EXPERIMENTAL ORAL CANCER IN THE RAT

Studies on 4-nitroquinoline-1-oxide induced changes in rat oral mucosal epithelium

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PRECIS

The purposes of this study were:

1. To establish an experimental model of oral cancer in rats using the water-soluble carcinogen 4-nitroquinoline 1-oxide (4NQO). This experiment if successful would verify the reproducibility of Wallenius and Lekholm's experiments (Wallenius and Lekholm, 1973a; Lekholm and Wallenius, 1976a).

2. To study in detail the macroscopic and histopathologic changes occurring in the palate and tongue of these animals during oral carcinogenesis induced by 4NQO. Detailed study of this nature has not been well documented for this cancer model.

The materials and methods used employed the protocol designed by Lekholm and Wallenius (1976a) but with necessary modifications. A 0.5% solution of 4NQO in propylene glycol was painted three times a week on the palate of 23 female Porton rats with their salivary flow temporarily inhibited by hyoscine butyl bromide. Propylene glycol was similarly applied to 12 animals while 4 other animals remained untreated. Groups of animals were sacrificed at 4, 8, 12, 16, 20 and 24 weeks. The macroscopic changes were studied with the aid of a dissecting microscope and the histopathologic changes were studied using optical microscopy.

The results of this study verified the reproducibility of Lekholm and Wallenius's work. Macroscopic and histopathologic study revealed changes passing through stages of increasing severity, resulting in carcinoma. Detailed descriptions of these changes were provided.

The findings indicate that the 4NQO-oral cancer model is suitable for the production and study of oral epithelial dysplastic and neoplastic change.

DECLARATION

This thesis is submitted in part fulfillment of the requirements for the Degree of Master of Dental Surgery in the University of Adelaide.

This thesis contains no material which, except where due mention is made, has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge, this thesis contains no material previously published or written by another person, except where due reference has been made in the text.

P.N.C. WONG

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INTRODUCTION

Pioneers in the field of experimental oral epithelial carcinogenesis consistently met with failure until Salley (1954) successfully produced carcinoma in the cheek pouch of the hamster. Since then the hamster has been the most popular choice of animal for experimental studies on oral carcinogenesis. Because of its peculiar anatomic position, environment and histology, some authorities have questioned the suitability of the cheek pouch for the investigation of oral carcinogenesis (Kolas, 1955; Levy, 1957; 1963; Stormby and Wallenius, 1964). The use of another experimental animal species could avoid such potential bias and controversy. In this regard, the laboratory rat is a logical choice in view of its availability,the adequate size of its oral cavity and its ease of handling. In Australia, where hamsters are regarded as vermin and therefore not available for experimental purpose, these features virtually dictate the use of rats for oral mucosal cancer experiments. However, chemical carcinogenesis of the oral epithelium of rats had been a formidable task until Wallenius and Lekholm (1973a) began using the water soluble carcinogen 4-nitroquinoline 1-oxide (4NQO). Surprisingly, however, similar experiments have not been documented outside Sweden. Further,although several papers and monographs have derived from Wallenius's group, there is little detailed documentation available on the macroscopic and histopathologic changes occurring during 4NQO-induced oral carcinogenesis in rats.

The purposes of this study were to establish an experimental model of oral cancer in rats using 4NQO and to study in detail the macroscopic and histopathologic changes occurring in the palate and tongue of these animals during oral carcinogenesis by 4NQO.

CHAPTER 1

GENERAL CONCEPTS OF CHEMICAL CARCINOGENESIS

Studies on the mechanisms of chemical carcinogenesis can be viewed as falling into two broad areas of interest. One is concerned with the understanding of the basic pathobiology of carcinogenesis. This includes the sequential events of tumour initiation and promotion, theories and hypotheses proposed to explain observed phenomena at sub-cellular levels and factors that can interact with or modify the behaviour of chemical carcinogens. The other is concerned particularly with the understanding of the metabolism of chemical carcinogens. This includes the metabolic activation of chemical carcinogens and the interactions between the activated forms of chemical carcinogens and the molecular constituents of the target tissues. The ultimate goals are to delineate which of these reactions is actually involved in carcinogenesis and to elucidate the means by which the critical interaction(s) cause the development of cancer (Miller and Miller, 1969).

A tremendous amount of research work has been done in an attempt to elucidate these mechanisms. However, the pathobiology of cancer formation is still not understood. Because of the diversity and heterogeneity of the different studies and findings, contradicting concepts and ideas exist. In studying the metabolism of chemical carcinogens, formidable problems are created by the extreme diversity in origin, nature and complexity of more than 3,000 pure chemical carcinogens that exist (Coombs, 1980). The purpose of this review is to knit together those essential ideas to form a conceptual framework on the mechanisms of chemical carcinogenesis which would contribute to better appreciation of experimental work on oral chemical carcinogenesis.

1. THE CONCEPTS OF INITIATION AND PROMOTION, COCARCINOGENESIS, COMPLETE CARCINOGEN AND MULTIFACTORIAL NATURE OF CARCINOGENESIS.

The earliest experimental studies of chemical carcinogenesis involved the local application of a carcinogenic substance to the skin of animals and the observation of the biologic changes subsequently occurring at the site of application. Friedwald and Rous (1944) coined the term "initiation and promotion" to describe the basic sequential events involved in epithelial tumour formation. Numerous experiments illustrating the two-stage process have been done, the most typical one being the application of a sub-threshold dose of a carcinogen on the skin of mice which by itself cannot result in overt cancerous lesions followed by sustained application of a promoting agent (promoter) which would lead to the appearance of carcinomas (Berenblum, 1941a, 1941b). The general working concept derived from these various experiments is that initiation is a rapid irreversible event, producing permanent change in the tissue, whereas promotion is a slow process, consisting of a progression of reversible effects. Also, initiation must take place before promotion, or it is without effect (Scribner and Süss, 1978).

Boutwell (1964) was able to demonstrate that promotion could be divided into two consecutive steps that can be fulfilled by the same or two different agents. Thus he established the operational concept of a three-step process in chemical carcinogenesis in that initiation accomplishes a lesser change in the cell which should be described as "initiated" and the early step in promotion, described as "conversion", is to convert the initiated cell to the "dormant" tumour cell which hitherto had been regarded as the stage accomplished by initiation. In the second step in promotion, described as "propagation", the dormant tumour cell is caused to proliferate into frankly malignant tumour. The three-step concept of Boutwell is summarized

in figure 1.1. As more is known about initiation and promotion, it will become evident that there are actually multi-stages involved in carcinogenesis (Miller, 1978).

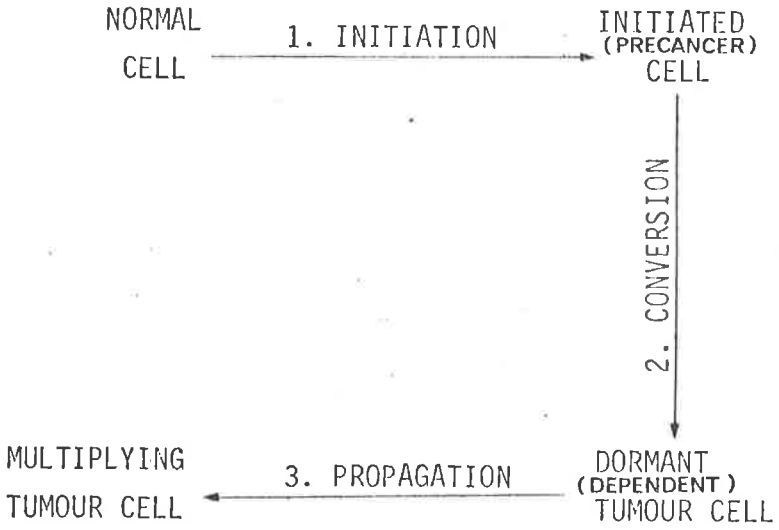


Figure 1.1. Diagrammatic representation of the steps in the formation of skin tumours. (Adapted from Boutwell, 1964).

The development of the two-stage or multi-stage concept of carcinogenesis was fully described by Miller and Miller (1979). Detailed discussion of the experimental aspects and controversial issues can be found in the excellent papers by Scribner and Süss (1978), Boutwell (1964) and Berenblum (1975). Although there are many pros and cons regarding the concept of skin carcinogenesis being a two-stage process of initiation and promotion, nevertheless, it remains to be a fundamental conceptual framework and newer concepts in fact are by and large amplification and further elaboration of the original two-stage concept.

Not infrequently carcinogens may be found that show complete carcinogenic properties where there is no need for a promoter to cause tumour

proliferation. To explain such phenomena, Boutwell (1964) was of the opinion that the same carcinogen having been retained in the tissue long enough, accomplishes both initiation and promotion (at least to the conversion stage), a concept agreed also by Berenblum (1975); or alternatively that promoting phases of carcinogenesis may proceed spontaneously at slow rate owing to intrinsic promoting factors (for example, the natural proliferative activity of young animals). In cases of tumour caused by relatively little amount of carcinogen, it was suggested that the carcinogen itself or a derivative thereof has high specific activity for promotion. On the other hand, Scribner and Süss (1978) commented that complete carcinogenesis may proceed by a mechanism that does not involve a significant level of promotion, or that occult promoters could well be present in the environment which enhance the effect of the carcinogens.

Scribner and Süss (1978) pointed out the distinction between cocarcinogenesis and promotion. An experiment in which an enhancing agent is given simultaneously with a carcinogen cannot be called an initiation-promotion experiment since it lacks the temporal separation of the two agents. Such a situation is called "cocarcinogenesis" and the enhancing agent is called a "cocarcinogen".

Though distinction of such important terms as complete carcinogen, incomplete carcinogen, promoter and cocarcinogen is to be encouraged, it is not always possible to absolutely categorize a particular agent as having one and only one type of action, especially when the target organs or tissues are exposed to several different carcinogenic stimuli - chemical, viral and physical agents - in various combinations. In addition, complex interactions of different agents given at different stages of carcinogenesis illustrate the complex pattern concerning the basic mechanisms of carcino-

genesis. It is increasingly accepted that in most cases carcinogenesis in man has to be viewed as a multifactorial process involving the synergistic action of several 'influences' or 'factors'. (Hecker, 1976; Harnden, 1980).

2. THEORIES OF CARCINOGENESIS

There are several working hypotheses or theories proposed to explain the observed phenomena in carcinogenesis at biochemical and molecular levels. The one that has gained much acceptance is the Somatic Mutation Theory. An enormous amount of research work on DNA in relation to carcinogenesis has been done under the assumption that this hypothesis is true. The concept was originally suggested by Boveri in 1914 (Trosko and Chang, 1978) that cancer might arise as a result of mutations in somatic cells. The details and evidence in favour of this theory were elaborated by Trosko and Chang (1978) and Coombs (1980).

Attempts to explain all observed phenomena in carcinogenesis by mutation are highlighted by Comings's General Theory of Carcinogenesis (Comings, 1973), in which it is stated that spontaneous, chemically induced, and radiation-induced tumours are due to double somatic mutations at the 'repressor gene' or 'regulatory gene ' loci, thus allowing a series of 'structural genes' (or 'transformation genes')to assume their activities which would lead to unrestrained cell proliferation ending in malignant tumour development.

While supportive evidence for the mutational theories of carcinogenesis is abundant, there are not infrequent findings to suggest that carcinogenesis proceeds via non-mutagenic pathways.

These have led to the formation of the Epigenetic Theory . Trosko and Chang (1978) summarized the main experimental evidence against the Mutagenic Theory and Rubin (1980) made a very comprehensive and critical appraisal of this theory which includes enlisting of further unfavourable experimental evidence.

To further develop this concept, Trosko and Chang (1978) ascribed the epigenetic events to gene modulation. Their Integrative Theory, proposed to link together the two seemingly contrasting concepts of mutation and epigenetic change , states that carcinogenic agents can induce a stable transformation of a cell by either mutation or epigenetic alteration in gene expression. Thus, the Integrative Theory explains all observed phenomena being due to events occurring around the genetic apparatus; even though some are non-mutational, they do affect the genes by their modulation effects. In fact, many events that do not involve a direct mutation do have strong genetic basis. As Miller (1978) commented, genetic information also determines the metabolism of chemical carcinogens, the synthesis and metabolism of hormones, and a variety of other factors that may affect tumour incidence without being directly involved in the initiation of cancer cells. An important example of these is genetic deficiency in the immunologic mechanism which reduces the immunologic surveillance to cancer cells.

3. BIOCHEMICAL AND MOLECULAR EVENTS IN CHEMICAL CARCINOGENESIS

Inasmuch as the concept of alteration of the genome is currently receiving much attention, investigations of the mechanisms of chemical carcinogenesis at the biochemical and molecular level naturally centre on genetically involved macromolecules of which DNA has received the most attention. Such work attempts to examine how a carcinogen chemically or physically reacts or binds with the DNA macromolecule

with the end result being a permanent mutation. Essentially, a carcinogen will cause damage to the DNA macromolecule and the natural consequence is to elicit certain repair mechanisms. Normally pre-replicational error-free repair by excision of any damaged portion of the DNA molecule will restore the DNA to its normal condition. If the repair mechanism goes wrong, as in post-replicational error-prone repair, the damage would be incorporated permanently in the DNA macromolecule, thus resulting in mutation which can be categorized as 'base-pair substitution', 'frameshift', 'deletion', and 'insertion' mutation. Subsequent transcription and translation of the mutated genetic information would result in derangement of biochemical activities that may eventuate in malignant growth behaviour. The mechanism of DNA damage and repair has been extensively investigated and a voluminous literature is available on this topic. Further elaboration is beyond the scope of this chapter. A very comprehensive treatise on DNA related events in chemical carcinogenesis by different leading authorities was edited by Grover (1979) and a comprehensive review was written by Irving (1973).

Besides reacting with DNA, chemical carcinogens also react with other macromolecules such as RNA and proteins. Since all these are electronegative (nucleophilic) molecules, naturally, the electrophilic (electron like) carcinogens would tend to be attracted to these molecules. Though not many studies have been done on these non-DNA reactions, authorities like Miller (1978) and Irving (1973) agree that the role played by RNA and proteins in the initiation and promotion of carcinogenic processes cannot be underestimated.

The biochemical activities of promoting agents have been studied extensively, especially with phorbol diesters of croton oil. Scribner and Süss (1978) and Boutwell (1974) provided detailed elaboration on various aspects of promotion. Trosko and Chang (1979) also presented a comprehensive review and Miller (1978) presented a concise summary. In brief, a promoter may affect the activities of certain enzymes and coenzymes such as ornithine decarboxylase, plasminogen activator, cyclic GMP and cyclic AMP and cytosol proteins all of which are involved in regulation of mitotic activities and/or gene expression. It may also exert its effects by interacting with cell membrane and DNA and suppressing the immune system.

4. MODIFYING FACTORS IN CHEMICAL CARCINOGENESIS

The multifactorial nature of carcinogenesis has been emphasized by Hecker (1976) and Harnden (1980). Therefore, other potentially modifying factors that are not conventionally discussed as a part of the arena of chemical carcinogenesis may also have their impact on the latter process.

Two major issues are viral carcinogenesis and cancer immunology both of which have received extensive studies. The etiology of virus in animal carcinogenesis has been much evidenced. Enthusiasts in this field interpreted all carcinogenic processes as having a viral etiology. The 'oncogene', 'infection' and 'protovirus' hypotheses on mechanisms of carcinogenesis proposed by Temin (1974) have their rationale based on the incorporation of the genes of the RNA virus into the host's genome. Such genes subsequently direct neoplastic behaviour of the transformed cell. It has also been suggested that the role of chemical carcinogens is to activate endogenous viral genes and/or oncogenes which play the main role in chemical

carcinogenesis (Huebner and Todaro, 1969).

The role of the body's immune system in the protection against tumour formation is well recognized. A voluminous literature on cancer immunology exists. However, in chemical carcinogenesis, the role played by the immune system is still very controversial. Only a small, relatively selected group of tumours induced by chemical carcinogens, mainly aromatic hydrocarbons and azo dyes, are detectably immunogenic and rejectable (Klein, 1976). The immunosuppressive effect of 9,10-dimethyl-1,2-benzanthracene (DMBA) has been demonstrated by Szakal and Hanna (1973). Absence of immunologic influence on chemical carcinogenesis has been widely demonstrated with 2-acetylaminofluorene (AAF) by Baldwin (1973), who investigated extensively the immunologic aspects of chemical carcinogenesis.

Some environmental factors such as ionizing radiation and UV-light background radiation which are also causative agents in environmental carcinogenesis may superimpose on chemical carcinogens. Genetic predisposition, which forms the basis for breeding susceptible strains of experimental animals for cancer research; hormonal influence, such as thyroxine and corticosteroids; interactions with other chemical carcinogens, promoters, inhibitors and anti-metabolites that are present insidiously in the internal milieu of the target organ or tissue; age and sex differences; and caloric restriction, may all affect the outcome of chemical carcinogenesis and should not be neglected though there are still no consistent patterns of such influences. A discussion of these factors influencing initiation and promotion was presented by Berenblum (1975).

5. BASIC CONCEPTS OF METABOLIC ACTIVATION AND MECHANISM OF ACTION OF CHEMICAL CARCINOGENS

The observation of some carcinogens, especially the aromatic amine derivatives, producing tumours at distant sites such as the liver and urinary bladder regardless of the route of administration and the finding of protein-bound derivatives of some carcinogens were the first clues for metabolic activation of carcinogens (Miller, 1978).

Much of our knowledge on the metabolism and mechanism of action of chemical carcinogens has been built up from the work of Elizabeth and James Miller in the late 60's (Miller and Miller, 1969; Miller 1970). They arrived at the general proposition that most carcinogens are really PRECARCINOGENS that are metabolized *in vivo* to ULTIMATE CARCINOGENS frequently via the intermediate formation of PROXIMATE CARCINOGENS, by drug metabolizing enzymes.

The enzymes that carry out these oxidations occur in the microsomal fraction (on the endoplasmic reticulum) of cells as members of the MIXED FUNCTION OXIDASE SYSTEM. This is a complex of numerous mono-oxygenases which require NADPH and molecular oxygen and which carry out aromatic ring oxidation, aliphatic hydroxylation, oxidative demethylation, N-hydroxylation, etc., on a great variety of organic compounds. Relatively high levels of these enzymes are present in liver and they are detectable in most other tissues. Their levels are inducible by their substrates and this inducibility is under genetic control (Coombs, 1980; Heidelberger, 1977).

The ultimate carcinogens are strong ELECTROPHILIC reactants (that is, they are deficient in electrons and would attack or be attracted to negatively charged or electron dense sites) that can combine covalently with cellular molecules which have NUCLEOPHILIC sites (that is, negatively charged sites or sites of high electron density). It is now axiomatic that such covalent binding between the carcinogen metabolite and cellular macromolecules leads to the initiation of the carcinogenic process (Heidelberger, 1977). However, these nucleophilic sites are relatively abundant in DNA's, RNA's and proteins and include certain oxygen and nitrogen atoms in the nucleic acids, and nitrogen, sulphur and oxygen atoms in proteins. Because some, and probably many, precarcinogens are metabolized to more than one ultimate carcinogen and because there are multiple nucleophilic sites in each macromolecule, multiple DNA-, RNA- and protein-bound derivatives of each carcinogen are possible and are frequently observed. Accordingly, basic problems of great importance today are the elucidation for each carcinogen of those informational macromolecule-bound products that are important in carcinogenesis and the identification of the specific role of each of these adducts in the carcinogenic process (Miller, 1978).

There are a few exceptions to the above fundamental trend in metabolic activation of chemical carcinogens. These are the DIRECT-ACTING CARCINOGENS such as the ALKYLATING AGENTS and NITROSAMIDES . They do not require enzymatic activation since their reaction with water and other cellular nucleophiles (such as sulfhydryl groups) results in the formation of electrophilic reactants which will alkylate to various degrees a wide variety of nucleophilic sites in the target cells (Miller, 1978). It has to be clarified at this stage that although the term ALKYLATING AGENTS is reserved for a group of direct acting carcinogens, in fact

most electrophilic reactants, whether formed spontaneously as with direct acting carcinogens or enzymatically through metabolic activation as with the rest of the carcinogens, are alkylating agents. Therefore, it is quite obvious that electrophilicity is a common property of ultimate carcinogens (Miller, 1978).

There are several major pathways of metabolic activation of chemical carcinogens: 1. Aromatic amines and azo dyes first undergo N-hydroxylation (hydroxylation at the nitrogen atom) and then esterification with an acid at the hydroxylated site to form an ester. The acidic group of the ester readily detaches leaving a highly electrophilic active site on the mother molecule (Figure 1.2) (Miller and Miler, 1969; Miller, 1970; Cramer et al, 1960; Lotlikar et al, 1966; DeBaun et al, 1970; Kriek et al, 1967; King and Philips, 1968; Kriek, 1974; Irving et al, 1971; Irving 1973; Conners, 1975). 2. Nitrosocompounds undergo C-hydroxylation (hydroxylation at a carbon atom). This is followed by demethylation and nitrogen removal resulting in an electrophilic alkylating agent (Figure 1.3) (Druckrey, 1975; Irving, 1973). 3. Polycyclic hydrocarbons and a large variety of miscellaneous carcinogens initially undergo C-hydroxylation in a benzene ring followed by epoxidation. The epoxide formed is very unstable and strained which can easily provide an electrophilic site (Figure 1.4) (Boyland, 1950; Pullman and Pullman, 1955; Miller, 1978; Sims et al, 1974; Wood et al, 1977). Besides the above major pathways, there are other less well characterized metabolic pathways for other types of carcinogens (Weisburger, 1978). Detailed discussion of the metabolic pathways is out of the scope of this review but can be found in the references cited above.

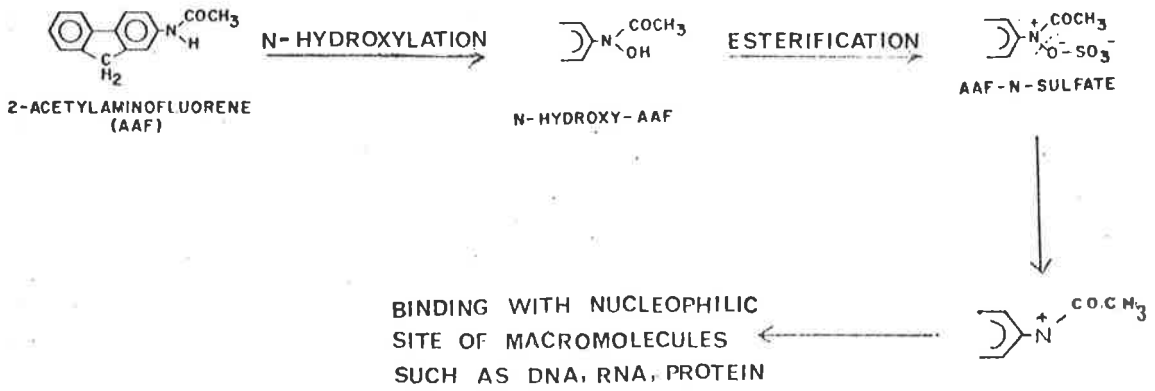


Figure 1.2 The pathway for the metabolic activation of an aromatic amine, 2-acetylaminofluorene (AAF). (Adapted from Miller, 1978).

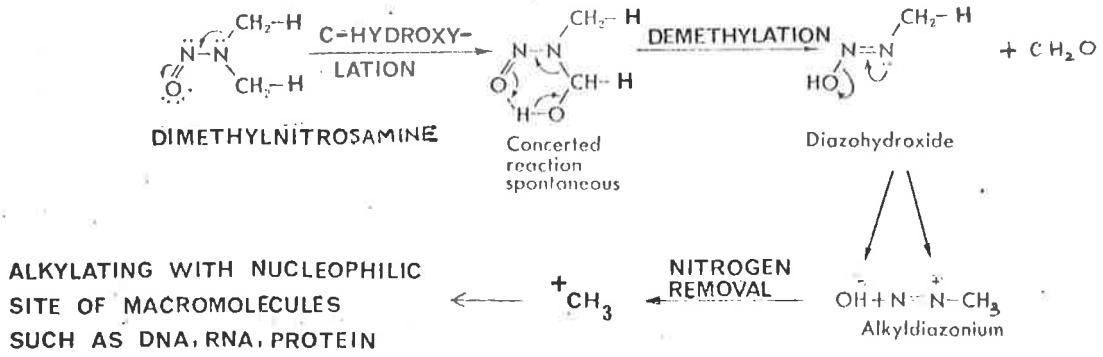


Figure 1.3 The pathway for the metabolic activation of a nitroso-compound, dimethylnitrosamine. (Adapted from Irving, 1973, and Druckrey, 1975).

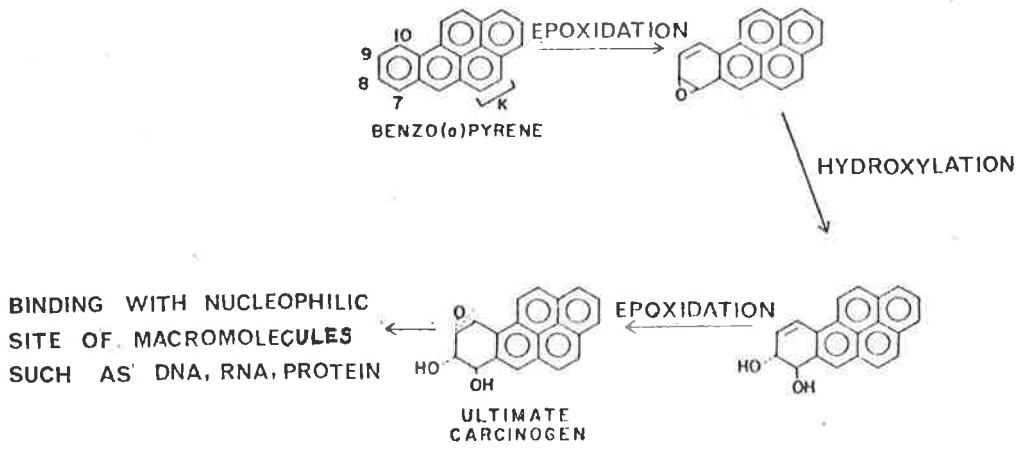


Figure 1.4 The pathway for the metabolic activation of an aromatic hydrocarbon, benzo(a)pyrene. (Adapted from Miller, 1978).

CHAPTER 2

EXPERIMENTAL WORK ON ORAL CANCER USING LABORATORY ANIMALS AND CHEMICAL AGENTS

Experimental oncogenesis studies of the oral tissues involved the oral mucosal epithelium, the connective tissue components, the supportive alveolar and jaw bone and other oral structures such as the salivary glands, teeth and tooth-forming tissues. Physical, biological and chemical agents have been utilized to produce or modify neoplastic changes. Experimental oncogenesis of the oral mucosal epithelium only by chemical means is to be explored in this review.

There have been relatively few (about 200) papers written on oral carcinogenesis in its short history. This quantity of research is in no way comparable to the voluminous literature, in the order of thousands of papers, on chemical carcinogenesis outside the oral cavity. However, these studies have touched on some interesting aspects of chemical carcinogenesis, with particular relevance to the oral mucosal epithelium. In this chapter, the work on chemical oral carcinogenesis is reviewed with the purpose of illustrating the spectrum of research activities in this field.

In this chapter, the terms "oral carcinogenesis", "chemical carcinogenesis" and "experimental carcinogenesis" are used interchangeably and the animal models mentioned refer to carcinogenesis in the oral mucosal epithelium unless otherwise specified.

1. EXPERIMENTAL ORAL CANCER

ANIMAL SPECIES

Early work on oral carcinogenesis showed that the oral mucous membrane of animals was very resistant to tumour induction. Except for some sporadic successes, oral carcinogenesis was by and large a failure (Bonne, 1927; Krebs, 1928; Prohaska, et al, 1939; Levy, 1948; Levy and Ring, 1950; Levy, et al, 1950; and Wantland, 1954). However, Salley (1954) reported the production of numerous benign and malignant neoplasms in the cheek pouch of hamsters repeatedly painted with 9,10-dimethyl-1,2-benzanthracene (DMBA), 3,4-benzpyrene and 20-methylcholanthrene. In addition carcinomas on the palate, buccal mucosa and tongue were incidental findings. Subsequently, the hamster has been repeatedly proved to be the animal species that is especially suitable for oral carcinogenesis studies (Chaudhry, 1955; Salley and Kreshover, 1959; and Morris, 1961).

There are pros and cons regarding the choice of the hamster cheek pouch as the model system for oral carcinogenesis. Arguments for using the hamster cheek pouch include: its similarity to the lining of the oral cavity proper, its ease of access, its isolation from the oral environment and absence of salivary gland tissues, and its storage capacity for carcinogens, details of which can be found in papers by Salley (1954), Peacock and Brawley (1959), Kreshover and Salley (1957), and Shklar et al (1979).

Arguments against the hamster cheek pouch are the following: Kolas (1955) pointed out that the cheek pouch of hamsters has no salivary glands and is not exposed to the same environmental influences

e.g. salivary flow, as the oral mucous membrane. Levy (1957, 1963) was of the opinion that the cheek pouch is not a true intraoral mucous membrane since it apparently has neither mucous glands nor any of the other types of appendages found in the oral mucous membrane. Since the cheek pouch is not physiologically equivalent to the oral cavity proper, together with its unresponsiveness to changes in the oral environment during carcinogenesis and its peculiar anatomy in having the buccal area reduced by the wide opening of the pouch, Stormby and Wallenius (1964) regarded the hamster as unsuitable for the investigation of experimental oral carcinogenesis.

In support of this view, Wallenius (1965a) showed that 30 percent of a group of rats developed oral squamous cell carcinomas after painting the palate repeatedly with DMBA for 16 months. In the subsequent year he was able to obtain 100 percent yield of carcinomas in rats after 11 months using the same carcinogen but with complete inhibition of salivary secretion (Wallenius, 1966). When the water-soluble carcinogen 4NQO (4-nitroquinoline 1-oxide) was used, Wallenius and Lekholm (1973a) were able to obtain a 100 percent yield of oral carcinomas after 7 months even without inhibition of salivary secretion. So far, Wallenius and his group are the only ones consistently using rats in the investigation of various aspects of oral carcinogenesis. In addition, a group of Japanese workers (Yamamura et al, 1975) were able to obtain a spectrum of precancerous and cancerous changes in the artificially created caecal pouch in the lower lip of rats with local application of DMBA and methylcholanthrene, and a wide range of mesenchymal tumours with N'-nitro-nitrosoguanidine.

Although Kreshover (1952, 1955) failed to produce cancerous changes in the oral mucosa of mice using tobacco smoke, this animal species has

occasionally been successfully used for oral carcinogenesis studies. Protzel et al (1964) repeatedly painted benzpyrene to the labial buccal mucosa of mice and succeeded in producing carcinomas in a considerable proportion of the mice, especially in those with artificially induced hepatic disturbances. Fujino et al (1965) applied 4NQO to the mucosa of the lower lip of mice and obtained a good yield of labial and lingual carcinomas which was further increased if mechanical injury was inflicted to the lower lip with a metal wire. Other than these few studies, however, mice have not been especially utilized.

A few studies utilizing monkeys have also been reported in the literature. These investigators were mainly concerned with the effects of long term chewing and contact of betel quid and tobacco (Cohen and Smith, 1967; Hamner and Reed, 1972; Cohen et al, 1971; Hamner, 1972). However, no significant changes were observed.

INTRAORAL SITE SUSCEPTIBILITY

Regarding the susceptibility of specific sites within the oral cavity to carcinogens , success has been reported in almost all sites. The universal susceptibility of the hamster cheek pouch is established beyond doubt (Salley, 1954). The tongue (Fujita et al , 1973; Marefat and Shklar, 1977), buccal mucosa (Salley, 1954), palatal mucosa (Salley, 1954; Salley and Kreshover, 1959) and the gingiva (Al-Ani and Shklar,1966; Mesrobian and Shklar, 1969; Suzuki, 1976) of the hamster have also been reported to undergo malignant change. However, invariable success of 100 percent is only possible with the cheek pouch and the tongue of the hamster.

The palate and the tongue of rats were shown by Wallenius and his

group to be invariably susceptible to carcinogenesis, especially using 4NQO (Wallenius and Lekholm, 1973a; Lekholm and Wallenius, 1976a).

Successful oral carcinogenesis using mice was reported in the labial, buccal, palatal and lingual mucosa (Fujino et al, 1965; Geary et al , 1970) and in the gum (Prohaska et al, 1939).

STUDIES EMPLOYING SUSPECTED CARCINOGENS

As a result of epidemiologic studies, substances such as tobacco smoke, alcohol, betel quid and lime have been incriminated as etiologic agents for oral cancer in man. The earliest animal studies in this area can be traced back to those dealing with tobacco. Roffo (1930) was first to apply tobacco products to the oral mucosa experimentally. No lesions were produced by applying extracts of nicotine and of tobacco to the gingiva of rabbits. However, leukoplakia was produced when whole tobacco smoke was applied.

Salley (1963) reported a series of studies with mice done by Kreshover and himself on the effect of tobacco smoke on the oral mucous membrane (Kreshover, 1952; Kreshover, 1955; Kreshover and Salley, 1957; Kreshover and Salley, 1958; and Salley, 1959). These authors found that tobacco smoke alone was not a carcinogen but that it was a cancer-promoting agent in DMBA-induced carcinogenesis. A similar cancer-promoting effect of cigarette smoke was demonstrated by Elzay (1969) in hamster.

Kendrick (1964) found no tumour induction in hamster cheek pouch lining when cigarette smoke condensate was applied over the life-span of the animals. Hyperplasia and dysplasia were however recorded. Similarly, Bastiaan and Reade (1980) only obtained irritational hyperplasia after

application of tobacco tar and heat to the lip mucosa of rats.

Henefer (1966) and Elzay (1966, 1969) demonstrated the cancer-promoting effect of alcohol on oral mucosal epithelium. However, their findings were inadequate to pinpoint the exact role of alcohol in carcinogenesis.

Besides tobacco smoke, other forms of tobacco and substances forming ingredients of tobacco mixtures have been studied in animal models. These models include: application of tobacco tar, tobacco tar plus heat to the hamster cheek pouch (Tabah et al, 1957; Moore and Miller, 1958; Saipogal et al, 1973); implantation of snuff and tobacco in hamster cheek pouch (Peacock and Brawley, 1959); application of ingredients of betel quids (tobacco, tobacco tar, black pepper, incense, nutmeg, etc.) to hamster cheek pouch (Dunham and Herrold, 1962); application of betel quid and tobacco to cheek pouches of monkeys (Cohen and Smith, 1967; Cohen et al, 1971); implantation of tobacco snuff into the gingivo-lingual folds of hamsters (Homburger, 1971); application of dimethyl-sulfoxide extract of betel nut and tobacco to hamster cheek pouch (Suri et al, 1971); application of betel quid (betel leaf, areca nut and lime) and tobacco on the oral mucosa of baboons (Hamner and Reed, 1972); application of NAS (tobacco, lime, ash, plant oil and water) to hamster cheek pouch (Kiseleva et al, 1976); application of shell lime and tobacco to hamster cheek pouch with the animals conditioned by vitamin A palmitate (Kandarkar and Sirsat,1977); treatment of hamster cheek pouch with lime (Dunham et al, 1966); and application of certain plant materials for chewing to hamster cheek pouch and upper gastrointestinal tract (Dunham et al, 1974). The experimental findings with these animal models are by and large inconsistent, ranging from completely negative findings to occasional squamous cell carcinoma,

and the roles of these various substances in oral carcinogenesis have not yet been clearly determined.

Oral carcinomas have also been incidentally obtained when various nitroso compounds have been administered to hamsters and rats via drinking water or via an intragastric route. These compounds include nitrosomethylaniline (NMA) and nitrosomethylcyclohexylamine (NMC) (Goodall et al, 1970), N-nitrosomorpholine (NM) and N-nitrosopiperidine (NP) (Mohr et al, 1974), N-butyl-N-nitrosourethan (BNUR) (Takeuchi et al, 1974), N-propyl-N-nitrosourethan (PNUR) (Maekawa et al, 1976), N-methyl-N-nitrosourea (NMU) (Herrold, 1968; Edwards,1978). Milievskaja and Kiseleva (1976) also demonstrated that local application of one of these compounds (NMU) to the hamster cheek pouch could result in tumour formation.

MACROSCOPIC AND MICROSCOPIC FEATURES

Various groups of workers have described the macroscopic and histologic changes in relation to their experimental work on chemical oral carcinogenesis, including those using DMBA and 4NQO (Levy et al, 1951; Salley, 1954; Salley, 1957; Reiskin et al, 1960; Morris et al, 1961; Stahl, 1963a; Stormby and Wallenius, 1964; Levij et al, 1967; Shklar, 1972; Wallenius and Lekholm, 1973a; Yamamura et al, 1975; Lekholm and Wallenius, 1976a, 1976b; Maekawa et al, 1976; Marefat and Shklar, 1977; Cottone et al, 1979; MacDonald, 1973; Eveson and MacDonald, 1978; Franklin and Smith, 1980; Delarue et al, 1971a, 1971b; Gohari and Johnson, 1974). To illustrate these changes, a few of these studies are described.

Salley (1954, 1957) and Salley and Kreshover (1959), using the DMBA/

hamster cheek pouch model, described the following macroscopic changes in the hamster cheek pouch: inflammation, oedema, leukoplakia, nodules, papillomas and carcinomas, presumably in sequence. Microscopically, the four preneoplastic stages were inflammation, degeneration and necrosis, regeneration and hyperplasia. The neoplastic stages were benign papilloma showing atypia, squamous cell carcinoma in situ (preinvasive) and squamous cell carcinoma.

Other groups who later described the macroscopic and microscopic changes in the hamster cheek pouch observed essentially the same features. However, some studies do contain further observations that are worth noting. Stormby and Wallenius (1964) using DMBA in hamster described the formation of leukoplakia and papillary carcinoma and the histologic features of hyperkeratosis, parakeratosis, acanthosis, basal cell hyperplasia and papilloma developing into papillary carcinoma. Santis et al (1964) using DMBA in hamster grouped their microscopic observations under the following headings: normal mucosa, chronic inflammation and epithelial hyperplasia, papilloma, hyperkeratosis and dyskeratosis, well differentiated epidermoid carcinoma, and poorly differentiated, anaplastic epidermoid carcinoma. In between normal mucosa and epidermoid carcinoma, there was almost consistently a transitional zone of "leukoplakia" characterized by hyperkeratosis and dyskeratosis with varying degree of underlying inflammatory infiltrate. Camilleri and Smith (1965) using DMBA in hamster describe minute red nodules or pale plaques as the earliest changes which subsequentl developed into papillomatous growths and then further into pedunculated and lobulated malignant growths. These investigators emphasized the frequent association of areas of carcinoma in situ and invasive carcinoma. Levij et al (1967) using DMBA in hamster emphasized the developmental progressive nature of lesions from hyperplasia through to benign and premalignant papilloma to carcinoma in situ through to squamous

cell carcinoma. These investigators however also noted that foci of intra-epithelial carcinoma (carcinoma in situ) appeared in non-papillomatous epithelium.

It is worth noting that while some workers (Camilleri and Smith 1965; Levij et al, 1967; Cottone et al, 1979; and Yamamura et al, 1975) have listed features of cellular atypia to describe "dysplasia" and carcinoma in situ, none has defined how many features of cellular atypia should be present for a diagnosis of dysplasia.

The gross and microscopic changes resulting from application of 4NQO to rat oral mucosa were first described by Wallenius and Lekholm (1973a). Early changes observed were slight swelling, reddening and increased keratinization of palatal mucosa during the 5th month. By the 6th month, ulcerations with discrete haemorrhages were found centrally in the keratinized areas. On the tongue nodular growths were observed. During the following two months (7th and 8th months), the clinical course of lesions was marked by expanding ulceration surrounded by hyperkeratotic, wall-like zones. The teeth showed, simultaneously, greater mobility and the clinical picture suggested malignancy in all animals. The lesions occurred preferentially on the right side of the palate. Some of the nodular lesions on the tongue later developed into papillomas. Also noted were superficial hyperkeratotic "plates". Microscopically, malignant tumours on the palate were highly differentiated squamous cell carcinomas with a more anaplastic periphery which infiltrated into the surrounding bone. It was typical for palatal carcinomas to be ulcerated and infiltrative. Squamous cell carcinomas from the base of the tongue belonged to the well/moderately differentiated category. Papillary carcinomas, papillomas and benign epithelial changes in the form of hyperkeratosis and acanthosis with moderately pronounced atypical cells were also observed. One feature that

deserves special mention is the presence of flattened lesions referred to as "plates" some of which showed areas of atypia with poor maturation of the epithelium towards the surface, atypical mitoses at varying levels of the epithelium, a tendency to erosion or fully developed ulceration and transformation to frankly invasive carcinomas.

When temporary xerostomia was induced by scopolamine during and after painting of 4NQO, Lekholm and Wallenius (1976a) observed some additional features compared to their previous experiment. The initial macroscopic change noted in xerostomia rats was inflammation of the palatal mucosa. This subsequently subsided. At the beginning of the second month, the palate again reacted with redness and swelling. These signs persisted for two months and were followed by the development of hyperplasia and hyperkeratosis, indicated by loss of the usual anatomic pattern of the rugae. By the beginning of the fourth month, some animals had palatal carcinomas. By five and a half month, all rats had palatal carcinomas. There were also small sessile nodes at the base of some tongues. Microscopic examination of tissues by these workers revealed oral changes in a larger number of cases than did the gross examination. While all animals had squamous cell carcinomas in the palate, the tongues exhibited a range of pathologic changes which included hyperkeratosis and acanthosis, benign papilloma formation, mild, moderate and severe epithelial dysplasia. In a subsequent study using 4NQO, Lekholm and Wallenius (1976b) classified the changes observed microscopically into four groups: hyperplasia including mild dysplasia; moderate dysplasia; severe dysplasia and carcinoma in situ; and infiltrative squamous cell carcinoma, following the WHO's system for the histologic classification of genital tract tumours (WHO, 1975).

From the different observations made by these workers (Wallenius and Lekholm, 1973a ;Lekholm and Wallenius 1976a, b) in their various experi-

ments using 4NQO in rats, it would appear, though it was not stated clearly, that some invasive carcinomas evolved through hyperplasia and papillomas while others arose from the flattened "plate"-like lesions, while still others developed from very non-specific lesions.

Yamamura et al (1975), in studying DMBA and methylcholanthrene induced intraoral carcinogenesis in rats, stressed the development of tumours could proceed as a result of basal cell proliferation giving rise to carcinoma in situ which later gave rise to infiltrative carcinoma. Alternatively, proliferation of the basal cells could be followed directly by the development of infiltrating carcinoma. Maekawa et al (1976), in their study of the carcinogenicity of PNUR in rats, emphasized that many carcinomas of the oral cavity and pharynx, especially the tongue, could develop without passing through a papillomatous stage since leukoplakia or papillomas were seldom observed in the early stage. More carcinomas than papillomas were observed in the late and final stages by these investigators.

A point of interest deriving from the studies just described is that the methods of assessment of histologic material by investigators appear to fall into one or a combination of the following methods: 1. a subjective and qualitative description of the morphologic and cellular features, using such terms as hyperkeratosis, parakeratosis, acanthosis, loss of polarity, cellular pleomorphism; 2. a subjective grading of lesions/changes by means of categorical terms, such as mild, moderate and severe dysplasia, carcinoma in situ, which are further qualified, subjectively, by descriptive cellular features; and 3. a subjective use of categorical terms, without defining those terms, and taking for granted that the terms are understood by the readers.

In a search for studies that were primarily intended to quantify

the histopathologic features of premalignancy and malignancy using animal oral cancer models, only a very small number of studies were found. These are MacDonald's (1973) evaluation of the prognostic values of the objective atypia scoring technique of Smith and Pindborg (1969), Eveson and MacDonald's (1978) method of grid-counting of projected microscopic sections on a screen for cell kinetic studies, and Franklin and Smith's (1980) stereoscopic method for assessment of epithelial atypia. The last one is by far the most complicated, tedious and mathematical method of assessment.

From the above studies, it would appear to be the rule rather than the exception to find different versions on the progressive changes that occur during carcinogenesis as characterized by macroscopic and microscopic features. The trend of changes differs with different animal species, different carcinogens and intraoral environment, different anatomic sites within the oral cavity, different investigators or the same investigator on different occasions. This strongly indicates that there is not a unique pattern of changes which characterizes tumour development in experimental animals.

There are very few studies available detailing the changes occurring in the mesenchyme beneath the epithelium during carcinogenesis of the oral mucous membrane. Delarue et al (1971a, b) studied the vascular changes associated with DMBA-induced carcinogenesis in the hamster cheek pouch. They observed a marked disorganization of the subepithelial vascular system in the pouch with the development of a vascular system of the tumour itself which consisted of its own newly formed vessels mixed with those of the host. Gohari and Johnson (1974) did a quantitative study on the inflammatory response in DMBA-induced carcinogenesis in hamster cheek pouches. Development of tumours, both benign and malignant, was accompanied by a

concomitant steep rise in the number of macrophages and a rise in the number of lymphocytes. These investigators concluded that the significance of this inflammatory cell pattern was not clear but might be related to host resistance to tumour development and to the rarity of metastasis in this experimental model.

There are only a very limited number of ultrastructural studies based on oral cancer models. Listgarten et al (1963) described widening of the intercellular spaces as the earliest change and later clumping of tonofibrils in the cytoplasmic periphery during DMBA-induced hamster cheek pouch carcinogenesis. White and Squier (1974) in their morphometric studies using DMBA/hamster model suggested that in malignant basal cells there is a decrease in intercellular attachment by means of desmosomes and hemidesmosomes. Woods and Smith (1969, 1970) using the same model found that in the "premalignant" state, basal cells formed pseudopodia extending through discontinuities in the basal lamina of the epithelium. In fully developed malignancies the basal lamina and pseudopodia were absent with the latter replaced by convoluted plasma membrane which was in contact with disintegrated connective tissues. Hattori (1970) using methylcholanthrene in mice reported that in malignant cells, the basement membrane, microvilli and pseudopodia of basal cells, desmosomes, mitochondria, tonofibrils, free ribosomes, Golgi apparatus and pinocytic vesicles exhibited marked irregularity in orientation, shape, number and size. The findings of Marefat et al (1979) using DMBA in hamster were essentially similar to those of Hattori.

CYTOLOGY

Experimental work on exfoliative cytology done in oral cancer models is primarily concerned with testing the ability of this diagnostic tool

to reflect the true state of affairs occurring during carcinogenesis. Findings are, by and large, not consistent. Studies showing a good correlation between the diagnoses from cytologic smears and from biopsy and thereby justifying its usefulness include those of Stahl (1963a, b), Camilleri and Smith (1964), Fischman and Greene (1966). The study of Chaudhry et al (1967) pointed out that error in cytologic diagnosis was greatest in the late stages of carcinogenesis when such errors were least desired. The study of Levij et al (1967) demonstrated the almost complete failure of cytology as a diagnostic tool in a hamster experimental model.

HISTOCHEMISTRY AND BIOCHEMISTRY

It is generally believed that changes in the molecular constituents within cells accompany malignant transformation of tissues although a cause and effect relationship cannot yet be proven. Histochemical and biochemical investigations may be utilized to detect cellular changes tending towards malignancy which are too subtle to be detected at the macroscopic and microscopic level. Histochemical studies and special tissue labelling techniques can also reveal changes in tissue structures which cannot be demonstrated by routine staining techniques.

Using a variety of histochemical stains and special techniques, Morris (1958) was able to demonstrate an increased thickness of basement membrane of the epithelium in the preneoplastic hyperplastic stage in a hamster model system. The basal lamina thinned out in papillomas and disappeared in areas of cellular invasion. Reiskin et al (1960) and Morris et al (1961) found that the sulfhydryl (SH) content in the cheek pouch mucosa of hamsters increased in tumourous condition. Among the three enzymes studied by Scott et al (1960, 1962),

namely hexokinase (Hex), glucose-6-phosphate dehydrogenase (G6PDH) and phosphogluconate dehydrogenase (PGD), the changes of concentration of the last enzyme showed the greatest degree of correlation with the observed neoplastic state. Histochemical studies on hydrolytic enzymes and dehydrogenases made by Mori et al (1962) demonstrated alkaline phosphatase activity was present to a striking extent on the surface of neoplastic epithelium induced by DMBA in hamsters. Santis et al (1964) demonstrated that the activities of acid phosphatase, alkaline phosphatase, beta-D-galactosidase increased from normal epithelium to leukoplakia to epidermoid carcinoma while the reverse was true for esterase. Shklar (1965) concluded that the consistent findings of an increase in lactic dehydrogenase activity and of a decrease in succinic dehydrogenase activity during carcinogenesis were highly suggestive of a fundamental alteration in metabolism from aerobic respiration to anaerobic glycolysis or fermentation. Likewise, Evans et al (1980) studied the activities of succinic dehydrogenase, G6PDH and lactate dehydrogenase and attempted to postulate their roles in oral carcinogenesis. Lekholm et al (1975) studied the lipid content in rat palatal epithelium during DMBA- and 4NQO- induced carcinogenesis and were able to demonstrate histochemically a focal loss of lipid stainability in the epithelium during the advanced stages of carcinogenesis.

