THE CO-CARCINOGENIC EFFECT OF TOPICAL VITAMIN A PALMITATE ON 9,10-DIMETHYL-1,2-BENZANTHRACENE (DMBA)-INDUCED CARCINOMA IN THE BUCCAL POUCH OF THE SYRIAN GOLDEN HAMSTER.

With a review of different aspects of hamster cheek pouch carcinogenesis and the function and action of Vitamin A in relation to epithelia.

by

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Thesis submitted for the degree of Doctor of Medicine, (M.D.) according to the requirements of the University of Cape Town Medical School, Cape Town, Republic of South Africa.

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The work was performed in the laboratories of the Department of Pathology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel. - Chairman, Professor, H. Ungar.

June 1969

To my dear wife LILY without whose constant enthusiasm and moral support, this work would not have been possible.

To my parents I extend my respect and gratitude.

ACKNOWLEDGEMENTS

I wish to thank my instructor, Dr. I.S. Levij, for the encouragement, assistance and valuable criticism offered throughout this work and whose efforts have been instrumental in the initiation, completion and continuation of this work.

I am indebted to the Hebrew University-Hadassah Medical School for providing Grants No. 34.707. 1968 which enabled me to perform these studies.

CONTENTS

INTRODUCTION

General:

Salley in 1954, (122) was the first worker to use the hamster cheek pouch as a model for experimental carcinogenesis and to produce squamous cell carcinomas in this organ. For a number of reasons, the pouch is most suitable for sequential studies of carcinogenesis, and these include the fact that it is easily accessible and can be everted simply, facilitating macroscopic examination. Furthermore, its anatomic situation makes it a simple model for topical application of any carcinogen. Each animal has two pouches, thus providing its own control. In addition, the pouch serves as a storehouse and is lined only by stratified squamous epithelium with no glands or hair follicles in its wall, thus rendering it less susceptible to cyclic changes than more complex tissues, in which accessory structures are present.

The cheek pouch epithelium appears to have only one stem cell population. These are the basal cells (112), which are in a steady state in the adult animal and facilitate an easier kinetic analysis than is possible in skin or other epithelial tissues. In addition, it is known that the rich vascular supply of the pouch assures adequate oxygen supply, which is most likely to be responsible for the tissues' ability to maintain a constant temperature, despite extremely variable experimental conditions (86). The combination of all the above factors makes the pouch a particularly well adapted organ for experimental work involving the topical application of carcinogenic agents.

A description of the anatomy and a review of the literature available on different aspects of the hamster cheek pouch and carcinogenesis in this organ is appropriate here, in order to provide the necessary background to the subject material.

Anatomical Considerations:

In Syrian golden hamsters (Cricetus auratus), two well developed pouches are present which are situated beneath the skin and superficial fascia along the lateral side of the head and neck. The distal end of the pouch is inserted into the lumbar fascia in the mid-dorsal line by a thin muscle slip. The buccal pouch opens into the oral cavity and the main function of the organ is to carry and store food. Anatomically and physiologically, the pouches do not appear to be related to the dentition and in fact they appear to be evaginations of the lateral buccal wall, devoid of glands and possessing no vascular papillae, except at the aperture (107). The pouch is also devoid of hair follicles, adnexae and salivary glands and consequently no complicating factors exist in the study of carcinogenesis in this organ in contrast to other mucosal surfaces where adnexal structures are present. However, Gillete (55) reported that while the main body of the pouch is devoid of glands the rim of the pouch is supplied by the parotid gland and two minor salivary glands. These open at the medial rim of the pouch, where the pouch wall joins the mucosa of the oral cavity proper. Thus it appears that the pouch is not completely devoid of saliva, a fact worthwhile bearing in mind in any

discussion comparing reactions to carcinogens in the mucosa of the pouch and of the oral cavity proper. Salley and Kreshover (126) have shown that in the pouch there is apparently no need for a portal of entry to initiate carcinogenesis, in view of the lack of lack of hair follicles and glands. Despite this, the pouch tends to retain the carcinogen applied in an area of painting longer than in a more exposed portion of the oral mucosa painted with the agent.

The pouch itself varies from 4 to 5½ cm in length and from 1 to 1½ cm, in breadth, but it can be distended to 6½ cm in length (53). Grossly, the epithelial lining is arranged in smooth longitudinal folds and occasionally minor transverse folds appear, similar to the oral buccal mucosa. The wall of the pouch consists of longitudinal strated muscle arising from the buccinator muscle which is inserted into the lateral aspects of the pouch, but not at its closed end. These muscles are instrumental in moving the posterior part of the pouch in the direction of the orifice, and thus food is removed from the pouch. The muscles in and around the pouch are innervated by branches of the facial nerve and the arterial supply is via the inferior labial artery, a branch of the external maxillary artery, and also via a branch of the external carotid artery. The venous drainage is through the anterior and posterior facial veins. Fulton et al (51) examined the rich vascular supply of the pouch using cinephotomicroscopic techniques in order to study small blood vessels and showed that due both to the action of sphincters at the arterial end of capillaries and to changes in the blood pressure differential between the two ends of the capillary, there is a very frequent spontaneous intermittency of flow in the small vessels of the pouch.

Embryology:

The buccal pouch epithelium arises from the ectoderm of the hamster embryo, and is derived from the same epithelium that produces keratinised epithelium, papillae of the tongue and gingival epithelium. The initial differentiation of the pouch epithelium results in a flattened type stratified squamous epithelium with minimal rete ridges. Between 24 - 180 days, numerous folds develop because of contraction of the muscles in its wall. However, when distended, it has a flattened surface throughout.

Histology:

The normal adult pouch has four major histologic divisions. The superficial stratified squamous epithelium consists of 3-5 cell layers with a thin zone of keratin on the surface. Small epithelial rete ridges may occasionally extend into the lamina propria but generally the epithelial-lamina propria junction is a straight plane. Beneath the mucosa there is a lamina propria composed of dense bundles of collagen, with fibroblasts, mast cells and elastic fibres, but no adnexal structures are present. Beneath the lamina propria there is a striated muscle layer more abundant nearer the orifice of the pouch and lacking at the closed end. The deepest layer of the pouch consists of loose areolar connective tissue. A rich capillary network is present in the connective tissue and muscular layers.

Electronmicroscopical observations by Allbright and Listgarten(1) have shown that there is a 360 $\mathring{A}$ thick basement membrane which separates the basal epithelial cells from the collagen fibres in the lamina propria. Both basal and stratum spinosum

cells contain many tonofibrils and fine granules, and numerous well preserved mitochondria . The stratum corneum contains less numerous mitochondria , and densely compact tonofibrils appear to be the main constituent of this layer.

Hamster Cheek Pouch Carcinogenesis:

In 1954 Salley produced the first experimental carcinoma in the hamster cheek pouch (122). In this study he attempted to determine the susceptibility of the pouch to the action of different chemical carcinogenic compounds and to establish the carcinogen of choice for subsequent investigations of initiation, development and metabolism of induced carcinomas. In this study the right cheek pouches of hamsters were painted with solutions of various hydrocarbons including 9,10-dimethyl-1,2-benzanthracene (DMBA), 20-methylcholanthrene and 3-4 benzpyrene, three times per week for 16 weeks. The solvents used in this study were benzene and acetone. According to Berenblum (11) the three carcinogenic compounds used in this study are the three most potent compounds available for epithelial carcinogenesis, in the order listed above. Salley concluded that an acetone solution of DMBA was the most potent of the carcinogens used. In the initial stages (2 weeks), an acute inflammatory reaction developed with subsequent necrosis and sloughing of the distal end of the pouch and some of his animals died. This initial stages was followed by reactive hyperplasia with subsequent tumor formation after 6-8 weeks. The first tumors to appear were benign papillomas, and thereafter carcinomas-in-situ and squamous cell carcinomas with local invasion appeared. In this study he found metastases to cervical lymph glands - a phenomenon not recorded since, in experimental hamster cheek pouch

carcinogenesis. Escape of the carcinogenic material into the oral cavity and surrounding skin and the forestomach produced lesions ranging from metaplasia and premalignant lesions to squamous cell carcinomas-in-situ. Later in 1955 (123), he showed that if one used a non-volatile solvent such as mineral oil, U.S.P., the tumor induction period was reduced to 4½ weeks as opposed to 6-7 weeks when using an acetone solvent. Control animals painted with the vehicle only for periods up to 50 weeks exhibited no neoplasia. He also demonstrated, using an in-vivo microscopic spread preparation, that the mineral oil vehicle containing the carcinogen was still distributed in the intercellular spaces of the basal epithelial layer, whereas the same hydrocarbon dissolved in acetone was left as a crystalline residue on the surface epithelium because of the rapid volatilization of the vehicle. It is of interest to note that in 1966 Elzay (39) showed that when DMBA was used in 50% ethyl alcohol, animals developed tumors of a larger size earlier when compared to animals painted with DMBA in mineral oil. These apparent contradictions in results can only be explained by strain differences.

In further studies in 1957 (124), Salley described the early histological changes associated with DMBA carcinogenesis in the buccal pouch and confirmed previous impressions that there were distinct phases of reaction before the actual appearance of definite neoplasmas in the pouch. These included inflammation, degeneration, and regeneration followed by hyperplasia. He also noted that different areas of the pouch appeared to react to the carcinogen at different times resulting in the appearance of lesions ranging from hyperplasia, to frankly malignant tumors, in the same

pouch. In these studies and in a further study in 1961 (127) he showed that epithelial carcinogenesis could proceed without a portal of entry (the presence of accessory structures like sebacceous glands, hair follicles) and saliva - requirements claimed to be neccesary in the initiation of skin carcinogenesis (25, 81, 136), for these were absent in the hamster cheek pouch and it is quite feasible that the carcinogen is brought to the cell by a process of simple absorption through the mucosal surface. Work performed in Salley's laboratory in 1961 (128) using fluorescent microscopy, indicated that DMBA was present both in and beneath the intact mucosa, thus providing further evidence in support of this absorptive phenomenon. In addition, when comparing the latent periods of DMBA and benzpyrene-induced skin and pouch mucosal carcinomas (126) it was found that pouch tumors were manifested earlier than ear lesions, despite the presence of adnexal structures in the ear. Thus the presence of adnexal structures does not appear to promote the carcinogenic response and it seems that the role of these epithelial structures in the process of carcinogenesis in oral tissues, is still open to discussion. In the latter study (126), local treatment with the carcinogen was discontinued after the first appearance of benign neoplasms, but there was no spontaneous regression of these tumors.

In a different study, specifically designed to determine the minimal period of treatment with DMBA required for the development of buccal pouch carcinomas, Morris and Reiskin (99) found that the critical changes took place in animals treated for 4 or more weeks and tumors were found in all animals, sacrificed 7 weeks after cessation of DMBA application. In our laboratory we noted in our strain of hamsters, that animals treated

for 6 weeks with DMBA and then left untreated for a period of 2 months, did not develop carcinomas. This finding was confirmed (78) in a study performed specifically to establish whether irreversible changes leading to carcinoma formation occur after 6 weeks application of DMBA. No ready explanation other than a difference in the response of different strains of animals used was offered to explain these apparently contradictory results in the literature. Similar regression and even disappearance of DMBA induced papillomas was also observed by Elzay in his study performed in 1966 (39).

In other studies, using similar techniques, (21, 100, 144) different authors confirmed Salley's sequence of events in the hamster cheek pouch and carcinomas developed in their animals after periods of time varying from 4-13 weeks, and followed initial epithelial hyperplasia and atypicality. In 1963, Listgarten et al., (82) studying the ultrastructural changes occurring in the pouch epithelium after DMBA application, found that there was widening of the intercellular spaces as early as the second day of application of DMBA. This may facilitate the penetration of the carcinogen into the stratum germinativum. The tumor cells showed clumping of the tonofibrillar elements to the periphery of the cytoplasm with a concomittant depletion of the tonofibrils in the main portion of the cytoplasm - changes related to the appearance of early dyskeratosis in these cells.

Studies on the carcinogenic effects of tobacco tars and cigarette smoke have also been performed on the hamster cheek pouch (30, 38, 94, 95, 147). Tabah (147) showed that 20% of the animals treated this way showed nodules and thickening of the distal end of the pouch. Histologically there was atypical fibroplasia without epithelial atypia.

Moore and Miller (95) also obtained negative results in their animals treated with cigarette tar and no tumors were seen. Histologically there was thickening of the epithelium and a chronic inflammatory reaction was present in the lamina propria. Later, Moore and Christopherson (94) painted exteriorised cheek pouches with cigarette smoke condensate and after 683 days, histological examination revealed hyperkeratosis and slight epithelial hyperplasia. In 1964, Kendrick (71) confirmed that cigarette smoke condensate elicited only transient inflammation and persistent hyperplasia and dysplasia of the pouch mucosa, but no tumors were induced.

Histochemical Studies in Hamster Cheek Pouch Carcinogenesis:

The pouch has also been used to record histochemical observations during experimental carcinogenesis. These studies have been a definite aid to defining the alterations in the activity of various enzymes of human neoplasms. Gardner (53) noted abundant acid phosphatase in the basal half of the stratified squamous epithelium of normal pouches with the heaviest concentration of the enzyme occurring in the basal layer itself and decreasing as cell maturation advanced, resulting in the eventual absence of the enzyme in the superficial layers. In studies in our laboratory this has been confirmed (106) and we have also noted prominent staining of this enzyme in the granular layer, in the surface keratin, and in mast cells present in the lamina propria.

Mori et al. (96) studied the distribution of various enzymes including alkaline phosphatase, acid phosphatase, esterase, β -glucuronidase, aminopeptidase, and succinic dehydrogenase during DMBA carcinogenesis in hamster pouches. They found that alkaline phosphatase activity was present on the surface of the neoplastic epithelium and in

inflammatory cells of the stroma. Normal hamster pouch epithelium is free of this enzyme and it appears that its presence is related to the inflammatory reaction rather than to the advancing neoplasia. Non-specific esterases and β -glucuronidase had moderate to high activity in the germinal layer of the neoplastic epithelium. Succinic dehydrogenase activity was present in the germinal layer only and the activity of malic and lactic dehydrogenase was greater than that of succinic dehydrogenase. The horny layers of the neoplasms were devoid of these three dehydrogenases. However, in this layer acid phosphatase activity was strikingly present. In addition, little or no activity of the above mentioned enzymes was noted in the stromal elements.

In 1958, Salley and Kreshover (125) studied hexokinase, glucose-6-phosphate dehydrogenase (G-6-PD) and phosphogluconate dehydrogenase (PGD) in extracts prepared from hamster pouches painted with DMBA for 1 week, 2-3 and 7 weeks - i.e., in the various stages of inflammation, hyperplasia, premalignant and neoplastic stages of carcinogenesis. They concluded that the changes recorded in PGD showed a greater correlation with the neoplastic state than with the hyperplastic state.

In 1960, Scott et al. (130) also determined enzyme activities in extracts of whole pouches or tumors and tissue from which tumors had been removed, after treatment with DMBA. They found minor fluctuations of all 3 enzymes mentioned above during the inflammatory period. Thereafter hexokinase and PGD fell to 40-50% of control levels and then slowly rose to control values during the period of hyperplasia and tumor formation. In tumors the hexokinase levels were 1.5 - 5.5 times the control levels, whereas in the residual mucosa both these enzymes ranged from 1-3.5 x the

normal values. The G-6-PD increased continuously from 4-5 weeks and activities were 2-5 x the control values in both tumors and surrounding tissues. In further studies (131) they also concluded that changes in PGD showed the greatest correlation with the neoplastic state as compared with the hyperplastic state.

Morris (97) showed that the basement membrane of the epithelium undergoing preneoplastic hyperplasia exhibited increased thickness and staining intensity with the PAS stain in contrast to the unaltered basement membrane prior to this phase. In the papilloma stage the basement membrane was less defined, thinner , and it stained less intensely than in normal and hyperplastic epithelium. In areas of cellular invasion no basement membrane could be demonstrated. In 1961 Morris et al. (100) showed that the sulfhydryl group (SH) concentration of cell free extracts of whole pouches of male hamsters treated with DMBA showed a bi-phasic curve with increased SH at 36 hours followed by a decrease at 60 hours. This was not evident in female pouches. The SH concentration remained low until tumor production and thereafter it rose slowly in the whole pouches.

Correlation of Cytology & Pathology in Hamster Cheek Pouch Carcinogenesis

This experimental model has also been used in studies attempting to correlate between cytological findings and histopathology of tumors, in an attempt to apply the results to human pathology and thereby ascertain whether exfoliative cytology is a reliable reflection of the progress of a clinical lesion. Stahl (144, 145) concluded that there was an accurate correlation between the cytological diagnosis and biopsy and the evolution of experimentally induced carcinomas and in his opinion cytology

reflected accurately the progress of the lesion, particularly in the early stages of carcinogenesis. He found that cytological specimens demonstrated cellular atypia prior to clinical recognition of the tumor. This impression was confirmed by Camilleri and Smith in 1964 (20). In 1965 (21) the same authors concluded that cytological and histological appearances corresponded at all stages carcinogenesis in the pouch. Fishman and Green (46) also confirmed these results. However, studies performed in this laboratory in 1967 (77), showed that cytology reflected the histologic findings during the early reactive changes in the pouch mucosa but that no consistent correlation existed in the critical phase when premalignant lesions and intraepithelial carcinoma were present. We concluded that this was probably due to the thick hyperkeratotic layer overlying these lesions, rendering positive cytology difficult. In the later phases, when infiltrating carcinomas were present, all cytological smears contained tumor cells, in accordance with the presence of malignant cells superficially in the tumor. Randall et al. in 1964 (110) also concluded, using a similar model, that exfoliated cells did not permit a reliable diagnosis regarding the stage of the lesions of the hamster cheek pouch as evaluated by histological criteria.

Factors influencing carcinogenesis in the pouch:

These factors were comprehensively studied by Morris in 1961 (98) in an effort to determine the extent to which some factors represent variables in the experimental production of buccal carcinomas. He concluded that the tissues of the cheek pouch of old hamsters are more resistant to carcinogenic stimuli than those of young hamsters.

No difference in the rate of carcinogenic response was seen between the ages of 3-9 weeks and 5 weeks appears to be the ideal age for hamsters to be used for experimental cheek pouch carcinogenesis. A 0.5% concentration of DMBA appears to be the optimal concentration for the rapid production of malignant tumors in the pouch. This concentration produces maximal response with a minimum latent period and no loss of animals because of toxicity. A shorter latent period is required for tumor development in animals exposed to a carcinogen 3 times per week than in those receiving the carcinogen twice a week. The response to the carcinogen was not related to the sex of the animals, and conditions of caging (3 or 4 hamsters per cage) had no apparent effect on the experimental results.

Co-Carcinogenesis in the Hamster cheek pouch.

Since the advent of Berenblum's concept of the two-phase theory of carcinogenesis (10) consisting of a stage of initiation followed by a stage of promotion, many workers have studied the effect of various agents on the initiation and promotion of experimental carcinogenesis. A number of agents have been clearly shown to be co-carcinogenic in the hamster cheek pouch. Dachi in 1961 and 1962 (26, 27) studied the role of polyoxyethylene sorbitane monostearate (Tween 60) as a promoting agent in DMBA induced carcinoma using this experimental model. He found that the latent period for tumor appearance was shortened by 2-3 weeks when DMBA was dissolved in Tween 60. Renstrup (113, 114) investigated the effect of mechanical irritation upon carcinogenesis. This irritation was provided by a twisted wire ligated around the first molar and projecting into the pouch. No tumors

occurred with chronic irritation alone after 18-24 weeks, but chronic irritation enhanced the appearance of chemically induced tumors which appeared as early as after 4 weeks of treatment when compared to 10 weeks in the animals treated with the carcinogen alone.

