STUDIES OF INITIATING STAGE

OF

EPIDERMAL CARCINOGENESIS

IN MICE

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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March, 1963

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PREFACE

The intention to study the nature of diseases by experiment led me to experimental pathology three years ago. It was hoped that experience of experimental pathology would give me better understanding of the many diseases which I deal with daily, as a pathologist. Among many subjects, my interest in the behaviour of human tumours stemmed from the observation of various tumours while training in surgical pathology.

The mysterious behaviour of human tumours resulted in an academic ambition to study the fundamental nature of tumours and the mechanism of neoplastic transformation. Thus, carcinogenesis became the subject of my study.

Despite the vast amount of factual material accumulated, a theoretical understanding of what occurs in tissue and cells during carcinogenesis is still lacking. The stimulus needed for this study is amply provided by any awareness of the human suffering and despair mirrored in the face of those with tumours.

Dr. G.C. McMillan, Strathcona Professor and Chairman of the Department of Pathology, McGill University, and Dr. A.C. Ritchie, then Miranda Fraser Associate Professor of Comparative Pathology, McGill University, accepted me at the Pathological Institute in Montreal. The kindness and encouragement extended by Dr. McMillan provided me with a unique atmosphere and a ready entry into the new field of research work.

Dr. Ritchie, with authority and wide experience in the field, guided my experimental work throughout the three years of my research training. The strict yet kind instruction and constructive criticism helped to orient my endeavour. His supervision always gave me the self assurance needed to face the many problems encountered in the experimental work. When Dr. Ritchie moved to Toronto as Professor and Head of the Department of Pathology, University of Toronto, it was very fortunate for me that I was able to join his department there and continue my work under him.

All the members of the staff of the Department of Pathology, both at McGill University and at the University of Toronto, aided me in many ways. Dr. J.W. Steiner, Assistant Professor of Pathology in the University of Toronto, helped me through conversation and co-operation. Dr. B.I. Wigensberg, Assistant Professor of Pathology at McGill University, gave me much help and advice in the work related to the chemical study. Dr. J.V. Frei, senior in our project, worked with me for two years in Montreal. His brilliance and energetic work always stimulated me. Dr. M.C. Patterson joined the project in Toronto, and became a consistent helpmate. My discussions with these colleagues, and their helpful suggestions were of immeasureable value.

Mr. H. Nye, Mrs. M. Passuka and Mrs. H. Foley, all of the Department of Pathology at McGill University, and Miss G.K.R. Boyd, Mrs. J. Cetkovski and Miss H. Williamson of the Department of Pathology at the University of Toronto aided me by their efficiency and kindness.

I would like also to extend my deep appreciation to the technical staff who worked on our projects. Without their help my work

would never have been completed. Miss H. Basillières, Mr. W. Kingsley, Mr. S. Muity and Mr. L. Sales at Montreal and Mr. P. Burton and Mr. B. Eichfuss at Toronto worked to keep the animals and the animal rooms in the best possible condition. They worked hard, were dependable, and have become good friends. Mrs. N. Geoffroy at Montreal and Miss L. Harrison at Toronto did most of the histological work, and Mr. H. Colleta at Montreal and Mr. H. Layne at Toronto did the photographical work. Mrs. H. Hardman of Toronto did the final typing. To all those people I am deeply indebted and am sincerely grateful.

Very humbly, I wish to dedicate this thesis to my mother, who unfortunately, did not live to see its completion. It was she who instilled in me the ambition and perseverance necessary to embark on this phase of my education. I will be forever grateful. To my wife, Maria Anthonine, no words can express my gratitude. Her deep understanding of my studies and never failing encouragement aided in the achievement of this goal.

The work described in this thesis was done in about three years. Needless to say, there is much more to be done if comprehension and mastery of the subjects which I have studied are to be gained. Experimental work has taught me a new approach to the study of pathology. It is my hope that the work has made also a small contribution to the vast world of experimental research.

This is not the end, but merely the beginning.

March, 1963

H. Shinozuka

PREAMBLE

The thesis consists of five parts, a review of literature, an account of the experimental work, a discussion of the findings, a bibliography and appendices.

The review of literature begins with a brief note on the history of epidermal carcinogenesis, and proceeds to discuss the development of the two stage theory of carcinogenesis, and our knowledge of the nature of initiation and promotion. Two stage carcinogenesis other than in mouse skin, and the immunological aspects of carcinogenesis are discussed briefly. The dose response of mice to carcinogen and the interaction of carcinogen with nucleic acids are also discussed.

The experimental work consists of eight experiments. In each case, a description of method is followed by a summary of the results. The discussion of the experimental results is reserved for the discussion, at which time, the findings are evaluated and compared.

In the appendices are tables and figures giving the complete and detailed experimental results, to augment the summaries given in the text. The bibliography lists the many works referred to in the text.

In the tables and figures, several abbreviations are used. 9,10-dimethyl-1,2-benzanthracene is referred to as DMBA, croton oil as CO, and orotic acid as OA.

I REVIEW OF LITERATURE

A) INTRODUCTION

The earliest description of cancer has been traced back to records on Egyptian papyri of the fifteenth century B.C. Following this, in the era of Hippocrates, in the fourth century B.C., the nature of cancer was described as the presence of a tumour, and the absence of any tendency towards healing. Hippocrates used the term "καρκινος ", "carcinos", for all indolent ulcers, and "καρκινωμα", "carcinoma" for progressive malignant tumours. In spite of many descriptions, and many personal views as to the origin of cancer in the following centuries, little came but mere speculation.

Perhaps, the one of the most notable events in the history of cancer research was the essay by Bernard Peyrihle(1735-1804) of Lyon, "Qu'est ce que le cancer?". In this essay, he described an attempt to identify the toxin of cancer and to trace the spread of the disease, in order to devise a better method of treatment. Though his experiment was a failure, he won the prize for the best essay from the Académie des Sciences et Belles-Lettres de Lyon, and has been rightly considered as the person who conducted the first systemic experimental investigation.

The microscope helped develop knowledge of cellular pathology. The early nineteenth century was occupied with a detailed study of the morphology of tumours, their separation from a variety of diseases, the elucidation of their histogenesis, and the writing of the natural

history of malignant neoplasms. Cancer research, as an experimental science, began at the close of the nineteenth century with the study of animal tumours, and of the transplantability of these tumours to a new host. The introduction of animal experiments into the field of cancer research has been of great value, for it enabled the investigators to study various characteristics of neoplastic disease at will, unhampered by the limitations of human subjects. The twentieth century opened as a real experimental era with systemic study. Although more than two hundred years have passed since Peyrilhe's attempt, research into the cause of cancer is still one of the most intricate and difficult scientific activities of modern times.

A large number of agents of many kinds are now known to be capable of inducing cancer in various animal tissue. They may be classified into three categories: physical, chemical and biological. The physical agents may be represented by X-ray and ultraviolet light, and biological agents by viruses and hormones. As for chemical agents, Hartwell(1941;1951), listed close to 1300 chemical substances which had been tested for carcinogenic activity. Some 200 were reported to possess carcinogenic activity. Furthermore, Shubik and Hartwell(1957), published a supplementary work reporting that many more carcinogenic substances had been found. It is, moreover, not improbable that as the number of compounds tested increases, yet more compounds will be found capable of inducing cancer in animals. It must be realized, however, that the primary purpose of these

investigators has not been merely to describe new carcinogens, but to find important relations among the known carcinogenic agents and to acquire information as their mode of action.

The field of cancer research is widespread. The efforts in numerous branches of medicine, such as clinical medicine, pathology, radiology, genetics, bacteriology, and endocrinology, have all been directed toward the common aim of elucidation and control of the neoplastic transformation. As the field is so widespread, no single individual is capable of mastering the entire field of cancer research, but no individual can work effectively at the modern level in any one of its component disciplines without understanding something of the work as a whole and appreciating its boundaries. Nevertheless it is not possible to discuss cancer research in its entirety in this review. The discussion will be confined therefore, to the development of experimental epidermal carcinogenesis.

Epidermal carcinogenesis is one of the oldest subjects in cancer research. Carcinogenic agents can be applied directly to the skin, and the subsequent changes followed step by step by direct visual observation. The knowledge of epidermal carcinogenesis has been markedly advanced by the huge accumulation of information from individuals and groups scattered throughout the world. One is confronted with a monstrous and amorphous literature, some of which is hastily ambitous, some controversial, and some containing hopeful hypotheses. In this review special attention will be given

to the development of our knowledge of carcinogenesis in the skin of mice. Other species of animals will, however, be considered from time to time, on a comparative basis.

There are many reasons for the use of the skin of mice for the investigation of epidermal carcinogenesis. The advantages of using mice in experimental cancer research was cited by Borrel(1909), "Avec les souris qui vivent trois ans au maximum et qui donnent des générations nombreuses, il est toujours possible de soumettre a l'épreuve expérimentale la notion de l'hérédité et dans des conditions de complète certitude. Un an d'observation sur les souris, c'est un siècle de vie humaine.

Avec les souris, d'un prix de revient minime, d'un entretien facile et peu onéreux, on peut grouper dans un espace restreint des milliers de sujets d'expérience: constituer des hameaux, des villages, des villes soumises à des régimes variés, à des conditions diverses et l'étude étiologique du Cancer se trouve vraiment possible!! This can be translated, as follows: As the mouse lives only three years at the most, and produces many generations of descendants, the question of heredity can be submitted to experimental investigation under definite and fixed conditions. One year of observation in the mouse, is as good as a hundred years in man. Cheap to buy, and easy to maintain, mice can be housed by the thousand in a small space, and hamlets, villages and cities of them subjected to the most diverse conditions. This statement still stands true, even at present, and for these

and other reasons, many investigations are performed using mice.

Excellent reviews of the history of cancer research, and of epidermal carcinogenesis are available, and were consulted in the preparation of this thesis. Valuable information was derived from such reviews, particularly about the early developments in the field. The following were especially valuable. For the historical review; Wolff(1907), Woglom(1913), Ewing(1940), Oberling(1943), and Willis(1960). For epidermal carcinogenesis; Woglom(1926), Watson(1932), Seelig and Cooper(1933), Greenstein(1954), Cowdry(1955), Huxley(1958), and Homburger and Fishman(1959).

B) EXPERIMENTAL TUMOURS OF THE SKIN

1) Early Work other than with Tar and Polycyclic Hydrocarbons

Near the end of the nineteenth century, the discovery of X-Rays(1895) by Roentgen and the discovery of radioactivity(1896) by Becquerel were soon followed by serious lesions on those exposed to the ionizing radiation. The carcinomata of the skin which occurred in many of the pioneer radiologists and physicists proved the association between prolonged exposure to radiation and cancer. The first case of cancer of the skin in man thought due to irradiation was reported by Frieben, in 1902, from Hamburg (Frieben, 1902). A thirty-three year old man employed in the manufacture of X-ray tubes tested the apparatus with his hand for four years, without adequate protection, and developed cancer on the skin of his hand, necessitating amputation of the arm.

Other reports soon followed, and the number of victims of X-ray cancer in the entire world, are presumed to be counted by the thousands (Oberling,1944).

In man, roentgen cancer never appears immediately after the exposure, but is always preceeded by chronic dermatitus which may not become manifest for years. It is known that the latent period, between the application of the noxious agent and the inception of cancer, may vary from four to fifteen years. In animals, Marie, Clunet and Raulot-Lapointe in 1910, (quoted by Marie, Clunet and Raulot-Lapointe 1912) reported the induction of sarcoma in a rat by X-ray. They irradiated rats until ulcers appeared and kept these from healing by renewed exposures. Two of the animals developed sarcoma, one nine months and the other two years after the beginning of the experiment. This is the first experimentally induced animal tumour recorded in literature. Following this, Bloch(1924) produced carcinoma of the skin by localized irradiation of rabbit ears with a dose amounting to about 3.8000r. in 32 months. In his study, the importance of dosage was clearly demonstrated. Light exposure was followed by superficial burns only, and very large doses by necrosis without subsequent tumours. Intermediate doses gave rise to cancer. Jonkhoff(1927) was the first to report the production of carcinoma of the skin in mice by radiation.

In old references, a number of convincing illustrations and descriptions of the carcinogenic power of sunlight are found, such as the relative frequency of cancer of the skin among those who pass their

lives in the open air, and the limitation of these neoplasms to the exposed parts of the body.

The production of skin tumours in mice and rats by ultraviolet rays from mercury vapour lamps was reported by Findlay(1928; 1930). It is not necessary to use an artificial source, as sunlight alone will suffice. Roffo(1935) exposed 600 white rats to the sun for five hours each day over a period of seven to ten months. Among the 235 survivors, seventy percent developed carcinoma or sarcoma on the hairless areas, such as the ears, eyelids, snout and paws.

The carcinogenic action of the radioactive emission from radium in animals was reported by Daels and Biltris(1931) who produced malignant epithelial and connective tissue tumours on mice and guinea pigs.

From this evidence drawn from the early stages of experimental research, it can be concluded that every form of electromagnetic radiation, X-ray and ultraviolet, is endowed with carcinogenic power. The search for the nature of the action of these rays has been a struggle against many difficulties. Speculation that the development of cancer is a result of some action exerted by the rays on the cell's nucleus, and particularly on the nuclear chromatin, was derived from the frequent observation of degenerative changes in the cell nucleus in irradiated cells. With the demonstration of the induction of germinal mutations by X-ray (Müller, 1927), the idea developed that the action of the rays on somatic cells was similar. The abnormal chromatin complexes, and the multipolar mitoses observed after radiation favour this idea. The hypothesis that X-ray cancer is the

production of a somatic mutation has long been an attractive conception.

The hormonal influence in the genesis of cancer is particularly clearly shown in mammary tumours. The sex-limited nature of the spontaneous occurrence of such tumours led directly to this speculation (Lathrop and Loeb,1913). Lacassagne(1932) was the first to induce mammary cancer in male mice by means of purified oestrogen. However, so far as epidermal carcinogenesis is concerned, the evidence for a direct action of hormones is scanty.

Bosc(1903), and Borrel(1903) first put forward the hypothesis that cancer is of viral origin, basing the theory on the proliferative effect on tissue sometimes exerted by a virus. In 1908, Ellerman and Bang(1908), published their classical observation of leukaemia in fowls. Leukaemia can be transmitted from one chicken to another by the injection of blood or organ extracts, even after they have been ultra-filtered. Two years later, Rous(1910;1911) studied a spontaneous sarcoma of subcutaneous tissue in Plymouth Rock hens, and showed its viral origin. At about the same time, Fujinami and Inamoto(1914) reported a filterable myxosarcoma of the fowl. Later, Shope(1933) described the skin papilloma which appeared on the skin covering the abdomen, and on the neck and shoulder of wild rabbits from Iowa and Kansas. These tumours could be transmitted with an ultra-filtrate to both wild and domestic rabbits, but not to rats, mice, guinea pigs, or cats. Subsequent studies by Rous and Beard(1934;1935) established the viral origin of these papillomata.

The old belief that "chronic irritation" is the cause of

cancer has been a constant point of controversy. Numerous clinical instances are cited in the old literature, suggesting a causative relationship between an irritant and cancer. Virchow, justly called the father of the irritation hypothesis, stressed the importance of chronic inflammation, which he thought might lead to cancer, whatever its type, be it from burns, soot or sunlight. The idea that trauma may be responsible for the development of tumours is also widely held by both the public and a number of those in the medical profession. Opinion is divided, some think that as many as 44.7% of all malignant tumours may be due to trauma (Löwenthal, 1895), others that not many tumours have been caused by trauma (Knox, 1929). In consequence, a variety of irritants, both chemical and physical have been studied by many investigators, with confusing results. For example, Burckhardt and Mueller(1923) failed to produce cancer by repeated scalding or burning; Daels(1923) found that eosin, haematoxylin gentian violet, picric acid, silver nitrate, phosphorus, arsenic, ichthyol and balsam of Peru were all without carcinogenic action; Leitch(1922a;b) succeeded in producing carcinoma in mice by crude shale oil containing paraffin and arsenic, although he found tobacco tar extract benzene, benzidine, xylol, aniline, various dyes, iodine, zinc chloride and creosol all to be without carcinogenic activity (Leitch, 1923); Derom(1924) was unable to excite malignant growths with tincture of iodine or with dilute phenol; but Narat(1925) succeeded in eliciting carcinomata in two strains of mice by painting with 3-6% potassium hydrochloride or 3-5% hydrochloric acid.

2) The Establishment of Tar Cancer

Even before the beginning of this century it had been known that those whose occupation exposes them to soot, tar, or similar substances are prone to develop cancer. The earliest paper in this field is perhaps that of Percival Pott, who, in 1775, reported in his "Chirurgical Observations" on the prevalence of scrotal cancer in chimney sweeps. He noted that "chimney sweepers disease", scrotal cancer, appeared at the age of twenty to twenty-five in the men who engaged in the chimney work, in thick and sooty chimneys during their boyhood. This indicated that the latent period for the appearance of cancer was about ten to fifteen years. However, only one hundred forty years after Pott's paper was tar subjected to adequate experimental examination. Earlier, Hanau(1889), had painted rats for months with gas tar and similar materials, but elicited only a chronic dermatitis. In his paper, he suggested that a long period of application might prove more successful in eliciting tumours. Cazin, in 1894, using a dog, failed to produce cancer after five months tarring (quoted by Goedel, 1923). Tobacco tar was applied to the ears of rabbits by Wacker and Schmick(1911) without the development of tumours. Haga(1913) similarly failed to induce tumours when he applied soot to the ears and scrotum of rabbits. Probably in these experiments, the irritants were applied for too short a period, to the wrong species, or were the wrong kind of irritant. The experiment by Bayon(1912) was considered to be the most promising of the early works. He reported abundant

epithelial proliferation four weeks after the injection of gas-work tar into the ears of rabbits. In spite of these many failures to produce epidermal tumours, in Germany, France, England, and Japan, the problem of inciting malignant tumours at will was on the eve of solution at the beginning of this century.

It was certainly good fortune that at least two Japanese investigators selected an animal sensitive to the irritant used, an appropriate irritant, and continued to apply the irritant for a sufficient length of time. In 1914, Yamagiwa and Ichikawa reported that they had been able to produce papillomata on the ears of rabbits by repeated applications of tar (Yamagiwa and Ichikawa,1916: The first complete description of their experiments, portions of which are illustrated in the Appendix VIII). In the following year they recorded the development of three cancroids in the tarred areas. By 1917, they had produced sixteen carcinomata and forty-nine benign tumours in rabbit ears (Yamagiwa and Ichikawa,1917). The shortest latent period for the production of a carcinoma was 103 days, and the longest 565 days. Soon after the publication of Yamagiwa and Ichikawa's work, Tsutsui(1918) painted the skin of the back of mice with coal tar every three or four days, and succeeded in producing cancer after one hundred days or more. Two of the mice had metastases to the lung. Tsutsui found mice even more susceptible than the rabbit, for the growths arose earlier and more frequently; indeed, with suitable technique, he was able to induce tumours in ninety to one hundred percent of his animals. Tsutsui's work was soon

confirmed by Fibiger and Bang(1920). In mice painted every two or three days, the first papillary outgrowth appeared on the eighty-sixth day, while all those that lived from 275 to 331 days developed malignant tumours. Wide confirmation of the work of Yamagiwa and Itchikawa and Tsutsui appeared in literature, and the first great period of experimental carcinogenesis began.

3) Analysis of Tar and Isolation of Carcinogenic Hydrocarbons

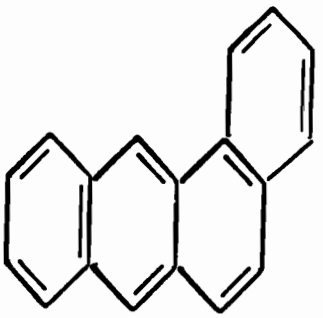
After the pioneer investigators had established the carcinogenic effect of coal tar, the next logical step was the fractionation of tar, and the isolation of the active material or materials. This work was met with considerable difficulties, for in early studies, several hundred substances were identified in coal tar, though not more than one hundred have been definitely isolated (Spielman,1924). Furthermore, the composition of tar varied considerably, depending on the coal from which it was distilled. An early observation was made by Bloch and Dreifuss(1921), in Germany, who stated that the carcinogenic factor in coal tar was contained in the high boiling fractions (370-440°C), and that it was soluble in benzol and contained no hydrocarbons, bases, or phenols of a low boiling point. This fraction caused extremely malignant tumours in one hundred percent of the mice in about four months. Following this, the summary of various investigations by Deelman(1922), was that carcinogenicity was associated with certain fractions, that the active constituent was soluble in benzol, and

that it was probably a cyclic hydrocarbon of high molecular weight. After these first tentative efforts, a systemic search for the isolation of the carcinogenic fraction of tar was instituted by a group of workers in the Royal Cancer Hospital in London; Kennaway, Cook, Hieger and others.

Kennaway(1925) was able to prepare a number of artificial carcinogenic tars, some of them containing only compounds of carbon and hydrogen. The fractionation of coal tar into carcinogenic fractions was facilitated by the discovery by Hieger(1930) that tar and oils known to produce cancer in experimental animals gave characteristic fluorescene spectra, with bands occurring at 4,000, 4,180 and 4,400 Å. Many known compounds with natural fluorescene were examined, among them, anthracene, but only 1,2-benzanthracene (Figure 1) was found to give bands near the wave lengths mentioned above. The substance proved to be weakly carcinogenic, but the resemblance of its spectrum to those of the more active carcinogenic tars stimulated the synthesis of polycyclic hydrocarbons related to 1,2-benzanthracene.