CELL KINETICS

Studies on cell kinetics are usually carried out by means of tritiated thymidine labelling and autoradiography. Using these techniques, it was demonstrated by Reiskin and Mendelsohn (1964) and Nagamine (1978) that during DMBA-induced carcinogenesis in the hamster cheek pouch the times of the various phases in the cell cycle as well as the cell cycle as a whole were shortened. In addition Toto and Swatske (1973) found that the number of basal cells were strikingly increased, reflecting more cells entering the DNA synthesis in the DMBA/ hamster system. A novel way

of studying cell kinetics during oral carcinogenesis was described by Eveson and MacDonald (1978). These investigators divided the oral epithelium into three horizontal compartments: the keratinized, maturation and progenitor compartments, and quantified their thickness, cell numbers and cell sizes. They found that when carcinogenesis was progressing, the latter two compartments increased in thickness owing to progressive increase in size as well as in number of the progenitor cells (the basal cells).

CYTOGENETICS

Cytogenetic studies are not normally done with oral tissue. However, Ahlström and Mark (1975) did carry out a comparative cytogenetic analysis of oral and cutaneous squamous cell carcinomas induced in rats by topical application of DMBA and 4NQO. Cells from the tumours produced were cultured and the chromosomes studied in vitro. It was found that there were non-random chromosomal patterns which were specific for the carcinogen used irrespective of the location of the tumours.

In another study using rats, Wallenius et al (1975) studied the cytogenetics and G6PDH activity in oral carcinomas induced by 4NQO and DMBA. There was usually a close relationship, starting from the dysplastic stage, between chromosomal changes and the simultaneous presence of morphologic atypia and metabolic atypia, the latter being represented by the aberrant G6PDH activity. Changes in X-chromosomes, which in humans code the synthesis of G6PDH molecules, could perhaps explain deviating metabolic reactions in premalignant cells.

2. MODIFYING FACTORS IN EXPERIMENTAL ORAL CARCINOGENESIS

Greenstein (1954) stated : " the carcinogenic potency of an agent does not reside in the nature of the agent alone but is a function of the following factors: the dosage, the nature of the vehicle, the mode and length of time of administration of the agent; the strain, the species, the sex, and the age of the test animals; the site of application, the presence of concomitant factors such as the level of essential dietary constituents and the number of animals kept in a cage, and perhaps still other as yet unknown conditions." By and large, his statement is still true.

THE CARCINOGEN AND VEHICLE

The concentration of the carcinogen, frequency of application, the solvent in which the carcinogen is dissolved and the promoting agent used in conjunction with the carcinogen can all exert certain influences, either singly or in combination, on the efficacy and behaviour of the carcinogen. For example, Morris (1961) found that a 0.5 percent concentration of DMBA solution was the optimal concentration for maximum oral tumour response in hamster with minimum latent period and no loss of animals due to toxicity. Further, application of the carcinogen three times per week rather than two resulted in a shorter latent period. Salley (1954) and Marefat and Shklar (1977) regarded acetone as the most suitable solvent for DMBA. Though it is most common to dissolve DMBA in mineral oil or acetone, combinationsof DMBA with liquid paraffin and benzene have also been employed with good success in hamster cheek pouch carcinogenesis (Salley, 1954; Milievskaja and Kiseleva, 1976; Franklin and Smith, 1980; Evans et al,1980). Dachi (1961) found that a 2 percent DMBA dissolved in the promoter Tween 60 shortened the

latent period for tumour appearance in hamsters and also the life-span of the animals when compared to 0.5 percent DMBA dissolved in mineral oil. Silverman and Shklar (1963) found that the promoter croton oil retarded the appearance of cheek pouch carcinoma in young hamsters but enhnaced carcinogenesis in older hamsters.

ANIMAL VARIABLES

Morris (1961) found that the tissue of the cheek pouch of old hamsters was more resistant to carcinogenic stimuli than that of young hamsters. Five week old animals appeared to be the ideal age for experimental oral carcinogenesis study from the standpoint of ease of manipulation and tumour production. The sex of the animals appeared to be irrelevant in the response of hamsters to oral carcinogens. There was a slight difference in the latent period when two different strains were used but no difference when different litters of the same strain were used.

Kreshover and Salley (1957) studied the predisposing factors that might affect oral carcinogenesis in mice and hamsters. These investigators found that factors such as sex, strain, as well as other variables including vitamin A and B deficiency, gonedectomy , increased gonadal function by addition of estradiol benzoate and testosterone propionate did not result in alteration of the intraoral tissue resistance to tobacco smoke and benzpyrene. The epidermis was however affected by such variables.

Eveson and MacDonald (1977) found that the hamster oral epithelium failed to respond to the carcinogenic action of 4NQO which is so effective, on the other hand, in rats. They ascribed the difference in behaviour to

species differences.

PORTAL OF ENTRY

Many early animal experiments on oral carcinogenesis were unsuccessful in producing changes in the oral mucous epithelium while tumours could be produced in the epidermis with ease when the same carcinogen was used (Levy et al , 1950 ; Levy and Ring, 1950; Kreshover, 1955).

The facts that carcinogens were shown in fluorescence studies to accumulate in the sebaceous glands of mouse skin (Simpson and Cramer, 1943, 1945) but not in scarred dermal tissue which was devoid of skin appendages (Lacassagne and Latarjet, 1946) or epidermis of new born mice that had not yet developed skin appendages (Cowdry, 1955) led to the belief that a carcinogen needs a pathway of entry into the epithelium. Levy et al (1951) proposed that the resistance of the oral mucosa to chemical carcinogenesis was due to the absence of sebaceous glands and hair follicles.

Supportive evidence for Levy's proposal included the success in producing malignant change when methylcholanthrene was applied beneath the oral mucosal epithelium but not when topically applied (Levy, 1958). Further support was provided by Goldhaber's (1958) study when it was demonstrated that the carcinogen remained localized in the superficial layers of the oral mucosal epithelium. Evidence against Levy's proposal included the success of oral carcinogenesis in hamsters using topical DMBA (Salley, 1954; Salley and Kreshover, 1959), the demonstration by fluorescence studies (Salley,1961) and carbon 14 labelling and autoradiography (Meskin and Woolfrey, 1964) of the penetration of carcinogens through oral mucosal epithelium to the lamina propria.

Further evidence against the portal of entry theory was provided by Wallenius (1965b) who demonstrated that rat's cheek skin containing normal skin appendages transplanted into the buccal cavity was resistant to chemical carcinogenesis. The lack of sebaceous glands and hair follicles could thus not explain the resistance of the oral mucosa to topically applied carcinogens.

SALIVA

Another theory proposed by Levy et al (1951) and supported by occasional studies such as that of Hill and Toto (1973) to explain the poor susceptibility of the oral mucosa to carcinogens was that the saliva might act as an inhibitory or protective agent. However, painting of saliva on the skin of mice or saliva contamination of perioral skin failed to protect the skin from chemical carcinogenesis (Kolas, 1955). As a result, it was suggested that the saliva did not possess specific anticarcinogenic action but rather that the protection was due to the cleansing and diluting effect of the saliva and tongue movement (Kolas, 1955; Kreshover, 1952, 1955). The oral mucosa was also shown to be resistant to chemical carcinogenesis after attempts to eliminate the influence of saliva by surgical extirpation of the major salivary glands (Goldhaber et al , 1956 ; Kreshover and Salley, 1957). Experimental evidence thus far tended to negate the concept of a specific protective effect of saliva as proposed by Levy et al (1951).

Newer ideas on the role of saliva were offered by Stormby and Wallenius (1964) following their success in obtaining a higher cancer yield in the palate of hamsters after surgical extirpation of the major salivary glands and upper cervical ganglion and the recently discovered (Kronman and Chauncey, 1964) fourth major salivary gland

in hamsters. These investigators considered that previous experiments mentioned above were done in inappropriate conditions in that the salivary secretion in the animals was not sufficiently reduced. In a subsequent study, Wallenius (1966),using rats which were painted with DMBA on the palate, observed that oral cancers invariably appeared earlier in animals having their major salivary glands extirpated and salivary secretion intermittently inhibited by scopolamine or inhibited by scopolamine alone. Interestingly, there was little difference between animals having salivary gland extirpation only and intact control animals. Wallenius (1966) therefore concluded that the protective capacity of the saliva seemed to be due to the presence of a mucous layer separating the carcinogen from the mucosal epithelium rather than the saliva having a cleansing effect. Wallenius (1966) also demonstrated the penetration of another carcinogen, benzpyrene, through the oral mucosal epithelium to the lamina propria in rats with scopolamine saliva inhibition. Similar desalivation experiments on hamsters were done by Smiler (1970) using DMBA as a carcinogen and comparable findings were obtained.

The above viewpoint regarding the role of saliva in carcinogenesis inhibition held by Wallenius (1966) was altered as a result of subsequent findings in a study using the water-soluble carcinogen 4-nitroquinoline 1-oxide (4NQO). Wallenius and Lekholm (1973a), on repeated application of this carcinogen onto the palate of rats, obtained carcinomas at the site of application in 100 percent of intact animals after 7 months. Carcinomas appeared in addition at the base of the tongue (75%), on the gingiva of the mandible (20%) and in the stomach (20%). The surprising high frequency of cancer and the efficiency of the carcinogen indicated that neither the dilution capacity nor the mechanical protective effect of saliva was of importance for this water-soluble carcinogen. Many previous difficulties in explaining carcinogenesis in the oral mucosa

could thus depend not so much on some general resistance possessed by the oral mucosal tissues, possibly enhanced by salivary protection, as on the fact that a carcinogen unsuited to the oral mucosa had been used.

In still later studies Wallenius and Lekholm (1973b) again revised some of their concepts regarding the role of saliva in experimental oral carcinogenesis. They found that a salivary layer applied to the auricle prior to the application of DMBA and 4NQO resulted in a 30 percent delay in the time for the development of carcinomas (Wallenius and Lekholm, 1973b) and also that temporary xerostomia produced by scopolamine greatly shortened the period for the development of intraoral carcinomas induced by 4NQO (Lehkolm and Wallenius, 1976a). These authors again concluded that a protective effect of the saliva existed whether the oral mucous membrane was attacked by water- or fat-soluble carcinogens. However; it was not known whether the protective effect of the saliva per se depended only on the presence of mucopolysaccharide material or if it was a combined effect of this material and other factors such as immunological products, trace elements, and enzyme systems (Lekholm and Wallenius, 1976a). Since these investigators also found that carcinogenesis was delayed longer in the case of 4NQO compared to DMBA when saliva was painted on the epidermis of rats while on the other hand 4NQO could assert itself so easily in the oral mucous membrane, Wallenius and Lekholm (1973b) considered that the structure of the two tissues or their different chemical characteristics could account for the difference in response to carcinogens of different nature (water-soluble or insoluble). Following this line, this group of workers (Lekholm et al , 1975; Lekholm and Wallenius, 1976b) started to investigate the fatty acid and phospholipid contents of the oral mucous membrane in relation to carcinogenesis but so far no conclusive statement has been made.

DIETARY FACTORS

Among the mineral elements in the diet, the role of zinc in oral carcinogenesis has been investigated. The first study was that of Poswillo and Cohen (Poswillo and Cohen, 1971; and Poswillo, 1973). These investigators found that hamster cheek pouch carcinogenesis was inhibited in animals supplemented with zinc. However, Edwards (1976) failed to demonstrate the inhibitory effect of zinc on oral carcinogenesis in a similar system. The study of zinc was further expanded by Wallenius's group (Wallenius et al , 1979; Mathur et al, 1978 unpublished). These workers concluded that supplementary zinc in rats delayed the onset of tumour formation whereas a low zinc diet facilitated the development of initial histologic changes. Once the protective barrier was overcome, however, the tumour development was accelerated by the high zinc diet.

Salley et al(1962) found that chronic thiamine deficiency decreased the time of latency of DMBA-induced carcinogenesis in the hamster cheek pouch.

In connection with oral carcinogenesis, vitamin A is the most widely studied vitamin. Rowe and Gorlin (1959) found that vitamin A deficiency in the diet increased the rate of DMBA-induced tumour formation in hamster cheek pouch carcinogenesis. Polliack and Levij (1967, 1969) observed that vitamin A palmitate applied topically to the hamster cheek pouch greatly enhanced malignant tumour formation by DMBA. Polliack and Levij (1969) concluded that such a promoting effect might be due to membrane-labilizing properties of vitamin A resulting in activation and release of lysosomal enzymes which could play an important role in the initial stages of cell division and tumorigenesis. On the contrary, Shklar et al (1980a, 1980b) found that systemic application of vitamin A analogue, 13-cis-retinoic acid through the oral route delayed carcinogenesis in the cheek pouch

and tongue of hamsters and that the tumours formed ultimately were smaller and less invasive. They suggested that immunoenhancement might be the possible mechanism through which vitamin A acts. McGaughey et al (1977) studied the effects of cyclic AMP and cyclic GMP and their corresponding nucleotides and nucleosides on vitamin A promotion of tumour formation induced by DMBA in hamster cheek pouch. The findings were highly inconclusive.

Rubin and Levij (1973) found that local application of vitamin D_2 and D_3 caused inhibition of tumour formation by DMBA in hamster cheek pouch. They explained that the vitamin D's might produce their effect by inducing the synthesis of alkaline phosphatase which binds calcium to the cell membrane thereby regulating better the flow of metabolites which are involved in the mitotic processes.

PHYSICAL FACTORS

Simultaneous application of mechanical trauma to the intraoral site and painting of a carcinogen has been shown invariably to result in enhancement of tumour development and improvement of tumour yield (Renstrup et al , 1961, 1962; Fujino et al, 1965; Fujita et al, 1973). On the contrary, application of trauma in the form of electrosurgery prior to the application of a carcinogen resulted in the paradoxical finding of a considerable decrease in tumour yield (Poswillo and Cohen, 1971).

Shklar (1968) studied the effects of trauma in the form of incision, massage and compression on well established carcinomas in the hamster cheek pouch and found that the behaviour of tumours was unaltered and that metastasis formation was not observed. These findings suggested that biopsy of a carcinoma does not produce greater spread of the lesion.

The effects of irradiation on established carcinomas in the hamster cheek pouch were studied by Brown (1970) who found that the cell cycle time of tumour cells was prolonged while that of normal cells was reduced. Horn et al. (1971) observed recurrence of carcinoma shortly after an initial destruction of the tumour by X-irradiation in hamster cheek pouch.

IMMUNOACTIVE AGENTS

Some interest has been placed on the immunosuppressive agent Antilymphocyte Serum (ALS). Systemic application of ALS through the subcutaneous route was demonstrated to enhance intraoral carcinogenesis and increase tumour yield in hamsters (Woods, 1969 ; Giunta and Shklar, 1971; Giunta et al , 1974). Neonatal hamsters pretreated with ALS were found to accept transplants of carcinomas readily Merk et al , 1979). On the other hand, the immunoenhancing agent Bacillus Calmette-Guérin (BCG) retarded carcinogenesis (Giunta et al , 1974). Experiments with ALS and BCG appear to have demonstrated the concept that immunosuppression enhances and immunostimulation delays carcinogenesis.

Eisenberg and Shklar (1977) studied the effects of levamisole, an antihelminthic drug having immunoenhancing properties on cell-mediated immunity, on hamster cheek pouch carcinogenesis. It was found that levamisole significantly retarded the initiation and progression of carcinogenesis. On the other hand, Cottone et al (1979) found that while levamisole retarded carcinogenesis in the hamster cheek pouch initially, such an effect was not maintained after 12 weeks of DMBA application.

Pyran copolymer, another immunoenhancing agent, was investigated

in DMBA/hamster model by Elzay and Regelson (1976). It was found that this substance inhibited the carcinogenic effect of DMBA during the initiation phase but did not influence the progression of tumours.

Dinitrochlorobenzene (DNCB), an immunostimulant as well as a potent allergen causing a delayed hypersensitivity reaction, was investigated by Marshack et al (1978) and Mohammad (1979). It was found that DNCB retarded carcinogenesis and decreased tumour yield in the DMBA/hamster model but had little effect on well established tumours.

The above studies on immunoactive substances support the general concept that tumorigenesis is related to some impairment of the tumour immunosurveillence system and that non-specific stimulation of the cell-mediated immune system can retard tumorigenesis (Mohammad, 1979).

In addition to the above studies concerning immunoactive substances, Gould (1976) was able to demonstrate, by haemagglutination methods, that DMBA itself exerted immunosuppressive effects on hamsters during induction of cheek pouch cancer. However, whether the DMBA or the neoplastic process itself caused such immunosuppression could not be established in such a small study.

CYTOSTATIC AGENTS

A number of cytostatic agents which are used for chemotherapy of human malignancies have been studied in hamster cheek pouch models. Shklar et al (1966) found that concomitant subcutaneous injection of methotrexate and painting of DMBA on hamster cheek pouches resulted in hastened cancer formation and yielded more anaplastic and

larger tumours. Similar effects were obtained by Shklar (1972) with 5-fluorouracil. A similar study by Weather and Halstead (1969) on the effects of 5-fluorouracil was inconclusive.

Levij et al (1970) carried out a comparative study in DMBA/hamster model using several cytostatic agents. Concurrent topical application of cyclophosphamide, methotrexate, or vinblastine with the carcinogen caused inhibition of tumour formation and suppression of further development of tumours to varying degrees, with vinblastine exerting the maximum effect.

Another antimetabolite, azathioprine, used as an immunosuppressant in human organ transplantation, was studied by Sheehan et al (1971). It was found that in animals treated with azathioprine through drinking water, carcinomas developed more rapidly and tended to be anaplastic. However, in the late stage of tumorigenesis, azathioprine apparently had a tumour destructive effect, causing most of the tumours to regress. These investigators concluded that azathioprine augmented carcinogenesis because of its immunosuppressive properties and then destroyed the tumour through its antimetabolic action.

MEMBRANE-ACTIVE AGENTS

The effects of vitamin A, which is regarded as a membrane-active agent, have already been reviewed in a previous section and will not be repeated here. Cortisone acetate, which is a corticosteroid hormone and which can also be regarded as a membrane-active agent, will be discussed under the section on hormones. Besides these two agents, the other membrane-active agents studied in oral cancer models are chlorpromazine (CPZ) and dimethylsulfoxide (DMSO).

The tumour inhibitory effect of chlorpromazine (CPZ) was demonstrated by Polliack and Levij (1972) in the DMBA/hamster model. Concurrent topical application of CPZ and DMBA to the cheek pouch resulted in marked inhibition of tumour formation. Pretreatment of the cheek pouch with CPZ resulted in almost complete inhibition of tumour formation. These investigators suggested that the inhibitory effect of CPZ was related to its stabilizing effect on biologic membranes.

Dimethylsulfoxide (DMSO) acts as a penetrant carrier of biologic membranes in that it facilitates the entry into the membranes of substances that are dissolved in it(Jacob et al,1964). When DMSO was used as the solvent for DMBA, Sanders and Dachi (1966) noted a shortening of the latent period and Elzay (1967) observed an increase in tumour yield in hamster cheek pouch carcinogenesis by DMBA. In contrast to the above findings, Shklar et al (1969) found that DMSO in combination with DMBA resulted in a retardation and decrease in tumour yield. Siegel and Shklar (1969) were able to reproduce the same findings in another experiment. Moreover, when DMBA, DMSO and triamcinolone were applied together, no tumours developed. The observation was considered to be a consequence of the tissue damaging effect of DMSO and triamcinolone.

HORMONES

Levij et al (1968) found that castration of hamsters had no effect upon the initiation of chemical carcinogenesis and did not delay the appearance of tumours. However, the subsequent rate of tumour growth was depressed and the number of tumours was reduced.

Polliack et al (1969, 1970a)studied the effects of systemically administered (i.m.)oestrogen and testosterone on DMBA-induced

carcinogenesis in the cheek pouch of castrated and intact hamsters. It was found that oestrogen potentiated and testosterone decreased carcinoma formation in intact animals only.

Sabes et al (1959) found that topical application of a combination of prednisolone acetate and DMBA on hamster cheek pouch hastened the development of tumours and resulted in a higher tumour yield compared to application of Tween 80 and DMBA or DMBA alone.

Shklar (1966,1967)studied the effect of systemically administered cortisone acetate through the subcutaneous route on hamster cheek pouch carcinogenesis induced by DMBA, and found that all stages in carcinogenesis were hastened. There were increases in tumour size and the extent of invasion in the connective tissueswhen compared to animal painted with DMBA only.

In contrast to the findings of Shklar of a tumour-enhancing effect of cortisone acetate, Polliack et al (1970b) found that concurrent topical application of cortisone acetate and DMBA to hamster cheek pouches resulted in a marked decrease in tumour formation.

SUMMARY

The earliest work on experimental oral carcinogenesis was concerned with the mere production of cancer in the oral cavity of experimental animals with well known potent carcinogens, just to see whether or not the oral mucous membrane was susceptible to carcinogens. When this type of investigation became successful, research started to ramify into different areas of interest. Studies that involved primarily the success or failure of cancer model production included: investigation

into the problems of species, strain difference and specific sites within the oral cavity with respect to their susceptibility to oral carcinogenesis. Animals known to be susceptible to oral carcinogenesis can be used to test the carcinogenicity of suspected carcinogenic agents such as tobacco smoke, betel snuff and alcohol. When a reliably reproducible model had been established, it became possible to utilize it to study various aspects of carcinogenesis. These include: characterization of the carcinogenic processes by means of morphology, histopathology, ultrastructure, histochemistry, biochemistry, cytology, cell kinetics and cytogenetics; investigation into the mechanism of carcinogenesis, particularly on how carcinogens gain access to the target in the oral mucosal epithelium and studies on the factors that can modify the carcinogenic processes with particular emphasis on carcinogenesis in the mouth. The last area is the most popular in oral carcinogenesis and may prove to be the most useful in cancer therapy.

CHAPTER 3

4-NITROQUINOLINE 1-OXIDE (4NQO)

- ITS CHEMISTRY, CARCINOGENICITY, METABOLISM AND MODE OF ACTION IN CARCINOGENESIS

4-Nitroquinoline 1-oxide (4-Nitroquinoline N-oxide, 4NQO) has been investigated extensively since its initial synthesis in 1942 (Ochiai et al, 1943). Literature on this compound and its derivatives exceeds three hundred papers, much of it produced by Japanese researchers. Key literature on 4NQO includes the papers by Nakahara and Sugimura (1957), Nakahara et al (1958), Nakahara and Fukuoka (1959,1960) and the monograph edited by Endo et al (1971b). More recent and similarly important papers relating to 4NQO include those by Nagao and Sugimura (1976), Tada and Tada (1972, 1976), and Kondo (1976). The purpose of this chapter is not to produce something of comparable dimensions to the above excellently written papers but to provide an overview of 4NQO with particular emphasis on its carcinogenicity, metabolism and postulated mode of action in experimental carcinogenesis.

1. CHEMISTRY

4NQO (Figure 3.1) was synthesized for the first time by Ochiai and his collaborators in 1942 (Ochiai et al, 1943). This group of organic chemists, specializing in the chemistry of quinoline compounds later synthesized various derivatives of 4NQO, an interest by and large stimulated by the subsequent demonstration of the carcinogenicity of 4NQO by Nakahara and his colleagues in the late nineteen fifties. By 1970, more than forty-seven derivatives of 4NQO had been synthesized some of which have been shown to be carcinogenic.

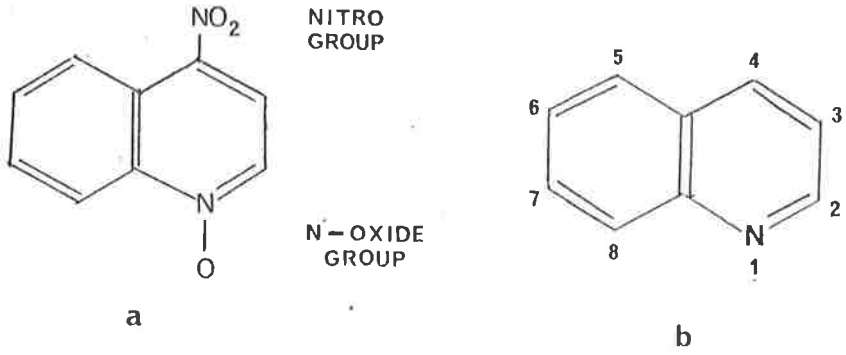


Figure 3.1. (a) Chemical structure of 4-nitroquinoline 1-oxide (4NQO), (b) Quinoline molecule with numbering of the ring atoms.

The organic and physical chemistry of these double-ringed aromatic amine oxides was exhaustively reviewed by Ochiai (1967). Physically, 4NQO is a stable yellow crystalline compound with a melting point of 155-157°F It is soluble in a large variety of solvents including water, acids, benzene, acetone, alcohols and propylene glycol. Structurally, it is characterized by two strong polar (electronegative) groups, i.e. the N-oxide and the nitro (NO_2) groups, in the molecule.

Two of the most representative chemical reactions of 4NQO and its derivatives are nucleophilic replacement (nucleophilic substitution) and reduction of the nitro group. As early as 1953, Okabayashi (1953b) reported the replacement of the nitro group by a sulfhydryl (SH) group. The nitro group is also known to undergo ready replacement with halogens, alkoxyl, aryloxyl, mercapto,

hydroxyl and amino groups. A newly attached group on the benzene ring at position 4 can in turn be replaced. For example, 4NQO reacts readily with acid chlorides, such as phosphoryl chloride (Ishikawa, 1945) or with concentrated hydrochloric acid (Okamoto, 1951) to give 4-chloroquinoline 1-oxide. 4-Chloroquinoline 1-oxide will in turn react with hydroxylamine to produce 4-hydroxy-aminoquinoline 1-oxide (4HAQO) (Ochiai et al,1957).

The nitro group in 4NQO readily undergoes hydrogenation (reduction) under various conditions. For example, 4-hydroxy-aminoquinoline 1-oxide (4HAQO) is prepared by the catalytic reduction (hydrogenation) of 4NQO with palladium charcoal in ethanol (Ochiai et al, 1957). The reason why hydrogenation stops at this reduction stage can be explained by the comparatively high stability of the 4HAQO and by its poor solubility in ethanol. Hydrogenation progresses beyond this stage only very gradually to produce 4-aminoquinoline 1-oxide (4AQO) and aminoquinoline (AQ) as a result of deoxygenation of the N-oxide group. These steps only proceed in a strong acidic medium (Figure 3.2). Deoxygenation of the N-oxide group only, can also be achieved but under very rigid experimental conditions for there is the tendency of the nitro group to be reduced far earlier than the rupture of the N-O bond of the N-oxide group and it cannot, therefore, be expected to eliminate the oxygen atom without a concomitant reduction of the nitro group (Kawazoe, 1971).

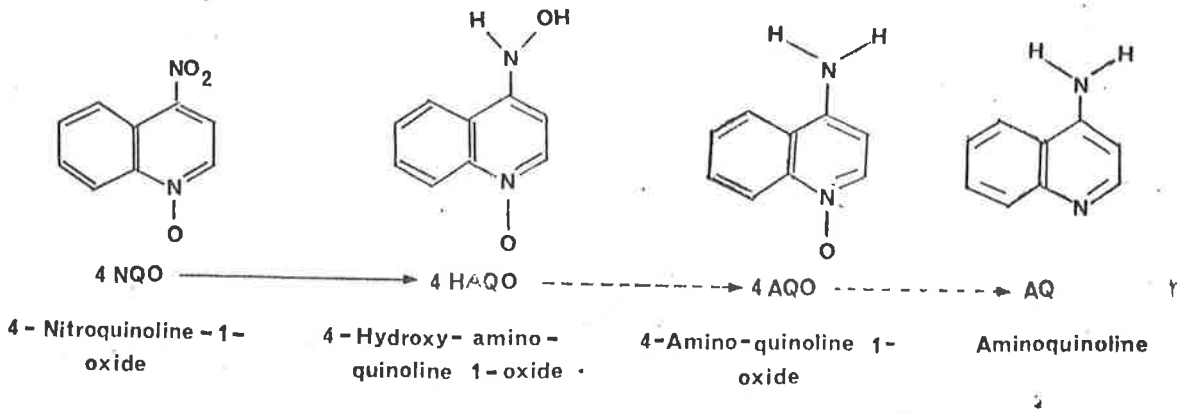


Figure 3.2. Schematic diagram of the stepwise reduction (hydrogenation) of 4-nitroquinoline 1-oxide.

The above information is only a very brief review of the chemistry of the quinoline compounds. Further elaboration is beyond the scope of this review. However, the chemical reactions, albeit briefly, do demonstrate the chemically active nature of the 4NQO molecule. These same sites on the 4NQO molecule are therefore likely to be important in a patho-biologic context in relation to the mechanisms of 4NQO carcinogenesis.

2. EARLY WORK BY NAKAHARA AND CO-WORKERS

The early findings of the tumoricidal/anticancer action of 4NQO on sarcoma cells in vitro and in vivo (Sakai et al, 1955), the fungicidal and mutagenic action of 4NQO on some fungal species (Sakai et al, 1957; Okabayashi, 1953a, 1955) and the postulate of Haddow et al (1948) that anticancer agent can also be carcinogenic and vice versa, had aroused the interest of Nakahara and co-workers in exploring the carcinogenicity of this compound in experimental animal models.

In their first experiment reported in 1957, Nakahara and Sugimura applied 0.25% solution of 4NQO in benzene to a depilated area of the skin of 20 mixed strain mice three times a week by means of a small glass pipette. The amount of the solution was about 1/50 c.c. (0.02 ml) for each application, equivalent to 0.05 mg of 4NQO. The treatment was discontinued when the macroscopic appearance of the tumours produced warranted the tentative diagnosis of malignancy.

The macroscopic changes that occurred in sequence were depilation of hair, papilloma and nodules formation occurring between 11 to 20 weeks, papillomas developing into dome-shaped growths and then to roundish infiltrative tumours occurring between 17 to 29 weeks. Keratin horns, reflecting the highly keratinizing nature of these growths were a prominent feature.

Histologically, the malignant tumours produced were infiltrative squamous cell carcinoma associated with superficial keratin horns and at times large keratinizing cyst and haemorrhagic cystic spaces. Judging from the photomicrographs, it appears that the two early squamous cell carcinomas reported to occur in macroscopically hyperplastic areas were in fact carcinoma in situ in modern terms.

Nakahara and Sugimura (1957) also noticed a small number of infiltrative fibrosarcomas among the tumours produced. At autopsy, only two mice showed metastasis, one from a carcinoma, the other from a sarcoma. While most of the internal organs showed no apparent changes, the liver exhibited amyloidosis.

Based on these findings, Nakahara and Sugimura (1957) concluded that 4NQO was the first bicyclic compound to be shown to have the carcinogenic activity of the magnitude approaching that of the most potent polycyclic hydrocarbons, for example 7,12-dimethyl-1, 2-benzanthracene (DMBA), 20-methylcholanthrene (MCA), a fact suggesting the possibility that a carcinogen of even greater activity might be found among its various derivatives. Although lacking proof these workers strongly suspected that the nitro radical might be very important in relation to the carcinogenicity of this class of compounds. Hence they called for the elucidation of the relationship between the chemical structure of 4NQO and its carcinogenic action.

Nakahara et al (1958) subsequently conducted a series of animal painting experiments using several selected 4NQO derivatives: 4NQO (I), 4-nitro 2-ethyl-quinoline 1-oxide (II), 4-nitroquinaldine 1-oxide (III), 6-chloro-4-nitroquinoline 1-oxide (IV), quinoline 1-oxide (V), 3-methylquinoline 1-oxide (VI), 4-nitroquinoline (VII), and 6-nitroquinoline (VIII) (Figure 3.3).The painting on mouse skin of these substances revealed that compounds (I), (II), (III), (IV) were carcinogenic and (V), (VI), (VII) and (VIII) were not, indicating that both the nitro group at position 4 and the N-oxide at position 1 of the quinoline nucleus were necessary for the carcinogenicity of 4NQO. Also the derivatives of 4NQO with C_2H_5, CH_3 or Cl at the position 2 or 6 showed the carcinogenicity of about the same order of magnitude as the original 4NQO.

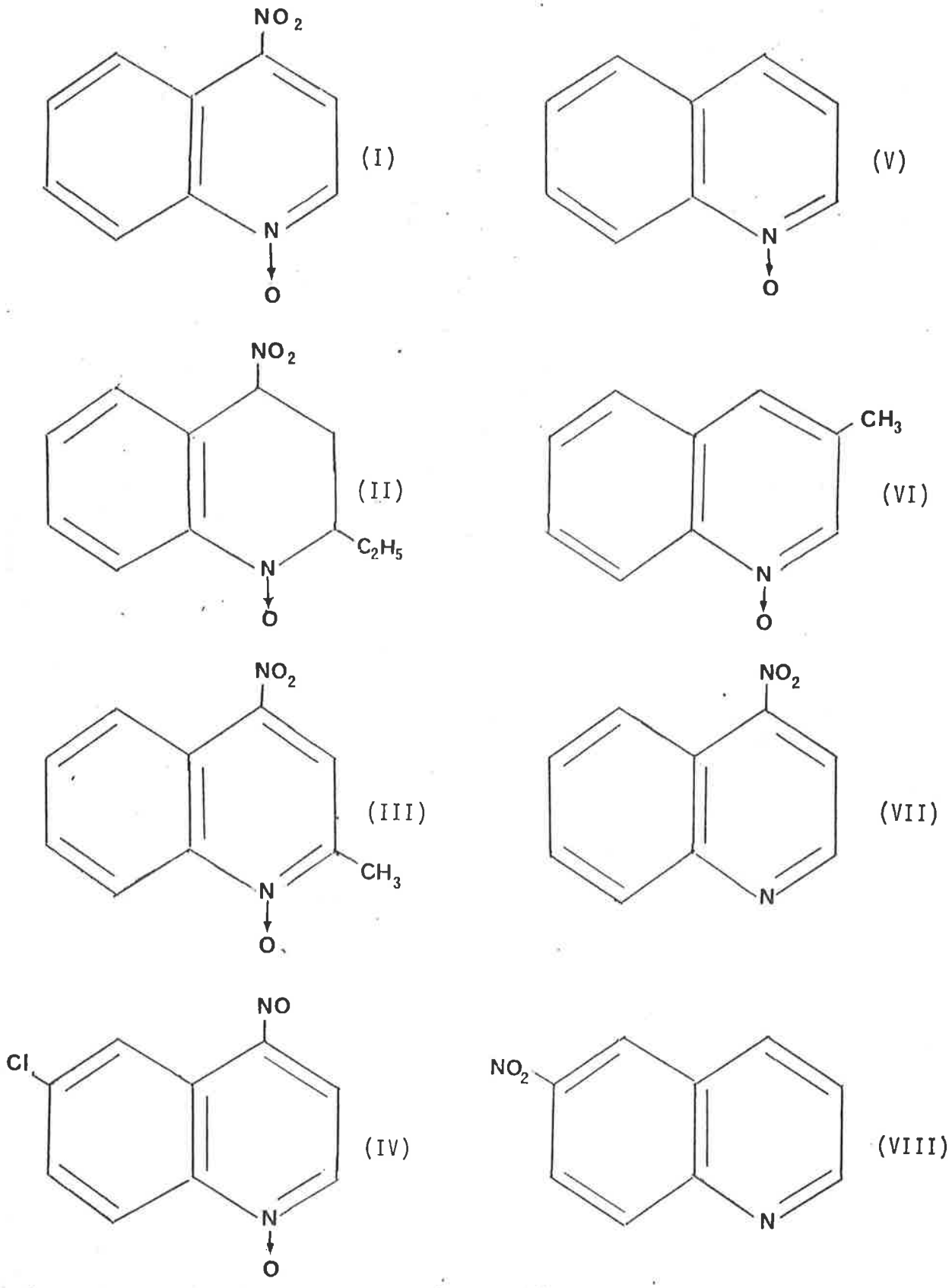


Figure 3.3. 4NQO and its derivatives. 4NQO (I), 4-nitro-2-ethyl-quinoline 1-oxide (II), 4-nitroquinaldine 1-oxide (III), 6-chloro-4-nitroquinoline 1-oxide (IV), quinoline 1-oxide (V), 3-methyl-quinoline 1-oxide (VI), 4-nitroquinoline (VII), and 6-nitro-quinoline (VIII).

Based on the findings in this series of experiments using multiple 4NQO derivatives, Nakahara and his group amended some of their earlier observations. In this series of experiments there were a few cases in which the early neoplastic changes took the form of surface erosions and ulcerated downgrowths without the usual piling-up of hornified material on top. It was also probable that some of the "sarcomas" described initially (Nakahara and Sugimura, 1957) might have been more properly diagnosed as spindle cell carcinomas.

The results of studies by Nakahara et al (1958) together with the already known chemical behaviour of the nitro and N-oxide groups (Nagata, 1971; Kawazoe, 1971) led to further experimentation of the substitution reactions between the nitro group on the 4NQO molecule and nucleophilic groups of tissue constituents, especially the SH groups.

The next series of experiments with 4NQO by Nakahara and Fukuoka (1959) were aimed at studying the carcinogenic mechanism of 4NQO. The experimental findings and concepts proposed by Nakahara and Fukuoka are summarized as follows. In contrast to other chemical carcinogens , the simplicity of structure and reactivity and the rapidity and regularity of tumour production by 4NQO were considered by Nakahara and Fukuoka (1959) to be desirable requirements of a model for the study of mechanisms of carcinogenesis. Because of the fact that substitution of the nitro group with SH groups takes place most readily within a physiologic pH range and without enzymatic intervention in microbial system (Okabayashi, 1953b) and because quinoline derivatives demonstrated to be carcinogenic generally show stronger reactivity of their nitro radical with SH compounds than those that are non-carcinogenic (Endo, 1958), Nakahara and

Fukuoka (1959) hypothesized that the very first chemical reaction which 4NQO undergoes *in vivo* could be a substitution reaction with SH compounds (Figure 3.4).

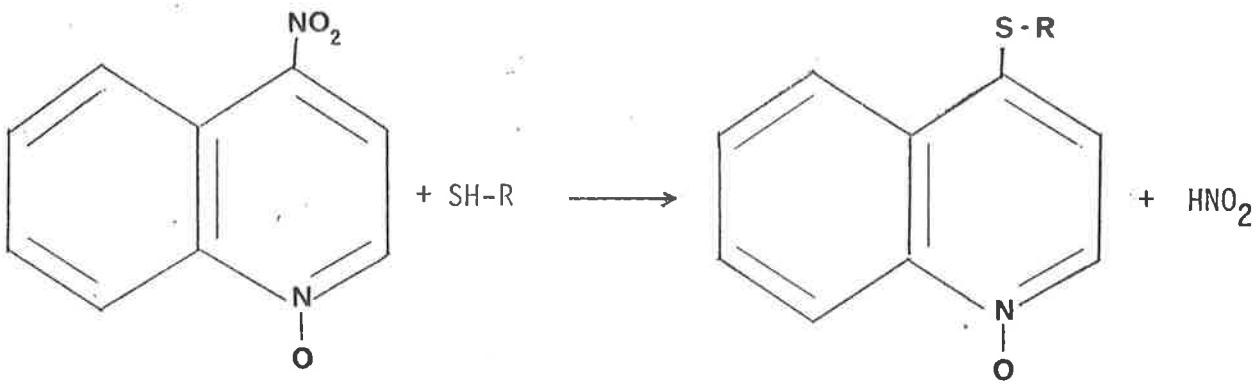


Figure 3.4. Substitution (replacement) reaction of 4NQO with SH (sulfhydryl) compounds.

The failure to demonstrate by subcutaneous injection the carcinogenicity of the reaction products from this substitution reaction ,however, led these two workers to postulate that the substitution reaction itself, and not the chemical products of the reaction, was responsible for the carcinogenic action of 4NQO. The energy released by the substitution reaction was the sole proximate cause of carcinogenesis. They further suggested that the energy released disturbed the normal replication process of the duplicants (DNA nucleic acid) by misdirecting the sequence of configurations which were being formed alongside the template, or by preventing one or more of the components of the duplicants from joining up to the forming chain. Cancerization would result from the summation of multiple irreversible changes of this nature , as suggested by Druckrey and Kupfmuller (1948). The summation theory of Druckrey and Kupfmuller (1948) stated that cancerization results from the summation of a multiple of irreversible alterations (now known as mutations) in the critical cell components - the duplicants (now known as nucleic acids).

Nakahara and Fukuoka (1960) further illustrated and gave supportive evidence to the summation theory by showing that sequential application of submanifestational doses of 4NQO and 20-methylcholanthrene or vice versa, either successively or separated by a time interval, resulted in comparable tumour yields. They further suggested that since their effects were capable of summation, the carcinogenic mechanism of these two chemically unrelated carcinogens might be qualitatively identical. Corollary, if they accomplished carcinogenesis through different mechanisms, they could not be capable of summation to each other. In their experiments, they also established that 10 to 20 applications three times a week of a 0.25% solution of 4NQO in benzene might

safely be regarded as a submanifestational dose of 4NQO.

The pioneering series of studies by Nakahara and colleagues reported from 1957 to 1960 are extremely interesting and of immense value. Starting with their initial discovery of the carcinogenic properties of 4NQO, they proceeded to comparative studies of its derivatives, relating chemical structures to carcinogenicity; exploration of the mechanism of chemical carcinogenesis and theories of carcinogenesis and thus opened up a spectrum of enquiries and studies that could possibly be carried out using 4NQO as a carcinogen. This early work in fact built the firm foundation for further studies which have since developed extensively and ramified into different branches of oncologic science. Though some of the concepts developed by Nakahara and colleagues have become outdated, their ingenuity in those early days cannot be overemphasized.

3. LATER STUDIES ON THE CARCINOGENICITY OF 4NQO

Since the early work of Nakahara and colleagues, more than seventy reports have been published on the carcinogenicity of 4NQO and its derivatives. Endo (1971) reviewed very comprehensively the work done on 4NQO carcinogenicity up to 1971. Later work was reviewed by Nagao and Sugimura (1976).

On the specificity of the carcinogenic action of 4NQO, Lacassagne et al (1961) reported that as far as painting of this compound on mouse skin was concerned, it appeared to be more sensitive to strain differences in animals compared to polycyclic hydrocarbons. Takayama (1960) obtained the quite unexpected finding that the painting of 4NQO on rat skin resulted in sarcoma formation.

Searle and Spencer (1966) showed that connective tissue tumours developed in larger numbers than epithelial tumours when small doses of 4NQO were applied to the skin of C57bl and C57blXIF mice. NZY mice, however, developed almost exclusively epithelial tumours with higher yields. Thus the type of tumour experimentally induced in mice by 4NQO would appear to depend on the strain of animals used and the dosage of the carcinogen.

Besides mice and rats the carcinogenic action of 4NQO was successfully demonstrated in birds (Nakahara, 1964), guinea pigs(Parish and Searle, 1966a) and hamsters (Searle and Woodhouse, 1963; Parish and Searle, 1966b).

Regarding the site of tumour formation, Mori and Yasuno (1961) succeeded in inducing multiple pulmonary adenomas by a single injection of 4NQO, with dd mice being more susceptible than C57bl mice. Mori (1961) using repeated subcutaneous injection of 4NQO in mice produced multiple pulmonary tumours, including well-established adenocarcinoma with metastasis. He also produced squamous cell carcinoma in the lung of rats under similar experimental conditions (Mori, 1962).

Experimental cancer formation by carcinogenic agents in the glandular stomach had been considered very difficult to achieve. Baba et al (1962) succeeded in producing adenocarcinoma in the glandular stomach in only one of 33 rats by directly applying 4NQO into the glandular stomach of rats through an artificial gastric fistula. Tumour formation was enhanced, however, when 20-methylcholanthrene was given cutaneously afterwards. Using mice, Mori (1967a) demonstrated, more successfully, the development of adenocarcinoma of the glandular stomach

and squamous cell carcinoma of the forestomach by using the technique of intragastric instillation of 4NQO. Mori and Ohta (1967) also demonstrated good success with 4-hydroxyaminoquinoline 1-oxide (4HAQO) in producing adenocarcinoma in the glandular stomach of mice using the intragastric instillation method. Later it was shown that 4HAQO.HCl induced a slightly higher tumour yield than 4NQO in mice as well as in rats (Mori et al, 1969c). All the stomach experiments with 4HAQO.HCl were done with the compound dissolved in ethanolic solution. Mori et al (1969c) postulated that ethanol solution first damaged the mucous barrier so that 4HAQO.HCl could penetrate into the mucosal layer of the glandular stomach and come into direct contact with epithelial cells.

Ito et al (1969) administered 4NQO suspended in olive oil, Tween 80 and water by intragastric instillation to rats after removal of the submaxillary and parotid salivary glands and were able to produce squamous cell carcinoma of the forestomach, poorly differentiated adenocarcinoma with metastasis formation and leiomyosarcoma of the glandular stomach. These investigators considered that saliva played an important role in preventing direct contact of the carcinogen with the stomach mucosa.A similar view was later put forward by Wallenius (1966) in relation to oral carcinogenesis. By introducing 4NQO dissolved in alkylbenzene-sulfonate and ethanol into rats, Takahashi (1970) was able to produce adenocarcinoma, haemangiosarcoma and haemangioma in the glandular stomach and papilloma and squamous cell carcinoma in the forestomach. Without the inclusion of alkylbenzenesulfonate, no adenocarcinoma was found, indicating that administration of alkylbenzenesulfonate certainly enhanced the induction of adenocarcinoma by 4NQO. N-methyl-N'-nitro-N-nitrosoguanidine

(MNNG) usually induces well differentiated adenocarcinoma. However, Sugiura (1973) demonstrated that the addition of a small amount of 4NQO in ethanol and alkylbenzenesulfonate following application of MNNG, both applied through drinking water,produced a good yield of undifferentiated adenocarcinoma. 4NQO treatment thus apparently converted well differentiated adenocarcinoma to undifferentiated types.

Regarding cancer production in the upper aerodigestive tract, the successful demonstration of labial and lingual carcinomas by Chino and Kameyama (1965) and Fujino et al (1965), of intraoral carcinoma of the palate and the tongue by Wallenius and Lekholm (1973a) and of oesophageal cancer by Horie et al (1965) is noteworthy. Oral carcinogenesis using 4NQO was discussed separately in chapter 2.

Regarding tumour induction in other sites, Hayashi and Hasegawa (1971) reported the induction of pancreatic tumours in rats using 4HAQO. HCl and Ito (1973) induced kidney transitional cell tumours in rats by implantation of cotton impregnated with 4NQO-beeswax.

In an attempt to observe the effect of age on tumour incidence, newborn, infant and young mice were treated with 4NQO by various groups of workers in the 1960's. It was found that pulmonary tumours, lymphoma and lymphatic leukaemia developed predominantly, especially in newborn mice, irrespective of the strain and the application route of the compound (Endo, 1971). Nomura and Okamoto (1972) injected 4NQO solution directly into the abdominal cavity of mouse fetuses. Injection of 4NQO on day 14 or 15 of gestation resulted in a high rate of abortions

and stillbirths. Injection on day 16 or 17 resulted in live offspring which all developed pulmonary tumours in the twenty-first week after birth. A single subcutaneous injection of 4NQO into pregnant mice per se on day 7 of pregnancy did not induce tumours.

One of the variables present in the various 4NQO studies carried out by various researchers is the vehicle in which the carcinogen was dissolved. Mori (1964) found that subcutaneous injection of 4NQO produced not only lung cancers in rats but also uterine sarcomas and carcinomas when the vehicle was changed from olive oil and cholesterol to olive oil and lecithin. Mori et al (1966) demonstrated a much higher tumour yield in the lungs of rats and mice using 10% lecithin in water as a vehicle compared to the other two combinations. Tanaka et al (1963) also observed that 4NQO dissolved in cotton seed oil induced sarcomas in C57bl mice with considerable frequency but, when 4NQO was dissolved in propylene glycol, no tumours were observed during a 300 days period. These results indicate that the vehicle changes not only the incidence of the tumour but also the target of the carcinogen.

Regarding the carcinogenic potencies of the derivatives of 4NQO, Takayama and Endo (1962) applied various derivatives of 4NQO to the skin of mice and showed that the carcinogenicities of the following compounds were in decreasing order of potency when compared: 6-chloro-4-nitroquinoline 1-oxide, 4-nitroquinoline 1-oxide and 4-nitroquinaldine 1-oxide. Lacassagne et al (1966) examined the sarcomagenic activity of three derivatives of 4NQO by repeated subcutaneous injection in mice ——6-methyl-4-nitroquinoline 1-oxide, 6,7-dimethyl-4-nitroquinoline 1-oxide and 6-fluoro-4-nitroquinoline

1-oxide. All three were shown to be carcinogenic, with the tumour incidence caused by 6, 7-dimethyl-4-nitroquinoline 1-oxide being higher than that caused by 4NQO. Thus, it is clear that appropriate substitution can enhance the carcinogenic activity of 4NQO, just as occurs with carcinogenic polycyclic hydrocarbons. Nakahara et al (1958) found that 4-nitroquinoline (4NQ) was not carcinogenic and that an oxygen atom on the ring nitrogen was essential for the strong carcinogenicity of 4NQO and its derivatives (Figure 3.3 VII). However, Mori et al (1969 a,b) reported the positive carcinogenicity of 2- and 4-nitroquinoline (2NQ and 4NQ) in mice, in which lung cancer and sarcoma were induced by subcutaneous injection. Recently, quinoline was proved to be carcinogenic by Ito (1976), who administered quinoline via the diet and was able to produce hepatoma and haemangiosarcoma in the liver of rats. Other derivatives of quinolines without the necessary presence of the oxide group have also been shown to be carcinogenic (Ito, 1976).

