	11	3	6	8	3	9
4. Basal cell hyperplasia	3	11	6	0	15	5
5. Loss of intercellular adherence	0	13	7	6	7	7
6. Loss of polarity	1	10	9	1	10	9
7. Hyperchromatic nuclei	8	11	1	10	10	0
8. Increased nucleo-cytoplasmic ratio	1	13	6	0	7	13
9. Anisocytosis and anisonucleosis	2	12	6	0	15	5
10. Pleomorphic cells and nuclei	0	12	8	1	14	5
11. Mitotic activity	3	5	12	2	14	4
12. Level of mitotic activity	15	5	0	7	13	0
13. Presence of bizarre mitoses	6	7	7	6	4	10

Table 4.3 Analysis of cellular atypia in human and hamster lesions indicating the numbers of cases in individual categories used to compute the atypia scores.

Drop shaped rete ridges	None	Slight + marked
Human	6	14
Hamster	14	6

$X^2 = 6.4$
$P < 0.02$

Loss of intercellular adherence	None	Slight + marked
Human	0	20
Hamster	6	14

By Fisher exact probability test P = 0.01

Increased nucleo-cytoplasmic ratio	None + slight	Marked
Human	14	6
Hamster	7	13

$X^2 = 4.91$
$P < 0.05$

Increased mitotic activity	None + slight	Marked
Human	8	12
Hamster	16	4

$X^2 = 6.67$
$P < 0.01$

Level of mitotic activity	Normal	Lower ½ only
Human	15	5
Hamster	7	13

$X^2 = 6.46$
$P < 0.02$

Table 4.4 Analysis of five histological features in which a significant difference between human and hamster lesions was demonstrated.

	VARIABLES	
DIAGNOSES	1	Squamous cell carcinoma
	2	Premalignant lesion
	3	Papilloma
	4	Papillary hyperplasia of palate
	5	Hyperplasia
HISTOLOGICAL FEATURES		
Keratinization	6	Hyperorthokeratosis
	7	Hyperparakeratosis
	8	Keratin pearls
	9	Premature individual cell keratinization
	10	Semikeratinization
Cellular Organisation	11	Acanthosis
	12	Focal atrophy
	13	Ulceration
	14	Basal cell hyperplasia and loss of polarity
	15	Basal cell budding
	16	Loss of orderly maturation above basal layer
	17	Anisocytosis in basal layer
	18	Anisocytosis above basal layer
	19	Anisonucleosis in basal layer
	20	Anisonucleosis above basal layer
	21	Intercellular oedema
	22	Intracellular oedema - basal layer
	23	Intracellular oedema - above basal layer
	24	Acantholysis
Mitotic Activity	25	Elevated mitotic activity
	26	Abnormal mitosis
	27	Normal mitosis above basal layer
Connective Tissue	28	Acute inflammatory cell infiltration
	29	Chronic inflammatory cell infiltration with very few or no plasma cells
	30	Chronic inflammatory cell infiltration with numerous plasma cells
Morphology	31	Papillary

Table 4.5 Histological features recorded in 48 human oral mucosal lesions.

CASE NUMBER	DIAGNOSES 1	2	3	4	5	HISTOLOGICAL FEATURES 6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1					+		+				+												+				+		+		
2		+				+	+	+	+	+	+		+	+	+	+	+	+	+	+		+	+		+	+	+	+		+	
3					+		+				+		+										+				+	+	+		
4			+			+		+	+		+			+		+						+	+					+		+
5					+	+			+			+				+	+	+	+	+	+	+	+						+		
6				+					+		+	+				+					+	+	+					+		+	+
7	+							+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	
8					+		+		+		+	+		+								+				+		+		
9	+							+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	
10					+	+	+		+			+					+		+		+	+						+		
11					+	+			+		+	+		+	+	+	+	+	+	+	+	+	+					+		
12					+	+						+									+	+						+		
13					+	+	+				+			+							+	+	+					+		
14	+					+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+		+	
15		+				+		+	+		+	+	+	+	+	+	+	+	+	+	+							+	+	+	
16					+		+		+	+	+										+	+							+		
17			+				+				+			+	+	+	+	+	+	+	+		+		+		+		+		+
18					+	+	+				+		+	+							+	+	+		+		+	+	+		
19					+	+	+		+		+			+	+						+	+	+		+	+	+		+		
20	+								+		+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+		+	
21					+	+			+		+	+				+					+	+	+			+			+		
22				+			+	+			+	+		+	+						+	+	+				+	+		+	+
23			+			+			+	+	+	+		+		+					+	+	+		+	+	+	+		+	+
24					+	+					+			+	+							+								+	
25			+			+	+			+	+			+												+	+		+		+
26					+		+		+		+		+	+		+	+		+										+		
27					+		+		+	+	+	+		+			+	+	+	+	+								+		
28	+					+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+			+			+	+		+	
29					+		+			+	+							+		+					+				+		
30					+	+			+	+	+			+	+	+		+		+	+	+	+		+				+		
31					+		+			+	+			+									+						+		
32					+	+	+			+	+	+											+					+	+		
33					+	+	+				+	+											+	+				+		+	
34					+	+			+	+	+		+	+		+	+		+				+					+		+	
35			+								+			+									+	+				+		+		+
36					+	+	+		+		+	+		+			+		+				+				+	+		+		+
37				+			+	+	+		+	+		+	+	+						+	+	+	+		+	+			+	+
38					+	+	+				+			+		+	+	+	+	+						+	+	+		+		
39					+	+			+	+	+			+														+		+		
40		+					+				+			+	+	+	+	+	+	+	+						+	+		+		
41	+					+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+		+	+	+
42	+							+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+		
43	+							+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+		
44		+				+	+	+	+	+	+	+		+		+	+	+	+	+	+	+	+	+	+	+	+	+	+			
45	+						+	+	+		+	+		+		+	+	+	+	+	+	+	+			+			+			
46	+					+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+		+		
47	+							+	+		+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+			+		
48	+					+	+		+		+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+			+		
TOTALS	12	4	5	3	24	27	29	16	32	13	45	28	15	37	21	28	25	23	25	23	28	36	33	9	15	22	34	18	27	21	10

Table 4.6 Features of 48 human oral mucosal lesions. A + indicates the presence of the feature.

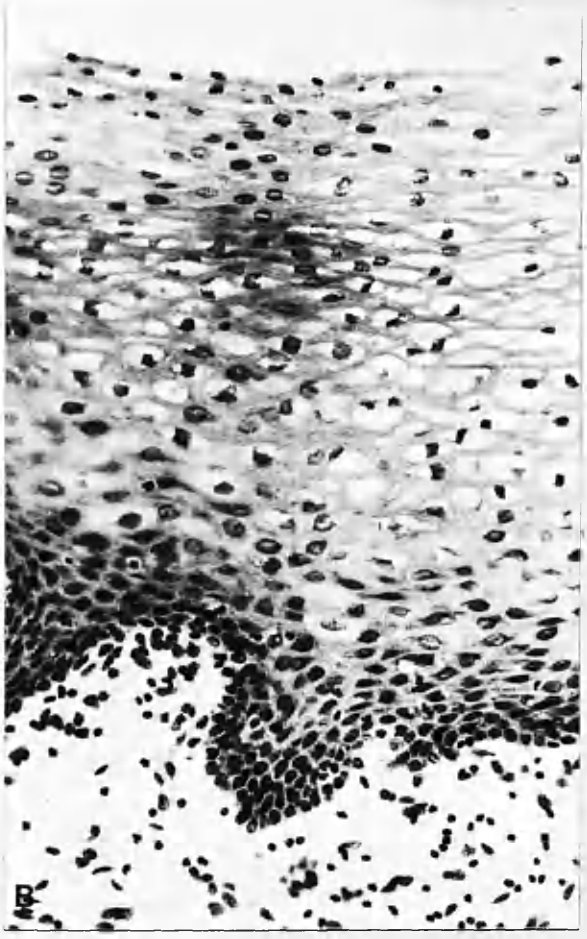
See Table 4.5 for details of the diagnostic groups 1 - 5 and histological features 6 - 31.

		CARCINOMA	PREMALIGNANT	PAPILLOMA	PAPILLARY HYPERPLASIA	HYPERPLASIA
Keratinization						
6	Hyperorthokeratosis				-.2927*1	
8	Keratin pearls	.6123*6				-.7372*6
9	Premature individual cell keratinization	.4082*5				-.3243*1
Cellular Organisation						
12	Focal atrophy	.4879*6				-.3876*4
13	Ulceration	.5449*6				-.3430*3
14	Basal cell hyperplasia	.3148*1				-.4237*5
15	Basal cell budding	.5576*6				-.4991*6
16	Loss of orderly maturation	.4879*6				-.5568*6
17	Anisocytosis in basal layer	.5537*6	.2891*1			-.3356*2
18	Anisocytosis above basal layer	.6019*6	.3143*1			-.4156*5
19	Anisonucleosis in basal layer	.5537*6	.2891*1			-.3356*2
20	Anisonucleosis above basal layer	.6019*6	.3143*1			-.4156*5
21	Intercellular oedema	.4879*6				-.3876*4
23	Intracellular oedema above basal layer	.3892*4				-.4667*6
24	Acantholysis	.5855*6				-.5008*6
Mitotic Activity						
25	Elevated mitotic activity	.4411*5				-.3430*3
26	Abnormal mitosis	.6276*5				-.6246*6
27	Normal mitosis above basal layer					-.3402*3
Connective Tissue						
28	Acute inflammation	.5465*6				-.6352*6
29	Chronic inflammation without plasma cells	-.5576*6			-.2927*1	.5832*6
30	Chronic inflammation with plasma cells	.5576*6			.2927*1	-.5832*6
Morphology						
31	Papillary			.6647*6	.5033*6	-.3294*2

The values indicated are r values and the * figure indicates the level of significance as under.

Levels of significance (P) *1 significant at P = 0.05
*2 " " P = 0.025
*3 " " P = 0.02
*4 " " P = 0.01
*5 " " P = 0.005
*6 " " P = 0.001

Table 4.7 Histological features which showed a significant correlation with the five diagnostic categories of human oral mucosal lesions.