In 1959, Rowe and Gorlin (117) showed that a vitamin A deficient diet promoted carcinoma formation in hamsters receiving topical application of DMBA. Silverman and Shklar (139) showed that the known co-carcinogenic qualities of croton oil in other experimental animals (12, 13) were also applicable to this experimental model. They noted, somewhat paradoxically, however, that in young animals (2-3 months old), the application of 1% croton oil retarded the appearance of cancer induced by DMBA, whereas in older animals it resulted in enhancement of carcinogenesis.

In 1965, Hamer (59) was unable to show the co-carcinogenic effect of single X-ray doses of 500-2000 R in young hamsters when used in conjunction with topical application of DMBA to the hamster cheek pouch. At present this aspect of hamster cheek pouch carcinogenesis is under investigation in our laboratory and the effects preceding and simultaneous irradiation on DMBA induced pouch carcinomas is being studied (80).

Elzay has shown recently (39) that 50% ethyl alcohol is co-carcinogenic and hamsters painted 0.5% DMBA in 50% ethyl alcohol, developed larger epithelial tumors earlier than those painted with DMBA in mineral oil. Histologically , these tumors displayed more aggressive qualities than the tumors in other control groups. It is possible that alcohol produces this effect either by acting directly as a local irritant because of its dehydrating action, or by altering the intracellular metabolism in some way , rendering the cells more susceptible to the carcinogen. On the other hand, it may be an excellent vehicle for carrying the carcinogen to the parabasal area.

Both Elzay (40) and Dachi et al. (28) have studied the effect of dimethyl sulfoxide (DMSO) on hamster cheek pouch carcinogenesis. Elzay (40) noted that there was no significant decrease in the latent period of tumors induced when DMSO was added to the DMBA when compared to the latent period of tumors induced by DMBA alone. However, the animals receiving DMSO developed extremely large tumors macroscopically and there was a higher incidence of squamous cell carcinomas in these animals. He attributed this effect to the effective transfer of the carcinogen through the horny layer into the active cellular layer, beneath.Dachi et al. (28), on the other hand, demonstrated that the use of DMSO as a vehicle for DMBA significantly reduced the latent period for tumor production when compared to the usual mineral oil vehicle.

Sabes et al. (119) in 1963, and later Shklar in 1966 and 1967 (134, 135), showed that topical and systemically administered cortisone hastened the development of initial epithelial dysplasia and eventual buccal pouch carcinomas in hamsters treated with DMBA, and although the tumors were not more anaplastic, they appeared to invade the underlying tissue and muscle more extensively . Shklar attributed this "enhancing action" of the cortisone either to its depression of immunologic reactivity, which facilitated deeper penetration of the lesion, or to its antianabolic action on connective tissue enabling easier invasion. However, despite these theories they were unable to demonstrate regional metastasis. In preliminary studies in our laboratory, aimed at assessing the effect of a number of different membrane and lysosomal stabilising agents like cortisone and chloroquine on cheek pouch carcinogenesis, we have been unable to confirm these results (105). On the contrary, cortisone appeared to inhibit and delay the

growth and development of these experimental tumors. This is also compatible with the results of the studies to be discussed in this thesis and with the hypothesis that lysosomes may be involved in the process of cellular proliferation and malignant change.

The influence of male and female sex hormones on this experimental model has not been extensively studied and are at present being investigated in our laboratory. Weatherred and Salley (153), however, have shown that estrogen treatment appears to shorten the latent period slightly, whereas castration appears to lengthen the latent period of tumor induction in male animals. Studies performed in this laboratory (79) have shown that castration of male hamsters prior to application of DMBA does not appear to shorten the latent period of these tumors but seems to inhibit the growth and development of tumors after 12 weeks of application of the carcinogen. After 16 weeks of DMBA application there are less tumors in the castrated animals than in the non-castrated animals. In addition, the injection of estrogen particularly in castrated male animals enhances carcinoma formation in the buccal pouch considerably whereas the injection of testosterone does not effect this process convincingly (104).

Chemotherapy of buccal pouch carcinomas:

Lately, Shklar et al. (133) have surprisingly shown that the simultaneous administration of an anti-tumor agent, methotrexate, enhanced DMBA-induced carcinogenesis in the pouch, and tumors appeared earlier, reached a greater size and appeared more anaplastic than in control groups. It is possible that these results were achieved by too small, obviously non-therapeutic, non-toxic doses of an anti-cancer agent which is known to alter cell metabolism.

In a comprehensive study of the effect of various anti-tumor agents, including methotrexate, vinblastine and cyclophosphamide on buccal pouch carcinogenesis performed in this laboratory, this effect of methotrexate was not confirmed (118). In this study, the therapeutic effect of these agents on intraepithelial and squamous cell carcinomas was studied and in addition these drugs were administered simultaneously with the carcinogen for 6 and 12 weeks. This work represented the first attempt to investigate the therapeutic effect of topically administered cytostatic agents, on both premalignant lesions and carcinomas of the hamster pouch. It was found that cyclophosphamide retarded the growth and development of carcinomas during prolonged concomittant application of the drug and the carcinogen. Vinblastine and methotrexate produced similar but more striking inhibitory and antineoplastic effects when applied in a similar fashion. As far as the therapeutic effects of these agents on pre-existing lesions of the pouch is concerned it was found that cyclophosphamide exhibited only a slight therapeutic effect, whereas the other two drugs produced almost complete regression of the in-situ lesions. When infiltrating carcinomas were treated in an identical manner it was found that methotrexate had no effect . The other 2 agents proved to be more effective therapeutically but nevertheless succeeded in producing only slight regression of tumor growth.

Privileged Immunologic Site:

It is of interest to note that the pouch is a known privileged site for both homo- and heterotransplants (15, 102) and both human and experimental tumors are successfully maintained in the pouch (56, 57, 75, 85). In addition, studies of the vascular pattern of

transplanted tumors, inserted into the pouch have also been performed using a specialised transparent chamber (58, 152). The Sanders-Shubik cheek pouch system for direct observation of the pouch, has also been used in observations concerning the behaviour of tumor cells into the pouch (32). The explanation of this unique immunological phenomenon offered is related to the loose areolar tissue component of the pouch which may prevent host immunologic factors from reaching the grafts. As yet, however, there is no really satisfactory explanation for this finding. Hertz (62) has shown marked suppression of choriocarcinoma transplants in the pouch, by vinblastine, and Goldenberg & Witte (57) have also produced temporary regression in the growth of human colonic carcinoma transplanted into the pouch, using cyclophosphamide and several other drugs.

DMBA - Considerations on its mechanism of function:

DMBA, a polycyclic aromatic hydrocarbon, is probably the most potent skin carcinogen known . The molecular and cellular mechanisms whereby these substances induce and initiate epithelial tumors after topical application is as yet not clear. According to Heidelberger and Giovanella (61) there are three possible mechanisms whereby biologically carcinogenic hydrocarbons initiate tumor growth, and these are: (1) Conversion of normal cells into cancer cells. (2) Selection of pre-existing clones of cancer cells. (3) Activation of a latent oncogenic virus.

The first mechanism is produced according to the authors, either by a genetic change, referred to as a somatic mutation or by some permanent metabolic alteration

not involving a somatic mutation . The latter appears to be more likely but there is no conclusive evidence to suggest that chemical carcinogens are not mutagenic (18). Trainin et al. (150) have also shown that none of a series of chemical mutagens could act as an initiator of carcinogenesis on mouse skin. According to Heidelberger (61) in order for hydrocarbons to cause their carcinogenic effect, they must interact directly with deoxyribonucleic acid (DNA) and he has demonstrated a close correlation between the process of carcinogenesis and the binding of hydrocarbons to a cytoplasmic protein and which has given rise to the attractive theory of protein deletion (103). This theory, however, was not accepted by all, for it was difficult to accept that an alteration in a cytoplasmic protein can be perpetuated and can explain the process of carcinogenesis. Nevertheless, the theories of Jacob and Monod (65), related to enzyme induction, repression and metabolic regulation were incorporated into this theory to provide a feasible and acceptable model for chemical carcinogenesis. This model explains how a perpetuated change could result from a transient interaction of a carcinogen and a cytoplasmic protein. In this system there is deletion of the "repressor" in which the carcinogen is bound. Thus it is possible that carcinogenesis may result from altered metabolic circuity and it seems that it is not essential to involve a direct mutational event in hydrocarbon carcinogenesis, as is so commonly assumed.

In vivo, carcinogens interact with DNA, RNA, and proteins in the target tissues, and Brookes and Lawly (17) have shown that the amount of binding of the hydrocarbons to DNA paralleled their carcinogenic activity. This binding occurs, in vivo, to mouse

skin DNA in the epithelial sheet and in the basal cells. Thus in different in vivo models it appears that the carcinogen is covalently bound to soluble proteins of the target tissue in a fashion quantitatively related to their carcinogenic activities. In addition, the proteins to which the carcinogens are bound, exhibit similar electrophoretic behaviour and are absent from the tumor that is induced.

Evidence has been accumulating to suggest an action of the carcinogen on nucleic acids (90). McCarter and Quastler (88, 89) have shown that DMBA inhibited incorporation of thymidine into DNA in the growing hair follicle of the mouse and the duration of the phase of DNA synthesis is much prolonged in skin cells treated with DMBA . In addition, it has been shown that it inhibited incorporation of cytidine into RNA of the cells of the epidermal basal layer of the mouse (141). These works point to the importance of nucleic acids in the early stages of carcinogenesis and apparently DMBA binds itself to the DNA, by means of co-valent bonds or by intercalation (76).

Recently, Allison in a number of studies (6, 7,) has suggested that it is possible that lysosomal enzymes, activated by diverse carcinogenic agents, may be instrumental in early carcinogenesis, and it appears that there is an increased malignant potential after lysosomal damage. Allison and Malluci (4) have shown that carcinogens enter the cell and are concentrated in preformed organelles (lysosomes). It is possible that they enter as micropinocytotic vacuoles which eventually fuse with the primary lysosomes. Thereafter there is either initial solution of the carcinogen or it is possible that no metabolic conversion takes place for some time (142). Thus the characteristic proteins, to which carcinogens become bound, described by different authors, may well be lysosomal. On the other hand, it appears that in some cells the carcinogens appear to decrease

the stability of lysosomal membranes as shown by the presence of acid phosphatase staining after the carcinogen is taken up. Allison (2, 3) has suggested that low concentrations of carcinogens could bring about a gradual release into the cytoplasm, over long periods of time, of enzymes such as RNAse, DNAse and protease which may interfere with the mechanism controlling division of cells, without impairing their capacity to divide.

In 1964, Meskin & Woolfrey (92), using DMBA labelled with carbon 14, found that immediately after topical application, there is a rapid absorption of the carcinogen through the intact pouch epithelium, and after 24 hours there is an increased amount of carcinogen in the submucosa and a decrease in the epithelium. The submucosal radioactivity was much lower than the surface epithelial radioactivity of 48 hours. Mononuclear cells tended to localise to the site of increased submucosal radioactivity after multiple paintings. The leucocytic infiltration bore no relationship to the radioactive foci.

In 1962, Delarue et al. (31) showed that the introduction of DMBA into the pouch first caused blocking of the circulation with white blood cells emerging from the vessels, and by agglutinating erythrocytes . Subsequently vasodilatation and edema appeared with the formation of a capillary network around the treated zone.

In studies of the proliferation kinetics in the epithelium of the cheek pouch during DMBA carcinogenesis , using tritiated thymidine, Reiskin and Berry (112) showed that there were quantitative correlations between the mean tumor latent period and the subsquent growth rate of the tumors which developed. There were also significant

differences in the mean latent periods and growth rates of tumors, in the different strains of hamsters used in the study.

Vitamin A and keratinisation and epithelial differentiation:

Vitamin A is a fat soluble vitamin known to influence the process of keratinisation and epithelial differentiation (93). One of the major functions of the vitamin is to maintain the normal epithelial structure and it is vital for the maintenance of the skin, corneal epithelium and epithelium of the genito-urinary and upper respiratory tracts. In 1925, Wolbach and Howe (159) described the tissue changes in animals following deprivation of vitamin A and these included xerosis, and keratinisation of epithelial tissues in the eye, salivary glands, respiratory tract and genito-urinary tracts. They also noted that the administration of the vitamin in advanced deficiency resulted in restoration of the altered tissues to normal . In vitamin A deficiency there is epithelial atrophy and substitution of the epithelium by metaplastic stratified squamous keratinising epithelium arising from the proliferation of basal cells. In other studies (160) the vitamin was administered to deficient animals and the process of repair was studied. This repair was abrupt and involved the removal and disposal of keratinised cells and and maturation of the deeper cells. In 1932, Harris (60) noted that infections occurred commonly in rats fed a vitamin A free or deficient diet, mostly in areas related to keratinisation, and particularly in the salivary glands where secretory epithelium lost it's normal character and underwent squamous metaplasia . These changes were reversible. Thus it appears that vitamin A maintains the normal status, health and function of mucous secreting surface.

In 1951 Sabella et al. (120) administered an excess of topical vitamin A to rat skin for 10 days and noted an increase in epidermal thickness, to about twice it's normal width, with a prominent increase in the stratum granulosum and a decreased rate of keratin formation . Estrogen did not counter the stimulatory effect of the vitamin. Bern et al. (14) treated the epidermis of male rats similarly with topical vitamin A in sesame oil and also produced acanthosis, a reduction of the intercellular bridges and mild parakeratosis. Lawrence and Bern (72) also showed rapid epidermal proliferation and hypertrophy of mouse epidermis and an increase in the mitotic index in response to excess vitamin A. This finding had been demonstrated earlier by Alov (8) and was also confirmed by Sherman (132) using albino rats in 1961 . He showed however that the administration of physiological amounts of the vitamin caused a significant increase in the mitotic index of epithelia while higher, toxic, doeses significantly diminished mitotic activity. A significant reduction of the mitotic rate was found in the epithelia of vitamin A deficient rats. Thus it appears that vitamin A is a definite factor in the regulation of the mitotic activity of epithelial cells.

Lawrence et al. (73) studied the effects of vitamin A pellets on the hamster buccal pouch mucosa and showed that it produced a thicker epithelium and granular layer with an increase in the number of keratohyaline granules. In addition the epithelial cells appeared more ovoid and there was an increase in mitoses. In response to moderate doses of the vitamin there was interference with keratinisation , less epithelial hypertrophy but more atypicality of the basal cells with hyperchromasia of nuclei, increased mitoses and mucoid metaplasia . In response to the highest doses

of the vitamin there was widespread epithelial necrosis and cell death with edema of the submucosa and an extensive infiltration with leukocytes. Lawrence and Bern also demonstrated the formation of mucoid metaplasia and mucous gland formation in keratinised adult epithelium treated with vitamin A (74). In 1963, Fitton Jackson and Fell (45) studied the fine structure of embryonic chicken skin during atypical differentiation induced by vitamin A. They found disorientation of the basal layer, the appearance of intercellular lacunae, disappearance of the tonofilaments, arrest of keratinisation, enlarged mitochondria and the presence of intercellular mucus secretions with microvilli.

In 1954 (68, 69) Kahn demonstrated that when vitamin A was applied topically to rat vagina it inhibited the estrogen-induced cornification of vaginal epithelium and resulted in metaplasia to a stratified cuboidal epithelium. SH activity was also inhibited. In other studies of vaginal rat epithelium, in tissue cultures, it was shown that vitamin A in the presence of estrogen did not prevent cornification but only delayed it (70). In 1953 Fell and Mellanby (41) using tissue cultures, demonstrated that chick ectoderm underwent mucoid metaplasia in the presence of excess vitamin A. This suppressed keratinisation and caused the ectoderm to differentiate into mucus secreting, often ciliated epithelium, in contrast to control cultures which produced keratinisation. This metaplasia is the reverse of the changes obtained in vitamin A deficiency in nasal epithelium where there is metaplasia of mucous secreting epithelium to keratinising squamous epithelium. Lately Cavalaris and Krikos (22) introduced paraffin rods containing excess amounts of vitamin A into the hamster cheek pouch and also caused inhibition of

keratinisation. After 10 days there was mucoid metaplasia with areas of hyperplasia and parakeratosis, cytoplasmic vacuolation and moderate acanthosis. After 15 days there was even more interference with keratinisation, and focal goblet cell metaplasia appeared. Rothberg (116) has also recently, successfully cultivated metatarsal skin of embryonic chicks for periods of up to 11 days and has shown that vitamin A when administered to the medium inhibited keratinisation and promoted mucopolysaccharide (MPS) synthesis. Vitamin A is also known to influence biological membranes including those of lysosomes and is important for regulating their stability. This effect is achieved by adsorption of the vitamin, which is hydrophobic, to the membranes and it's importance and significance will be reviewed in detail during the discussion of our results (pages 56-60). Furthermore vitamin A, because of it's anti-keratinising action has been used in the therapy of certain oral and skin diseases characterised by abnormal or exaggerated keratinisation. This aspect and what is known about the effect of the vitamin on experimental tumorigenesis will also be reviewed during the discussion of the results of these studies (page 60-65).

These established facts about the role of this mitogenic vitamin in epithelial differentiation, the process of keratinisation and it's regulating influence on cellular and subcellular membranes together with the lack of information and understanding of it's mechanism of action during carcinogenesis, stimulated the present series of studies which were begun in 1967.

PURPOSE OF THE STUDIES

The purpose of these studies was to assess the effect of vitamin A, a mitogenic agent and membrane and lysosomal labiliser which influences epithelial differentiation and keratinisation, on experimental chemical carcinogenesis. The model chosen to be used in these studies was the hamster cheek pouch an organ with which the author was well acquainted, and one which for reasons already discussed is particularly suitable for the study of experimental carcinogenesis. The carcinogen selected was 9, 10-dimethyl-1, 2-benzanthracene (DMBA) one of the most potent of the carcinogens used in experimental skin tumorgenesis and one known to produce infiltrating carcinomas in the hamster cheek pouch, by topical application, after a relatively short latent period (up to 12 weeks). It was hoped that these studies would throw new light on the role of vitamin A in experimental carcinogenesis and provide information on the mechanism of action of this agent during carcinogenesis.

Prior to the execution of the above studies it was necessary to establish the response of our local stock of hamsters to topical application of DMBA and to assess the sequence of events in the pouch mucosa after exposure to the carcinogen (Experiment 1). Thus hamster pouches were treated with a 0.5% solution of DMBA during periods of 2, 4, 6, 8, 10, 12 and 14 weeks. A similar study was performed, using different concentrations of vitamin A palmitate (10 and 20% solutions), in order to assess the changes recorded after topical application of this agent to the pouch mucosa and to establish which concentration of the vitamin would be most suitable for our purposes with the least toxicity. Accordingly,

pouches were painted topically with 10% and 20% vitamin A palmitate during periods of 2, 4, 6, 8, 10 and 12 weeks (Experiment 2). At the same time the effect of the vehicle used , in this case paraffin oil, was also determined in our animals, in order to study the possibility that some of the changes to be recorded were due to this agent.