The critiques and summary of the earlier experiments concerning the summation theory of carcinogenesis as it relates to 4NQO were presented by Nakahara (1961) in a very detailed paper. The summation effect of carcinogenesis is independent of the route of application. Takayama (1961) painted 4NQO and 20-methylcholanthrene on the skin of rats previously fed with azo dye in a submanifestational dose. He observed the development of hepatoma, while no hepatoma arose in any of the other groups which were treated with 4NQO or 20-methyl-cholanthrene alone by painting, or in combination with azo dye feeding in the reverse order. Hoshino et al (1968) observed the skin tumour response in female mice of ddN strain treated with various doses of 4NQO and β-rays. The

combination of the two carcinogens, both in submanifestational doses, applied in any order, showed the summation effect in skin tumour formation. Searle and Woodhouse (1963, 1964) found contradictory data in connection with the summation theory. They showed that the rate of appearance of papillomas on the skin of mice treated with 3,4-benzpyrene was markedly retarded by 4NQO. Moreover, they also observed the same inhibition by 4NQO in 1,2,5,6-di-benzanthracene carcinogenesis.

In vitro carcinogenesis of 4NQO and its derivatives have been investigated. Sato and Kuroki (1966) and Kamahora and Kakunaga (1966) succeeded independently in demonstrating a malignant transformation of hamster embryonic cells by treatment with 4NQO in vitro. Ishikawa and Endo (1967) in their study with bacteriophage T4 demonstrated that the actual duration of the transforming effect of 4HAQO might be less than 15 minutes. The process of malignant transformation by 4NQO was carefully analyzed by Kakunaga (1974). A31-714 cells did not produce transformed foci when kept under non-replicating conditions after 4NQO treatment. However cells that underwent one cell replication within 24 hours after 4NQO treatment retained the ability to transform when subsequently kept in a non-growing state. Pretreatment of 3T3 cells with 4NQO was shown to significantly enhance the efficiency of transformation by SV40 virus treatment (Kakunaga and Miyashita, 1972). Many other studies of in vitro carcinogenesis by 4NQO have been carried out and were summarized by Nagao and Sugimura (1976).

In connection with the in vitro carcinogenesis of 4NQO and derivatives, some work on the cytologic effects of these compounds has been done. Endo et al (1959) found in tissue culture that 4NQO produced characteristic intranuclear inclusion bodies in resting cells and also demonstrated a degree of parallelism between carcinogenicity and the ability to form nuclear inclusions among 4NQO and its derivatives. 4HAQO was also shown to produce the same nuclear inclusions (Endo and Kume, 1963a). The inclusion bodies were shown by Endo et al (1961) to be ribonucleoprotein. Endo (1962) observed formation of polynuclear and giant cells in cell culture treated with 4NQO. Non-specific nucleolar changes in the form of decrease in size, fusion of nucleoli and redistribution of the nucleolar components into caps were observed by Reynolds et al (1963).

Yoshida et al (1967) analyzed chromosomes of cells which were transformed in vitro by 4NQO and 4HAQO and found aneuploidal, deletion, trisomy, heteroploidal and diploidal changes, thus indicating their possible relationship to cancerization by 4NQO. There are many other studies done on in vitro transformation with 4NQO, discussion of which is out of the scope of this chapter.

In vivo studies of chromosomal changes caused by 4NQO were done by Moriwaki et al (1963) and Yoshida et al (1965). Changes observed included: abnormal elongation of chromatids and presence of chromatid gaps; chromatid breakage (chromatid intrachange) and chromatid translocation (chromatid interchange). Combined with the carcinogenic and mutagenic activities of 4NQO, these characteristic effects on chromosomes suffice to classify 4NQO among the so called radio-

mimetic agents. Fibroblasts from patients with Xeroderma Pigmentosum were very sensitive to induction of chromosome aberrations by 4NQO (Stich et al, 1973), further indicating its radiomimetic effects.

Regarding the effects of 4NQO and its derivatives on the immune system, the immunosuppressive activity of 4NQO has been well documented. Humoral dose-dependent immunosuppression was observed after subcutaneous or intraperitoneal injection of 4NQO by Jerne et al (1963) and Onoue et al (1963). Philips (1972) reported that rapidly dividing bone marrow cells were affected most by 4NQO. Outzen and Prehn (1973) demonstrated the immunosuppressive effect of 4NQO on the cell-mediated immune response by observing prolongation of allograft survival time after subcutaneous administration of 4NQO.

Since the mutagenic activities of 4NQO on fungi were first observed by Okabayashi in 1955, a tremendous amount of work has been done in this area with 4NQO and its derivatives. Work carried out during the three decades after their initial synthesis was reviewed in detail by Nagao and Sugimura (1976). Mutational effects of 4NQO and derivatives include base-pair change, frameshift, deletion, mitotic gene conversion, loss of cytoplasmic factor and phage induction from lysogenic bacteria. The DNA repair mechanism as illustrated by 4NQO has been extensively studied and was reviewed by Kondo (1975, 1976). Further elaboration is beyond the scope of this chapter.

4. METABOLISM AND MODE OF ACTION

4.1 METABOLISM

As described earlier, following their discovery of the carcinogenicity of 4NQO, Nakahara and colleagues were the first to study the carcinogenic mechanism of 4NQO. These investigators hypothesized that the carcinogen acts through energy release as a result of a non-enzymatic substitution reaction with SH-compounds in biologic systems which disturbs the normal replication process of genetic material (Nakahara and Fukuoka, 1959). The hypothesis lost popularity as more work on the metabolism of this carcinogen was done resulting in the discovery that the original compound 4NQO was in fact metabolized through several intermediate compounds. It was found that 4NQO was reduced enzymatically to 4-hydroxyaminoquinoline 1-oxide (4HAQO), 4-aminoquinoline 1-oxide (4AQO) and 4-aminoquinoline (4AQ) (for chemical structures see Figure 3.2) in sequence, by microorganisms (Okabayashi and Yoshimoto, 1962) as well as by mammalian cells (Toriyama, 1965; Sugimura et al , 1965). It was also discovered that the biologic actions exerted by 4NQO such as mutation in microorganisms (Okabayashi et al, 1965) and nuclear inclusion body formation in tissue culture cells (Endo and Kume, 1963a) were also exhibited by 4HAQO but not by 4AQO and 4AQ. A mutagenic action on T4 phage was also demonstrated by 4HAQO but not by 4NQO or 4AQO (Endo and Kume, 1963b). These findings suggested that 4NQO might exert its carcinogenic activity via its reduced form namely 4HAQO.

The carcinogenicity of 4HAQO was examined initially by Shirasu and Ohta (1963) and Endo and Kume (1963c) who succeeded in demonstrating fibrosarcoma formation by subcutaneous injection of 4HAQO into rats. If 4HAQO was an active intermediate in 4NQO carcinogenesis, it would be expected that the 4-hydroxyamino

compound would be more potent than 4NQO. To test this point, Endo and Kume (1965) administered both compounds to rats by single injection, in an equimolar dose and found induction of sarcoma only in the group of 4HAQO-treated animals. Similar findings have been reported by Shirasu (1963) using mice. Furthermore, Kawazoe et al (1967b) in their study on the structure-carcinogenicity relationship among derivatives of 4NQO and 4HAQO, found that the carcinogenic property depended on the production of 4-hydroxyamino derivatives. Mori et al (1967) reported the induction of lung cancer and other tumours in mice injected with 4HAQO. Mori (1967b) also demonstrated that 4HAQO is a more powerful carcinogen than 4NQO in cancer induction in the glandular stomach. The results of such studies thus supported the view that the carcinogenic effect of 4NQO seems to be developed through its conversion into the active metabolite, 4HAQO.

To elucidate the metabolism of 4NQO, in vitro enzyme studies were also carried out. Sugimura et al (1965) found that the incubation of 4NQO with liver homogenate under anaerobic conditions resulted in the production of predominantly 4-aminoquinoline 1-oxide (4AQO), a step beyond the production of 4HAQO. Hydrogen donors, such as β-hydroxybutyrate, ethanol or isocitrate, and the corresponding reductases with hydrogen carriers, either NAD or NADP, were required. Besides 4AQO, 4HAQO was also formed as detected by thin-layer chromatography and U.V. fluorescence. The reduction reaction was mainly found to occur in particulate fractions including microsomes and mitochondria. Matsushima and Sugimura (1971) noted that the enzyme activities required to form 4AQO from 4NQO in lung and skin were one-third and one-tenth of those in liver respectively.

The enzyme catalyzing the reduction of 4NQO up to 4HAQO only, was found in the supernatant fraction after centrifugation of homogenate of rat livers and hepatomas (Sugimura et al, 1966). Either $NADH_2$ or $NADPH_2$ could serve as hydrogen donor. This enzyme was identified by column chromatography as DT-diaphorase (Ernster, et al, 1962). The liver, stomach and lung of the rat showed especially high activity of this enzyme (Sugimura et al, 1966). Whether or not the reduction of 4NQO to 4HAQO and of 4HAQO to 4AQO was catalyzed by the same enzyme remained unclear (Takamiya, 1967).

Since it appeared to be a widely held view that 4HAQO was the ultimate carcinogenic form of 4NQO, later studies of the mode of action of 4NQO in carcinogenesis centred on the direct binding of 4HAQO to macromolecules, especially DNA. The finding that although 4HAQO would bind to nucleic acid in vivo but that the binding rarely occurred in buffer solution prompted Tada and Tada (1972) to suspect that 4HAQO needed activation for binding. These workers indeed demonstrated the presence of an activating enzyme in extracts of rat ascites hepatoma cells and baker's yeast (Tada and Tada, 1972). This enzyme was found in the cytosol fractions of these cells. It catalyzed the binding of 4HAQO to nucleic acid or protein in the presence of ATP and Mg^{2+} (Tada and Tada, 1972) and the amino acid, L-serine (Tada and Tada, 1974). Further studies on the reaction mechanism of 4HAQO activation by this enzyme revealed that the purified enzyme from baker's yeast was seryl-tRNA synthetase (Tada and Tada, 1975) which belongs to the group of aminoacyl-tRNA synthetases. The reaction of most aminoacyl-tRNA synthetases has been considered to occur in two consecutive

steps:

Amino acid + ATP + Enzyme $\rightleftharpoons$ Aminoacyl-AMP-enzyme + PPi (diphosphate)

Aminoacyl-AMP-enzyme + tRNA $\rightleftharpoons$ Aminoacyl-tRNA + AMP + Enzyme

In the first reaction aminoacyl-AMP is synthesized on the enzyme molecule and in the second reaction the intermediary complex transfers amino acid to tRNA. Following this reaction pattern, Tada and Tada (1975) assumed that 4HAQO is acylated by seryl-AMP formed on seryl-tRNA synthetase and that seryl-4HAQO ultimately reacts with nucleic acid. The assumed mechanism of the reaction is as shown below:

Serine + ATP + Enzyme $\rightleftharpoons$ Seryl-AMP-enzyme + PPi

Serine-AMP-enzyme + HAQO $\longrightarrow$ Seryl-4HAQO + AMP + Enzyme

Seryl-4HAQO + Nucleic acid $\longrightarrow$ 4HAQO-nucleic acid + Serine

To prove this assumption, Tada and Tada (1975) isolated the seryl-AMP-enzyme complex and examined its ability to activate 4HAQO in vitro. The isolated complex was able to transfer serine to tRNA and was also able to activate 4HAQO to bind to nucleic acid in the absence of raw serine and ATP in the reaction mixture. The binding of 4HAQO to nucleic acid even occurred by the addition of synthetic seryl-AMP in the absence of the enzyme. This non-enzymatic 4HAQO-binding reaction also occurred with other kinds of aminoacyl-AMP such as leucyl-AMP. These results thus supported the assumption that 4HAQO is activated through acylation by seryl-AMP formed as the intermediary complex in the seryl-tRNA synthetase reaction.

Since many aminoacyl-tRNA synthetases are known to form aminoacyl-AMP-enzyme complexes as their reaction intermediates, it would be expected that these aminoacyl-tRNA synthetases could also be effective for 4HAQO activation. This in fact was demonstrated by Tada and Tada (1976) who found that synthetic aminoacyl-AMPs were generally able to activate 4HAQO and further that a few

particular aminoacyl-tRNA synthetases were able to activate 4HAQO. They also pointed out that these aminoacyl-tRNA synthetases might participate *in vivo* in the metabolism not only of 4HAQO but also of some other N-hydroxy compounds through their aminoacylating capacity.

4.2 MODE OF ACTION

4.2.1 INTERACTION WITH NUCLEIC ACIDS

The most widely held concept regarding the carcinogenic mode of action of 4NQO concerns its interaction with nucleic acid. Effects on proteins have also been implicated. Both *in vitro* studies using synthetic and biologic materials as well as *in vivo* studies have contributed to the knowledge of interaction of 4NQO and related compounds with DNA.

The 4NQO molecule, without being metabolized to 4HAQO, was shown *in vitro* to be able to combine with the bases of the DNA molecule by virtue of the electropositive sites on the 4NQO molecule which would be attracted to electronegative centres on the bases of the DNA molecule (for explanation, refer to the section on Chemistry). Such a reaction was described as charge transfer complex formation between 4NQO and DNA and would result in erroneous base pairing which then became a self-perpetuating process in further DNA replications (Karreman, 1962). Relatively good correlation between charge transfer quantity and carcinogenicity of 4NQO and its derivatives was in fact demonstrated by Nagata et al(1963). Nagata et al (1966b) and Malkins and Zahalsky (1966) were able to demonstrate that the sites of binding on the DNA molecule were the purine bases, i.e. adenine or guanine. Nagata (1971) regarded such binding was non-covalent

but whether the 4NQO molecule was intercalated between the DNA bases or not remained unclear. Nevertheless, Paul et al (1971) were able to construct a model of *in vitro* complex formation in which the 4NQO molecule lay alongside the guanine base of the DNA molecule, a picture that was similar to that suggested previously by Nagata et al (1966b).

Besides charge transfer complex formation, it was also demonstrated *in vitro* that UV-radiation on the 4NQO molecule gave rise to free radicals (Kataoka et al, 1966) which photodynamically damage the guanine base in the DNA molecule (Kodama and Nagata, 1967).

The 4NQO molecule per se was also shown by fluorescence studies to combine covalently with DNA *in vivo* (Matsushima et al, 1967).

Therefore, there was some evidence that the intact 4NQO molecule without being metabolized could have a role in interacting with cellular macromolecules. However, the main focus of interaction with celluar macromolecules soon shifted to the metabolite 4HAQO. Although Tada et al (1967) demonstrated in fluorescence studies covalent binding of 4HAQO to DNA *in vivo*, the general concensus of opinion supported by abundant experimental evidence was that the biologic action of 4HAQO, such as carcinogenicity and inactivation of transforming DNA, might be related to its ability to generate free radicals, directly or indirectly, by oxidative processes. These radicals in turn damaged the genetic material (Nagata et al, 1966a; Kataoka et al, 1967; Ishikawa and Endo, 1967; Tanooka et al, 1968; Takahashi et al, 1978; Hozumi, 1969).

However, the concept of free radicals was challenged by Tada and Tada (1972). Their findings of the further metabolism of 4HAQO to aminoacyl-4HAQO prior to binding with nucleic acid (Tada and Tada, 1975) and other similar findings (Kawazoe and Araki, 1967a; Enomoto et al, 1968) led Tada and Tada (1976) to suggest that it might be the aminoacylated 4HAQO that attacked the nucleophilic centre of adenine and quanine in DNA. However, they have not as yet further elaborated on how the aminoacylated 4HAQO carries out its attack.

By means of in vitro and in vivo studies of the reactions of 4HAQO with natural and synthetic nucleic acids (Tada and Tada, 1971, 1972; Kawazoe et al, 1975), the chemical structure of an adduct between 4HAQO and adenine of the DNA molecule was worked out to be either 3-(N^6-adenyl)-4-aminoquinoline 1-oxide or 3-(N^1-adenyl)-4-aminoquinoline 1-oxide (Figure 3.5) (Kawazoe et al, 1975).

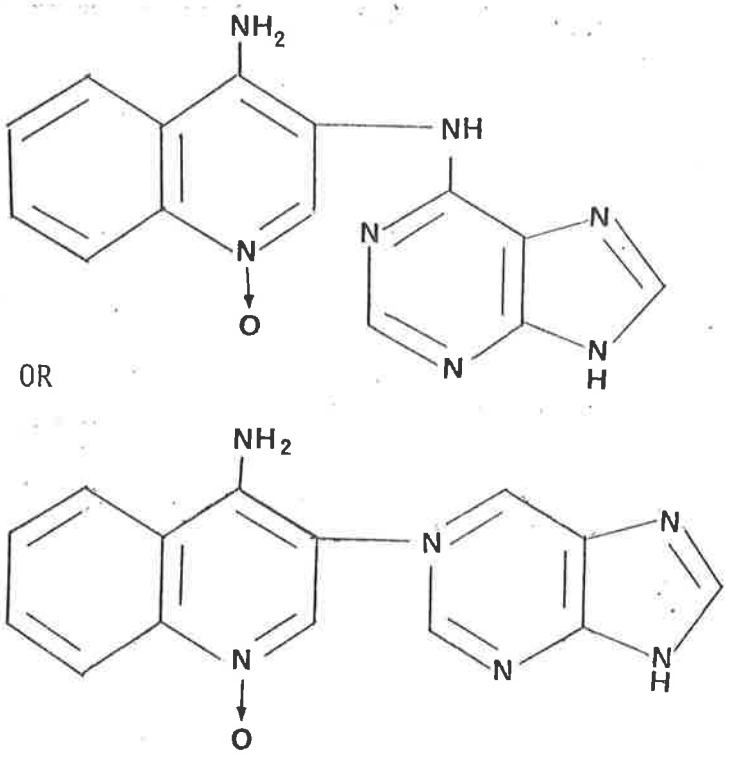


Figure 3.5. Proposed structure of quinoline-adenine adduct (QA_{II}) (Tada and Tada, 1976).

Interaction of the carcinogens 4NQO and 4HAQO with RNA has not received much attention, though it is likened to DNA. However, effects resulting from the interaction have been much investigated and were summarized by Endo et al (1971 a). In brief, 4NQO and its derivatives inhibit RNA synthesis of whole cells. The length of RNA chains transcribed by affected DNA is much smaller, about one-tenth of normal, but more RNA chains are formed. The amino-accepting ability of tRNA can also be altered.

4.2.2 INTERACTION WITH PROTEINS

Okabayashi (1953b) found that the 4-nitro group of 4NQO can easily be substituted by the SH group of cysteine or thioglycolic acid under physiologic conditions. After reaction with the SH compounds, antifungal activity to Aspergillus niger was lost, suggesting a causal significance of the nitro group in the antifungal action of this compound. Hayashi (1959) had verified that 4NQO actually reacts with the SH group in skin *in vivo* by demonstrating, both histochemically and spectrophotometrically, a definite decrease of intraepithelial SH content in the skin after a single application of 4NQO. Takayama and Ohta (1961) also demonstrated a definite correlation between the histochemical localization of SH groups and autoradiographic localization of the carcinogen by painting mouse skin with ^{3}H-4NQO. The carcinogen was shown to be trapped in the superficial layers which were rich in SH content. Andoh et al (1971) incubated ^{3}H-4NQO with mouse fibroblasts and found that the radioactive 4NQO bound to many soluble proteins.

4HAQO differs from 4NQO in that it does not undergo substitution reactions with SH compounds, but it does have the capacity to catalytically oxidize SH containing compounds such as glutathione and cysteine with the formation of disulphide bonds (Hozumi et al, 1967) and concomitant reduction of cytochrome c (Matsushima et al, 1968). Sulfhydryl enzymes have been reported to be inactivated via oxidation of the SH groups by 4HAQO (Hozumi, 1968). The extent of these oxidoreduction reactions with 4HAQO in vivo and the role of these reactions in carcinogenesis are still obscure.

4NQO has been found to inhibit protein synthesis. This effect is achieved through impairment at the transcriptional or translational level of protein synthesis. Miscellaneous effects on protein synthesis were summarized by Endo et al (1971a).

To summarize, this chapter has provided an overview of the chemistry, carcinogenicity, metabolism and suggested modes of action of the synthetic aromatic heterocyclic compound 4NQO. The early work by Nakahara and his colleagues is particularly noteworthy for it provides very practical, basic information on how this carcinogen behaves in experimental models. It also provides considerable direction as to how this carcinogen can be utilized in oral carcinogenesis studies. The results of a larger number of studies on 4NQO (and some of its derivatives) indicate that this single chemical compound perhaps has very diverse carcinogenic effects in different experimental biologic systems. Its carcinogenicity is potent and reliably reproducible and its metabolism and mode of action have been relatively well characterized when compared to other carcinogens.

CHAPTER 4

MATERIALS AND METHODS

MATERIALS AND METHODS

1. ACQUISITION AND CONDITIONING OF EXPERIMENTAL ANIMALS

Thirty-nine four weeks old female albino rats of the Porton strain, weighing an average of 110 grams each, were acquired from the Central Animal House (SPF Animals), Waite Institute of Agriculture, South Australia. The animals were kept in standard polypropylene cages with four animals in each cage and were fed standard pellets and tap water ad libitum (Appendix I). They were kept in the Animal House, Dental School, the University of Adelaide for the whole experimental period. These animals were manipulated gently by hand three times a week. Two weeks later, in addition, they were anaesthetized with nitrous oxide-oxygen-halothane three times a week. One week later, experimentation with the animals commenced. Therefore, the animals had been conditioned to the new environment, human handling and general anaesthesia episodes before the real experiment began by which time they were seven weeks old, each weighing an average of 174 grams.

Twenty-three of the 39 rats were included in the experimental group to which the carcinogen was applied. The remaining 16 rats served as controls and were divided into the treated control and untreated control groups.

2. PREPARATION OF THE CARCINOGEN

A 0.5% solution of 4-nitroquinoline-1-oxide (4NQO) in propylene glycol was prepared by dissolving 250 mg of the carcinogen in 50 ml of propylene glycol at room temperature (Appendix II). The stock solution was kept in a tinted glass bottle and stored in darkness. Two ml

at a time was transferred to a small vial for actual application. In this manner, it could avoid contamination and the possibility of photoactivation of the carcinogen.

2. PREPARATION OF THE ANTISIALOGOGUE

A 1 mg/ml solution of Hyoscine Butyl Bromide in normal saline was prepared by diluting a concentrated solution of 20mg/ml 20 fold . The concentrated solution came in the form of a 1 ml vial of "BUSCOPAN" (Boehringer Ingelheim Pty Ltd - Fher, N.S.W., Australia).

3. SALIVARY INHIBITION, ANAESTHETIZATION AND PAINTING PROCEDURE

At 9:00 AM each rat was given a subcutaneous injection of diluted Buscopan at a dosage of 1.5 mg/Kg body-weight. The site of needle entrance was in the lower abdomen-inner thigh region. Four rats forming the untreated control group were not injected.

The procedures from now on were carried out in a fume cupboard (Figure 4.1). Four rats at a time were anaesthetized with a combination of nitrous oxide, oxygen and halothane in a closed perspex box of size 31 cm X 14 cm X 14 cm. The N_2O/O_2 ratio was 2:3 by volume. The mixture flowed past a reservoir of halothane at a rate of five litres per miniute into the closed perspex box. The induction period for a group of four animals took an average of two minutes. When a rat was fully anaesthetized, it was taken out from the perspex box and was placed back-down on a metal tray padded with absorbent tissue. The mouth was opened with a pair of specially designed atraumatic forceps bearing two loops on its tips(Figure 4.2).The larger loop engaged onto the lower incisors with

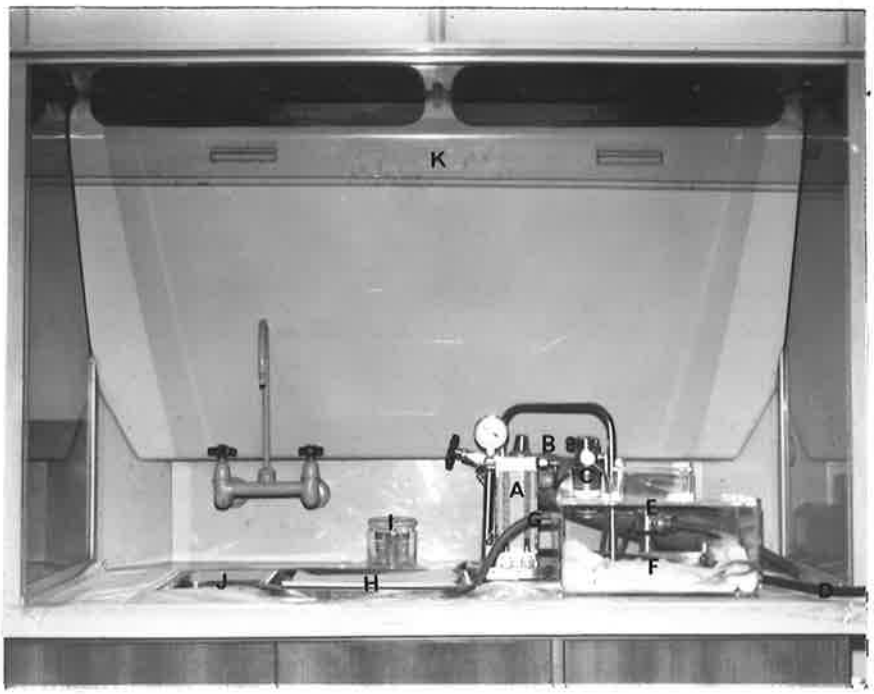


Figure 4.1: Laboratory setting for anaesthetization and painting procedures. A - Anaesthetic machine. B - Nitrous oxide cylinder. C - Reservoir of halothane. D - Connection to oxygen cylinder. E - perspex box. F - Rat. G - Outlet for anaesthetic gases. H - Padded tray. I - Carcinogen, contained in a small vial within the big jar. J -Receptacle for waste. K - Glass window of the fume cupboard, which was pulled down during the actual procedures.

the tongue beneath it. The smaller loop engaged onto the upper incisors. When the arms of the forceps were forced open with finger pressure, the jaws of the rat were forced apart with the tongue simultaneously depressed by the bigger loop. In this manner, there was free access to the palate. Looping onto the upper and lower incisors had also the advantage of immobilizing the jaws in the open position; the animal could not move its head away from the constraint of the loops if it happened to regain partial consciousness. By the time the mouth was fully opened, the Buscopan had already assumed its full pharmacologic effects of inhibition of salivary flow.

The palate was then swabbed clean with a cotton bud. A No. 2 synthetic hair brush was dipped into the vial of carcinogen, the excesses being removed from the brush by touching on the wall of the glass vial. The carcinogen was then painted on the palate along the midline in a one-stroke rotating movement, from the soft palate to the incisive papilla (Figure 4.3). The pair of forceps was then disengaged from the incisors and the painted animal was returned to its cage with the ventral body surface facing down. This positioning of the animal automatically closed its mouth. Twenty-three animals forming the experimental group were painted with the carcinogen while 12 animals forming the treated control group were painted with propylene glycol only. Four animals constituting the untreated control group were left unpainted and unanaesthetized.(Table 4.1).

The whole procedure from taking the rat out from the perspex box to returning it to the cage lasted only 20 seconds.The animal regained consciousness in a further 20 seconds.

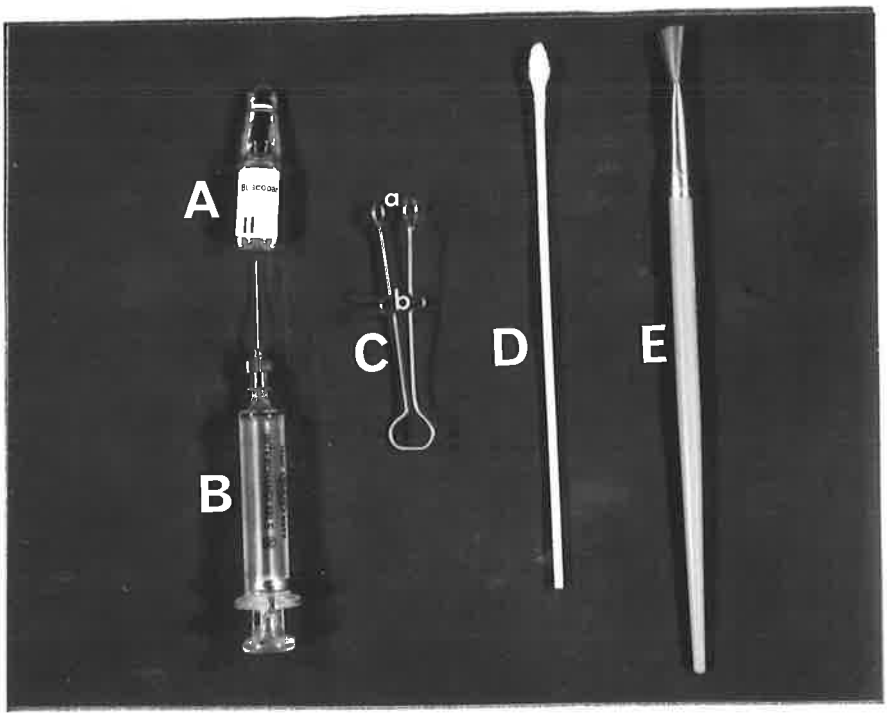


Figure 4.2: Instruments for injection of Buscopan and painting procedure. A - Ampule of Buscopan. B - Dermal syringe. C - Atraumatic forceps (a - loops, b - finger rests). D - Cotton bud. E - No. 2 hair brush.

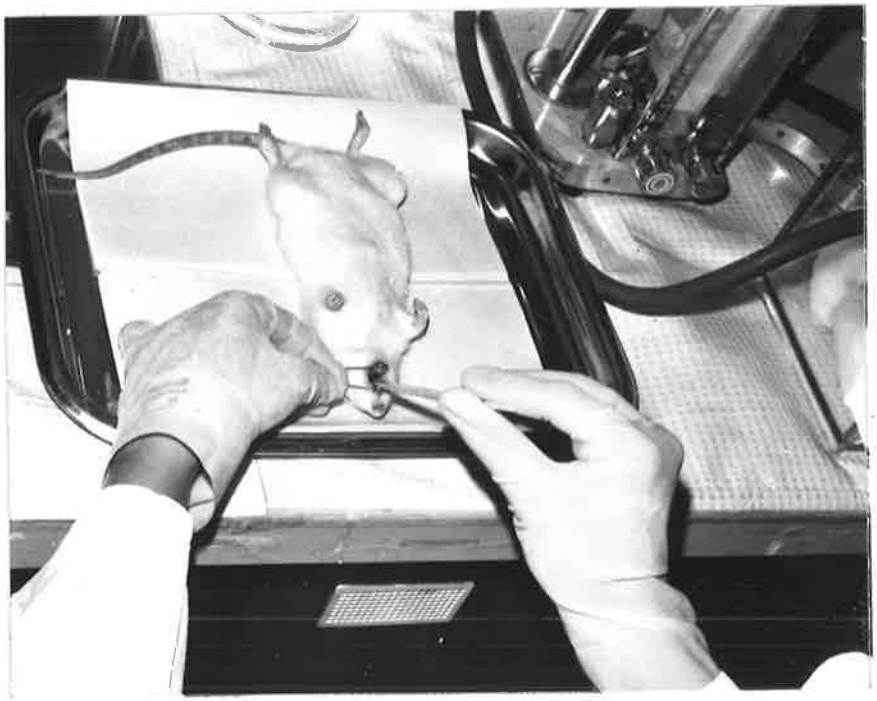


Figure 4.3: Demonstration of the painting procedure.
The anaesthetized animal was lying back-down. 4NQO was painted on the palate when the mouth was opened with the pair of atraumatic forceps.

Table 4.1. Grouping of animals.

GROUPING	NO. OF RATS	SUBSTANCES APPLIED
EXPERIMENTAL GROUP	23	0.5% 4NQO in propylene glycol, Buscopan (1.5 mg/Kg s.c.)
TREATED CONTROL GROUP	12	Buscopan (1.5 mg/Kg s.c.)
UNTREATED CONTROL GROUP	4	None

Table 4.2. Schedule for sacrifice of animals.

DURATION (WEEKS)	NO. OF ANIMALS SACRIFICED		
	EXPERIMENTAL	TREATED CONTROL	UNTREATED CONTROL
4	2	2	1
8	2	2	0
12	3	2	1
16	3	2	0
20	6	2	1
24	7	2	1

Drinking water was withdrawn until 5:00 PM on the same day which was approximately 7 hours from the time the animal was painted. This was to allow the mouth to remain in a dry condition following carcinogen application.

The same experimental procedures were repeated three times a week (on Monday, Wednesday and Friday) for a maximum period of 24 weeks.

4. MONITORING OF THE ANIMALS' HEALTH CONDITION

The activities of the animals, the lustre of their fur and the amount and quality of the stool were observed approximately three times a week when they were caged and also at the time they were being subjected to the experimental manoeuvre. They were weighed fortnightly. The average weight changes throughout the experimental period are shown in Appendix III.

5. SCHEDULE FOR SACRIFICE OF ANIMALS, METHODS OF DISSECTION OF SPECIMENS AND POST-MORTEM EXAMINATION

Groups of animals were sacrificed at 4, 8, 12, 16, 20 and 24 weeks after the first painting. Since it was anticipated more changes would appear later, fewer animals were sacrificed at earlier time intervals, with the number increasing toward the end of the experimental period. Thus, for the experimental group, two animals at a time were sacrificed at 4 and 8 weeks; three animals at a time were sacrificed at 12 and 16 weeks; and 6 and 7 animals were sacrificed respectively at 20 and 24 weeks. For the treated control group, two animals at a time were sacrificed at 4, 8, 12, 16, 20 and 24 weeks. For the untreated control group, one

animal at a time was sacrificed at 4, 12, 20, 24 weeks (Table 4.2).

The animal was first anaesthetized with N_2O/O_2 and halothane and then killed with an injection (i.p.) of 60 mg of pentobarbitone sodium.

When the animal was dead, which took approximately 5 minutes, a midline incision was made on the ventral surface from the chin-point down to the genitalia and a circumferential incision was made around the neck (Figure 4.4a). The head skin was stripped off to facilitate dissection of the jaws.

The cheek on both sides including the masticatory muscles was cut across and the pterygoid and temporalis muscles were freed from the joint area to facilitate dislocation of the mandible which was achieved by pulling apart the maxilla and the mandible forcefully (Figure 4.4b). The rest of the muscle /tissue attachments around the ramus of the mandible, the upper oesophagus, larynx and submandibular muscles were severed to entirely separate the mandible from the head (Figure 4.4c). Then the head was decapitated at the neck.

The whole tongue was excised from the mandible at its base. The whole palate was separated from the calvarium by forceful cutting through the bones.

The abdominal cavity and the thoracic cavity of the decapitated animal were opened for post-mortem gross examination. The lung, heart, other internal organs, thoracic and peritoneal cavities were thoroughly examined for the detection of neoplastic growth. The body surface and the neck were palpated and examined for the detection of neoplastic lumps.

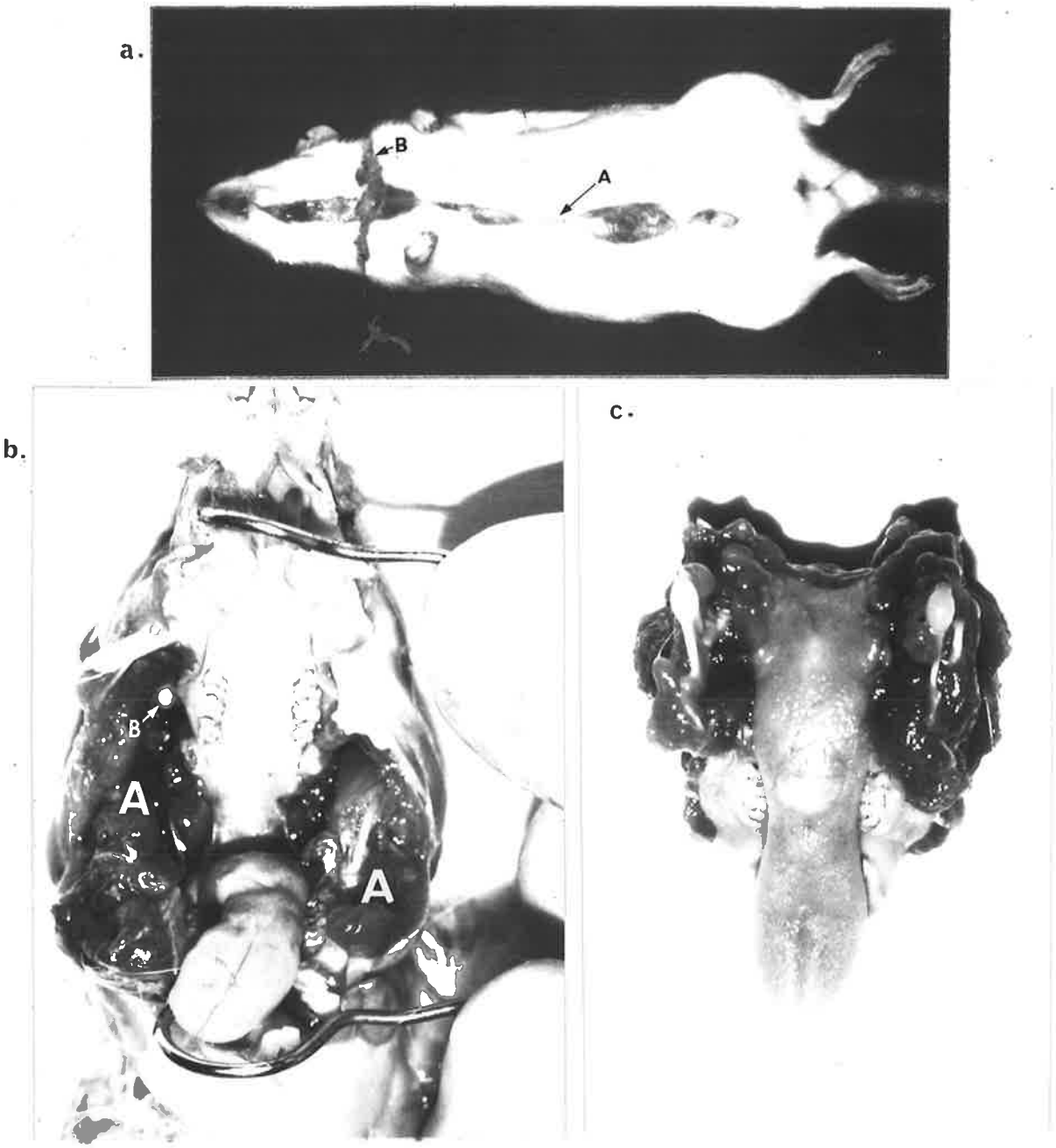


Figure 4.4: Illustration of dissection procedures.

a. Skin incisions were made along the midline of the ventral surface (A), and circumscribing the neck (B). b. The cheek and masticatory muscles (A) were dissected to free the mandible. The condyle (B) was dislocated.

c. The whole mandible was separated from the head.

After examination, the head structures and the internal organs were placed in two jars of 10% formalin-saline fixative.

6. METHOD OF MACROSCOPIC STUDY

The palate and the tongue were arbitrarily divided by imaginary lines into areas to facilitate observation and recording of findings. Thus, a line across the anterior surfaces of the first molars and a line across the junction of the hard and soft palate landmarked by a semilunar pad divided the whole palate into the ANTERIOR PALATE, MIDDLE PALATE and POSTERIOR PALATE. The GINGIVAL AREA was segregated from the middle palate by a line approximately 1 mm below the free gingival margin and running parallel to the contour of the gingival margin. The area so segregated included the free and attached gingiva (Figure 4.5a). A line drawn across the tongue anterior to the crescentic pad of hypertrophied lingual papillae divided the tongue into the ANTERIOR TONGUE and POSTERIOR TONGUE (Figure 4.5b).

These different areas of the palate and the tongue as well as the oral mucosa were examined once a week when the animals were anaesthetized for the painting procedure in the beginning of a week. Likewise the four animals from the untreated control group were also anaesthetized and examined weekly.

When the animals were sacrificed and their jaws dissected, these areas were again examined. In addition, the palate and the tongue were examined under a dissecting microscope at high and low magnifications. These specimens were examined again when they were well fixed at which time morphologic appearances were recorded by black and white photography at microscopic magnifications of x5 and x9 for the palate

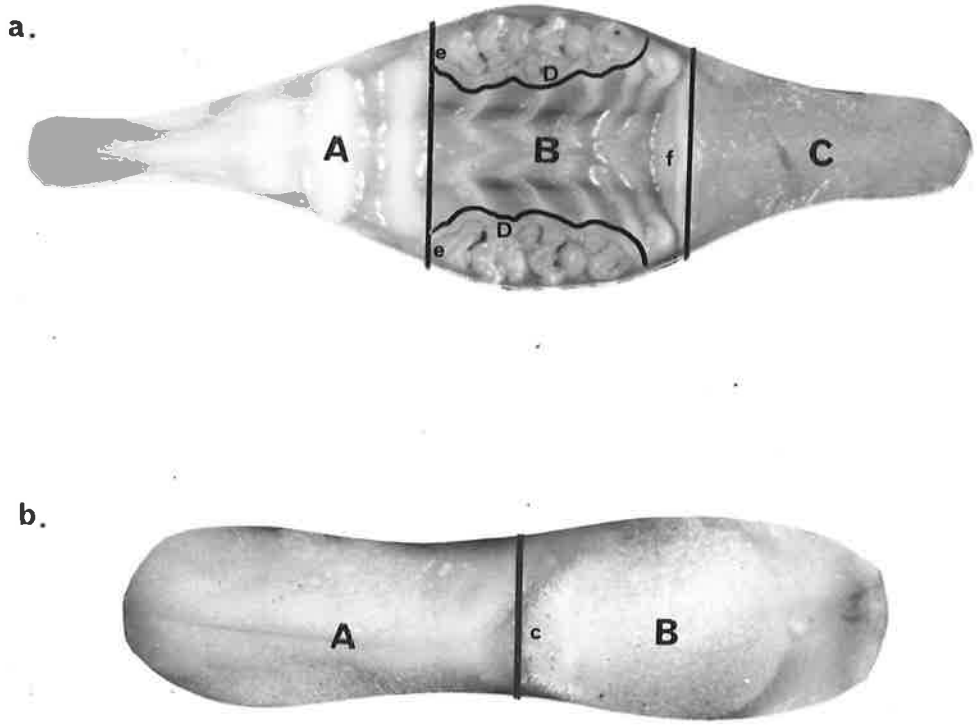


Figure 4.5: Territorization of the palate and tongue.

a. The palate: A - Anterior palate. B - Middle palate. C - Posterior palate. D - Gingival area. Important landmarks are the anterior surfaces of the first molars (e) and the semilunar pad (f).

b. The tongue: A - Anterior tongue. B - Posterior tongue. The important landmark is the crescentic pad of hypertrophied lingual papillae (c).

Magnification, a x 5, b x 4.

and X4 and X9 for the tongue. The black and white prints were also examined at a later time. The various observations carried out at different times were recorded in writing and then later assimilated and recorded in special data charts (Appendix IV).

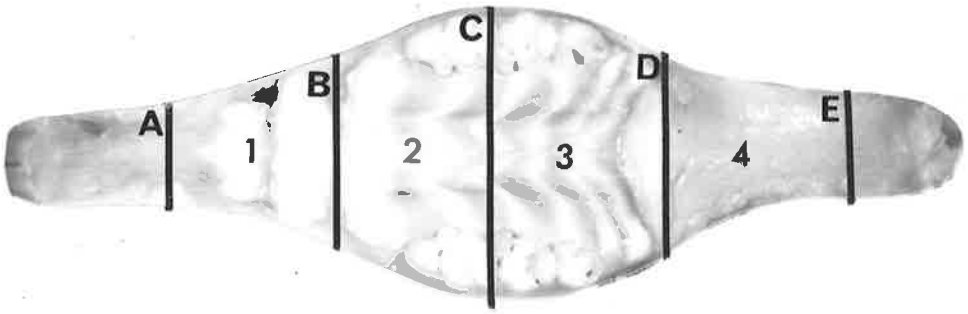
7. PREPARATION OF SPECIMENS FOR HISTOLOGIC STUDY

The formalin-fixed palates were decalcified in buffered formic acid (Appendix V). The degree of decalcification was monitored by radiographing the specimens. After decalcification was completed, the specimens were neutralized in 5% sodium sulphate overnight. The specimens were then ready for processing.

Five equally spaced, transverse cuts were made on the decalcified palate, dividing it into four segments of approximately the same length. The first cut was at half-length between the incisor and the incisive papilla. The second cut was just anterior to the second ruga of the anterior palate. The third cut was between the first and the second molars. The fourth cut was just posterior to the semilunar pad at the junction between the hard and soft palate. The fifth cut was in an area at the posterior end of the soft palate (Figure 4.6a).

The various segments were processed for double embedding in paraffin wax. Since each segment had its own characteristic morphology, it could easily be identified. When being blocked the segments were oriented with their anterior edge facing down and the palatal plane positioned vertically so that when sections were examined under the microscope, they appeared as if they were being viewed from the front. All the four segments were embedded in the same block following a definite order (Figure 4.6b).

a.



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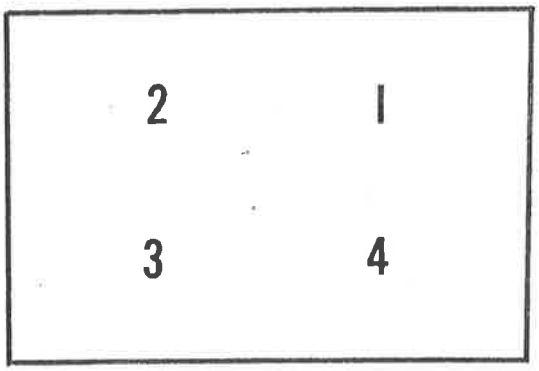

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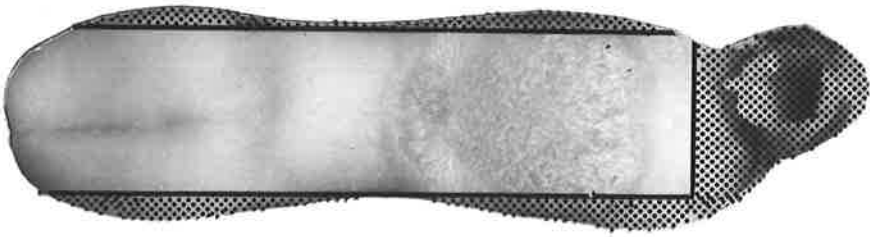


Figure 4.6: Preparation of specimens for histopathologic study.

a. The palate was divided by five cuts (A, B, C, D and E) into four segments (1, 2, 3 and 4).

b. This shows the order of arrangement of the palatal segments for blocking in paraffin wax.

c. The sides and laryngeal portion of the tongue were trimmed prior to embedding in paraffin.

The tongues were not subjected to decalcification since they were pure soft tissues. The laryngeal portion was cut off and the sides of the tongue were trimmed flat to facilitate orientation when blocking. Thus, the tongue assumed the shape of a long rectangle (Figure 4.6c). The tongue specimens were then processed for single embedding in paraffin wax. When being blocked, the specimens were oriented with their trimmed left side facing downward so that when sections were examined under the microscope they appeared as if they were being viewed from the left side.

Serial histologic sections of the palate and the tongue specimens were cut at a thickness of 7 microns. The first and every 20th section were mounted and stained with haematoxylin and eosin (Appendix VI). The sections were then ready for microscopic examination. On average, 20 to 30 sections were examined from a palate and 15 to 20 sections from a tongue, the number being dependent on the quality of the specimens.

8. METHOD OF MICROSCOPIC STUDY

The palate and the tongue were divided into areas similar to those defined for the macroscopic examination. All serial sections prepared were examined microscopically at low and high magnifications ranging from X40 to X400. The various histologic features observed were recorded in writing. Selective sections at widely spaced levels, not less than 5 in number, were re-examined. Newer findings, if any, were recorded and incorrect findings discovered were discarded. The findings were then assimilated and recorded in special charts (Appendix VII).