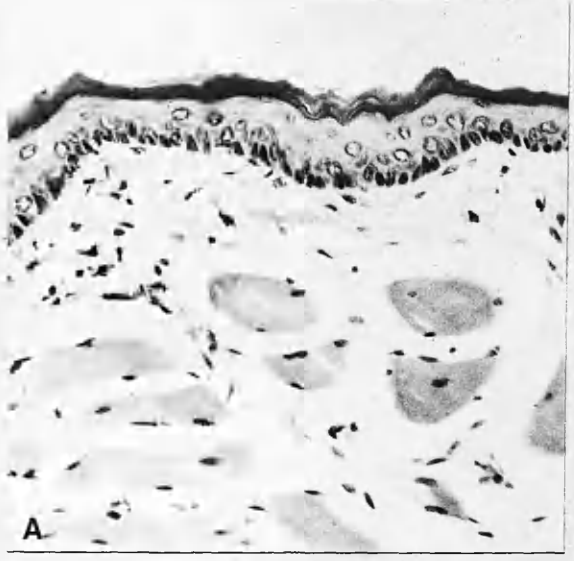


Fig. 4.3 Comparison of normal hamster cheek pouch (A) with normal human buccal mucosa (B) and normal human palatal mucosa (C). These prints are all at a magnification of x 230.

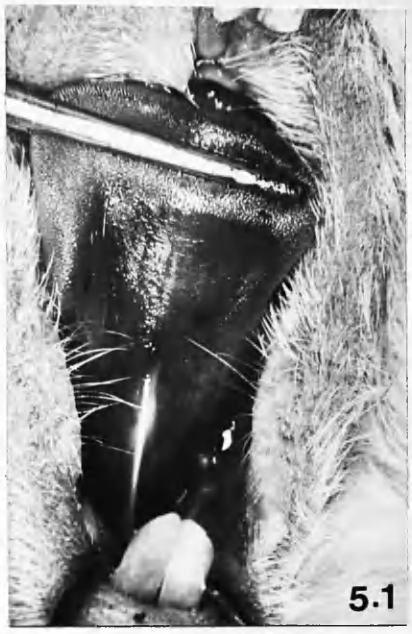
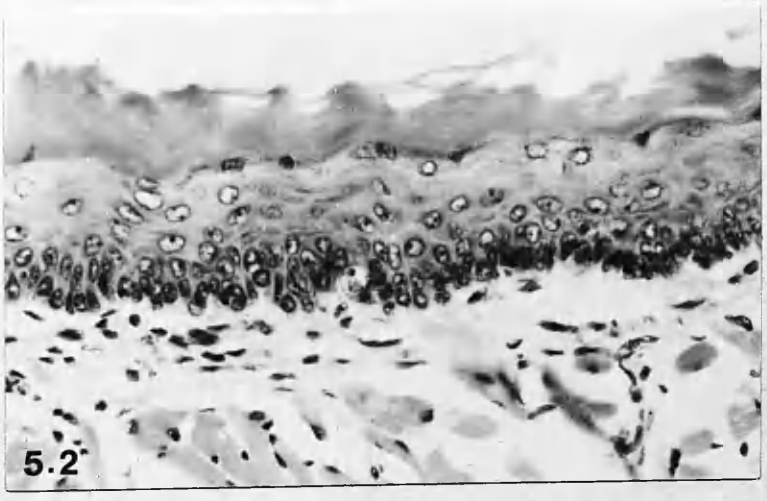
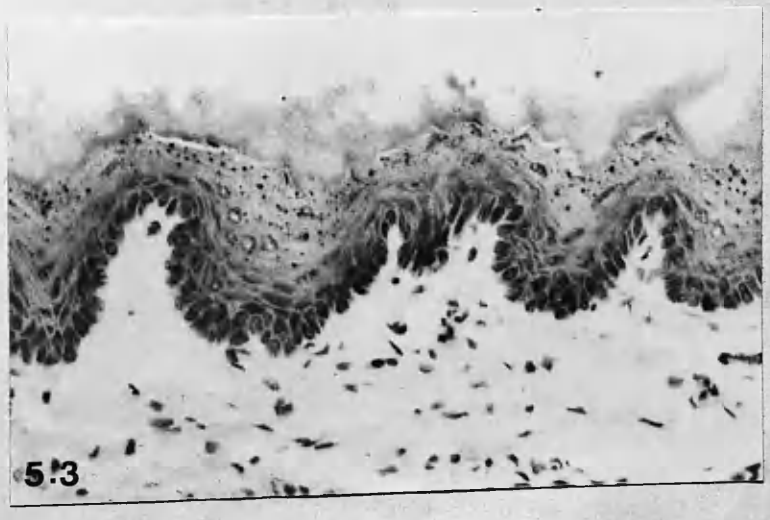


Fig. 5.1 Ventral surface of normal hamster tongue (with tattoo marks).

Fig. 5.2 Ventral surface of normal hamster tongue. x 310.

Fig. 5.3 Normal lingual gingiva of hamster. x 310.





A

HAMSTER No.	CARCINOGEN (weeks)	ACANTHOSIS	HYPERKERATOSIS	INFLAMMATION	ATYPIA SCORE
1	6	+		+	7
2	6	+		+	15
3	7	+		+	34
4	7	+		+	26
5	8			+	23
6	9			+	13
7	9	+	+	+	22
8	10	+	+	+	43

B

ANIMAL	CARCINOGEN EXPOSURE (weeks)	RANK 1	ATYPIA SCORE	RANK 2	d RANK 1 - 2	d^2
1	6	1.5	7	1	0.5	0.25
2	6	1.5	15	3	-1.5	2.25
3	7	3.5	34	7	-3.5	12.25
4	7	3.5	26	6	-2.5	6.25
5	8	5	23	5	0	0
6	9	6.5	13	2	4.5	20.25
7	9	6.5	22	4	2.5	6.25
8	10	8	43	8	0	0

$$r_s = 1 - \frac{6 \sum_{i=1}^{N} d_i^2}{N^3 - N}$$

$$= 1 - \frac{6 \times 47.5}{504}$$

$$= 0.4345$$

$$P > 0.05$$

Table 5.1 A. Histological changes observed after 6 - 10 weeks of carcinogen applications to the ventral surface of the tongue.

B. Correlation of atypia scores of ventral tongue epithelium with duration of carcinogen exposure.

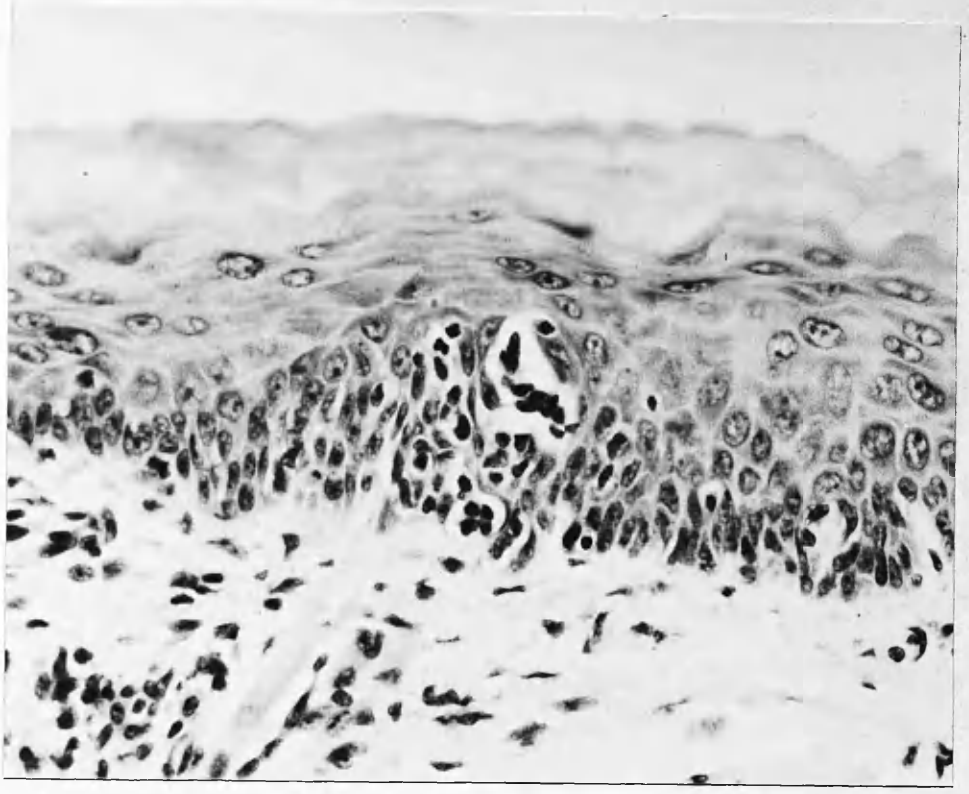


Fig. 5.4 Ventral surface of hamster tongue showing infiltration of mononuclear cells following carcinogen applications. x 440.

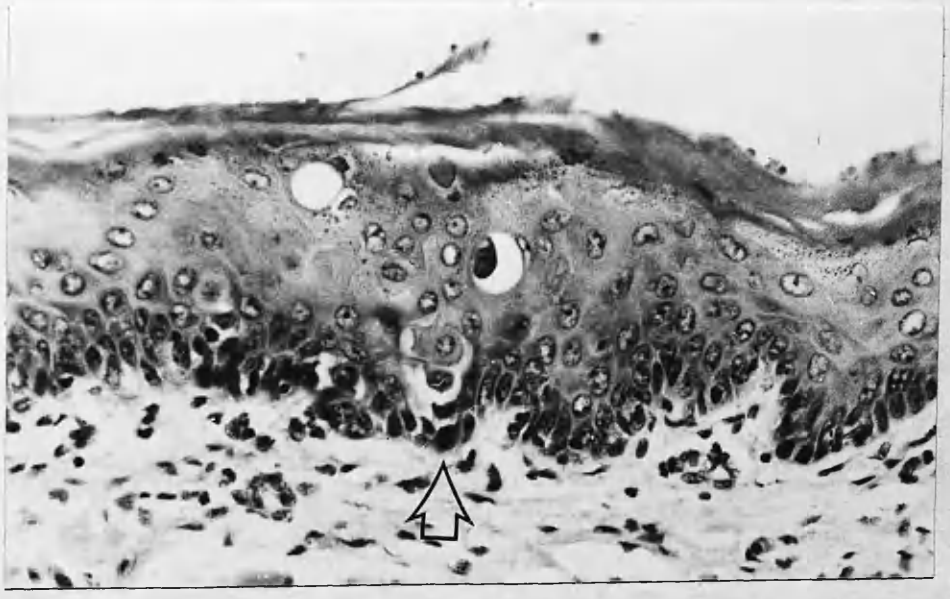


Fig. 5.5 Focus of acantholysis and premature keratinization (arrow) observed in ventral tongue epithelium after 6 weeks of carcinogen applications. x 390.