In order to assess the effect of vitamin A on carcinogenesis a series of different experiments were designed to determine:-

(a) The effect of simultaneous application of the vitamin and the carcinogen on the process of carcinogenesis (Experiment 3). Furthermore, in the light of the fact that the vitamin is used therapeutically, in humans in a topical manner, we felt it important to determine:-

(b) The effect of topical application of the vitamin on premalignant lesions of the pouch, induced by the application of DMBA during a period of 6 weeks . (Experiment 4).

(c) The effect of similar therapy on benign hyperkeratotic lesions of the pouch (comparable to leukoplakia in the human), induced by 4 weeks of DMBA application. (Experiment 5).

(d) The effect of pretreatment of the pouch mucosa with topical vitamin A during 10 weeks, on subsequent DMBA induced carcinogenesis. (Experiment 6).

It was hoped that after these studies we would be able to conclude whether the vitamin had any effect on the initiation and promotion of DMBA carcinogenesis in the hamster

cheek pouch and if so what the underlying mechanism of this influence was. Furthermore it was felt that these studies might have some clinical and therapeutic significance in relation to human pathology despite the obvious inadequacy of a direct application of results obtained in animals to human conditions.

MATERIALS & METHODS

The animals used in all these studies were male Syrian golden hamsters (Cricetus auratus) of the local stock of the Hadassah-Hebrew University Medical School, Jerusalem, Israel. A total of 234 animals, weighing 55-65 grams and aged 1.5-2 months old, were used in all the experiments. They were kept 3-5 in a cage, fed Purina Lab. Chow and tap water ad libitum. The right cheek pouches were always used throughout the experiments and were painted three times per week with the different solutions, using a no. 4 camel's hair brush. The left cheek pouches served as untreated controls. The painting was performed by the same worker in all studies, in the following manner:- the brush was dipped into the solution, excess was allowed to drip off and then the pouch was stroked firmly several times along its entire length. It was estimated that an average of 0.2 ml. paraffin could be introduced into the pouch on each painting of the pouch.

The carcinogen - 9.10 dimethyl - 1,2 benzanthracene - DMBA (7, 12 Dimethyl (a) anthracene, 1,4 - dimethyl - 2,3 - benzphenanthrene,) Sigma Chemical Co., was dissolved in liquid paraffin (USP) of specific gravity of 0.88 - 0.91.A 0.5% (500 mg/100 ml paraffin) concentration of the DMBA was prepared monthly and was kept in a tightly closed dark container, at room temperature. Vitamin A Palmitate (E. Merck A/G,

Darmstadt; 1.7 million units ≠ g.) was used and prepared as a 10% or 20% solution in liquid paraffin on a weight to volume basis. This solution was also stored in a tightly closed dark container at room temperature.

When both substances were applied together, a solution was prepared containing both 0.5 g. DMBA and 10 or 20 g. Vitamin A palmitate per 100 ml. of liquid paraffin.

At the end of all the experiments, complete autopsies were performed. Both pouches were examined and the regional lymph nodes were dissected out with special care. The organs were fixed in 10% formalin or Bouin's solution, embedded in paraffin and stained with hematoxylin and eosin.

A series of different experiments were planned as follows:-

Experiment 1. Designed to assess the sequence of events during DMBA carcinogenesis, in the pouches of our local stock of hamsters.

Groups of 6 male Syrian golden hamsters were used, and the right cheek pouches were painted with DMBA 3 times per week, for maximal periods of 14 weeks. At bi-weekly intervals after the initial painting, groups of 6 animals were killed and autopsies were performed. Two groups, both painted for 14 weeks, were allowed to survive for an additional 2 months after the final paintings.

Experiment 2. To assess the sequence of events after the application of different concentrations of vitamin A palmitate in the pouches of our local stock of hamsters. 42 animals were used and the right cheek pouches were painted 3 times per week with one of the following materials:

(1) Vitamin A palmitate 10% in liquid paraffin (18 animals)

(2) " " 20% " " (18 animals)

(3) Liquid paraffin only (6 control animals)

Three experimental animals of each group and one control animal were sacrificed after treatment for 2, 4, 6, 8, 10 and 12 weeks respectively.

Experiment 3. To assess the effect of simultaneous application of vitamin A and DMBA on the process of hamster cheek pouch carcinogenesis.

54 animals were used and the right cheek pouches were painted 3 times per week with one of the following substances or combinations dissolved in liquid paraffin.

1) 0.5% DMBA only (18 animals, DMBA group)

2) 0.5% DMBA combined with 10% vitamin A palmitate (18 animals, DMBA-A group)

3) 10% vitamin A palmitate only (18 animals, A group)

3 animals from each group were sacrificed after treatment for 2, 4, 6, 8, 10 or 12 weeks respectively.

Experiment 4. To study the effect of topical application of vitamin A on established premalignant lesions of the pouch, induced by topical application of DMBA during a period of 6 weeks.

The right cheek pouches of 24 animals were painted 3 times per week for 6 weeks with a 0.5% solution of DMBA in liquid paraffin. After discontinuation of DMBA, 6 animals (Group 1) were sacrificed immediately. The remaining 18 animals were

sacrificed 2 months later. During this 2-months' period, the right cheek pouches of 6 animals (Group II) were painted 3 times per week with a 10% solution of vitamin A palmitate in liquid paraffin and the pouches of 6 other animals (Group III) were painted 3 times per week with liquid paraffin only. The remaining 6 animals (Group IV) were left without treatment throughout the 2-months' period.

Experiment 5. To study the effect of topical application of vitamin A palmitate on benign hyperkeratotic lesions of the pouch, (comparable to human leukoplakia), induced by 4 weeks application of DMBA.

The right cheek pouchs of 24 animals were painted 3 times per week with a 0.5% solution of DMBA in liquid paraffin. After cessation of DMBA, 6 animals (Group 1) were sacrificed immediately. The remaining 18 animals were sacrificed 2 months later. During this period the right cheek pouches of 6 animals (Group 2) were painted 3 times per week with a 10% solution of vitamin A palmitate in liquid paraffin and 6 other animals (Group 3) were treated in a similar fashion with liquid paraffin only. The remaining 6 animals were left without treatment throughout the 2 months' period. (Group 4).

Experiment 6. To study the effect of pretreatment of the pouch mucosa with topical vitamin A during 10 weeks, on subsequent DMBA induced carcinogenesis.

The right cheek pouches of 24 animals were painted three times per week for 10 weeks with 10% vitamin A palmitate in liquid paraffin, Immediately thereafter 6 animals were sacrificed (Group 1). The remaining 18 animals were sacrificed after an additional period of 6 weeks. During this period, 6 animals were left untreated (Group 2). In 6 other

animals (Group 3), the right cheek pouches were painted 3 times per week with a 0.5% solution of DMBA in liquid paraffin. The right cheek pouches of the remaining 6 animals were painted in a similar manner as described above with liquid paraffin alone (Group 4).

In addition, another group of 12 male Syrian golden hamsters, 1.5 to 2 months' old and weighing 55-65 gm. were left untreated for a period of 10 weeks, while Groups 1-4 were treated with vitamin A. Subsequently the right cheek pouches of 6 of these animals (Group 5) were painted three times per week during 6 weeks with a 0.5% solution of DMBA in liquid paraffin, and the right cheek pouches of the remaining 6 animals were painted during the same period, in a similar fashion, with liquid paraffin only (Group 6). The animals were sacrificed immediately after this 6 week period.

RESULTS

Experiment 1:

Histologically, the normal cheek pouch has a lining consisting of keratinised squamous epithelium, four to six cell layers thick. Rete pegs were almost absent. (Fig. 1).

After 2-4 weeks of painting, histology revealed inflammatory changes in the pouch mucosa and submucosa. The inflammatory infiltrate consisted of neutrophils, lymphocytes and mononuclear cells. On occasion ulceration and necrosis of the epithelium were seen.

After 4-6 weeks of painting, histology showed areas of hyperplastic epithelium

(Fig. 2), with parakeratosis, hyperkeratosis, irregular acanthosis and presence of rete pegs. In addition, there was marked inflammation of the epithelium and of the submucosa, with increased vascularity.

In animals sacrificed after 7-8 weeks of painting, histology revealed lesions similar to those described in the previous group. In the same pouches there were benign papillomata (Fig. 3) and areas with hyperkeratosis, parakeratosis, acanthosis with loss of polarity of cells, nuclear atypicality and a small number of mitoses. These areas were considered as early premalignant lesions.

Histology in animals sacrificed after 9 and 10 weeks demonstrated the changes described in the previous groups. Furthermore, all animals showed areas of acanthosis, prominent loss of polarity of cells, nuclear atypicality, numerous abnormal mitoses and dyskeratosis with an intact basal layer (Fig. 4-8). Pronounced hyper-and para- keratosis was a constant finding . This lesion was diagnosed as carcinoma in situ (intraepithelial carcinoma). In addition, there were areas of carcinoma in situ in papillomata (Fig. 9).

Infiltrating squamous cell carcinoma was found for the first time in animals sacrificed after 11 weeks of painting. In addition, these animals showed lesions similar to those found in the previous groups. The basal layer of the carcinomata was always irregular and small groups of malignant cells were seen invading the submucosa (Figs. 10 & 11). These lesions were covered by a prominent layer of keratin, but occasionally small groups of malignant cells had reached the surface.

All the animals sacrificed after 12-14 weeks had squamous cell carcinoma and

invariably malignant cells had reached the surface of the lesions . The tumors were large, with marked necrosis, ulceration and inflammation. Smears taken at this stage showed malignant cells with large nuclei, irregular chromatin clumping, accentuation of the irregular nuclear borders, basophilic cytoplasm, indefinite cell outlines and an increased nuclear-cytoplasm ratio. Other malignant cells had irregular markedly hyperchromatic nuclei and eosinophilic cytoplasm.

Animals kept alive for a period of 2 months after 14 weeks of painting showed large necrotic and ulcerated malignant tumors. There was no metastasis to cervical lymph nodes or to other organs. The oesophagus and fore-stomach showed marked hyperkeratosis but no premalignant lesions were found.

Experiment 2:

The results are summarized in Table I. No pathological changes were found in the animals treated with liquid paraffin only. The changes to be described were present in all animals of each group at the various stages of the experiment.

Animals Treated with a 10% Solution of Vitamin A

Macroscopically, no abnormalities were seen in the pouches of the animals at any stage of the experiment.

After 2 weeks, no histological changes were present. By the 4th week there was focal, mild regular acanthosis, focal parakeratosis, and slightly decreased keratinization. After 6 weeks, there was more pronounced epithelial hyperplasia, and focal, dense lymphocytic infiltrates were present in the lamina propria.

Marked acanthosis with mild cellular pleomorphism and occasional rete peg formation were noted by the 8th week, and there was a thin layer of pale staining keratin on the surface. After both 10 and 12 weeks there was also marked acanthosis, but the epithelial atypicality was more obvious, and in some areas cells with enlarged, hyperchromatic nuclei were present on the epithelial surface.

No mucoid metaplasia was found after the application of 10% vitamin A.

Animals Treated with a 20% Solution of Vitamin A

No changes were noted after 2 weeks. After 4 weeks, there was a diffuse, subacute inflammatory infiltrate in the lamina propria consisting of neutrophils, lymphocytes, and plasma cells. There was focal acanthosis with formation of rete pegs and mild pleomorphism was noted, with occasional irregular hyperchromatic nuclei. (Fig. 12). A thin layer of pale staining keratin was present in some areas, but keratinization was mostly absent.

By the 6th week there was a diffuse, dense chronic inflammatory infiltrate in the lamina propria consisting of lymphocytes, histiocytes, plasma cells and numerous mast cells. Marked focal acanthosis was present, with moderate epithelial atypicality. (Fig. 13). Rete peg formation was prominent. Some areas showed epithelial atrophy. A striking feature was the presence of mucoid metaplasia, with large vacuolated columnar cells, resembling goblet cells, in the epithelium, and gland-like structures in the lamina propria (Fig. 14). Generally, there was little keratin on the surface.

After 8 weeks, the lesions were essentially similar, but there was more cellular atypia. Throughout the entire epithelium, and occasionally on the surface, there were

epithelial cells showing irregular hyperchromatic nuclei (Figs. 15, 16). Elongated rete pegs containing atypical cells extended into the lamina propria, but infiltrative growth was never found. However, in one area a histological picture compatible almost with early infiltrating carcinoma was encountered (Fig. 17). Keratinization was absent, but keratohyaline granules were prominent. Mucoid metaplasia was found, as after 6 weeks. The diffuse inflammatory infiltrate had increased in intensity, and in some areas it had acquired a nodular pattern. These infiltrates were dense, and consisted of lymphocytes, plasma cells, and histiocytes, some of which had atypical nuclei (Figs. 18-23). The lesions after 10 and 12 weeks were identical to those seen after 8 weeks.

Table I. Results of local treatment of hamster cheek pouches for varying periods of time with 10% and 20% solutions of vitamin A palmitate in liquid paraffin

Duration of treatment	Percent. of vitamin A	Inflammation	Mucoid metaplasia	Keratinization	Acanthosis	Cellular atypia
2 weeks	10%	—	—	normal	—	—
	20%	—	—	normal	—	—
4 weeks	10%	—	—	slightly decreased	focal mild	—
	20%	moderate subacute	—	decreased	focal marked	mild
6 weeks	10%	moderate chronic	—	decreased	focal moderate	—
	20%	severe chronic	+	decreased	focal marked	moderate
8-12 weeks	10%	moderate chronic	—	decreased	focal marked	mild to moderate
	20%	severe chronic	+	decreased	focal marked	marked

Experiment 3:

The results are summarized in Table 2.

Two weeks

DMBA group: There was severe acute inflammation with focal ulceration of the mucosa, which extended into the lamina propria. Occasionally there was mild localized para-and hyperkeratosis, acanthosis and a striking increase in vascularity in the lamina propria. Some small blood vessels were occluded by fibrin thrombi.

DMBA-A group: Essentially similar changes were seen but there were only a few areas of mild hyperkeratosis and acanthosis. Most of the epithelium was atrophic and covered by a thin layer of keratin.

A group: No changes were noted.

Four weeks

DMBA group: Hyperkeratosis was now prominent in some areas. Acanthosis was present with occasional rete peg formation (Fig. 24). In one of the pouches there was a mild degree of pleomorphism of the epithelium. The submucosa showed proliferation of granulation tissue with residual inflammation.

DMBA-A group: In general there was atrophy of the epithelium and only mild hyperkeratosis and inflammation. However, there were focal areas of marked acanthosis and loss of polarity, with prominent epithelial atypicality and dyskeratosis. Occasionally cells with large hyperchromatic irregular nuclei reached the surface of the lesions. These areas were interpreted as intraepithelial carcinoma (Fig. 25). An

interesting finding was the presence of nodular areas of hyalinized connective tissue, surrounded by granulation tissue with many fibroblasts.

A group: There was focal mild regular acanthosis, focal parakeratosis, and a thin layer of keratin on the surface.

Six weeks:

DMBA group: Macroscopically, there were a few small papillary tumors present, Histology showed foci of prominent hyperkeratosis and acanthosis, benign papillomas and occasional areas of intraepithelial carcinoma.

DMBA-A group: The most striking feature was the presence of many foci of intraepithelial carcinoma and occasional areas of infiltrating squamous cell carcinoma (Fig. 26). Both in tumorous and nontumorous epithelium, there were groups of cells which showed mucoid metaplasia, with vacuolization of the cytoplasm (Fig. 27) and formation of gland-like structures (Fig. 28-30).

A group: There were foci of acanthosis with overcrowding of epithelial cells. Occasionally there was a dense lymphocytic infiltrate in the lamina propria.

Eight weeks:

DMBA group: The mucosa was irregular and small papillomatous tumors, 2-3 mm in diameter, were present. Histologically the lesions included epithelial hyperplasia with prominent hyperkeratosis, benign papillomas and intraepithelial carcinoma. There were occasional foci of infiltrating carcinoma.

DMBA-A group: There was diffuse irregularity of the mucosa and large tumors, 3-5 mm in diameter, were seen. These tumors were larger and more irregular than those in the

DMBA group. Histologically, the most outstanding feature was the presence of many foci of intraepithelial carcinoma and of infiltrating squamous cell carcinoma with marked pleomorphism and atypicality. There was mucoid metaplasia with formation of gland-like structures in the infiltrating tumors. Nontumorous areas showed diffuse epithelial hyperplasia and some foci of mucoid metaplasia, but the marked hyperkeratosis present in the DMBA group was absent and the epithelium was covered by a thin layer of pale staining keratin. The submucosa showed a dense infiltrate with many mast cells and fibroblasts.

A group: The pouch epithelium showed areas with marked acanthosis, hyperplasia and mild cellular pleomorphism. In most areas there was only a thin layer of pale staining keratin.

Ten and twelve weeks:

In the DMBA-A group there were more and larger tumors present than in the DMBA group. The diameter of the largest tumor was 0.8 cm in the DMBA group (Fig. 31) and 1.5 cm in the DMBA-A group (Fig. 32). In areas showing no gross tumors, lesions of a nature similar to those described after 8 weeks were present.

In the A group there were foci of marked acanthosis and moderate atypicality of cells (Fig. 33). In some acanthotic areas cells with large hyperchromatic nuclei were present on the surface (Fig. 34). No foci of mucoid metaplasia were present.

No spontaneous deaths occurred throughout the experiment.

TABLE 1. Incidence of Intraepithelial and Infiltrating Carcinoma in Hamster Cheek Pouches after Treatment with DMBA and with Vitamin A Alone or in Combination for Varying Periods of Time

Treatment	4 weeks	6 weeks	8 weeks	10 and 12 weeks
DMBA	Focal acanthosis in all three pouches, with mild pleomorphism in one pouch	Minute benign papillomas and occasional foci of intraepithelial carcinoma in all three pouches	One or two papillary tumors, 2 to 3 mm in diam., with multiple areas of intraepithelial carcinoma and rare foci of invasive growth in all three pouches	One to three papillary tumors, 2 to 8 mm in diam., with intraepithelial and invasive carcinoma in all six pouches
DMBA + vitamin A	Atrophy of epithelium; marked focal acanthosis and foci of intraepithelial carcinoma in all three pouches	Minute papillomas, multiple foci of intraepithelial carcinoma and rare foci of invasive growth in all three pouches	One to three papillary tumors, 3 to 5 mm in diam., with many foci of intraepithelial and invasive carcinoma in all three pouches	One to three papillary tumors, 5 to 15 mm in diam. in all pouches, all showing extensive infiltrative growth of carcinoma
Vitamin A	Mild regular focal acanthosis in all three pouches	Mild focal acanthosis in all three pouches	Marked focal acanthosis with mild pleomorphism in all three pouches	Marked focal acanthosis with moderate pleomorphism in all six pouches

Experiment 4:

The findings are summarized in Table 3.

Group I: In all treated pouches there was irregularity of the mucosa with contraction of the distal end of the pouch, and 1 or 2 papillary tumors, 1-2 mm in diameter, were found in every animal (Fig. 35).

Histologically, there was diffuse marked hyperkeratosis, and focal acanthosis which was at times irregular, with mild epithelial atypicality. There was rete peg formation and inflammation with fibrosis of the lamina propria. In addition, small foci of intraepithelial carcinoma were present in all pouches, with irregular acanthosis, severe cellular atypicality and loss of polarity, but without invasive growth (Fig. 36). The macroscopic tumors were all benign papillomas,

some of which showed mild epithelial atypicality. No infiltrating carcinoma was found in any of the animals.