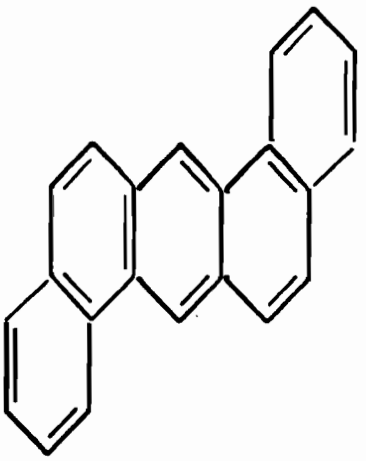
Among the compounds related to 1,2-benzanthracene studied was 1,2,5,6-dibenzanthracene (Figure 2), which proved to be a highly active carcinogenic substance, and was the first pure substance to induce cancer in a large proportion of the experimental animals. In 1933, Cook, Hewett, and Hieger(1933), succeeded in isolating an unknown hydrocarbon in pure form from coal tar, and subsequently this was shown to be 3,4-benzpyrene (Figure 3). This compound possessed a fluorescence spectrum very similar to that of some of the original tars, was present in the original tar to the extent of at least 0.003%, and was powerfully

Figure 1



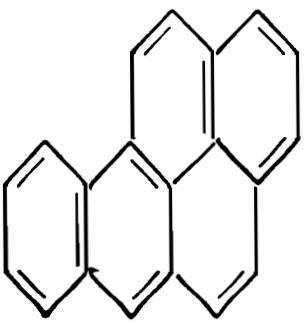
1,2-Benzanthracene

Figure 2



1,2,5,6-Dibenzanthracene

Figure 3

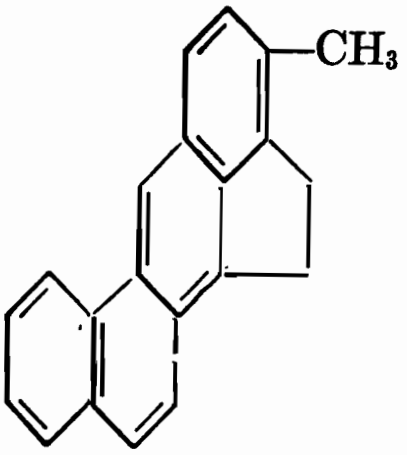


3,4-Benzpyrene

carcinogenic. Benzpyrene became one of the most widely employed carcinogens in experimental cancer research, and it is of particular interest because of its wide distribution. For instance, it has been identified in the exhaust of internal combustion engines, in the soot of industrial cities (Goulden & Tipler,1949), in processed rubber (Falks, Steiner, Goldfein, Breslow and Hyks,1951), in carbon black (Falks and Steiner,1952), and in the atmosphere (Waller,1952). The isolation of 3,4-benzpyrene solved the mystery of the carcinogenic property of coal tar but in the course of the synthetic studies, it was observed that many other hydrocarbons other than 1,2,5,6-dibenzanthracene possess carcinogenic activity. Not long after the isolation of 3,4-benzpyrene, another potent carcinogenic hydrocarbon, 20-methylcholanthrene (Figure 4) was produced by Wieland and Dane(1933) and Cook and Haslewood(1934) from derivatives of 1,2-benzanthracene, and by degradation from deoxycholic acid. One of the interesting facts about methylcholanthrene is the nature of the starting materials for its synthesis. Fieser(1936) derived the methylcholanthrene from cholic acid which is a natural component of the bile. Furthermore, it is chemically related to the sterols, including vitamin D and sex hormones.

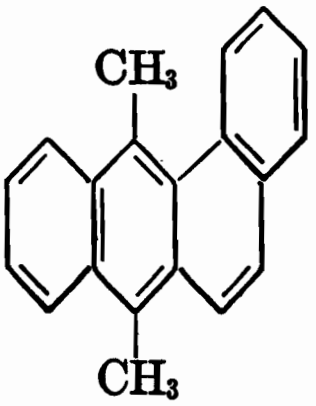
After the initial success in the isolation of chemical carcinogens, two important groups of investigators paid particular attention to the examination of the carcinogenic activity of the pure substances isolated. In London, Kennaway, Cook, Hieger and others started their investigation in the 1920's and the work continued into the early 1940's. In Boston, Shear, Fieser, Andervont and others,

Figure 4



Methylcholanthrene

Figure 5



1,4-Dimethyl-2,3-benzphenanthrene
(9,10-Dimethyl-1,2-benzanthracene)

embarked, in the early 1930's on a series of investigations of the carcinogenecity of the polycyclic hydrocarbons of the type isolated from tar by Kennaway and his colleagues. Thus, over a period of roughly ten years, two major efforts overlapped.

Many synthetic hydrocarbons were synthesized and tested for carcinogenecity. Another group of compounds that are derived from 3,4-benzphenanthrene was studied by the Kennaway group (Badger, Cook,Hewett,Kennaway,Kennaway, and Martin,1942). One of these was 2,3-benzphenanthrene (1,2-benzanthracene). With further substitution of the methyl group, at 1,4 position, thus gives 1,4-dimethyl-2,3-benzphenanthrene (9,10-dimethyl-1,2-benzanthracene). (Figure 5) This material is one of the most potent carcinogen known for mouse skin, and is commonly used for experimental studies at the present time.

It is worthwhile to notice that the London group of workers consistently tested compounds for carcinogenic potency by painting these on the backs of stock mice as a solution in benzene. However, in 1932, Burrows, Hieger, and Kennaway(1932), did dissolve 1,2,5,6-dibenzanthracene in lard, and gave it by subcutaneous injection to experimental animals, obtaining sarcomata at the site of the injection. The use of stock mice of mixed unknown genetic characteristics by the London group, interposed several hazards in the accurate estimation of carcinogenic potency.

The Boston group used an inbred strain of mice of known susceptibility, but used the technique of subcutaneous inoculation

of solutions of hydrocarbons in fatty vehicles, so the tumours produced were mainly subcutaneous sarcomata. It is important to notice that findings by these different routes of administration were different. For example, the London group reported that by the skin painting technique, 10-methyl-1,2-benzanthracene was non-carcinogenic (Cook, Robinson and Goulden,1937), but by the subcutaneous injection method the Boston group reported the substance carcinogenic (Shear, 1938). Such discrepancies have become even more obvious as experiments with different routes of administration and with different species, have grown.

Subsequent studies extended in many directions. The structure of the various active substances, biological factors such as susceptibility of the different strains and species, the effect of solvents, the effect of dosage and many other facets were investigated.

4) Modification of Carcinogenic Action

After it became obvious that tar and pure polycyclic hydrocarbons were effective in the production of skin tumours, but that other irritants were not, the old belief that chronic irritation is the cause of cancer grew faint. However, the question remained, whether irritation as such is associated with carcinogenesis or not. For instance, in occupational tumours in man and in experimentally produced tumours in animals hyperplasia often preceeds the development of the tumours. Irritation, both physical and chemical is well known to elicit a continued state of reparative hyperplasia. The trend of investigation

moved in a new direction, to consider the study of the influence of non-carcinogenic or weakly carcinogenic substances on the action of tar or pure chemical carcinogens. Shortly after the establishment of the carcinogenic power of tar, many attempts were made to shorten the latent period and increase the tumour yield by adding adjuvants.

For the convenience of the discussion, the experiments can be divided into three groups. Firstly, those in which a non-carcinogenic or weakly carcinogenic substance is applied concurrently with a carcinogenic substance; secondly, those in which the substance is applied for a period preceeding the application of the carcinogen; and thirdly, those in which the substance is applied after the application of the carcinogen.

As examples of the modification of the carcinogenesis that can be induced by the administration of a non-carcinogenic or weakly carcinogenic substance together with the tar or polycyclic hydrocarbon, one could quote many examples. The results varied from pronounced inhibition of tumour formation to pronounced augmentation. For example, Findlay(1928) applied ultra-violet light concurrently with tar to the skin of mice and recorded that it augmented the tar carcinogenesis. Dormanns(1934) reported a similar effect. Other investigators found that ultra-violet light had no significant effect on tar carcinogenesis (Kohn-Speyer,1929;Teutschlander,1937), and Taussig, Cooper and Seelig (1938) found it without effect on carcinogenesis by benzpyrene, and Rusch, Kline and Baumann(1942) found that it did not modify carcinogenesis by methylcholanthrene or 9,10-dimethyl-1,2-benzanthracene. Yet again,

Doniach and Mottram(1940) reported that strong sunlight weakened the carcinogenic activity of benzpyrene. Berenblum(1929) applied frozen CO_2 to mouse skin concurrently with tar, and found an augmenting effect on tar carcinogens at the periphery of the zone of the CO_2 application. Similarly, the non-carcinogenic basic fractions of tar were reported to have an augmenting effect on benzpyrene carcinogenesis in mice when they were injected subcutaneously or painted on the skin together with benzpyrene (Shear,1938;Cabot,Shear,Shear and Perrault,1940). Several substances, such as mustard gas or cantharidin, had, when applied concurrently, on the contrary, an inhibitory effect on tar carcinogenesis in the skin of mice. The inhibitory effect of heptaldehyde when applied to mouse skin together with methylcholanthrene was reported by Carruthers(1940).

In the second category, many non-carcinogenic substances were thought able to modify the carcinogenesis when applied before the application of the carcinogens. Except in a few instances, attempts to induce an augmenting action have been unsuccessful. Derom(1924) heated an epilated area in mice, using a lead shield heated to a temperature of 50, 60, or 70°C. by immersion in water. The thickened and scarred skin resulting from the application of the shield at 60 or 70°C. was more resistant to tar than normal skin. Burning, applied before the tar, does not favour the development of tar tumours (Parodi,1923). However, Deelman(1923) found that light scarification before the first application of tar hastened the appearance of cancer. An opposite result was obtained by Ludford(1929) who employed scarification by

sandpaper before the application of the carcinogen. In view of what is to follow, Mottram's experiment is of particular interest (Mottram, 1944b). He claimed that applications of croton oil before the application of benzpyrene augmented the incidence of tumour formation.

The most significant effect of non-carcinogenic substances or procedure upon epidermal carcinogenesis was obtained by their application after the application of the carcinogen. Deelman(1924) and Deelman and Van Erp(1926) made a single incision in the skin of mice after the application of tar. They recorded the appearance of many benign and malignant tumours along the scar of the incision. In contrast, Ludford(1929) scarified mouse skin with sandpaper after the application of tar without observing any augmenting effect. The application of frozen CO_2 after tar application to the skin of mice (Berenblum,1930), and light cauterization after the application 3,4,5,6-dibenzacridine to the skin of mice (Rodoni & Corbellini,1936) were both reported to have an augmenting effect upon carcinogenesis. Mottram(1938) reported that after the application of benzpyrene to the back of mice, irradiation with doses of 800 to 2800r. was followed by the appearance of many warts and carcinomata, while a dose of 6000r. supressed cancer formation.

Many other experiments of the same sort were carried out during this period, including the actions of substances on the carcinogenesis of subcutaneous tissue. These studies illustrated that the response of animals to carcinogenic agents may be profoundly modified by extraneous factors, administered simultaneously with, or subsequently to the carcinogenic agents. If these modifications lead to an augmentation

in tumour formation, the modification is called "co-carcinogenic". If the modification produces an inhibitory effect on tumour production, it is called "anti-carcinogenic". The term "co-carcinogenic" was originally introduced by Shear(1938), to describe an enhancement by a basic tar fraction of the carcinogenic effect of benzpyrene, has since been used to cover a wide range of phenomena. The study of co-carcinogenesis eventually produced the important discovery of the two stage theory of epidermal carcinogenesis.

C) TWO STAGE THEORY OF EPIDERMAL CARCINOGENESIS

1) Series of Experimental Work

The theory that carcinogenesis might be a discontinuous process consisting of multiple stages with different characteristics, had its origin in speculation by several investigators working independently. Though the concept of the two stages of epidermal carcinogenesis is now widely accepted, many steps in experimental work were required for its establishment. The pioneer work in this field began in early 1940 and many investigators contributed in subsequent years.

a) Work by Berenblum

As has been discussed previously, certain irritants, which are not carcinogenic in themselves, can augment the carcinogenic effect of tar or hydrocarbons on mouse skin when applied concurrently or following applications of carcinogen. Berenblum(1941a) reported an

important series of experiments. He found that the carcinogenic potency of benzpyrene was significantly dimished if it was applied in dilute solution. For instance, applications of 1% benzpyrene in acetone on mouse skin yielded a tumour incidence of 53%-66% of the mice at the 20th week, while applications of 0.05% benzpyrene in acetone yielded tumour incidence of only 0%-6%. If croton oil was applied concurrently with 0.05% benzpyrene, the tumour yield increased to 26%-37%. If croton resin (a constituent of croton oil) was used similarly, the tumour yield reached to 80%. In other experiments, the concurrent applications of 30% turpentine with 0.05% benzpyrene yielded only 0%-3% tumour bearing mice, and the concurrent applications of xylene only 8%. Berenblum thought the effect of croton oil or croton resin could not be explained by a summation of the effect of two weak carcinogens, since croton oil or croton resin alone gave no evidence of carcinogenecity for the skin of mice. Nor could the effect be attributed to a non specific irritative action on the part of croton resin, since other skin irritants such as xylene or turpentine failed to augment carcinogenesis under similar conditions. Berenblum proposed that the co-carcinogenic action of croton oil constituted a specific reaction distinct from the carcinogenic process. In a subsequent series of experiments (Berenblum 1941b), no carcinogenic effect was observed when croton resin was applied to the skin and benzpyrene was injected, nor was it possible to augment the carcinogenic effect of benzpyrene on subcutaneous tissue by injection of croton resin at the same site. The preliminary treatment with croton resin for a period of 26 weeks failed to influence the response to subsequent applications of benzpyrene.

But most importantly, the effect of croton resin on mouse skin previously treated with benzpyrene for a limited period was significant. After eight weeks of applications of 1.0% benzpyrene in acetone, subsequent treatment with applications of 0.025% croton resin produced tumours in 86% of the mice. Subsequent treatment with 30% turpentine gave tumours in 44% and subsequent treatment with acetone in 18%. This indicated that when croton resin is applied to skin which has already been rendered preneoplastic by carcinogen, it is capable of precipitating the development of warts in a high proportion of cases. There was also evidence that when croton oil was allowed to act on warts already established it seemed able to facilitate their conversion into malignant tumours. On the basis of these experiments, Berenblum classified the biological changes occurring in the epidermis after the application of carcinogen into three stages, preneoplastic stage, stage of papilloma, and the stage of malignancy, and the biological actions of the carcinogen as precarcinogenic, epicarcinogenic and metacarcinogenic. He summarized the new concept of carcinogenesis in the following five themes.

"1.) Precarcinogenic, epicarcinogenic and metarcarcinogenic action represent independent processes in carcinogenesis."

"2.) True carcinogens are capable of producing all three actions, though to a different degree in different species."

"3.) Croton resin differs from a true carcinogen in lacking the power of pre-carcinogenic action but resembles them in possessing both epi- and metacarcinogenic action."

"4.) It is possible that other kinds of incomplete carcinogens may exist, possessing, for instance metacarcinogenic, but not pre- or epicarcinogenic action."

"5.) The rarity of response to carcinogenesis of different tissue may possibly be attributed to a deficiency in one part of the chain reaction rather to a greater or lesser responsiveness to carcinogenesis as a whole."

Perhaps the important postulate is the necessity of dissociation of the process of carcinogenesis into component parts, the whole process consisting, as it were, of a chain of essentially independent processes.

b) Work of Rous and his Collaborators.

At the same period, Rous and Kidd(1941) engaged in a series of experiments similar to Berenblum's. They tarred the inner surface of rabbit ears twice weekly for periods varying six weeks to three months. When warts appeared, they stopped the tarring. Most of the warts then disappeared, but reappeared quickly when the tarring was resumed. There was evidently an increased response to the subsequent tarring. Retarring called forth warts in greater and greater profusions and called them forth earlier. Moreover, each tarring constituted an

additional carcinogenic stimulus, turning more cells into potential wart cells. The reappearance of warts, and crops of new warts, were also produced by replacing the second tarring by painting with turpentine or by making punch holes on the ear. The papillomata which turpentine and punch holes brought forth on skin previously tarred, were less fleshy and had thinner papillae than those evoked on the same animals by tarring, but otherwise there were no essential differences. Rous and Kidd thought that the stimulus to hyperplasia whether by re-tarring, by turpentine, or by wound healing caused abnormal cells lying dormant in the skin to develop into visible tumours. They thought that these abnormal cells were induced by the previous tarring. They also noted that the ability to form warts promptly on renewed tarring persisted for at least six months after the growths had vanished. In conclusion they postulated that all of the warts induced by tar are conditional in nature, fully dependent on aid for enduring survival.

In other experiments, MacKenzie and Rous(1941) tarred the ear of rabbits twice weekly for four weeks, then after an interval of five weeks made punch holes. The essential point was to tar the skin of rabbits for a period somewhat less than is ordinarilly required to elicit growths, and afterwards to subject it to non-carcinogenic stimulation. In their experiment, one growth appeared in each of two rabbits after the tarring before the holes were punched, but after the holes had been punched, thirteen tumours appeared in five of the seven rabbits. Seven of the tumours were at the place where repair was under way and six were elsewhere on the ears. When tarring was resumed four to eleven weeks after making the punch holes, there

was a growth of many new tumours, and they were more than three times as numerous where healing had taken place. In control experiments, the healing of holes in normal ears never called forth tumours. A significant point is that the growths which appeared while the discs were healing occurred only on the ears that had been previously tarred. MacKenzie and Rous emphasized the need for a sharp distinction between tumour inception and tumour formation, and between those factors which render cells neoplastic and those affecting their subsequent behaviour. They suggested that carcinogenic tar applied to rabbit skin renders many more epidermal cells neoplastic than declare themselves by forming tumours. Such latent tumour cells may be present in large numbers, and present for a considerable time after a brief tarring, yet give rise to no growths unless encouraged by other means.

The idea of separating epidermal carcinogenesis into different stages was more specifically formulated in publications by Friedewald and Rous(1944a;1944b). Their studies extended the experimental analysis of factors affecting the effectiveness of tar, benzpyrene, and methylcholanthrene when applied to rabbit skin. Friedewald and Rous thought the carcinogen acted in two ways: firstly it changed normal cells into neoplastic cells, an activity Friedewald and Rous called "initiation", and secondly it encouraged the multiplication of the initiated cells, an activity they called "promotion". Using similar experimental models as in their previous work, they applied benzpyrene or methylcholanthrene to rabbit ears, and subsequently punched holes in the treated areas, obtaining many tumours. They also found that the applications of chloroform to rabbit ears previously treated with

methylcholanthrene was able to elicit many tumours. However, while the power to encourage tumour formation and growth was exhibited very strikingly by tar, methylcholanthrene had little and benzpyrene had less such power on rabbit skin. Friedewald and Rous speculated on the nature of promoting influence, saying that anything which induced hyperplasia, turpentine, irritant secretions, trauma, or chloroform, might cause growths to appear if latent tumour cells were present in the epidermis.

c) Work of Mottram

Mottram proposed that three factors played a part in the production of tumours (Mottram,1944a & 1944b), a "sensitizing" factor which renders cells hyper-responsive to subsequent treatment, a "specific cellular reaction", the essential carcinogenic reaction, and a "developing" factor, concerned with the bringing into being of a visible wart. In order to illustrate a developing factor, Mottram painted benzpyrene in acetone on both flanks of a group of six mice for four days, and both flanks of a second group of six mice for fourteen days. Subsequently, the right flanks of all twelve mice were painted with croton oil, and left flanks with acetone. No tumours appeared on the left flanks, but many tumours on the right flanks. He claimed that in these experiments the benzpyrene acted as an agent which caused a specific cellular reaction, and that the croton oil enhanced the development of visible tumours. He stressed the importance of a developing factor in the experimental production of

tumours. In order to demonstrate the sensitizing factor, twelve mice were painted only once with benzpyrene on both flanks followed by a twenty week treatment with croton oil. On the right flanks, the painting with benzpyrene was preceded by a short period of croton oil treatment, on the left no such preparatory treatment was given. A great increase in tumour production was noted on the side where croton oil was applied prior to the benzpyrene application. Mottram claimed that the preliminary applications of croton oil acted as a sensitizing factor. He also noted that a developing factor is required even when a sensitizing factor is used. During these experiments, Mottram found that a single application of 0.3% benzpyrene in acetone is adequate to induce the specific cellular reaction so that subsequent applications of croton oil or croton resin were able to induce numbers of tumours in the prepared skin. Mottram's experiments are very significant for the demonstration of the separation of epidermal carcinogenesis in separate stages, and also for the simplification of the experimental procedure. The induction of tumours by a single application of carcinogen followed by repeated croton oil treatment enabled the various phases of carcinogenesis to be analysed in a more accurate and quantitative fashion.

d) Work of other investigators

Other experiments which suggest the necessity for the separation of epidermal carcinogenesis into stages are the studies of Tannenbaum.

In his studies on the effect of under-feeding on the initiation and promotion of tumours, Tannenbaum found that the initial changes in the epidermis due to the application of carcinogen occur regardless of whether the mice are on a full diet or a calorie restricted diet. On the other hand, a full diet promotes the subsequent development of tumours and calorie restriction inhibits such development. (Tannenbaum, 1940;1942;1944a;1944b). Tannenbaum concluded that the initiation and growth may be controlled by different factors.

The experiment of Kline and Rusch(1944) is also worth mentioning. After a single initial application of methylcholanthrene, mice were painted with croton resin, croton oil, naphthylquinone, sodium sulfide or were mechanically wounded. Croton resin, croton oil, naphthylquinone and mechanical wounding caused a significant incidence of tumours. These findings are in harmony with the general idea that cancer formation occurs following a sequence of biological changes. Rusch and Kline classified the series of changes into (a) an induction period, during which neoplastic cells are formed; (b) a critical or reversible period, during which the neoplastic cells may proliferate, die or lie quiescent; (c) a progression period, in which the neoplastic cells gain ascendency over the forces that hold them in control.

e) Formulation of the Theory and Its Terminology

Thus, by the end of 1944, many investigators had adopted the idea of the separation of epidermal carcinogenesis into separate stages.