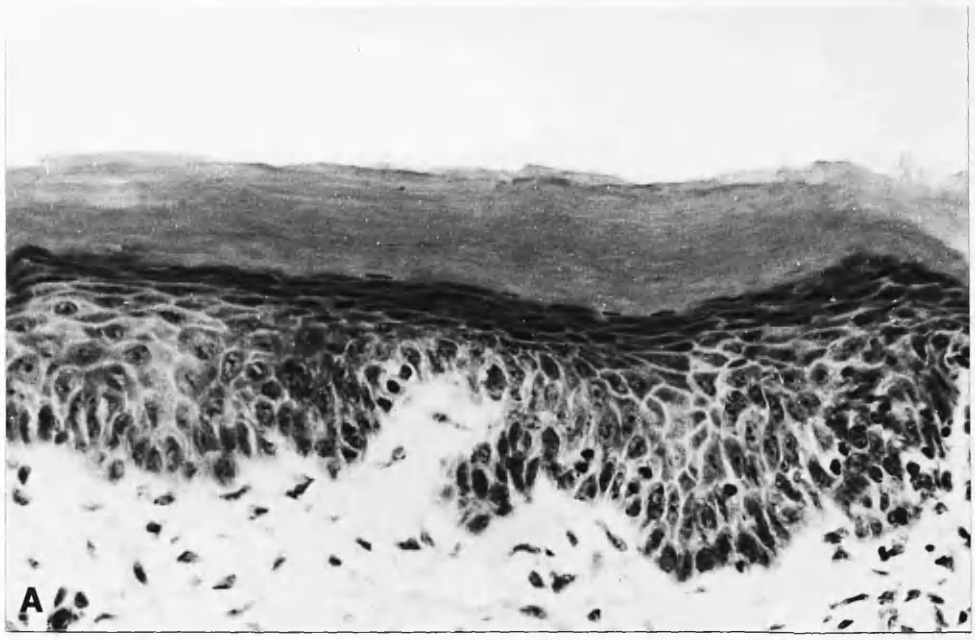


Fig. 5.6A Orthokeratotic patch of ventral tongue epithelium following 9 weeks of carcinogen applications. Basal cell hyperplasia, increased mitotic activity, irregular stratification and loss of polarity are evident. x 350.

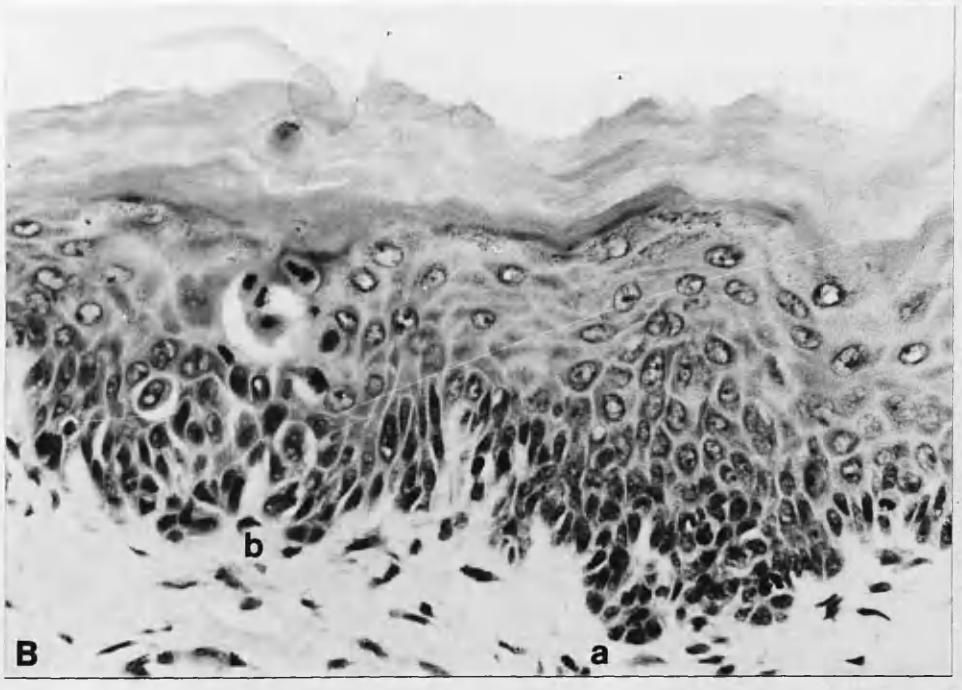


Fig. 5.6B Ventral tongue epithelium after 10 weeks of carcinogen applications. Basal cell hyperplasia, loss of polarity and irregular stratification are evident above a and premature keratinization and acantholysis are present above b. x 400.

ANIMAL	CARCINOGEN (weeks)	ATROPHY G	ATROPHY T	ACANTHOSIS G	ACANTHOSIS T	HYPERKERATOSIS G	HYPERKERATOSIS T	INFLAMMATION G	INFLAMMATION T	ATYPIA G	ATYPIA T	ATYPIA SCORE G	ATYPIA SCORE T
1 A	6							+	+				
B	6							+	+				
2 A	6							+	+				
B	6					+	+	+	+	+		13	
3 A	7	+						+	+		+		18
B	7							+	+				
4 A	7			+				+	+	+	+	12	7
B	7			+			+	+	+	+	+	5	3
5 A	8								+	+		22	
B	8							+	+		+		10
6 A	9					+				+		5	
B	9							+	+	+	+	16	5
7 A	9						+	+	+		+		8
B	9							+	+		+		33
8 A	10			+	+	+	+	+	+	+	+	14	12
B	10						+	+	+		+		19

Table 5.2 Histological features seen in lingual gingiva and lateral surface of tongue following 6 - 10 weeks of exposure to carcinogen.

For each animal A is the left side and B the right side.

For each histological feature a + indicates the presence of that feature in the lingual gingiva - G - or the lateral surface of tongue - T.

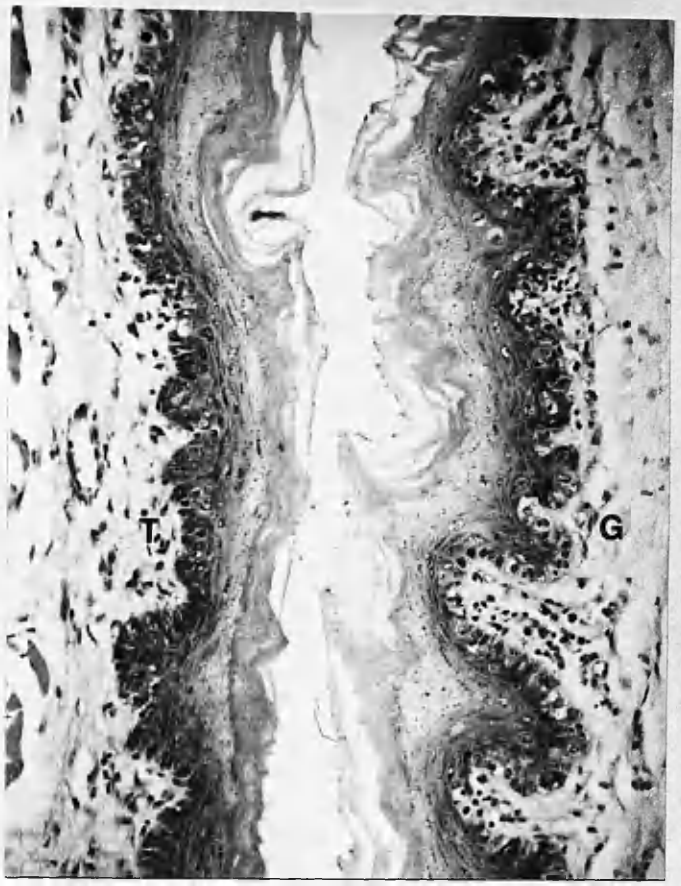


Fig. 5.7 Mononuclear infiltrate in lamina propria of lateral surface of tongue (T) and lingual gingiva (G). This infiltrate is more prominent in the gingiva. x 220.

Fig. 5.8 Papilloma on lingual gingiva following 10 weeks exposure to carcinogen. x 150.