Group II. In all treated pouches 2 to 6 irregular tumors were present, up to 8 mm in diameter (Fig. 37). The non tumorous mucosa was irregular.

Histologically all tumors were markedly pleomorphic squamous cell carcinomas with extensive infiltrative growth (Fig. 38). Many tumors were partially necrotic, ulcerated and hemorrhagic. Occasional tumor cells showed ballooning and vacuolization of the cytoplasm. Many tumor cells were present at the surface of the tumors, and there was less mature keratin present than in Group I. In addition, multiple foci of intraepithelial carcinoma were found. Other areas showed acanthosis with less keratinization than in Group I.

Group III: In all treated pouches there was irregularity of the mucosa. One animal showed a single papillary tumor, 3 mm in diameter, in the proximal end of the pouch (Fig. 39). No tumors were seen in the remaining 5 animals.

Histologically the general finding in all pouches was diffuse, marked epithelial atrophy (Fig. 40). The tumor seen microscopically was a papilloma with areas of marked atypicality and loss of polarity, but without invasive growth. These areas were regarded as intraepithelial carcinomas (Fig. 41). In the grossly nontumorous mucosa there were occasional foci of irregular acanthosis with moderate cellular atypicality, but without loss of polarity. These foci were therefore not regarded as intraepithelial carcinoma. No infiltrative growth was present.

Group IV: In all treated pouches there was irregularity of the mucosa, with contraction of the distal end. Solitary papillary tumors, 1-2 mm in diameter, were seen in 2 pouches (Fig. 42). One animal showed 2 tumors of the same dimensions. No tumors were seen in the remaining 3 animals.

Histologically, all pouches showed areas of epithelial atrophy with hyperkeratosis. There was focal acanthosis, with occasional cellular atypicality (Fig. 43). Less pleomorphism was present than in Group I or II, and no loss of polarity was seen. These lesions were not regarded as intraepithelial carcinomas. The macroscopic tumors were all benign papillomas, 1 of which showed foci of moderate cellular atypicality. There were no intraepithelial or infiltrating carcinomas.

Table 3: Results of local treatment of hamster cheek pouches with DMBA, followed by a treatment-free interval or by local application of vitamin A or paraffin (Each group consisted of 6 animals).

Group	Treatment	Macroscopic appearance	Number of tumors	Diameter of tumors	Microscopic appearance
I	DMBA 6 wk, killed immediately	Irregular mucosa; small papillary tumors in all animals	1-2	1-2 mm	Diffuse hyperkeratosis and focal acanthosis benign papillomas; foci of intra-epithelial carcinoma
II	DMBA 6 wk, followed by vitamin A for 2 months	Irregular mucosa; multiple large tumors in all animals	2-6	5-8 mm	Anaplastic infiltrating squamous cell carcinomas; multiple foci of intra-epithelial carcinoma.
III	DMBA 6 wk, followed by paraffin for 2 months	Irregular mucosa; small papillary tumor in 1 animal	0-1	3 mm	Epithelial atrophy and focal acanthosis with occasional cellular atypicality; papilloma with intra-epithelial carcinoma.
IV	DMBA 6 wk, no further treatment, killed 2 months later	Irregular mucosa; small papillary tumors in 3 animals.	0-2	1-2 mm	Epithelial atrophy and hyperkeratosis; focal acanthosis with occasional cellular atypicality; benign papillomas in 3 animals.

Experiment 5:

The findings are summarized in the Table 4.

Group I: In all the treated pouches irregularity of the mucosa was present, but no macroscopic tumors were seen. Histologically there was marked diffuse hyperkeratosis, focal parakeratosis, and regular acanthosis without cellular atypicality. There was mild chronic inflammation and fibrosis of the lamina propria. These lesions were regarded by us as compatible with leukoplakia (Fig. 44).

Group II: In all the treated pouches 1 to 6 irregular tumors from 1 mm to 6 mm in diameter were present (Fig. 45). The nontumorous mucosa was irregular. Histologically the tumors were either infiltrating squamous cell carcinomas (Fig. 46) or papillomas with intraepithelial carcinoma. Tumor cells were occasionally seen on the surface and frequently showed ballooning and vacuolization of the cytoplasm. Multiple foci of intraepithelial carcinoma were present in macroscopically non tumorous areas, and generally the epithelium showed lack of keratinization, and atrophy with chronic inflammation and fibrosis in the lamina propria. A striking feature was the appearance of dense infiltrates in the lamina propria and submucosa. Consisting of lymphocytes and other nononuclear cells. These lesions were occasionally nodular or polypoid and were often present in the region of infiltrating carcinoma.

Group 3: In all the treated pouches there was irregularity of the mucosa (Fig. 47) but no tumors were present. Occasionally there was contraction of the distal end of the pouch due to fibrosis in the lamina propria. Histologically the epithelium was of normal thickness, with areas of atropy. There were foci of leukoplakia with hyperkeratosis

but with less pronounced acanthosis than in Group 1 (Fig. 48).

Group 4: All the treated pouches had a smooth mucosa except for 1 that showed irregularity, contraction and ulceration of the distal end (Fig. 49). Histologically, there were no areas of epithelial atypicality, and no carcinoma were present in any of the pouches. Occasional lesions identical to those described in Group 3 were present (Fig. 50) but in general the epithelium was of normal thickness, with areas of atrophy.

Table 4: Results of local treatment of DMBA-induced Benign Hyperkeratosis in the hamster cheek pouch with vitamin A, compared with results in animals left untreated, or treated with paraffin (Each group consisted of 6 animals).

Group	Treatment	Macroscopic appearance	Number and size of tumors	Microscopic appearance
I	DMBA for 4 wk., killed immediately	Irregular mucosa	None	Leukoplakia
II	DMBA for 4 wk., vitamin A for 2 months	Irregular mucosa; multiple large tumors	1-6 tumors in each pouch, 1-6 mm. in diameter	Infiltrating squamous cell carcinomas; multiple foci of intraepithelial carcinoma
III	DMBA for 4 wk., paraffin for 2 months	Irregular mucosa	None	Epithelium generally of normal thickness: areas of atrophy and focal leukoplakia
IV	DMBA for 4 wk., no further treat- for 2 months	Smooth mucosa in 5, irregularity and contraction of distal end in 1	None	Epithelium generally of normal thickness; areas of atrophy and occasional leukoplakia

Experiment 6:

The results are summarized in Table 5.

Group 1: Macroscopically the pouch mucosa appreared smooth. Histologically there were foci of mild to moderate acanthosis, and occasionally slightly atypical epithelial cells were noted. In some areas there was a dense inflammatory infiltrate present in the lamina propria, consisting of lymphocytes and mononuclear cells.

Group 2: Macroscopically the mucosa was smooth. Histologically there was focal epithelial atrophy with a thin layer of keratin on the surface. Generally the mucosa appeared normal. Occasional foci of fibrosis were present in the lamina propria.

Group 3: Macroscopically there was irregularity of the pouch mucosa in all animals. One animal had 2 papillary tumors, 2 mm in diameter, in the distal end of the pouch. Another animal exhibited a solitary tumor, 7 mm in diameter, in the center of the pouch. Histologically, these 3 tumors were infiltrating squamous cell carcinomas. In addition, there were occasional foci of intraepithelial carcinoma present in all pouches. These foci exhibited acanthosis with loss of polarity and with nuclear atypia and hyperchromasia in all layers of the epithelium. However, the basement membrane appeared intact, and no infiltrative growth was present. The main finding in the non-tumorous epithelium was diffuse hyperkeratosis and acanthosis, with occasional focal moderate epithelial atypicality. A mononuclear inflammatory infiltrate was present in the lamina propria.

Group 4: Macroscopically the pouch mucosa was smooth, and no tumors were present. Histologically, a few areas of epithelial atrophy were present, but generally the mu-

cosa appeared normal and no atypicality was seen.

Group 5: Macroscopically no tumors were present, but there was slight irregularity of the mucosa. Histologically, no intraepithelial or invasive carcinomas were present. All sections examined showed diffuse hyperkeratosis and acanthosis, and occasional mild focal epithelial atypicality.

Group 6:

Macroscopically the pouch mucosa appeared normal and histologically no epithelial changes were noted.

Table 5: Enhanced carcinogenic effect of DMBA in cheek pouches of Syrian golden hamsters pre-treated with topical vitamin A palmitate (Each group consisted of 6 animals).

Group	Treatment				Resulting epithelial change
	Weeks 1 - 10		Weeks 11 - 16		
1	Vitamin A in paraffin	S	----------		+ (6 animals)
2	Vitamin A in paraffin		No treatment	S	- (6 animals)
3	Vitamin A in paraffin		DMBA in paraffin	S	+++ (6 animals) ++++ (2 animals)
4	Vitamin A in paraffin		Paraffin	S	- (6 animals)
5	No treatment		DMBA in paraffin	S	++ (6 animals)
6	No treatment		Paraffin	S	O (6 animals)

O = normal; - = generally normal, with focal atrophy; + = focal acanthosis and focal atypia; ++ = diffuse acanthosis and focal atypia; +++ = focal intraepithelial carcinoma; ++++ = infiltrating carcinoma; S = sacrifice.

DISCUSSION

The mechanism by which vitamin A produces mucoid metaplasia is not yet clearly understood. Flesch (48) studying the mode of action of the vitamin concluded that its effect on epithelium was probably local, and non-specific and due to interference in sulfhydryl metabolism os the epidermis. Later he postulated (49) that the hard keratin with no heratohyaline was altered to soft keratin rich in keratohyaline and eventually to mucin under the influence of vitamin A. The keratohyaline present probably reflects epidermal mucopolysaccharide formation and is the precursor of mucoprotein matrix present in softer keratin. It is also possible that because of an increase in permeability of lysosomal membranes, (to be discussed later), in response to vitamin A, certain enzymes are released which may inhibit keratin formation and provoke mucous metaplasia (22, 34). This is probably achieved by stimulation of other enzymes that are required for the biosynthesis of mucopolysaccharides (MPS) and cause incorporation of sulphate into MPS. Fell (42) has shown that there is, under the influence of vitamin A, an inhibition of keratinisation because of excessive utilisation of tyrosine and cystine in other processes and less incorporation of these amino acids into the superficial layers of the epithelium and as a result inorganic sulphate is incorporated into excess sulphated MPS and not into keratin. The electron-microscopical studies of Fitton Jackson and Fell (45) have indicated that the basal cells of embryonic chicken skin in culture show a reduction of their fine keratinous fibres, tonofilaments and

tonofibrils when exposed to excess vitamin A. This may be due to an inhibition of the synthesis of basic protein molecules or of their aggregation into tonofilaments rather than to the process of digestion of prekeratinous protein by lysosomal enzymes facilitated by the action of vitamin A on lipoprotein membranes (36, 84, 155). However the exact mechanism of this process of inhibition of keratinisation by vitamin A is not known and it is possible that mucous secretions are an automatic result of this process of inhibition. In addition to the findingsof Rothberg (116) who also demonstrated that vitamin A inhibits keratinisation and promotes the synthesis of MPS, works by Wolf and his associates (146, 151, 161, 162) indicate that vitamin A plays an important role in the activation of sulphate in the biosynthesis of sulfated mucopolysaccharides.

As far as the results of the present study are concerned: In experiment 1, after treatment of hamster cheek pouches with DMBA, a recognisable sequence of events was observed: After 2-4 weeks of painting, there was an inflammatory phase with focal ulceration and necrosis of the epithelium. By the fourth to the sixth week, there was regeneration of the damaged epithelium and epithelial hyperplasia in some animals. The latter finding was more prominent by the 8th week. At this stage of carcinogenesis, DMBA appeared to have a varying effect on the epithelium, producing in the same pouch lesions ranging from hyperplasia and benign papillomata to premalignant lesions. Yet, in general, the first neoplastic lesion seemed to be a benign papilloma which in our experience first appeared after 7-8 weeks of painting. The lesion later developed into

a papilloma with carcinoma in situ changes, but at the same stage foci of intra-epithelial carcinoma appeared in non-papillomatous epithelium . Squamous cell carcinoma with invasion appeared in some animals by the eleventh week, and in all by the twelfth to fourteenth week. As in other experiments (21, 77), there was no regression of the tumors after painting was discontinued. On the contrary, the tumors increased in size rapidly, became infected and bled easily. However, there was no metastasis to lymph nodes or to other organs.

As we have seen from data reviewed earlier, vitamin A affects cell proliferation and differentiation and influences keratinisation. Changes recorded after administration of excess vitamin A in varying concentrations include mucoid metaplasia of squamous epithelium, epithelial hyperplasia, rapid epidermal proliferation disorientation of the basal layer, an increase in the mitotic rate and decreased keratinisation (8, 14, 72, 132).

In experiment 2, the occurence of mucoid metaplasia was confirmed, but only after the use of 20% vitamin A. However, the most striking result of treatment with vitamin A was the appearance of focal epithelial hyperplasia, which progressed with the duration of the experiment, was more prominent with the higher concentration of the vitamin and eventually led to the development of foci of marked epithelial atypia and even in isolated areas to the appearance of lesions resembling very early infiltration of the lamina propria by epithelial cells . In addition there was decreased keratinisation and the formation of rete pegs. An additional finding of note was the appearance in the later stages of treatment of dense and nodular infiltrations of mononuclear cells

with occasional nuclear atypia, in the lamina propria. This finding was particularly prominent after the use of 20% vitamin A. The fact that in the present studies, more striking results were obtained with 20% vitamin A palmitate, supports other observations (73, 74) which have recorded more epithelial atypicality, hyperchromasia and an increase in the mitotic rate when larger concentrations of the vitamin were used.

The use of vitamin A therapeutically:

Vitamin A has been used both topically and systemically in the treatment of various skin and mucous membrane disorders characterised by altered keratinisation. Savitt and Obermayer (129) obtained improvement in cases of acne and senile keratosis treated with vitamin A. After topical application of vitamin A ointment to 6 patients with ichthyosis, Flesch (47) noted mild improvement and a decrease in scales and in the sulfhydryl content of these scales. This effect was related to the influence of the vitamin on keratinisation. However, follow up histological studies over a long period are absent in these cases. In 1953 Reiss and Campbell (111), administered topical vitamin A ti senile skin and it showed some suppressive influence and corrective effect on faulty keratin formation. Changes included a disappearance of follicular hyperkeratosis, an increased thickness of the epithelium and a more prominent granular layer with a thinner and less eosinophilic stratum corneum. Burgoon et al. (19) recorded clinical improvement in patients with keratosis follicularis, ichthyosis, psoriasis, pityriasis rubra pilaris and congenital ichthyosiform erythroderma. Histologically there was an accentuation of the granular layer immediately after treatment but once again there were no biopsies recorded to show the late epithelial changes after clinical

improvement. Getzler and Flint (54), have recorded successful treatment of a family with keratosis follicularis with parenteral administration of water soluble vitamin A (10,000-15,000 I.U. day) for 3-5 months. Futton et al. (53) have also produced remissions in Darrier's disease by topical vitamin A acid and histology of the lesions showed a reduction in acantholytic and dyskeratotic processes.

Other workers have recorded their experience with vitamin A in oral leukoplakia. Fryer in 1961 (50) failed to demonstrate improvement in his patients on vitamin A therapy. However. earlier, in 1958, Molay and Urbach (101) recorded marked improvement in 7 of 10 patients with oral leukoplakia treated with vitamin A troches (150,000 units per oral troches), 2-3 x daily for 2-6 months. The remaining 3 patients showed no or only slight improvement. Silverman et al. (137) induced total or partial clinical remissions in 7 of 16 patients with oral leukoplakia. This improvement was accompanied by microscopic changes including a mucoid metaplasia and less hyperkeratosis. In all instances, after the withdrawal of the vitamin there was either partial or complete recurrence of the clinical leukoplakia. Continuation of this work by the same authors (138, 140) showed similar results in 15 of the 19 patients treated. Johnson et al. (67) also recorded improvement in half of their cases of leukoplakia treated with the vitamin. Hyams et al. (64) have also recorded partial relief of leukoplakia vulvae with adequate doses of vitamin A and after therapy there was thinning of the keratin layer and the entire epidermis with less acanthosis and loss of the rete pegs.

Vitamin A and Experimental Carcinogenesis:

Little is known about the effect of vitamin A on experimental carcinogenesis and nothing at all is known of the topical effect of the vitamin on hamster cheek pouch carcinogenesis. In 1965, Chu & Malmgren (23) demonstrated that the addition of 0.5% vitamin A palmitate to the diet prevented the development of DMBA or benzo(a)-pyrene induced carcinomas of the gastro-intestinal tract of hamsters. Furthermore they showed that the addition of vitamin A palmitate to the DMBA used for painting the cervix and vagina prevented the development of carcinoma in these areas, but this treatment did not inhibit the development of carcinoma of the perineal skin. McMichael (91) also produced retardation of growth and delay in the initial appearance of the Shope rabbit papilloma when vitamin A was administered systematically in high doses. If such treatment was continued long enough it caused atrophy of the fleshy portions of the papillomas and in some animals this was followed by shedding of the keratinised portion of the papilloma and macroscopic disappearance of the tumor. Davies (29) also showed that Rhino mice fed a diet containing vitamin A, developed fewer papillomas following a single topical dose of DMBA than mice fed a diet deficient in vitamin A. The difference was due, at least in part, to more rapid loss of papillomas in the vitamin A supplemented animals.

Lately Prutkin (108) also demonstrated that topical application of vitamin A to rabbit keratoacanthomas resulted in viscous secretion from the tumor as a result of mucinogen droplets produced in the tumor cells with a concomitant reduction of tonofibrils. In addition the tumors had regressed considerably in size. Saffioti et al. (121) have also shown that vitamin A inhibited the induction of tracheobronchial squa-

mous metaplasia and carcinomas induced by benzo(a)pyrene. In a later study Prutkin(109)showed that vitamin A acid applied in lotion form to DMBA induced skin tumors in rabbits resulted in an increased number of keratoacanthomas which exuded a thick viscous secretion, and cessation of the vitamin treatment resulted in reversal to the usual form of tumor. This phenomenon was attributed to the synergistic action of the vitamin related to transfer of electrons in membranes, which might be a factor in increased carcinogenesis. Lately March and Biely (87) noticed that an increased dietary vitamin A palmitate administered to white leghorn cockerels resulted in an increased incidence of avian leukosis in the birds which they attributed to an alteration of membrane structure in a manner favourable to tumorigenesis by latent leukosis virus.

As far as vitamin A and the hamster cheek pouch is concerned, little at all is known other than the results of Rowe and Gorlin's study (117) which demonstrated that systemic hypovitaminosis appeared to enhance pouch carcinomas by approximately 26%. This is the only study on the effect of vitamin A in this experimental model, available in the literature and it does not deal with topical vitamin A application.