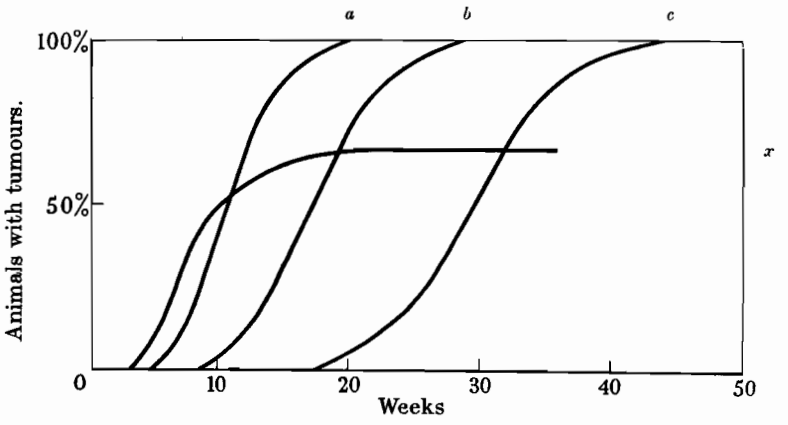
There were, however, still many conflicting points and certain differences in the usage of terminology and expression. A more detailed analysis of the component phases of carcinogenesis by a quantitative approach was therefore undertaken by Berenblum and Shubik.

Mottram's(1944b) idea of a sensitizing factor was questioned by Berenblum and Shubik(1947a). Using a single application of benzpyrene or 9,10-dimethyl-1,2-benzanthracene as an initiator on the skin of mice, they showed that the tumour yield induced by subsequent applications of croton oil was not significantly influenced by the application of croton oil before the application of carcinogen. This was in direct contradiction to Mottram, and as Berenblum and Shubik's experiment was on a much larger scale, their view has prevailed. The matter has not, however, been further studied.

Berenblum and Shubik(1947b) found also that when different carcinogens are applied repeatedly to the skin of mice without croton oil, and the percentage of mice bearing tumours is plotted against time, the tumour incidence curve will be as a, b, and c as shown in figure 1.

When a carcinogen is applied for suboptimal period of eight weeks or only once, and the skin is thereafter treated with croton oil, the tumour incidence curve will be as x in figure 1. Though tumours begin to appear as early as, or even earlier than in the mice given a continuous treatment with carcinogen, the tumour incidence curve rapidly reaches a level well below 100% and remains at that level, even though croton oil treatment is continued. Berenblum and Shubik thought that this phenomenon could be explained by considering the preliminary

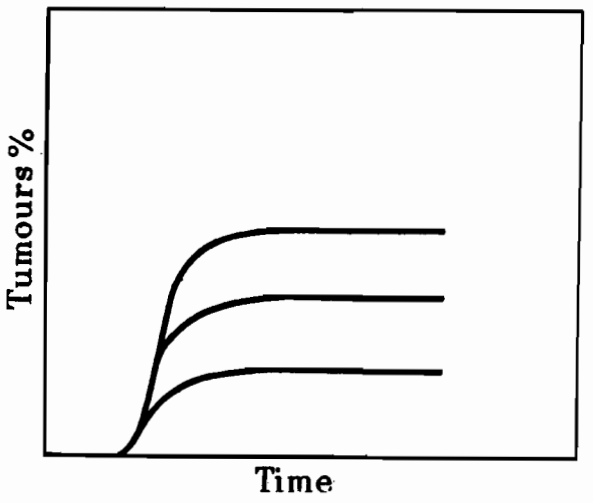
Figure 1



A comparison between the effect of painting skin continuously with carcinogen of high potency(a), moderate potency(b), and low potency(c). A single painting with sub-optimal dose of potent carcinogen followed by repeated painting of croton oil(x).
(Berenblum and Shubik 1947b)

carcinogenic action to cause an irreversible conversion of a few normal cells into a few latent tumour cells, and by assuming that the subsequent applications of croton oil convert all these latent tumour cells into visible tumours. The height of the curve x could thus be taken as a measure of the carcinogenic action of the single application of carcinogen. This relationship was illustrated when it was shown that if the croton oil treatment was kept constant but different carcinogens (benzpyrene, 1,2,5,6-dibenzanthracene or 9,10-dimethyl 1,2-benzanthracene) were used for the initial painting, the tumour incidence varied from group to group as shown in figure 2.

Figure 2.



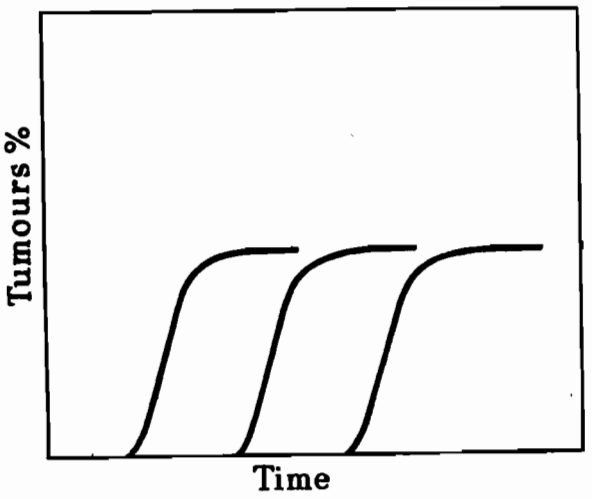
Different carcinogen used for initiation
(Berenblum and Shubik 1947b)

The average latent period in these various groups remained the same, suggesting that this measure was controlled by promotion.

On the other hand, when initial painting with carcinogen was kept constant but the beginning of croton oil treatment was delayed for different periods, the tumour incidence remained the same, but the latent period varied according to the length of the interval. It was, however, also constant if measured from the beginning of croton oil treatment as shown in figure 3. Using 1.5% 9,10-dimethyl 1,2-benzanthracene as initial painting, croton oil treatment was started after interval of 3 days, 5 weeks, 10 weeks or 20 weeks. The tumour incidence and the latent period as measured from the start of croton oil painting remained constant.

That initiation and promotion are different in character is

Figure 3.



When carcinogen is kept constant and croton oil treatment delayed for different periods.
(Berenblum and Shubik 1947b)

shown by the necessity of giving the two agents in the right order, namely initiation first followed by promotion. A single application of 9,10-dimethyl 1,2-benzanthracene followed by repeated applications of croton oil produced tumours in half of the treated animals, whereas repeated applications of croton oil followed by a single application of the carcinogen produced tumours in only two out of twenty animals (Berenblum & Haran-Gehra,1955a). Roe(1958) painted the skin of mice with 5% croton oil once, then repeatedly with 0.01% 9,10-dimethyl 1,2-benzanthracene in one group, and with 0.0025% of the same carcinogen in a second group. No tumours were produced in the second group only a few in the first group. The number of tumours in the first group was indeed less than were produced by 0.01% of the same carcinogen alone.

This clearly demonstrates that the reversal of the order of carcinogen and croton oil abolished their combined carcinogenic action.

From these studies, Berenblum and Shubik(1947b) stressed the following three points in the formulation of the two stage theory of carcinogenesis.

a) The ultimate number of tumours is predetermined by the single preliminary application of carcinogen.

b) The latent tumour cells produced by it remain indefinitely, until stimulated to further activity.

c) The effect of croton oil treatment is to convert all the latent tumour cells into visible tumours.

The first stage, in which it was postulated that a conversion of a few normal cells into a few latent tumour cells occurs, was called by Mottram(1944a) "specific cellular reaction", by Kline and Rusch(1944) an "induction period", and by Friedewald and Rous(1944a) an "initiating process". The second stage, in which it was suggested that latent tumour cells were converted into visible tumours, was called by Mottram "developing phase", by Kline and Rusch a "progression period" and by Friedewald and Rous a "promoting phase". Berenblum and Shubik proposed the adoption of terms "initiation" and "promotion" to describe the separate two stages in the formation of epidermal tumours.

2) Initiation

a) Introduction

After the postulation of the theory that the carcinogenic

process in the epidermis can be separated into two stages, attempts were made to study the nature of "initiation" and "promotion". We have seen that initiation or the initiating process is, according to Berenblum and Shubik(1949a), a sudden and irreversible change affecting a small minority of the cells of the treated area, and giving rise to isolated "latent tumour cells", apparently indistinguishable morphologically from the surrounding normal non-neoplastic cells. Initiation can be achieved by the administration of a specific substance or agent called an "initiator".

b) Different kinds of Initiator

The experiments so far discussed show that carcinogens such as tar, benzpyrene, methylcholanthrene, 1,2,5,6-dibenzanthracene and 9,10-dimethyl-1,2-benzanthracene are able to act as initiators on mouse skin. Other kinds of initiators for mouse skin have been reported. Perhaps the most interesting and unexpected discovery, was the initiating action of urethane on mouse skin (Salaman and Roe,1953 and Graffi,Vlamynch,Hoffman and Schulz,1953). A detailed discussion of the carcinogenic action of urethane will be found later in this review, but briefly, urethane has a significant degree of initiating action on mouse skin, yet most workers found it incapable of inducing skin tumours by itself (Salaman & Roe,1953;Berenblum & Haran-Ghera,1955b). A few reports are to the contrary. Lindsay(1956) produced a small number of skin tumours on male NCZ mice by the application of urethane, and Deringer(1962) produced a few papillomata and carcinomata on HR/De

mice by painting with urethane in ethyl glycol twice weekly. These are both unusual strains of mice and it should be noted that Deringer also obtained skin tumours in control groups painted with ethylene glycol alone. It thus remains that for most investigators, and unlike the hydrocarbons so far mentioned as showing initiating power, urethane has proved as non-carcinogenic for mouse skin. Thinking it to be non-carcinogenic but capable of initiating action, Salaman and Roe(1953) called urethane an incomplete carcinogen for skin, or pure initiator.

Subsequently, urethane was found able to induce its initiating action on mouse skin not only when applied topically, but also when given orally (Haran-Ghera & Berenblum,1956), or by intraperitoneal injection (Ritchie,1957;Berenblum & Haran Ghera,1957a). Moreover, though urethane is a potent initiator for the skin of mice, it does not produce any significant histological alterations in mouse skin after a single or repeated applications or injections (Salaman & Roe,1953; Ritchie,1957).

Other kinds of carcinogen are also able to induce the initiating changes in the skin when given by injection. Graffi, Scharsach and Heyer(1955) are able to induce initiating change in the skin of mice by intravenous or intraperitoneal injections of 9,10-dimethyl-1,2-benzanthracene. This observation was confirmed by Berenblum and Haran-Ghera(1957b), who also demonstrated that mouse skin could be initiated by intravenous or intraperitoneal injections of methylcholanthrene, benzpyrene or 1,2,5,6-dibenzanthracene.

Numerous substances related to urethane either in chemical

composition or in biological character, and many other substances, have been tested for their initiating action. Many initiators were found. Among them are triethyl melamine (T.E.M.) (Roe & Salaman,1955), 1,2-benzanthracene (Roe & Salaman,1955), beta-propiolactone (Roe & Glendenning,1956) and N-N-DI (2 chloroethyl) p-aminophenyl butylic acid (CBI348) (Salaman & Roe,1956c). Acetylaminofluorene, a potent carcinogen for many organs, was shown to have initiating action on mouse skin when given orally (Ritchie & Saffiotti,1955). Not only chemical substances, but physical agents such as beta radiation were shown capable of producing the initiating change in mouse skin, preparing it so that the subsequent application of croton oil becomes able to elicit skin tumours (Shubik,Goldfarb,Ritchie & Lisco,1953). Even mechanical irritation such as burning can induce a significant number of tumours when followed by repeated applications of croton oil (Shubik & Saffiotti,1956a).

c) Biological characters of initiation

It is known that a single application of a suitable carcinogen can elicit skin tumours in mice if given in a large enough dose and under favourable conditions. Findlay(1925) succeeded in producing epitheliomata on mice after a single application of tar. Mider and Morton(1939) and Cramer and Stowell(1943a) demonstrated the occurrence of benign and malignant tumours of mouse skin after a single application of methylcholanthrene. Similarly, 9,10-dimethyl-1,2-benzanthracene is able to elicit tumours of mouse skin after a single application.

(Law,1941;Roe,1956a;Terracini,Shubik & Della Porta,1960).

The significant point in two stage carcinogenesis is not that a single dose of carcinogen can induce the initiating change, but that the dose necessary to convert normal cells into latent tumour cells is much less than is needed to induce tumours. A single application of a suboptimal dose of carcinogen, a dose too small to induce tumours alone, is able to initiate the skin so that tumours can be elicited by subsequent applications of croton oil. As little as 0.16 ug of 9,10-dimethyl-1,2-benzanthracene has been shown to be capable of preparing the skin so that significant numbers of tumours appear when repeated applications of croton oil follow (Klein,1956). At least 100 ug is required to induce skin papillomata by a single unaided application of 9,10-dimethyl-1,2-benzanthracene (Terracini,Shubik & Della Porta,1960).

As previously mentioned, the number of tumours elicited by a single application of the carcinogen followed by repeated applications of croton oil depends upon the potency of the carcinogen. Berenblum and Shubik(1947b) used 1,2,5,6-dibenzanthracene, 3,4-benzpyrene or 9,10-dimethyl-1,2-benzanthracene, as initiator and found that when croton oil followed that the yield of tumours varied with the strength of the carcinogen. In a subsequent experiment, Berenblum and Shubik(1949a) painted mice once with different concentrations of 9,10-dimethyl-1,2-benzanthracene and they gave a standard course of croton oil treatment. An increase in the concentration of the initiator produced an increase in the tumour yield. The tumour yield varied more or less

with the logarithm of the concentration of the carcinogen. A quantitative study of the initiating action of a single dose of urethane by Berenblum and Haran-Ghera(1957a) also demonstrated approximately logarythmic relation between dose and tumour yield. If one adopts Berenblum and Shubik's assumption that all latent tumour cells appear as papillomata, this means that different strengths of the carcinogen must produce different numbers of latent tumour cells.

The theory that a single application of carcinogen leads to production of a definite number of latent tumour cells in epidermis, however, has been questioned. Friedewald and Rous(1950) noted that after a limited treatment with methylcholanthrene, there was a gradual accumulation of tumours on the ears of rabbits for as long as five years. They demonstrated that this gradual increase was the result of a steady appearance of new tumours and a steady, but slow, disappearance of old ones. They concluded that the stage of initiation was better described as the creation of a latent neoplastic potentiality in the skin.

The tumours produced by a single application of carcinogen followed by repeated applications of croton oil differ in many respects from those produced by repeated applications of carcinogen (Shubik,1950b; Shubik,Baserga & Ritchie,1953;Salaman & Roe,1956b). Tumours produced by a single application of carcinogen followed by croton oil, are mostly benign papillomata, and show a high rate of regression. The tumours produced by repeated applications of carcinogen are papillomata at first, but often turn into malignant tumours. Moreover, various histological

types of papillomata are produced by an application of carcinogen followed by croton oil. To explain this variety, Shubik(1950b) suggested that the process of initiation should be considered as a graded one, inducing graded degree of growth potentialities in the latent tumour cells. Shubik, Baserga and Ritchie(1953) agreed with this explanation of the variety of characteristics seen in the tumours produced by initiation and promotion in mouse skin. They, too, suggested that latent tumour cells are not all similar, but differ over a range of potentialities, giving rise for example, to tumours of different histological structure, growth rate or invasiveness.

The assumption that the latent tumour cells produced by initiator remain indefinitely has been further supported. Berenblum and Shubik(1947a;1949b) showed that the yield of tumours produced by a single application of 9,10-dimethyl-1,2-benzanthracene followed by repeated applications of croton oil after intervals of five weeks, fifteen weeks, twenty weeks, or forty-three weeks was the same as that obtained when the interval was three days, and that the rate of papillomata production was unaltered. Roe and Salaman(1954) also demonstrated that the initiating action of urethane on mouse skin persists for at least 24 weeks. Thus the specific change or changes produced by the initial treatment of carcinogen remains unchanged for a long period.

d) Histological changes by initiator

There has been much speculation about the cellular changes

underlying the initiating action. Histological studies were performed by many investigators in order to find some specific change, or changes, that occurred in the skin after the application of carcinogens. Pullinger(1940,1941) investigated the effect of methylcholanthrene, benzpyrene and 9,10-dimethyl-1,2-benzanthracene on mouse skin. She described characteristic and possibly specific reactions after the application of carcinogen. These included swelling of squamous epithelial cells and nuclei, and vacuolation of cytoplasm. These changes were not observed after treatment of skin with non-carcinogenic hydrocarbons or other nonspecific irritants.

Cramer and Stowell(1942) after histological studies of mouse skin treated with methylcholanthrene, concluded that the carcinogenic properties of a polycyclic hydrocarbon for the skin of mouse depended on its ability to inflict on the tissue an injury of a specific kind which lead to epithelial proliferation. They also noted that an area of skin exposed to carcinogen does not react equally, but shows considerable variation in the degree of response. Glücksmann(1945) counted resting, differentiating, dying and dead cells in the epidermis of mice treated with benzpyrene or non-carcinogenic irritants. He observed an increase in both the relative and absolute number of resting cells after treatment with carcinogen but not after non-carcinogenic treatment. He proposed that the hyperplasia resulting from carcinogenic treatment involved a delay in the maturation of the epithelium. Davibhadhana(1952), in experiments similar to those of Glücksmann, counted basal, spinal, and granular cells and dividing, degenerating

or dying cells in mouse skin treated with methylcholanthrene. He observed a prompt and continued rise in the granular cell population in the epidermis. Dividing and degenerating cells also become quite numerous. He concluded that the epidermal hyperplasia following carcinogen application was induced in a different manner from that of the usual type of regenerative hyperplasia, since the increased mitotic activity appeared to be permanent and to result in structural alterations in all cell types. Berenblum(1954), discussing Glückmann's histoquantative study, stressed this theory that initiation is an irreversible change in a few cells only. Therefore he thought any more general changes in the skin must be irrelevant.

A definite localized epidermal and follicular alteration was observed in methylcholanthrene treated mouse epidermis by Liang(1948). The first indication of an epidermal change was the presence of cell clusters which usually appeared at the junction of hair follicles with the basal epidermal layer. Later, extensive multiplication of these cells caused disruption of the follicular pattern and eventual ulceration in the center of the follicular area.

Since epidermal hyperplasia is one of the characteristic features after topical applications of carcinogen, the mitotic activity in the epidermis after carcinogen application was studied by many investigators. Cooper and Reller(1941) initially reported that methylcholanthrene acted as a stimulant to cell division, but that this effect later waned. Subsequent studies revealed that the first effect of hydrocarbons on mouse skin was to decrease mitotic activity. A marked increase followed

(Heller & Cooper,1944;Iversen & Edelstein,1952). The time of maximum decrease in mitosis, and the degree of decrease is seemingly different with different carcinogens, and with different concentrations of the same carcinogen (Evensen,1962).

In contrast with these widely accepted findings, Dammert(1961b) reported that increase of mitotic frequency on mouse skin after applications of 20 ug 9,10-dimethyl-1,2-benzanthracene in acetone is due to the action of acetone alone. He stressed that 9,10-dimethyl-1,2-benzanthracene acts constantly as a mitotic inhibitor. There appears to be an inverse relationship existing between the rate of mitosis induced by carcinogens and the length of induction periods of tumours (Daoust,1959).

Setälä, Merenmies, Stjernvall, Aho and Kajanne(1959) reported on the changes seen in the skin after the application of methylcholanthrene and 9,10-dimethyl-1,2-benzanthracene, using electron as well as light microscopy. A single application of a carcinogen produced a toxic alteration and reparative hyperplasia in the mouse epidermis. Continuous treatment caused cellular and nuclear atypia, and a severe disturbance in organization and differentiation. Setälä and his colleagues claimed that these changes were completely different from those produced by non-carcinogenic irritants. However, the importance of the changes is still in doubt. The apparent lack of histological alterations in the skin when carcinogens were administered by systemic routes, or when urethane was given, cast a question on their significance. Recently, however, moderate hyperplasia of the epidermal cells has been reported to follow the

oral administration of 9,10-dimethyl-1,2-benzanthracene (Setälä,Niskanen, Merenmies,Nyholm and Stjernvall,1961). Further studies are apparently necessary to evaluate the significance of the epidermal hyperplasia produced by carcinogen.

The dermal changes in the skin after applications of carcinogens were particularly studied by Orr. In his earlier experiments(Orr,1934; 1938), the development of dermal fibrosis with resulting ischaemia was stressed as a precipitating factor in skin carcinogenesis. Other changes occurring in the dermis during skin carcinogenesis, include; conversion of the parallel bundles of collagen fibers to loosely disoriented fibrils, alteration in the elastic tissue, and passive congestion of the blood vessels. In subsequent experiments, he and his colleagues studied the autologous transplantation of methylcholanthrene treated epidermis to a normal site, and of normal epidermis to a site denuded of epithelium but in which the epithelium had been painted with this carcinogen. Tumour developed in the latter case, but not in the former (Billingham, Orr & Woodhouse,1951;Merchant & Orr,1953). From studies of skin grafting and skin histology, Orr concluded that the evidence gave no support to the view that carcinogenic hydrocarbons cause a direct intracellular preneoplastic change in the epithelial cells themselves. He thought that the essential changes occurred in the deeper tissue of the dermis (Orr,1955;1958;1961). Others have remained skeptical of these claims (Berenblum,1959). They remark particularly on the technical difficulties in Orr's work, and the likelihood of contamination. Furthermore, Ashley(1961) claimed that one of the ultimate steps in

carcinogenesis was inhibited by skin transplantation. Experiments with whole thickness skin grafts by Salaman(1959) and Cowen(1959) failed to support Orr's theory. One group of mice was given an initiating application of carcinogen. After three weeks, skin from these mice was grafted onto untreated mice of the same strain and sex. Similarly, untreated skin was grafted onto mice pretreated with carcinogen. After the croton oil treatment, the mice which were carcinogen treated and had an untreated skin graft showed a large number of tumours on the host skin, but few on the grafted skin. The mice which were untreated but carried carcinogen treated skin graft showed a few tumours on the host skin but a considerable number on the grafted skin. From these experiments, it was concluded that the state of initiation is a property inherent to the skin. It should be noted that by using whole thickness grafts, Salaman and Cowen greatly modified Orr's experiment.

e) Chemical changes by initiator.