9. USE OF CHARTS

It has to be pointed out that in scoring, at both the macroscopic and

microscopic levels, a positive score against a listed feature only indicated the presence of that feature. It did not reflect on the frequency of occurrence of that feature. Frequency was only commented in the written text of the results.

CHAPTER 5

RESULTS:

PART A. DESCRIPTION

PART B. ILLUSTRATIONS, TABLES AND FIGURES

RESULTS

PART A

I. ANIMALS'HEALTH CONDITION

Throughout the experimental period all animals appeared to be in a good state of health as evidenced by their physical mobility, good fur lustre, adequate amount of stool and gradual increase of adult weight (Appendix III). However, towards the end of the experimental period, the average weight of the experimental animals became relatively static while the control animals continued to increase their weight. There was no mortality throughout the entire experiment.

II. POST-MORTEM EXAMINATION

No animal exhibited neoplastic growth in the internal organs and body cavities. There were no palpable or observable lumps detected on the external body surface. Histopathologic examination of the internal organs was not carried out.

III. MACROSCOPIC OBSERVATION

Due to the small size of the rat oral cavity, there were limitations to naked eye observation for the detection of changes on the oral mucosa. Other than relatively gross features, subtle changes could not readily be detected. Nevertheless, such weekly observation of all animals did reveal the progressive nature of the changes occurring on the palate and the tongue. Thus starting from the 11th week, the surface of the posterior tongues of a few animals appeared thickened. As the experiment proceeded, more tongues exhibited the gross feature of surface thickening and irregularity. By 20 weeks the tongues of most animals exhibited a severely thickened and patchy papillary surface. When the experiment terminated at 24 weeks, an ulcer was present on the posterior tongue of one animal. Changes on the palate were first detected at 19 weeks as surface thickening of the mucosa. In subsequent weeks, more palates became involved and the thickening became more pronounced with marked surface irregularities developing. There appeared to be some exophytic growths in the gingival area by 20 weeks. The oral structures of the control animals appeared to maintain their normal appearances throughout the entire experimental period.

Retrospectively, when these findings were compared with those obtained with the aid of a dissecting microscope, the limitations of naked eye observation became evident. Therefore, the macroscopic observations described below are those obtained with the aid of a dissecting microscope on post-mortem specimens.

CONTROL ANIMALS

Macroscopically, all palates and tongues of control animals sacrificed at different time intervals exhibited essentially the same features characteristic of normalcy. Therefore the following description applies, in general, to all specimens from the control groups of animals.

A: PALATE

1. Middle Palate

The middle palate exhibited fine and distinct V-W shaped rugae on a shiny smooth surface. The palatal mucosa was very adherent to the bony vault and thin such that the nasal passages were partially shown through it under trans-illumination. The semilunar pad forming the posterior limit of the middle palate was finely papillated. High power view showed that each of the rugae on the middle palate was composed of successive units of palisading short ridges elevated from the surface of the palate. (Figure 5.1).

2. Gingival Area

The gingival area also had a fine and smooth surface texture. The gingival margin fitted snugly onto the tooth surface, outlining the contour of the lingual aspect of the three molars. No gingival pocket was present and the crevices appeared clean and healthy. (Figure 5.1).

3. Anterior Palate

The anterior palate contained two very thick and prominent rugae and an incisive papilla in front. The surface of these rugae and the incisive papilla as well as the interrugal area was smooth and shiny. (Figure 5.1).

4. Posterior Palate

The posterior palate had a nodular and finely creased surface. Each nodule was well defined and represented the excretory opening of the underlying minor salivary gland tissues. (Figure 5.1).

B: TONGUE

1. Posterior Tongue

The posterior tongue exhibited a fine, hairy surface consisting of numerous fine projections of the lingual papillae. At the anterior extent of the posterior tongue, the crescentic pad remained unaltered. At the posterior limit, the foramen caecum was present.(Figure 5.24).

2. Anterior Tongue

The anterior tongue exhibited a coarse, hairy surface consisting of numerous well defined, thicker and blunter projections of the lingual papillae when compared to those on the posterior tongue. There was a shallow median groove running from the tip of the tongue to about half the length of the anterior tongue. (Figure 5.24).

Topographically, both the anterior and posterior tongue presented an even surface, giving it a linear and smoothly curved contour on side view. (Figure 5.24).

4NQO-TREATED ANIMALS

A: PALATE

Four Weeks

The palate did not exhibit any overt abnormal changes when examined macroscopically.

Eight Weeks

The middle palate exhibited mild thickening with partial loss of definition of the rugae. The shiny and smooth surface became lustrousless and roughened. The gingival area was similarly affected. Gingival pockets were not yet observed though some food fibres were impacted in the gingival crevices.

The anterior and posterior palate also exhibited mild thickening.

Twelve Weeks

Different palatal areas exhibited changes essentially the same as those occurring at eight weeks. (Figure 5.6).

Sixteen Weeks

The middle palate exhibited more pronounced thickening; the rugae had almost all disappeared. The surface was further roughened, showing irregularities which trapped much amorphous white material, presumably dislodged keratin.

The gingival area was similarly thickened with the presence of very deep gingival pockets around the molars. The anterior and posterior palate remained mildly thickened. (Figure 5.8).

Twenty Weeks

The middle palate was severely thickened with exaggeration of all those features mentioned for the 16 weeks observation period. Some of the surface irregularities now presented short, non-specific ridges and papillary growths. The gingival area was similarly affected. In addition, there were small, exophytic growths from the depth of the gingival pockets. Though arousing much suspicion, these exophytic growths alone could not warrant

the diagnosis of carcinoma . (Figure 5.10). However, in one animal, the exophytic growths occurring bilaterally were very exuberant, attained a large size , and had a raised and rolled-in periphery. The exuberant growth together with the characteristic periphery strongly suggested that the lesions were carcinomas arising from the gingival areas . (Figure 5.12). The anterior and posterior palate remained mildly thickened.

Twentyfour Weeks

The middle palate and the gingival area exhibited changes essentially the same as those observed at 20 weeks. However, there were more lesions in the gingival area that could be recognized as gingival carcinomas (Figure 5.15),some of which were relatively small(Figure5.16).The anterior and posterior palate remained mildly thickened.

The various macroscopic changes on the palate are summarized in Figures 5.45 and 5.46 and in Table 5.1.

B: TONGUE

Four Weeks

The posterior tongue exhibited mild thickening of the filliform papillae. The crescentic pad of papillae still maintained its definition. The anterior tongue also exhibited mild thickening of the papillae. (Figure 5.27; 5.29).

Eight Weeks

The posterior and anterior tongue exhibited changes essentially the same as those observed at four weeks. In addition there were small

areas without papillary projections, thus presenting a flattened surface. Such lesions were identified as"Plaques". (Figures 5.27).

Twelve Weeks

The posterior tongue exhibited irregular hyperplasia of papillae, giving the surface of the posterior tongue a patchy and uneven topography. The plaques that existed assumed more prominence and increased in size. They remained smooth, devoid of the papillary projections and apparently became slightly elevated from the adjacent surface of the posterior tongue. The crescentic pad had lost much of its definition. (Figure 5.31).

The anterior tongue remained mildly thickened.

Sixteen Weeks

The posterior tongue exhibited severe thickening with more plaques in evidence. The crescentic pad of papillae had disappeared. The anterior tongue remained mildly thickened. It also contained some plaques. (Figure 5.33a)

Twenty Weeks

The severe changes observed previously on the posterior tongue became more exaggerated. It appeared very patchy and full of plaques, giving the surface a very raised topography. The anterior tongue remained mildly thickened, and more plaques developed in more animals. (Figure 5.33b,c;5.37). Two animals exhibited exophytic growths in the form of raspberry-like collections of short finger-like processes on a sessile base. These were regarded as papillomas. (Figure 5.43).

Twentyfour Weeks

The changes on the posterior tongue were essentially the same as those observed at 20 weeks. In addition, one animal exhibited a very large crateriform ulcer on the posterior tongue. Adjacent to it was another deep ulcer of a smaller size. The crateriform ulcer had an indurated base full of debris and a raised and rolled-in periphery. The characteristics of these lesions suggested that they were infiltrative carcinomas. (Figures 5.39) . In another animal there was a small ulcer or break observed on the surface of the posterior tongue. However, when being probed, it was found to extend very deep into the tongue. Several deep grooves were present on the same tongue. Though these deep , pit-like ulcers and grooves were very suspicious-looking,their clinical features did not warrant the diagnosis of a carcinoma. (Figure 5.41).

Two animals (one with the tongue showing the pit-like ulcer), exhibited exophytic growths in the form of bunches of long finger-like processes and raspberry-like collections of shorter processes. These were both regarded as papillomas.(Figure 5.41;5.43).The anterior tongue remained mildly thickened containing a few plaques.

The various macroscopic changes occurring on the tongue are summarized in Figures 5.47 and 5.48.

IV. MICROSCOPIC OBSERVATION

CONTROL ANIMALS

Histologically all palates and tongues of control animals sacrificed at different time intervals exhibited essentially the same features characteristic of normalcy. Therefore the following description applies in general to all specimens from the control groups of animals.

A: PALATE

1. Middle Palate

Since the histologic sections were cut transversely across the palate and the palatal rugae on the middle palate took the form of a V or W shape topographically, an average histologic section of this area of the palate exhibited a smooth surface outline coupled with one, two or three well defined ridge-like/spiky projections above the surface on each side and/or in the middle. These projections represented sectioning through the small ridges comprising the palatal rugae. (Figures 5.2a,b).

The epithelium was composed of a superficial layer of orthokeratin and the cellular compartment beneath. The orthokeratin layer had a rather uniform thickness in the interrugal area. It increased gradually towards the rugal ridges. The orthokeratin layer was about one-half or more the thickness of the cellular compartment. (Figures 5.2c).

The cellular compartment was made up of multiple strata of squamous cells typical of stratified squamous epithelium. The number of cell layers ranged from about 13 to 17. Towards the base of the epithelium, the squamous cells were abruptly succeeded by a single cell layer of low columnar, palisading basal cells. (Figure 5.2 d) . The

baseline of the epithelium was thrown into successive units of rete pegs composed of basal cells only, even when the rete pegs were long. The height of these rete pegs ranged from being very shallow to as much as one-fourth to one-third of the thickness of the cellular compartment. The basal cells in the rete pegs were oriented as if they flared out from a central core. There were present a few mitotic figures confined mostly to the basal cell layer; as many as six mitoses per high power field (x400) were observed. The uniform thickness of the cellular compartment and the orthokeratinized layer contributed to the smooth contour of the mucosal surface and the keratin-cellular compartment junction.

Beneath the epithelium was the fibrous connective tissue compartment consisting of a zone of dense fibrous connective tissues immediately beneath the epithelium and a more distant zone of loose tissues containing neurovascular elements which were more abundant in the lateral aspect of the palate. Beyond this connective tissue compartment which amounted to the thickness of the epithelium in its narrowest width, there was the bony vault of the palate composed of dense lamellar bone, which had a smooth surface outline. (Figures 5.2 a) .

2. Gingival Area

The non-sulcal gingival epithelium exhibited features essentially the same as those of the middle palate except that it was thicker and had more prominent rete pegs. The sulcal epithelium was much thinner, having less than 10 cell layers; it tapered towards the gingival attachment. A good attachment of the sulcal epithelium to the root surface was often seen. There were no rete pegs associated with the sulcal epithelium; its base formed a smooth curve. Not infrequently,

the sulcal epithelium was partially keratinized and mildly infiltrated with mononuclear cells, signifying the presence of chronic inflammation. (Figure 5.3).

3. Anterior Palate

The epithelium of the anterior palate exhibited features that were essentially the same as those of the middle palate except that there were no rugal ridges and the orthokeratin layer, cellular compartment and the rete pegs were all of smaller dimensions. It had abundant loose and vascular fibrous connective tissues separating the epithelium from the nasal structures. (Figure 5.4) .

4. Posterior Palate

The posterior palate was lined by a thin orthokeratinized, stratified squamous epithelium which was slightly undulating. Beyond a thin layer of subepithelial dense fibrous connective tissues, there was an abundant amount of mucous salivary gland tissues. (Figure 5.5).

B: TONGUE

1. Posterior Tongue

The surface of the posterior tongue exhibited a continuous array of closely packed lingual papillae which constituted the epithelium. Each lingual papilla was long and slender and lined by a single layer of cuboidal basal cells. Despite its narrow width, stratification of the squamous cells in the core of the papilla was clearly discernible.The squamous cells transformed superiorly into granular cells containing numerous keratohyaline granules. Above this granular layer was the superficial orthokeratin layer. The latter assumed a spiky or villous configuration, each projection being associated with one lingual papilla.

Beneath the epithelium there was a thin zone of dense fibrous connective tissues which extended upward in between adjacent epithelial lingual papillae which were therefore very much accentuated in configuration. Beyond this zone of fibrous connective tissues were the muscles of the tongue, the fibres of which were arranged into fascicles running in different directions. Towards the posterior limit, there were numerous serous and mucous salivary gland tissues among the muscle fibres. The consecutive lingual papillae extended to the same depth into the connective tissue compartment such that a straight line or smooth curve could be drawn across the bases of these papillae. Similarly the villous processes of the surface orthokeratin projected upwards to approximately the same height such that a straight line or smooth curve could also be drawn across their tips. (Figures 5.25a,b).

Towards the anterior end, a group of papillae assumed very prominent sizes in which all the above mentioned features were exaggerated. This corresponded to the anatomic crescentic pad, which delineated the boundary between the anterior and the posterior tongue. (Figure 5.25c).

2. Anterior Tongue

Although the epithelium here was also composed of repeating units of lingual papillae, they were much broader and less well defined when compared to the posterior lingual papillae. They were more akin to broad rete pegs in oral mucosal epithelium. However, the stratification of the squamous cells into layers was more pronounced and better defined. The base of the epithelium was also lined by a single layer of palisading basal cells. The epithelial surface was covered by a thick layer of orthokeratin from which horny processes projected upward, each corresponded roughly to a unit of the underlying papillae. The fibrous

connective tissue zone and the muscular bulk of the tongue were the same as those in the posterior tongue except that there were no salivary gland tissues. (Figures 5.26).

4NQO-TREATED ANIMALS

A: PALATE

Four Weeks

The whole palate did not exhibit any abnormal changes.

Eight Weeks

The middle palate exhibited generalized hyperorthokeratosis and hyperplasia of the cellular compartment in the form of acanthosis, elongation and broadening of rete pegs which now contained also squamous cells in addition to the basal cells (Figure 5.7b). In one animal the elongation of the rete pegs took the form of apparent branching of the rete pegs which was termed "Arborization". (Figure 5.7a). Though generalized, the hyperplastic growth did not appear to be very uniform resulting in an irregular surface outline on top of the orthokeratin layer as well as an irregular junction between the orthokeratin layer and the cellular compartment. These histologic appearances were reflected in the macroscopically observed mild thickening and roughness on the middle palate. In focal areas, there was keratinization of the full thickness of the epithelium, the keratinized cells extending to the basal layer of the epithelium. The individual keratinized cells still retained their pyknotic nuclei. (Figure 5.18). Features of cellular atypia were observed. These included keratinization of single cells, reduction in cellular cohesion, and cellular pleomorphism.

Since all these features, except the full thickness keratinization, were confined to the basal one-third of the cellular compartment and appeared to be a mild deviation from normal, the general disturbance in the epithelium as a whole was regarded as mild dysplasia. (Figure 5.18). The gingival area, anterior and posterior palate did not exhibit abnormal changes.

Twelve Weeks

Changes in the middle palate were essentially the same as those at eight weeks. In addition there were also focal areas showing cellular atypia involving two-thirds or more of the thickness of the cellular compartment and the cellular features deviated more from normal. Hence the disturbance in the epithelium in such areas was regarded as moderate to severe dysplasia. Since it was a very subjective decision to draw the line between moderate and severe, these two conditions were combined in one descriptive phrase -"Moderate to Severe Dysplasia." Various features of dysplastic epithelium are illustrated in figure 5.20, 5.21.

The gingival area, especially the sulcal epithelium, exhibited mild dysplasia similar to that described for the middle palate.

The anterior and posterior palate did not exhibit abnormal changes.

Sixteen Weeks

At 16 weeks , the middle palate exhibited more severe hyperkeratosis and hyperplasia in that the pattern of the rugal ridges

could no longer be identified. This was reflected in the macroscopically observed severe thickening of the palate which had an uneven surface. Against a general background of mild dysplasia, there were more focal areas of moderate to severe dysplasia showing more bizarre features of cellular atypia. Thus, keratinization of single cells and cell groups, full thickness keratinization and cellular pleomorphism were all accentuated.(Figure 5.21). In one animal there was a large area of moderate to severe dysplasia about the midline. This area showed marked reduction of cellular cohesion in which individual cells separated from each other creating cleft-like spaces. There was also extensive keratinization of cell groups. In this midline area, the base-line of the epithelium was distinctly below that of the adjacent epithe-lium.The epithelial processes advanced at approximately the same level, forming a convex pushing margin. The palatal bone facing this advancing front, though separated from the latter by fibrous connective tissues, exhibited resorption such that the resulting irregular outline assumed a concavity corresponding to the convexity of the epithelial front. There was a mild degree of chronic inflammation in the dense fibrous connective tissues separating the epithelium and the palatal bone. (Figure 5.9).

The above histologic appearance of the midline area strongly suggested that the epithelium in this area had an aggressive behaviour. However, the size and early nature of the lesion precluded accurate diagnosis although the possibility of early verrucous carcinoma was very much in favour.

Hyperorthokeratosis and epithelial hyperplasia extended from the middle palate to the non-sulcal gingival epithelium in the gingival area. The sulcal epithelium of the gingiva exhibited marked keratinization and hyperplasia from which epithelial processes arose and extended deep into the alveolus.(Figure5.22).The epithelial processes frequently exhibited mild, moderate to severe dysplasia and were associated with a heavy mixed inflammatory cell infiltrate in the subjacent fibrous connective tissues.

Occasionally, abscesses were formed. Gingival pockets were pronounced. Despite the proliferative changes, frank invasion by infiltrating epithelial islands, cords or nests was not present. Such and similar gingival changes without obvious invasion are described as "Gingival Proliferative Changes" and are illustrated in Figure 5.11.

The anterior and posterior palate did not exhibit abnormal changes.

Twenty Weeks

The histologic changes exhibited by the middle palate were essentially the same as those at 16 weeks. Two out of the six animals sacrificed exhibited the probable verrucous carcinoma described above. The lesions were positioned off the midline.

In the gingival area, the proliferative changes became more pronounced. In addition to more severe downward proliferation giving rise to a folded appearance of the epithelium, there were upward outgrowths from the depth of the gingival pocket composed of a lining epithelium and

central coresof fibrous connective tissues. Sometimes the gingival pocket was filled with overwhelming amounts of orthokeratin (Figure 5.11). Apparently some of this keratin material was occasionally circumscribed by the surrounding epithelial lining, giving rise to inclusion cysts resembling the epidermoid cyst of the skin or odontogenic keratocyst . (Figure 5.23). Despite extensive growth and destruction of the alveolar bone,no infiltrating epithelial processes were observed. In view of the absence of such invasive signs of the epithelium and the fact that alveolar bone is known to be prone to resorption, there was not enough evidence to regard such proliferative changes as constituting an invasive carcinoma.

In some animals, there were signs of malignant behaviour exhibited by the proliferative epithelium which included very extensive destruction of the alveolar bone to the extent of leaving almost no bone support to the tooth;and very exuberant growth and deep ingrowth of the epithelium into the alveolus. These appearances could not be accounted for by just an innocent hyperplasia of the gingival epithelium or by any known periodontal disease. In other instances the proliferation began with infiltrating epithelial cords and nests.Such changes were also seen at the periphery of solid columns of proliferative epithelium. These essentially well differentiated squamous cell carcinomas were termed "Gingival Carcinoma" to differentiate them from verrucous carcinomas of the middle palate . (Figures 5.13, 5.14). The anterior and posterior palate exhibited a very mild degree of thickening of the keratin layer and the underlying cellular epithelium.

Twentyfour Weeks

The histologic changes of all palatal areas were essentially the same as those at 20 weeks, except that more animals (six out of seven sacrificed) exhibited gingival carcinomas and palatal verrucous carcinomas. Some of the latter had caused complete erosion of the

bony vault of the palate. These verrucous carcinomas exhibited more classical changes including marked epithelial proliferation with downgrowths of bulbous rete pegs.(Figure 5.17).

The various histologic changes in the palate of animals sacrificed at different time periods are summarized in Figures 5.49 and 5.50 and Tables 5.2, 5.3 and 5.4.

B: TONGUE

Four Weeks

Both the posterior and anterior tongue did not exhibit abnormal changes.

Eight Weeks

The posterior tongue exhibited a mild degree of hyperorthokeratosis and hyperplasia of the cellular compartment in that the lingual papillae were thickened and elongated. (Figure 5.28). In one of the two animals sacrificed, there was a group of lingual papillae showing the distinguishing features of hypochromatism, very wide papillae and excessive hyperorthokeratinization with replacement of the surface configuration by a flat surface outline. High power view demonstrated enlarged nuclei and nucleoli in the prickle cells and basal cells. Their sizes were rather uniform. A few nuclei had dense clumps of chromatin resembling nuclei undergoing mitosis. The epithelial disturbance was regarded as mild dysplasia. The lesion having the above distinguishing histologic features is termed a "Plaque" (Figure 5.28) and corresponded to the smooth plaque noted macroscopically.

In the other animal, there was a focal area of keratinization of the full thickness of the epithelium. Features of full thickness keratinization are illustrated in Figure 5.35.

The anterior tongue also exhibited a mild degree of hyperkeratosis and hyperplasia of the cellular compartment similar to those described for the posterior tongue.(Figure 5.30).

Twelve Weeks

The histologic changes in the posterior and anterior tongue were essentially the same as those exhibited at eight weeks. Features of cellular atypia were observed in the lingual papillae of the posterior tongue . These included reduction of cellular cohesion, cellular pleomorphism, keratinization of single cells, and irregular epithelial stratification, all occurring to a very mild degree and confined to the basal one-third of the epithelium. The disturbance in the epithelium was regarded as mild dysplasia. (Figure 5.32). More plaques were observed and they exhibited essentially the same histologic features described previously.

Sixteen Weeks

The posterior tongue showed more pronounced hyperorthokeratinization and hyperplasia. Against a background of mild dysplasia there were focal areas of more severe cellular atypia which included irregular epithelial stratification, keratinization of single cells and cell groups, reduction of cellular cohesion and cellular pleomorphism. Since these atypical features were more bizarre and involved two-thirds or more of the thickness of the epithelium, the epithelial disturbance was regarded as moderate to severe dysplasia. Features of moderate to severe dysplasia are illustrated in Figure 5.36.

A papilloma was observed in one animal. It consisted of exophytic papillary processes on a sessile base projected above the surface of the epithelium. Only very mild atypia was exhibited. Features of papillomas are illustrated in Figure 5.44.

The anterior tongue showed predominantly mild hyperkeratosis, hyperplasia and mild dysplasia. However in one animal moderate to severe atypia was very prominent.

Twenty Weeks

At twenty weeks, changes were essentially the same as those at 16 weeks except that in the posterior tongue, areas of moderate to severe dysplasia were more pronounced and occurred more frequently. The anterior tongue of one animal exhibited a plaque. The rete pegs of this plaque were very much widened and thickened, and it was covered by a thick layer of orthokeratin having a flat surface outline. Cellular features were essehtially normal. (Figure 5.38).

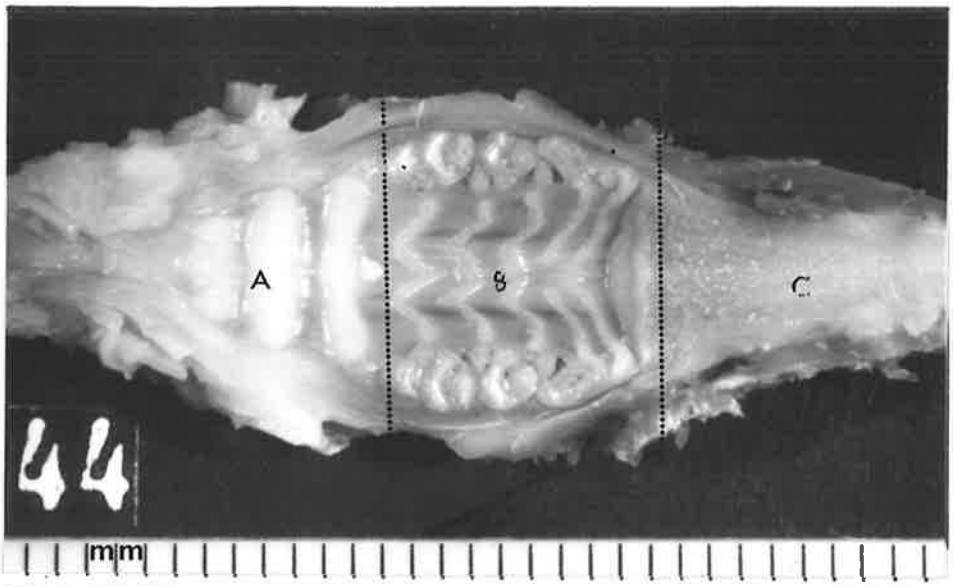
Twentyfour Weeks

Changes in the anterior and posterior tongue were essentially the same as those at 20 weeks. In addition, three animals had carcinomas in the posterior tongue. These carcinomas were infiltrative in nature in that they invaded deep into the musculature of the tongue without showing any exophytic growth above the level of the tongue surface. However, undermining of adjacent epithelium by the proliferative tumour mass had resulted in an elevated periphery of the lesion. The surface of the tumour was ulcerated in that the continuity of the surface epithelial lining was broken with the base of the ulcer being the friable and rugged superior limit of the tumour. The main mass of the tumour was composed of long columns of well differentiated squamous

epithelium with extensive whorls and pearls of parakeratin. Features of cellular atypia were present at the periphery of the epithelial processes. The invasive front demonstrated infiltrating cords, nests and islands of epithelial cells. Very heavy chronic inflammation and fibrosis circumscribed the invading periphery of the carcinoma. In at least two animals, two adjacent carcinomas not far from each other were observed. The overall impression was that they were essentially well differentiated squamous cell carcinomas. (Figures 5.40, 5.42).

The various histologic changes occurring in the tongue of animals sacrificed at different time periods are summarized in Figures 5.51, 5.52 and 5.53 and Tables 5.5, 5.6 and 5.7.

5.1 a.



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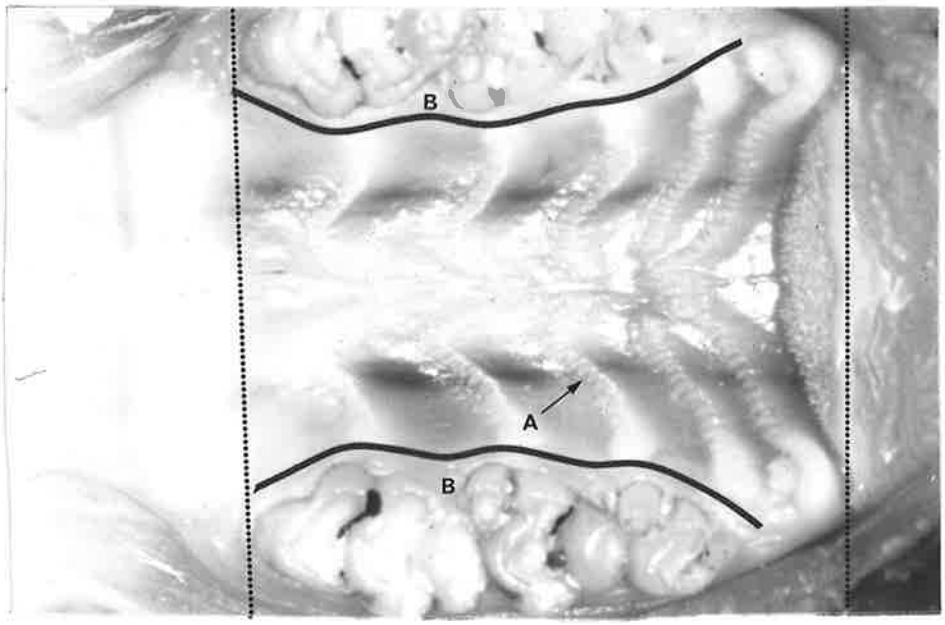


c.

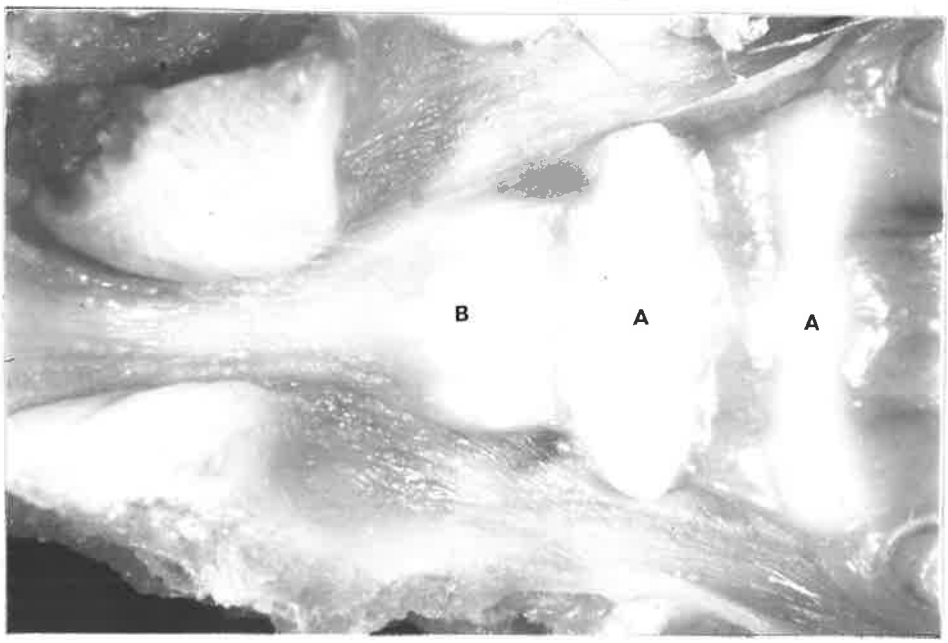


Figure 5.1: The morphology of a control palate.

a. The anterior (A) and middle (B) palate show a shining and smooth surface associated with well defined rugae. The posterior palate (C) shows a finely nodular surface.

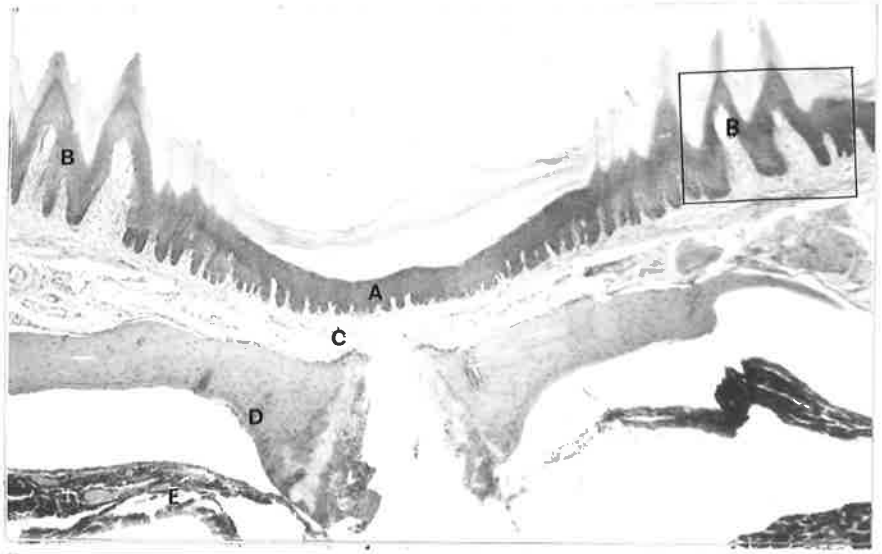
b. Higher magnification of normal middle palate and gingival area. Small ridges comprising the palatal V-W shaped rugae are clearly demonstrated (A). The gingival area (B) is delineated by the solid line.

c. Higher magnification of normal anterior palate. Transverse rugae (A) and the incisive papilla (B).

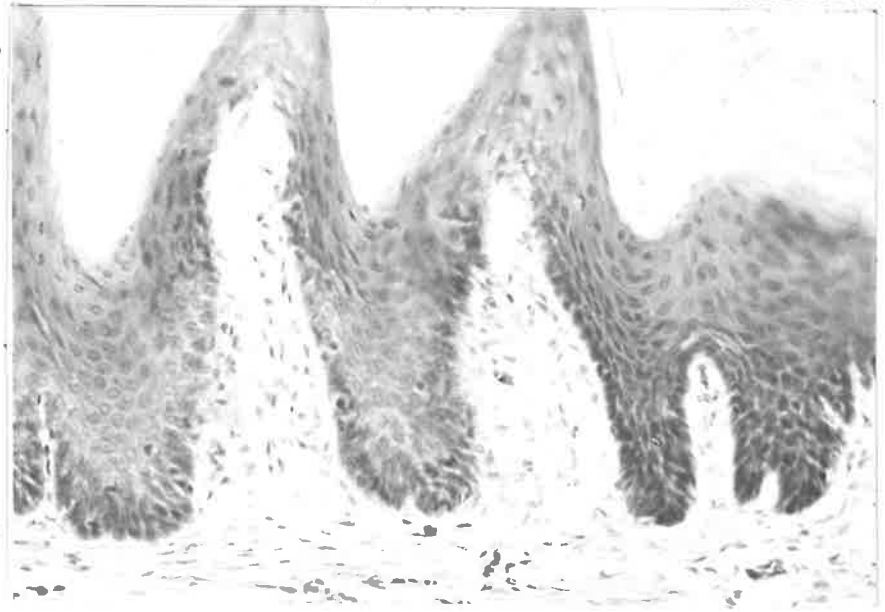
d. Higher magnification of posterior palate.

Magnification, a x 5, b x 9, c x 9, d x 9 ; treated control at 8 weeks.

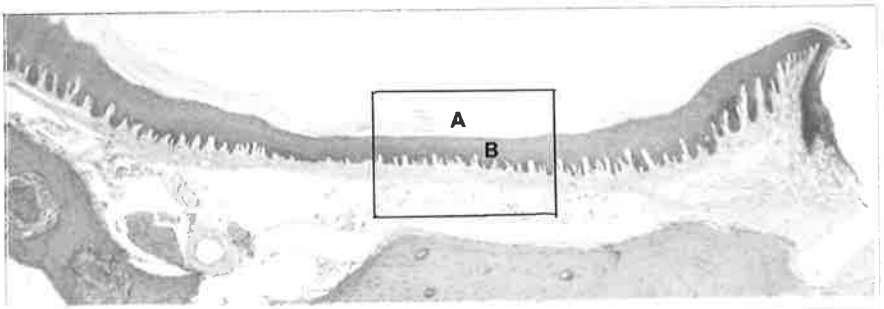
5.2 a.



b.



c.



d.

Figure 5.2: Histology of control middle palate.

a. The microscopic architecture shows a uniform epithelial lining (A) containing rugal ridges (B), a subepithelial fibrous tissue zone (C) and a bony palatal vault(D) beyond which are the nasal structures (E).

b. Higher magnification of the rugal ridges (framed area in a.)

c. This view demonstrates the uniform thickness of the keratin layer (A) and the cellular compartment (B).

d. Higher magnification of the palatal epithelium (framed area in c).

Magnification, a x 40, b x 200, c x 40, d x 200; a and b, untreated control at 20 weeks; c and d, treated control at 24 weeks.

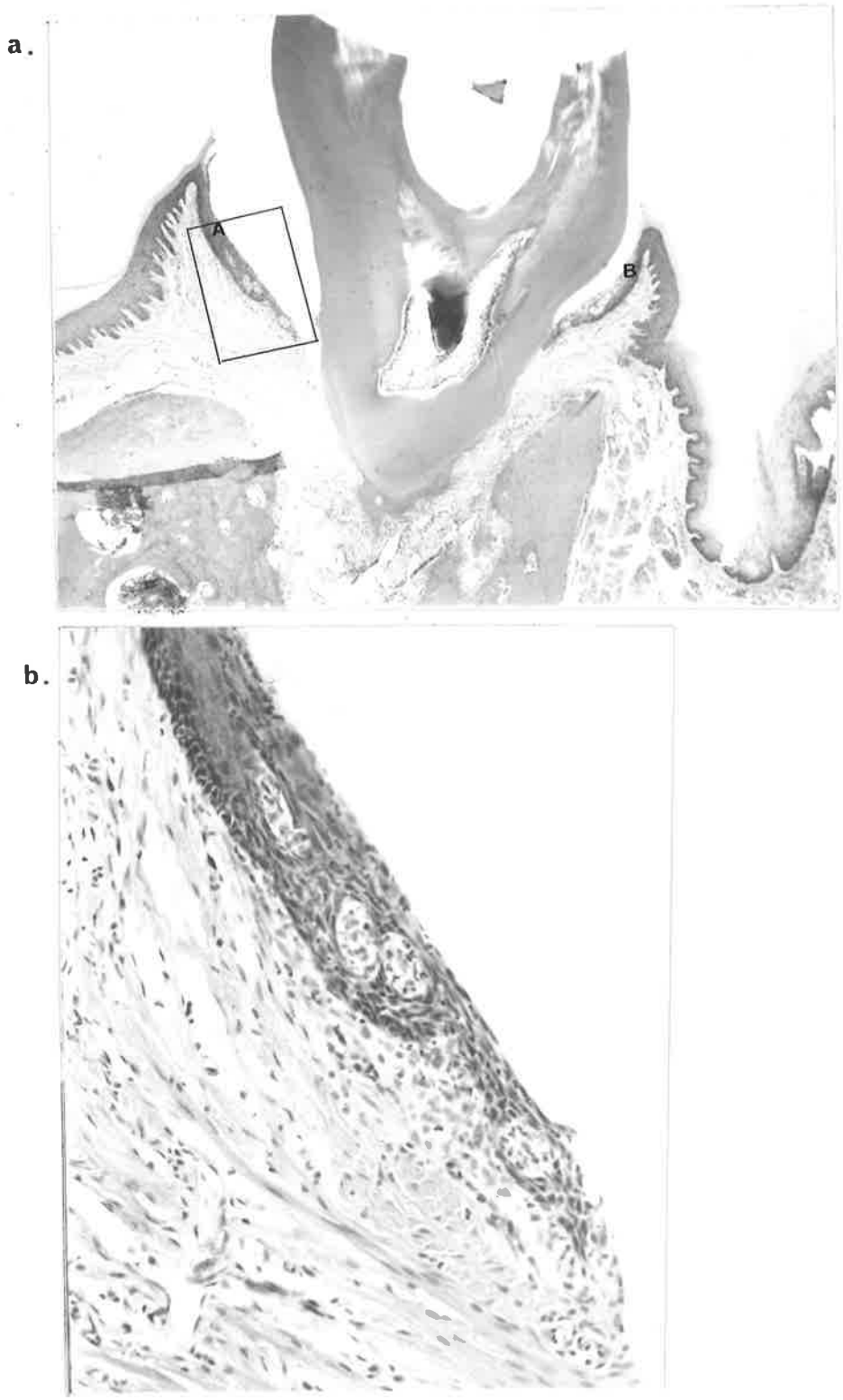


Figure 5.3: Histology of control gingival area.

a. Both the palatal (A) and buccal (B) sulcal epithelium are thin and smooth and partly keratinized.

b. Higher magnification of the gingival area. (Framed area in a.) The sulcal epithelium is mildly inflamed and has a smooth basal outline.

Magnification, a x 40, b x 200; treated control at 20 weeks.

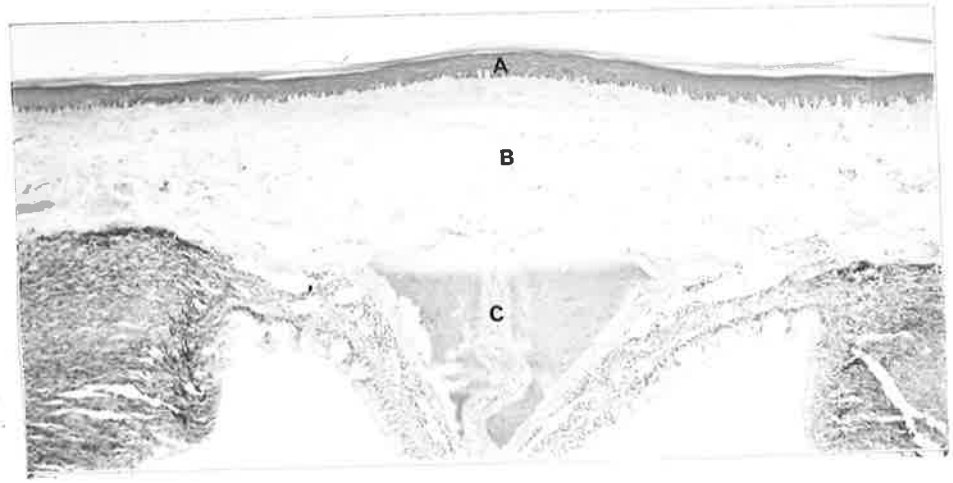


Figure 5.4: Histology of control anterior palate. The lining epithelium (A) is similar to that of the middle palate but thinner. Fibrous connective tissues (B). Nasal structures (C).
Magnification, x40; treated control at 4 weeks.

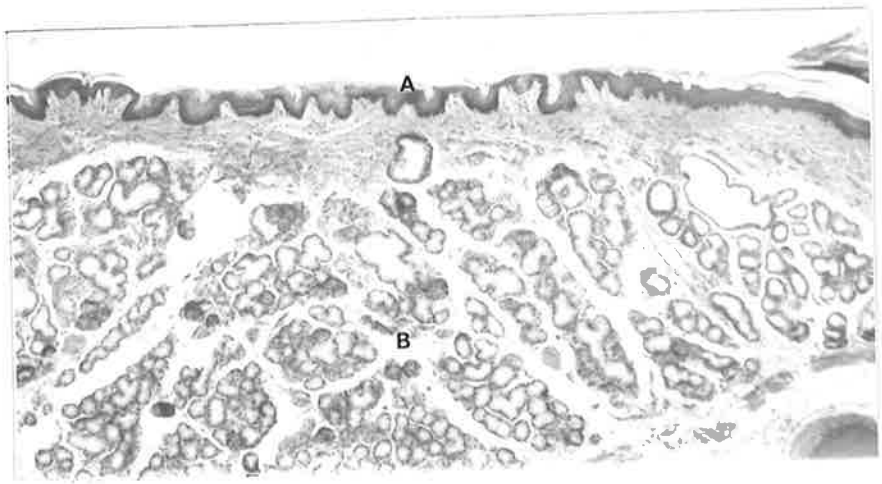


Figure 5.5: Histology of control posterior palate.
Epithelium (A). Mucous salivary gland tissues (B).
Magnification, x40; treated control at 24 weeks.

a.

b.

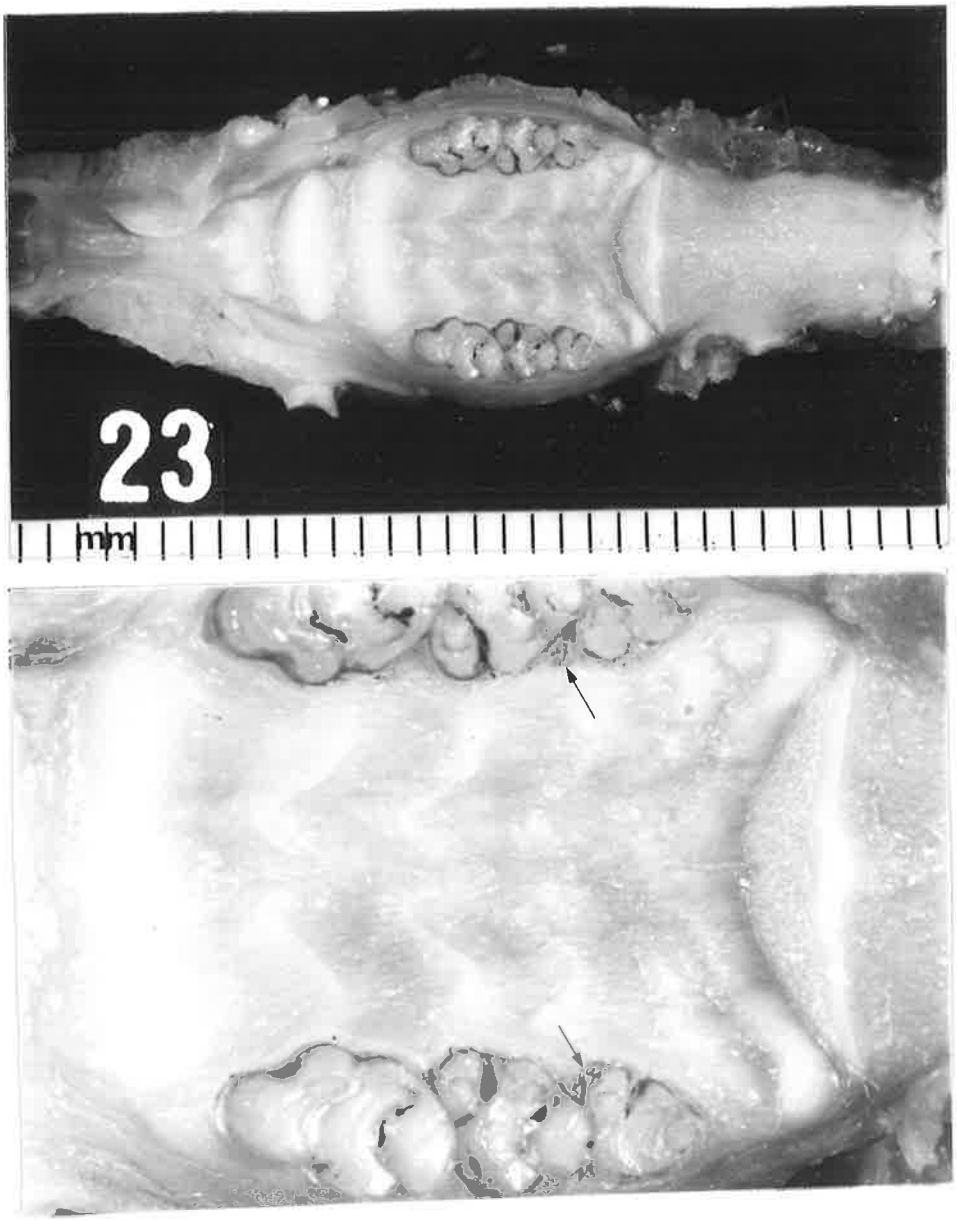


Figure 5.6: Mild thickening of the palate.

a. This represents the early changes in the palate observed at 8 and 12 weeks. The normally shiny and smooth surface of various areas has become lustrousless and roughened.

b. Higher magnification of the middle palate and gingival area. Rugae have partially lost their definition. Gingival pockets are not yet observed though some food fibres are impacted in the gingival crevices (arrows).

Magnification, a x 5, b x 9; experimental animal at 12 weeks.

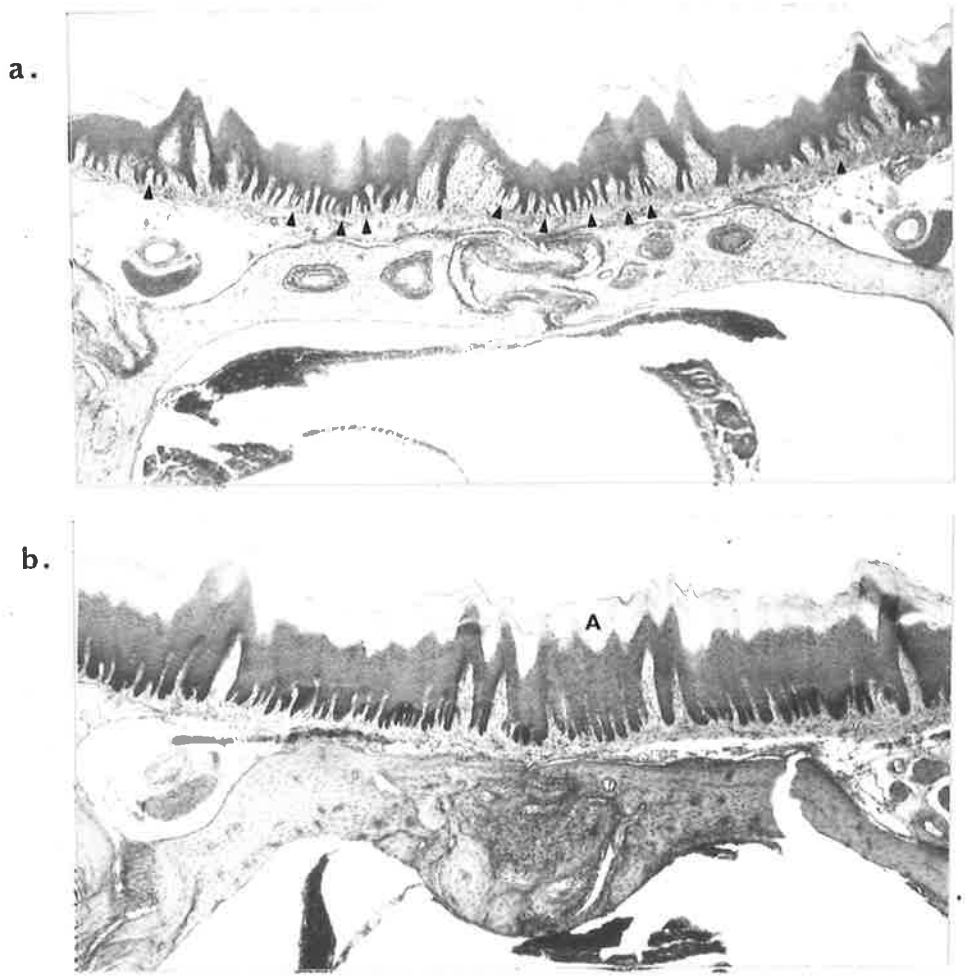


Figure 5.7: Early hyperkeratosis and epithelial hyperplasia of the middle palate.

a. Arborization of the rete pegs (arrows) is a rather common feature. The corresponding gross morphology of this palate is shown in Figure 5.6.

b. Acanthosis and elongation of the rete pegs. In both a and b , there is marked thickening of the orthokeratin layer (A). The surface outline is irregular.