Vitamin A and its Influence on Biological Membranes:

In the last 20 years much new information regarding the effect of vitamin A on biological membranes has accumulated. Today it has been established conclusively that vitamin A is important for regulating the stability of biological membranes. A normal concentration of the vitamin seems to ensure optimum stability but large

doses labilise membranes both in vivo and in vitro. This effect is important in a number of different membrane systems, both cellular and subcellular, and include the outer membrane of the erythrocyte and mitochondrial and lysosomal membranes (84, 115). Blough (16) was able to produce filamentous forms of the predominantly spherical viruses - Influenza A and NDV with vitamin A alcohol. The vitamin altered the packing of the bimolecular lipid leaflet of the cell membrane, thus resulting in filamentous forms, The latter effect is achieved by absorption of the vitamin, a hydrophobic substance to the cell surface. In 1964, Lucy and Dingle (84) demonstrated the ability of vitamin A to penetrate lipid films, in studies of rabbit etythrocytes in contact with excess vitamin A. Because of the excess of the vitamin the membrane became unstable and there was lysis of the cell after 15 minutes at 37^{o}C. The lysis of cells was preceded by identations of the membrane with the formation of pinocytotic vesicles, and associated with damage to the mitochondrial membranes and endoplasmic reticulum. Dingle and Lucy (35) have also shown that fibroblasts grown in the presence of excess retinol (Vitamin A) undergo gross changes on electron microscopy, including a loss of endoplasmic reticulum , a marked increase in the number of free ribosomes, and small invaginations of the plasma membrane. These changes are followed by mitochondrial swelling and a decrease in respiratory activity with an increase in the number of cytolysosomes. These changes are induced by penetration of the membranes by the vitamin A and are due to interaction between the vitamin and the phospholipid membrane.

Vitamin A and Lysosomes:

Vitamin A is also known to influence lysosomes, as a result of its effect on the lysosomal membrane, in a manner related to that described above. According to de Duve 1959 (30) the lysosomes are specific cytoplasmic organelles containing a number of acid hydrolases including ribonuclease, deoxyribonuclease, phosphatases, sulphatases, glucuronidase, and cathepsin. The properties of these organelles have been summarised at a recent Ciba Foundation Symposium (24). Studies (34, 43, 83, 149, 154) on the dissolution and disintegration of cartilage matrix, (chondroitin sulphate) by vitamin A both in vivo and in vitro indicated that the vitamin acted by releasing endogenous acid proteases from the cartilage cells resulting from rupture of the lysosomal membranes. In 1961, Dingle (33) showed that particulate preparation of rat liver treated with vitamin A alcohol, released a proteolytic enzyme (acid protease). This particulate fraction contained the bulk of acid hydrolases intimately bound to lysosomes. Other studies (44, 158) showed that lysosomal stabilisers, like cortisone, retarded the harmful action of excess vitamin A upon cartilagenous bone rudiments and liver fractions in vitro and blocked the effects of other lysosomal labilising substances like endotoxins and UV irradiation (66, 156, 157).

Some authors (49) have used the above hypothesis to explain the effects of the vitamin on keratinisation. Other workers have described the role played by lysosomes in the process of cell division, using the tissue culture technique. As mentioned earlier the mitogenic effect of the vitamin has been recorded

previously in the literature (8, 132). Hirschhorn et al. (63) noted an increase in acid phosphatase in the cytoplasm of human lymphocytes cultured in the presence of phytohemagglutinin before the process of cell division, and Allison and Paton (5) showed chromosomal damage in diploid cells following activation of lysosomal enzymes by a photosensitising agent - acridine orange. This chromosomal damage was due to structural alterations in the chromosomes due apparently to the activation of lysosomal enzymes (DNase) which split the DNA bonds. These observations tend to support the hypothesis that lysosomes can react with and produce lasting changes in organic nuclear material resulting in malignant change (6). In other studies Allison and his associates (4) have suggested that carcinogens may produce their carcinogenic effect by way of a gradual release into the cytoplasm over long period of times, of lysosomal enzymes such as DNAse, RNAse and proteases, capable of damaging the mechanism controlling cell division but without impairing the capacity of cells to divide.

Allison and Young (2) have also shown that vitamin A alcohol produced intensification of lysosomal fluorescence when incubated with monkey kidney cells, showing that it is specifically concentrated in the lysosomes. Later Allison and Malluci (3) confirmed the observations recorded by Hirschhorn et al. (63) and showed that lysosomes increased in number when cells enlarged prior to cell division, showing that the release of lysosomal material may act as a "trigger" initiating cell division. Basset and Packer (9) also showed alterations in the membrane of isolated lysosomes in response to vitamin A alcohol at 35^{o}C, as a result of incorporation of the vitamin

into the membrane. Lately Dresser has shown (37) that vitamin A may act as an adjuvent probably as a result of its effect on lysosomal membranes; in this way it stimulates cell division. The work of Takayama and Ojima (148) also tends to support the concept that lysosomal activation could represent an important contributary mechanism in the occurence of somatic mutation leading to carcinogenesis. Most recently Allison (6, 7) has summarised the great significance of lysosomes in carcinogenesis and has concluded that they are associated with an increased malignant potential in cultured cells.

Discussion of Results of Experiments 3-6 and Conclusions.

In experiment 3, intraepithelial carcinomas developed in the DMBA group by the sixth week and infiltrating carcinomas were present by the eighth week. In the DMBA - A group the early changes were similar to those in the DMBA group. However, atypical epithelial changes were already present in a number of animals after 4 weeks. By the sixth week occasional foci of invasive carcinoma were found. By the eighth week there were larger and more anaplastic squamous cell carcinomas than in the DMBA group. This finding was confirmed in the 10-and 12-week groups. Thus, tumors had appeared earlier and had reached a larger size in the animals receiving DMBA and vitamin A simultaneously than in those treated with DMBA alone. Furthermore the tumors in the DMBA-A group showed more extensive infiltrative growth and a greater degree of anaplasia. Pouches treated with vitamin A alone showed acanthosis and eventually moderate epithelial pleomorphism. Epithelial changes of a similar nature have been reported after treatment with vitamin A. alone

as in experiment 2 .

In the present study application of vitamin A alone for up to 12 weeks did not cause mucoid metaplasia of the cheek pouch epithelium. However, non-tumorous epithelium and tumors originating in cheek pouches treated for similar periods with combination of vitamin A and DMBA regularly showed mucoid metaplasia. The mucoid cells occurred singly as well as in groups forming glandular structures. DMBA thus appeared to enhance the metaplastic effect of vitamin A on squamous epithelium. The earlier development and increased incidence of carcinomas in animals treated with a combination of vitamin A and DMBA may be the result of an additive effect of an agent known to influence the mitotic rate and capable of producing epithelial proliferation and even atypia, and the carcinogenic action of the DMBA. The latter effect of the vitamin is probably due to the labilisation of lysosomal membranes which may result in the release of lysosomal enzymes known to influence nuclear metabolism and cell division. Alternatively the increase in permeability induced by vitamin A may facilitate quicker and more effective permeation of DMBA into the cell and sub-cellular organelles, thereby potentiating its carcinogenic effect.

In Experiment 4: after 6 weeks of treatment with DMBA, all animals showed intraepithelial carcinomas, areas of hyperkeratosis, and focal acanthosis with epithelial atypia. In addition some animals also had benign papillomas. When the pouches were left untreated for 2 months after cessation of DMBA-application, no infiltrating carcinomas developed. On the contrary, there were fewer

areas of epithelial hyperplasia, and histologically these showed no signs of malignancy. Thus, partial regression of the epithelial changes had taken place after the treatment with DMBA was discontinued. The findings in the animals treated for 2 months with paraffin after discontinuation of DMBA were basically the same as those in the animals receiving no further treatment. The presence of areas of intra-epithelial carcinoma in the single papilloma found suggests lack of spontaneous regression of a DMBA-induced premalignant lesion.

However, when lesions induced with DMBA during 6 weeks were subsequently treated with a mild topical excess of vitamin A (10%) for 2 months, large anaplastic carcinomas with marked invasive growth, and many foci of intra-epithelial carcinomas were found.

In Experiment 5, after 4 weeks of treatment with DMBA, all pouches showed diffuse hyperkeratosis and regular acanthosis, without cellular atypicality (lesions comparable to human leukoplakia). There were no papillomas or intraepithelial carcinomas. When the pouches were left untreated for 2 months after cessation of DMBA, no carcinomas developed , and the lesions were similar to and not more advanced than those seen in animals sacrificed immediately after cessation of DMBA. Most of the pouch epithelium was of normal thickness, with areas of atrophy, in contrast to the hyperplastic appearance of the epithelium immediately after cessation of DMBA. These findings suggest that the cellular changes induced by DMBA regressed partially after withdrawal of the carcinogen.

However, when lesions induced by DMBA during 4 weeks were subsequently treated with 10% vitamin A for 2 months, many foci of intraepithelial and infiltrating carcinoma developed , and large tumors were present macroscopically. Thus it appears that vitamin A stimulated the growth and development of these carcinomas. These results confirm the findings of experiments 3 and 4, in which the potentiating effect of the vitamin during carcinogenesis was demonstrated . The fact that no malignancies developed in the animals treated with paraffin only, after cessation of DMBA, excludes the possibility that the results obtained with vitamin A dissolved in paraffin were caused by the vehicle.

Experiment 6, shows that reversal of the sequences of the previous experiments also results in potentiation of DMBA carcinogenesis in the hamster cheek pouch and this experiment was specifically designed to assess the effect of preliminary application of the vitamin on the process of initiation of chemical carcinogenesis. The increased tumor formation, encountered in the animals in experiments 4 and 5, treated initially with DMBA for 4 and 6 weeks and subsequently with vitamin A and in the animals of experiment 6 in which the pouches were pretreated with vitamin A, cannot only be explained by an increased permeability state induced by the vitamin which facilitates easier penetration of the carcinogen into the epithelium, as in experiment 3. The results are more likely to be due to the changes induced by vitamin A and already described in detail earlier, in epithelium already altered by a carcinogen and to the additive effect of both these compounds on epithelium. These results appear to strengthen Allison's concept (6, 7) of the role of lysosomes in carcinogenesis

and cell division which has been reviewed extensively in the discussion. As mentioned earlier, he and his associates have suggested that lysosomal labilisation may be an important factor in the initiation of carcinogenesis and he has demonstrated (5) structural alterations in the chromosomes, probably due to the activation of lysosomal DNAse which enters the nucleus after lysosomal damage and leads to malignant transformation. Furthermore it has been suggested (3, 4, 6) that lysosomal enzymes can release cells from mitotic inhibition and may be involved in the initiation of cell division. Hirschhorn et al. (63) have also noted an increase of lysosomal acid phosphatase in human lymphocytes prior to cell division and lately Dresser (37), has suggested that vitamin A may stimulate the division of lymphocytes. Thus from these studies it is feasible to suggest that lysosomal labilisers may play an important role in the process of cell division through the release of lysosomal enzymes which influence nuclear metabolism. The results of experiment 3 - 6 can be explained on this basis. This hypothesis may also explain the co-carcinogenic effect of substances like Tween 60, DMSO, and Croton oil which are also known to affect the stability of cellular and subcellular membranes (26, 27, 28, 40, 139). It is of interest to note that the epithelial proliferation and eventual malignancy induced by DMBA may be achieved in a similar manner as the epithelial changes induced by vitamin A i. e. by the release of lysosomal enzymes which may result in somatic mutations and eventual malignancy. An argument in favour of this supposition is the observation that hydrocarbon carcinogens which have been brought into contact with cell cultures, are selectively concentrated in the

lysosomes and remain there for relatively long periods of time (4). Similar conclusions were drawn by Takayama and Oyima (148) from a study of lysosomes on the effect of 3-methylcholanthrene on Hela cells.

The results of the experiments recorded here, offer conclusive evidence that vitamin A palmitate causes an increase in the rate of tumor formation in the cheek pouches of hamsters treated with DMBA. This co-carcinogenic effect exists when the vitamin is applied in excess before, after or at the same time as the carcinogen. These facts may be of clinical and therapeutic significance but their application to human pathology is at present speculative. Nevertheless it appears worthwhile to note that, in the hamster cheek pouch, prolonged treatment with vitamin A enhances the formation of carcinoma in epithelium altered by a carcinogen.

SUMMARY

The effects of topical application of vitamin A palmitate during DMBA (9, 10 dimethyl 1-2 benzanthracene) carcinogenesis in the hamster cheek pouch, are described. A series of different experiments were designed in order to assess the influence of vitamin A, a mitogenic agent known to influence epithelial differentiation and keratinisation, on the initiation and promotion of carcinogenesis in the hamster cheek pouch. The application of DMBA as a 0.5% solution in paraffin oil produces a recognised sequence of events in the hamster cheek pouch resulting in an initial phase of necrosis followed by regeneration and reactive epithelial hyperplasia after 4-6 weeks application. Thereafter benign tumors (papillomas) and premalignant lesions including

intra-epithelial carcinoma appear and this is followed by the development of multiple fungating and invasive squamous cell carcinomas after approximately 6 to 12 weeks of application of the carcinogen. No regional or distant metastases were encountered.

When topical 10% or 20% vitamin A palmitate is applied to the pouch mucosa, histologic changes are noted mainly after 6 to 12 weeks and these alterations are most striking with 20% vitamin A. The epithelial changes recorded included decreased keratinisation, focal acanthosis, formation of rete pegs, and eventually marked atypia. Mucoid metaplasia was only noted after the use of 20% vitamin A palmitate. In addition, a dense lymphocytic and histiocytic infiltrate with occasional nuclear atypia was present in the lamina propria in the later stages of the experiment . The epithelial changes may be the result of gradual liberation of lysosomal enzymes over a long period, with consequent structural alterations of nuclear material, and decreased inhibition of mitotic activity. After establishing the effects of both DMBA and vitamin A on the pouch mucosa of our strains of animals, the influence of vitamin A on the process of DMBA carcinogenesis was investigated.

Initially the right cheek pouches were painted with a combination of 10% vitamin A palmitate and 0.5% DMBA for periods from 2 to 12 weeks and these were compared with pouches treated with carcinogen only for identical periods of time. After DMBA alone for 6 weeks intraepithelial carcinoma was present. However, after only 4 weeks' application of the combination of DMBA and vitamin A, intraepithelial carcinomas were found and after 6 weeks infiltrating carcinomas were present. After 8 weeks infiltrating

carcinomas were also present in pouches treated with DMBA alone but at this stage, as well as after 10 and 12 weeks, the combination of DMBA and vitamin A had induced more anaplastic and larger tumors than DMBA alone.

In the light of the above results and considering the fact that topical vitamin A is used therapeutically in certain oral and skin diseases characterised by hyperkeratosis and acanthosis, (comparable to experimental lesions obtained after 4-6 weeks of carcinogen application), further studies were designed to assess the effect of topical application of vitamin A to DMBA-induced benign papillomas and intraepithelial carcinomas A (after 6 weeks DMBA) and to benign hyperkeratotic acanthotic lesions of the pouch (after 4 weeks DMBA).
In the former experiment - foci of intra-epithelial carcinoma and benign papillomas up to 2 mm. in diameter were present in the cheek pouches of all 6 hamsters sacrificed immediately after completion of the local treatment with 0.5% DMBA during a period of 6 weeks. Among 6 hamsters treated similarly but sacrificed 2 months after discontinuation of the local application of DMBA, only 3 showed benign papillomas, and no carcinomas were present. Of the 6 animals receiving DMBA for 6 weeks, followed by local application of paraffin for 2 months, only 1 showed a papilloma with intra-epithelial carcinoma, and in the remaining 5 animals no tumors were found. All 6 hamsters receiving similar treatment with DMBA during 6 weeks, followed by topical administration of 10% vitamin A palmitate in paraffin during 2 months, showed infiltrating squamous cell carcinomas.

The epithelial changes induced with DMBA during 6 weeks thus regressed partially when application of the carcinogen was discontinued. However, subsequent treatment of these lesions with vitamin A induced an increase in size and malignancy.

In a similar study designed to assess the effect of the vitamin on an earlier stage of hamster cheek pouch carcinogenesis i.e. after 4 weeks of DMBA application, it was found that in all 6 hamsters sacrificed immediately after the completion of this treatment, benign hyperkeratotic lesions compatible with leukoplakia were present. In 6 hamsters allowed to survive for 2 months without further treatment, and in 6 other animals treated locally with paraffin for 2 months, the cheek pouches were generally normal, but showed areas of atrophy and focal leukoplakia. In all 6 animals where application of DMBA was followed by local treatment with 10% vitamin A palmitate in liquid paraffin for 2 months, squamous cell carcinomas developed.

These 2 studies confirm the results obtained with simultaneous treatment of the pouch by the carcinogen and vitamin A and demonstrated the enhancing potential of the vitamin in promoting experimental chemical carcinogenesis initiated by DMBA. These findings may have clinical implications in view of the fact that topical vitamin A has been used in the treatment of oral leukoplakia and other skin disorders, but application of experimental results to human pathology is at present merely speculative.

In a further experiment designed to assess the effect of pretreatment of the pouch by topical 10% vitamin A palmitate for 10 weeks, prior to DMBA application, in order to establish whether, in this way, the vitamin could enhance carcinogenesis, it was found that carcinomas developed in hamster cheek pouches after topical treatment with vitamin A palmitate during 10 weeks, followed by the application of DMBA for 6 weeks. No carcinomas were found after treatment with DMBA during 6 weeks, without preceding application of vitamin A palmitate, and the most advanced lesion was acanthosis with slight epithelial atypia. Thus the carcinogenic action of DMBA was enhanced by preceding topical treatment with vitamin A. From these experiments it seems that, contrary to most experimental data available in this field, vitamin A acts as a cocarcinogen during chemical carcinogenesis in the hamster cheek pouch.

The discussion of the results includes an extensive review of the influence of vitamin A on epithelium and keratinisation, biological membranes including lysosomes, experimental carcinogenesis and other aspects of the vitamin's action. The possible underlying mechanisms of action of the vitamin in our experimental model is discussed . It is suggested that the results are related to the direct mitogenic effect of the vitamin on epithelial cells previously, simultaneously or subsequently altered by the carcinogen - DMBA. This enhancing effect of the vitamin is probably related to its recognised influence on cell membranes, lysosomes and nuclear activity. It also appears feasible to suggest that in the experiment where the vitamin and carcinogen were applied simultaneously, the labilising action of the

vitamin increased permeability and facilitated easier entry of the carcinogen into the cell, thereby potentiating its carcinogenic activity. However, the results of the later experiments are more likely to be related to the labilising effects of the vitamin on lysosomes causing release of certain enzymes, such as DNAse, known to influence nuclear metabolism, cell proliferation and the process of cell division.

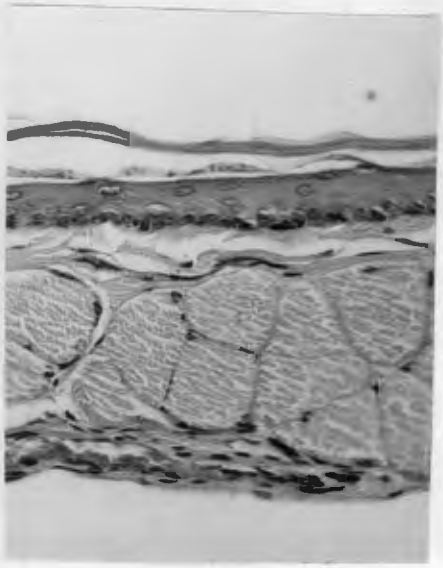
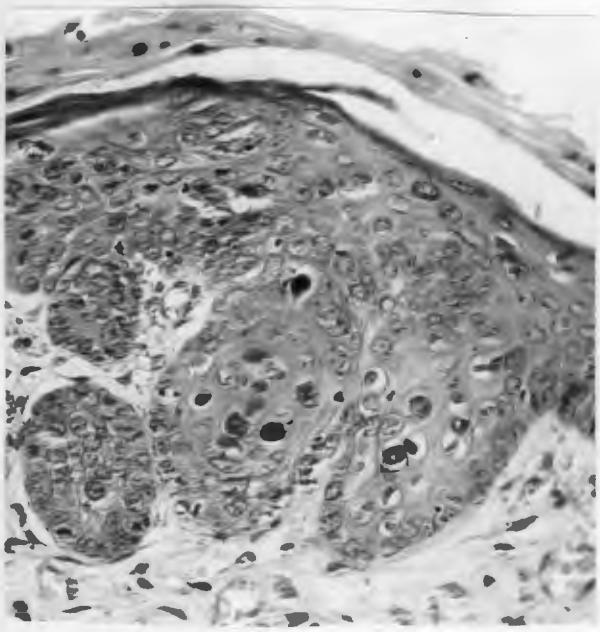


Fig. 1: Histological section of normal hamster cheek pouch mucosa. Note thin layer of keratin on the surface, absence of rete pegs, delicate connective tissue of lamina propria and beneath it a layer of smooth muscle (H & E x 270).