The changes in the skin after application of carcinogen have also been studied chemically. The group of investigators represented by Cowdry should be particularly mentioned in this respect. They analysed the changes in skin after carcinogen treatment by histochemical methods and by direct chemical analysis. They hoped to find some chemical changes which occurred in the skin during transformation from normal to the neoplastic state. Mice were used as experimental animals, and methylcholanthrene as the carcinogen. The number of subjects which they investigated was numerous, including desoxyribonucleic acid,

ribonucleic acid, alkaline phosphatase, mineral components, S-H groups, iron-nucleoprotein phosphorus ratio, citric acid metabolism, cholesterol-protein nitrogen ratio, lipid-protein nitrogen ratio, vitamins, enzymes such as succinic dehydrogenese, cytochrome oxidase, adenylpyrophosphatase, cytochrome C and arginase, and many amino acids. The results were summarized by Cowdry(1945;1951;1955) and Carruthers(1950). No clear lead has emerged. Hieger(1961), while admiring Cowdry's works, states "when the dictum that scientific method consists in measurement is taken too literally the result is sometimes a mere complication of numerical data; how to fit findings into other line of investigation on the subject are a much more difficult task". Another group of investigators headed by Baumann engaged in a systemic study of the changes in sterols which occur in the skin after carcinogen treatment. Their significant finding was that there is a decrease in the concentration of Δ 7 cholestanol in carcinogen treated skin whereas there is some increase in amount of cholesterol (Kandutsch & Baumann,1955;Brooks & Baumann,1956).

Another group of investigators headed by Miller and Heidelberger engaged in another interesting line of chemical studies. They have investigated the interaction of carcinogenic hydrocarbons with the tissue components of skin following a single application of carcinogen, in the belief that they were studying the key reaction which led to the induction of tumours. Their study stemmed from the important observation by Miller and Miller(1952), who demonstrated that during azodye hepatocarcinogenesis carcinogenic azodye was bound to liver protein. Miller(1951) studied the binding of carcinogenic chemicals

to skin protein. After painting benzpyrene on mouse skin, the content of benzpyrene bound to protein was estimated by the fluoroscent method. Binding was maximum at 24 hours after the last application of benzpyrene, remained for several days, then gradually declined, and none could be detected three weeks later. Methylcholanthrene, although a more potent carcinogen than benzpyrene, was protein bound to a lesser degree. These observations were confirmed by Weiss and Heidelberger(1953) employing C^{14} labeled benzpyrene. C^{14} labelled dibenzanthracene was also bound to the protein of the skin of mice after topical application. There proved to be an excellent correlation between the extent of protein binding and carcinogenic activity of hydrocarbons for the skin with only one exception, namely the non-carcinogenic 1,2,3,4-dibenzanthracene which is bound to protein (Heidelberger and Moldenhauer,1955). Moodie, Reid & Wallick(1954) reached the same conclusion, employing spectrophotometric methods for their determination. They observed that non-carcinogenic hydrocarbons such as 1,2-benzanthracene, anthracene, fluoranthene and phenanthrene were much less effectively bound to skin protein than were benzpyrene, 1,2,5,6-dibenzanthracene and 9,10-dimethyl-1,2-benzanthracene. Studies by Woodhouse(1954,1955) were opposed. Using fluoroscopic techniques, he found that the amount of hydrocarbon bound to the skin protein was the same whichever the hydrocarbon, be it strong carcinogen, weak carcinogen, or non-carcinogen. On the basis that there is a considerable amount of non-metabolic zero-time binding of the non-carcinogenic hydrocarbons to mouse skin protein, and also significant zero-time binding of 9,10-dimethyl-1,2-benzanthracene,

Hadler, Darchun and Lee(1959) also questioned the validity of the correlation between protein binding and carcinogenicity of hydrocarbons. Heidelberger opposed Hadler's view after demonstrating no zero-time binding of 9,10-dimethyl-1,2-benzanthracene to skin protein, using a finer method of detection than Hadler's (Heidelberger & Davenport,1961).

The significance of protein binding of carcinogens in terms of carcinogenesis is not well established. Miller and Heidelberger's demonstration of protein bound carcinogen led to the formation of the "deletion-hypothesis" of carcinogenesis. (Potter,1958;Heidelberger,1959). Briefly, it postulates that among the proteins to which the carcinogen is bound there are enzymes important to the control of growth. As a result of the binding, the enzymes are inactivated, and in subsequent cell division, a few cells are produced which lack these enzymes. These cells which lack the growth controlling mechanism become tumour cells.

There is some evidence which suggests that carcinogenic hydrocarbons are bound to the nucleic acids of epidermal cells (Heidelberger & Davenport,1961;Boyland & Green,1962), and the carcinogenic action might be related to this interaction (Evensen,1961). The matter will be reviewed elsewhere in this thesis.

There is much controversy over Warburg's thesis (Warburg,1956), that the initial change of cells undergoing malignant transformation was damage to cellular respiration. According to his theory, due to damage of respiration, some cells die, but others become capable of replacing respiration by glycolysis as the main energy producing reaction,

and are changed into cancer. His evidence was based on some parallelism between carcinogenic action and respiration inhibitory properties of carcinogens, and on high anaerobic glycolysis in cancer cells. The view is not widely accepted because there is some discrepancy, such as that some normal tissues are capable of anaerobic glycolysis almost as high as that of tumours, and that it is not clear whether a high anaerobic glycolysis is a cause or a result of carcinogenesis.

Cell metabolism during skin carcinogenesis was also studied by Iversen(1960,1962), who investigated the endogenous dehydrogenase in the epidermis of hairless mice after a single application of various carcinogenic and non-carcinogenic substances. He employed the tetrazolium reduction method as a rough measure of the metabolic rate of cells. The results show a close correlation between the carcinogenic potency of a substance and an increased value of formazan deposit on the epidermis two days after the applications. However in his experiments, the type of reaction after the application of 9,10-dimethyl-1,2-benzanthracene was about the same as that after an application of croton oil, and that the action of urethane was similar to that of non-carcinogenic substances. This brought forth questions as to the significance of his work to initiating action.

Calcutt(1961a;1961b) and Calcutt and Coates(1961) concentrated on the change in sulphydryl levels in mouse skin after the application of carcinogens, on the assumption that a rise in target tissue sulphydryl levels during the tumour induction period is a prerequisite of tumour formation. There was an apparent rise of SH levels in skin

after applications of carcinogenic hydro-carbons, but their results were not consistent.

Thus, though so much work has been done on the chemical alterations in the skin, no conclusive evidence has been obtained to account for the specific change that the carcinogen induces on the skin.

f) Somatic mutation hypothesis

Berenblum and Shubik(1949a) stressed that the initiating action exhibits three striking features: its specificity, its speed of action, and its irreversibility. Since these three features are also characteristic of mutation, the mutation hypothesis was put forward to explain the underlying mechanism of initiation.

Somatic mutation as an origin of cancer had been proposed for many years. The theory postulates that the transformation from normal to malignant cells or tissues is analogous to the formation of mosaics of aberrant elements in normal tissue. The pleomorphism of cancer cells and the irregular atypical mitosis, so characteristic of their nuclei, had been observed and studied since the middle of the nineteenth century. The presence of multipolar mitosis and other abnormal mitotic figures in tumours was introduced in the idea of von Hansemann(1890) of the anaplasia of cells in malignant tumours. Boveri's theory (1911) on the nature of the origin of cancer stemmed from his observations on atypical mitosis on sea urchin eggs. The irregular distribution of chromosomes to daughter cell in the case of polyspermy led him to compare

this cell with an abnormal chromosomal complex to cancer cells. When aneuploidy occurred, he noted that the cells frequently did not develop normally but divided independently to yield atypical groups of cells. Boveri stated "the essence of my theory is not the abnormal mitosis but a certain abnormal chromatin complex no matter how it arises!" The theory has since been refined and supported by Bauer(1928), Lockhard-Mummery(1934) and Strong(1949).

The most vigorous attempts to test the mutation hypothesis have been made by comparing the mutagenic effects of substances with their carcinogenic effects. Among the physical agents, Müller(1927) found that x-rays are mutagenic and later Koller(1934) described chromosomal damage following irradiation. The mutagenic action of ultra-violet light was described by Altenburg(1933). The first convincing demonstration that chemical agents may be mutagenic was reported by Auerbach and Robson(1946), who used mustard gas to obtain lethal mutation in Drosophila. Tatum(1947) studied various types of mutation induced with methylcholanthrene using neurospora as an indicator. Testing various carcinogenic and non-carcinogenic hydrocarbons on drosophila, Demerec(1948) was able to find some correlation between carcinogenicity and the power to produce mutation, though the correlation was not absolute. Latarjet(1948) in similar experiments failed to find any correlation between mutagenic and carcinogenic potency. Further support for the somatic mutation hypothesis was the demonstration of an increased incidence of mutations occurring in mice treated with methylcholanthrene by Strong(1945), and with 1,2,5,6-dibenzanthracene by Carr(1947). Obviously, these investigations show that while there

is a large degree of overlapping of the two properties, mutagenicity and carcinogenicity, there are also many cases where the two do not run parallel. Burdette(1955) reviewed this matter, and he favoured the view that there is no parallelism between these properties. Nevertheless, as Schultz(1959) pointed out, the partial correlation of mutagenicity and carcinogenicity was at least circumstantial support to the somatic mutation hypothesis for the origin of cancer. The cytological studies of experimental tumours and tumour cell strains cultured in vitro revealed that in respect to chromosome number and morphology, these tumours are composed of a highly heterogenous cell population. Furthermore, it was noted that neither a chromosome number nor a chromosome pattern has so far been found which was characteristic and specific for any neoplastic cell type. This was interpreted by Koller(1960) to mean that the chromosome abnormalities are secondary phenomena which follow malignant change and occur independently.

Among the difficulties in the somatic mutation theory of cancer, is that mutation is thought to be a comparatively rare event, whereas neoplastic transformation occurs more frequently, and that mutation is of an instantaneous nature whereas tumour formation is remarkably slow. Berenblum and Shubik avoided these conflicts by saying that only the initiating stage of carcinogenesis is mutational. However, Berenblum (1954) later stated "while the mutation hypothesis remains the most attractive and plausible explanation of the initiating action, it can not yet be said to have been established". He stated further "the more

we learn about carcinogenesis, the less plausible the somatic mutation hypothesis appears, and the more attention we have to pay to the alternative concept of the neoplastic transformation being the nature of an abnormal irreversible differentiation".

The term "mutation" implies one of the three possibilities: a change in the nuclear genes, or in the cytogenes, or in the structural arrangement or completeness of the chromosomal system. Darlington(1948; 1959) offered the plasmagen theory of the origin of Cancer, emphasizing the cytoplasma as the source of the change leading to cancer. He described self propagating particles in the cells similar to those of the nucleus, but consisting of ribonucleic acid protein instead of deoxyribonucleic acid protein. These particles which he called plasmagenes were, he thought, the basis of development and differentiation. Changes in these particles could be held responsible for the origin of all types of tumour. The theory that neoplastic characteristics are due to extrachromosomal factors was reviewed by Haddow(1944), who mentioned the possibility that mechanism which caused continued growth in tumours may reside at least in part in the cytoplasm.

The recent spectacular progress in nucleic acids chemistry, brought evidence that nucleic acids constitute the probable repository of genetic information. From the chemical basis, reasonable evidence is accumulating to support the concept that alkylating agents act directly on the nucleic acid molecules, and that their mutagenic and carcinogenic actions may be similar. However, there is no substantial evidence that polycyclic hydrocarbons or other agents which are able

to produce initiating action act directly on nucleic acid molecules (Goldthwait,1960), though it has been suggested how they might do so. The subject will be discussed elsewhere in this review.

Thus, while the somatic mutation theory remains attractive, particularly as an explanation for initiation, it also remains unestablished.

3) Promotion

a) Introduction

Thus, the initiating phase of carcinogenesis is believed to be an irreversible conversion of normal cells into latent tumour cells, and the subsequent phase is that in which the dormant tumour cells become visible tumours. This second stage is called the "Promoting Stage", and the substances which induce it are called "Promotors".

b) Different kinds of promotor

As has been discussed, croton oil is a powerful promotor for mouse skin. Croton oil is expressed from seeds of croton tiglium, L.Euphorbiaceae. It is known to be powerful vesicant, and was formerly used as drastic purgative and cathartic. The vesicant action of croton oil was studied by Cohnheim(1889) in his work on the vascular reaction in the early phases of inflammation. Ledoux-Lebard(1885, quoted from Wolff,1907) reported the induction of epitheliomata of lung in dogs by intravenous injections of a croton oil emulsion. Despite these early

CORRECTION

Due to the considerable difficulty in obtaining a copy of the original work by Ledoux-Lebard(1885), his work was reported as quoted by Wolff(1907). It was found that there is a discrepancy between the original description by Ledoux-Lebard, and that by Wolff. Ledoux-Lebard describes an experiment by Martin saying "Ayant injecté dans la veine jugulaire d'un lapin de l'huile d'amandes douces rendue irritante par l'addition d'un ou deux centiemes d'huile de croton tiglium, il produisit dans les poumons des lésions qui rappelaient completement cette forme d'épithélioma decrite par Malassez, sous le nom d'épithélioma muquoide, et par leurs caractères anatomique et par leur mode de développement."

It can be translated "Having injected into the jugular vein of a rabbit, sweet almond oil made irritant by the addition of one or two hundredths of croton tiglium oil, it produced in the lungs, lesions which completely recall the form of epithelioma described by Malassez, under the name of mucoid epithelioma, both in their anatomical character and their mode of development."

ref.) Ledoux-Lebard,A.: Le cancer, maladie parasitaire.
Arch. generales de medicine. Series 7, vol. 1 -
413-441, 1885

reports, croton oil was seldom mentioned in experimental work until Berenblum(1941a) demonstrated its strong promoting action for mouse skin. Various other agents have been reported to have promoting power in initiated mouse skin. In testing substances for promoting power, a model experiment, a single application of carcinogen followed by repeated applications of the test substance, has usually been employed. It is not always easy to choose a suitable concentration of the substance tested, to determine the proper interval spacing between applications, or to choose the solvent to be used. However, a large number of substances, including agents previously regarded as harmless, have proven to have some promoting power in mouse skin, though very few have anything like the potency of croton oil. Iodoacetic acid and chloroacetophenone (Gwynn and Salaman,1953); phenol (Rusch,Bosch & Boutwell, 1955;Salaman and Glendenning,1957); several fractions of catalytically cracked mineral oil; and the straight chain hydrocarbon, n-dodecone (Shubik,Saffiotti,Feldman & Ritchie,1956;Horton,Denman & Trosset,1957) all showed some promoting power in mouse skin.

A new but not physically well defined group of promoting agents were reported by Setälä, Setälä and Holsti(1954) and by Setälä(1956). These are polyoxyethylene sorbitan compounds, such as Tween 40 (sorbitan monopalmatate), Tween 60 (sorbitan monostearate), and Span 20. The promoting activity of Tween 60 was confirmed by Della Porta, Shubik, Dammert and Terracini(1960) and Ritchie(1958). Like croton oil, Tween 60 was initially thought to be a pure promoting agent, but later, it was found that Tween 60 also produces an appreciable number of skin

tumours when applied repeatedly. (Setälä,1956:Della Porta,Shubik Dammert & Terracini,1960).

Intradermal injections of sclerosing agents like proflavin and ethanolamine acetate are also shown to act as promotor on mouse skin initiated with carcinogen (Salaman & Glendenning,1957).

Twort and Twort, (1939), showed that continuous local treatment, five times per week, with undiluted oleic acid given after preparatory administration of a carcinogenic hydrocarbon favoured the formation of local skin tumours which in a number of fatty acids, including oleic acid, were later reported to have promoting power, provided that they are applied with sufficient frequency (Holsti,1959). Shubik(1950a), however, applied oleic acid twice weekly and showed no tumour promoting activity.

Other new promotors are the phenolic fractions of cigarette smoke (Gellhorn,1958:Roe,Salaman & Cohen,1959), and the acidic fractions of cigarette smoke (Wynder and Hoffman,1961).

Citrus oils derived from the skins of the orange, lemon and grapefruit have also found to be promotors. These oils contain a terpene fraction and terpeneless fraction. The former consists mainly of monoterpene, such as d-limonene, and the latter of oxygenated substances such as methyl anthranilate, citral and decanal. It was found in the case of orange oil that only the terpene fraction had tumour promoting ability (Roe,Salaman & Cohen,1959:Salaman,1961). Latices of Euphorbia ingens, a plant belonging to the same family as croton tiglium, the source of croton oil was also shown to be a potent promotor for mouse skin. (Roe & Peirce,1961).

It is known that croton oil, though it is potent promotor on mouse skin, is not effective on the skin of rabbits, guinea pigs and rats (Shubik,1950a). Similarly, wound healing and chloroform are potent promotor on rabbit skin, but are not effective on mouse skin (Friedewald & Rous,1944a). These facts lead further complexicity for the study of the nature of promotion.

c) Biological Characters

How the promoting action brings about the change to progressive neoplastic growth, and what kind of biological processes are involved, is still unknown. Only a few facts are known. In order to elicit visible tumours after the treatment with initiator, repeated applications of promotor are necessary. For instance, various concentrations (1.25%, 2.5% & 5%) of croton oil applied once only to mouse skin after the initiating treatment by 9,10-dimethyl-1,2-benzanthracene failed to elicit any tumours (Salaman,1952). Or again, as discussed previously, a single application of 100 ug of 9,10-dimethyl-1,2-benzanthracene in acetone produces only a few tumours on mouse skin, while 200 ug produces a significant number. 100 ug is, however, an adequate initiating dose (Klein,1956). A single application of 0.2% or 2% of croton oil given after 100 ug of 9,10-dimethyl-1,2-benzanthracene did not cause any significant increase of tumour yield (Tomatis,Terracini & Shubik,1962). Similar results were reported by Klein(1953b), who painted mouse ears once with methylcholanthrene. Thereafter, croton oil was applied three times a week to the same site. Although few or no tumours were observed

following one, five, ten, or twenty paintings with croton oil, a definite effect was obtained when the number of applications was increased to thirty. Not only must the promotor be applied long enough, it must be applied often enough. Shubik(1950a) ahowed that twice weekly applications of undiluted oleic acid have no promoting action on initiated mouse skin. Holsti(1959), while confirming Shubik's observation, showed that twice daily or once daily applications of undiluted oleic acid did have a potent promoting action on mouse skin. Holsti(1960) also applied 0.2% croton oil twice daily, once daily and twice weekly on the initiated mouse skin, and found an increasing potency of tumour promotion with the increasing frequency of applications. A similar relationship was demonstrated when Tween 60 was used as promotor for mouse skin. (Della Porta,Shubik,Dammert & Terracini,1960).

The change induced by the promotion is not permanent. Salaman(1952) compared the effect of continuous and intermittent applications of croton oil. Two groups of mice were initiated with a single application of carcinogen. One group then received weekly applications of croton oil from the sixth week after the carcinogen application to the 34th week. The other group received croton oil from the 6th to the 13th week after the application of carcinogen, and from the 22nd to the 34th. No croton oil was given between the 14th and the 21st weeks. During the interruption, no new tumours appeared. When croton oil treatment was restarted new tumours again began to appear, but only after a latent period of about the same length as noted when croton oil was first started. Salaman concluded that the change produced by croton oil in

mouse skin previously treated with carcinogen is a gradual one, requiring repeated treatment and that it is reversible.

Klein(1953a) also studied the influence of continued and intermittent painting of croton oil after a single application of methylcholanthrene. He observed that new tumours continued to appear on the tumour bearing mice for a time after the cessation of croton oil. Klein agreed that repeated applications were necessary to induce visible tumours after a single application of methylcholanthrene, but thought that once a certain stage in tumour promotion had been reached, further applications of croton oil were unnecessary.

As mentioned above, in the earlier experiments on two stage carcinogenesis, treatment by croton oil alone produced no tumours, or only an occasional one (Berenblum,1941a;1941b & Shubik,1950b). Later several investigators found that croton oil induced a few tumours after a long period of treatment (Roe & Salaman,1955;Roe,1956b;Ritchie,1957). The question of whether the promoting action of croton oil was related to its low grade carcinogenic activity arose. Saffiotti and Shubik (1956a) painted groups of mice with a single application of a strong carcinogen and followed by repeated applications of 0.01% 9,10-dimethyl-1,2-benzanthracene in one group, and by repeated croton oil treatment in another. The group treated with a low concentration of carcinogen showed a considerable number of tumours with high rate of malignant transformation, while the croton oil treated group showed a large number of benign tumours with a low ratio of malignant transformation. Saffiotti and Shubik concluded that the primary action of

croton oil could not be explained by its feeble carcinogenic action. The observation of Setälä(1961b), that the histological, cytological and chemical reactions of mouse skin treated with carcinogen and promotor are different, gave further support to Saffiotti and Shubik's view. Allsopp(1951) suggested that croton oil inhibited the production of malignant tumours, but later Salaman and Roe(1956a) were unable to confirm this suggestion. On the contrary, they found that simultaneous application of croton oil and carcinogen actually increased the yield of malignant tumours in mice treated with repeated applications of 9,10-dimethyl-1,2-benzanthracene.

The tumour yield is directly related to the frequency of the promoting treatment (Holsti,1960; Della Porta, Shubik, Dammert & Terracini,1960), and to the concentration of the promotor (Merenmies, 1959;Frei & Ritchie,1962). In experiments by Frei and Ritchie(1962), groups of mice were initiated by the same concentration of carcinogen but were given repeated applications of different concentrations of croton oil. The tumour yield increased as the concentration of the croton oil increased. Several possibilities were proposed in order to explain these findings. One of the suggestions was that croton oil might be able to act on the initiated cells in more than one way. One or more of these actions might lead to the growth of the initiated cells into papillomata, while another or others might prevent their growth. A stronger concentration of croton oil favours the chance of tumourous change, while a weaker concentration favours the other possibility.

d) Histological Studies

Histoquantitative studies of the effect of croton oil on mouse skin initiated by a small dose of a chemical carcinogen were carried out by Salaman and Gwynn(1951). The skin treated with croton oil for 1.5 to 3 months after previous treatment with carcinogen showed an increase in the percentage of the resting cells, and this increase was maintained. In normal skin given croton oil applications only, the percentage of the resting cells rose slightly at first, but returned to a normal level. Salaman and Gwynn concluded that the effect of repeated applications of croton oil on skin initiated by an application of carcinogen is similar to the effect of repeated applications of carcinogen and different from the effect of croton oil on uninitiated skin.