Magnification, a x 40, b x 40; a, experimental animal at 12 weeks ; b , experimental animal at 8 weeks.

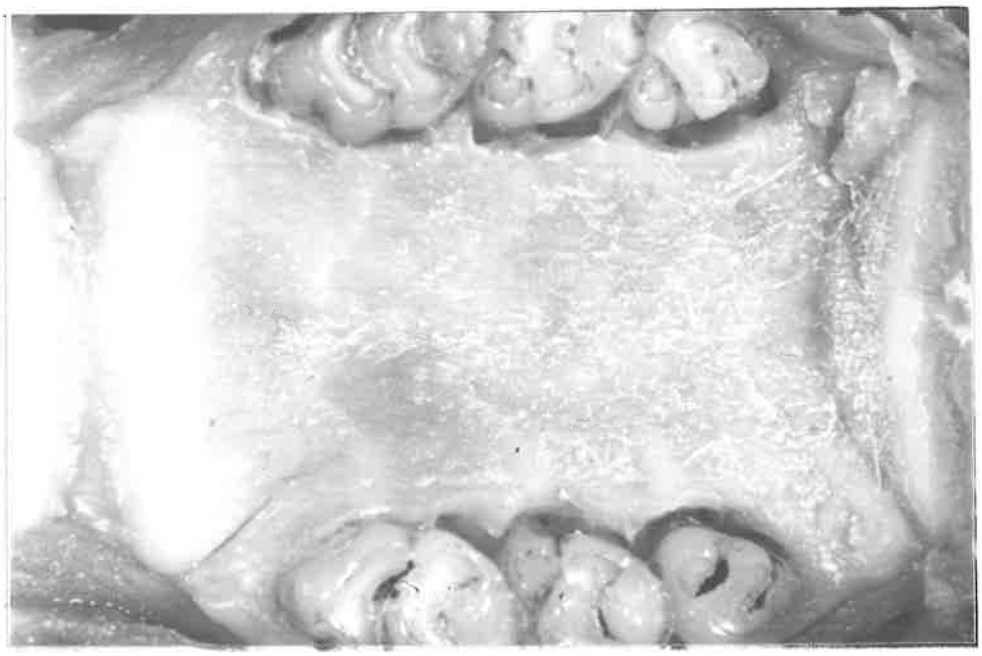
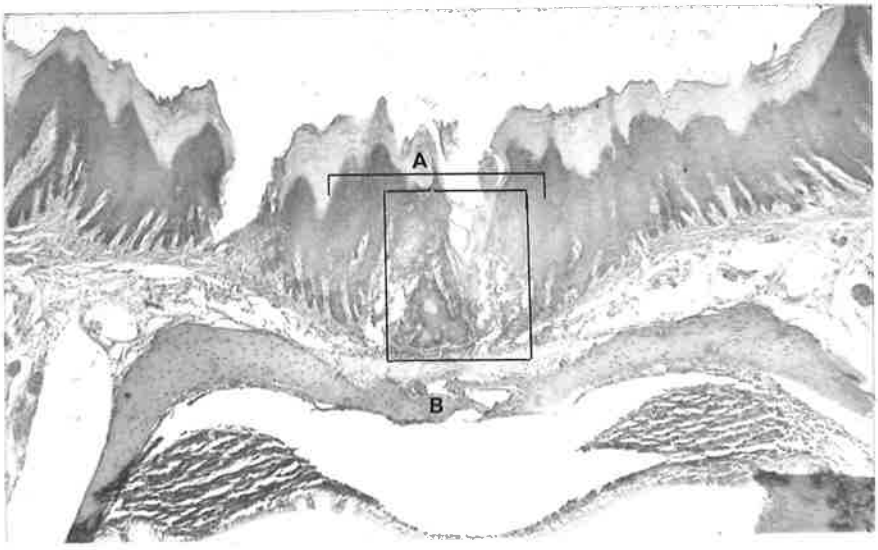


Figure 5.8: Severe thickening of the middle palate and deep gingival pockets in the gingival area. The palatal rugae have almost disappeared.

Magnification, x9; experimental animal at 16 weeks.

5.9 a.



(For legend see opposite page.)

b

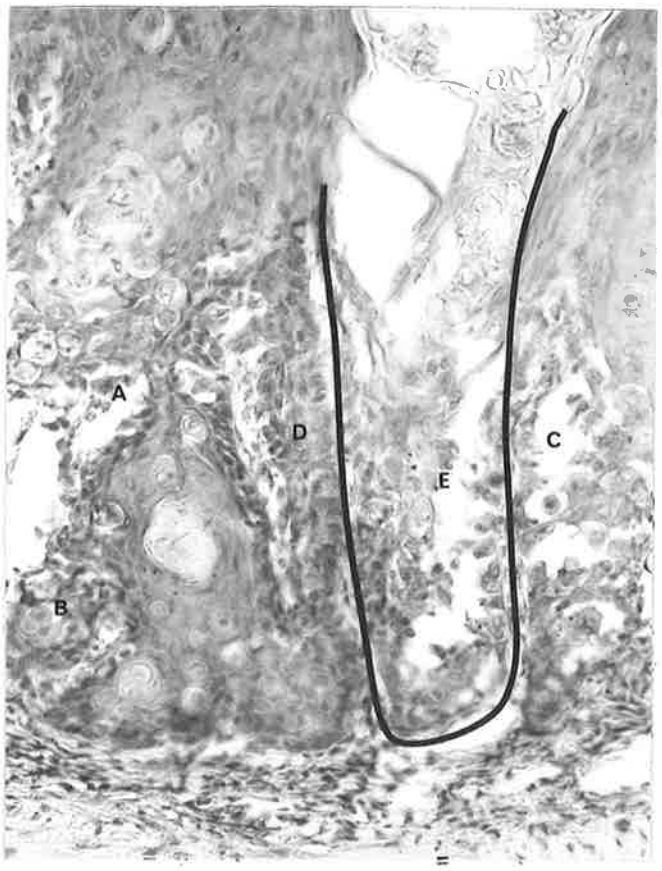


Figure 5.9: Severe hyperkeratosis and epithelial hyperplasia and possible early verrucous carcinoma of the middle palate.

a. The possible early verrucous carcinoma (A) has an advancing front, forming a pushing margin below the baseline of adjacent epithelium. There is resorption of palatal bone (B). The exophytic component is masked by hyperplasia of adjacent epithelium.

b. Higher magnification of the framed area in a. There are features of severe cellular atypia which include keratinization of single cells (A) and cell groups (B), marked reduction in cellular cohesion (C) and cellular pleomorphism (D). The cell column (E) exhibits full thickness keratinization.

Magnification, a x 40, b x 200; experimental animal at 16 weeks.

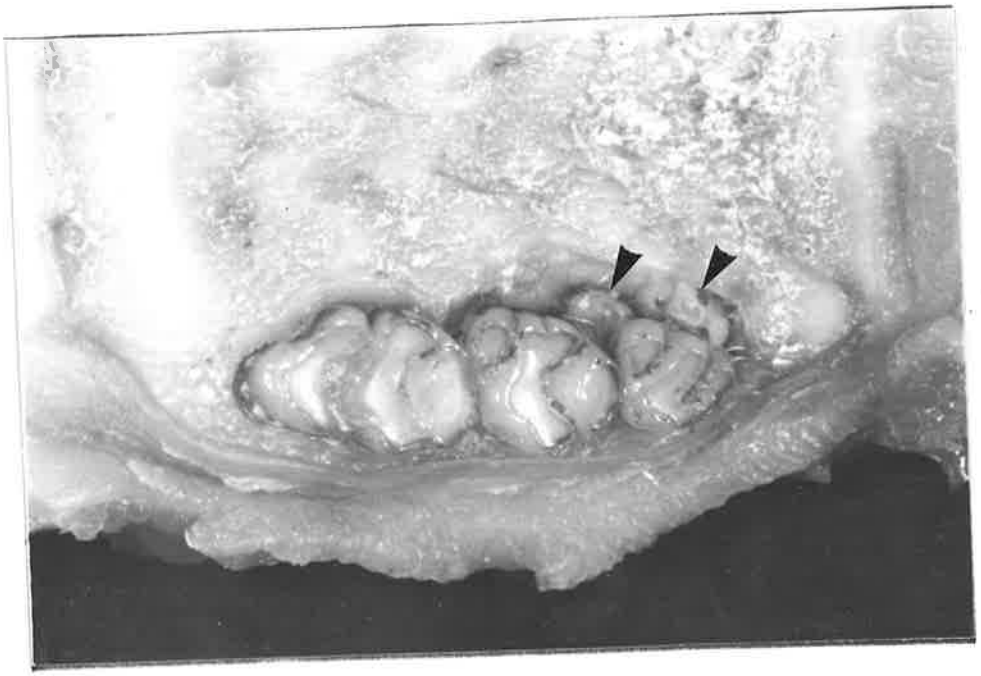
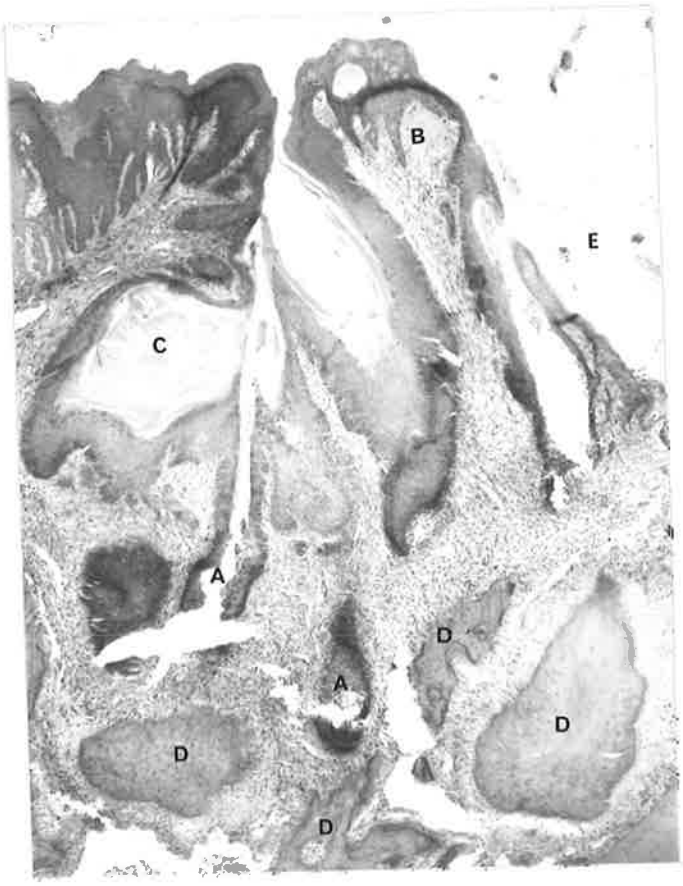


Figure 5.10: Deep gingival pocket with exophytic growth in the gingival area (arrows) and severe thickening of the middle palate.

Magnification, x 9; experimental animal at 20 weeks.

5.11 a.



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b.

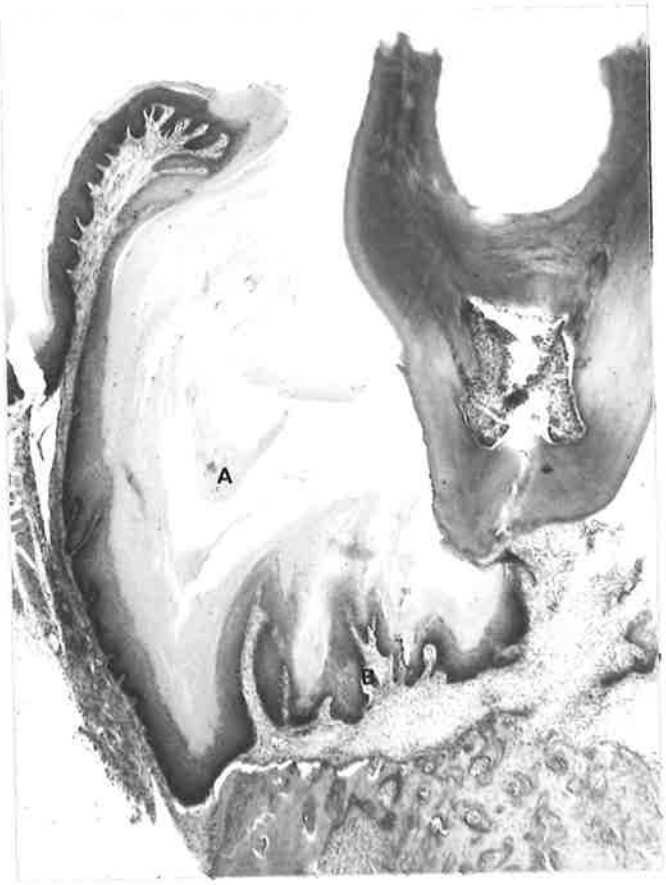
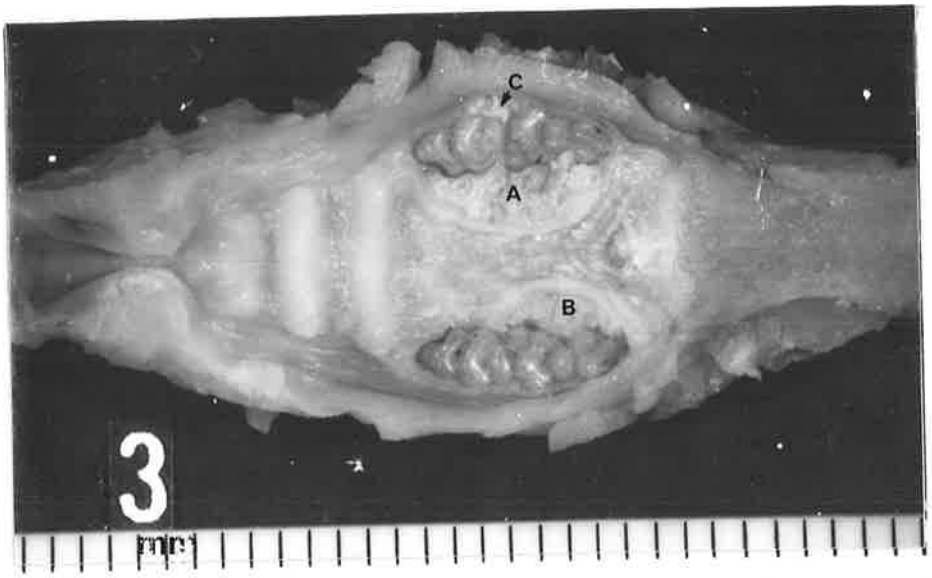


Figure 5.11: Gingival proliferative changes.

a. There is severe downward proliferation of the gingival epithelium (A), as well as exophytic growth (B). The epithelium is hyperkeratotic with the keratin occluding the epithelial lined sac (C). The alveolar bone is grossly resorbed (D). The tooth is not in view. E indicates the periodontal pocket. The corresponding macroscopic appearance of this palatal gingival lesion is shown in Figure 5.10.

b. This is a buccal lesion. The deep gingival pocket is filled with keratin (A). The epithelial downgrowth forms a very solid advancing front (B). Magnification, x 40; experimental animal at 20 weeks.

a.



b.

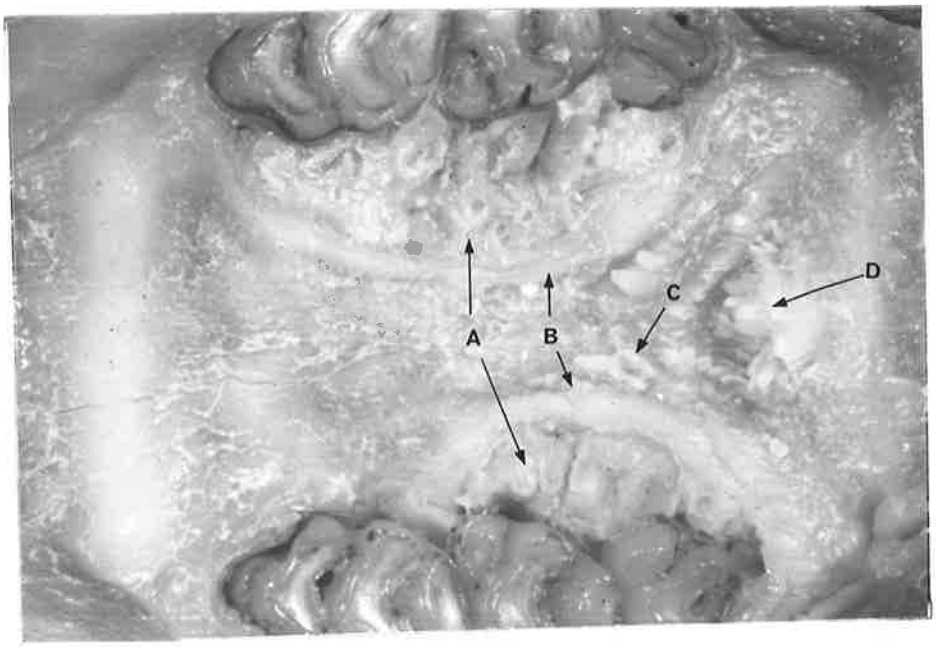


Figure 5.12: Gingival carcinomas and severe thickening of the middle palate. a. The middle palate is laterally expanded. There are two large carcinomas in the palatal gingiva (A,B) and a small one in the buccal gingiva (C). b. Higher magnification of the gingival carcinomas and the palatal lesions. The carcinomas have irregular exuberant outgrowths (A) having an elevated and rolled-in periphery (B). The irregular surface of the middle palate contains ridges (C) and papillary exophytic growths (D). Such changes were commonly observed at 24 weeks.

Magnification, a x 5, b x 9; experimental animals at 20 weeks.

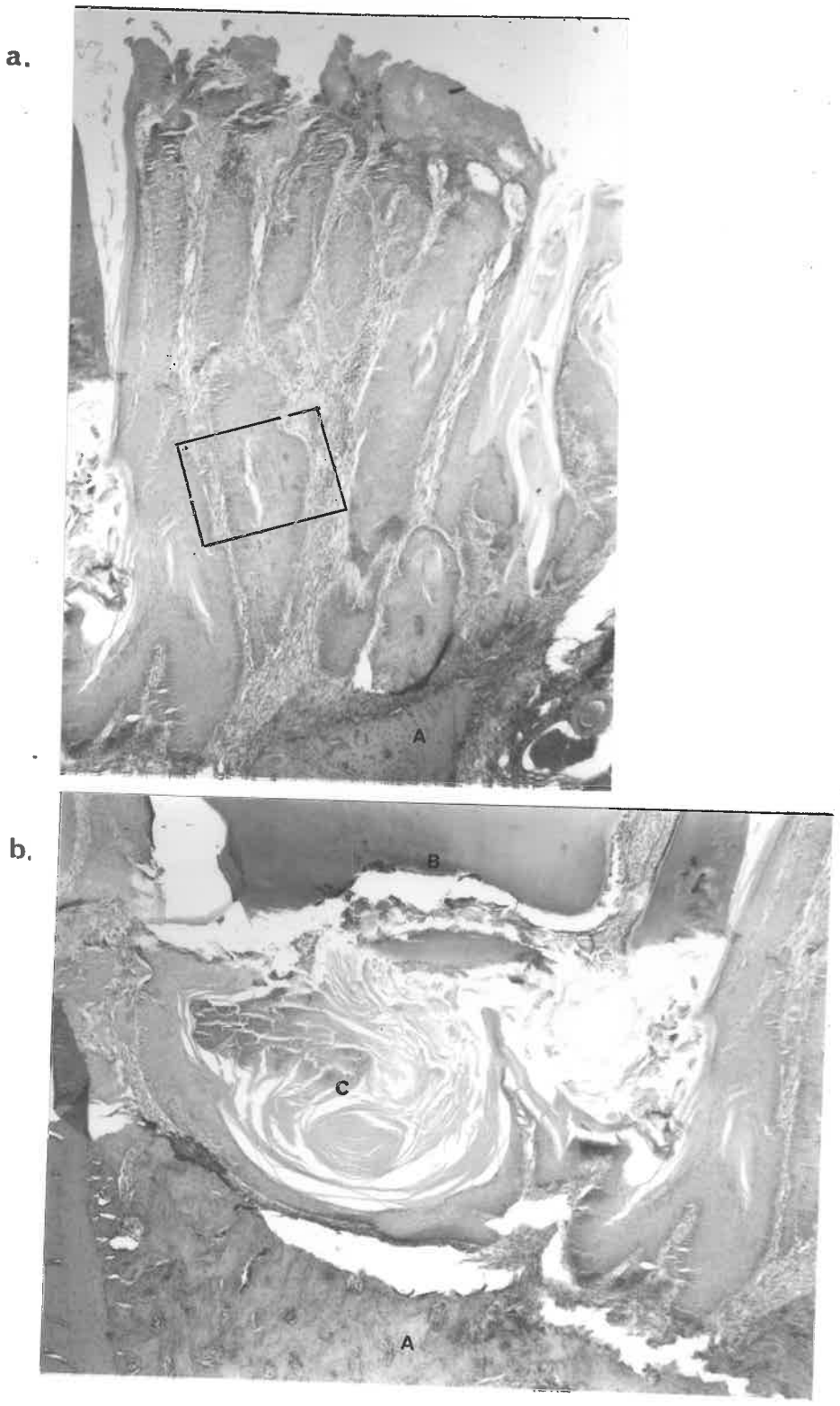


Figure 5.13: Gingival carcinoma.

a. The carcinoma is composed predominantly of long columns of infiltrati epithelium. It has caused very extensive destruction of the alveolus (A)

b. The same carcinoma has undermined the supporting alveolus (A) underne the tooth (B). In this area a very large cyst-like inclusion of the keratin has occurred, giving the appearance of a keratocyst (C).

c.

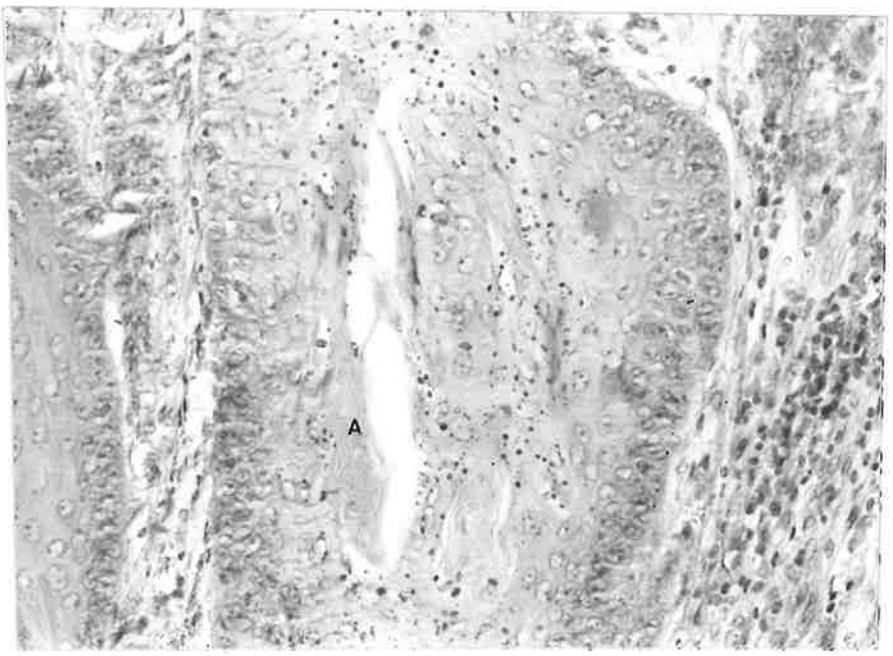


Figure 5.13: (cont.)

c. Higher magnification of the gingival carcinoma (framed area in a). The epithelium is well differentiated with keratin formation (A). The fibrous stroma is chronically inflamed. Such changes were commonly observed at 24 weeks. The corresponding gross morphology is shown in figure 5.12 a. & b.

Magnification, a, b x 40, c x 200; experimental animal at 20 weeks.

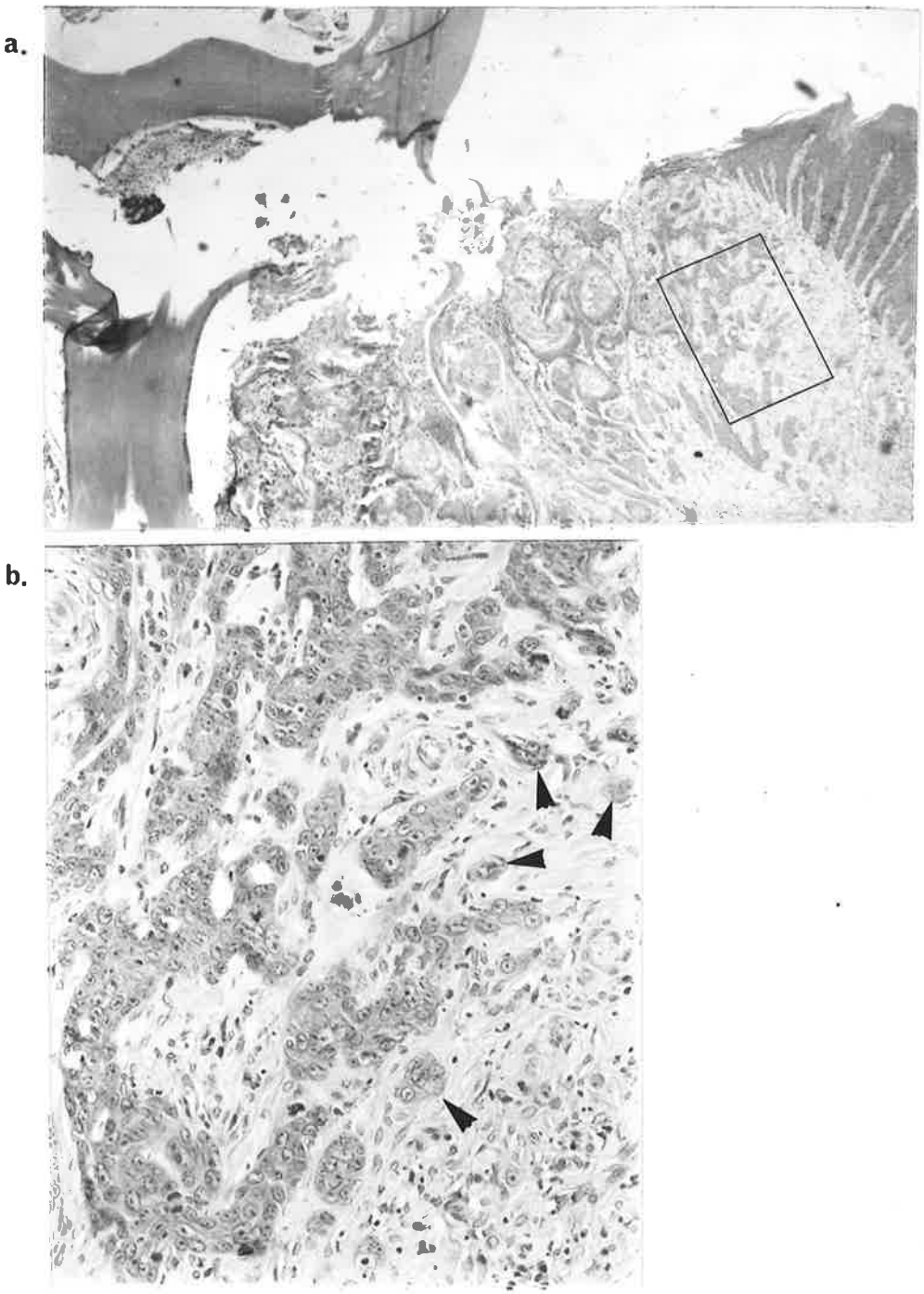


Figure 5.14: Gingival carcinoma.

a. This is an example of the gingival carcinoma which presented as a very invasive growth consisting of severe branching of the epithelium into very infiltrative processes. Macroscopically, it presented with deep gingival pockets containing some friable tissues which could not be identified clinically as carcinoma.

b. Higher magnification of the gingival carcinoma (framed area in a) showing infiltrating processes. They take the form of ramifying nests and cords as well as cell islands composed of only a few cells (arrows).

Magnification, a x 40, b x 200; experimental animal at 24 weeks.

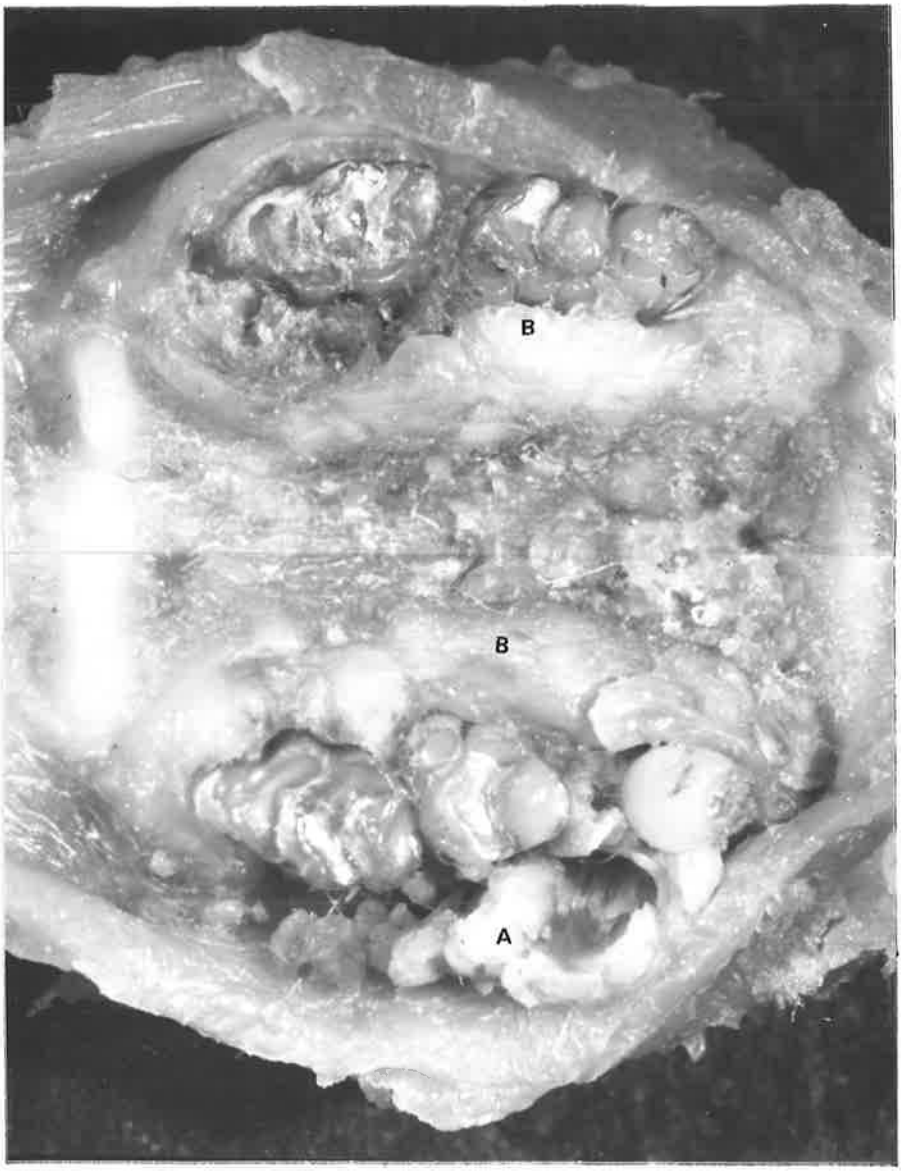


Figure 5.15: Gingival carcinomas and severe thickening of the middle palate. This is a variant of the gingival carcinoma showing basically the typical features of malignant growth (A). Compared to the lesions shown previously, these have a thickened periphery (B) partially concealing the exophytic growth on the palatal side. The middle palate is severely thickened and undulating. Histology showed verrucous carcinoma in the middle palate. Magnification, x 9; experimental animal at 24 weeks.

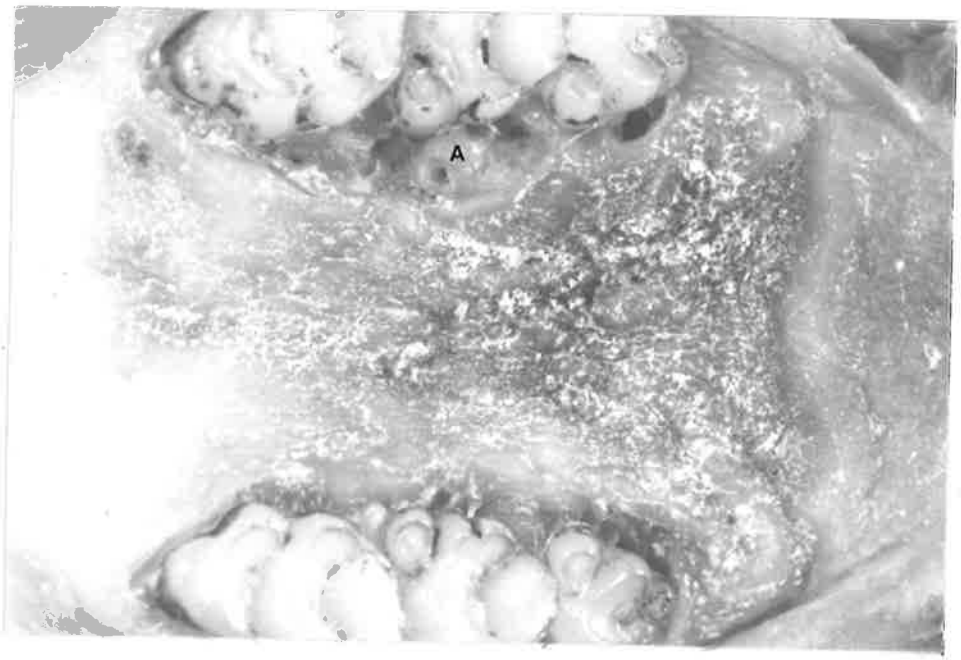
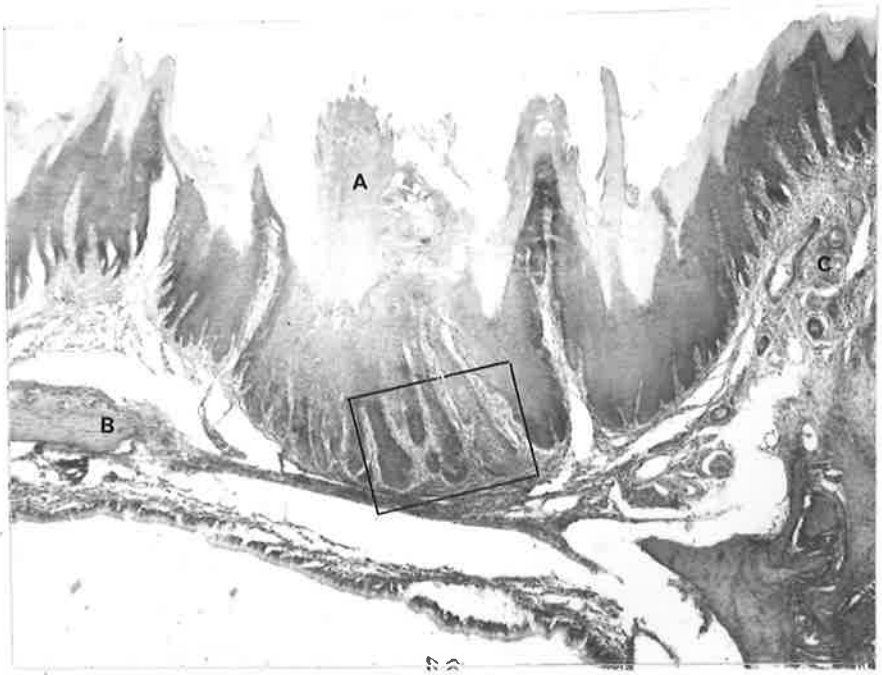


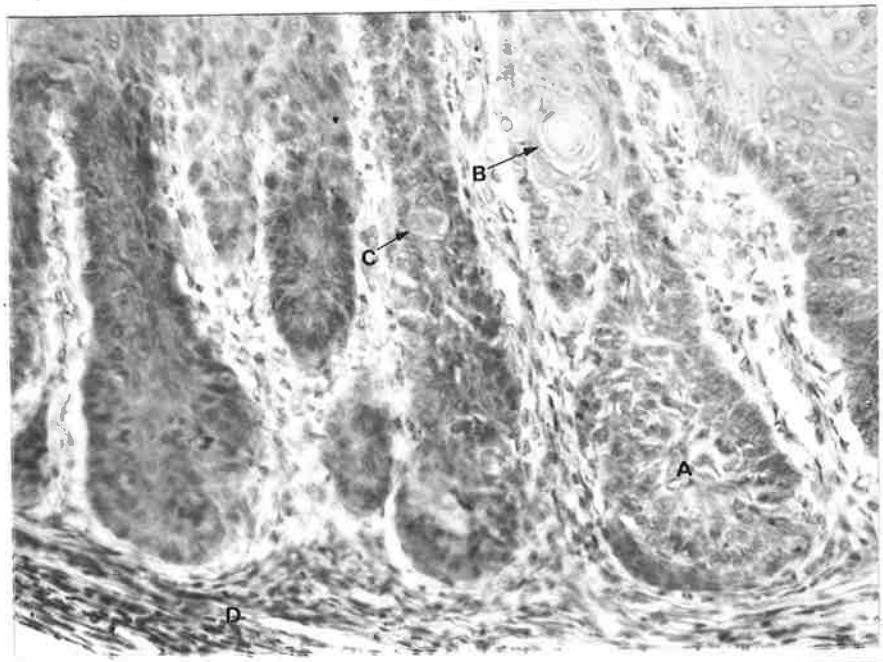
Figure 5.16: Severe thickening of the middle palate and gingival carcinoma. In this case the gingival carcinoma (A) has not attained a remarkable size. However, exophytic growth, much of which was dislodged in handling of the specimen, and a thickened periphery are recognizable.

Magnification, x 9; experimental animal at 24 weeks.

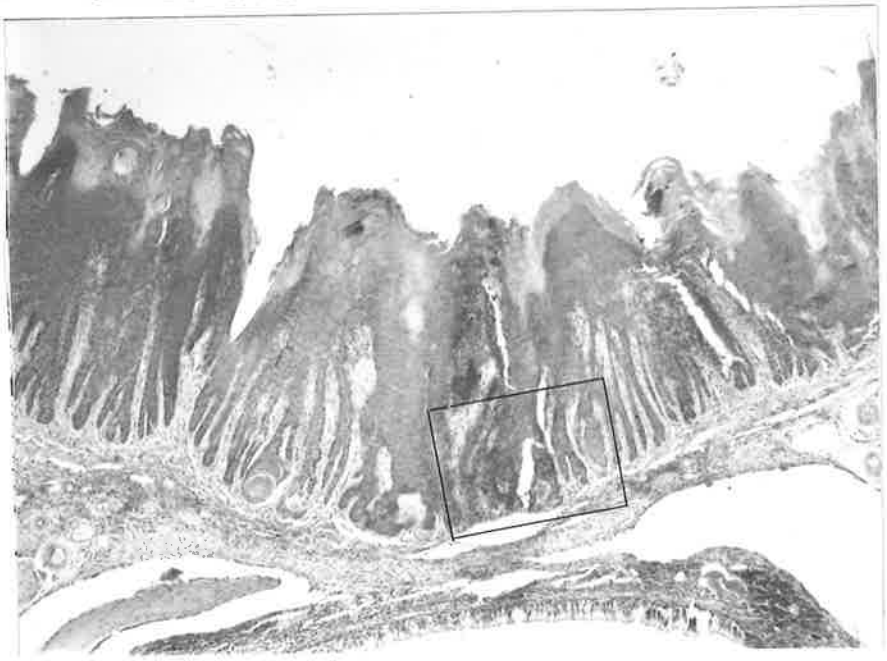
5.17 a.



b



c.



d.

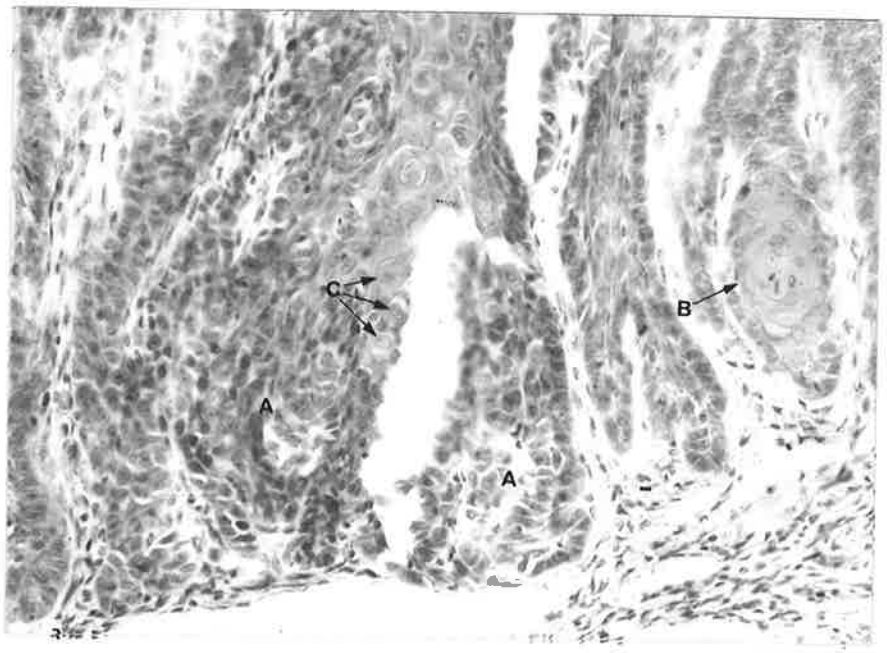


Figure 5.17: Verrucous carcinoma of the middle palate.

a. The verrucous carcinoma shows very elongated, bulbous epithelial processes and piling up of orthokeratin into horn-like structures(A). It has completely eroded the bony vault of the palate, remains of which are seen at B. Infiltrating epithelial islands from an adjacent gingival carcinoma are seen at C.

b. Higher magnification of the verrucous carcinoma (framed area in a) demonstrates epithelial processes some of which show features of cellular atypia including irregular epithelial stratification, reduction of cellular cohesion and cellular pleomorphism (A), keratinization of cell groups (B) and single cells (C). There are apparent fibrosis and a dense mononuclear cell infiltrate subjacent to the epithelial processes (D).

c. Another example of verrucous carcinoma.

d. Higher magnification of framed area in c, demonstrating features of cellular atypia that are similar to those described in b.

Magnification, a x40, b x200, c x 40, d x200;

a, b, c, d, experimental animals at 24 weeks.

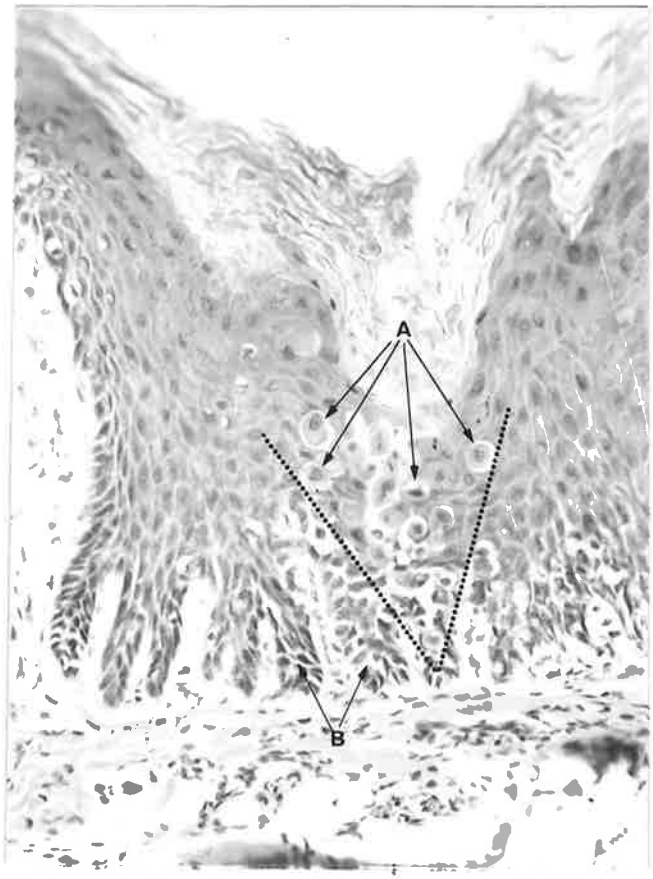


Figure 5.18: Mild dysplasia and full thickness keratinization. This is an illustration of full thickness keratinization of the epithelium occurring in a cleft-like pattern (dotted line) extending right down to the base of the epithelium. "A" indicates some keratinized cells. There are features of cellular atypia which include mild degrees of cellular pleomorphism and reduction of cellular cohesion (B).

Magnification, x 200; experimental animal at 8 weeks.

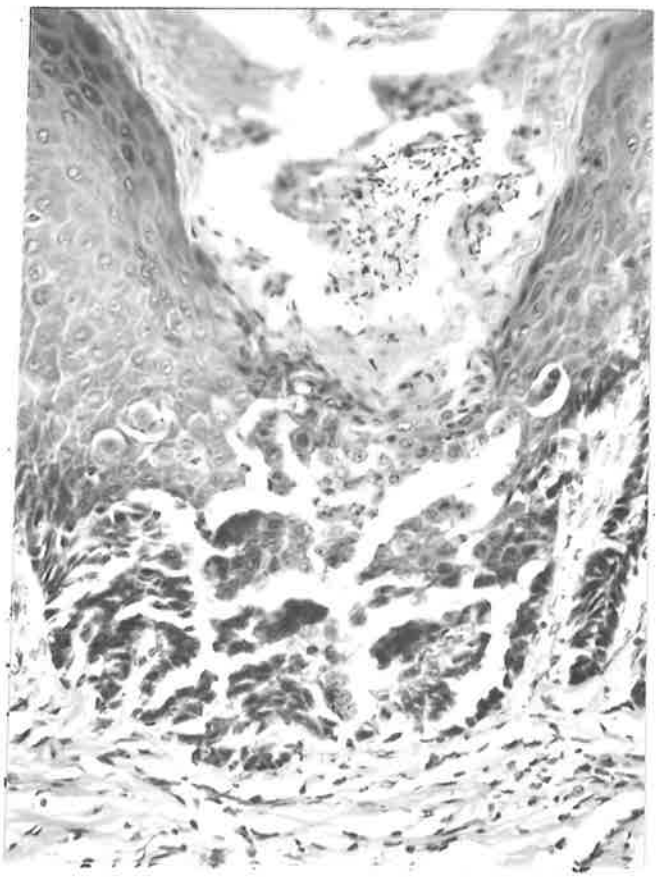


Figure 5.19: Full thickness keratinization.

This is another example of full thickness keratinization. Keratinization of single cells extends throughout the whole thickness of the epithelium right down to the basal layer. The histology is masked by very prominent loss of epithelial cell cohesion.

Magnification, x 200; experimental animal at 16 weeks.