Fig. 2: Cheek pouch mucosa after 4 weeks application of DMBA showing hyperkeratosis, focal parakeratosis and regular acanthosis with absence of atypicality. (H & E x 270).

Fig. 3: Benign papilloma in hamster cheek pouch treated with DMBA for 8 weeks (H & E x 270).

Fig. 4: Intraepithelial carcinoma in a pouch painted with DMBA for 9 weeks. (H & E x 112). For cellular detail see other figures.



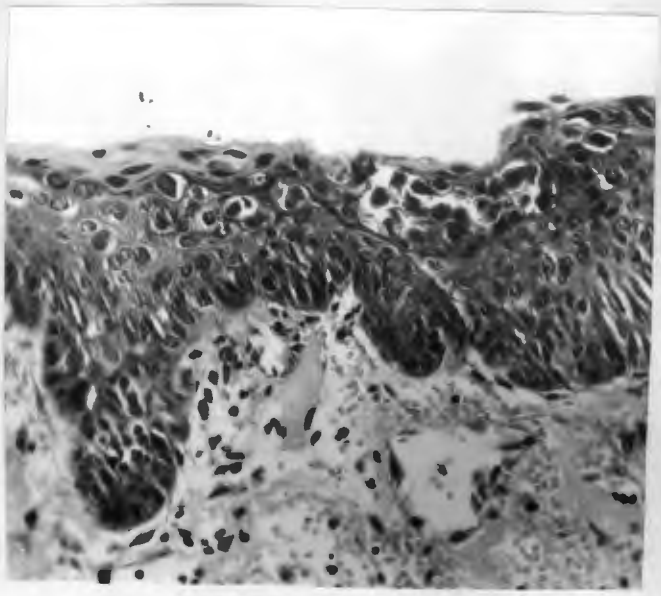
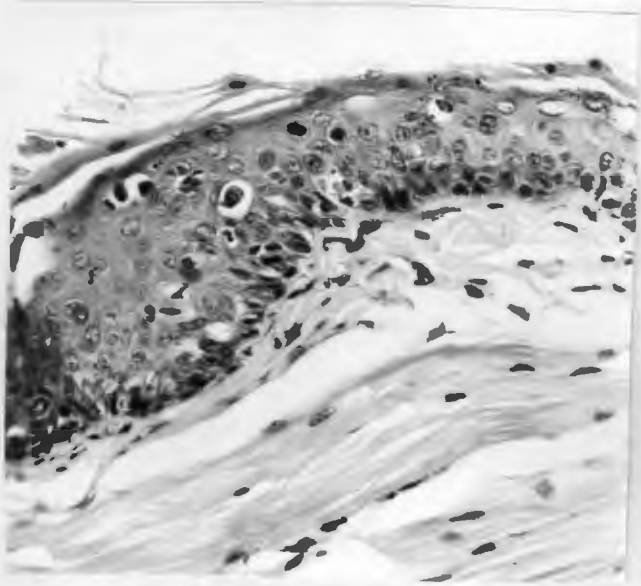
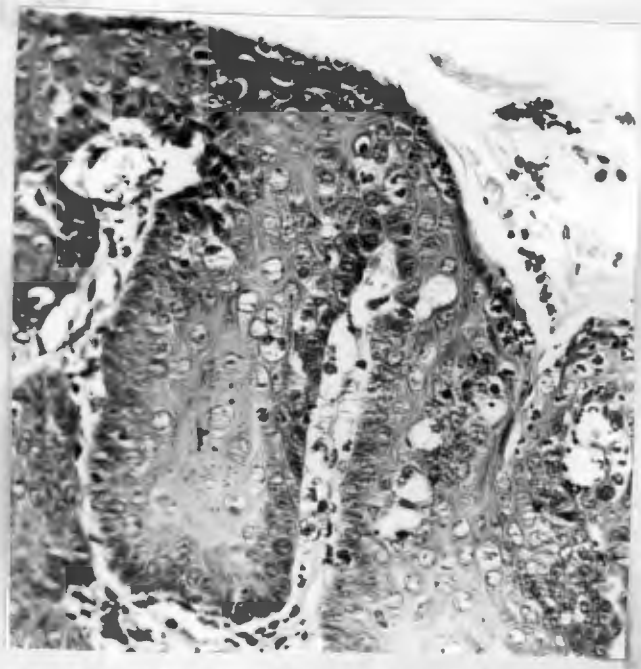


Fig. 5: Higher power of intraepithelial carcinoma. Note loss of polarity and hyperchromasia of nuclei (H & E x 270).





Figs. 6-8: Foci of intraepithelial carcinoma after 9 weeks application of DMBA (H & E x 270).

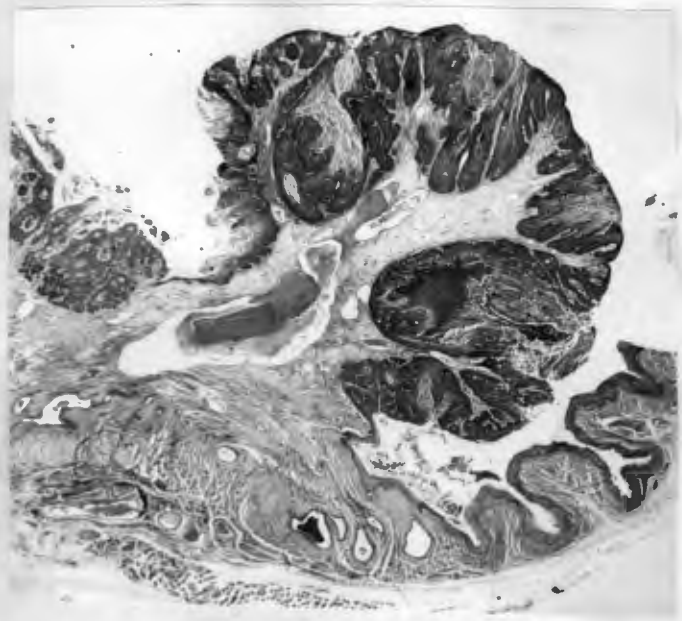


Fig. 9: Papilloma with intraepithelial carcinoma after 9 weeks application of DMBA . (H & E x 43).

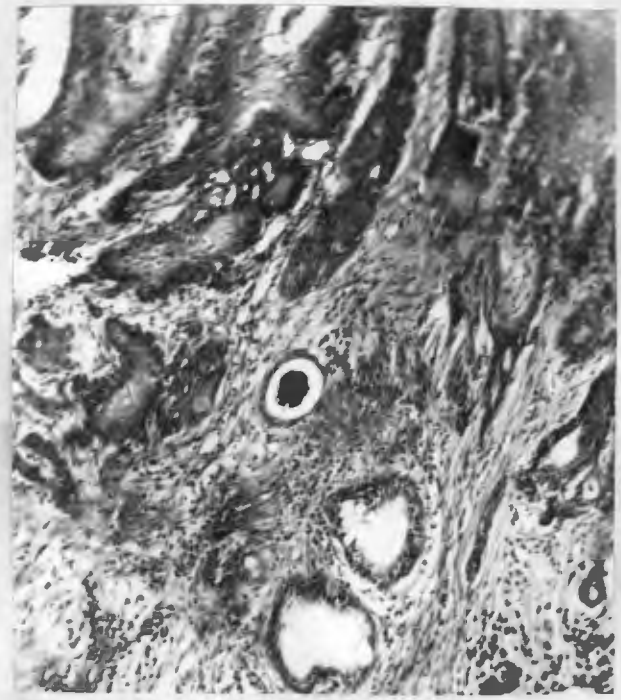


Fig. 10: Squamous cell carcinoma with invasion of the underlying tissue in a pouch painted for 11-12 weeks with DMBA. (H & E x 108).

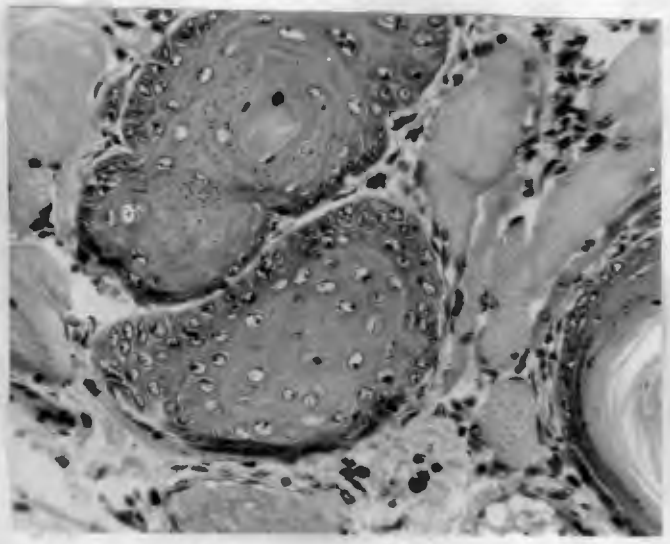


Fig. 11: Higher power of carcinoma infiltrating the muscle of the pouch painted with DMBA for 12 weeks (H & E x / 260).

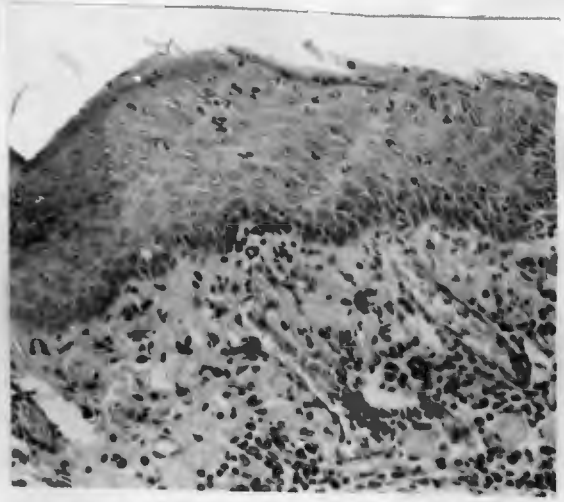


Fig. 12: Hamster cheek pouch epithelium after 4 weeks application of 20% Vitamin A. There is regular acanthosis and a chronic inflammatory infiltrate in the lamina propria (H & E x 168).

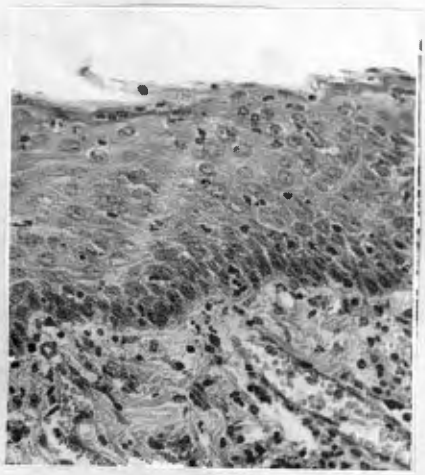


Fig. 13: Same as above, but after 6 weeks application of Vitamin A 20% (H & E x 270).

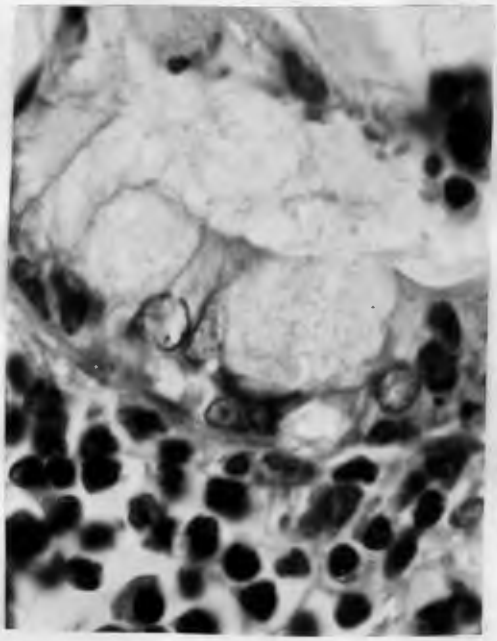


Fig. 14: Buccal pouch epithelium of hamster treated for 6 weeks with 20% vitamin A, showing part of glandlike structure lined by metaplastic vaculated columnar cells. H & E, 1080.

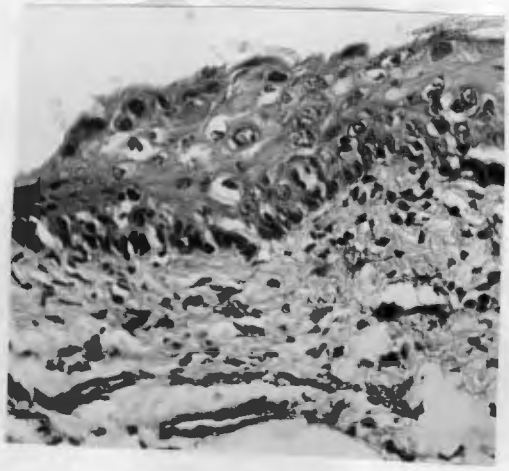


Fig. 15: Hamster cheek pouch epithelium treated with 20% vitamin A for 8 weeks, showing an area of irregular acanthosis with marked atypia H & E. x 270.

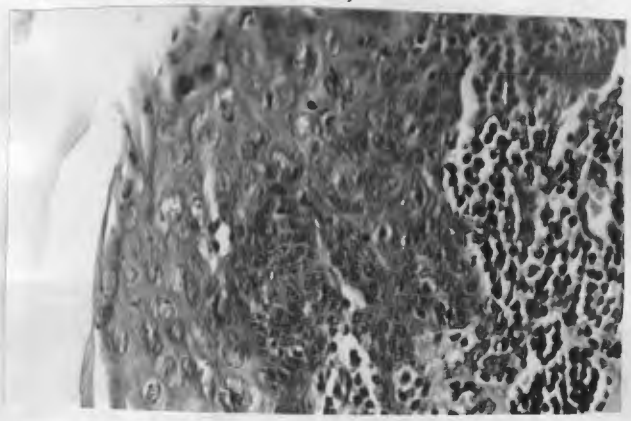


Fig. 16: Another area of the cheek pouch shown above. There is focal acanthosis, and cells with irregular hyperchromatic nuclei are present on the surface. H & E, x 270.

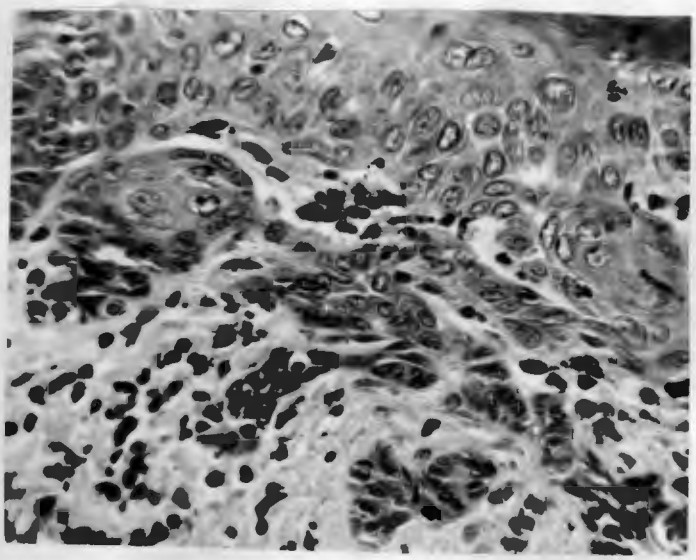


Fig. 17: Hamster cheek pouch after 10-12 weeks application of 20% Vitamin A showing atypical epithelial changes and irregular proliferation of the basal layer. (H & E x 260).

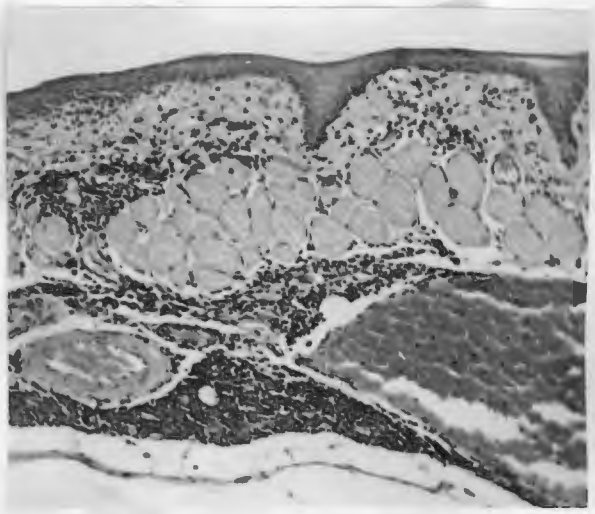


Fig. 18: Hamster cheek pouch treated with 20% Vitamin A for 10 weeks showing diffuse, dense perivascular mononuclear cell infiltration (H & E x 43).

Fig. 19: As in Fig. 18 showing nodular polypoid "lymphoma-like" lesions in the lamina propria. (H & E x 42).

Fig. 20: As above, showing dense mononuclear cell infiltration of the lamina propria (H & E x 105).