Histological studies of the effect of Tween 60 on mice initiated by 9,10-dimethyl-1,2-benzanthracene were carried out by Dammert(1961b). The characteristic reaction of mouse skin to this sequence of treatment was that immediately after the commencement of Tween 60 treatment there was a huge rise in the mitotic frequency, accompanied by inflammatory changes, with hyperplasia and hypertrophy of the epidermal cells, and with an increase in the dermal tissues. Following this, the mitotic frequency dropped rapidly, and continued to decline in spite of the continuous treatment. Despite the decreasing mitotic rate, the hyperplasia was maintained. If Tween 60 treatment was started four weeks after a single application of 9,10-dimethyl-1,2-benzanthracene, the epidermis reacted to the promoting treatment with Tween 60 with the

same degree of mitotic increase and hyperplasia as when it was started after an interval of one week.

The changes produced in skin by Tween 60 differ from those caused by similarly applied carcinogens (Setälä,Merenmies,Stjernvall, Aho & Kajanne,1959). Treatment with Tween 60 did not produce retrogressive alterations such as a disturbance of organization or differentiation in the epidermal cells. Rather the reaction is an intense cell multiplication which commences immediately after the applications begin. Continuous treatment with Tween 60 caused neither cellular nor nuclear atypia, and no disturbance in organization, but the number of cells in differentiation increased significantly. Setala and his colleagues concluded that tumour promotion is not a phase of the carcinogenic process itself, but is an intense, continuous, reparative cell multiplication. The following table (Setälä,1961b) illustrates the opposite reactions of carcinogen and promotor.

Electron microscopic studies of mouse skin treated with carcinogen and with Tween 60 or Span 20 further confirmed these observations (Setälä,Merenmies,Niskanen,Nyholm, & Stjernvall,1960; Setälä,Merenmies,Stjernvall,Nyholm & Aho,1960).

As discussed previously, Orr(1955), emphasized the importance of the dermis in connection with epidermal carcinogenesis. He suggested that the promoting action may result from an indirect effect on the dermis such as ischaemia or fibrosis, and claimed that intradermal injections of ephedrine augmented carcinogenesis by repeated applications of carcinogen (Orr,1935). However, Ritchie(1952) was unable to show

TABLE

Difference in Reaction between Carcinogen and Promotor

		Carcinogen	Promotor
1.	Tumours	tumour development	usually no tumour development
2.	Epidermal hyperplasia	irregular, progressive	regular
3.	Hair Follicle Sebaceous Gland	destroyed	intact
4.	Cells	increase in basal cells	increase in resting cells
5.	Cell Morphology	atypia and anaplasia	no atypia or anaplasia
6.	Nucleo-Cytoplasmic Ratio	increased	no change
7.	Cell Volume	decreased	increased
8.	Cytoplasma	high ultra-structural change	no change
9.	Mitochondria	accumulation of electron dense bodies	no change
10.	Cell Contact	tight	considerable intercellular spaces
11.	Localisation of Mitosis	all layers of cell with nuclei	only in basal layer
12.	-SH concentration	increased	no change
13.	-S-S concentration	decreased	no change
14.	Keratinization	slow and irregular	secondary delay
15.	ATP action	promote keratinization	no action

(Setälä,1961b)

that the injection of ephedrine beneath the painted areas during promotion influenced tumour promotion.

e) Chemical Studies

The chemical analysis of the croton seed was first undertaken by Cherbuliez and Bernhard(1932). From a purely chemical standpoint, it contains a nucleoside, (9-ribofranosylisoguanine), which is isometric with guanosine and gives on acid hydrolysis D-ribose and isoguanine (Chargaff & Davidson,1955), and glycerides of stearic, palmitic, myristic, lauric and tiglic acids.

Several attempts have been made to isolate chemically from croton oil, the active principle responsible for its tumour promoting action. Gwynn(1955) separated a number of fractions from croton oil chromatographically, all of which, like the parent oil, produced gross epidermal hyperplasia but only two of which had promoting power. Lijinsky(1958) has submitted methanolic extracts of croton seeds to silica-gel chromatography and obtained fractions, comprising about one percent of the whole oil, that appear to have all its promoting activity. Sicé(1958) succeeded in extracting at least two different tumour promoting principles from croton oil by chromatography. They were labile to mild alkylis, which implies the probability of an ester structure, an essential requirement for activity.

The effects of Lijinsky's fraction on the active transport of glucose by the isolated surviving guinea pig intestine was studied Du Ruisseau and Quastel(1959). The croton oil fractions were found to

depress the absorption of glucose under aerobic conditions to that observed under anaerobic conditions. Other oils were without effect. This finding was interpreted as shown that croton oil depresses the yield from oxidative phospharylation of the adenosene triphosphate required for the active transport of glucose. Du Ruisseau and Quastel claimed that there is a correlation between tumour promoting activity of croton oil and its ability to depress the glucose absorption of isolated guinea pig intestine.

The fractions from croton oil responsible for tumour promotion were studied by Allison and Lightbown(1961). They investigated the effects of the fractions on cellular respiration using rat liver homogenate, rat liver mitochondria, mouse skin and heart muscle. The results show that these fractions inhibit the cellular respiration mediated through cytochromes. Allison and Lightbown assumed that initiated cells are more able to utilize anaerobic glycolysis than are other cells, and envisaged promotion as a process by which the small number of tumour cells produced by an initiator are placed at a competitive advantage over normal cells, the growth of which are handicapped by respiratory inhibition. Under the continued influence of the promotor, it was supposed that tumour cells could form clones sufficiently large to become self-perpetuating.

They extended their hypothesis to explain the action of some co-carcinogenic viruses on chemical carcinogenesis (Duran-Reynolds,1962; Tanaka & Southam,1962), suggested thus, the viruses too inhibited respiration, so placing the initiated cells at an advantage because of

their greater glycolytic ability.

Endogenous dehydrogenase in the epidermis as measured by the amount of formozan deposit by the tetrazorium reduction methods shows an initial increase after a single croton oil treatment, followed by a decrease (Iversen,1962). The reaction is similar to that obtained with carcinogens, such as methylcholanthrene or 9,10-dimethyl-1,2-benzanthracene.

The estimation of sulphydryl groups in mouse skin epidermis after the application of croton oil showed that there is a constant increase of S-H levels regardless of whether or not the skin was treated initially by a carcinogen (Calcutt,1961b).

In the dermis of the rat skin, after the injection of croton oil, there is a loss of acid mucopolysacharide and insoluble glycoprotein, with a small decrease in the amount of sialic acid (Houck, Jacob & Vickero,1962).

f) Hyperplasia vs. Promotion

The most obvious reaction of the skin to most promotors, croton oil, turpentine, Tween 60, and wound healing is marked epithelial hyperplasia. It was thus quite natural to think that a nonspecific stimulation of growth, by encouraging the dormant cells to divide, might be responsible for the promoting action. Indeed, Friedewald and Rous(1944a), in their study of initiation and promotion in rabbit skin, concluded that anything which induced hyperplasia in the epidermis, turpentine, irritant secretions, mechanical trauma or chloroform, was capable of

acting as a promotor. In the case of mouse skin, the matter is much more complicated.

Shubik(1950a) undertook an investigation to test the promoting activity of several substances which are able to induce epidermal hyperplasia. The various substances were tested on mouse skin prepared by an application of 1.5% 9,10-dimethyl-1,2-benzanthracene as initiator. The hyperplastic response and the promoting power of the various substances were compared. Turpentine, acridine, fluorene, phenanthrene, castor oil, oleic acid and silver nitrate were tested. None of them had any tumour promoting effect, but acridine and silver nitrate produced a significant degree of epidermal hyperplasia, though not as great as that produced by croton oil. Shubik concluded that epidermal hyperplasia and tumour promoting activity are not related in any simple fashion. Holsti(1959) threw some doubt on this work by suggesting that the test for promoting power was not delicate enough. However, Gwynn(1955) also failed to show a correlation between promoting power and the ability to induce epithelial hyperplasia. Among the substances he tested, iodoacetamine, podophylin, and 1,4-naphthoquinone produced significant epidermal hyperplasia, but lacked promoting action. Gwynn expressed the view that hyperplasia might be a necessary, but was not a sufficient condition for carcinogenic action. Sicé(1958), isolated two different tumour promoting principles from croton oil. No parallelism was found between promoting activity and epidermal hyperplasia or dermal inflammation.

Despite these negative correlations between hyperplasia induced on skin and promoting potency, several groups of investigators favour the

view that hyperplasia is the essential element in promotion. While studying the tumour promoting activity of citrus oils, Roe, Salaman and Cohen(1959) found that the oils produced and maintained active hyperplasia. Subsequently it was shown that the terpene fraction of orange oil is a strong hyperplasia inducing substance and is a potent promotor, but the terpeneless fraction lacks hyperplasia inducing capacity and also lacks promoting power (Salaman,1961).

Similarly, in the study of the tumour promoting action of the latices of Euphorbia ingens, Roe and Peirce(1961) tested nine species of Euphorbia. On the whole, the tumour promoting effect seemed to be directly related to the degree of hyperplasia. However, in one case, the latex of Euphorbia obovalifolia produced a significantly high degree of epidermal hyperplasia, but its tumour promoting effect was very weak. No explanation for this paradox can be given.

The promotors Tween 60, and Span 60 produce a degree of epidermal hyperplasia which correlates well with the promoting power of the agents. (Setälä,Merenmies,Stjernvall,Aho & Kajanne,1959). Setälä(1961a) presented the following conclusions concerning the role of epidermal hyperplasia in tumour promotion. "1) All tumour promotors tested so far have caused a high degree of hyperplasia; 2) This hyperplasia is of a regular type and tissue preserving, and it lasts as long as the treatment. Morphologically, that is, light microsopically, electron microscopically, and histo-quantitatively, it does not in any way differ from simple reparative cellular proliferation, for instance from that obtained by painting with water. 3) The degree of hyperplasia induced by treatment

with dipole-type substances shows positive correlation with the frequency of treatment and with the concentration of the substance in an aqueous solution. 4) Similarly, the degree of hyperplasia induced by treatment with dipole-type agents has a positive correlation with the tumour promoting efficacy of the substance in question."

The difference between simple reparative hyperplasia and the hyperplasia induced by croton oil was discussed by Berenblum(1954). Reparative hyperplasia is merely an enhancement of the normal growth cycle, where the rate of division of the stem cells is balanced by the maturation and the death of the cells at the surface. Thus, the increased rate of division in simple reparative hyperplasia is attended by a comparable increase in maturation and death, so that a new equilibrium is reached involving an increase in maturing and dying cells, but not of stem cells. Berenblum postulated, in contrast, that promoting action evoked an increase in the number of stem cells including those which, through previous initiating action, had become dormant tumour cells. Since the piling up of stem cells is normally prevented by the process of maturation, it follows that promoting action is essentially a process of delayed maturation. Delayed maturation alone is insufficient, however, to explain promoting action, as it fails to account for the fact that the resulting tumour, once established, usually continues to grow without the need for further promoting action. Berenblum, suggested that the most far reaching implication of the delayed maturation hypothesis was that the evolution of a tumour may have little or nothing to do with the problem of growth. The initiating action results in a sudden,

permanent change in the potentiality of a few normal cells. Promoting action, by delaying maturation, allows a sufficient number of undifferentiated daughter cells of the altered cell to accumulate, and thus reach a critical sized colony. Once this critical colony size has passed, the tumour can grow and survive independently of promotion.

The difficulty in accepting non-specific proliferation as an explanation of promoting action led to several other hypotheses. One is that promoting action is a successive mutation, either in an adjoining cell, or in the same cell (Rusch,1954). By the mathematical analysis of the age incidence of cancer in man, Fisher and Hollomon(1951) and Armitage and Doll(1954;1957) supported the theory that a sequence of mutations is the origin of cancer. However, the fact that tumours arise when promoting action follows initiating action but not when promoting action comes first, argues strongly against the successive mutation theory of promotion. Danielli(1952) proposed an explanation of the two stage theory of carcinogenesis based on the concept of cytoplasmic genes (plasmagenes). He thought the deletion of a chromosomal gene by initiation would produce no effect so long as plasmagene reproduction continues, but if the plasmagene is eliminated by promotion, an abnormality would quickly appear. Thus, Danielli suggested that the initiating action of carcinogenesis may be due to a deletion of a chromosomal gene and promoting action to a deletion of a plasmagene.

g) Summary

More studies are necessary to bring a clear theoretical

explanation of promotion. From our present knowledge, however, it seems reasonable to think that the hyperplasia produced by many promotors plays at least some part in promotion. Other actions, such as successive mutations or alterations in tissues other than epidermal cells may, however, play a major part in the process of promotion.

D) URETHANE (ETHYL CARBAMATE) AS A CARCINOGEN

The narcotic action of urethane has been known from the time of Schmiedeberg(1885). It has frequently been employed when a prolonged mild anaesthesia is desired, and has been found particularly useful for keeping animals quiet during long exposure to X-rays. Phenyl-urethane has been shown to arrest the division of the sea urchin egg at metaphase (Warburg,1910). Vogt(1948), described sex linked mutations in Drosophila after administration of urethane, and Bryson(1949) described mutagenic action of urethane on bacteria. Its leukopenic action in animals has been well known since Hawkins and Murphy's(1925) description. This action of urethane later had its application in the treatment of human leukaemia. After a series of experiments in animal leukaemia and lymphosarcoma (Murphy & Strum,1946), Paterson, ApThomas, Haddow and Watkinson(1946) introduced urethane for the treatment of human leukaemia. Due to certain side effects on human beings (Meacham,Tillotson, & Heinle, 1952), and the development of new chemotherapeutic agents, the use of urethane for antileukaemic therapy has diminished. It is listed as the first choice for the treatment of certain forms of tumours, such as multiple myeloma (Rundles & Coonrad,1957).

In 1943, Nettleship and Henshaw found that repeated minimal anaesthetizing doses of urethane given by intraperitoneal injection increased the incidence of lung tumours in C3H female mice from less than 5% to more than 75%. This finding was confirmed by Henshaw and Meyer(1944,1945), who noted that urethane was also capable of inducing pulmonary tumours in mice when administered by the oral route or by local implantation. Rats were also susceptible to urethane's carcinogenic influence on the lung (Jaffe,1947). After the establishment of the carcinogenic action of urethane (ethyl carbamate) on the lung, a series of esters of carbamic acids were tested in a similar fashion, and it was found that the high carcinogenic activity of the ethyl derivative was low in the isopropyl and n-propyl derivatives and absent in methyl, n-butyl and isomyl derivatives (Larsen;1947). The lung tumours that urethane produces are mostly benign adenomas. Their histological structure was well described by Orr(1947). Klärner and Gieseking(1960), demonstrated by electron microscopy cytoplasmic inclusion bodies in the cells of urethane induced lung tumours, and regarded these bodies as viruses of possible aetiological significance. However, the existence of such viral particles could not be confirmed by Svoboda(1962).

The initiating action of urethane on mouse skin has been mentioned already. Salaman and Roe(1953) painted the skin of the backs of mice once or a few times with doses of between 60 and 360 mg. of urethane in watery solution, and then gave repeated applications of 0.5% croton oil for twenty weeks. They found thirteen papillomata on the eight survivors at the sixteenth week, and eleven papillomata on

the five survivors at twenty weeks. In further studies, using twenty-six mice, they applied 240 mg. of urethane (120 mg. on the first day and 120 mg. on the eighth day), and followed on with croton oil to produce 115 tumours on twenty-two survivors. When urethane was applied in the same dose without croton oil, no tumours were produced. In order to test for a possible promoting action of urethane, mice were painted with 0.3 mg. 9,10-dimethyl-1,2-benzanthracene once, and then with 60 mg. urethane weekly for eighteen weeks. No tumours were produced. In the same year in Germany, Graffi, Vlamynch, Hoffman and Schultz(1953), tested the effect of various substances on mouse skin, applying the substances alternately with croton oil, for a period of twelve months. They found that urethane applied with the croton oil produced significantly greater numbers of tumours than did croton oil alone. In contrast, the administration of urethane together with 9,10-dimethyl-1,2-benzanthracene in the initiating stage was reported to reduce the tumour yield produced by the subsequent repeated applications of croton oil (Gwynn,1956;Shubik, Della Porta, Spencer & Pietra,1958).

Urethane is also able to initiate skin tumours not only when applied to the skin, but when given by oral administration (Haran-Ghera & Berenblum,1956), or intraperitoneal injection (Ritchie,1957; Berenblum & Haran-Ghera,1957a).

At present, most of the known initiating agents are able to produce skin tumours alone when applied in large doses or repeatedly. In contrast, urethane is usually said to possess only initiating action on the skin, and to be unable to produce any tumours alone. It is

sometimes called an incomplete carcinogen, or pure initiator for the skin (Roe & Salaman,1954). However, as mentioned before, Lindsay(1956), applied urethane once or twice to male mice (NCZ) and found a total of twenty-four neoplastic skin lesions in eleven of fifteen mice treated. There was one squamous cell carcinoma, fourteen papillomata and six sebaceous adenomata. More recently, Pietra, Rapapport and Shubik(1961), injected 0.04 mg. urethane intraperitoneally to twelve hour old mice and noted one carcinoma and one papilloma in the surviving mice.

In contrast to the incomplete carcinogenic action of urethane on the skin, urethane is a complete carcinogen for the lungs. A search for the possible promoting factors active in the formation of lung tumours by urethane was suggested (Boyland,1960). For that purpose, Dipaola(1959) investigated the significance of the oxygen concentration in the air after the administration of urethane, and found a high tumour yield in a group which was exposed to hyper-oxygenic environment. The opposite was reported by Mori-Chavez(1962), who compared the incidence of lung tumours induced by urethane in mice at sea level and high altitude. A high tumour yield was obtained in the group kept at high altitude. Mori-Chavez attributed the increased yield to the deficiency of oxygen.

The initiating effect of 240 mg. urethane on mouse skin is approximately equal to that of 0.03 mg. 9,10-dimethyl-1,2-benzanthracene (Salaman & Roe,1954). On the assumption that a lower dose of urethane might prove to be relatively or absolutely more effective, Roe and Salaman (1954) extended their work on the quantitative analysis of the initiating

power of topically applied urethane on mouse skin. The backs of mice were painted with different doses of urethane, 240 mg., 180 mg., 50 mg., 10 mg., or 2 mg., and eighteen weekly applications of 0.5% croton oil followed. The dose of 240 mg. and that of 180 mg. could not be given in a single dose as were the others. The former was given as two applications of sixty mg. urethane with fifteen minute intervals on the first and eighth days, the latter as three applications of 60 mg. given at two hour intervals. When the tumour yield was expressed as the average number of tumours per survivor, the relationship between the total dose and the tumour incidence was a linear. This linear relationship between dose and tumour yield is in contrast to the logarithmic relation noted with carcinogenic hydrocarbons given in one dose. The difference might be due in part to the administration of the larger doses as separate small doses. Roe and Salaman(1954), also investigated the effect of altering the interval between applications on the initiating activity of 180 mg. of urethane. One group received 180 mg. urethane as three applications of 60 mg. urethane given at two hour intervals, and another the same dose given with four day intervals. The tumour incidence was demonstrated to be about the same. Roe and Salaman also demonstrated that the initiating effect of urethane persists about twenty-four weeks.

A quantitative analysis of the initiating action of urethane was also carried out by Berenblum and Haran-Ghera(1957a). They studied the effect of: a) varying the total dose of urethane given once by mouth; b) varying the number of urethane feedings given but keeping the overall dose

constant; c) different routes of administration; and d) variations in the interval between the end of initiating action and the commencement of promoting action. The results are summarized in the following tables:

Effect of dose of urethane, administered orally, as initiating agent

Initiating Agent	Mice bearing Papilloma/ Survivor	Av. no. of Papilloma per Mouse	Av. Latent Period
Single dose by Mouth			
Urethane 64mg	24/24	5.0	8
16mg	15/24	1.2	14.5
4mg	6/14	0.4	20.5
1mg	3/24	0.25	16
0mg	2/20	0.1	15.5
Different numbers of urethane feedings, totalling the same overall dose			
64mg in 1 dose	24/24	5.0	8
64mg in 2 doses	37/37	8.0	6
64mg in 5 doses	17/20	2.6	10.5
64mg in 20 doses	21/24	2.2	11
Urethane action by oral, subcutaneous, and intraperitoneal routes			
64mg P.O.	24/24	5.0	8
50mg I.P.	28/28	5.7	8
50mg S.C.	22/22	4.3	10

(Berenblum & Haran-Ghera,1957a)

As we see from the table, the findings are in agreement with those found using polycyclic hydrocarbons. With a single dose the relation between dose and tumour incidence is approximately logarithmic, but divided doses were less effective than one large single dose. Urethane was more or less equally effective given orally, intraperitoneally or subcutaneously. Varying the interval between the initiation and the commencement of croton oil treatment from thirty minutes to fifty-six days did not significantly alter the tumour yield. The results of these experiments are consistent with Berenblum and Shubik's original hypothesis (1947b) that the number of tumours produced is a function of the initiating action, while the latent period is a function of the promoting action.

In a more complicated recent experiment by Pound(1962), mouse skin was initiated by feeding urethane orally, for five consecutive days. The tumour yield was higher if the croton oil started on the second day of urethane feedings rather than if it was started a week after the urethane feedings.

A carcinogenic action of urethane other than on the skin and lung of mice and other species has been reported. Tannenbaum and Silverstone (1958) and Tannenbaum(1961) described urethane as a multipotential carcinogen for mice, reporting the formation of pulmonary adenomata, mammary carcinomata, malignant mesenchymal tumours, cystoadenomata of lachrymal glands, blood cysts of the liver, and Harderian gland adenomata. Papillomata of the forestomach may follow oral administration of urethane in mice (Berenblum & Haran-Ghera,1957c).