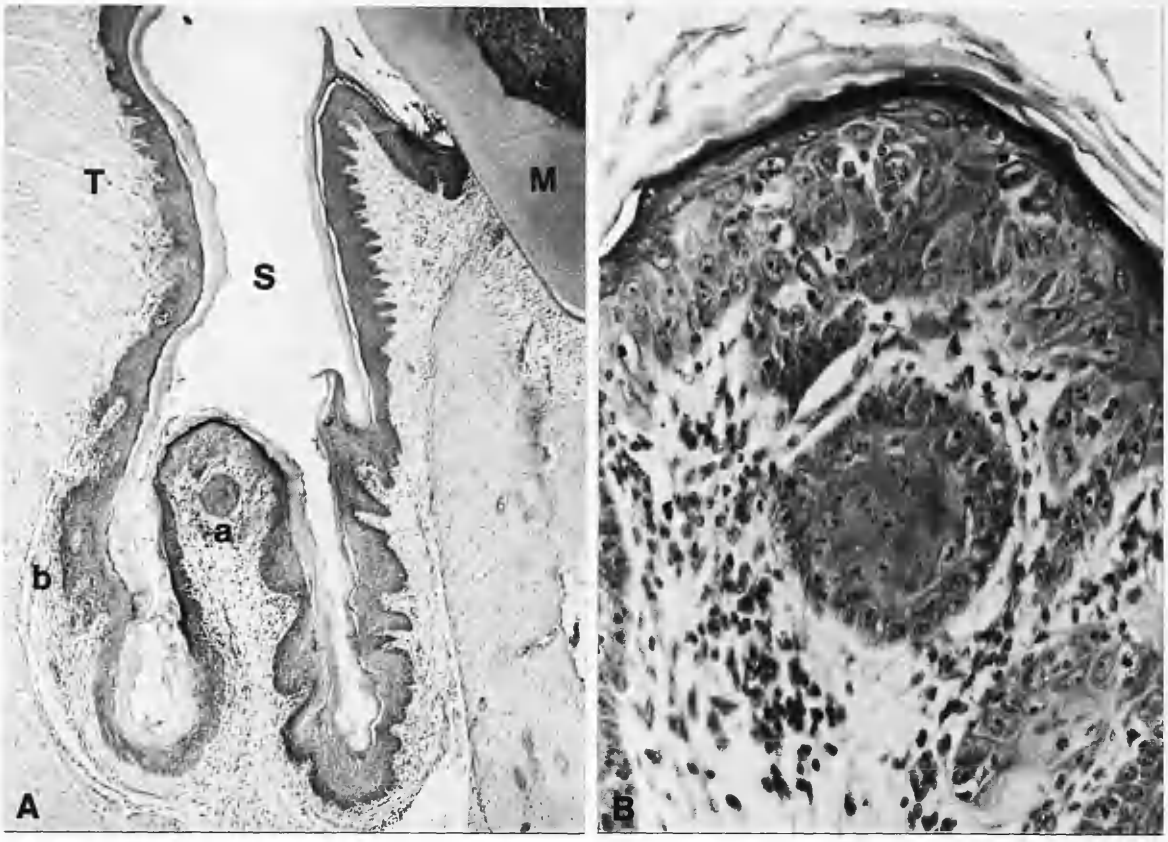
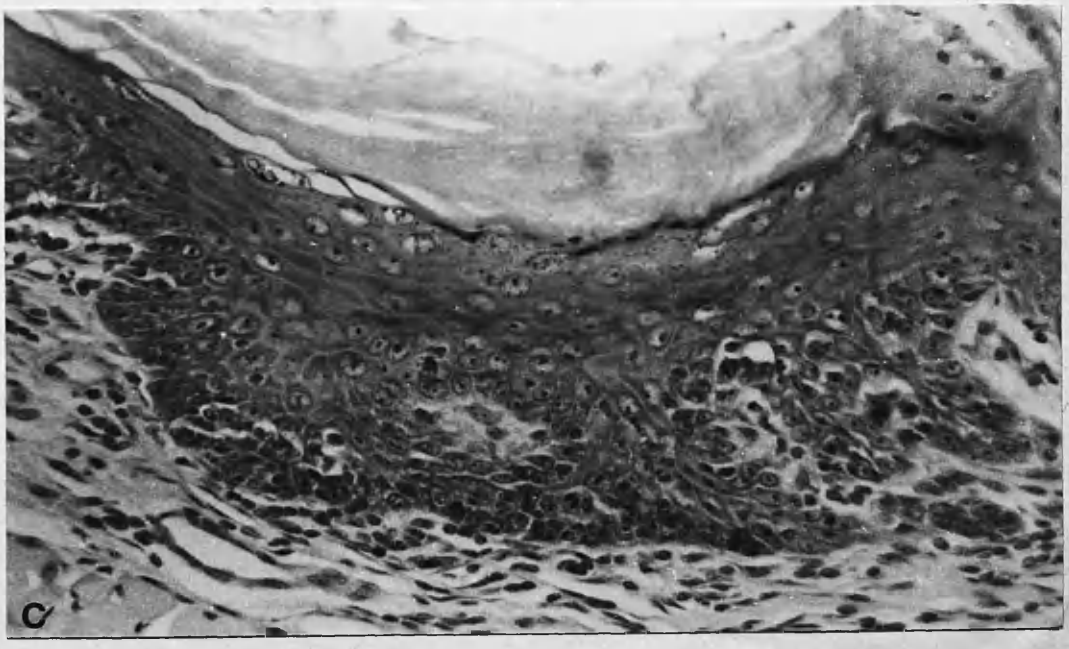


Fig. 5.9 A. Lingual sulcus (S) with adjacent molar tooth (M) and tongue (T). Note areas a and b. x 50.

B. High power of area a in Fig. 5.9A showing moderately severe cellular atypia. x 320.

C. High power of area b in Fig. 5.9A showing moderate cellular atypia. x 340.



Frequency of occurrence of cellular atypia in drainage area

	With atypia	Without atypia
Lower lingual gingiva	7	9
Lateral surface of tongue	9	7

$X^2 = 0.5$
$P > 0.3$

	With atypia	Without atypia
Lower lingual gingiva	7	9
Ventral surface of tongue	8	0

By Fisher exact probability test $P < 0.01$

	With atypia	Without atypia
Lateral surface of tongue	9	7
Ventral surface of tongue	8	0

By Fisher exact probability test $P < 0.01$

Comparison of atypia scores by Mann-Whitney U test

Lower lingual gingiva v lateral surface of tongue $P = 0.3745$

Lower lingual gingiva v ventral surface of tongue $P = 0.0409$

Ventral surface of tongue v lateral surface of tongue $P = 0.1736$

Table 5.3 Comparison of the frequency of occurrence of cellular atypia and atypia scores in three intraoral drainage sites in the hamster.

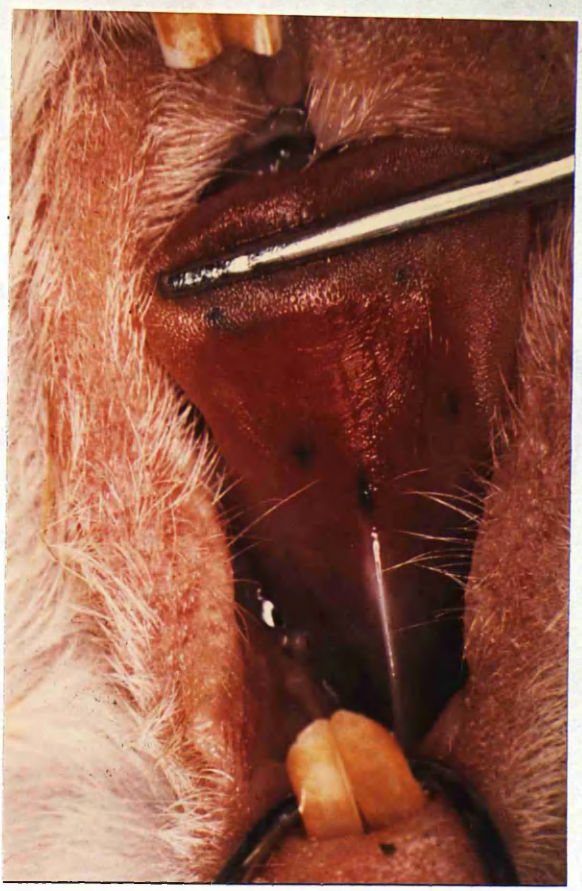


Fig. 6.1 View of ventral surface of hamster tongue showing experimental area demarkated by black tattoo marks.

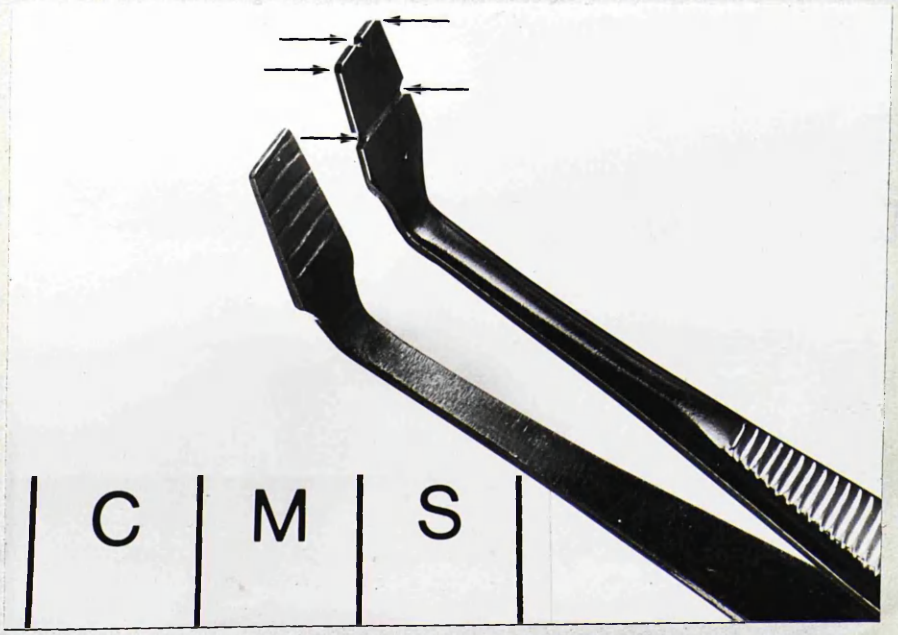


Fig. 6.2 Modified slide forceps for holding tongue and locating the needle for five tattoo points (arrows).

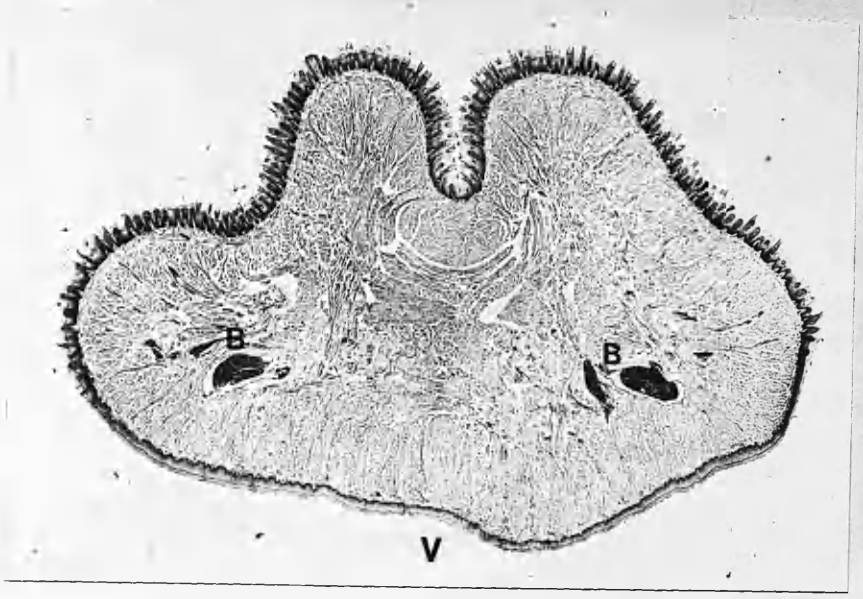


Fig. 6.3 Cross section of hamster tongue. The ventral surface is marked V. Note the prominent thin walled blood vessels (B). x 16.