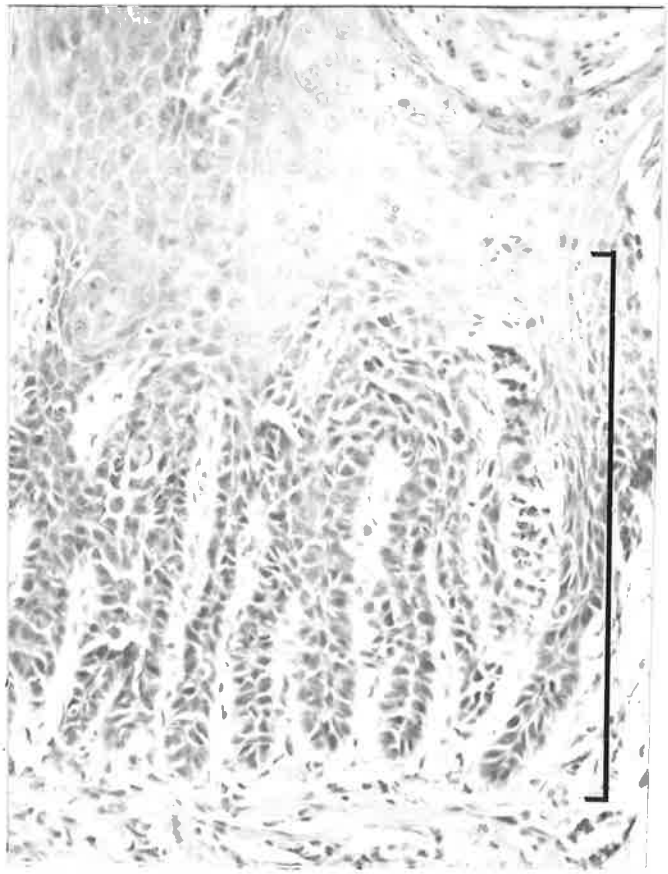


Figure 5.20: Moderate to severe dysplasia. The features of cellular atypia stand out prominently and involve two-thirds of the thickness of the cellular compartment (bracket).

Magnification, x 200; experimental animal at 24 weeks.

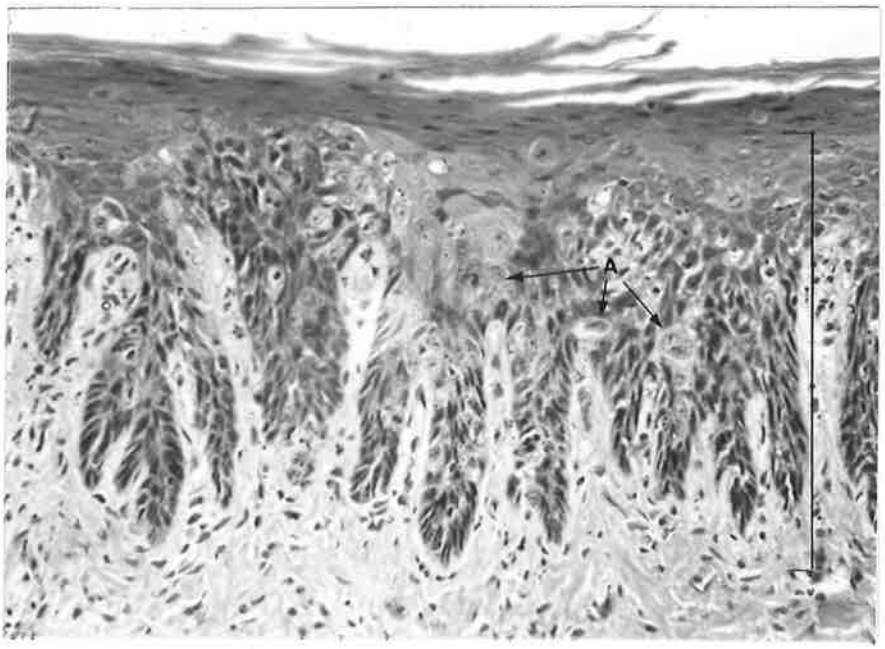


Figure 5.21: Moderate to severe dysplasia.

Features of cellular atypia extend to the upper level of the cellular compartment (bracket). There are severe degrees of cellular pleomorphism with the cells assuming widely different sizes and shapes (A) and variation in stainability of cells some of which appear paler. Reduction in cellular cohesion and irregular epithelial stratification are in evidence. Magnification, x 200; experimental animal at 16 weeks.

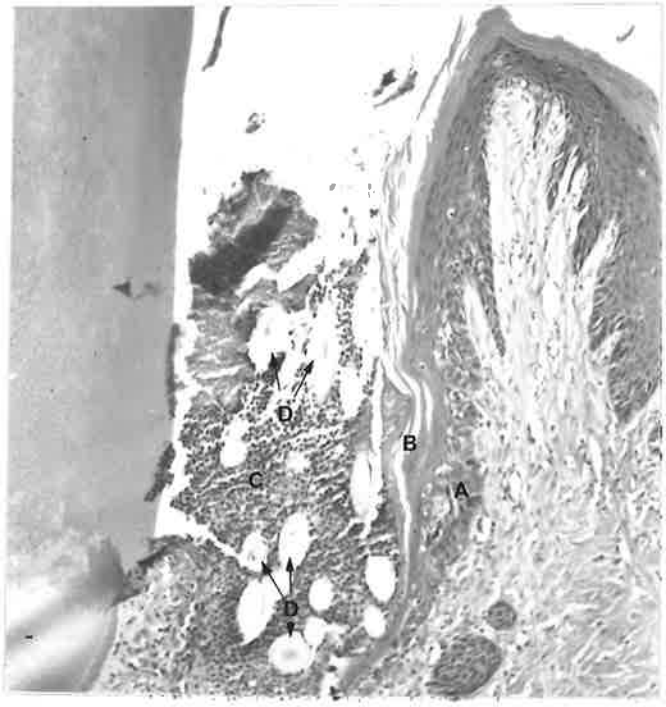


Figure 5.22: Early proliferation of sulcal epithelium and gingival pocket formation. The sulcal epithelium exhibits features of cellular atypia involving more than half of the cellular compartment (A) and associated with hyperorthokeratinization (B). The gingival pocket contains pustular materials consisting of degenerating polymorphs (C), and food fibres (D).

Magnification, x 100; experimental animal at 16 weeks.

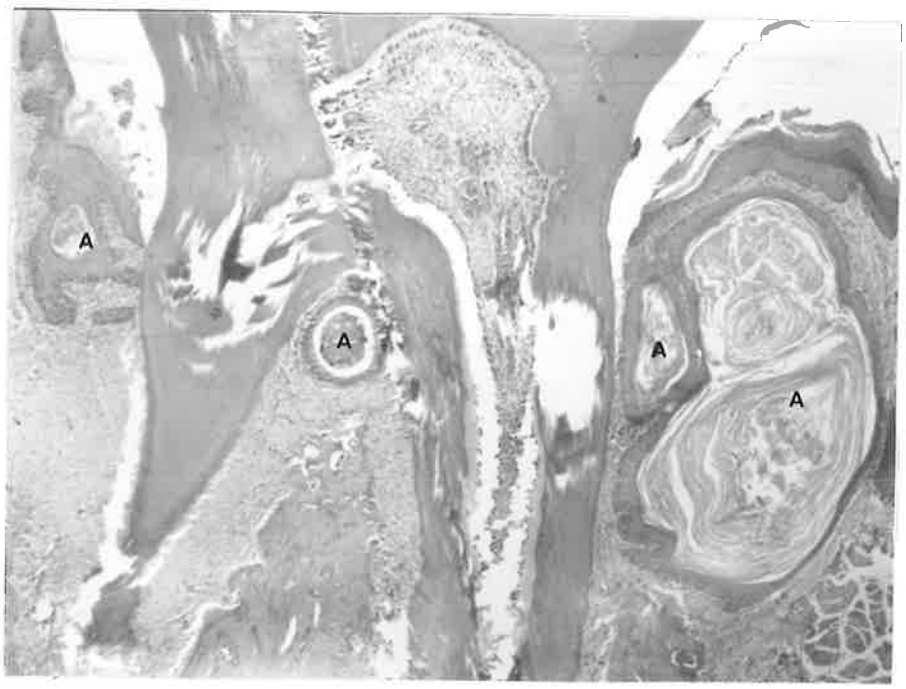
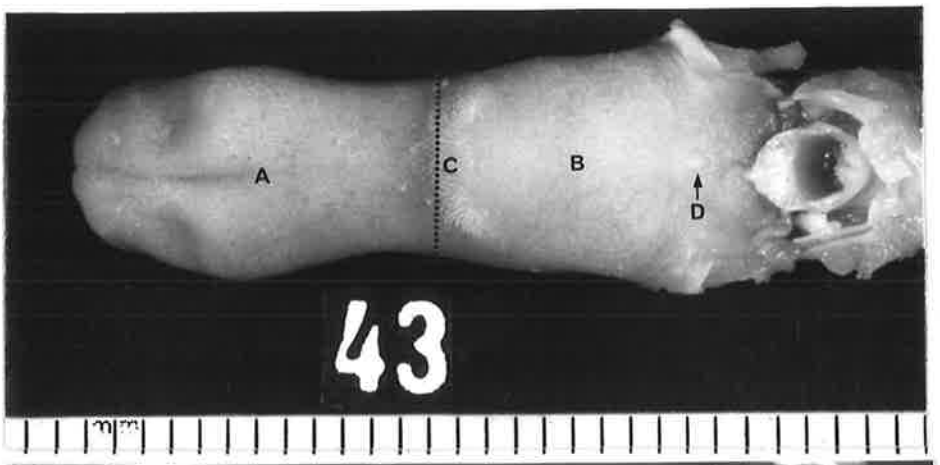


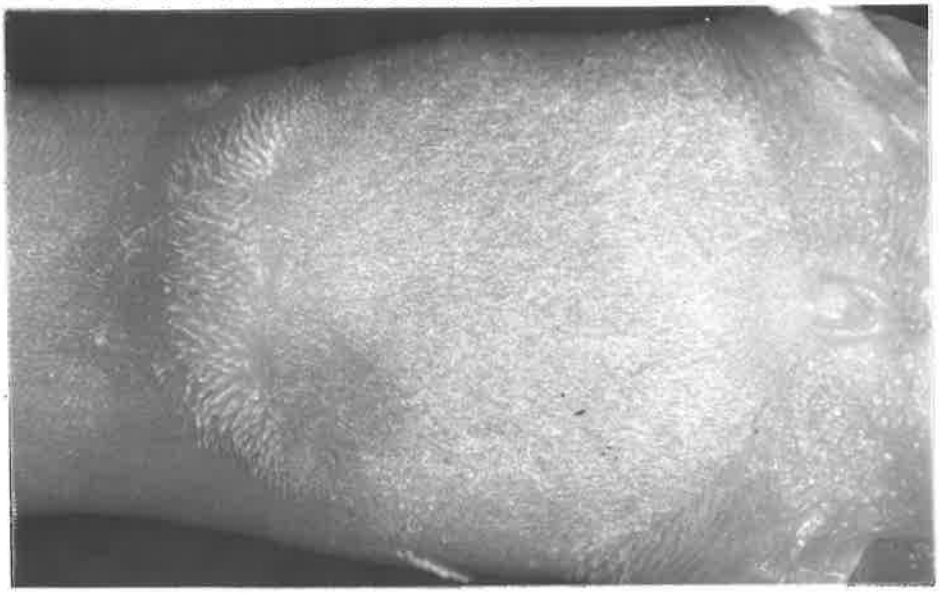
Figure 5.23: Keratocyst formation in the proliferative gingival epithelium. Very prominent keratocysts are observed (A).

Magnification, x 40; experimental animal at 24 weeks.

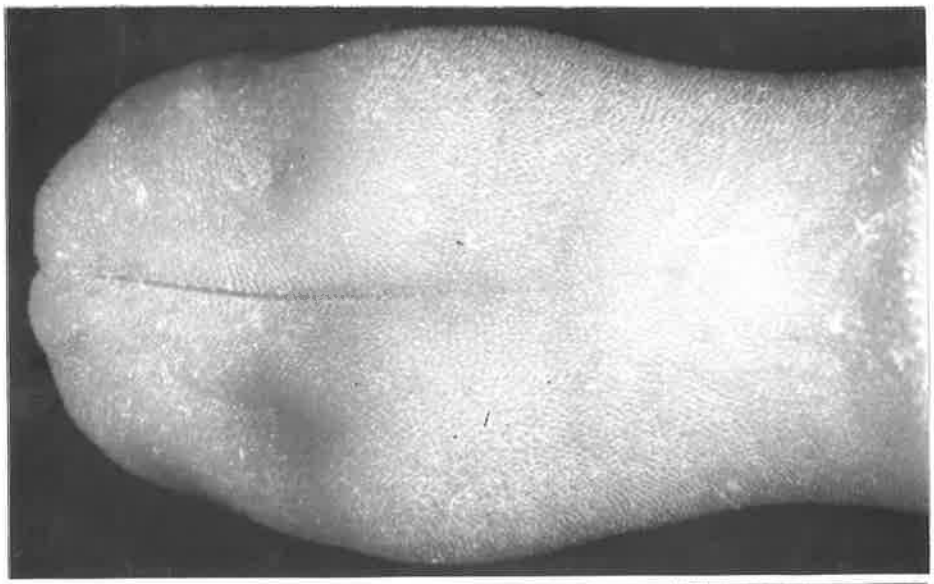
5.24 a.



b.



c.



d.

Figure 5.24: The morphology of a control tongue.

a. Dorsal view of the whole tongue. The crescentic pad of thick papillae (C) delineates the tongue into anterior (A) and posterior (B) tongue (dotted line). The foramen caecum (D) is situated at the posterior limit of the tongue.

b. Higher magnification of the posterior tongue.

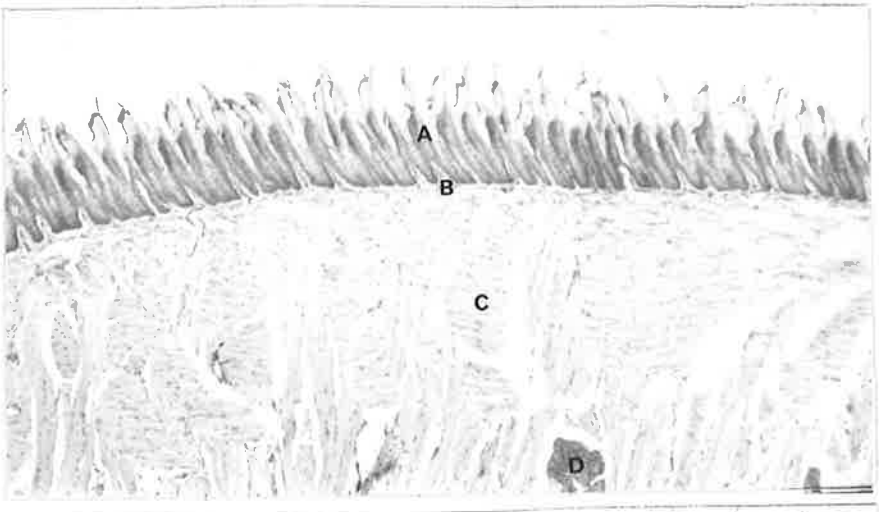
c. Higher magnification of the anterior tongue.

d. Lateral view of the normal tongue. It demonstrates the streamlined contour of the surface of the tongue.

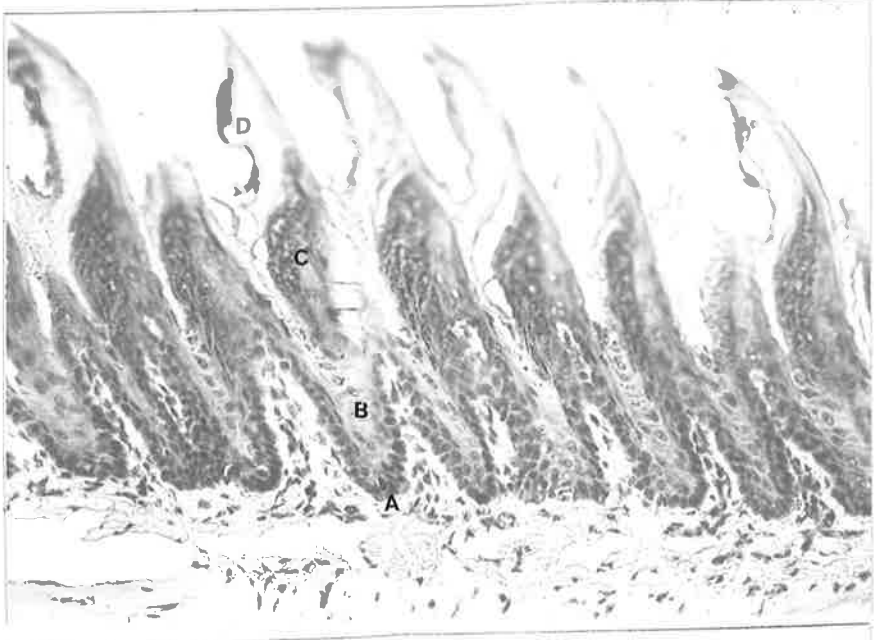
Magnification, a x 4, b x 9, c x 9, d x 5;

a,b,c, untreated control at 12 weeks; d,treated control at 16 weeks.

5.25 a.



b.



c.

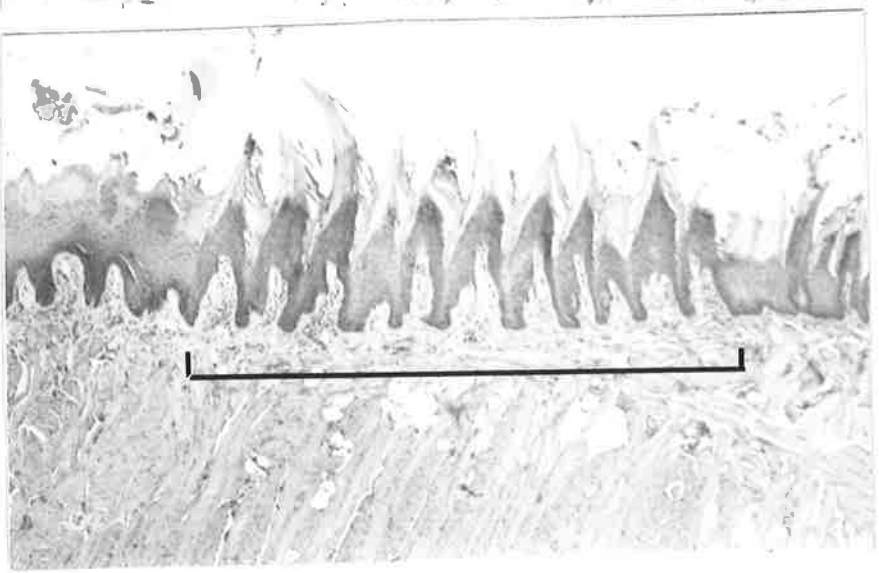


Figure 5.25: Histology of a control posterior tongue.

a. Microscopic architecture shows an array of lingual papillae(A), fibrous connective tissues (B), muscle fibres (C) and salivary gland tissues (D).

b. Higher magnification of the posterior tongue showing basal cells(A), stratified squamous cells (B), granular cell layer (C) and orthokeratin layer (D).

c. Histology of the crescentic pad of hypertrophied lingual papillae (bracketed area).

Magnification, a x 40, b x 200,c x 40; treated control at 4 weeks.

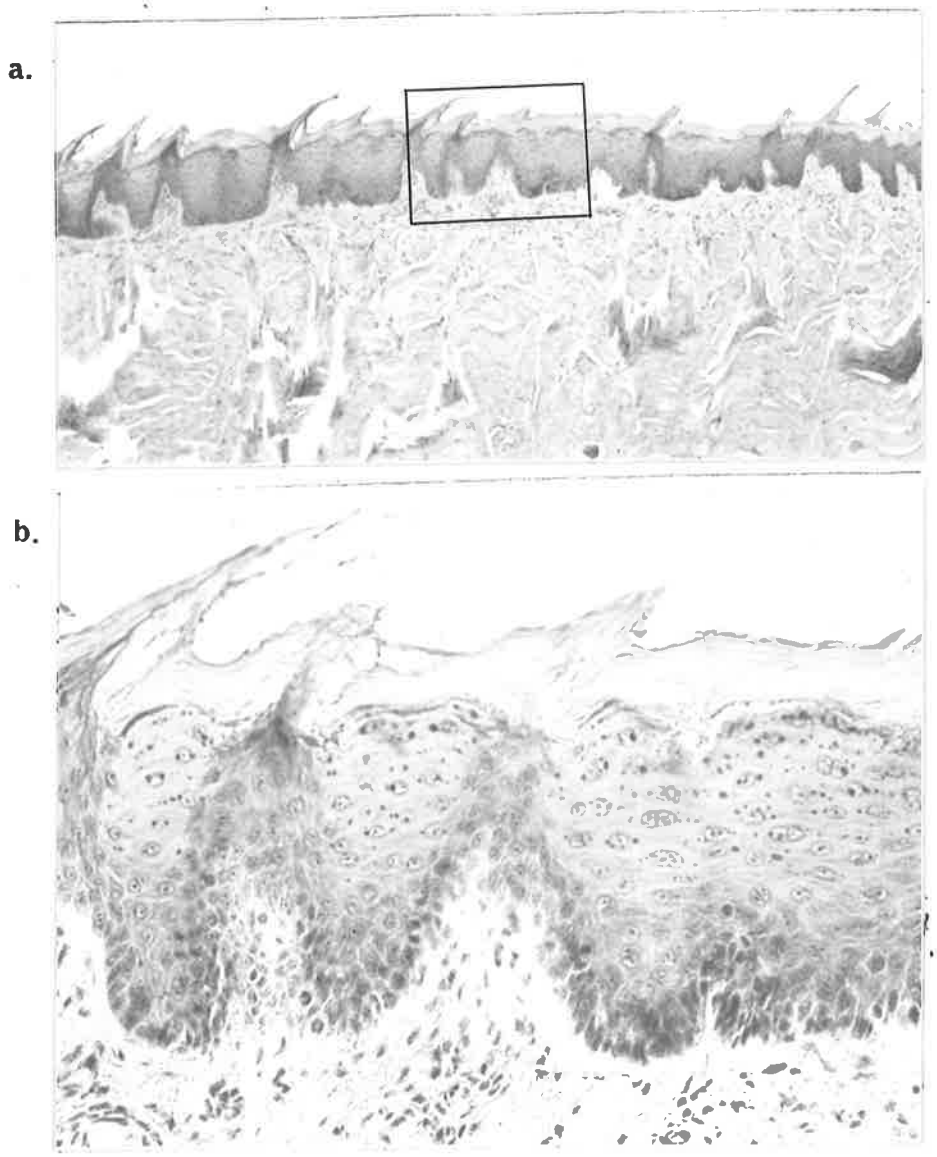


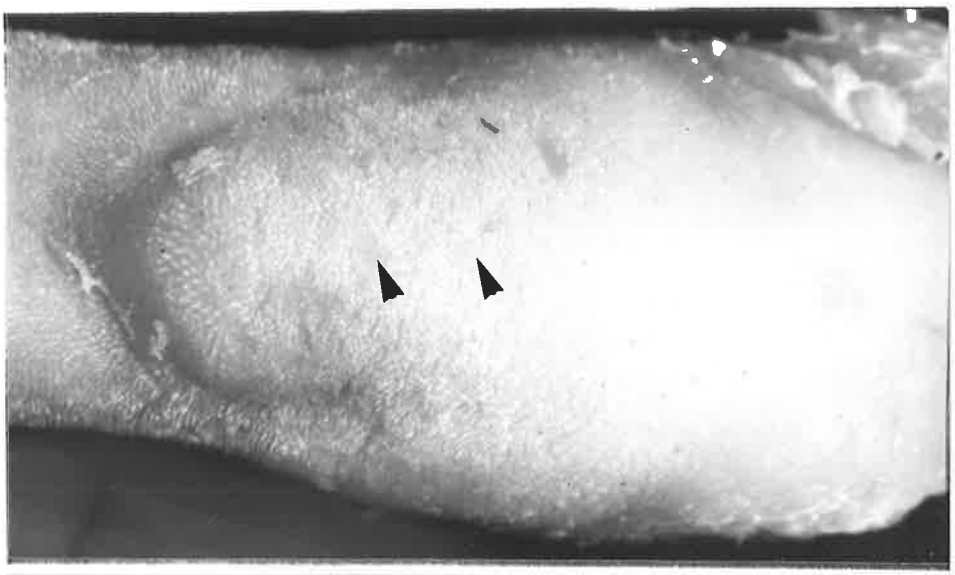
Figure 5.26: Histology of a control anterior tongue.

a. Architectural view.

b. Higher magnification of the anterior tongue (framed area in a). The broad papillæ show very regular stratification of the squamous cells.

Magnification, a x 40, b x 200; treated control at 4 weeks.

a.



b.

Figure 5.27: Mild surface thickening of the posterior tongue.

a. There are early, small plaques presenting as areas denuded of papillary projections (arrows). Though thickening was observed at 4 and 8 weeks, plaques did not appear until 8 weeks.

b. Lateral view of the same tongue demonstrating the elongated and thickened lingual papillae on the posterior tongue surface.

Magnification, a, b x 9; experimental animal at 8 weeks.

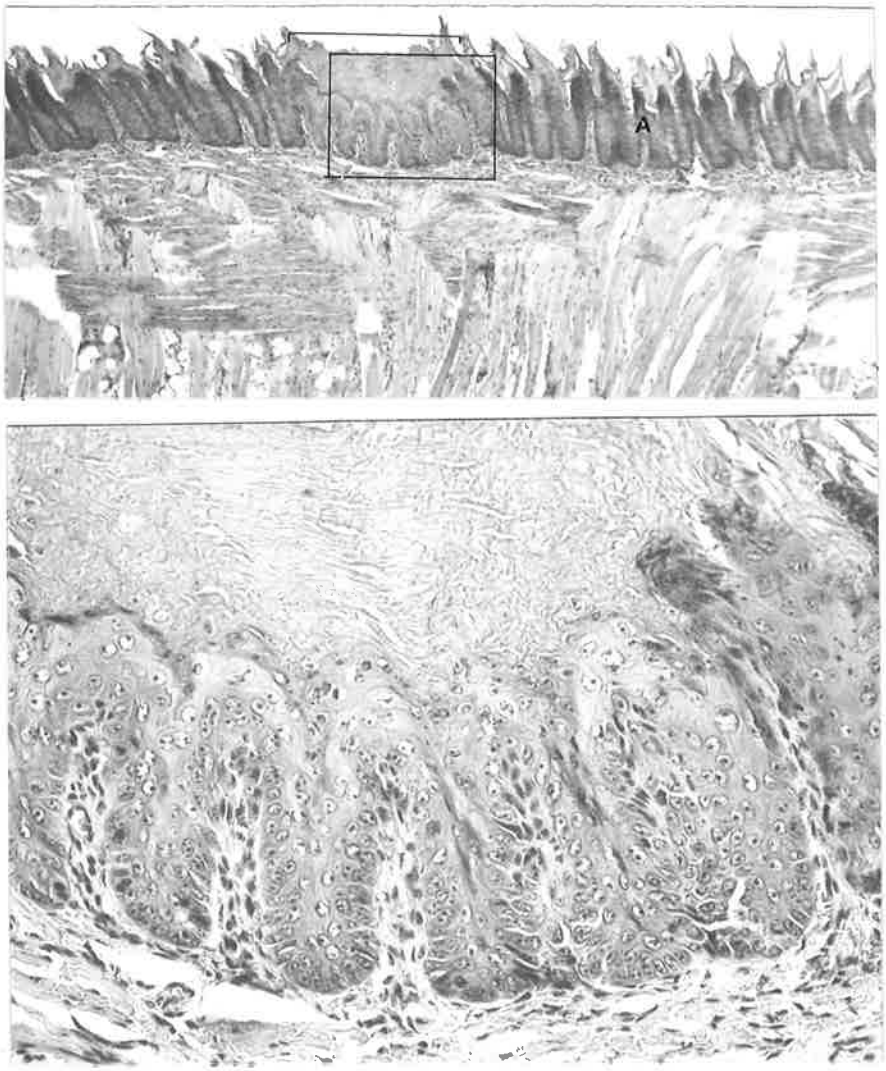


Figure 5.28: Mild hyperkeratosis and hyperplasia and plaque. Tongue.

a. The lingual papillae(A) and orthokeratin layer are thickened and elongated. In the middle (bracket) a group of papillae are replaced by a plaque. Hyperplasia and hyperkeratosis were observed at 4 weeks but the plaque did not appear until at 8 weeks. The corresponding macroscopic appearance of a plaque is shown in Figure 5.27.

b. Higher magnification of the plaque (framed area in a). The atypical cellular features are uniform enlargement of the nuclei and nucleoli. Though larger plaques appeared at later time intervals, they showed essentially the same cellular features.

Magnification, a, b x 200; experimental animal at 8 weeks.

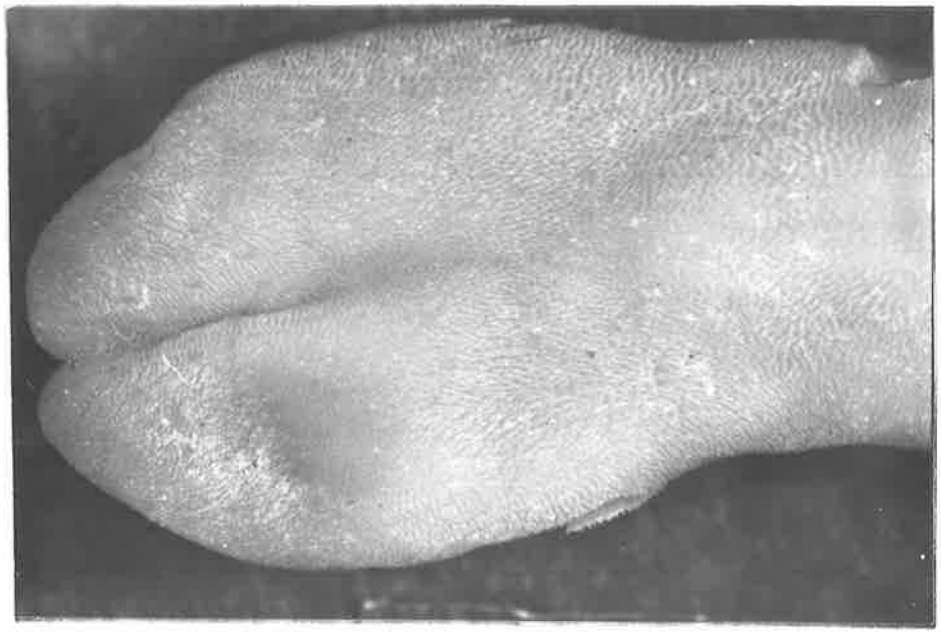


Figure 5.29: Mild thickening of the anterior tongue papillae. These changes were exhibited by the anterior tongue at all time intervals throughout the experimental period .

Magnification, x 5; experimental animal at 4 weeks.

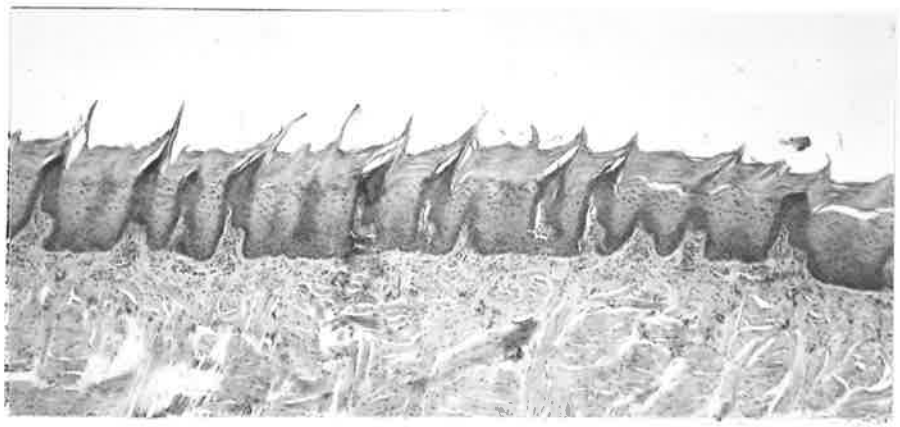


Figure 5.30: Mild hyperkeratosis and hyperplasia of anterior tongue. The orthokeratin layer and the lingual papillae are thickened compared to those of the control tongue. The same degrees of changes were maintained at different time intervals throughout the experimental period. The corresponding macroscopic appearance is shown in Figure 5.29.

Magnification, x 40 ; experimental animal at 4 weeks.

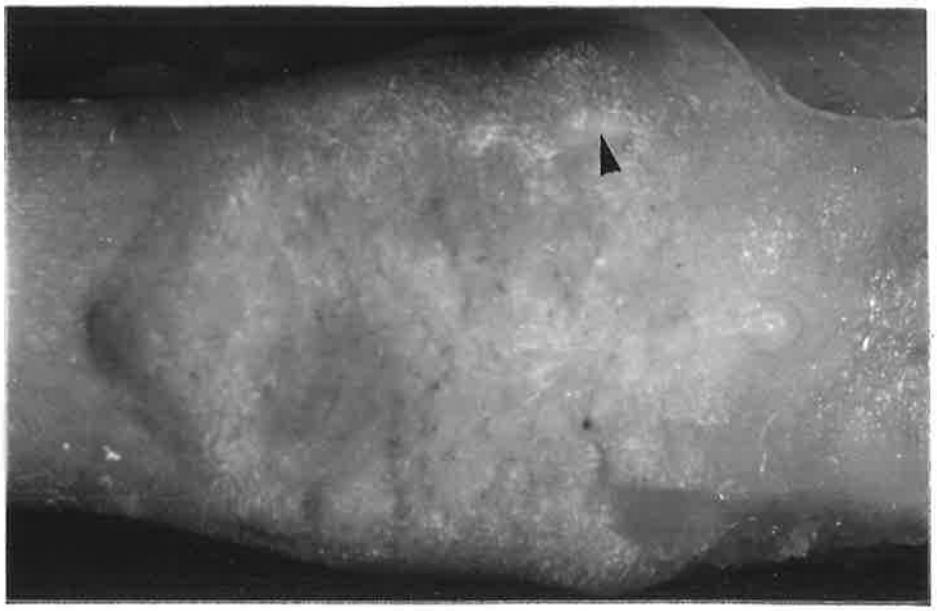


Figure 5.31: Mild surface thickening. Tongue.

At 12 weeks the posterior tongue exhibited more progressive thickening compared to earlier periods. Arrow indicates a large plaque.

Magnification, x 9; experimental animal at 12 weeks.

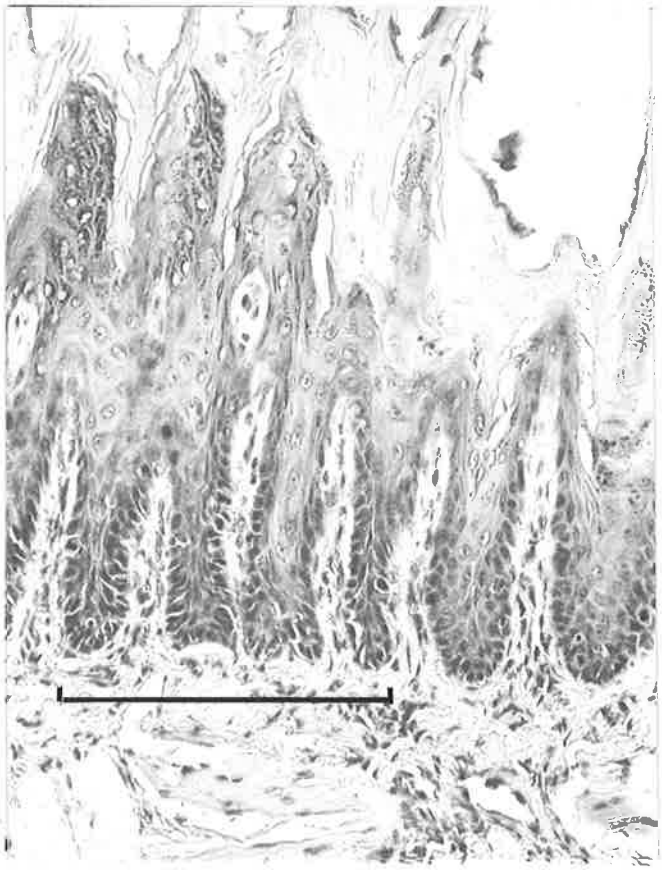


Figure 5.32: Mild dysplasia. Tongue.

There are mild degrees of cellular pleomorphism and reduction in cellular cohesion confined to the basal area of the epithelium (bracket). There are also hyperorthokeratinization and hyperplasia of the lingual papillae. Such changes were exhibited initially at 8 and 12 weeks.

Magnification, x 200; experimental animal at 8 weeks.

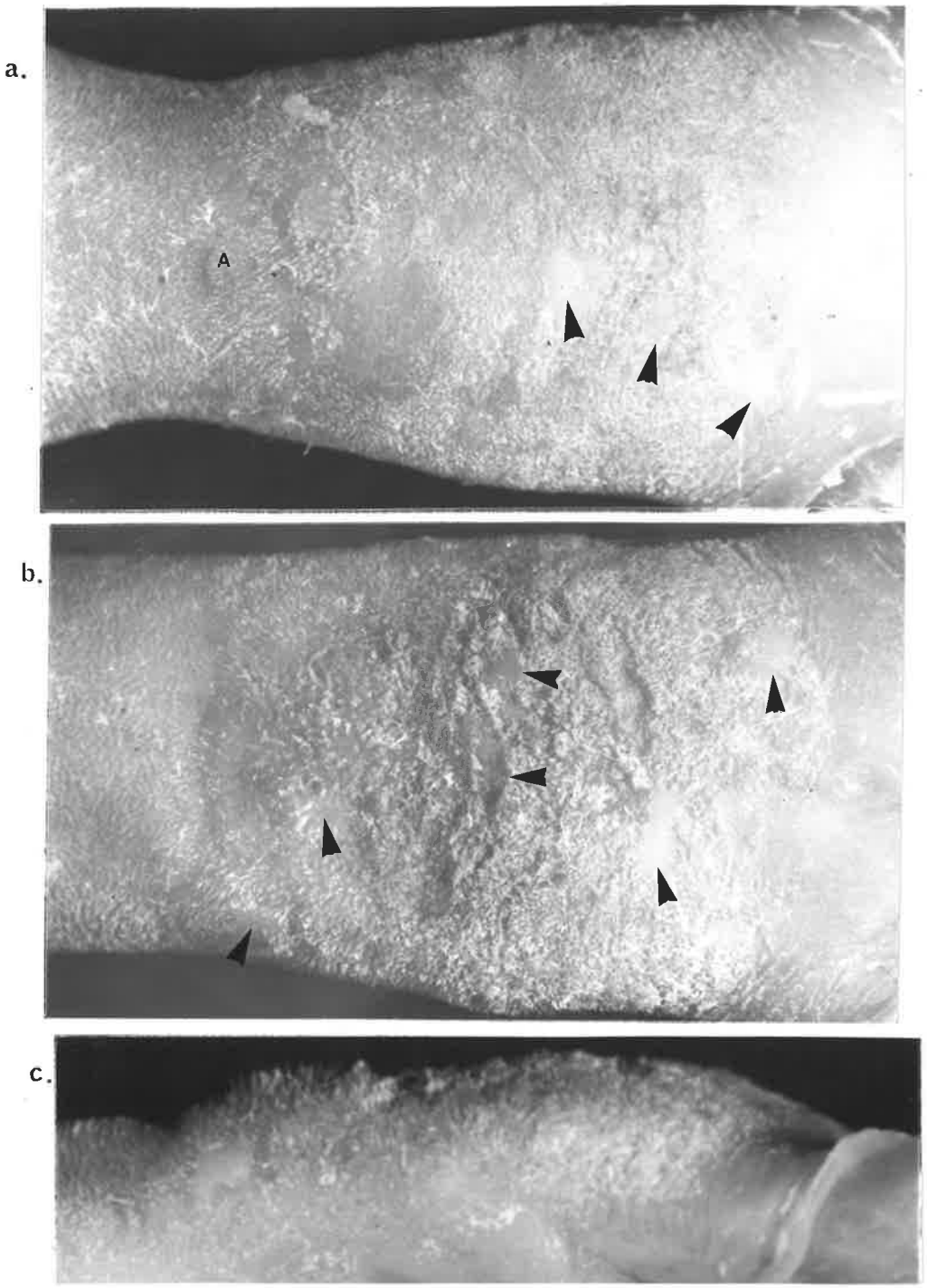


Figure 5.33: Severe surface thickening of the posterior tongue.

a. At 16 weeks, further surface thickening of the posterior tongue resulted in the disappearance of the crescentic pad of papillae (A). More plaques appeared (arrows).

b. This represents the severe surface thickening of the posterior tongue commonly seen at 20 and 24 weeks. The changes are more exaggerated in that the tongue appears patchy and exhibits numerous plaques (arrows).

c. Side view of the posterior tongue illustrating the irregular surface contour. Magnification, a,b,c x 9; experimental animals at 16 weeks (a) and 24 weeks(b,c).

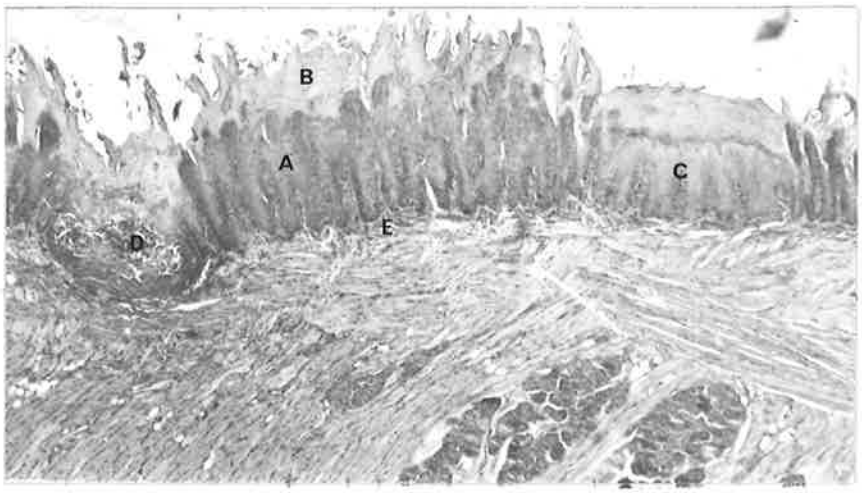


Figure 5.34: Severe hyperkeratosis and hyperplasia and plaque. Tongue. Changes are hyperplasia of lingual papillae (A), hyperorthokeratosis (B), plaque (C), full thickness keratinization (D) and mild chronic inflammation (E). Such changes were commonly observed from 16 weeks onwards. The corresponding macroscopic appearances are shown in figure 5.33. Magnification, x 40; experimental animal at 24 weeks.

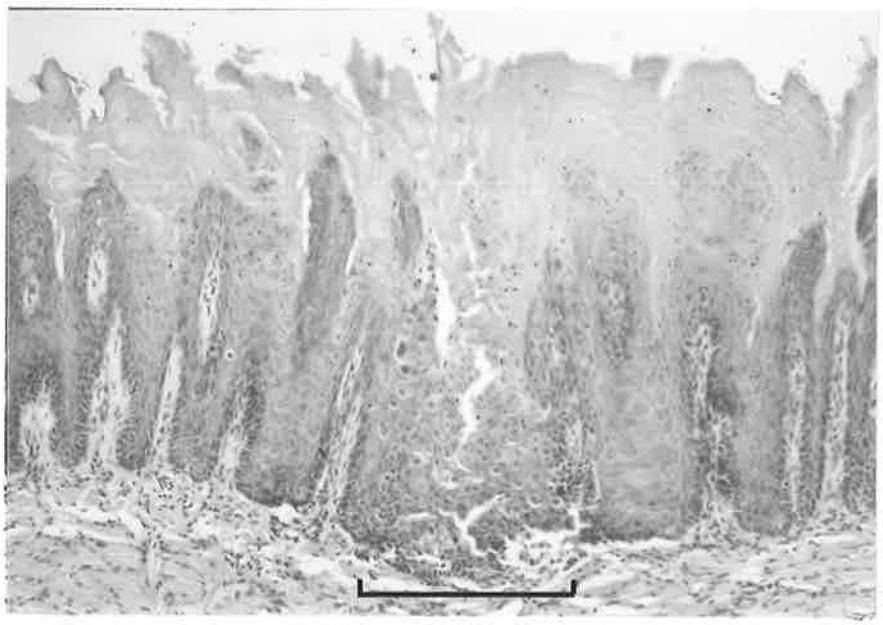
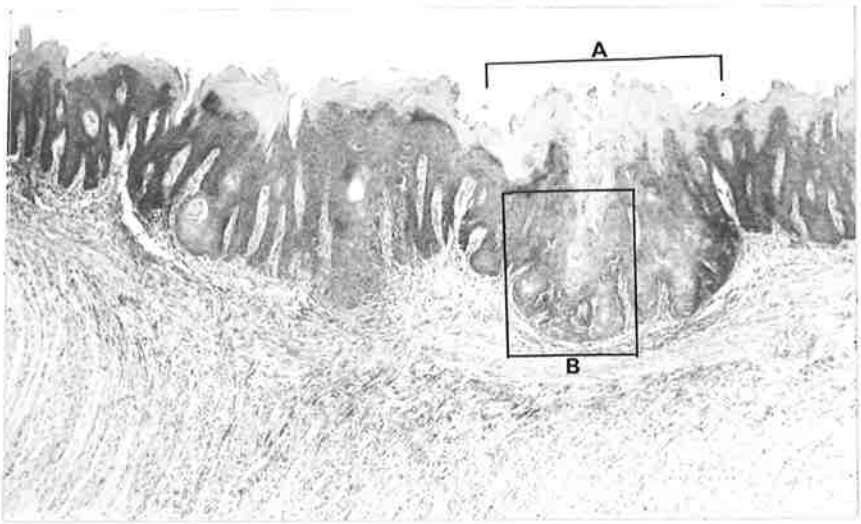
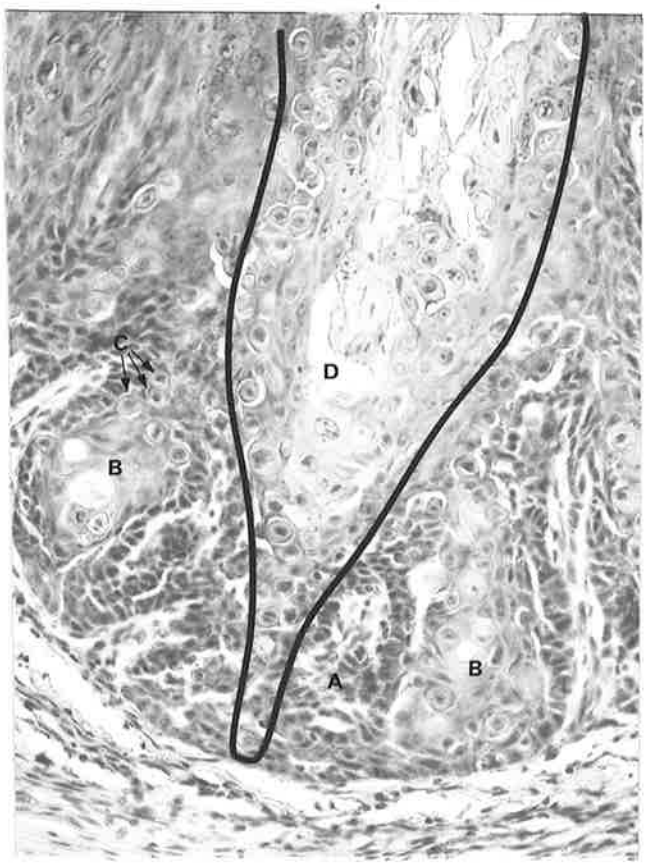


Figure 5.35: Full thickness keratinization (bracket). Tongue. The keratinized cells still retain their pyknotic nuclei. Magnification, x 100; experimental animal at 20 weeks.

5.36 a



b.



c.