The induction of hemangiomata in the liver, intestine and pancreas in mice of the AkR strain and of the C58 strain was reported by Kawamoto, Kirschbaum, Ibanez, Trentin and Taylor(1961). A leukaemogenic action of urethane, and its power to potientate the leukaemosenic action of X-rays, oestrogens and methylcholanthrene have also been reported. (Kawamoto, Kirschbaum & Taylor,1958). Similarly, the formation of lymphoma (Toth, Tomatis and Shubik,1961) and thymic lymphoma (Doell,1962) by oral feeding of urethane have been reported. Della Porta and Shubik(1960) and Toth, Tomatis and Shubik(1961) reported the multipotent carcinogenic action of urethane in the Syrian Golden hamster. Melanotic tumours of the skin, papillomata and carcinomata of the forestomach, adenomata polyps of the caecum, pulmonary adenomatosis, mammary tumours, hepatoma and hemangiosarcoma were all produced.

The mechanism of the action of urethane was investigated from two points of view, its carcinogenic action and its tumour suppressing effect. Haddow and Saxton(1946) described the inhibitory action of carbamic esters on Walker rat tumours, and suggested that urethane might belong to the group of caryoclastic substances, and that inhibition of growth and mitosis might be due to an upset in purine metabolism. The in vitro effect on the growth and mitosis of normal and malignant cells was interesting. All cultures obtained from normal tissue showed a reduction of growth and a fall in the number of cell division. However, in the cultures of malignant tumours, a stimulation of growth and mitosis was observed (Lasnitzki,1949). Decreased mitotic activity and cellular degeneration in the crypts of Lieberkuhn in mice (Dustin,1947), and

inhibition of all stages of mitosis in rat cornea (Guyer & Claus,1947) have also been reported. In connection with the suggestion made by Haddow and Saxton, Cowen(1949) reported the inhibitory action of pentose nucleotides upon urethane induced pulmonary tumours. He postulated that pentose nucleotides might act by directly supplementing the normal cell purine, and so rendering the urethane ineffective, or might produce a leukocytosis which neutralized the leukopenic action of urethane. The changes in nucleic acid metabolism induced by urethane were further discussed by Boyland(1952) and Boyland and Koller(1954). In their study of the effect of urethane on mitosis in the Walker rat carcinoma, they observed that urethane produced abnormalities of nuclei, with chromosome fragmentation and anaphase bridges, in a portion of the dividing cells in the tumour. The frequency of these abnormal mitoses was reduced, and recovery was accelerated by the simultaneous administration of thymine. Thymine did not affect the frequency of abnormal mitosis produced in the Walker rat tumour by nitrogen mustard. Boyland and Koller suggested that urethane might interfere with cellular synthesis of thymine, by inhibiting the methylation of uracil to thymine, so leading to a deficiency of thymine. Roe(1955) thought that if suitable purine precursors were supplied in high concentration at the time of urethane administration, the carcinogenic action of urethane might be reduced. He found that urethane's initiating action on skin tumour formation in mice was inhibited if formate and glycine were both administered together with it, though neither had any effect when administered alone.

The suggestion that urethane might exert its carcinogenic action

by disturbing the synthesis of nucleic acids was further strengthened by a series of experiments by Rogers(1957a;1960). The cellular nucleic acids are known to be important carriers of genetic information, and so might be expected to be involved whether the neoplastic change occurred through an alteration of previously existing cellular information or through the mediation of a virus bringing new information into the cells. Rogers attempted to modify the response of the animals, as measured by the production of lung tumours induced by a single injection of urethane. He administered known precursors of nucleic acids synthesis, and found an inhibitory effect when pyrimidine or pyrimidine precursors were given. In particular, orotic acid had a striking inhibitory effect on urethane carcinogenesis in the lungs. Orotic acid is known to enter the pathway of synthesis of both ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) (Carter,1956). However, thymine, which is known to be incorporated only in DNA, also had an inhibitory effect, which suggested that the action of urethane is on DNA synthesis. Thus, Roger's results taken together indicate that urethane initiated the neoplastic change in the lung by interfering with pyrimidine synthesis. The inhibitory influence of orotic acid and other pyrimidine precursors point toward a site of action below orotic acid, as probably being the site of the primary carcinogenic action of urethane.

Recently, Bresnik(1960) reported that urethane in vitro is an antagonist of the enzyme converting carbamyl phosphate to ureidosuccinate. From the point of inhibitory action on certain

tumours, Elion, Bieber, Nathan and Hitchings(1958), postulated an interference with the amylation of the uracil moiety.

Another study was concerned with the metabolic degradation of urethane, on the assumption that a metabolite is responsible for its carcinogenic action. The chemical formula of urethane is relatively simple. It is known to be hydrolysed quickly after injection into the body, giving end products such as ethanol, carbon dioxide, and water. It is distributed rapidly throughout all organs of the body in equal proportion, (Archer, Chapman,Rhoden & Warren,1948;Boyland & Rhoden, 1949), and is almost completely excreted from the body within twenty-four hours (Skipper,Bennet,Bryan,White,Newton & Simpson,1951). Therefore, it would seem that the carcinogenic action of urethane is over within twenty-four hours. A number of derivatives and analogues of urethane were tested for carcinogenic power in the lung and for initiating power in the skin, in the hope that in this way an active metabolite might be identified. Seven compounds with a modification of the carbamyl portion, four phosphorylated derivatives and four compounds of a complex of ketoacid with urethane were tested (Berenblum,Ben-Ishai, Haran-Ghera,Lapidot,Simon & Trainin,1959). Most of the compounds proved to be inactive, and those that did give positive results were weak. None of them seemed to be the sought for metabolite. Injection into mice of rabbit plasma obtained from rabbits injected with urethane $2\frac{1}{2}$ hours previously, increased the incidence of lung adenomata, as well as producing the initiating change in the skin, so that papillomata arose at the site of subsequent croton oil applications (Berenblum, Kaye & Trainin,1960). These results were not obtained if the rabbit

plasma was lyophylized or dessicated, removing of free urethane. The condensed vapour collected during the process of lyophylization, on the other hand was carcinogenic. These results could be entirely accounted for on the supposition that the carcinogenic effect was due to urethane itself, without participation of any metabolite. Supporting the evidence for this view presented by Kaye(1960a), he chromatographed plasma from rabbits given an injection of C^{14} labelled urethane $2\frac{1}{2}$ or $3\frac{1}{2}$ hours previously, and plasma from mice given an injection of C^{14} labelled urethane $2\frac{1}{2}$, 4, or 6 hours previously, and a urethane solution in which mouse lung, liver, and skin had been incubated. In all cases a single migrating component was found, and its rate of migration and volatility corresponded to those of free urethane. The result again suggests that no metabolite is involved in urethane carcinogenesis. In the study of the relationship between the rate of urethane catabolism and carcinogenesis, Kaye(1960b) found a greater retention of urethane in the blood of young mice than in older ones. He concluded that the greater response to the carcinogenic action of urethane in young mice suggests that the length of time urethane remains in the body is a critical factor in determining the tumour yield.

E) TWO-STAGE CARCINOGENESIS OTHER THAN IN MOUSE EPIDERMIS

The two stages of carcinogenesis have been well demonstrated in the skin of mice. There is also evidence that initiation and promotion are involved in the induction of tumours other than in mouse skin. Indeed, Rous and Kidd(1941) and MacKenzie and Rous(1941), were among the first to

postulate a two stage theory. From the studies of the behaviour and structure of tumours induced by repeated applications of tar to the skin of rabbits, they deduced that warts produced by tar are conditional, and depend for their growth on continued extraneous stimulation. Tumours that regress during an intermission of tarring, recur at the same site when tarring is resumed. Moreover, wound healing or turpentine can evoke growths from previously tarred skin. Rous and his colleagues distinguished the two separate elements in carcinogenesis in rabbit skin by tar, and, as has been mentioned, called them initiation and promotion (Friedewald & Rous,1944b). The carcinogenic action of 9,10-dimethyl-1,2-benzanthracene on rabbit skin was demonstrated by Berenblum(1945;1949), and Shubik(1950a) applied this carcinogen to rabbit skin in a sub-optimal dose and followed it by mechanical wounding, turpentine applications or croton oil applications. Only wound healing acted as a promoting agent. It is of interest to note that croton oil, the most potent promotor on mouse skin, was not effective for promotion in rabbit skin. Roe(1956c) also reported the ineffectiveness of croton oil to promote tumours on the rabbit skin after the applications of 9,10-dimethyl-1,2-benzanthracene. However, a promoting effect of croton oil on rabbit skin initiated by the applications of 9,10-dimethyl-1,2-benzanthracene was claimed by Graffi(1951).

A more difficult problem is whether two stage mechanism is peculiar to skin carcinogenesis, or whether it also operates, perhaps in a modified form, in other organs. The evidence on this subject is rather scanty. The reasons are perhaps more technical than basic. It

is relatively easy to design suitable experiments for this purpose in the case of skin, but it is difficult to do so for other organs.

In several instances, induced hyperplasia of a particular organ has been shown to enhance the power of a carcinogen acting on that organ. Partial hepatectomy has been reported to hasten the appearance of hepatomata in rats on a diet containing butter yellow (Glinos,Bucher, & Aub,1951). Law(1956) reported a similar result in rats fed acetylaminofluorene. In these cases, there were no significant differences in the tumour incidence. Various procedures which lead to hyperplasia of the thyroid, partial thyroidectomy (Bielschowsky,1949), or the administration of allyl-thiourea (Bielschowsky,1944;1945) result in the development of multiple thyroid tumours in rats fed acetylaminofluoren. A very small dose of acetylaminofluorene is able to accelerate greatly the appearance of thyroid adenomata in rats subsequently fed thyouracil (Hall,1948;Hall & Bielschowsky,1949). It seems reasonable to assume that in these experiments the carcinogen, butter yellow or acetylaminofluorene might act as initiator, while induced hyperplasia had a promoting function. In experimental hepatocarcinogenesis, subcarcinogenic doses of dimethylaminoazobenzene were able to elicit tumours in rats when they were followed by painting of the skin by tar (Muta,1943 quoted by Odashima, 1959), methylcholanthrene (Odashima,1959) or 4-nitro quinoline N-oxide (Takayama,1961). In these instances, the action of the promoting agents is obscure. Maltoni and Prodi(1960) reported that carbon tetrachloride acts as a promoting agent in liver carcinogenesis if p-dimethylaminoazobenzene in noncarcinogenic dose is used as the initiating stimulus.

Cytochemical studies of carcinogenesis by azodyes in rat liver indicate that separate processes occur during development of tumours. Maini and Stich(1961) studied the action of several hepatocarcinogens, with particular regard to their ability to provoke a proliferation of hepatic cells; to induce the formation of highly polypoid cells; and to cause mitotic irregularity. There was a correlation between the carcinogenic activity of substance and its ability to induce both proliferation and a relatively large number of mitotic irregularities in the precancerous liver. They concluded that hepatomata are only produced when two factors are present, namely, an injury to the chromosomes and an active cell proliferation. Also, Fiala, Fiala and Glinsmann(1961) claim that hepatocarcinogenesis in rats induced by 3-methylaminoazobenzene consists of two phases. They proposed induction period in which sudden and irreversible changes in many cell parometers occur during a critical period, and then an unrestricted proliferation period.

A few instances of yet other organs aside from the liver which indicate a two stage mechanism in tumour formation will be mentioned. A summarized list of experiments relevant to this matter will be found at the end of this chapter (Table). Berenblum and Trainin(1960) postulated a possible two stage mechanism in experimental leukaemogenesis, based on the augmenting effect of urethane on leukaemogenesis by X-ray, when urethane follows radiation but not when the sequence is reversed. X-ray might act as the initiator, and urethane as the promotor. In an experiment by Peirce(1961), tumours of the stomach in mice were induced by oral administration of benzpyrene as an initiator

followed by lime oil feeding as the promotor.

Dao(1959) made an interesting observation while inducing mammary tumours in rats by feeding methylcholanthrene. When pregnancy follows methylcholanthrene feeding, there is an augmentation of tumour induction, though when methylcholanthrene is given to the pregnant rats there is no augmentation. On the basis of these observations, Dao claimed that the first stage in the induction of mammary tumours is a chemical one, but the second stage is physiological one governed by some hormonal effect. Kim and Furth(1960) showed that although a single dose of methylcholanthrene, or the administration of mammotropes alone failed to induce mammary carcinomata in rats, a single dose of methylcholanthrene with mammotrope induces a high incidence of carcinomata. Perhaps methylcholanthrene acts as the initiator, the mammotrope as the promotor. The effect of mammotrope was studied by Yokoro and Furth (1961) on the induction of mammary tumours on rats by radiation. It was shown that while a single dose of 50r. or 150r. of radiation was unable to induce mammary tumours, subsequent administration of mammotrope gave rise to carcinomata. Thus, in sum, this work shows what appears to be a genuine promoting action of hormone in mammary tumour induction in the rat.

In mice there was no apparent promoting action of mammotrope on the mammary glands initiated by methylcholanthrene (Haran-Ghera,1961). This apparent difference between mice and rats is attributed to the different characteristics of mammary tumours in the two species. Mammary tumours in rats are in most cases hormone-dependent, in mice

they are not. Recent studies by Yokoro and Furth(1962) have, however, disclosed that in mice mammotrope acts as promotor for the induction of mammary tumours initiated by virus. In the C 57L/J X A/HeJ strain of mice, treatment with mammary tumour virus (Bittner's), mammotrope or diethylstilbesterol alone failed to induce mammary tumours, but treatment with the mammary tumour virus followed by mammotrope administration induces high incidence of tumours.

Finally, we should consider the critical question; whether or not two stage carcinogenesis has any significant role in human carcinogenesis. There are interesting facts in the problem of age and human cancer which are relevant to this topic. Nordling(1953) and Stocks (1953) independently suggested that the development of cancer was the end result of a series of discrete cellular changes. Nordling(1953) studied the frequency distribution of cancer in adults in relation to age, and found that the log of death rate increased in direct proportion to the log of age, but about 6 times more rapidly. He suggested that this relationship could be explained if the cancer cells are the results of 7 successive mutations. Armitage and Doll(1954) took up this statistical approach in more detail, considering different kinds of human cancer individually. They later offered an explanation of data in terms of the two stage hypothesis (Armitage and Doll,1957). Haenszel, Loveland and Sirken(1962) engaged in the epidemiological investigation of lung cancer. They found a high urban to rural ratio of incidence and mortality from lung cancer. The higher risk for certain groups with a variety of residencial exposures, and the obvious presence of

more than additive effects in the ratio for specific combinations of smoking and residence histories, together with the possibility of multiplier effects, were interpreted as suggestive of a multi-stage hypothesis for the aetiology of lung cancer. From the genetic point of view, Court Brown(1962) expressed the opinion that a two stage model has value in the consideration of the human carcinogenesis. He states "One may postulate that the initiating agent, whatever it may be, has to act through the inception of genetic damage. One may further postulate that under the influence of promoting agents, cells carrying the initial damage will proliferate and that during this stage of proliferation, the initial damage itself may lead to an increased liability to the establishment of further genetic errors - some of these may become obvious in the appearance of cell lines with visible chromosomal re-arrangements or with abnormal chromosomal numbers".

The value of these hypotheses is difficult to assess, and it is obvious that more investigations are necessary.

TABLE

TWO STAGE CARCINOGENESIS OTHER THAN SKIN

Investigator, (year reported)	Target organ	Animal	Initiator	Promotor
Muta (1943)	liver	rat	dimethyl aminoazo benzene	tar
Hall (1948)	thyroid	rat	2-acetyl aminofluorene	allyl thyourea
Glinos, Bucher & Aub (1951)	liver	rat	4-dimethyl aminoazo benzene	partial hepatectomy
Law (1956)	liver	rat	2-acetyl aminofluorene	partial hepatectomy
Odashima (1959)	liver	rat	"	methylcholanthrene
Berenblum & Trainin (1960)	blood (leukaemia)	mouse	X-ray	urethane
Maltoni & Prodi (1960)	liver	rat	4-dimethyl aminoazo benzene	carbontetra-chloride
Takayama (1961)	liver	rat	"	4-nitro quinoline-N oxide
Peirce (1961)	stomach	mouse	benzpyrene	lime oil
Murphy (1961)	endocervix	mouse	methyl cholanthrene	estrogen
Kim & Furth (1960)	breast	rat	methyl cholanthrene	mammotrope
Yokoro & Furth (1961)	breast	rat	X-ray	mammotrope

F) IMMUNOLOGICAL THEORY OF CARCINOGENESIS

The immunological theory of cancer evoked much interest recently. Over many years, much has been written about cancer immunology. The first era of immunological study in cancer research was mainly concerned with a search for a tumour specific antigen, with the hope of the production of an anti-cancer antibody. The second era was concerned with tumour transplantation. Changes in the antigenic make-up of transplantable tumour is a common observation, and it is clear that many immunological characteristics of transplanted tumours are the result of the genetic difference between tumour cells and cells of the host. The analysis of tissue specific antigens, or self markers, lead to a further understanding of immunological processes. More recently, the suggestion that an immune reaction might be conceived in the process of carcinogenesis itself has been raised.

Recent studies provide evidence that chemical carcinogenesis might involve the gain and persistence of new antigenic properties. Prehn and Main(1957) found that methylcholanthrene induced sarcomata, but not spontaneous fibrosarcomata, produced immunity in subsequent transplants. This indicated that the antigenicity of induced tumours is in fact tumour specific, since spontaneous tumours do not show such antigenicity. Further studies (Prehn,1959;1960) showed that the removal of a methylcholanthrene induced fibrosarcoma in an inbred strain of mice reduced greatly the incidence of sarcomata resulting from a further injection of methylcholanthrene. It was assumed that methylcholanthrene had altered some cellular constituents so that they had become antigenic

to their own immune system. Révész(1960) also showed an antigenic differences between transplanted methylcholanthrene induced sarcomata of recent origin and their isologous hosts. In his experiments, mice were twice given before tumour transplantation, injections of irradiated cells derived from the tumour to be transplanted. In the pretreated mice, the tumours were accepted in fifteen percent of cases, as compared with fifty-six per cent of takes in the control mice. The immunization of the primary host to its own methylcholanthrene induced sarcomata was reported by Klein, Sjörgrene, Klein and Hellstrom(1960). They demonstrated the immunization in the primary host after excision of sarcoma, followed by serial pretreatment of the host with irradiated tumour cells and a series of subsequent challenges with viable cells derived from the animal's own sarcoma. A direct immunological method to analyse the antigenecity of these chemically induced tumours is, however, not well established. Furthermore, the host recognition of such antigens must be reflected in an immune response if the change in the immunological properties is related to the carcinogenic process. Though Narcissov and Abelew (cited by Zilber,1958) claimed to have found complement fixing antibodies in sera of rats carrying tumours induced by 9,10-dimethyl-1,2-benzanthracene or methylcholanthrene, the evidence for such an immune response in animal carrying primary tumours has been inconclusive.

In 1954, Green(1954) first presented his immunological theory of cancer. His theory postulates that when normal tissue is treated with a chemical carcinogen, the carcinogen combines with a protein which

confers tissue specificity, thus forming a marker-carcinogen complex. This combination behaves as an antigen and elicits an antibody response unfavourable to the cell containing the antigen. By an adaptive process, a new type of cell appears, which is lacking in the tissue specific antigen or self marker capable of combining with carcinogen. As a result, the cells are no longer identified and can proliferate regardless of tissue boundaries. The starting point of his hypothesis was the observation that the tumour inhibitory power was present in some non-carcinogenic hydrocarbons, which was the same nature as induced by carcinogens. He assumed that these tumour inhibitory powers were due, not to a cytotoxic action in the tumour, but to an enhancement of immunity to homologous tumour tissue. According to a more recent report (Green,1961), cancer is due to the loss of tissue specific antigens. Green speculated that the tissue specific antigens are lipo-proteins, located in the endoplasmic reticulum, and that the binding of chemical carcinogens to such lipo-proteins, is the first stage of chemical carcinogenesis. Speculation goes further to explain the process of initiation and promotion in carcinogenesis. An initiated cell is one showing a marginal loss of identity protein, as the result of an immune reaction to cyto-specific auto-antigen. Hyperplasia of such cells may tend to select the most deficient, which will have a competitive advantage, as they are already less susceptive to the normal growth inhibitory control. Green considered this process as promotion. On this basis, initiation would not be a completely separate process from promotion, but is the first stage of deletion of tumour specific antigen, a process which is

continued if repeated applications of carcinogen or promotor are administered. The initiated cells would be a qualitatively feeble counterpart of the malignant cells, and would remain in this stage indefinitely, provided a normal environment prevailed.

Due to a considerable lack of supporting experimental evidence, Green's hypothesis was not widely supported (Hieger,1961). Experimental demonstration that some chemical carcinogens are bound to protein of the cells in a target organ (Miller,1951:Miller & Miller,1953). lead to the "deletion hypothesis" of chemical carcinogenesis. This hypothesis considers the key change in the formation of malignant cells the deletion of some specific protein or enzyme, by the binding of the carcinogen to protein, and is in accord with Green's view.

G) DISTURBANCES OF NUCLEIC ACID METABOLISM AND ITS RELATION TO CARCINOGENESIS AND INITIATION

Nucleic acids play an important role in cell physiology and biochemistry, because of their unique ability to transfer information. The foundation of the present knowledge on the importance of the nucleic acids, as significant cellular constituents, was laid by Miescher(1897) and Kossel(1891) at the end of the last century. These workers showed for the first time that the nucleoprotein is an essential component of all the cells of the living organism. One of the fundamental contributions in this field was the finding that there two types of nucleic acid in the cell, namely deoxyribonucleic acid (DNA), and ribonucleic acid (RNA). In spite of chemical similarity, the biological properties and roles of

DNA and RNA are strikingly different. The RNA which is present in the cytoplasm, nucleoli, and nuclei of the cells is a labile and active substance which undergoes a considerable quantitative change during cell metabolism and plays an important role in protein synthesis. Conversely, DNA which in normal cells is confined to the nuclei and chromosomes only, is remarkably stable and is more or less independent of cell metabolism. The idea of the extreme importance of DNA for cell life and cell continuity, and the intimate association of DNA, chromosome components and the genetic material can be considered as derived mainly from the following three fundamental observations. Firstly, the chromosome complement is a constant characteristic of each cell for each species and carries the genetic material (Morgan,1919). Secondly, the chromosomes have DNA as an essential building stone (Feulgen and Rossenbeck,1924). Thirdly, DNA is an essential chemical constituent of the genetic material (Avery, MacLeod & McCarthy,1944).