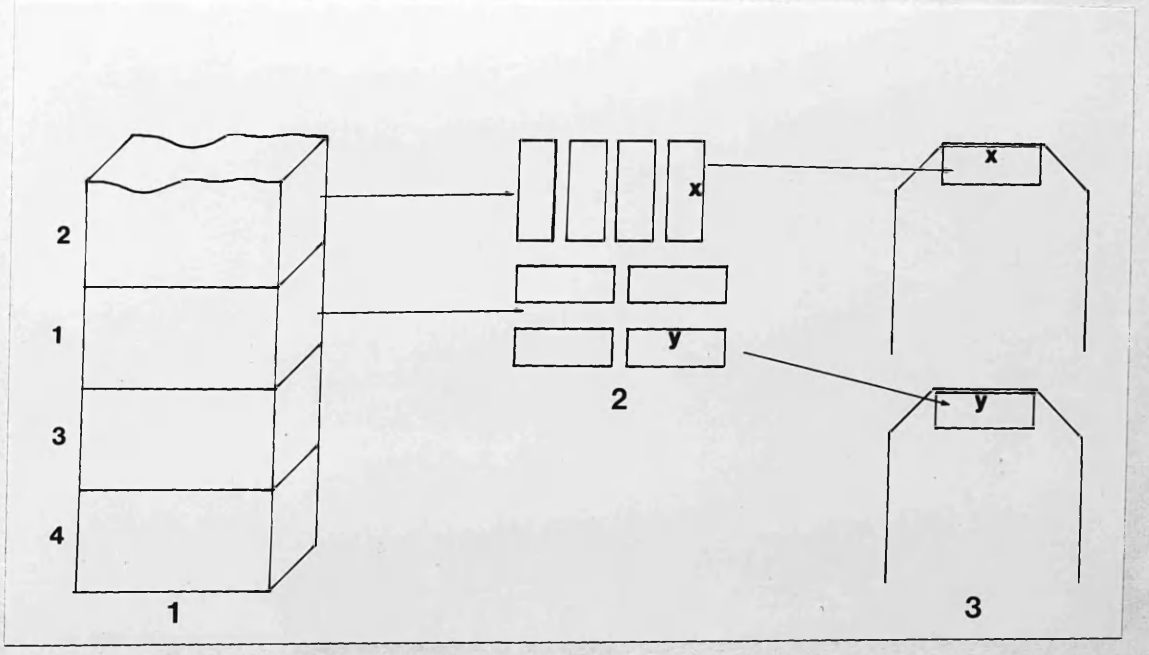


Fig. 6.4 Diagram showing the procedure for taking the blocks from the strip of tongue mucosa (1) taken in the long axis of the tongue and then trimming these into precisely orientated blocks (2) which were embedded to give sections in the long axis (x) or the short axis (y) of the tongue.

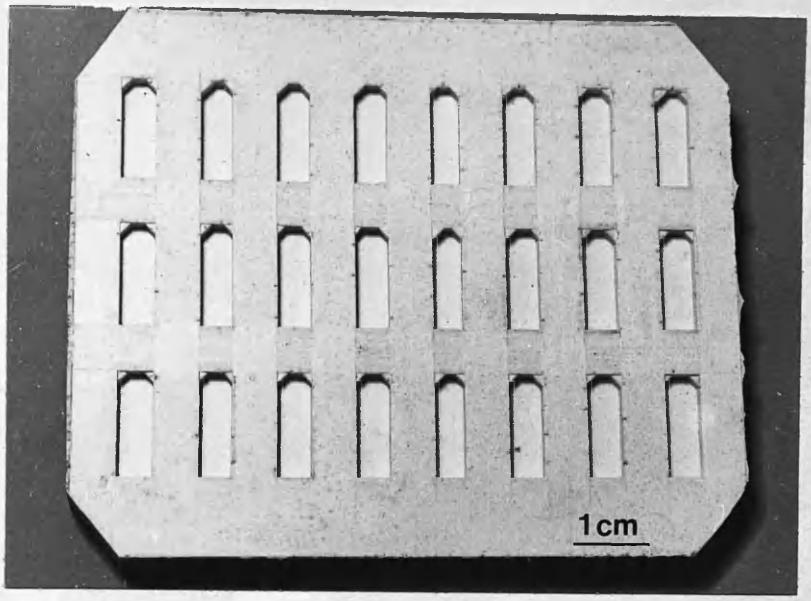


Fig. 6.5 Latex mould for flat embedding specimens in araldite.

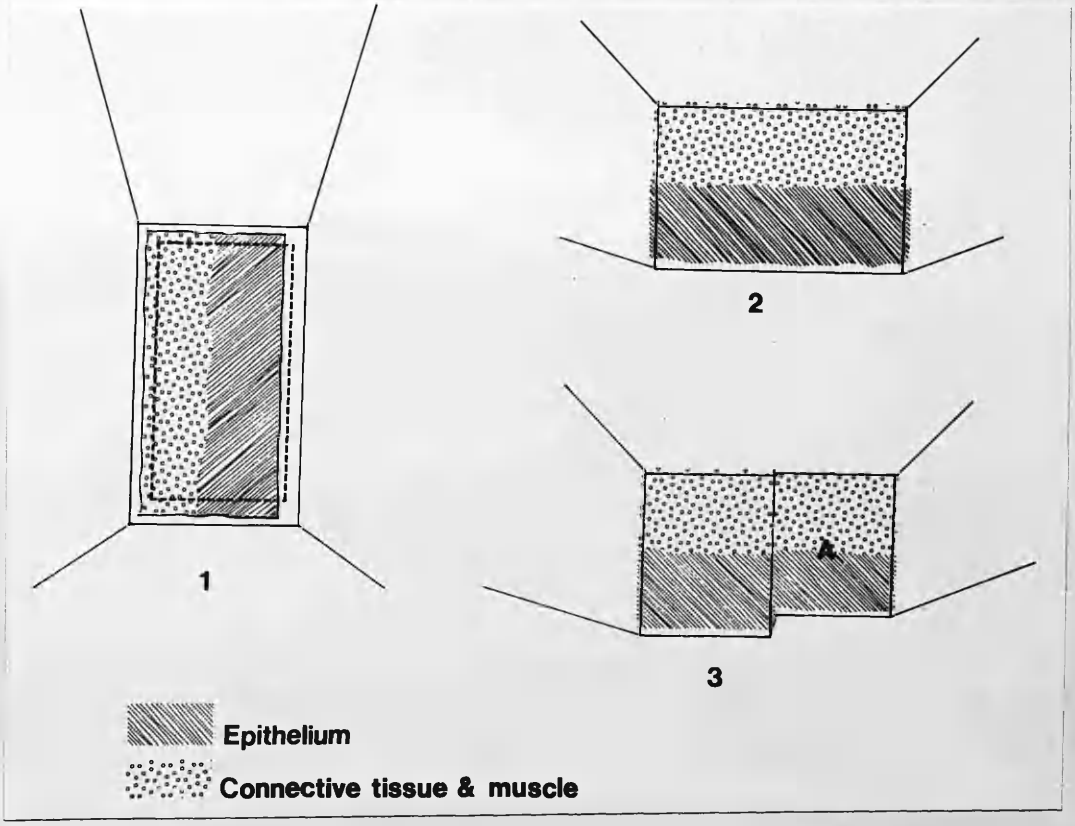


Fig. 6.6 Procedure for section preparation. The block is initially trimmed in the ultromicrotome with the epithelial surface vertical (1). This allows the knife to be set perpendicular to the keratinized surface. The block is then turned (2) and sections for light microscopy obtained. Approximately half the face is then cut back (3,A) and thin sections prepared from the remaining block face.

Fig. 6.7 Electron microscope section placed on copper grid to allow viewing of a column through the epithelium at right angles to the keratinized surface (K). x 600.

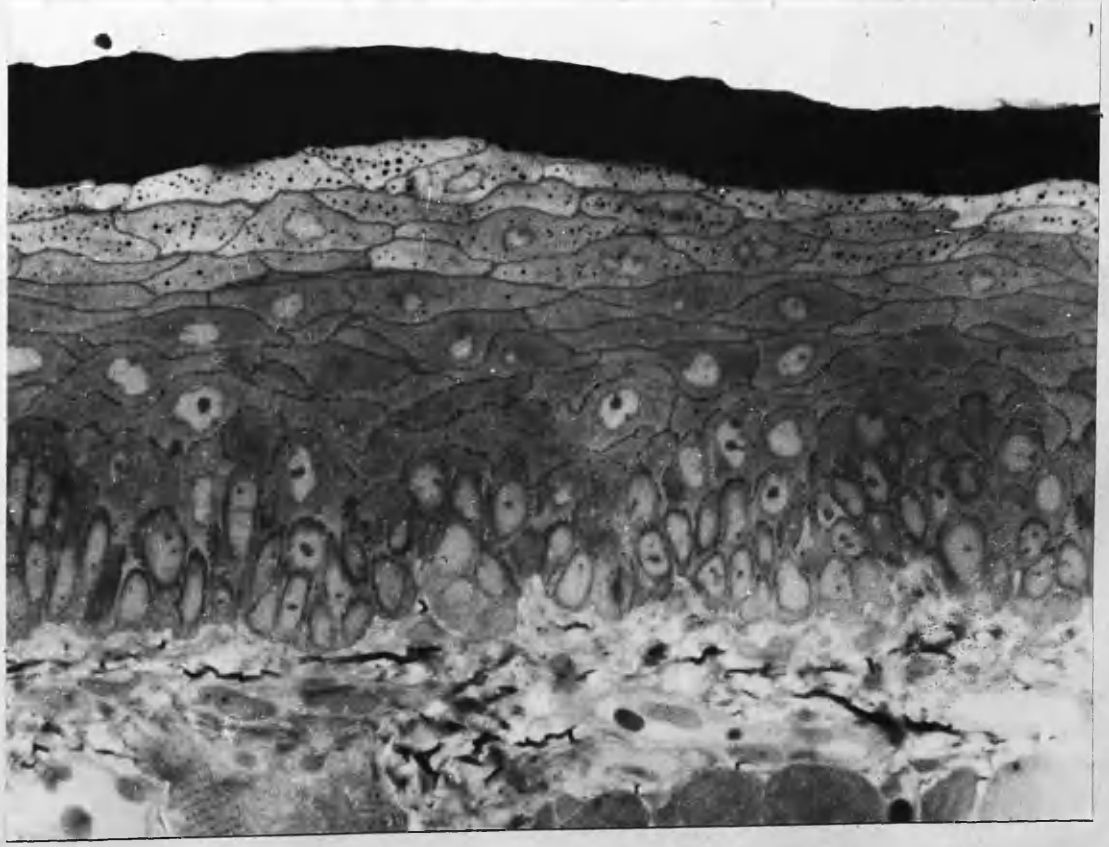


Fig. 6.8 Light microscopic picture of 0.6 µm araldite section of ventral surface of normal hamster tongue. Stained methylene blue in borax. x 1000.