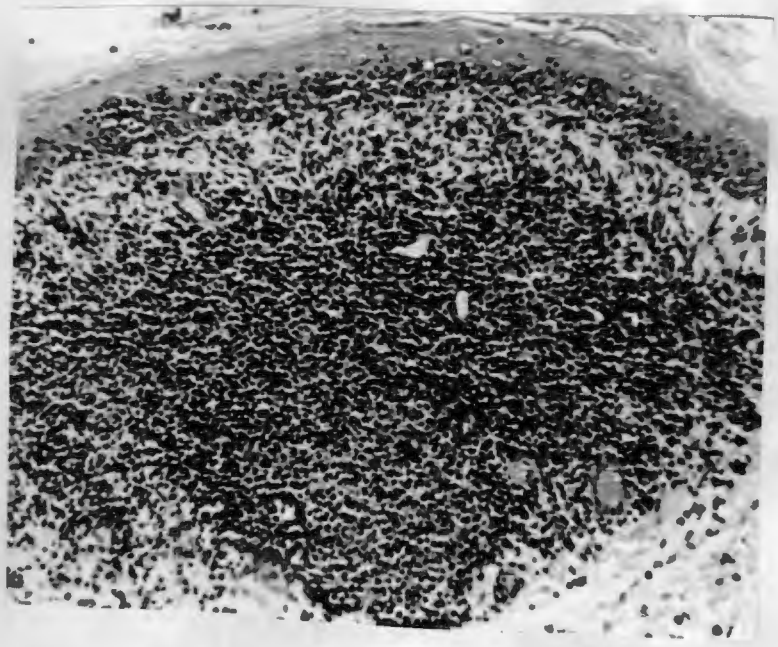
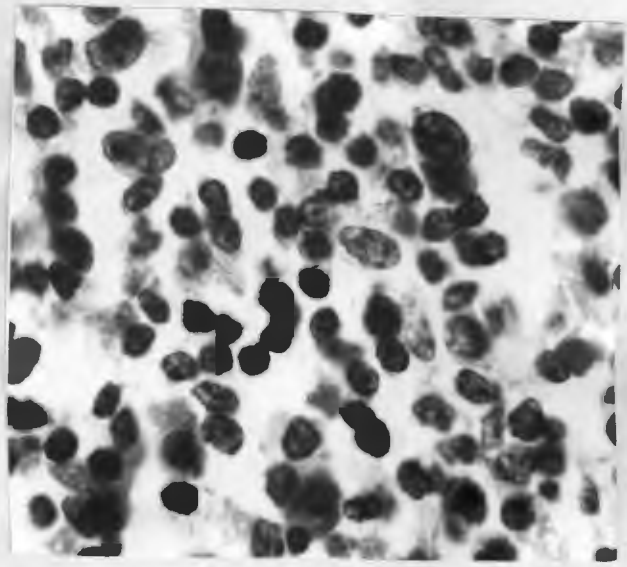
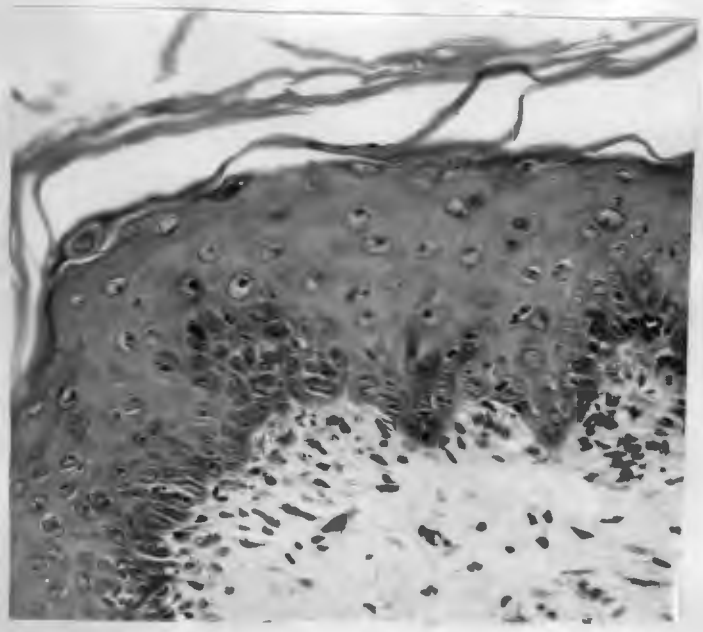


Fig. 21: As above, showing well circumscribed mononuclear cell infiltrates in the lamina propria. (H & E x 168).



Figs. 22-23: High power photomicrograph of dense inflammatory infiltrate consisting of mononuclear cells with irregular nuclei. (H & E x 1080).



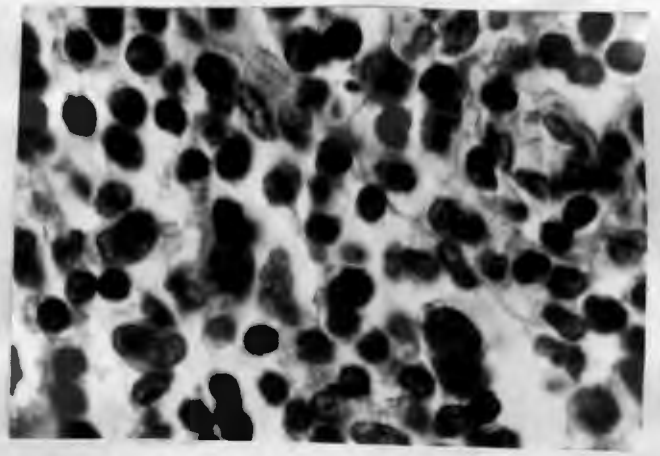


Fig. 24: Buccal pouch epithelium after 4 weeks of DMBA application . There is mild hyperkeratosis and marked acanthosis (H & E. x 270).

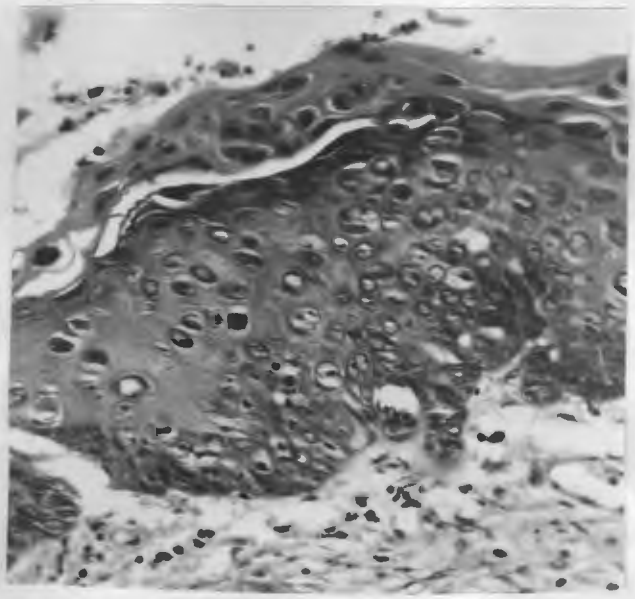


Fig. 25: Intraepithelial carcinoma in buccal pouch epithelium after 4 weeks' application of DMBA and 10% vitamin A (H and E, x 270).

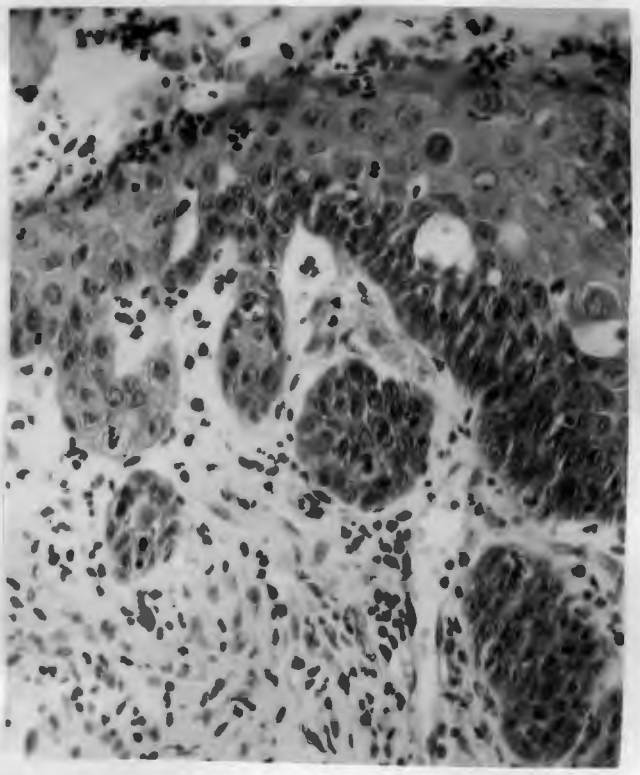


Fig. 26: Edge of infiltrating squamous cell carcinoma from pouch treated with DMBA and 10% vitamin A for 6 weeks (H and E. x 270).

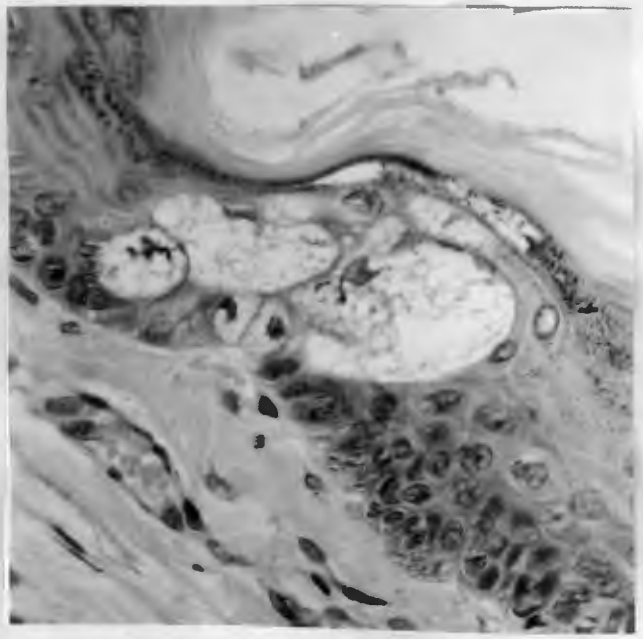


Fig. 27: Buccal pouch epithelium after 6 weeks' application of DMBA and 10% vitamin A, showing mucoid metaplasia in non-tumorous epithelium (H and E, x 430).

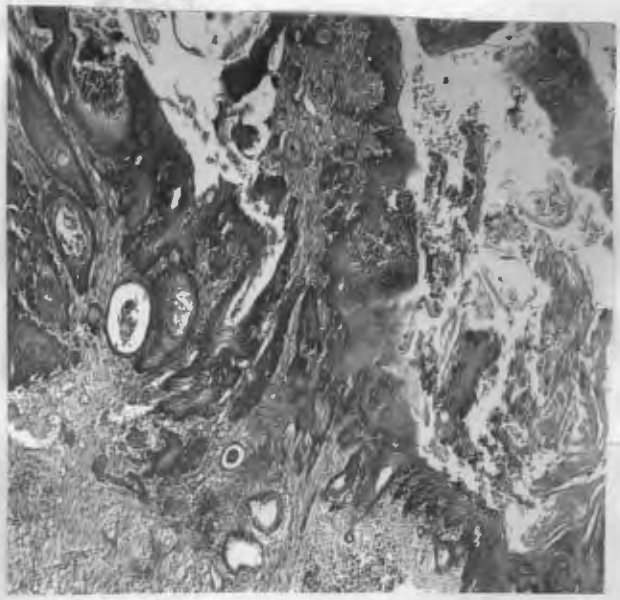


Fig. 28: Buccal pouch with infiltrating squamous cellcarcinoma after 8 weeks' application of DMBA and 10% vitamin A. Note gland-like structures in the depth of the tumor (H & E, x 43).

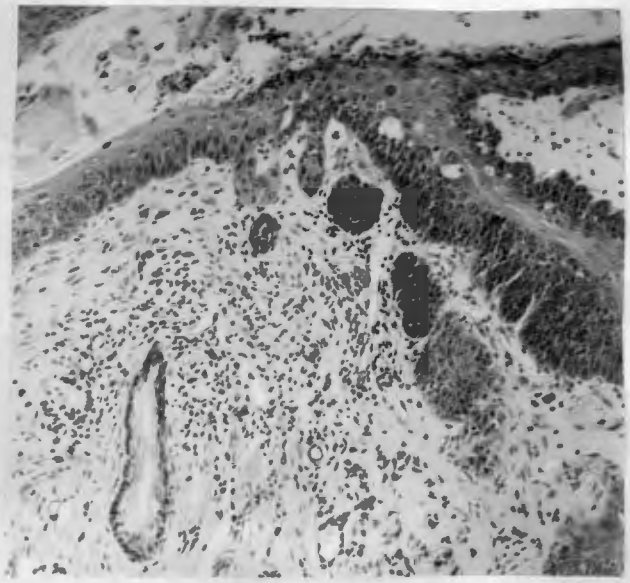


Fig. 29: Buccal pouch with infiltrating squamous cell carcinoma after 8 weeks application of DMBA and 10% Vitamin A showing tubular gland-like structures. (H & E x 108).

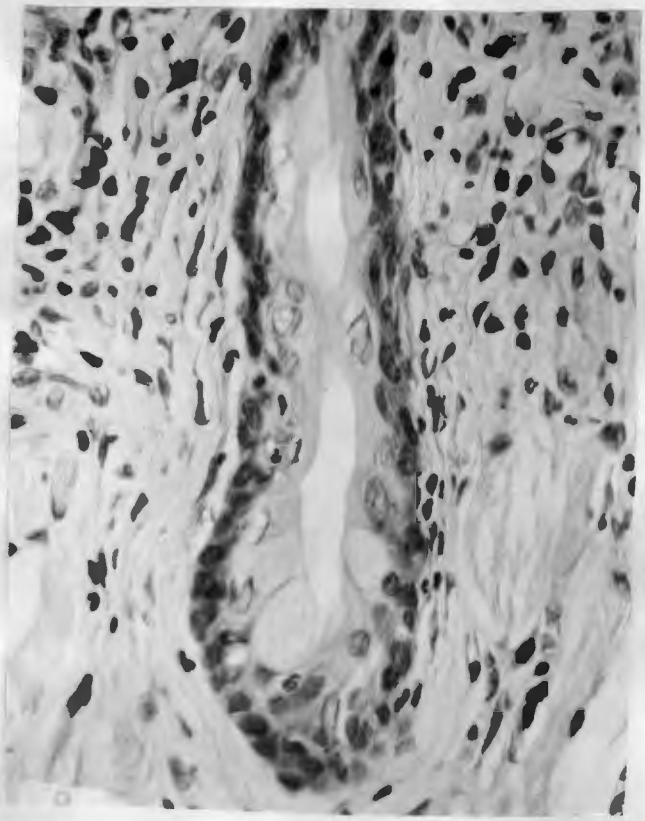


Fig. 30: High power photomicrograph of gland-like structures shown in Fig. 29 (H & E x 430).

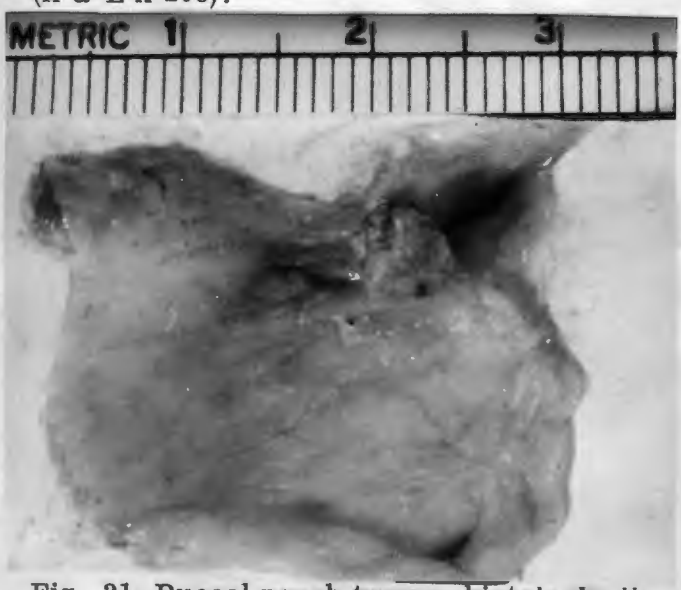


Fig. 31: Buccal pouch tumor, histologically infiltrating carcinoma, after 12 weeks' application of DMBA only.

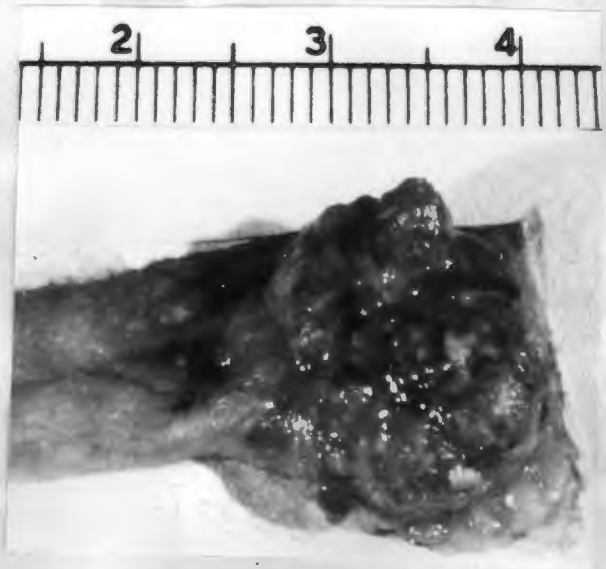


Fig. 32: Buccal pouch tumor, histologically infiltrating carcinoma, after 10 weeks' application of DMBA and 10% vitamin A.

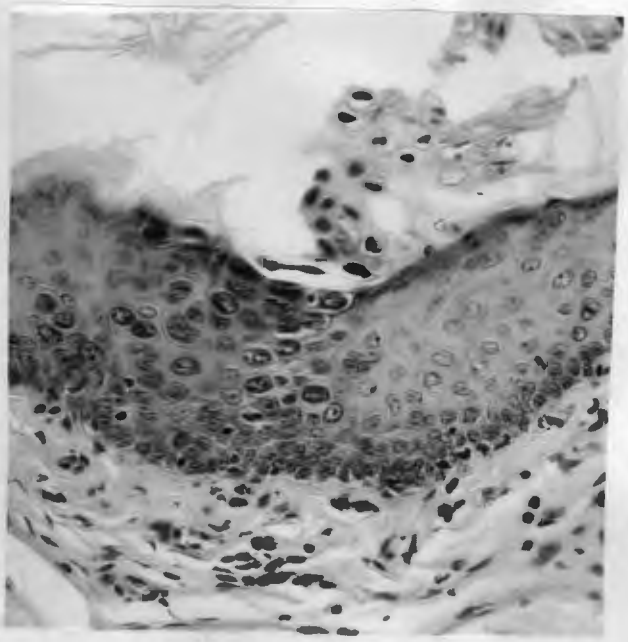


Fig. 33: Buccal pouch epithelium after 10 weeks' application of 10% vitamin A only, showing acanthosis and moderate atypicality (H & E, x 270).

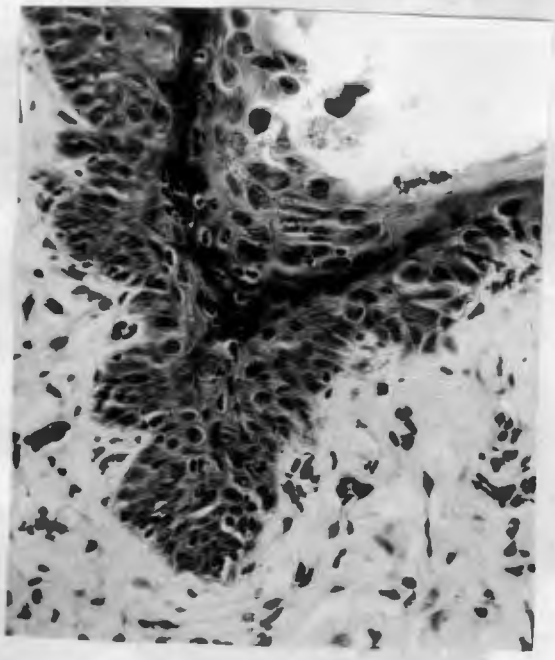


Fig. 34: Buccal pouch epithelium after 10 weeks' application of 10% vitamin A only, showing atypical cells at the surface of an area of acanthosis (H & E, x 270).

immediately after completion of local treatment with DMBA during 6 weeks, showing irregularity of the mucosa, contraction of the distal end of the pouch, and a small papillary tumor.