On the basis of x-ray diffraction studies of DNA from a variety of sources, Watson and Crick(1953) constructed a DNA model and made the classic proposal that DNA consists of two helical polynucleotide chains of opposite polarity which are twined around one another. The two chains are held together by hydrogen bonds between their bases, each base being joined to a companion base on the other chain. The pairing is specific, adenine binding with thymine and guanine with cytosine. The RNA of animal cells, bacteria and viruses appear to be single stranded macromolecules containing helical regions and intramolecular hydrogen bonding (Kit,1960). According to Watson and Crick(1953), in duplicating

its structures, the twin stranded DNA molecule partially unwinds and each base attracts a complimentary free nucleotide already available for polymerization within the cell. These nucleotides, whose phosphate groups probably already possess the free energy necessary for poly-esterization would then link up with one another after being held in place by the parental template chain to form a new polynucleotide molecule of the required nucleotide sequence. Thus, each DNA strand serves as template for the synthesis of a complimentary strand. With the idea of structure and synthesis of DNA as described, there comes the hypothesis that a change in the DNA sequence of one or a few nucleotides would be mutagenic. Mechanisms for spontaneous mutation and experimentally induced mutation have been suggested on the basis of this concept.

The cancer cells possess the property of more or less uncontrolled growth in the host. Since the uncontrolled growth is a hereditary property of the cancer cell, it is possible to consider the nature of this cell in terms of an alteration in the normal transfer of information at the subcellular levels, namely genes, chromosomes and DNA.

There is some evidence which suggests that the carcinogenic substances act on the DNA of living cells, however, the question of whether the neoplasms result from a disturbance in the replication and function of nucleic acid is not completely answered. Since there are so many agents capable of inducing tumours, the action of all these substances cannot be resolved by any generalization. The nature of the disturbance of nucleic acid metabolism might not be the same, and the

initial action may vary, but the end-result would be a modification of the genetic structure and function. It is, of course, only assumed that a disturbance of the nucleic acid metabolism may in several instances be the basis for their carcinogenic action.

The carcinogenic action of ultra-violet light on skin has been observed in many species and the incidence and latent period has been studied as a function of the intensity and duration of the dose (Blum, 1959). The action spectrum of ultra-violet light for carcinogenesis is below 320 mu., while that for mutation in microorganisms has an optimum at 260 mu., which is the point of maximum absorption by nucleic acid. Because of the action spectrum, as well as the inactivation of transforming DNA by ultra-violet light, the production of mutant forms in bacteria has been considered to be due to an alteration of nucleic acid structure. The action of ultra-violet light in carcinogenesis could perhaps be similar.

Ionizing radiation is also known to be carcinogenic. Its action has been related to its direct damage to, and deletion of specific genetic material. It is known that ionizing radiation will produce morphological abnormalities of cell nuclei. Following radiation, a series of chromosomal abnormalities, breaks, stickiness and crosses can be observed, as well as deletion of specific chromosomal segments. (Biesele,1958 & Puck,1958). In vitro experiments, it was shown that irradiation induces a partial degradation of DNA, characterized by a change in viscosity (Shooter,1957), and a change in its chromatographic profile (Bendich,Pahl & Beiser,1956). vonEuler and Hevesy(1942) first reported that x-radiation inhibited the incorporation of radioactive

phosphate into the DNA of Jensen sarcoma, a finding of which was interpreted as an inhibition of DNA synthesis. Subsequent studies show that radiation inhibits the synthesis of DNA in microorganisms (Lwoff, 1953), and in a variety of animal cells (Kelly,1957;Beltz & Applegate, 1959) by inhibition of a key enzyme, or by damage to the template.

Alkylating agents, such as nitrogen mustard, sulfur mustard, triethylene melamine and diepoxide etc., are widely known as cancer chemotherapeutic agents. It is of interest that some of these substances also act as carcinogens. Boyland and Horning(1949) demonstrated that nitrogen mustard is able to induce lung tumours, lymphosarcomata, and spindle cell sarcomata when given by subcutaneous injection to mice, and Roe and Salaman(1955) demonstrated the initiating action of triethylene melamine on mouse skin. Since chromosomal abberations in dividing cells are an outstanding feature of mustard intoxication, most hypotheses as to their mechanism of action postulated that the target site in the cell was the genetic material, nucleic acid. Auerbach and Robson(1946) observed that nitrogen mustard was the mutagenic agent in drosophila. Nitrogen mustard reacts with DNA in vitro to decrease its viscosity, which is evidence of breaks in the double helical structure of the molecule (Conway,Gilbert & Butler,1950). The effects of the alkylating agents on the biosynthesis of nucleic acids have been investigated by many workers. It is generally agreed that biosynthesis was impaired by alkylating agents (Goldthwait,1952;Davidson & Freeman,1955;Drysdale, Hopkins,Thomson,Smellie & Davidson,1958). Many kinds of polynucleiotide-mustard reaction are cited, such as an attack on the 3', 5' linkage of

DNA with disruption of the polymer, alkylation of two adjacent phosphoryl hydroxyls within the polymer, alkylation of purine or pyrimidine bases with production and incorporation of "base analogs" into the polymer, and alkylation of guanine at 7-position with subsequent opening of the ring, loss of base and formation of an a-purinic acid-like structure. The mechanism of these actions is a complex one, and furthermore, there are suggestions that, targets other than nucleic acid, are attacked by these agents and partially responsible for the biological effects produced (Trams,Nadkarni & Smith,1961a & 1961b).

Urethane is known to possess multipotential carcinogenic action as well as initiating action on the skin of mice. Experiments on urethane induced pulmonary tumours by Rogers(1957a,1960) was cited in a separate chapter of this review. It had been mentioned that a disturbance of nucleic acid metabolism may be critically involved in carcinogenesis by urethane.

Among polycyclic hydrocarbons, benzpyrene and methylcholanthrene are known to induce chromosomal translocation and fragmentation (Biesele, 1958). There is less evidence for the interaction between hydrocarbons and nucleic acids. Using dibenzanthracene on mouse skin, Weiss and Heidelberger(1953) obtained no evidence of interaction of this carcinogen with the nucleic acid of mouse skin. However, recent studies by Heidelberger & Davenport(1961) demonstrated that there is a firm binding of 1,2,5,6-dibenzanthracene to DNA and RNA in mouse skin painted with isotopically labeled carcinogen. This finding is unlike the weak association between hydrocarbon and nucleic acid that Boyland and his

associates (Booth,Boyland & Orr,1954) demonstrated in an in vitro experiment. However, more recent studies by Boyland and Green(1962) on the solubilization of benzpyrene by calf thymus DNA, and the fluorescene quenching of benzpyrene by DNA, showed that there is an appreciable amount of binding of benzpyrene to nucleic acid. They estimated that about fifty benzpyrene molecules are bound per DNA molecule. It was also shown that this characteristic is abolished by the destruction of double helical structure of DNA by heat denaturation. They suggested that the binding results from intercalculation of the hydrocarbon between base-pairs of DNA molecules. The significance of this binding in relation to carcinogenesis of the skin is still not clear.

With the technique of radioautography using the radioactive DNA precursor thymidine, Evensen(1961) showed that the incorporation of thymidine into DNA of an epidermal cell is significantly decreased after the application of methylcholanthrene to mouse skin. He proposed that methylcholanthrene interferes with the synthesis of DNA in the epidermal cells. Further studies (Evensen,1962) demonstrated that a similar decrease of DNA synthesis occurs shortly after the application of 9,10-dimethyl-1,2-benzanthracene and benzpyrene on mouse skin, but not after the non-carcinogenic agents.

The importance of viruses as carcinogens may be related to the fact that viruses contain nucleic acids. Some tumour viruses have been shown to contain DNA and no RNA, others contain RNA and no DNA. Some are seen to reside in the nucleus, others in the cytoplasm and still others at the cell membrane. One can easily see that virus infection could

allow the introduction of information into the cell in the form of virus nucleic acid. The evidence that nucleic acids constitute the probable repositories of genetic information (Butler & Davison,1957), and the discovery that the replication of an entire virus can be initiated following cell infection with viral nucleic acid alone (Fraenkel-Conrat, Singer & Williams,1957) led to the idea that action of viruses in carcinogenesis is very much similar to that of other carcinogens. Tumour viruses will doubtless be found to act through a variety of mechanisms (Kaplan,1962), but one model of virus carcinogenesis has been propounded in which the cell-virus relationship is conceived in terms of lysogenic host and provirus (Luria,1960). That is, the virus is believed to be integrated with the host genome, and the DNA altered by virtue of the incorporation of the viral genetic material. Alternatively, viruses might act as mutagens through interference with, or competition for DNA synthesis pathways, or through "activation" of latent mutator genes in the cell genome. A virus might be a stable independent particle which acts as an added template for the synthesis of new molecules of nucleic acids or protein. As for the RNA viruses in the cytoplasm, it is possible to conceive of integration of a RNA tumour virus into the ribosomal particles of the endoplasmic reticulum, and that it might then direct the synthesis of a new molecules of RNA or protein.

Thus, the oncogenic viruses leads to important suggestions to solve the mystery of the intracellular events leading to the neoplastic transformation.

The production of neoplasms by the injection of nucleic acids

has been recently reported. Latarjet, Rebeyorette and Moustacchi(1958) innoculated nucleic acids (DNA & RNA) isolated from AKR leukaemic cells into newborn mice. Seven out of eighty innoculated mice developed multiple tumours including parotid gland tumours, subcutaneous sarcomata and carcinomata. Bielka and Graffi(1959) were able to induce leukaemia in 10% of their mice with a RNA preparation isolated from myeloid leukaemia. The induction of anaplastic tumours in mice innoculated with RNA extracted from Ehrlich ascites mouse tumour has also been reported (Lacour,Lacour,Havel & Huppert,1960). There is also good evidence that nucleic acid, sensitive to DNA-ase but insensitive to RNA-ase, can be extracted from polyoma virus and can cause tumours in mice (DiMayorca, Eddy,Stewart,Hunter,Friend & Bendich,1959). As for the skin tumours, Ito and Evans(1961) succeeded in inducing skin papillomata in domestic rabbits, after the innoculation of DNA preparation extracted from the papillomatous tissue of wild cotton tail rabbits. The activity of the preparation could be completely abolished by exposure to DNA-ase. Antisera against Shope virus papilloma did not block the oncogenic activity.

These experiments are further evidence that the production of neoplastic change involves nucleic acids, and that this mechanism must be related in some way to the problem of information transfer within the cell. The extraction of a partially purified virus preparation also yielded extracts with omogenic potency. Although speculation that the introduction of nucleic acid molecules into a cell could alter the metabolic pathways so as to transform the normal cell into a neoplastic cell is plausible, the precise mechanism is not fully understood.

H) DOSAGE OF CARCINOGEN AS A MODIFYING FACTOR OF CARCINOGENESIS

Among the many factors that modify the response of animals to carcinogenic stimuli, is dosage. In general, two different aspects should be considered when evaluating an epidermal carcinogenic response. One is the host condition, such as genetic type, sex, age, the phase of the hair cycle at the time of administration of the carcinogen. The other is the extrinsic state, such factors as dietary condition, and environmental conditions. All play an important role in the induction of tumours in experimental animals.

Before entering into a discussion on the effect of dosage, brief mention should be made on the effect of the solvent. In the early days of chemical carcinogenesis with tar, the solvent was considered a simple diluent need to render the tar more manageable for skin painting. Later, it was considered a differential solvent to extract the active component. Still later, it was thought to be an adjuvant which increased the irritating quality of the tar and perhaps its carcinogenic potency.

Various solvents have been used for chemical carcinogens. Among them chloroform, ether, sesame oil, olive oil, lanolin and tricaprylin were in common usage. When pure chemical carcinogens were used, the choice of the solvent became narrowed to one that would dissolve the relatively insoluble chemical in a sufficient concentration. Among the volatile solvents used for skin painting, Orr(1938) concluded that acetone was best, as it was the least damaging to the skin. Stowell and Cramer (1942) observed that epidermal carcinogenesis was more rapid if acetone was used as a solvent for methylcholanthrene, than if benzene was used.

But on the whole, the problem of choosing a satisfactory solvent from the volatile agents for skin carcinogenesisis not a difficult one, and usually any of the commonly used solvents, such as acetone, benzene, ethanol, ether, chloroform, were satisfactory and had little influence on the potency of the carcinogenic hydrocarbons.

For the quantitative study of skin carcinogenesis, the application of a constant effective concentration is obviously desirable. For this purpose, a fatty vehicle such as mineral oil (liquid paraffin) is frequently used. When the carcinogen is dissolved in a volatile solvent and applied to the skin, the solvent rapidly evaporates, giving a very high concentration of the carcinogen. On the other hand, when a carcinogen dissolved in a non-volatile solvent is applied to the skin, the solvent remains for a certain period, giving more constant concentration of carcinogen. Clearly, the quantity of carcinogen needed is much greater when it is applied in a fatty solvent. For instance, in a two stage experiment in the mice using Tween 60 as a promotor, 25 ug of 9,10-dimethyl-1,2-benzanthracene dissolved in acetone was equivalent to 250 ug of the same carcinogen dissolved in mineral oil, as judged by tumour incidence (Della Porta, Shubik,Dammert & Terracini,1960).

When lanolin was employed as a solvent, no skin tumours were induced on mice given repeated applications of methylcholanthrene. (Simpson & Cramer,1943;Simpson,Carruthers & Cramer,1945). Simpson and Cramer postulated that lanolin was anti-carcinogenic. However, Berenblum and Schoental(1947) attributed the apparent anti-carcinogenic effect of lanolin to the retention of the carcinogen in the lanolin, so that the skin

was exposed only to the low concentration in the lanolin, instead of to the very high concentration left on the skin as a volatile solvent evaporated. However, the matter is still in some doubt. Plaut and Sobel (1949) investigated the effect of benzene, lard, human sebum, and lanolin as solvents for methylcholanthrene in mouse skin carcinogenesis. They observed no tumours when lanolin was used although tumours were numerous with the other solvents. Gillman, Hathorn and Penn(1956) applied lanolin to areas of skin previously treated with methylcholanthrene and found that lanolin delayed the onset of tumours and diminished the total incidence of tumours. They postulated that lanolin may act metabolically by replacing skin lipid which is known to be diminished by the application of carcinogens.

Substances which possess both lipid soluble and water soluble properties have been introduced as solvents of carcinogens. Polyethylene oxide introduced by Stamer(1945) and glycol carbowax introduced by Setälä (1949) are examples of these substances which facilitate the transfer of the fat soluble hydrocarbons to the aqueous phase within the living cells. They have no particular advantage, and do not greatly alter the carcinogenic process. The initiating action of urethane, for example, is not altered by substitution of the viscous solvent, carbowax, for acetone (Roe & Salaman,1954).

In order to evaluate, in quantitative terms, the response of animals to the action of carcinogenic agents, dosage must be taken into consideration. In most of the early works on epidermal carcinogenesis, arbitrary doses large enough to ensure that the experimental animals

responded to the more active carcinogens were employed. It soon became obvious that several factors, such as the total amount of carcinogen applied, the duration of the application of carcinogen, and the interval between the applications, were significant. The importance of the duration of the exposure of the carcinogen was demonstrated in the work of Bang (quoted by Woglom,1926), who, in 1922 showed the following relationship between the duration of painting with tar and the response of the animals:

One month's painting with tar - No carcinomata in fourteen mice

Two month's painting with tar - 3 carcinomata in sixteen mice

Three month's painting with tar - 9 carcinomata in thirteen mice

Four month's painting with tar - 12 carcinomata in twelve mice

The importance of the interval between the applications of the carcinogen was discussed by Deelman(1924), who gave the following summary:

> "Twenty-two applications were required when tar was applied at two days interval; nineteen applications with four days interval; seventeen applications with six days interval; eighteen applications with seven days interval."

He stated that the development of carcinomata depended on the summation of the quantities of the carcinogen applied, but also that when this cumulative effect has reached a certain point, malignant tumour formation proceeds inexorably, whether the irritation by the carcinogen is continued or not.

Cramer and Stowell(1941;1943b) also studied the effect of varying the interval between applications of the carcinogen used to paint the mouse skin. Groups were painted with methylcholanthrene once every two weeks, every three weeks, or every four weeks. The total dose necessary to induce carcinomata in 100% of the animals diminished as the exposure of the skin to the carcinogen became increasingly infrequent. It was also noted that if the paintings were performed thrice weekly, the amount of the carcinogen necessary to produce the first tumour was as large, or larger than was required to produce tumours in all of the mice painted every two, three, or four weeks. Varying the concentration of the carcinogen used within the limits of 0.3 to 1.0% had relatively little effect on the neoplastic response. (Hieger,1936;Beck & Peacock, 1940). Fieser(1938) stated that 0.3% of polycyclic hydrocarbons in benzene is the lowest standard concentration for the carcinogenic agents. Many other reports with similar findings could be noted.

To express the response of animals to carcinogenic agents, several formulae are in general use. Tumour incidence and the latent period are among the most popular. The tumour incidence may be expressed in various ways, as the average number of tumours per survivor, the average number of tumours per tumour bearing animal, or percentage of survivors bearing tumours. Latent periods can be expressed in two ways. One is the latent period of all tumours, and the other, that of tumour bearing animals. On the basis of such measures, a quantitative relation has been established between the dose of the carcinogen and the neoplastic response. However, different methods of measurement led to different conclusions. For instance, when methylcholanthrene, benzpyrene and

1,2,5,6-dibenzanthracene are compared on the basis of the minimum quantity used to induce tumours when injected subcutaneously, their order of potency is dibenzanthracene, methylcholanthrene and benzpyrene; whereas, if they are compared on the basis of the minimal latent period, this order is changed to methylcholanthrene, benzpyrene and dibenzanthracene (Bryan & Shimkin,1943).

The development of the idea of two stages of carcinogenesis brought a clearer quantitative relationship of dose of carcinogen to response in epidermal carcinogenesis. In the two stage experiment, the number of tumours produced after a single sub-optimal dose of the carcinogen, followed by repeated croton oil treatment, was thought to be determined by the potency of the initiating dose of carcinogen. When a single application of 0.3% benzpyrene, 0.3% dibenzanthracene or 1.5% 9,10-dimethyl-1,2-benzanthracene were followed by croton oil, the tumour incidence varied according to the potency of the carcinogen used, but the latent period remained the same (Berenblum & Shubik,1947b). Furthermore, if different concentrations of the same carcinogen were used, as an initiator in a standard two stage experiment, the final tumour yield differed according to the logarithm of concentration of the carcinogen. After a single application of 0.06%, 0.17%, 0.5% or 0.5%, 9,10-dimethyl-1,2-benzanthracene, the tumour incidence expressed in terms of tumours per tumour bearing mouse, was 1.1, 2.4, 2.7, and 3.9. The increase in the concentration of the initiator brought an increase also in tumour incidence expressed as percentage of survivors bearing tumours, or as the average number of tumours per tumour bearing animal (Berenblum &

Shubik,1949a). McCarter(1956) and McCarter, Szerb and Thompson(1956) used a modified technique for the application of the carcinogen, to avoid the possibility that the mice might lick it off. They found that the tumour yield in the skin of mice with 9,10-dimethyl-1,2-benzanthracene in liquid paraffin as an initiator followed by repeated applications of croton oil as promotor, varied directly with the area of skin covered by the carcinogen, directly with the time allowed for its absorption, but with the logarithm of the concentration of the carcinogen applied. Later, McCarter(1959) suggested that the tumour incidence is related to the amount of benzpyrene that penetrated the area of skin in a given time following the application of a solution in acetone, and in subsequent studies Ball and McCarter(1960) stressed that the yield of tumours was determined by the amount of the carcinogen absorbed by the skin, rather than the amount applied. By the chemical analysis of the amount of 9,10-dimethyl-1,2-benzanthracene absorbed into the skin after the initial painting, they showed that the absorption of the carcinogen increased as the amount applied increased from 7.5 to 75 ug then decreased to become constant when 300 to 1500 ug were applied. This is illustrated in the following table:

Concentration (in acetone)	DMBA Applied (ug)	DMBA in Skin (ug)
0.005%	7.5	0.50
0.01 %	15	0.81
0.05 %	75	3.48
0.10 %	150	2.44
0.20 %	300	1.87
0.30 %	400	1.38
0.70 %	1050	1.55
1.0 %	1500	1.88

The amount of carcinogen absorbed into the skin also varied with time. For instance, when 75 ug of 9,10-dimethyl-1,2-benzanthracene in acetone was applied to the skin, chemical analysis shows an increasing absorption up to five hours. In the following table are the results using 75 ug of 0.5% 9,10-dimethyl-1,2-benzanthracene in acetone.

Time after application of Solution (in hours)	Amount of DMBA in skin
0.25	0.15 ug
0.50	0.41 ug
1	0.89 ug
2	1.91 ug
3	3.48 ug
5	4.75 ug

On this basis, the final tumour yield after various applications of carcinogen followed by standard croton oil treatment was determined. Tumours per survivor increased as a logarithmic function of the amount of carcinogen absorbed. It is of interest to notice that the amount of 9,10-dimethyl-1,2-benzanthracene absorbed by the skin became constant when the applications are more than 300 ug and the final tumour yield also becomes constant after the applications of more than 300 ug (Ball & McCarter,1960).