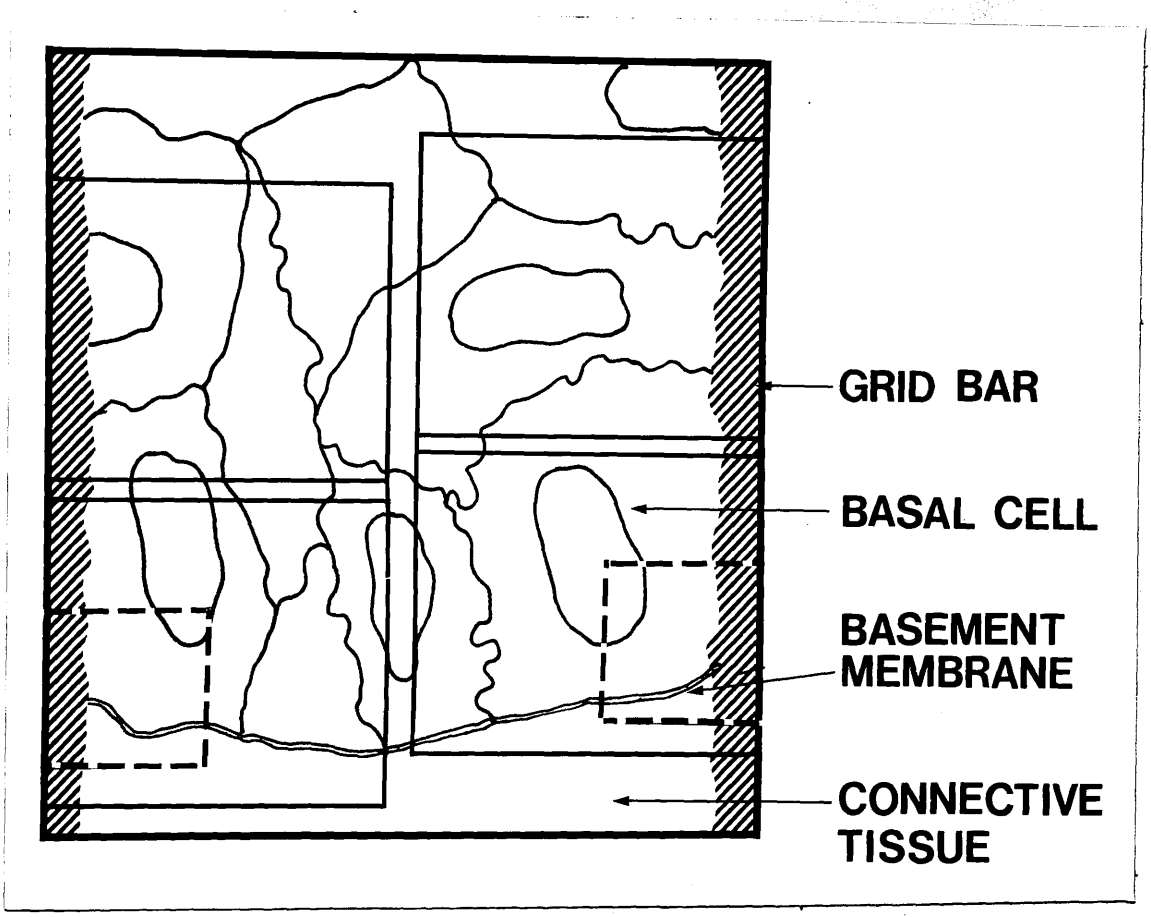


Fig. 6.9 Diagram to show sampling for ultrastructural study. An area of basal cells between adjacent grid bars is depicted and the bold outlines show the areas photographed at a magnification of 5,000 for the level 2 study. The pecked lines indicate the areas photographed at a magnification of 20,000 for the level 3 study.

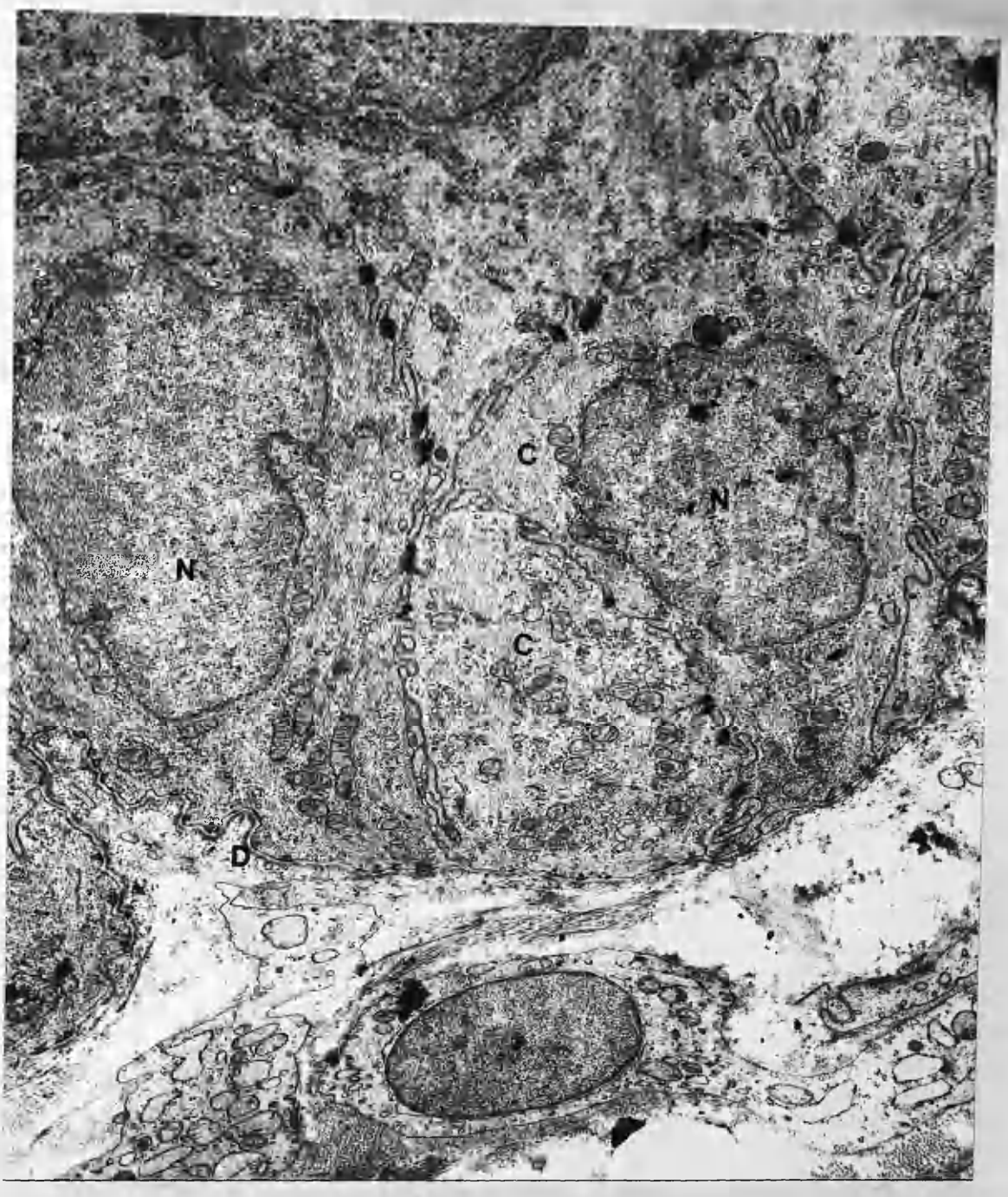


Fig. 6.10 Electron micrograph for level 2 ultrastructural study showing basal epithelial cells. The nuclei (N), cytoplasm (C) and lamina densa (D) are clearly distinguished. Normal hamster tongue ventral epithelium. x 12,500.