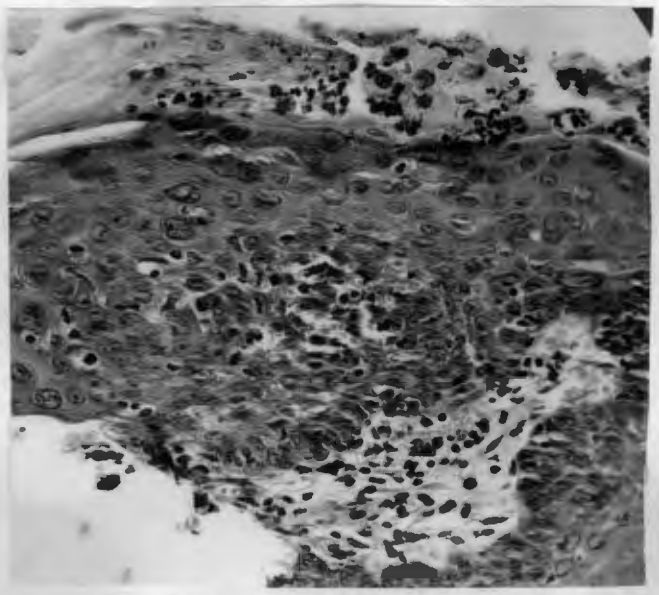


Fig. 36: From cheek pouch shown in Fig. 35 Area of intra-epithelial carcinoma with pleomorphism and loss of polarity but without infiltrating growth (H & E, x 270).

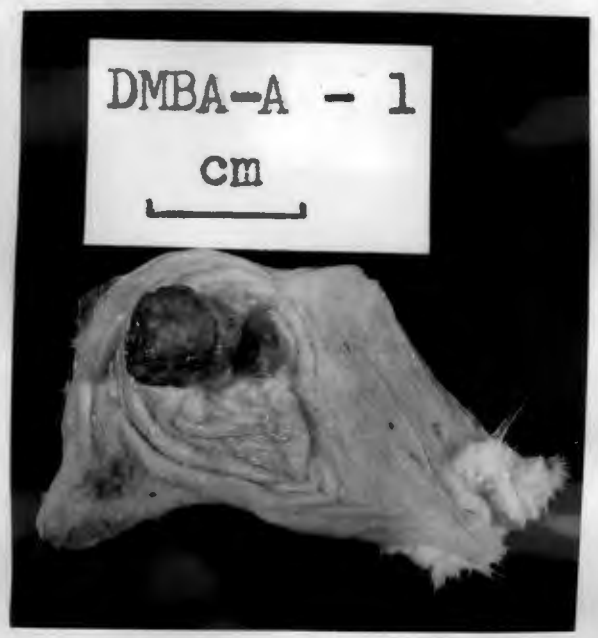


Fig. 37: Cheek pouch of hamster sacrificed after treatment with DMBA during 6 weeks, followed by the application of vitamin A during 2 months, showing irregularity of the mucosa, and 2 large tumors in the distal end of the pouch.

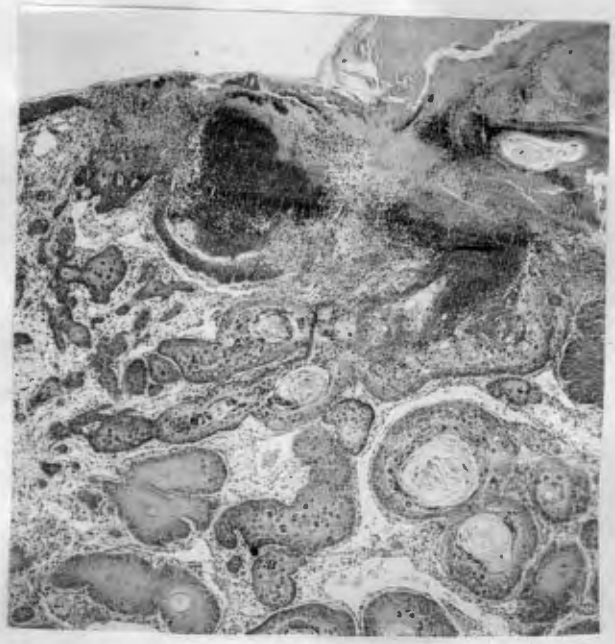


Fig. 38: From cheek pouch shown in Fig. 37 Infiltrating keratinizing squamous cell carcinoma with necrosis, ulceration and hemorrhage (H & E, x 270).

Fig. 39: Cheek pouch of hamster sacrificed after treatment with DMBA during 6 weeks, followed by paraffin for 2 months, showing irregularity of the mucosa, and a papillomatous tumor. This was the only tumor found in this group of animals.

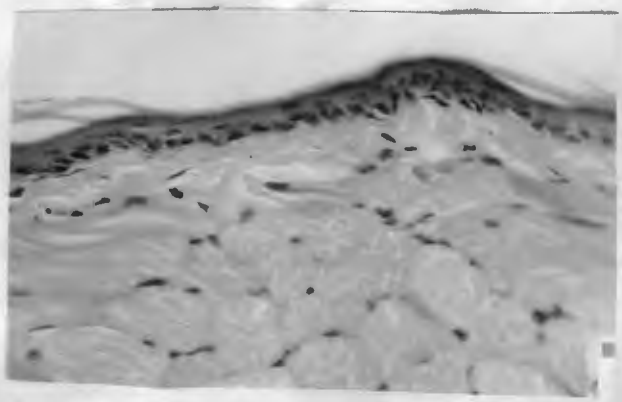


Fig. 40: From cheek pouch of hamster treated with DMBA for 6 weeks, followed by paraffin for 2 months, showing diffuse epithelial atrophy. This was the general appearance of the mucosa in both Groups III and IV (H & E, x 270).

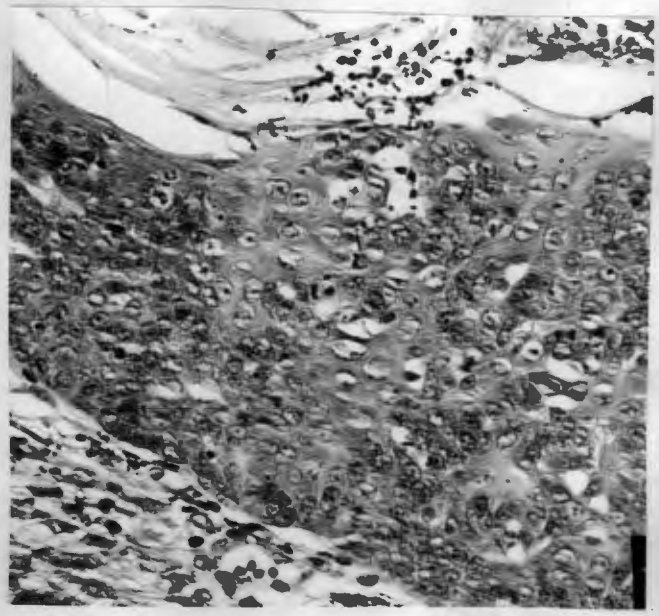


Fig. 41: Area of intraepithelial carcinoma from tumor shown in Fig. 39. This was the most advanced lesion found in this group (H & E, x 270).

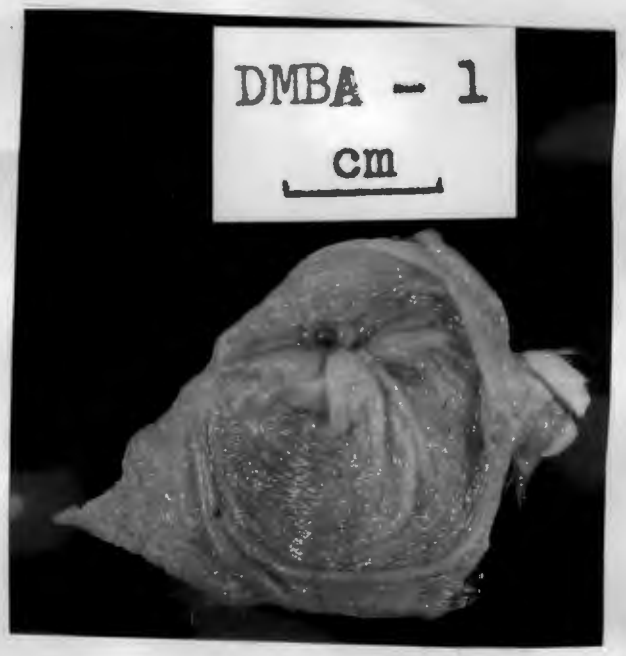


Fig. 42: Cheek pouch of hamster sacrificed 2 months after the completion of treatment with DMBA during 6 weeks, without treatment in the intervening period, showing irregularity of the mucosa, slight contraction of the distal end of the pouch, and small papillary tumor.

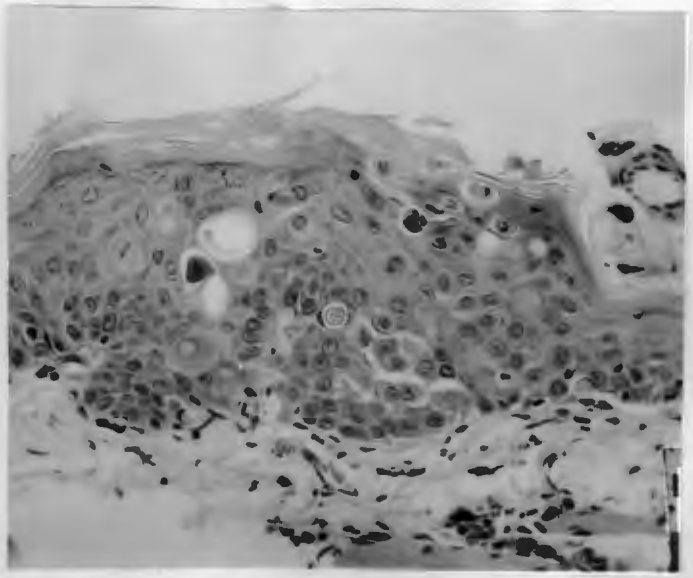


Fig. 43: From non-tumorous cheek pouch mucosa shown in Fig. 42. Area of acanthosis with cellular atypicality and occasional dyskeratosis but with preserved polarity. This was the most advanced lesion found in this group (H & E, x 270).

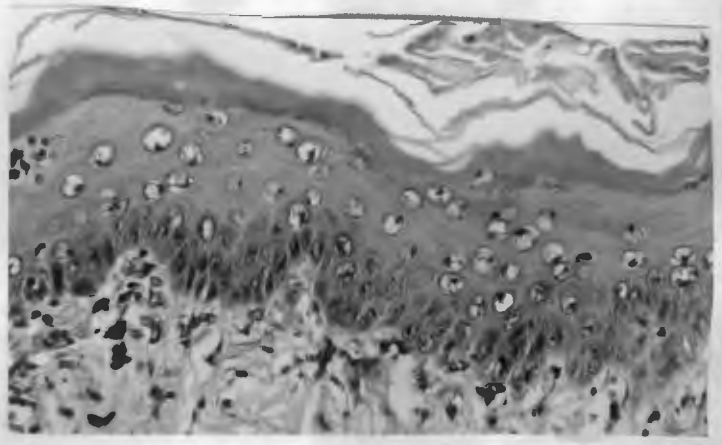


Fig. 44: Hamster cheek pouch immediately after cessation of treatment with DMBA during 4 weeks, showing leukoplakia (H & E, x 260).

Fig. 45: Cheek pouch of hamster sacrificed after completion of local treatment with DMBA for 4 weeks, followed by Vitamin A for 8 weeks, showing at least 1 large fungating tumor, 5 mm. in diameter, a number of smaller tumors and gross irregularity of the pouch mucosa.

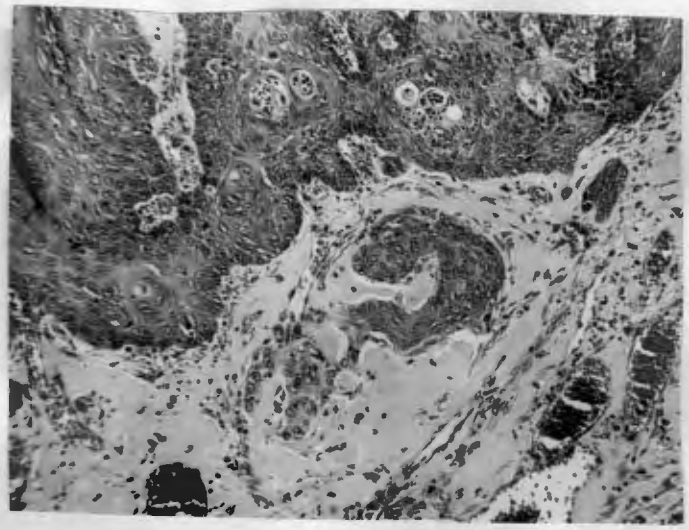


Fig. 46: Infiltrating squamous cell carcinoma from cheek pouch treated with DMBA during 4 weeks followed by Vitamin A during 2 months (H & E x 105).

Fig. 47: Cheek pouch of hamster sacrificed after cessation of local treatment with DMBA for 4 weeks, followed by paraffin oil only for 8 weeks, showing irregularity of the mucosa. No gross tumors are visible.

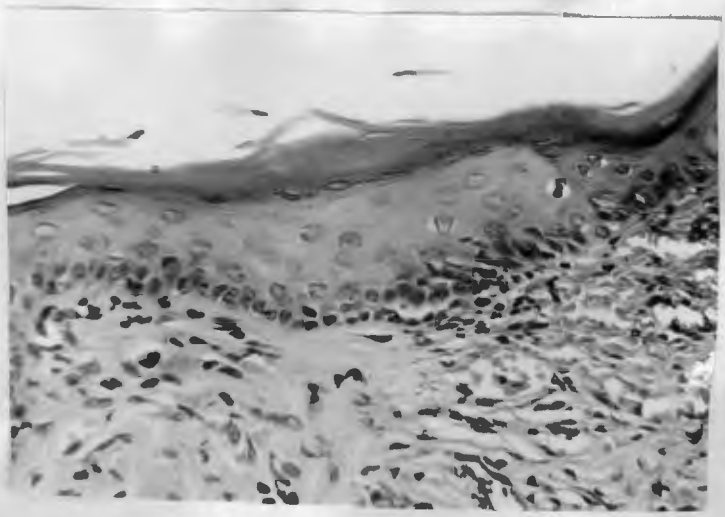


Fig. 48: Area of moderate acanthosis and hyperkeratosis from cheek pouch treated with DMBA during 4 weeks, followed by paraffin during 2 months. (H & E, x 260).

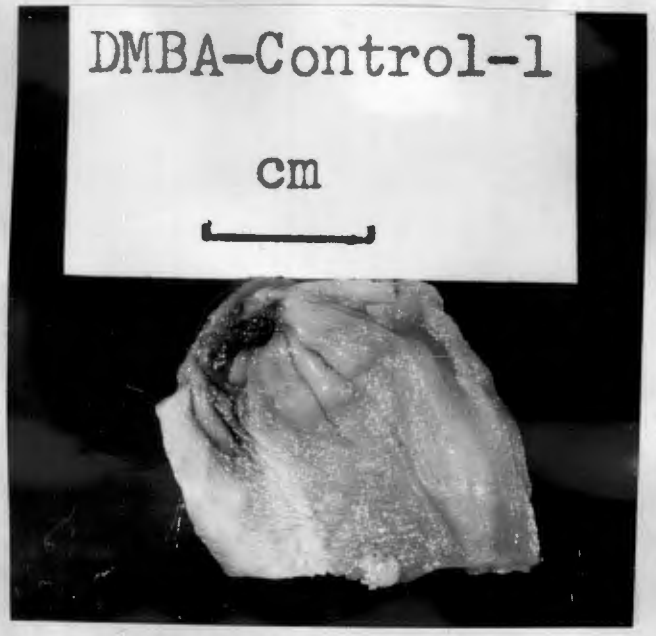


Fig. 49: Cheek pouch of hamster sacrificed after completion of local treatment with DMBA for 4 weeks, followed by a treatment-free period of 2 months, showing mild irregularity of the mucosa and an area of haemorrhage, in the distal end of the pouch.

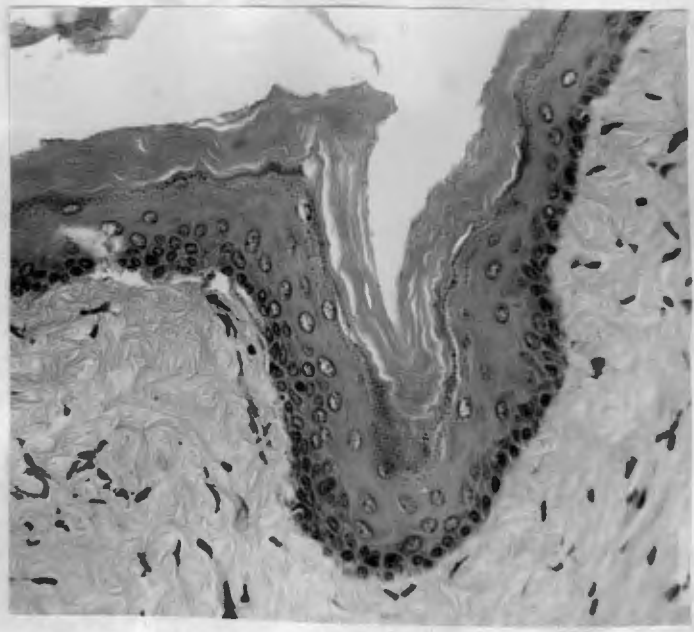


Fig. 50: Area of moderate acanthosis and hyperkeratosis from cheek pouch treated with DMBA during 4 weeks, followed by a treatment-free interval of 2 months (H & E x 260).

Some of the results of this studies have been published in the following journals:-

1) Polliack, A and Levij, I.S. Increased incidence of carcinoma induced by DMBA in the hamster cheek pouch in response to vitamin A. Nature, 216: 187-188. 1967.

2) Levij, I.S. and Polliack, A. Potentiating effect of vitamin A on 9-10 dimethyl 1-2 benzanthracene - carcinogenesis in the hamster cheek pouch Cancer: 22: 300-306. 1968.

3) Polliack, A. and Levij, I.S. Epithelial atypia in the hamster cheek pouch, induced by topical application of Vitamin A. Oncology, 22: 129-136. 1968.

4) Polliack, A. and Levij, I.S. The effect of topical vitamin A on papillomas and intra-epithelial carcinoma induced in the hamster cheek pouches with DMBA. Cancer Research, Feb. 1969.

5) Polliack, A., Rwomushana, J., Levij, I.S. Treatment of experimental benign hyperkeratotic lesions of the hamster cheek pouch by topical vitamin A palmitate (in press).

6) Levij, I.S., Rwomushana, J., and Polliack, A. Enhancement of chemical carcinogenesis in the hamster cheek pouch by prior topical application of vitamin A palmitate. Journal of Investigative Dermatology (in press).

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15) Billingham, R.E., Ferrigan L.W. and Silvers, W.K. Cheek pouch of the Syrian hamster and tissue transplantation immunity. Science. 132:1488.1960.

16) Blough, H.A. The effect of vitamin A alcohol on the morphology of Myxoviruses. 1. The production and comparison of artificially produced filamentous virus. Virology 19:349. 1963.

17) Brookes P. and Lawley P.D. Evidence for the binding of polynuclear aromatic hydrocarbons to the nucleic acids of mouse skin: Relation between carcinogenic power of hydrocarbons and their binding to deoxyribonucleic acid. Nature. 202:781. 1964.

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