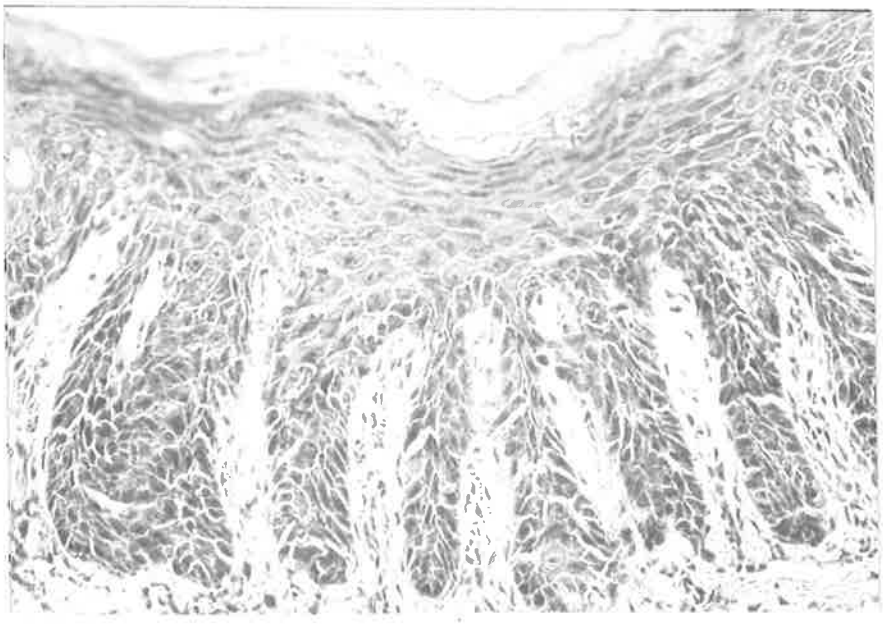


Figure 5.36: Moderate to severe dysplasia. Tongue.

a. Against a background of hyperplasia and hyperorthokeratosis, a focal area of epithelial dysplasia (A) has dropped below the level of adjacent epithelium and incited marked subepithelial fibrosis and chronic inflammation (B).The lesion is too small to predict its outcome.

b. Higher magnification of the epithelial dysplasia (framed area in a). Features of cellular atypia include loss of epithelial stratification, cellular pleomorphism and reduction in cell cohesion (A),keratinization of cell groups (B) and single cells (C). A wedge of full thickness keratinization (D and solid line) can be traced amongst the area of epithelial dysplasia.

c. This illustrates another pattern of severe dysplasia. The prominent features of cellular atypia involve two-thirds of the epithelial thickness. The normal morphology of the lingual papillae is lost and is replaced by epithelial rete pegs.

Magnification, a x 40, b,c x 200; a,b,experimental animal at 20 weeks; c, experimental animal at 16 weeks.

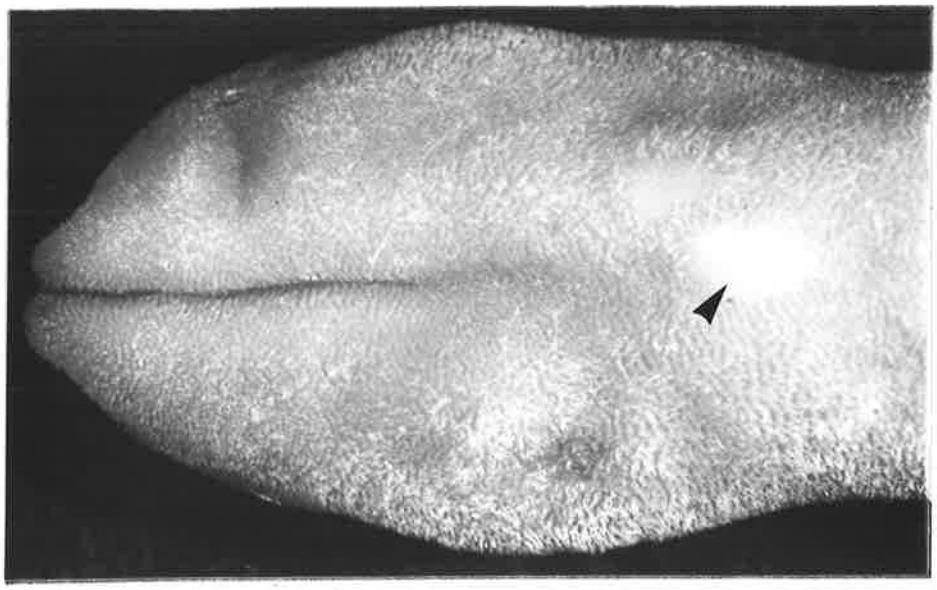


Figure 5.37: Mild thickening and a large plaque (arrow) of the anterior tongue.

Magnification, x 9; experimental animal at 24 weeks.

Figure 5.38: Histology of a large plaque on the anterior tongue. The cellular features are essentially normal.

Magnification, x 40 ; experimental animal at 24 weeks.

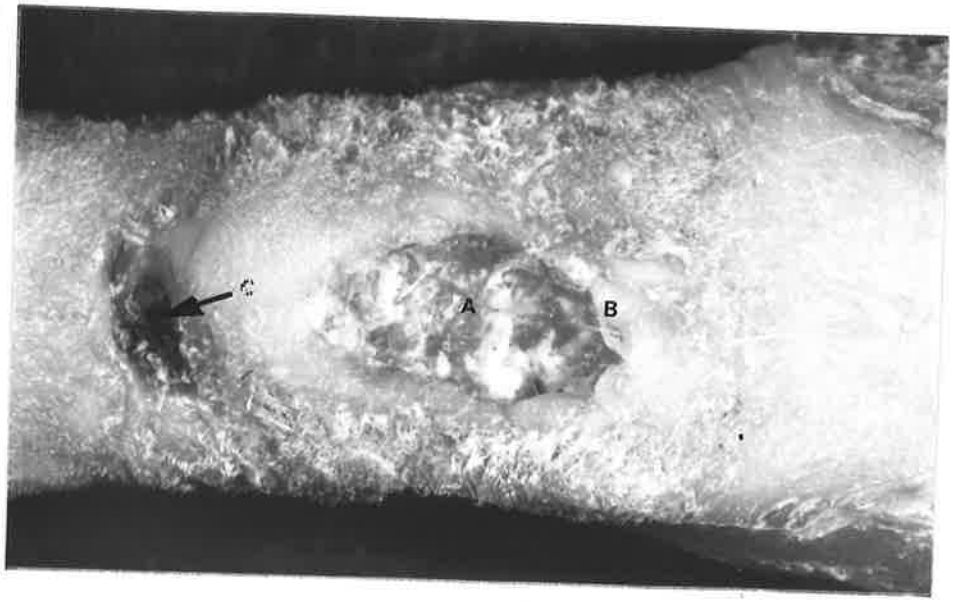
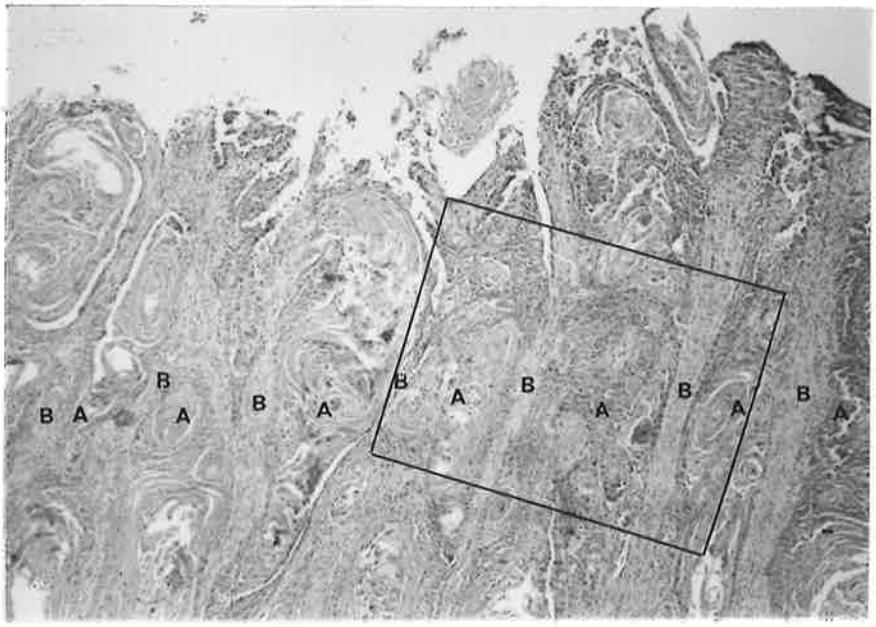
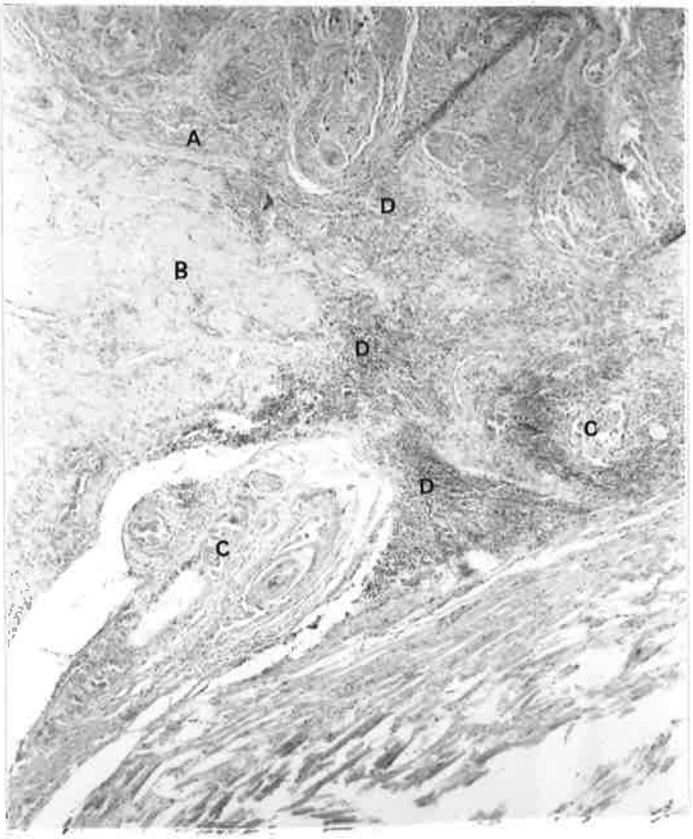


Figure 5.39: Carcinomas of the posterior tongue. In the centre, there is a big crateriform ulcer having an indurated base (A) and an elevated, rolled-in border (B) that are characteristic of an infiltrative carcinoma. The smaller deep ulcer in front (C) is less characteristic but still highly suggestive of a penetrating malignancy. Magnification, x 9; experimental animal at 24 weeks.

5.40 a.



b.



c.

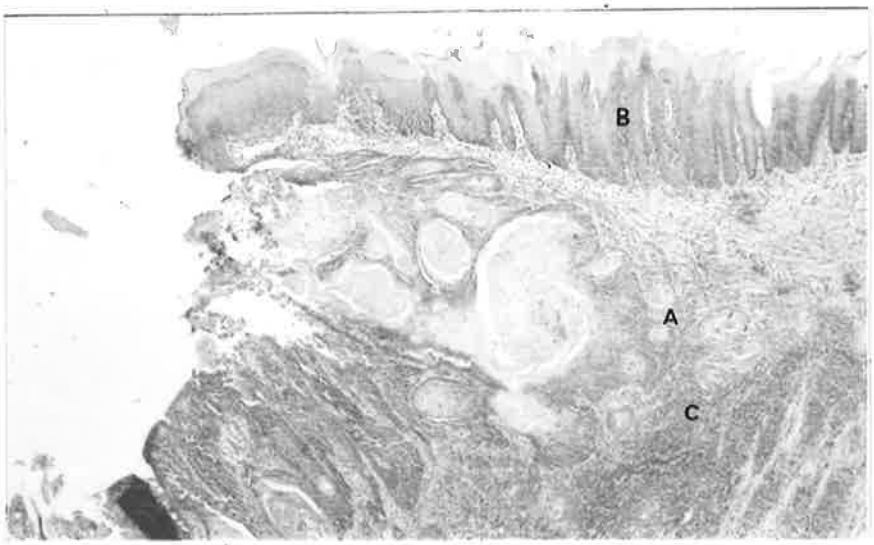


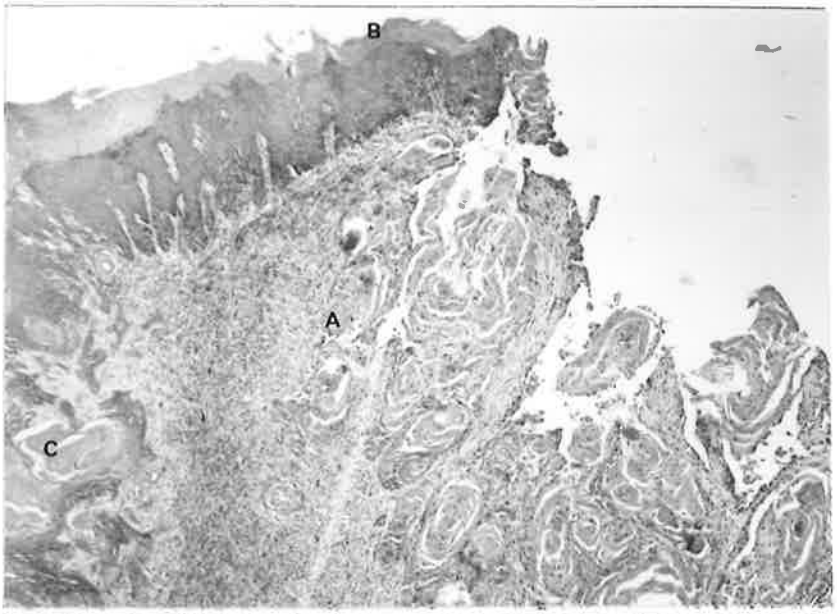
Figure 5.40: Carcinomas of posterior tongue.

a. The centre of the tumour consists of infiltrating columns of well differentiated epithelial cells (A) separated by narrow strips of fibrous tissues(B).

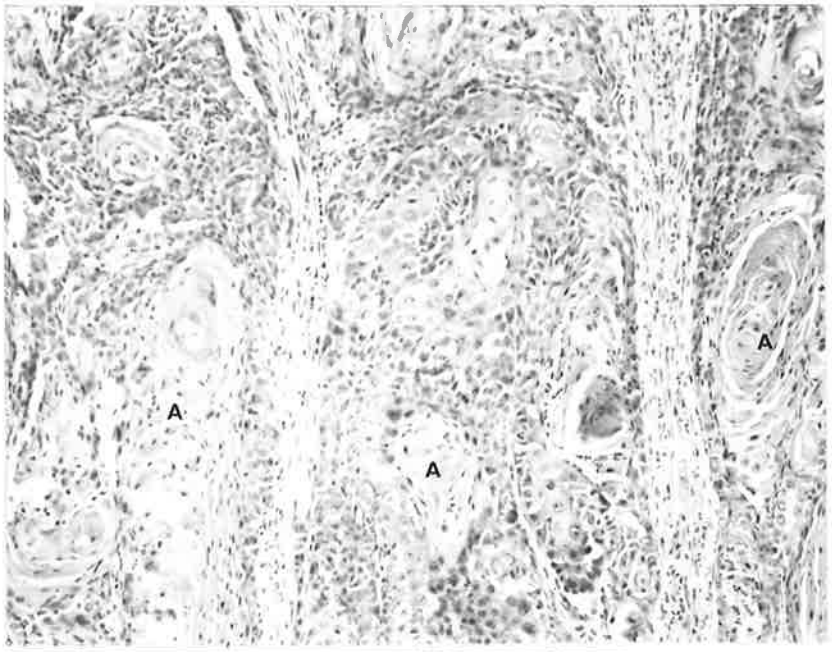
b. The invasive front of the tumour (A) has penetrated very deep into the tongue and encroached upon a large nerve (B). Some epithelial cell masses (C)have passed beyond the nerve and are situated in the ventral side of the tongue. A mononuclear cell infiltrate is present in the stromal connective tissues (D).

c. The posterior border of the tumour (A) has undermined the adjacent surface epithelium which shows marked hyperkeratosis and hyperplasia (B). It has also invaded the salivary gland tissues situated posteriorly (C).

5.40 d.



e.



f.

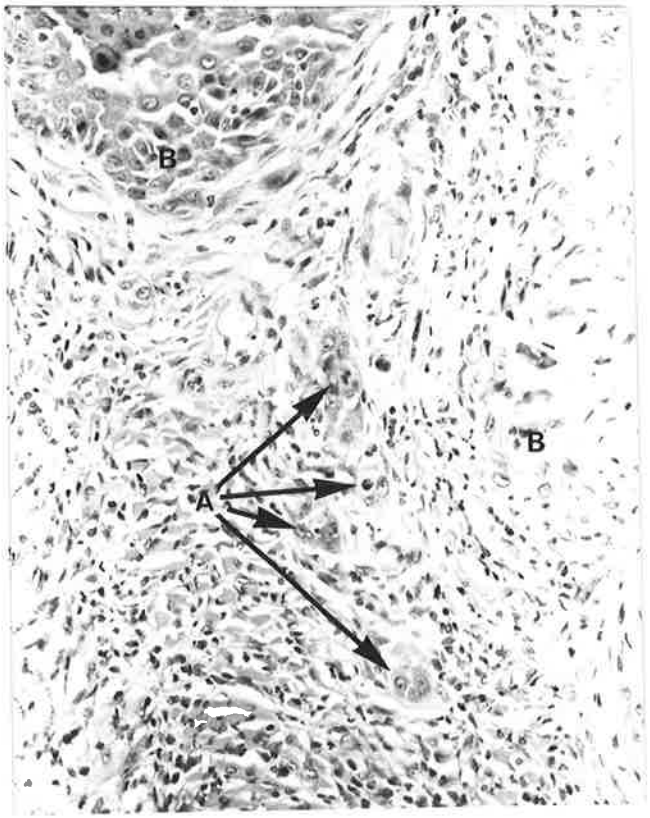


Figure 5.40: (cont.)

d. The anterior border (A) of the tumour also shows undermining of the surface epithelium producing a markedly elevated surface contour (B). There is another carcinoma (C) anterior to the main lesion.

e. Higher magnification of the carcinoma (framed area in a) showing well differentiated squamous epithelium producing abundant amounts of parakeratin (A). Though the carcinoma is considered well differentiated on account of its keratin forming capacity, there are features of cellular atypia, especially at the periphery of the epithelial columns.

f. Higher magnification of the invasive front of the carcinoma demonstrating isolated small islands of epithelial cells (A) in front of and adjacent to the solid epithelial columns (B), infiltrating the connective tissues. Magnification, a,b,c,d x 40, e x 100, f x 200; experimental animal at 24 weeks.

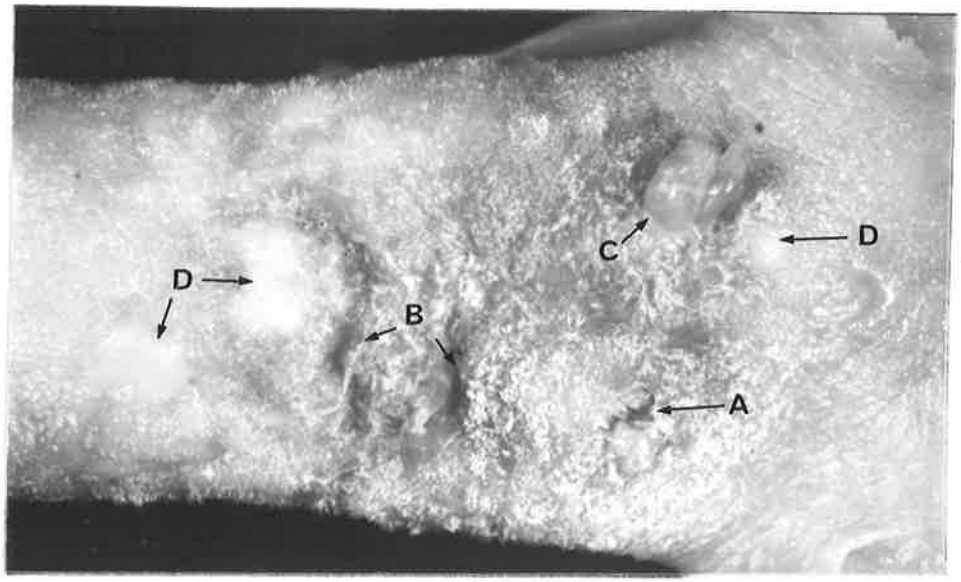
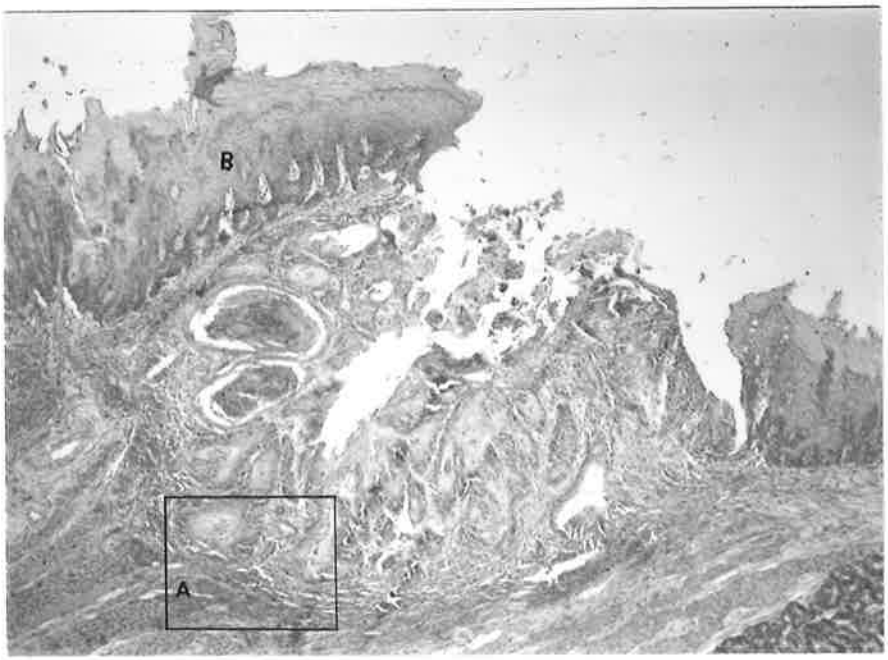


Figure 5.41: Severe surface thickening of the posterior tongue. This demonstrates the very unremarkable surface ulcer/opening (A) which when probed was detected to extend deep into the tongue. Histologically, it was a carcinoma. There are two deep transverse grooves (B) apparently produced by irregularities of the tongue surface. Finger-like processes are identified as papilloma (C). Large plaques are present (D).
Magnification, x 9 ; experimental animal at 24 weeks.

5.42 a



(For legend see opposite page.)

b.

c.

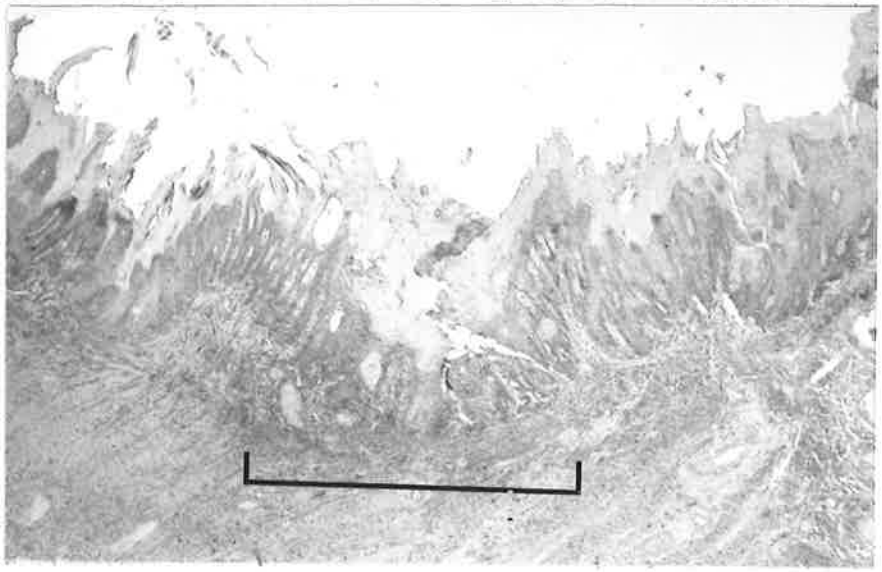


Figure 5.42: Infiltrative carcinomas of the posterior tongue.

a. This is the carcinoma with the grossly insidious appearance shown in Figure 5.41 (A). Microscopically, it has already advanced deep into the salivary gland tissues (A) and undermined the hyperplastic surface epithelium (B).

b. High power view of the carcinoma showing the invasive front destroying salivary gland tissues (A). In between two invading processes (B) are seen remnants of the ductal elements of the salivary gland tissues (C).

c. A very early carcinoma (bracket) was found anterior to the larger carcinoma .

Magnification, a x 40, b x 200, c x 40; experimental animal at 24 weeks.

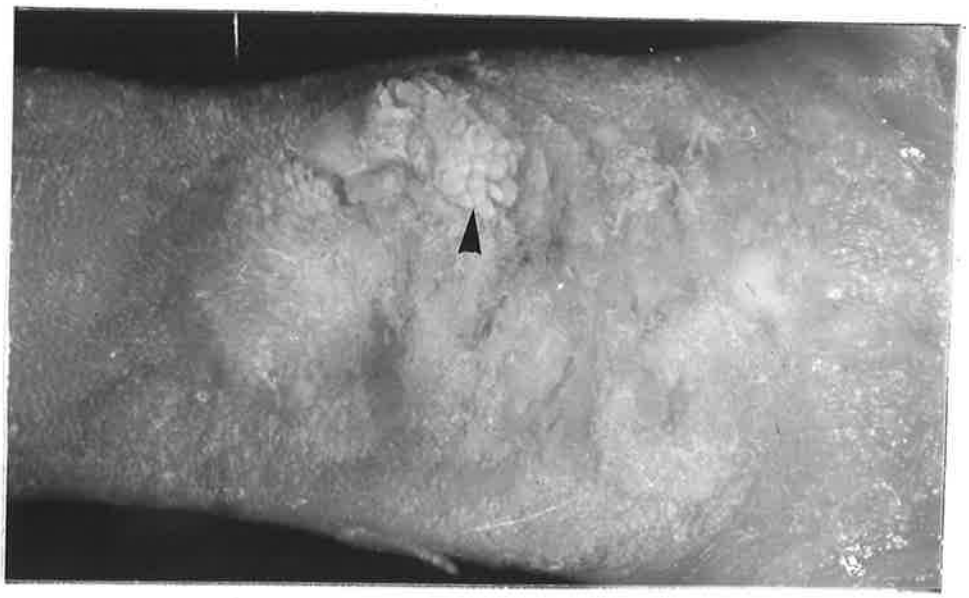
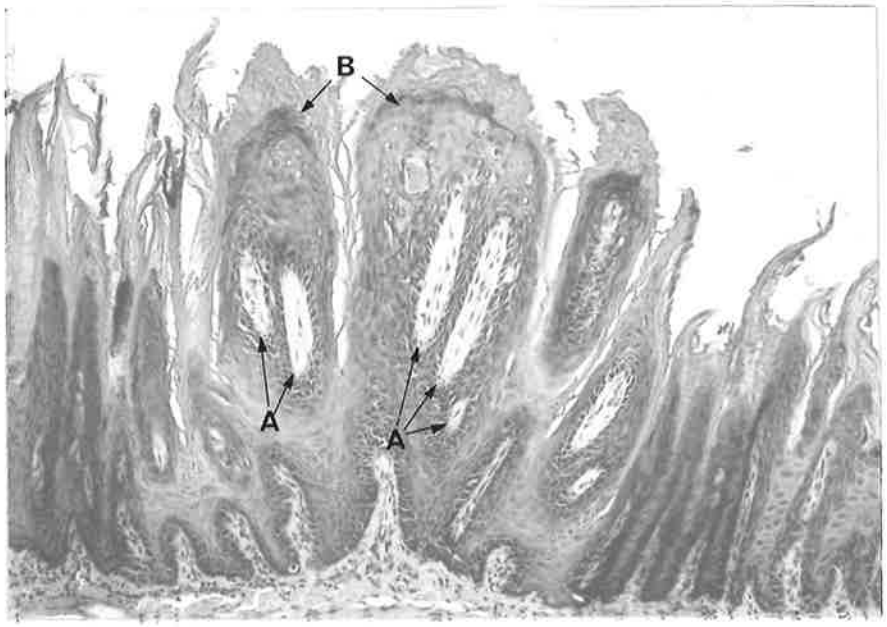


Figure 5.43: Papilloma.

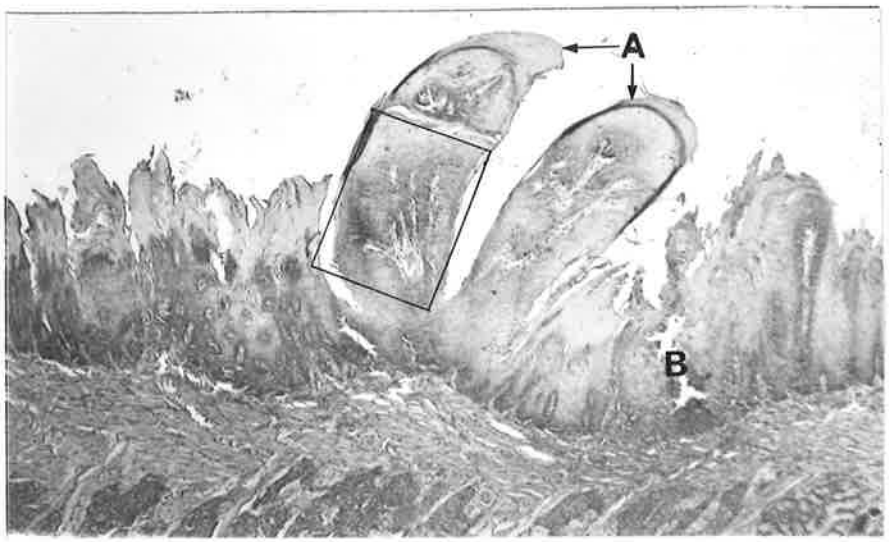
This is to illustrate another pattern of papilloma (arrow) observed on the posterior tongue. It consists of a collection of short exophytic processes on a sessile base.

Magnification, x 9; experimental animal at 20 weeks.

5.44 a.



b.



c.

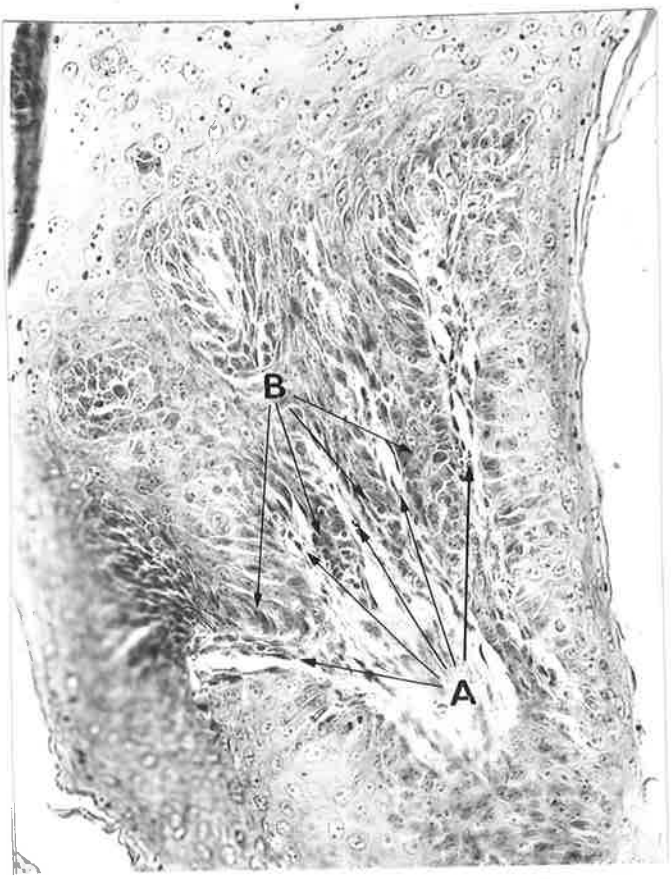


Figure 5.44: Papillomas. Tongue.

a. This illustrates the histologic features of the papilloma shown in Figure 5.43. An exophytic process containing more than one core of lamina propria (A) implies that more than one papillary process has fused to form an exophytic process (B). No features of cellular atypia are observed although an oblique cut in the basal area gives a false impression of atypia.

b. Histology of the papilloma shown in Figure 5.41. There are two outstanding exophytic processes (A). There is also an adjacent focal area of full thickness keratinization (B).

c. Higher magnification of the framed area in b shows multiple cores of lamina propria (A) implying fusion of many papillary processes. There are features of mild cellular atypia in the epithelial processes (B).

Magnification, a x 100, b x 40,c x 200; a , experimental animal at 20 weeks; b,c, experimental animal at 24 weeks.

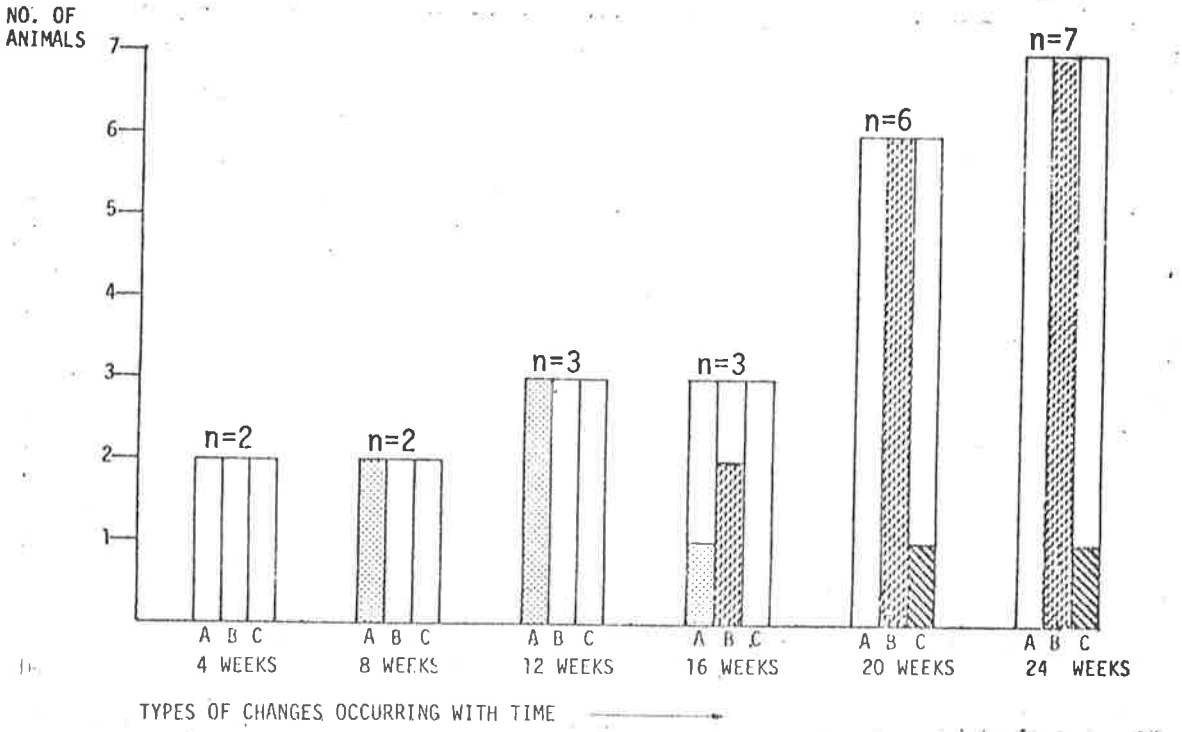


Figure 5.45: Macroscopic changes on the middle palate of 4NQO-treated animals. A - Mild mucosal thickening; B - Severe mucosal thickening; C - Exophytic growth.

For Figures 5.45 - 5.53:

The hatched portion of a bar represents the number of animals exhibiting the specified change.

The clear portion of a bar represents the number of animals without the change.

The whole bar represents the total number of animals sacrificed.

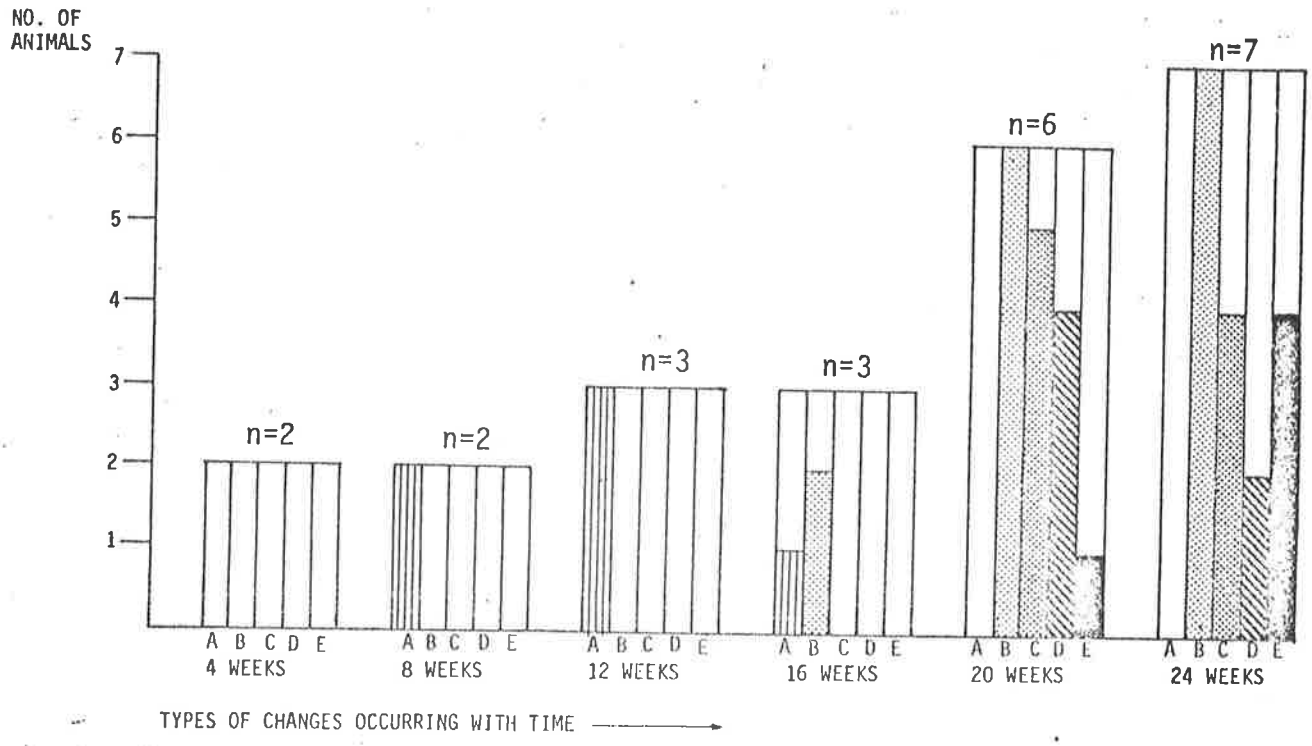


Figure 5.46: Macroscopic changes in the palatal gingival area of 4NQO-treated animals. A - Mild mucosal thickening; B - Severe mucosal thickening; C - Exophytic growth; D - Deep gingival pocket; and E - Carcinoma.

Table 5.1: Distribution of macroscopic palatal gingival changes in 4NQO-treated animals according to side.

Macroscopic Features		Duration in Weeks 4 n=2	8 n=2	12 n=3	16 n=3	20 n=6	24 n=7
Mild Mucosal Thickening	GL		2	3	1		
	GR		2	3	1		
	G		2	3	1		
Severe Mucosal Thickening	GL				2	6	7
	GR				2	6	7
	G				2	6	7
Exophytic Growth	GL					2	1
	GR				1	4	4
	G				1	5	4
Deep Gingival Pocket	GL					4	2
	GR						2
	G					4	2
Carcinoma	GL					1	4
	GR					1	4
	G					1	7

For Tables 5.1 - 5.7:

The fractional figure represents

$$\frac{\text{Number of animals sacrificed exhibiting a change}}{\text{Total number of animals sacrificed (n)}}$$

at the end of a specified time interval.

The integral figure represents the number of animals sacrificed exhibiting a change at the end of a specified time interval.

A - Anterior palate

M - Middle palate

P - Posterior palate

GL - Left gingival area

GR - Right gingival area

G - Gingival area (either left, right or bilateral)

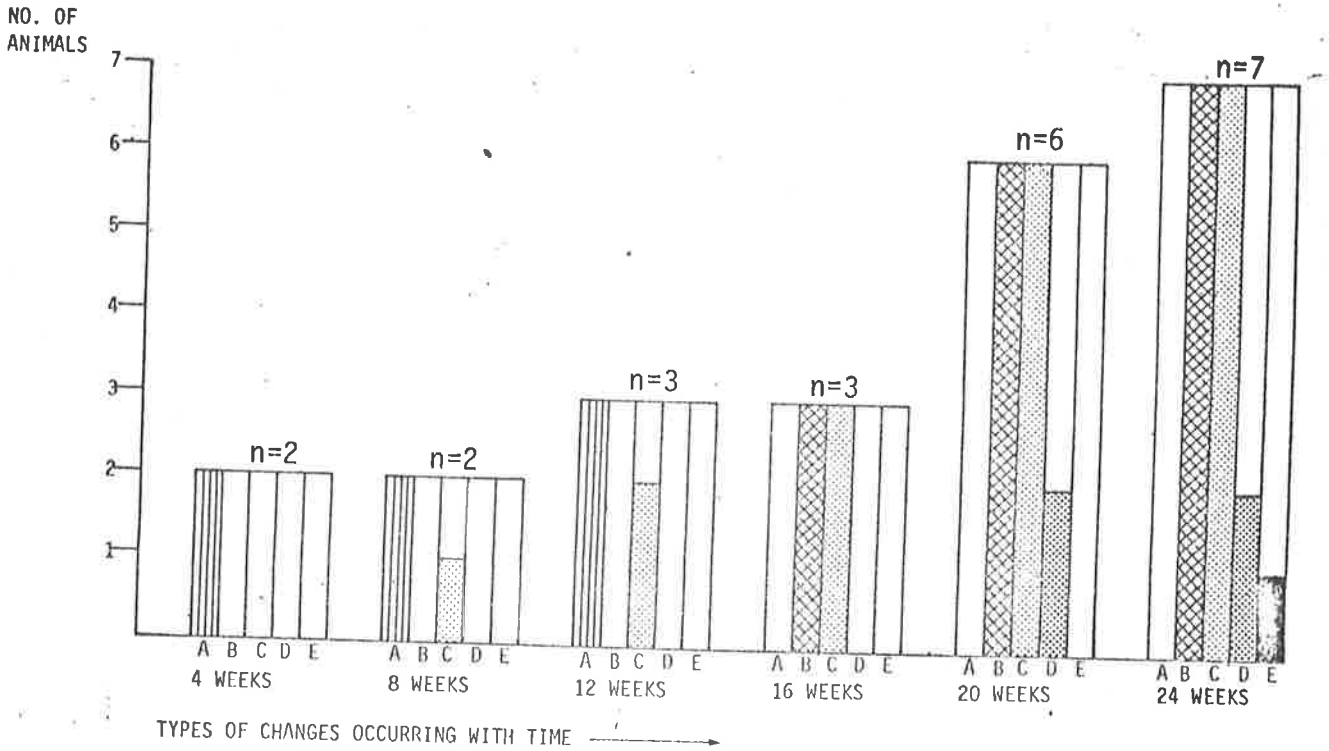


Figure 5.47: Macroscopic changes on the posterior tongue of 4NQO-treated animals. A - Mild mucosal thickening; B - Severe mucosal thickening; C - Plaque; D - Exophytic growth; and E - Carcinoma.

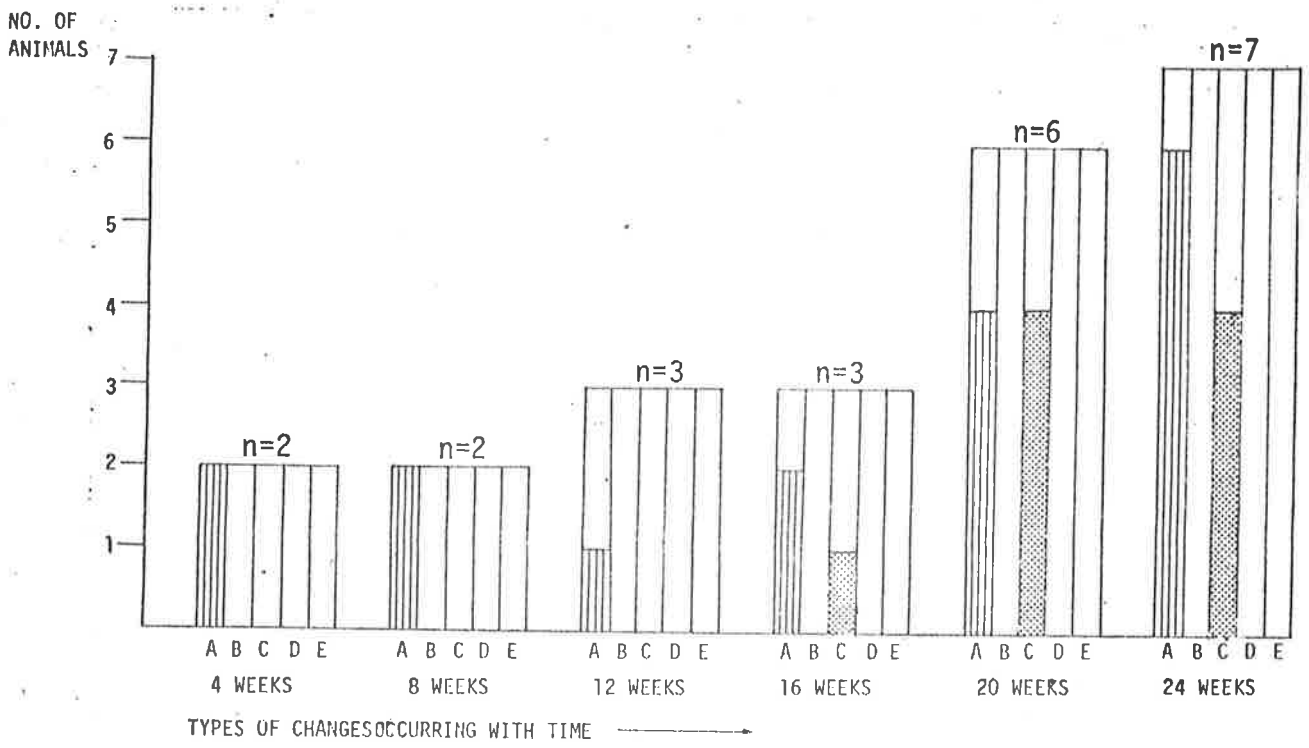


Figure 5.48: Macroscopic changes on the anterior tongue of 4NQO-treated animals. A - Mild mucosal thickening; B - Severe mucosal thickening; C - Plaque; D - Exophytic growth; and E - Carcinoma.

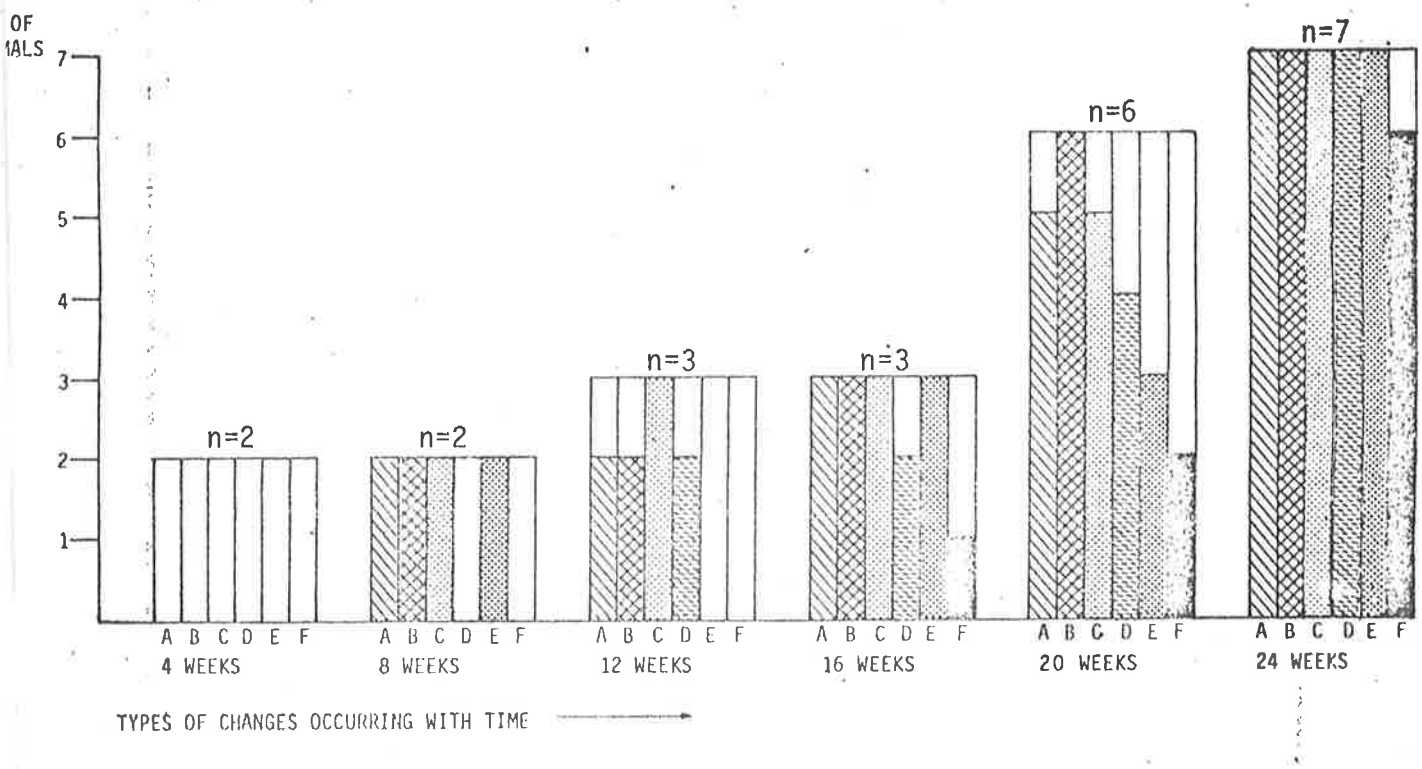


Figure 5.49: Histologic changes occurring in the middle palate of 4NQO-treated animals. A - Hyperkeratosis; B - Epithelial hyperplasia; C - Mild dysplasia; D - Moderate to severe dysplasia; E - Full thickness keratinization; and F - Palatal carcinoma.