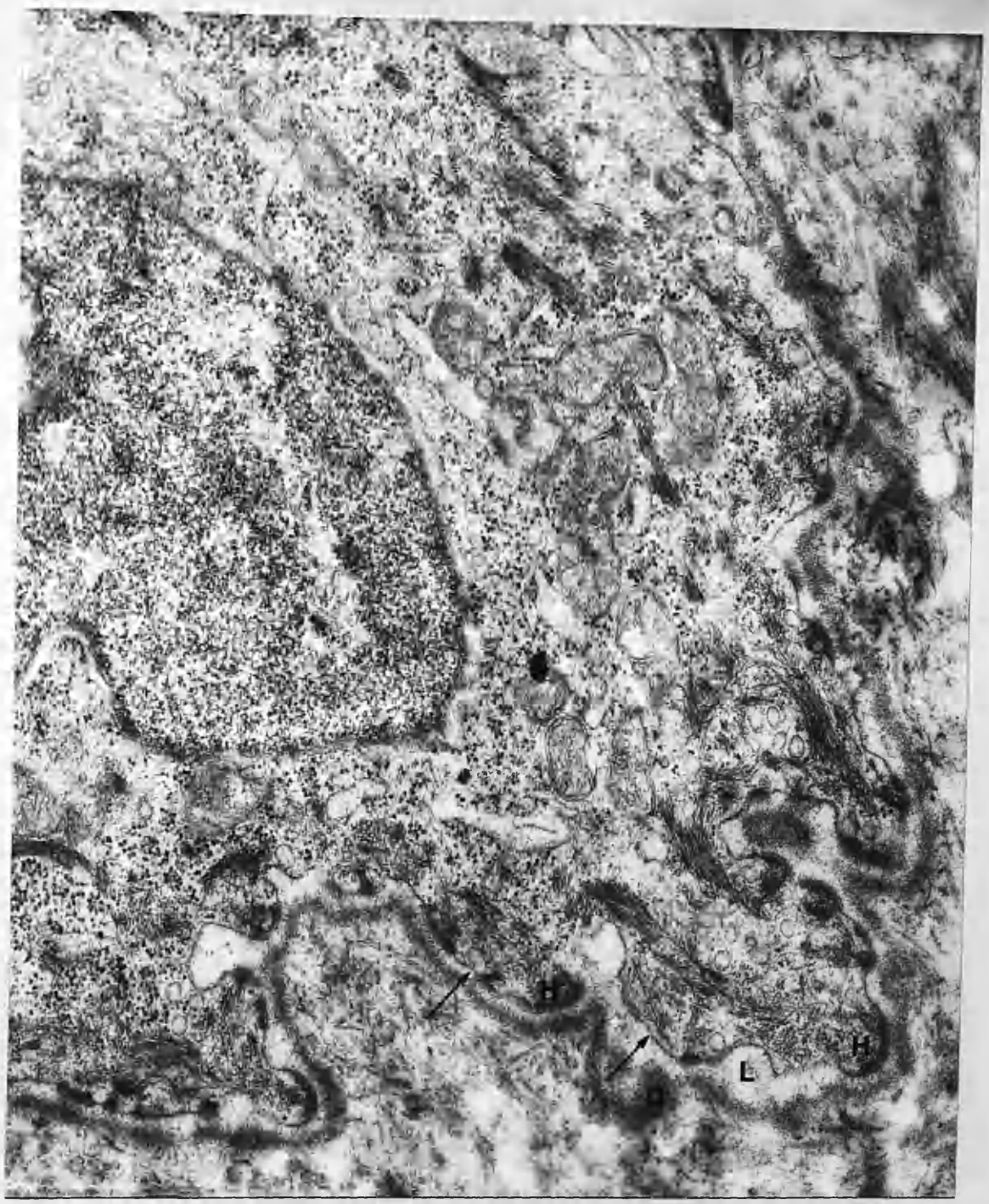


Fig. 6.11 Electron micrograph of basal end of basal cell from normal hamster ventral tongue epithelium, showing lamina densa (D), lamina lucida (L), hemidesmosomes (H) and basal plasma membrane (arrows).
Level 3 ultrastructural study. x 50,000.

Fig. 6.12 Electron micrographs of carcinogen treated hamster ventral tongue epithelium.

A. Pseudopodia (P) in intercellular space between basal cells. x 34,300.

B. Pseudopodia (P) in widened area of lamina lucida. x 37,500.

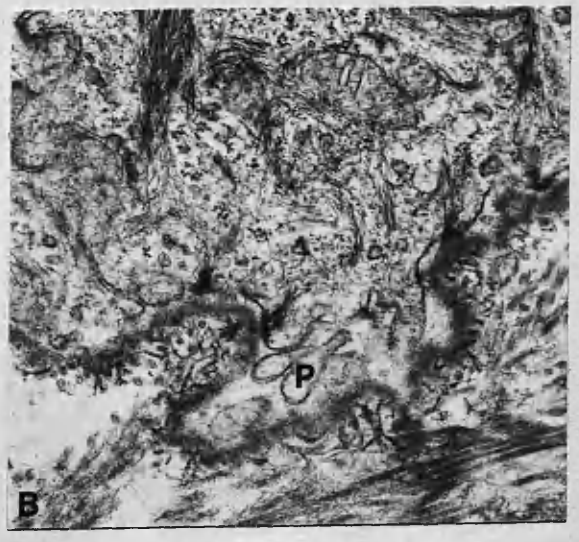


Fig. 6.13A Irregular epithelial connective tissue interface in carcinogen treated hamster ventral tongue epithelium. The lamina densa (D) and lamina lucida (L) are of variable thickness. Reduplication (R) of lamina densa is present and a possible gap (G) in the lamina densa is present.
x 39,250.

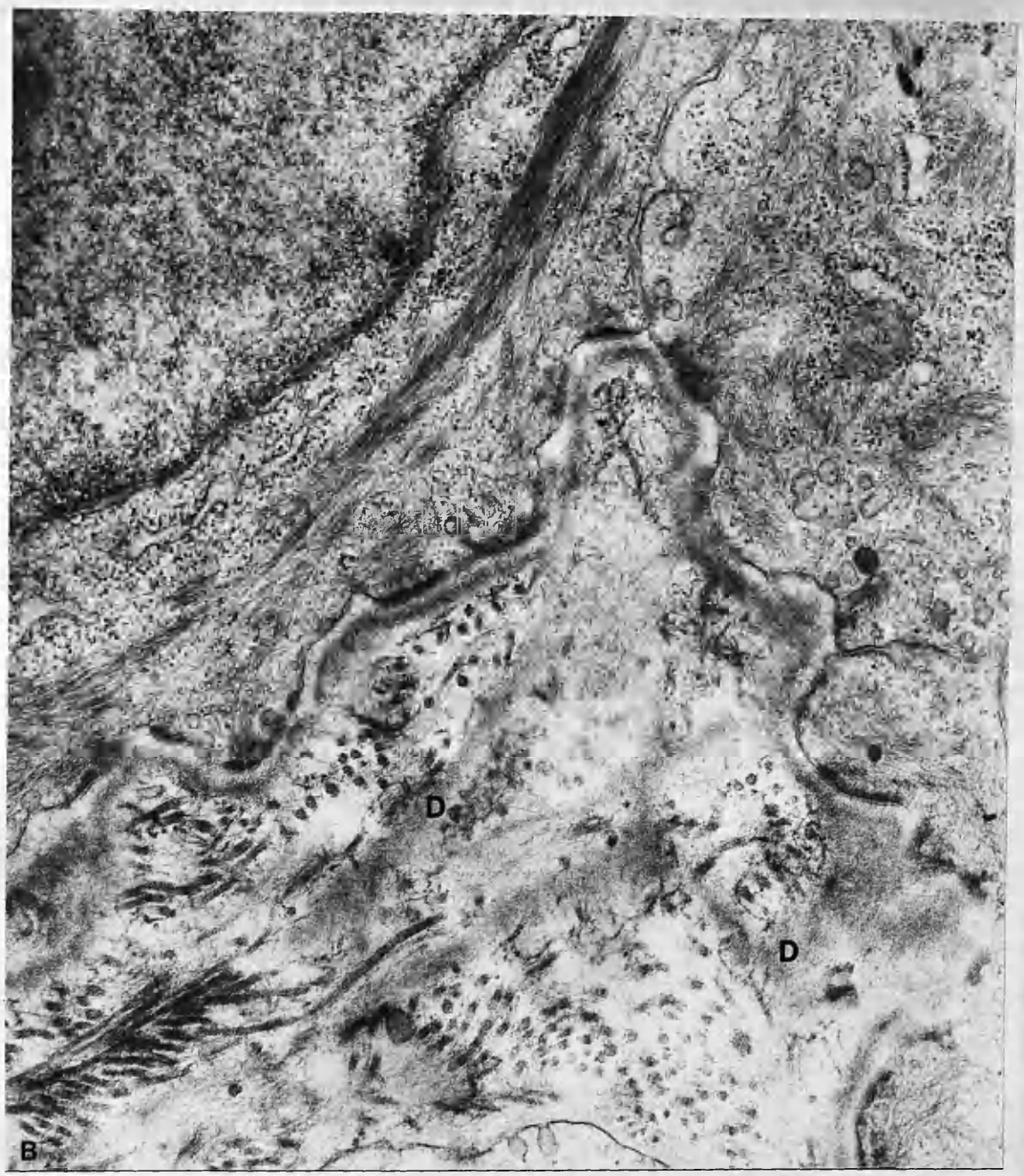


Fig. 6.13B Basement membrane zone of carcinogen treated hamster ventral tongue showing large area of reduplicated lamina densa (D). x 64,500.

Specimen	N		P_k	P_m	P_p	P_b	k+m+p	$\frac{p}{k+m+p}$	$\frac{b}{k+m+p}$	$\frac{b}{p}$
normal short axis	3	$\bar{x}$	60	139.333	102.667	48.667	302	0.33	0.165	0.475
		SD	11.269	35.005	11.59	6.807	47.286	0.081	0.042	0.048
normal long axis	3	$\bar{x}$	60.667	154	97	53.333	311.667	0.321	0.171	0.497
		SD	10.408	15.133	21.633	3.786	12.897	0.046	0.012	0.033
sham short axis	4	$\bar{x}$	51	142.25	106.25	46.75	299.5	0.355	0.157	0.441
		SD	9.416	6.292	6.449	4.573	21.977	0.006	0.022	0.055
sham long axis	4	$\bar{x}$	55.25	134.25	87	43.25	276.5	0.316	0.158	0.546
		SD	19.449	14.523	17.569	2.986	40.731	0.054	0.018	0.12
all control short axis	7	$\bar{x}$	54.857	141	104.714	47.571	300.571	0.351	0.161	0.456
		SD	10.479	20.753	8.321	5.192	31.442	0.033	0.029	0.051
all control long axis	7	$\bar{x}$	57.571	142.714	91.286	47.571	291.571	0.318	0.164	0.502
		SD	15.285	17.124	18.409	6.188	35.189	0.047	0.016	0.085

Table 6.1 Results of light microscopic stereologic study (level 1) of normal and sham treated controls.

N = number of specimens
k = keratinized compartment
m = maturation compartment
p = progenitor cell compartment
b = basal cells
k+m+p = total thickness of epithelium
$\bar{x}$ = mean value
SD= standard deviation

Specimen	N		P_k	P_m	P_p	P_b	k+m+p	$\frac{p}{k+m+p}$	$\frac{b}{k+m+p}$	$\frac{b}{p}$
control	7	$\bar{x}$	54.857	141.	104.714	47.571	300.571	0.351	0.161	0.456
		SD	10.479	20.753	8.321	5.192	31.442	0.033	0.029	0.051
carcinogen biopsy	9	$\bar{x}$	52	123.333	106.333	55.778	281.667	0.378	0.198	0.524
		SD	9.605	17.364	13.36	8.526	33.808	0.018	0.018	0.034
carcinogen sacrifice	9	$\bar{x}$	54.444	102.222	81.667	44.222	238.333	0.344	0.186	0.543
		SD	9.989	20.993	11.937	7.067	30.34	0.044	0.028	0.053
control v carcinogen biopsy	16	t	0.561	1.812	0.297	2.376	1.154	1.951	2.961	3.086
		P				0.032			0.01	0.008
control v carcinogen sacrifice	16	t	0.08	3.689	4.544	1.092	3.988	0.056	1.737	3.366
		P		0.002	0.0005		0.001			0.005
carcinogen biopsy v carcinogen sacrifice	18	t	0.529	2.325	4.13	3.131	2.862	2.146	1.082	0.95
		P		0.034	0.001	0.006	0.011	0.048		

Table 6.2 Results of level 1 stereologic study comparing combined normal and sham treated controls with carcinogen treated specimens showing values from 't' test and probability, P. Only P values less than 0.05 are shown.