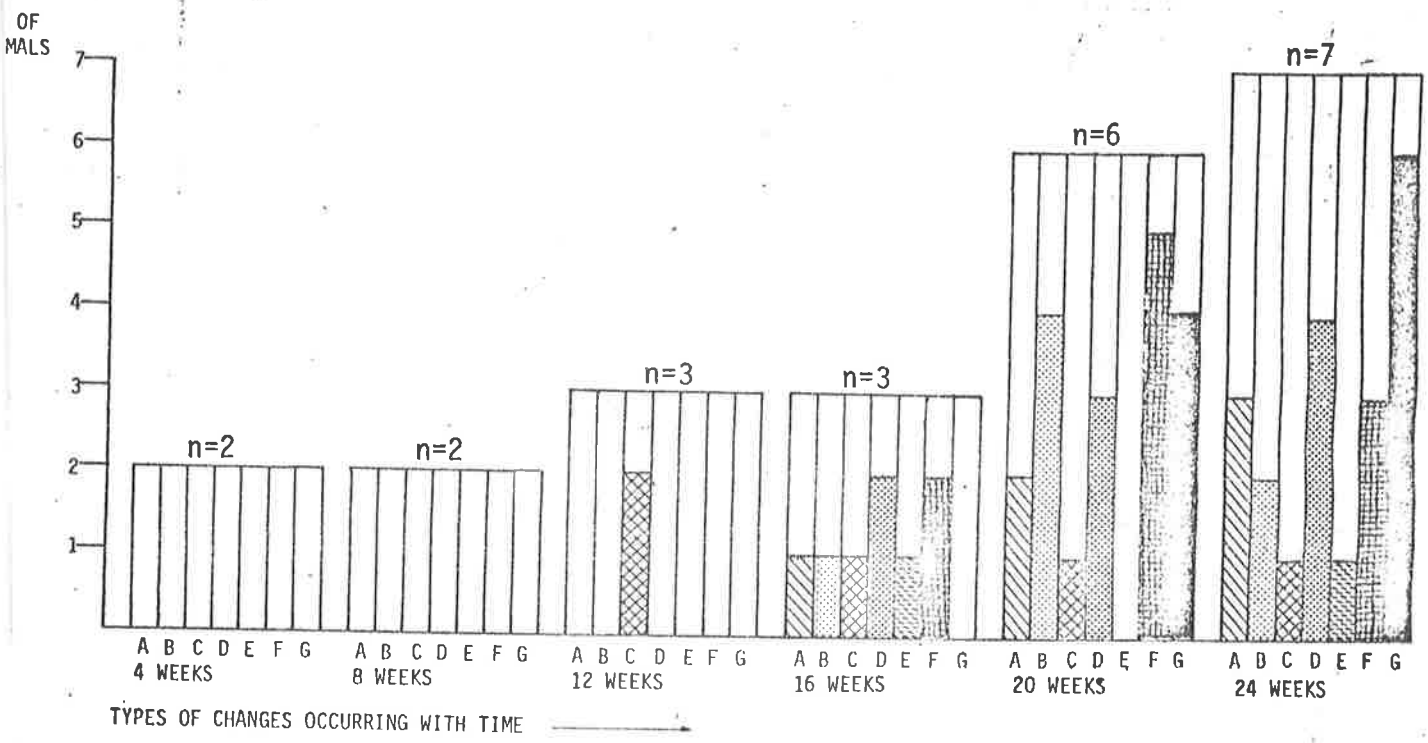


Figure 5.50: Histologic changes occurring in the palatal gingival area of 4NQO-treated animals. A - Hyperkeratosis; B - Epithelial hyperplasia; C - Mild dysplasia; D - Moderate to severe dysplasia; E - Full thickness keratinization; F - Gingival proliferative changes; G - Gingival carcinoma.

Table 5.2: Distribution of microscopic palatal gingival changes of 4NQO-treated animals according to side.

Histologic Features		Duration in Weeks 4 n=2	8 n=2	12 n=3	16 n=3	20 n=6	24 n=7
Hyperkeratosis	GL					1	1
	GR				1	2	2
	G				1	2	3
Epithelial Hyperplasia	GL					2	2
	GR				1	4	1
	G				1	4	2
Mild Dysplasia	GL			2	1		
	GR			1	1	1	1
	G			2	1	1	1
Moderate to Severe Dysplasia	GL		1		2	3	4
	GR				1	2	2
	G		1		2	3	4
Full Thickness Keratinization	GL				1		1
	GR						
	G				1		1
Gingival Proliferative Changes	GL				2	3	2
	GR				2	3	1
	G				2	5	3
Gingival Carcinoma	GL					3	4
	GR					2	5
	G					4	6

Table 5.3: Features of cellular atypia observed in middle palate and palatal gingival area of 4NQO-treated animals.(For definitions of terms, refer to Smith and Pindborg (1969) and Pindborg (1980).)

	Duration in Weeks					
	4	8	12	16	20	24
Irregular Epithelial Stratification					5/6	6/7
Keratinization of Single Cells		1/2	2/3	3/3	5/6	6/7
Keratinization of Cell Groups		1/2		1/3		3/7
Reduction of Cellular Cohesion		1/2	2/3	2/3	5/6	7/7
Cellular Pleomorphism		1/2	2/3	3/3	5/6	7/7

Table 5.4: Histologic features of palatal gingival carcinomasin 4NQO-treated animals.

	Duration in Weeks					
	4	8	12	16	20	24
Well Differentiated					3/6	5/7
Moderately Differentiated					1/6	1/7
Inflammation					4/6	6/7
Keratin Plug and Cyst					2/6	6/7
Early					2/6	
Advanced					2/6	6/7

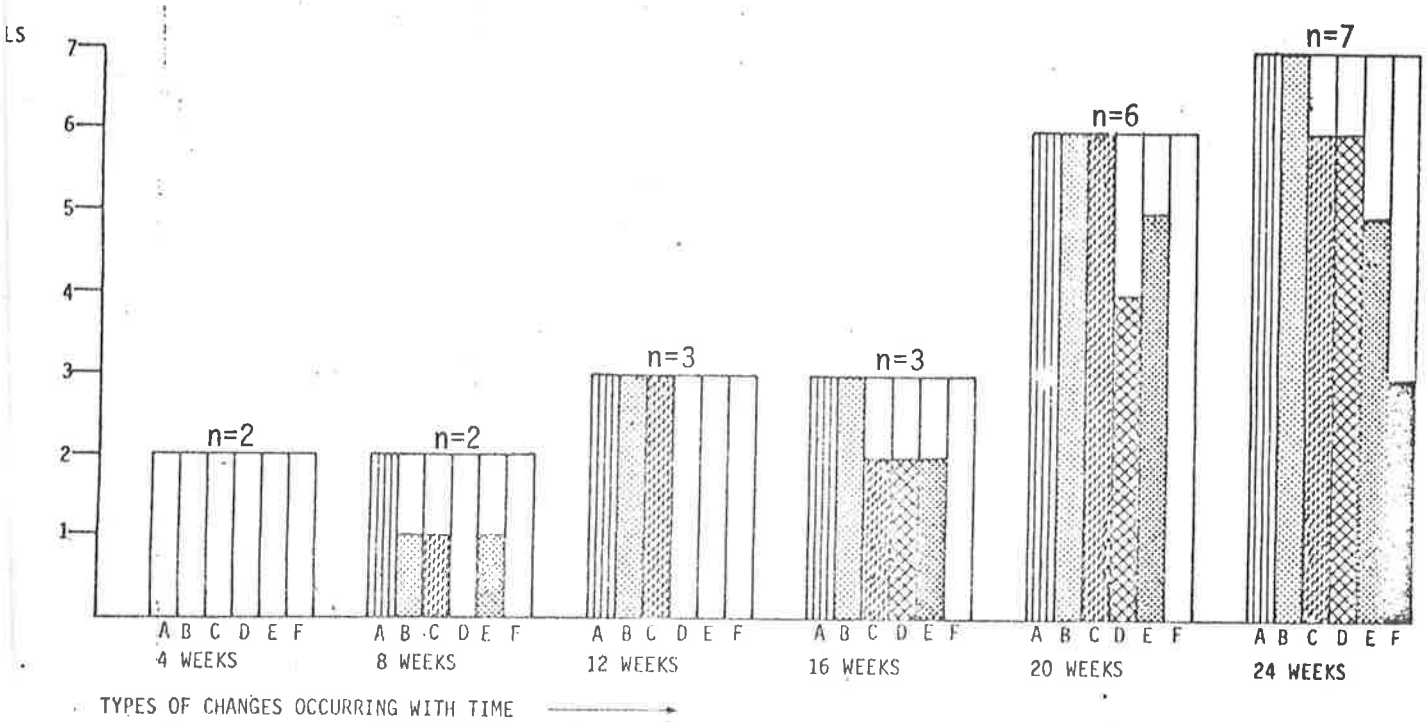


Figure 5.51: Histologic changes in the posterior tongue of 4NQO-treated animals. A - Hyperkeratosis; B - Epithelial hyperplasia; C - Mild dysplasia; D - Moderate to severe dysplasia; E - Full thickness keratinization; and F - Infiltrative carcinoma.

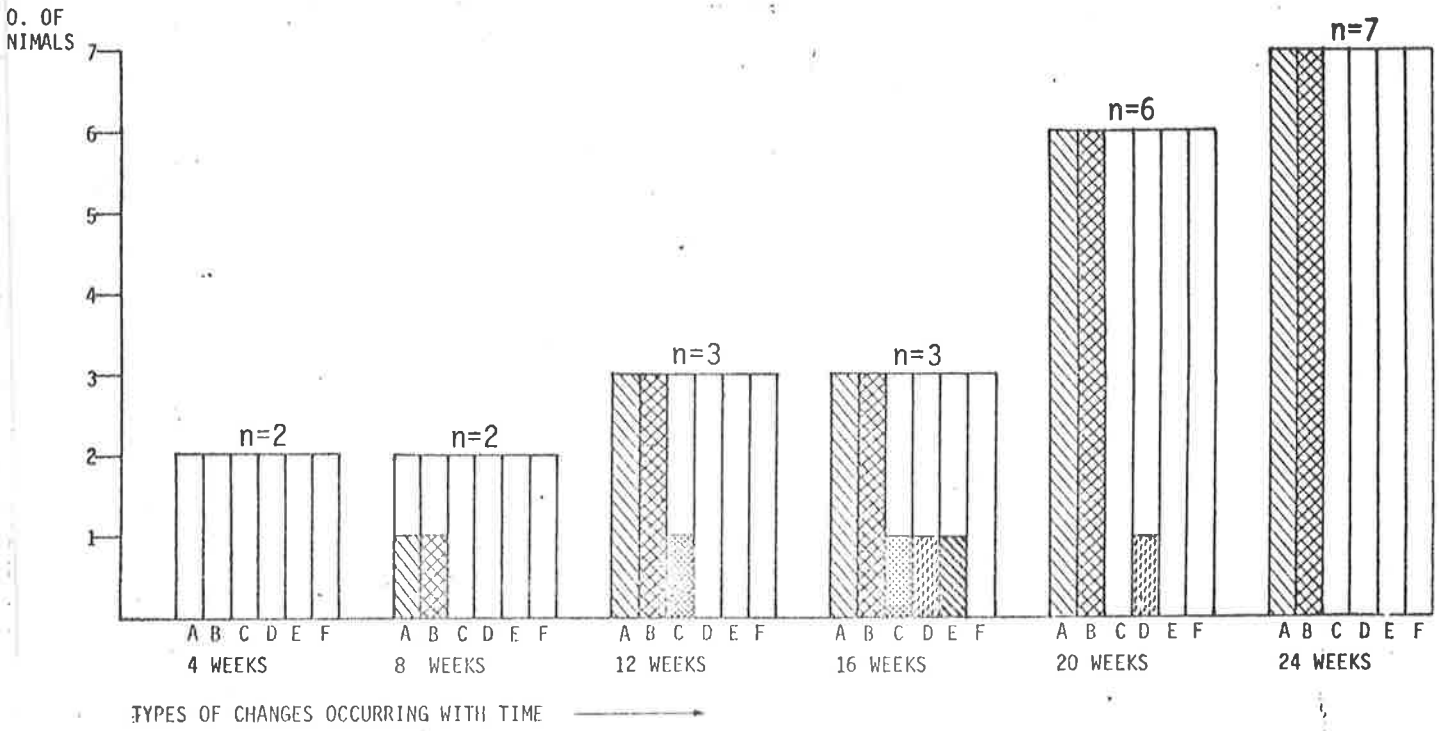


Figure 5.52: Histologic changes in the anterior tongue of 4NQO-treated animals. A - Hyperkeratosis; B - Epithelial hyperplasia; C - Mild dysplasia; D - Moderate to severe dysplasia; E - Full thickness keratinization; F - Infiltrative carcinoma.

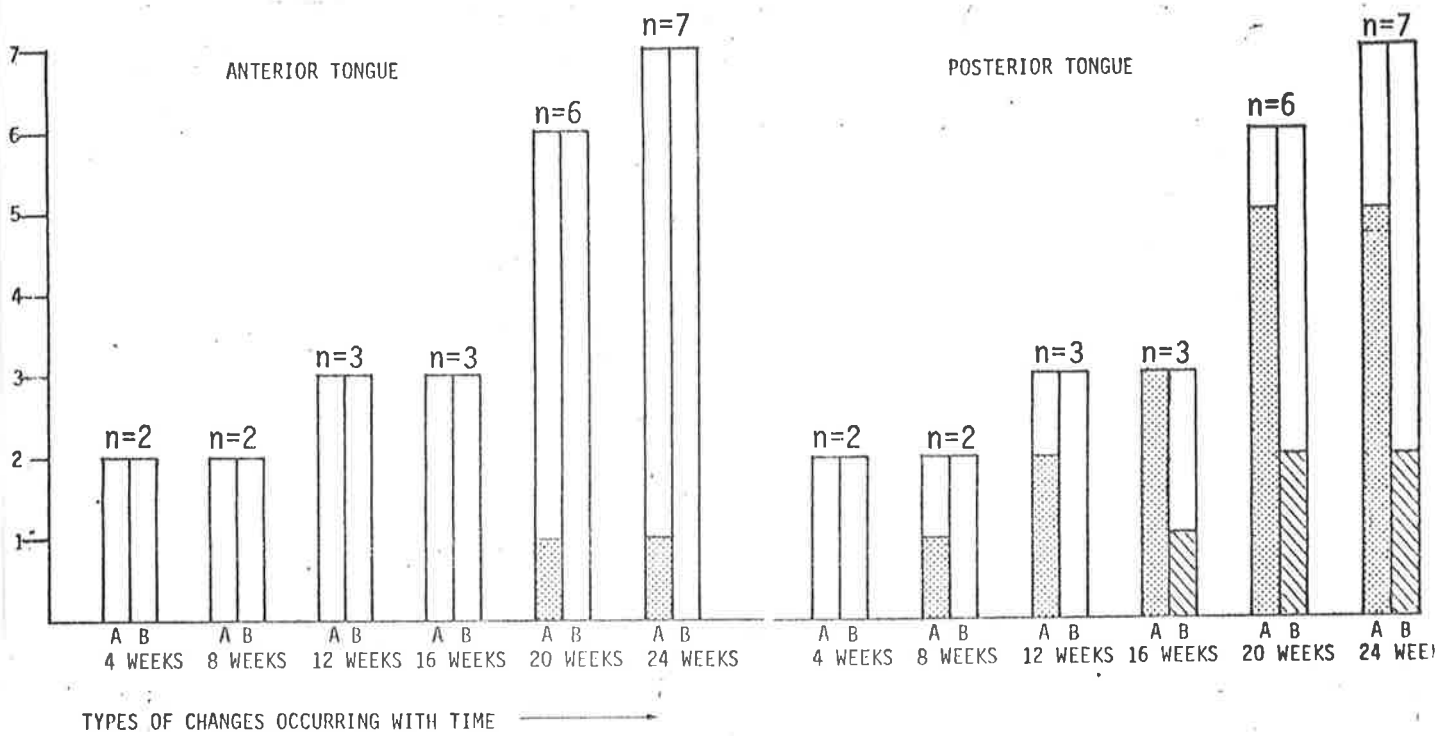


Figure 5.53: The occurrence of histologic plaques and papillomas on anterior and posterior tongues of 4NQO-treated animals. A - Plaque; B - Papilloma.

Table 5.5: Features of cellular atypia observed in the tongues of 4NQO-treated animals.

	Duration in Weeks					
	4	8	12	16	20	24
Irregular Epithelial Stratification			1/3	2/3	3/6	3/7
Keratinization of Single Cells			2/3	3/3	5/6	7/7
Keratinization of Cell Groups				1/3	2/6	7/7
Reduction of Cellular Cohesion			1/3	2/3	4/6	7/7
Cellular Pleomorphism			2/3	2/3	4/6	6/7

Table 5.6: Histologic features of plaques and papillomas occurring on the tongues of 4NQO-treated animals.

	Duration in Weeks											
	Plaque						Papilloma					
	4	8	12	16	20	24	4	8	12	16	20	24
Widening of rete pegs		1/2	2/3	3/3	5/6	5/7				1/3		
Elongation of rete pegs		1/2	2/3	3/3	5/6	5/7				1/3	2/6	2/7
Dysplasia		1/2	2/3	3/3	5/6	5/7				1/3	2/6	2/7

Table 5.7 : Features of Infiltrative Carcinomas in the tongues of 4NQO-treated animals.

Well differentiated	3/7
Moderately differentiated	1/7
Inflammation	3/7
Early	2/7
Advanced	3/7

(Total number of animals showing CA = 3)

CHAPTER 6

DISCUSSION

DISCUSSION

MATERIALS AND METHODS

The protocol for this study was modified from that originally used by Lekholm and Wallenius (1976a) (Appendix VIII). The modifications were made out of necessity and related to rat strain used, anaesthesia and the antisialogogue employed.

The Porton strain of albino rat was used because it was the closest available strain to Sprague Dawley. In their 1976(a) study, Lekholm and Wallenius used unanaesthetized rats during the experimental procedures. In the present experiment, all animals were anaesthetized to facilitate handling and also to prevent experimental hazards relating to accidental carcinogen contamination.

The absence of mortality, the good state of health of the animals during the experiment and the success of the experiment itself support the view that the method of anaesthetization used did not produce undesirable side effects. The time from induction to the regaining of consciousness took approximately three minutes. When compared to anaesthetization using barbiturates or other injectable forms of anaesthetics, this method of anaesthetization is highly recommended for short procedures dealing with small experimental animals. The pre-experimental handling of the animals for a period of three weeks and gentle handling of the animals during the experimental period are also considered to be important factors in relation to the matter of the good state of health of the animals in this study.

The antisialogogue, hyoscine butyl bromide,was well tolerated. This

drug was the closest available agent to the methylscopolamine nitrate used by Lekholm and Wallenius (1976a). Because of its higher molecular weight, a correspondingly higher dose of Buscopan was used. This was found to produce effects essentially similar to methylscopolamine nitrate though there were individual variations in the extent of salivary inhibition. In a small number of animals, a small amount of saliva was present at the time of carcinogen painting, but, by and large, there was no copious flow of saliva.

Wallenius and Lekholm (1973a) and Lekholm and Wallenius (1976a) mentioned that in each painting, 0.13 mg of 4NQO was delivered to the palate. However, it was observed in the present study that the hair brush appeared to deliver a variable amount of the carcinogen even though efforts were made to remove any excess carcinogen by touching the wall of the vial before painting the palate. It was also observed that the animals when painted usually made several reflex swallowing and tongue movements. Therefore, the amount of the carcinogen retained on the palate would be much less than the amount actually delivered. The minimum effective dose of the carcinogen was not established in the present study, nor is such information available in the literature pertaining to 4NQO-induced oral neoplasia, or 4NQO-induced neoplasia in other sites.

RESULTS

The macroscopic and microscopic changes occurring in the palate and tongue of Sprague Dawley rats during 4NQO-oral carcinogenesis were first described by Wallenius and Lekholm (1973a), Lekholm and Wallenius (1976a) and Wallenius et al (1979) but not in any great detail. The macroscopic and microscopic changes reported in the present study

are in broad agreement with those reported by these workers. However, additional findings and some points of difference between the present and previously reported studies were noted.

In the present investigation, at eight weeks mild macroscopic thickening of the middle palate was associated microscopically with epithelial hyperplasia, hyperkeratosis and mild dysplasia. At 12 weeks the above relationship was still maintained with the addition of occasional areas showing moderate to severe dysplasia. At 16 weeks severe thickening of the palate was associated with microscopic evidence of more severe hyperkeratosis, epithelial hyperplasia and atypical cellular features comprising moderate to severe dysplasia. There was one instance of a microscopic palatal carcinoma underlying the rather non-specific macroscopic appearance of severe thickening of the palate.

Though the gingiva did not exhibit pocketing at this stage, there were early proliferative changes occurring in the sulcal epithelium of the gingiva observed microscopically. At 20 weeks essentially the same palatal changes as those occurring at 16 weeks were noted. However, the gingival proliferative changes were demonstrable macroscopically as deep gingival pockets associated with exophytic growths. In contrast, the palatal carcinomas which occurred in some animals remained occult at the macroscopic level.

At twenty-four weeks the thickening on the middle palate was severe with the presence of irregular and rather non-specific exophytic growths. Corresponding microscopic examination demonstrated severe hyperkeratosis, epithelial hyperplasia, severe dysplasia and areas of carcinoma. The latter were not evident macroscopically. In contrast,most gingival carcinomas diagnosed microscopically showed up distinctly as carcinomas at the

macroscopic level. On the whole, the histology revealed more carcinomas than macroscopic examination could demonstrate.

At four weeks, though the tongue papillae appeared mildly thickened, there were no histologic changes that could be described as abnormal. At eight weeks, the mild hyperplasia of the posterior tongue papillae was associated with histologic changes consisting of mild hyperkeratosis, epithelial hyperplasia and mild atypia. A plaque identified macroscopically had its surface features duplicated histologically. At 12 weeks, there was complete association between macroscopic mild thickening of tongue papillae and hyperkeratosis, hyperplasia and dysplasia noted microscopically. The same was true for the macroscopic and histologic plaques. At 16 weeks, severe thickening of the posterior tongue was associated with more pronounced hyperkeratosis and hyperplasia and moderate to severe dysplasia. Concordance between macroscopic and histologic plaques was again evident.

At 20 weeks, the same correlations as those noted at 16 weeks were observed. In addition, macroscopically identified papillomas corresponded to histologic papillomas. At 24 weeks, there was again good correlation between gross severe thickening and severe hyperkeratosis, hyperplasia and moderate to severe dysplasia when tissues were observed microscopically. The same held true for plaques and papillomas. However, carcinomas diagnosed histologically in three tongues at 24 weeks were only demonstrable in one tongue at the macroscopic level.

Throughout the different time intervals, the anterior tongue exhibited only mild thickening of papillae. This corresponded to mild hyperkeratosis and epithelial hyperplasia at the microscopic level. Mild dysplasia was exhibited only in a few anterior tongues. Though macroscopic plaques started to appear at 16 weeks, they were not clearly demonstrated

histologically until 20 weeks.

The initial mucosal inflammation occurring in the first month and redness and swelling presenting in the second and third month reported by Lekholm and Wallenius (1976a) were not observed in the present study. These workers also mentioned the appearance of hyperplasia and hyperkeratosis and three palatal carcinomas in the fourth month and reported that all palates exhibited carcinomas in the sixth month. In the present study, severe thickening of the palate occurred in the fourth month but there was no macroscopic carcinoma. The first occurrence of palatal gingival carcinoma was in the fifth month with more carcinomas appearing in the sixth month. In the present study, six out of seven animals developed gingival carcinoma and verrucous carcinoma of the palate after six months. At macroscopic level, therefore, Lekholm and Wallenius's (1976a) study produced carcinomas earlier and in a better yield.

Lekholm and Wallenius did not report the occurrence of tongue cancer in their animals when xerostomia was induced (1976a). However, these workers in an earlier study (Wallenius and Lekholm, 1973a) did report that 75% of their animals exhibited well differentiated squamous cell carcinomas of the posterior tongue. In the present study only three out of seven animals (43%) exhibited histologic carcinomas of the tongue at 24 weeks. If the present experiment had been carried out longer than 24 weeks, more carcinomas would possibly have developed. It is also possible, however, that differences in animal strains used and technique differences between the present study and those reported by Wallenius and Lekholm could account for the variation in tumour frequency on the tongue.

The palatal carcinomas described by Wallenius and Lekholm (1973a) and Lekholm and Wallenius (1976a) were unilateral and localized

to the region closest to the molar teeth. In the present experiment, two animals, one at 20 and the other at 24 weeks, exhibited macroscopic bilateral gingival carcinomas. In those animals with unilateral carcinomas, the gingival pockets on the contralateral side looked macroscopically suspicious and in fact a greater number of bilateral carcinomas were seen microscopically. This implies that the oncogenic changes took place bilaterally on the gingivae in contrast to the apparently unilateral process described by Wallenius and Lekholm (1973a) and Lekholm and Wallenius (1976a).

Wallenius and Lekholm (1973a) and Lekholm and Wallenius (1976a) described sessile nodulary lesions on the posterior tongue which subsequently developed into papillomas. They also reported superficial hyperkeratotic plates firmly attached to the underlying structures (Wallenius and Lekholm, 1973a). In a different study, Wallenius et al (1979) further mentioned whitish, firmly adherent patches with small nodules that developed into exophytic papillomas. The latter in turn developed into ulcerative papillary cancers. These progressive changes were not observed in the present study. In this study, the five small papillomas observed from 16 weeks onward which might have been equivalent to the nodular lesions described by Wallenius and Lekholm (1973a) and Lekholm and Wallenius (1976a) could not be traced to any specific precursor lesion, nor could they be shown to develop further into more aggressive lesions. Their histologic appearances were rather benign. It seems unlikely therefore that the papillary lesions observed in the present study were precursors of carcinomas.

The plaques observed in this study would appear to correspond to the plates and patches described by Wallenius and colleagues in the above-mentioned studies. The plaques developed early and, though increasing in size, were not observed to develop into other

types of lesions. Histologically, they remained relatively benign lesions, showing static histologic features, throughout the experimental period.

Besides changes in the palate and tongue, Wallenius and Lekholm (1973a) and Lekholm and Wallenius (1976a) also incidentally found a few carcinomas around the mandibular molars and thickening of the gingiva and buccal mucosa. Such changes were not observed in the present study.

The palatal cancer around the molars described by Wallenius and Lekholm (1973a) and Lekholm and Wallenius (1976a) would appear to be equivalent to the gingival carcinoma described in the present investigation. No mention was made by these authors of the histologic changes occurring in the middle palate of their experimental animals. The finding of occult verrucous carcinoma in the middle palate of animals at 20 and 24 weeks in this study was therefore surprising and is considered significant. The diagnosis of verrucous carcinoma was based on the exhibition of the classical histologic features identified with human verrucous carcinoma. These are, marked epithelial proliferation with downgrowths of broad, blunt and bulbous rete pegs into the connective tissues but without a pattern of true invasion, the tendency for these epithelial processes to advance at approximately the same level forming a "pushing margin", an epithelium that is well differentiated but may exhibit features of cellular atypia, parakeratin-lined clefts and parakeratin plugs extending deep into the epithelium (Ackerman, 1948; Cooke, 1969; Shafer, 1972; Jacobson and Shear, 1972; Pindborg, 1980). The pattern of bone resorption produced by the lesions resembled that described by Kraus and Perez-Mesa (1966) in which the tumour tended to destroy the bony tissue on a broad front, eroding with a sharp margin

rather than infiltrating into the marrow spaces. Grinspan and Abulafia (1979) described three types of verrucous carcinoma. Type I has essentially benign cellular features. Type II shows features of cellular atypia. Type III exhibits the picture of a classical penetrating carcinoma but shows other features of verrucous carcinoma. The verrucous carcinomas observed in the present study would be type II tumours. Since the rat is a species different from human, one could not expect all features observed in these tumours to be identical to those found in the human situation.

One very interesting feature observed in this investigation was the early occurrence (at 8 weeks) of keratinization of the full thickness of the epithelium with the keratinized cells retaining their pyknotic nuclei. This feature was later found to exist by itself as well as in association with areas of mild, moderate to severe dysplasia and verrucous carcinoma. When it occurred in a verrucous carcinoma, it resembled the parakeratin clefts and plugs described for human verrucous carcinoma. However, the early appearance of this essentially dyskeratotic parakeratin could not be interpreted as an early signal for the development of verrucous carcinoma since it was also associated with so many other changes.

A practical difficulty encountered in the present investigation was the grading of features of epithelial atypia and their categorization into dysplasia. In this study the criteria and terminology of Smith and Pindborg (1969) and Pindborg (1980) for atypia-dysplasia were used as a basis for assessment of epithelial histologic features. However, it was found, as indicated in the results, that because of the absence of or difficulties encountered in recognizing some features of atypia which contribute to the diagnosis and grading of dysplasia that the system described by Smith and Pindborg (1969) was not entirely satisfactory for the purposes of

this study.

Studies specifically devoted to chemical induction of gingival carcinoma were reported by Al-Ani and Shklar (1966), Mesrobian and Shklar(1969) and Suzuki (1976). The two former groups using hamsters and topical application of dimethylbenzanthracene (DMBA) reported epithelial hyperplasia, dysplasia, papilloma and well differentiated squamous cell carcinoma developing almost invariably in the epithelium facing the oral cavity while sulcal epithelium remained relatively unaffected. These workers explained that the negative response of the sulcal epithelium was due to the fact that the carcinogen only entered the gingival sulcus in small quantities and that mastication removed the carcinogen from the gingival surface and sulcal area. In contrast, Suzuki (1976) using hamsters and systemic (intragastric) application of N-nitrosomethylurea (NMU) observed active downward proliferation of the sulcal epithelium associated with keratocyst formation, cellular atypia and accompanied by destruction of the alveolar bone and periodontal tissues. The oral aspect of the gingival epithelium exhibited only proliferative changes late in the experiment. The progressive changes in the sulcal epithelium observed in the present study are not unlike those reported by Suzuki (1976). The changes noted in the present investigation began with epithelial dysplasia, progressed to proliferative changes then to overt infiltrative squamous cell carcinoma. It must be admitted that in some cases it was hard to draw a line of distinction between proliferative changes which were not yet invasive, and infiltrative squamous cell carcinoma.

Suzuki (1976) tried to explain the sensitivity of the sulcal epithelium to carcinogens in terms of the active cell turnover in the attachment epithelium. It is premature to draw conclusive statements regard-

ing the mechanism of carcinogenesis involving the sulcal epithelium in the light of the present experimental study which included a small sample and only morphologic methods of analysis. However, in the light of past and present concepts of oral carcinogenesis, the following possibilities are suggested. The preference for carcinomas to develop in the sulcal epithelium as observed by Suzuki (1976) and in the present study suggests that carcinogens may be provided with a rapid portal of entry into the inner epithelial cells as a consequence of chronic inflammation affecting sulcal epithelium permeability. The gingival sulcus could also act as a reservoir for carcinogens.

The occurrence of verrucous carcinoma in the middle palate shows that the epithelium at this site is also susceptible to 4NQO-induced carcinogenesis. However, the type of carcinoma observed was of lower grade malignancy compared to the more aggressive gingival carcinomas. Although the middle palate was the site on which the carcinogen was directly applied, its smooth surface texture and the cleansing action of the tongue might have contributed to the displacement of the carcinogen to other areas, the gingival area being the most adjacent. Accordingly, the amount and duration of the carcinogen in contact with the middle palate might be less than that in contact with the gingival area. This factor could contribute to the observed difference between middle palate and gingival carcinomas. Another possibility is that the epithelia of the middle palate and the gingiva might have differing intrinsic biologic behaviour towards carcinogenic stimuli.

It was not surprising to observe carcinomas in the posterior tongue since it was in frequent contact with the middle palate, thus contaminating itself with the carcinogen. In addition, the papillary surface of the tongue would favour the retention of the carcinogen.

The occurrence of neoplastic changes at and around the site of oral application of 4NQO indicates that the carcinogen is a locally active carcinogen which does not require systemic metabolism to acquire its active form. It implies that either the oral mucosal epithelium of the rat or the oral environment, possibly the oral flora, might contain the enzyme system(s) needed for the metabolic activation of 4NQO to its active form(s).

SUMMARY AND CONCLUSION

An attempt was made to establish an experimental model of oral cancer in rats using the water-soluble carcinogen 4-nitroquinoline 1-oxide (4NQO). The experimental protocol employed was based on Lekholm and Wallenius (1976a) with some modifications. Detailed study of the macroscopic and histopathologic changes occurring in the palate and tongue at different time intervals was carried out and the results discussed particularly in relation to the work reported in the literature by Wallenius and co-workers.

The experimental model was successfully established. The reproducibility of Lekholm and Wallenius's experiment was thus verified.

The findings derived from the macroscopic and histopathologic study were as follows:

1. Macroscopic morbid changes in the palate progressed gradually from mild, innocuous thickening of the mucosa to severe thickening which had a markedly corrugated and rough surface. Overt gingival carcinomas developed late in the experimental period (20-24 weeks). This was preceeded by deep gingival pocket formation.
2. The tongue underwent similar macroscopic changes. However, only one specimen resulted in the formation of grossly observable carcinoma. Localized, keratotic plaques appeared early in the experimental period (starting from 8 weeks) and persisted without progressing to other lesions.
3. Histologically, the palate exhibited hyperkeratosis, epithelial hyperplasia and dysplasia beginning at 8-12 weeks. These features increased in severity with time. Gingival proliferative changes preceeded

the appearance of gingival carcinomas. The late changes on the palate were highlighted by the appearance of well differentiated gingival squamous cell carcinomas and lesions on the middle palate which were classed as verrucous carcinoma.

4. The tongue went through similar stages of histologic change. Some specimens resulted in the formation of infiltrative squamous cell carcinomas which were essentially well differentiated lesions. The plaques observed were characterized by special histologic features including depapillation and alteration in the epithelial cells.

5. Papillomas and papillary growths in both the tongue and palate were not observed to be precursors of carcinoma. Histologically such lesions were observed to be benign.

6. An unexplained phenomenon of keratinization of the full thickness of the epithelium was exhibited by the palate and the tongue beginning at 8 weeks and persisting throughout the experimental period. Such a change was frequently not associated with neoplastic epithelium.

Detailed study and correlation of the macroscopic and histopathologic changes occurring in the rat palate and tongue during 4NQO-induced oral carcinogenesis were not reported in depth by previous workers. The results of this investigation provide further useful macroscopic and microscopic data describing the changes in rat oral mucosa following repeated application of 4NQO.

In view of its reproducibility and the nature of the lesions produced, the 4NQO-rat oral cancer model is considered particularly suitable for the study of infiltrative gingival carcinomas and palatal carcinomas of an apparent verrucous type.

MANUFACTURER'S SPECIFICATIONS OF DIET AND CAGE

DIET (PELLET)

CHARLICKS FEEDS
MOUSE RATION

Min. Crude Protein.....21.0%
Min. Crude Fat.....3.5%
Max. Crude Fibre.....6.0%
Min. Vitamin A.....10000 I.U./Kg.
Min. Vitamin D.....2000 I.U./Kg.
Max. Added Salt.....0.5%
Ethoxyquin.....100 p.p.m.

Ingredients:
Wheat, Barley, Bran, Pollard, Lucerne, Meat Meal, Soyabean Meal, Yeast, Cottonseed Meal, Fish Meal, Salt, Dicalcium Phosphate, Vitamins, Minerals and Antioxidant.

Manufactured by:
Wm. Charlick Ltd.
South Australia.

CAGE

NORTH KENT PLASTIC CAGES LTD. ENGLAND.

RB3 cage
Polypropylene
Stainless steel top
Size: 45cm X 28cm X 22cm

MANUFACTURER'S LABELLING AND SPECIFICATIONS OF 4NQO AND PROPYLENE GLYCOL

4NQO

FLUKA AG, BUCHS SG 199347 578 K 7019-/116/1./5
73265 laä/KHB/P↓:1
250 mg PURUM
4-Nitroquinoline-N-oxide F 155-157°
Made in Switzerland

$C_6H_4NO:CHCH:CNO_2$
MG 190.16

PROPYLENE GLYCOL

LABORATORY UNILAB REAGENT
Manufacturer:
AJAX CHEMICALS LTD.
SYDNEY-MELBOURNE
Serial # 11697

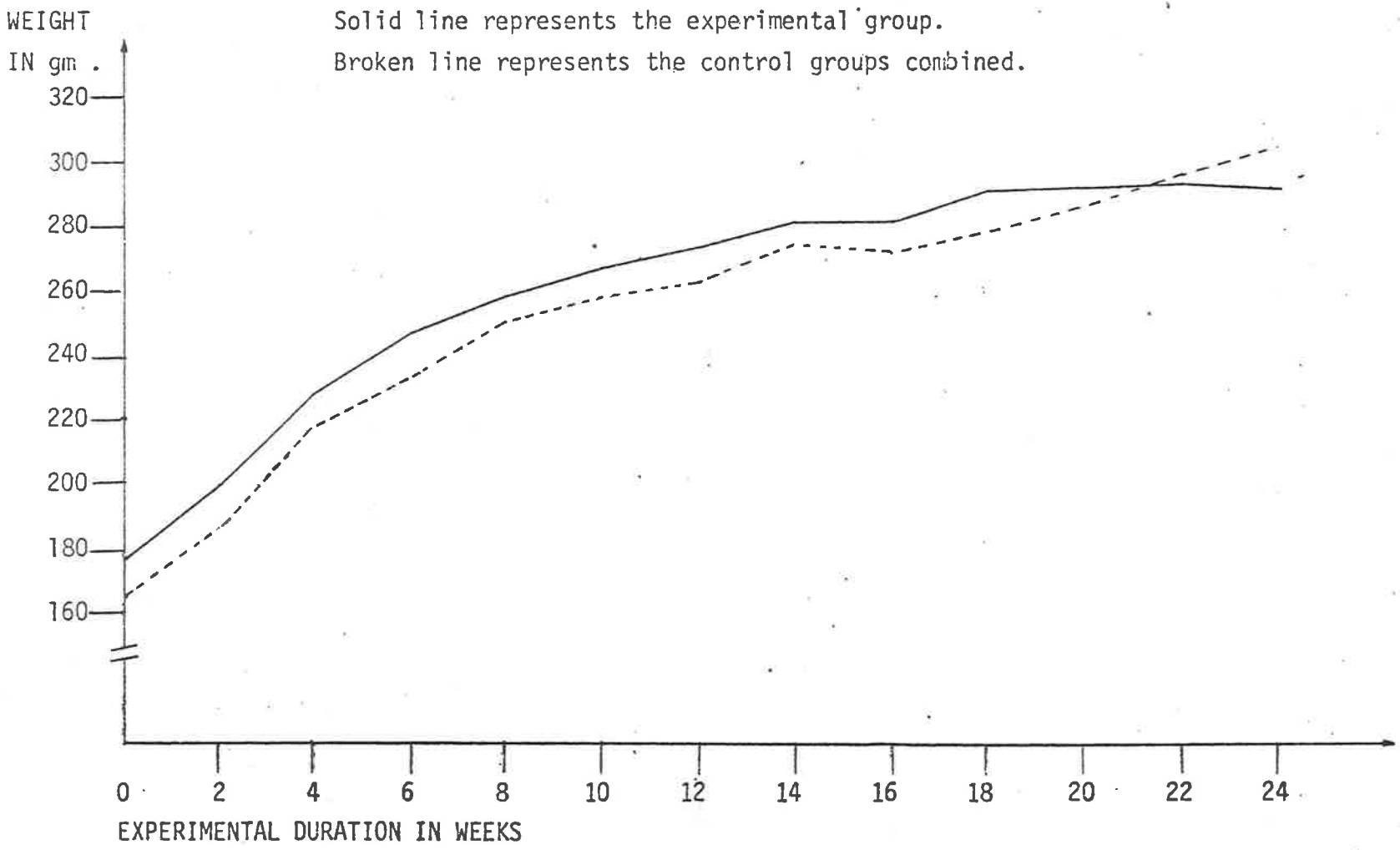
PROPANE-1,2-DIOL
(PROPYLENE GLYCOL)

$CH_3.CH(OH).CH_2.OH$ = 76.10	500 ml
Boiling range (95% min.)	185.0° to 189.0°C.
Refractive index @ 20°C	1.432 to 1.433
Wt/ml @ 20°C	1.037 to 1.040 g.

MAXIMUM LIMITS OF IMPURITIES (per cent):

Acidity (as CH_3COOH)	0.005
Sulphated ash	0.005
Water	0.3

AVERAGE WEIGHT CHANGES OF ANIMALS



APPENDIX III a

AVERAGE WEIGHT CHANGES (In gm.)

DURATION (WEEKS)	0	2	4	6	8	10	12	14	16	18	20	22	24
EXPERIMENTAL	177	201	228	245	259	267	273	282	281	290	292	292	291
CONTROL COMBINED	167	186	219	233	251	257	262	274	272	279	284	295	303

Average weight calculated by: $\frac{\text{Total weight of all surviving animals}}{\text{Total No. of surviving animals}}$.

Average weightsof experimental group and control group combined were separately calculated, and the values obtained for each group are separately listed in the table.

APPENDIX IIIb

CHART 1.

MACROSCOPIC OBSERVATIONS

PALATE

Animal No.
Experimental
Control

TYPES OF CHANGES		DURATION (WEEKS) 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
MILD MUCOSAL THICKENING	A	
	GL	
	GR	
	M	
	P	
SEVERE MUCOSAL THICKENING	A	
	GL	
	GR	
	M	
	P	
EXOPHYTIC GROWTH	A	
	GL	
	GR	
	M	
	P	
DEEP GINGIVAL POCKET	GL	
	GR	
CARCINOMA	A	
	GL	
	GR	
	M	
	P	

OTHER OBSERVATIONS

LEGEND: A = Anterior palate GL = Gingival area, left side GR = Gingival area, right side M = Middle palate P = Posterior palate
A positive finding is scored by an 'X' in the box provided.

CHART 2.

MACROSCOPIC OBSERVATIONS

TONGUE

Animal No. ☐☐
Experimental ☐
Control ☐

TYPES OF CHANGES		DURATION (WEEKS)																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
MILD MUCOSAL THICKENING	A																								
	P																								
SEVERE MUCOSAL THICKENING	A																								
	P																								
PLAQUE	A																								
	P																								
EXOPHYTIC GROWTH (PAPILLOMA)	A																								
	P																								
CARCINOMA	A																								
	P																								

OTHER OBSERVATIONS

LEGEND: A = Anterior tongue P = Posterior tongue
A positive finding is scored by an 'X' in the box provided.

METHOD OF DECALCIFICATION

RECIPE FOR DECALCIFYING FLUID

68 gm of Sodium formate

1660 ml of Distilled water

340 ml of Formic acid

makes 2 litres of fluid.

PROCEDURE:

1. Specimen placed in decalcifying fluid.
2. Specimen radiographed to check the degree of decalcification.
3. When no radiopacity is present, decalcification is completed.
4. Leave in the fluid for another 24 hours to ensure complete decalcification.
5. Specimen then placed in 5% Sodium sulphate for neutralization overnight (24 hours).
6. Specimen ready for processing.

RESULT:

After 72 hours (3 days), radiograph detected complete decalcification of the palate specimen.

HAEMATOXYLIN AND EOSIN STAINING

METHOD:

1. Sections to water,
2. Hillie/Mayer Haematoxylin, 4½ minutes,
3. Blue in running water, 5 minutes,
4. Differentiate in 1% Hydrochloric acid in 70% Alcohol,
5. Blue in running water,
6. Eosin, 30 seconds,
7. Rinse in water,
8. Dehydrate, clear and mount.

RESULTS:

The various elements are stained as follows:

Nuclei - blue,

Keratin - red to pink,

Collagen - pink,

Other elements - various shades of red.

CHART 3.

MICROSCOPIC OBSERVATIONS

PALATE

Animal No. | | |
Duration(Weeks) | | |
Experimental | |
Control | |

HISTOLOGIC FEATURES

HYPERKERATOSIS	A	
	GL	
	GR	
	M	
	P	
EPITHELIAL HYPERPLASIA	A	
	GL	
	GR	
	M	
	P	
MILD DYSPLASIA	A	
	GL	
	GR	
	M	
	P	
MODERATE TO SEVERE DYSPLASIA	A	
	GL	
	GR	
	M	
	P	
FULL THICKNESS KERATINIZATION	A	
	GL	
	GR	
	M	
	P	
VERRUCOUS CARCINOMA	A	
	GL	
	GR	
	M	
	P	
GINGIVAL PROLIFERATIVE CHANGES	GL	
	GR	
CARCINOMA	A	
	GL	
	GR	
	M	
	P	
PAPILLARY GROWTH	A	
	GL	
	GR	
	M	
	P	

FEATURES OF CELLULAR ATYPIA

IRREGULAR EPITHELIAL STRATIFICATION	
KERATINIZATION OF SINGLE CELLS	
KERATINIZATION OF CELL GROUPS	
REDUCTION OF CELLULAR COHESION	
CELLULAR PLEOMORPHISM	

FEATURES OF CARCINOMA

WELL DIFFERENTIATED	
MODERATELY DIFFERENTIATED	
KERATIN CYST	
EARLY	
ADVANCED	
INFLAMMATION	

FEATURES OF VERRUCOUS CARCINOMA

ELONGATION OF RETE PEGS	
MILD DYSPLASIA	
MODERATE TO SEVERE DYSPLASIA	
FULL THICKNESS KERATINIZATION	
INFLAMMATION	

OTHER OBSERVATIONS

LEGEND: A = Anterior palate GL = Gingival area, left side GR = Gingival area, right side M = Middle palate P = Posterior palate
A positive finding is scored by an 'X' in the box provided.

APPENDIX VIIb

CHART 4.

MICROSCOPIC OBSERVATIONS

TONGUE

Animal No. ☐☐
Duration(Weeks) ☐☐
Experimental ☐
Control ☐

HISTOLOGIC FEATURES

HYPERKERATOSIS	A P	
EPITHELIAL HYPERPLASIA	A P	
MILD DYSPLASIA	A P	
MODERATE TO SEVERE DYSPLASIA	A P	
FULL THICKNESS KERATINIZATION	A P	
INFILTRATIVE CARCINOMA	A P	
PLAQUE	A P	
PAPILLARY GROWTH (PAPILLOMA)	A P	

FEATURES OF CELLULAR ATYPIA

IRREGULAR EPITHELIAL STRATIFICATION	
KERATINIZATION OF SINGLE CELLS	
KERATINIZATION OF CELL GROUPS	
REDUCTION OF CELLULAR COHESION	
CELLULAR PLEOMORPHISM	

FEATURES OF CARCINOMA

WELL DIFFERENTIATED	
MODERATELY DIFFERENTIATED	
EARLY	
ADVANCED	
INFLAMMATION	

OTHER OBSERVATIONS

LEGEND:

A = Anterior tongue

P = Posterior tongue

A positive finding is scored by an 'X' in the box provided.

PROTOCOL OF LEKHOLM AND WALLENIUS'S EXPERIMENT (1976a)

CARCINOGEN	0.5% 4-Nitroquinoline 1-oxide (4NQO) in propylene glycol
CONTROL AGENT	Propylene glycol
ANIMAL SPECIES	Rat
STRAIN	Sprague Dawley
AGE	45 days (6 3/7 weeks)
WEIGHT	150 gm .
SEX	Female
METHOD AND SITE OF APPLICATION	One stroke painting from the soft palate to the incisive papilla with a No.2 sable hair brush.
METHOD OF SALIVA INHIBITION	Subcutaneous injection of methylscopolamine nitrate at a dose of 1 mg/Kg body weight.
FREQUENCY OF APPLICATION	Three times weekly until animals were sacrificed.
DIET	Standard pellet, tap water ad libitum.
CAGING	Standard cage, 5 rats/cage.
NO. OF EXPERIMENTAL ANIMALS	15
NO. OF CONTROL ANIMALS	5
SCHEDULE OF SACRIFICE	Animal sacrificed when gross carcinoma appeared on the palate.
METHOD OF EXAMINATION	Gross examination and light microscopy

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