Animal	P_n	P_c	I_{ld}	P_n+P_c	$\frac{P_n}{P_c}$	A_{bc}	L_{ld}	$\frac{A_{bc}}{L_{ld}}$
34	16	108	178	124	0.148	238.08	74.582	3.192
35	28	96	102	124	0.292	238.08	40.224	5.919
36	40	109	186	149	0.367	286.08	77.934	3.671
$\bar{x}$					0.269	254.08	64.274	4.261
SD					0.111	27.713	20.872	1.456

Table 6.3 Level 2 stereologic study of normal control sections in short axis of tongue showing the derivation of the nucleo-cytoplasmic ratio and the area of basal cells subtended by unit length of lamina densa.

n = nucleus
c = cytoplasm
$\frac{P_n}{P_c}$ = nucleo-cytoplasmic ratio

Units of measurement

A_{bc} μm^2

L_{ld} μm

ld = lamina densa
bc = basal cell

$\frac{A_{bc}}{L_{ld}}$ = area of basal cells subtended by unit length of lamina densa

Specimen	N		$\frac{P_n}{P_c}$	A_{bc}	L_{ld}	$\frac{A_{bc}}{L_{ld}}$
normal short axis	3	$\bar{x}$	0.269	254.08	64.274	4.261
		SD	0.111	27.713	20.872	1.456
normal long axis	3	$\bar{x}$	0.411	401.28	58.032	6.007
		SD	0.073	76.872	0.296	1.362
sham short axis	4	$\bar{x}$	0.519	263.5	56.987	4.659
		SD	0.246	56.964	14.842	0.254
sham long axis	4	$\bar{x}$	0.573	313.92	54.575	5.866
		SD	0.091	64.227	8.838	1.507
all control short axis	7	$\bar{x}$	0.412	259.474	60.097	4.488
		SD	0.229	43.634	16.444	0.886
all control long axis	7	$\bar{x}$	0.504	351.36	60.516	5.926
		SD	0.116	78.821	14.221	1.326
normal short axis v sham short axis	7	t	1.803	0.288	0.515	0.468
		P				
normal long axis v sham long axis	7	t	2.612	1.595	0.782	0.129
		P	0.048			
normal short axis v normal long axis	6	t	1.851	3.536	0.518	0.74
		P		0.024		
sham short axis v sham long axis	8	t	0.412	1.175	0.279	1.58
		P				
all control short axis v all control long axis	14	t	0.948	2.698	0.051	2.386
		P		0.019		0.034

Table 6.4 Results of level 2 stereologic study of normal and sham treated controls.

n = nucleus
c = cytoplasm
$\frac{P_n}{P_c}$ = nucleo-cytoplasmic ratio

Units of measurement

A_{bc} μm^2

L_{ld} μm

ld = lamina densa
bc = basal cell

$\frac{A_{bc}}{L_{ld}}$ = area of basal cells subtended by unit length of lamina densa

Specimen	N		$\frac{P_n}{P_c}$	A_{bc}	L_{ld}	$\frac{A_{bc}}{L_{ld}}$
all control	7	$\bar{x}$	0.412	259.474	60.097	4.488
		SD	0.229	43.634	16.444	0.886
carcinogen biopsy	9	$\bar{x}$	0.367	344.747	69.461	5.011
		SD	0.089	68.626	12.921	0.782
carcinogen sacrifice	9	$\bar{x}$	0.46	282.667	79.982	3.613
		SD	0.116	80.536	25.148	0.74
all control v carcinogen biopsy	16	t	0.492	3.024	1.238	1.232
		P		0.009		
all control v carcinogen sacrifice	16	t	0.506	0.736	1.906	2.104
		P				
carcinogen biopsy v carcinogen sacrifice	18	t	1.908	1.76	1.116	3.896
		P				0.001

Table 6.5 Level 2 stereologic study comparing sections in the short axis of the tongue from controls and carcinogen treated animals.

n = nucleus
c = cytoplasm
$\frac{P_n}{P_c}$ = nucleo-cytoplasmic ratio

Units of measurement

A_{bc} μm^2

L_{ld} μm

ld = lamina densa
bc = basal cell

$\frac{A_{bc}}{L_{ld}}$ = area of basal cells subtended by unit length of lamina densa

Specimen	N		HD%	mean L_{hd}	$\frac{N_{hd}}{L_{1d}}$
normal short axis	12	$\bar{x}$	39.704	0.128	3.695
		SD	4.283	0.035	1.391
normal long axis	12	$\bar{x}$	41.375	0.148	2.803
		SD	5.203	0.011	0.435
sham short axis	16	$\bar{x}$	35.662	0.12	3.016
		SD	7.525	0.022	0.49
sham long axis	16	$\bar{x}$	37.392	0.143	2.646
		SD	8.372	0.028	0.568
all control short axis	28	$\bar{x}$	37.394	0.129	3.307
		SD	6.563	0.028	1.02
all control long axis	28	$\bar{x}$	39.099	0.145	2.715
		SD	7.348	0.02	0.513
normal short axis v sham short axis	28	t	1.796	0.695	1.617
		P			
normal long axis v sham long axis	28	t	1.546	0.65	0.855
		P			
normal short axis v normal long axis	24	t	0.859	1.888	2.108
		P			0.047
sham short axis v sham long axis	32	t	0.615	2.584	1.973
		P		0.015	
all control short axis v all control long axis	56	t	0.916	2.46	2.744
		P		0.017	0.008

Table 6.6 Level 3 stereologic study showing comparison of normal and sham treated controls.

N = number of prints studied
HD% = percentage of basal plasma membrane forming hemidesmosomes

L_{hd} = length of hemidesmosomes in μm

$\frac{N_{hd}}{L_{1d}}$ = frequency of hemidesmosomes per μm of lamina densa

Specimen	N		HD%	mean L_{hd}	$\frac{N_{hd}}{L_{pm}}$
all control	28	$\bar{x}$	37.394	0.129	3.307
		SD	6.563	0.028	1.02
carcinogen biopsy	36	$\bar{x}$	36.174	0.124	3.061
		SD	7.403	0.031	0.661
carcinogen sacrifice	36	$\bar{x}$	34.971	0.152	2.295
		SD	10.267	0.026	0.596
all control v carcinogen biopsy	64	t	0.697	0.676	1.108
		P			
all control v carcinogen sacrifice	64	t	1.146	3.363	4.667
		P		0.001	$<0.001^{*}$
carcinogen biopsy v carcinogen sacrifice	72	t	0.545	2.183	5.164
		P		0.032	$<0.001^{\times}$

$^{*}P = 0.00002$
$^{\times}P = 0.000002$

Table 6.7 Results of level 3 stereologic study showing comparison of sections in the short axis of the tongue from normal and carcinogen treated animals.

N = number of prints studied
HD% = percentage of basal plasma membrane forming hemidesmosomes

L_{hd} = length of hemidesmosomes in μm

$\frac{N_{hd}}{L_{ld}}$ = frequency of hemidesmosomes per μm of lamina densa

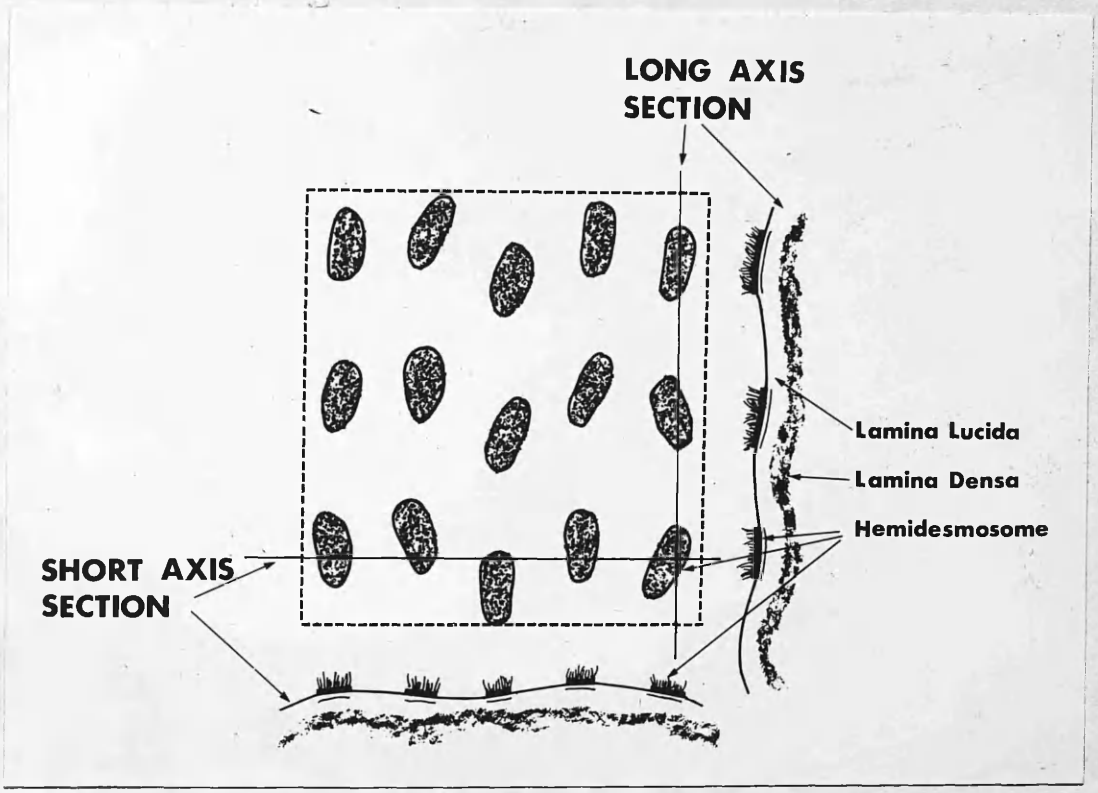


Fig. 6.14 Diagrammatic representation of a possible arrangement of hemidesmosomes in epithelium from the ventral surface of tongue, showing a plan view and the appearances which would be obtained in sections through the basement membrane area.