A similar dose response is noted when a single application of the carcinogen is applied to the skin of mice and no other treatment is given. A single dose of 9,10-dimethyl-1,2-benzanthracene in concentrations of 0.04%, 0.1%, 0.2%, 0.4%, 0.5%, or 1.0% in acetone, representing 20, 50, 100, 250, 500 or 1000 ug was applied to the skin of mice. With doses of 20 and 50 ug no tumours were produced. A small number of tumours were produced with 100 ug. With 200 ug, benign and malignant tumours were

produced in large numbers. With doses of more than 200 ug, there was no increased effect (Terracini,Shubik & Della Porta,1960).

When 9,10-dimethyl-1,2-benzanthracene is dissolved in a non-volatile solvent, the highest concentration possible is approximately 1.5%. In order further to increase the dose of the initiating carcinogen, Shubik and Ritchie(1953) modified the method of application of the initiator by applying the same concentration of the carcinogen one, two, or three times with an interval of one week between applications. In this way, the mice were initiated with different amounts of the carcinogen of the same concentration and the standard croton oil treatment followed. Two experiments were performed, one with 1.0% and the other with 0.2% 9,10-dimethyl-1,2-benzanthracene in liquid paraffin as the initiator. In both, the number of tumours produced after croton oil decreased, as the number of applications of the initiator increased. This unexpected result was interpreted as possibly due to the necrotizing effect of the carcinogen upon the skin (Haddow & Robinson,1939;Pullinger, 1940), or to a refractory state produced by the first application of the carcinogen which made the skin no longer susceptible to the further applications of the carcinogens.

The influence of severe ulceration of the skin was studied by Dammert(1961a). Mice were given five ulcerative doses of alkyl-dimethylbenzylammoniumchloride (Zephiran) after initiation by 20 ug of 9,10-dimethyl-1,2-benzanthracene in acetone, Tween 60 followed daily. The ulcerating doses of zephiran enhanced the tumour incidence by shortening the latent period, but lowered the tumour incidence as measured

by tumour bearing animals and the total number of tumours.

Similar experiments to those of Shubik and Ritchie were carried out by Vesselinovitch and Gilman(1957). The tumour incidence in mice which received one application of 1.5% 9,10-dimethyl-1,2-benzanthracene in mineral oil followed by repeated applications of croton oil was compared to that in mice which received two such applications of the carcinogen at an interval of thirty days and then croton oil. The tumour incidence of the former group was 5.7 tumours per tumour bearing mouse, but in the latter, it was 10.0. Vesselinovitch(1958) extended the experiment, giving the carcinogen once, twice or thrice at intervals of one month. Croton oil followed. The group which received a single application of the carcinogen had an incidence of 3.0 tumours per survivor; the group which received two applications had 5.08, and the group which received three applications had 6.32. Vesselinovitch though that this result demonstrated a logarithmic relationship between the amount of the initiator applied and the tumour incidence. It should be noted that the interval between applications in these experiments is different to that used by Shubik and Ritchie. The effect of altering the interval between carcinogen applications in this way is complex. Salaman and Roe(1956b) investigated the effect of varying the time interval between applications of carcinogen in mice painted with a small dose of 9,10-dimethyl-1,2-benzanthracene followed by a course of croton oil treatment. Two applications of the carcinogen were given at intervals of from one hour to ten weeks. Unfortunately, no comment was made on the number of benign papillomata produced in the experiment, but the results suggested

that the incidence of malignant tumours was greater if the two doses were given with an interval of four days or less. The malignant tumours in groups given the two doses at intervals of three weeks or ten weeks, tended to arise as early or earlier than in those given the doses at an interval of one day or four days.

In the experiments of Vesselinovitch and Gilman(1957), and Vesselinovitch(1958), another interesting point that influences tumour yield was discussed. That is the modification of the time of the commencement of croton oil treatment. After one, two, or three applications of carcinogen at monthly intervals, the croton oil treatment was usually commenced seven to ten days after the last application of the carcinogen. But when croton oil treatment was started seven days after the first application of carcinogen in groups which received two or three applications of the carcinogen, the tumour yield was significantly higher than in groups which received croton oil only after the last application of the carcinogen. As mentioned earlier, Pound(1962) performed a similar experiment using urethane as initiator, and reported a similar result.

I) SUMMARY OF THE REVIEW OF LITERATURE

The historical development of our knowledge of epidermal carcinogenesis was summarized at the beginning of the review. The establishment of the two stage theory of epidermal carcinogenesis was discussed, and various studies and theories on the nature of initiation and promotion were described. It was noted that the precise mechanism of initiation and promotion is not yet understood. The multipotential carcinogenic action of urethane was also noted.

The possible significance of a disturbance of nucleic acid metabolism in relation to carcinogenesis was reviewed. It was shown that there is an increasing amount of evidence suggesting that the primary effect of a carcinogen is on nucleic acid.

Finally, the dose response of animals to carcinogen was considered. It was noted that increasing the number of applications of an initiator does not always increase the tumour yield. The theory that initiation may induce a refractory state or necrotizing reaction in the skin was discussed.

II EXPERIMENTAL WORK

A) INTRODUCTION

The progress of our knowledge of experimental epidermal carcinogenesis has been discussed. A vast amount of literature and much information has been accumulated from numerous fields of scientific work. Despite the ever increasing number of reports from the various fields, the fundamental mechanism of carcinogenesis is not yet completely understood. There are many theories, as to how normal tissue transforms itself from the normal into the neoplastic state. In each discipline of science, be it chemistry, biochemistry, virology, biology, or pathology, there are still a considerable number of controversial matters and a great deal that is yet unknown. The experimental work in this thesis deals with some of the points currently under discussion.

One of the matters in doubt, the dose response of mice to the carcinogenic stimuli used as initiator in the two stage mechanism, was chosen as one of the subjects of the present investigation. A complicating factor is that the dose response is influenced by several conditions: The amount of carcinogen applied, the interval between the applications, the number of doses given, and the use of different carcinogens. The investigation further extends to the estimation of the amount of the carcinogen in the blood, and the analysis of a possible promoting action exerted by the carcinogen used as initiator.

The refined methodology of biochemistry has led to the remarkable advance in the study of the structure and function of nucleic acid. The suggestion has been made that the malignant transformation of normal tissue

might be related to an alteration in the nucleic acid. The question of whether the change induced during the initiating stage of epidermal carcinogenesis is related to an alteration in nucleic acid metabolism was undertaken as another subject of the present experimental work. The information derived from the experiments previously reported by several investigators was particularly helpful in the design of the present experiments.

The general methods used will be discussed first, and then the experiments themselves.

B) MATERIAL AND METHODS

1) Location of Animal Room

Experiments were performed in two locations. One in the Pathological Institute, McGill University, Montreal, and the other in the Banting Institute, University of Toronto, Toronto.

2) Experimental Animals

Swiss mice, bred in the Pathological Institute, McGill University, or purchased from Rockland Farms, (New City, N.Y.), were used. At McGill University, the mice were housed in a well ventilated room, kept at a temperature of between twenty and twenty-five degrees centigrade. The lighting was controlled automatically, so that the room was lit between 6 a.m. and 6 p.m. and dark for the remaining twelve hours. Groups of ten mice were kept in plastic cages. The bottom of each cage was covered by a layer of wood shavings, which were changed at least twice

per week. The mice were given free access to water and Rockland Mouse Diet. (A.E. Stanley Manufacturing Co., Chicago, Illinois.)

At the University of Toronto, mice were housed in similar experimental rooms, but without lighting control. Groups of six mice were kept in metal cages. The bottom of each cage was covered by a layer of wood shavings, which was changed once a week. The mice were given free access to water and "Purina Chow" mice cakes (West End Feed Company, 3348 Dundas Street West, Toronto, Ontario.).

When mice were purchased from the outside, they were isolated for at least three or four weeks before the commencement of an experiment. Only virgin female mice were used for the experiments designed to evaluate tumour incidence. The reason for avoiding the use of the male in this sort of experiment is that males readily fight one another, resulting in an undesireable scarification of their backs. This scarification might modify the tumour yield. The mice were usually six to seven weeks old at the start of the experiment. The animals were weighed before each experiment and only animals which weighed over eighteen grams were used. The younger mice and the underweight mice generally tolerated poorly the initial paintings with carcinogen or the injection of an initiator. The mice were randomly distributed in each cage, according to weight. The skin on the backs of the mice was clipped, by means of an electric clipper (Oster, Model 22, No. 50), the day before the first painting, and then once a week, until the end of the experiment. Male mice from seven to eight weeks old were used for the chemical determination of the quantity of the carcinogen in the blood.

3) Initiator

Two initiators were used. The hydrocarbon 9,10-dimethyl-1,2-benzanthracene (7,12-dimethylbenz (α) anthracene, Eastman Organic Co., Rochester, N.Y.) was dissolved in purified mineral oil (Nujol, Plough, (Canada) Ltd., 36 Caledonia Road, Toronto) by shaking overnight on a mechanical shaker, after the coarser lumps had been broken up by means of a number four camel's hair brush. The concentration of 9,10-dimethyl-1,2-benzanthracene was calculated on a weight per volume basis. Two different concentrations were used, 1.5% and 0.1%. The carcinogen was applied to the backs of the mice by two parallel strokes of a number four camel's hair brush dipped in the solution and briefly wiped on the inner free surface of the container. Special care was taken to paint the skin of the mice uniformly, and to give each mouse approximately the same amount of the carcinogen. The two brush strokes delivered approximately 1.0 to 1.5 mg. of 9,10-dimethyl-1,2-benzanthracene when a 1.5% solution was used. In order to give each mouse a more accurate quantity of carcinogen, the dropping method, employing a syringe was used in some experiments. By this method, the carcinogen was applied to the mid-line of the back of the mouse, by dropping, using a number twenty gauge needle attached to a tuberculin syringe. Each mouse received five or six drops of the carcinogen, amounting to exactly 0.1 mg. The amount of the carcinogen given to each mouse was 1.5 mg. if the concentration was 1.5%, and was 0.1 mg. if the concentration was 0.1%.

Urethane (ethyl-carbamate, Eastman Organic Co., Rochester, N.Y.) was also used as an initiator. It was dissolved in distilled water as a ten percent solution (weight per volume). It was given intraperitoneally

using a tuberculin syringe with a number twenty-five gauge needle. Each mouse received 0.3 ml. of urethane solution.

4) Promotor

Croton oil (Oleum croton, B.P.C., Boots Pure Drug Co. Ltd., Nottingham, England) was used as the promotor as a 5% solution in Nujol (volume / volume). A single brushful was painted over the desired area in three or four strokes, using a number 4 squirrel's hair brush. The croton oil was applied twice per week, commencing one week after the last application of initiator.

5) Other Chemicals

Orotic acid (Nutritional Biochemical Corp., Cleveland, Ohio) was given to mice by adding it to their drinking water. When orotic acid was given during the period of initiation, it was added to the drinking water for two days prior to the day of initiation, given on the day of initiation, and for three days afterwards, that is for a total of six consecutive days. When given during the period of promotion, it was added to the drinking water, seven days after the initiation, and maintained for the remainder of the experimental course. The orotic acid was dissolved in water, and sodium hydroxide was added to give a final pH of 6.8. The concentration of orotic acid given during the promotion period was 0.05%.

Thymine (Nutritional Biochemical Corp., Cleveland, Ohio) was

given by intraperitoneal injection, one day prior to the initiation, on the day of initiation, and one day after the initiation. It was given as a four percent suspension in distilled water. Each mouse received 0.4 ml. of the suspension.

6) Charting the Tumours

The mice were inspected once per week throughout each experiment. Any changes, such as areas of epilation or areas of ulceration, were carefully noted on special charts. When tumours appeared, they were charted as to their position and size. As there is considerable difficulty in identifying new tumours as papillomata until they have persisted for more than two weeks, only tumours that were present for two or more weeks were considered as tumours. The mice were identified by an ear punching code, or by toe clipping. All dead mice were autopsied, and if necessary, sections were examined histologically for the detection of the cause of death or the nature of the tumours.

7) Histology

In preparing the skin for histology, the hair on the back of the mice was clipped one day before sacrifice. After killing by cervical dislocation, a flap of skin from the back was placed on cork board and pinned at the corners. The tissue was then placed in ten per cent neutral formalin, or buffered neutral formalin, for twenty-four

to forty-eight hours. Three blocks were taken from each mouse. Sections were cut 7 mu, and stained with haematoxylin and eosin.

Other tissues from autopsies were also fixed in ten per cent formalin, cut, and stained.

EXPERIMENT I

Epidermal carcinogenesis induced in mice by one or three applications of 1.5% 9,10-dimethyl-1,2-benzanthracene followed by repeated applications of croton oil

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Introduction

The number of tumours produced in mouse skin by a single application of carcinogen followed by repeated applications of croton oil is determined by the potency of the initial application of carcinogen, and the tumour yield is roughly in logarithmic proportion to the concentration of the carcinogen (Berenblum & Shubik,1947b;1949a). However, when the concentration of the carcinogen was kept constant, but the number of applications of the carcinogen was increased from one to two or three given at intervals of one week, the number of tumours produced by subsequent applications of croton oil did not increase. Indeed, the number of tumours induced decreased as the number of applications of carcinogen increased (Shubik & Ritchie,1953). Similar experiments in which one, two or three applications of carcinogen were given at intervals of one month instead of intervals of one week did, however, show a

significant increase in the tumour yield (Vesselinovitch & Gilman, 1957;Vesselinovitch,1958). Shubik and Ritchie suggested two explanations of their failure to increase the yield of tumours by increasing the number of applications of carcinogen when the applications were given at weekly intervals. They suggested that the repeated applications of 9,10-dimethyl-1,2-benzanthracene at intervals of one week might produce a necrotizing reaction in the mouse skin, so that the latent tumour cells induced by the carcinogen were destroyed. Alternatively, they suggested that mice might develop a refractory state after the first application of the carcinogen, so that the second and third applications of the carcinogen were not able to induce more latent tumour cells. Vesselinovitch and Gilman suggested that the interval of one month used in their experiments might be sufficient to outlast the refractory state produced by the first application of carcinogen, so that the second and third applications of carcinogen were able to give an additive effect.

The present experiment was planned to clarify these points, and, in particular, to determine if the interval between applications of carcinogen is important when tumours are induced in mouse skin by three applications of 9,10-dimethyl-1,2-benzanthracene followed by repeated applications of croton oil.

Method

A total of 390 Swiss mice bred in the Pathological Institute of McGill University were used. They were divided into nine groups as shown in Table 1. Groups I and VI received one application of the

carcinogen. Groups II and VII received three applications of the carcinogen at daily intervals. Groups III and VIII received three applications of the carcinogen at intervals of one week. Groups IV and IX received three applications of the carcinogen at intervals of four weeks. Groups I to IV were given croton oil twice weekly starting a week after the last application of the carcinogen. Group V received croton oil treatment only, the croton oil starting on the same date as did croton oil in Group I. Groups VI to IX received no croton oil.

The experiments were staggered, starting at seven different times. On each occasion, either 90 or 50 mice were initiated, so that at least 10 of each experimental group was started.

The carcinogen used was 9,10-dimethyl-1,2-benzanthracene at a concentration of 1.5% in pure mineral oil. The experimental Groups (Groups I to V) consisted of sixty mice, except for group IV, which consisted of thirty mice. The control groups (Groups VI to IX) consisted of thirty mice each.

Results

The results of Experiment I are summarized in the Tables I to VII and figures I to VI in Appendix I.

Various methods of mensuration have been used to express the yield of tumours. The most common measures used in this sort of experiment are the average number of papillomata per survivor, the average number of papillomata per papilloma bearing mouse, and the percentage of mice bearing papillomata. All three were used in this experiment. Two latent

Table 1

Scheme of Treatment of Mice

Group		Initiating Agents	Interval between application of DMBA	Promoting Agent
I	GAA	1 application of DMBA	--	croton oil
II	GAB	3 applications of DMBA	1 day	croton oil
III	GAC	3 applications of DMBA	1 week	croton oil
IV	GAD	3 applications of DMBA	1 month	croton oil
V	GAE	nil	--	croton oil
VI	GAF	1 application of DMBA	--	nil
VII	GAG	3 applications of DMBA	1 day	nil
VIII	GAH	3 applications of DMBA	1 week	nil
IX	GAI	3 applications of DMBA	1 month	nil

DMBA 1.5%

CO 5%

periods are given for each group. The "Average Latent Period of Papillomata" has been obtained by considering all papillomata, and the "Average Latent Period of Papilloma Bearing Mice" has been obtained by considering only the first papillomata to appear on each mouse. In each case, the latent period is the average time between the first application of croton oil and the appearance of the papillomata. The various figures are given because different authors have used different measures of the latent period.

The mortality rate in this experiment was rather high, particularly in the groups painted with the carcinogen three times daily or three times weekly (Appendix I, Fig. IV). Judging from the mortality curve, the best time for the evaluation of the tumour yield is at the thirteenth or fifteenth week of croton oil treatment, when the experiment had proceeded long enough to give a reliable result, but the death rate was not yet too high.

In general, the reaction of the skin to croton oil after three applications of the carcinogen given at intervals of one day or one week was more severe than after a single application. This was manifested by prolonged epilation and a high degree of focal ulceration or necrosis of the skin (Appendix I, Fig.V). The skin reaction to croton oil after three applications of the carcinogen at monthly intervals followed by croton oil applications was not so severe, being of the same order as that seen after a single application of carcinogen.

The single application of the carcinogen followed by twice weekly croton oil treatment (Group I) resulted in the usual pattern of tumour formation. Papillomata began to appear after five weeks of croton

oil treatment, and there was a steady increase in the yield of tumours until the twelfth or fourteenth week. This is true, whether the yield is expressed as the number of papillomata per survivor, the number of papillomata per papilloma bearing mouse, or the percentage of mice bearing papillomata. However, the flattening of the curve fourteen to sixteen weeks after the start of the croton oil treatment was not as striking as one might have expected. Papillomata kept appearing at a slow rate until the end of the experiment. The tumours produced in this group were all benign.

The groups which received three applications of the carcinogen at intervals of one day, one week, or one month and then repeated applications of croton oil showed somewhat similar curves for the appearance of tumours. However, papillomata began to appear four weeks after the start of the croton oil treatment in the group with one day intervals, two weeks after the croton oil treatment in the group with one week intervals, and only one week after the croton oil treatment in the group with a one month interval. The initial increase in the tumour yield in these three groups was steeper than in groups given a single application of the carcinogen. It reached its peak after about thirteen to fifteen weeks of croton oil treatment. After this period the curves of tumour yield in groups given three applications of carcinogen dissociate without any constant pattern. This dissociation of curves is probably due to the small number of survivors. The tumours produced in the groups given three applications of the carcinogen were mostly benign papillomata. A few carcinomata appeared in groups which received three applications

at intervals of one week or one month.

The following table (Table 2) gives a summary of the tumour yield in each group at the thirteenth and fourteenth weeks of croton oil treatment, expressed by the three different measures.

Table 2

Summary of Tumour Yields

Groups	Papillomata per survivor			Papillomata per Papilloma Bearing Mouse			Percentage of mice bearing Papillomata		
	week of CO Treatment			week of CO Treatment			week of CO Treatment		
	12	16	20	12	16	20	12	16	20
DMBA 1x & CO	2.5	3.6	4.9	4.9	5.3	6.0	50	67	71
DMBA 3x daily &CO	7.0	6.8	7.3	8.9	9.2	10.0	79	78	75
DMBA 3x weekly &CO	7.4	9.5	7.0	10.2	12.1	9.8	76	77	71
DMBA 3x monthly &CO	8.1	8.0	9.0	11.4	8.9	11.5	67	90	80
CO only	0	0	0.3	0	0	1	0	0	3
DMBA 1x	0	0	0	0	0	0	0	0	0
DMBA 3x daily	0.1	0.1	0.1	1.0	1.0	1.0	1	1	1
DMBA 3x weekly	0.3	0.4	0.7	3.0	2.0	2.0	24	19	37
DMBA 3x monthly	0	0.1	0.2	0	1	1	0	10	16

It can be seen that the yield of papillomata was much the same in the three groups given three applications of the carcinogen and then croton oil, and that it was greater in these groups than in the group given a single application of the carcinogen and then croton oil. The ratio of the yield of tumours in the groups given three applications of the carcinogen to that in the group given only one application is 3:1, if the papillomata per survivor are considered, 2:1 if the papillomata per papilloma bearing mouse are considered, and 1.5:1 if the percentage of mice bearing papillomata are considered.

The average latent periods of the papilloma bearing mouse and of all papillomata are shown in the following table (Table 3).

Table 3

Average Latent Periods

Group	Average latent period of papillomata bearing mice in weeks	Average latent period of papillomata in weeks
DMBA 1x & CO	11.1	13.9
DMBA 3x daily & CO	6.1	9.8
DMBA 3x weekly & CO	4.8	8.3
DMBA 3x monthly & CO	3.6	8.1
		week

The latent periods are shorter in the groups given three applications of the carcinogen than in the group given only one, and grow progressively shorter as the interval between applications lengthens. This is because papillomata began to appear in the first week of the croton oil treatment in the group given three applications of the carcinogen at monthly intervals, in the second week in the group given three applications at weekly intervals, in the fourth week in the group given three applications at daily intervals, but only in the fifth week in the group given a single application.

The groups which received no carcinogen but only repeated croton oil applications produced only one papilloma on one mouse.

The groups which received one application of three applications of carcinogen at intervals of one day, one week or one month, but received no croton oil application, produced few tumours except in the group given three applications of the carcinogen at intervals of one week. In this group, papillomata began to appear two weeks after the last application of the carcinogen, and reached a maximum in the fifth and sixth week after its last application. Twenty-seven papillomata were observed on nine mice. Many of these papillomata turned into carcinomata (Appendix I, Fig. VI).

Summary

Groups of mice were initiated by a single application of 1.5% 9,10-dimethyl-1,2-benzanthracene in pure mineral oil, or three applications of the same carcinogen at intervals of one day, one week, or one month.

Twice weekly treatment with 5% croton oil followed. Three applications of the carcinogen followed by croton oil, produced more tumours than did a single application of the carcinogen followed by croton oil. Tumours began to appear earlier when three applications of the initiator were given, than when a single application was given. The control group given three applications of the carcinogen at intervals of one week, but no croton oil, showed a moderate tumour incidence with a high rate of malignant transformation. Control groups given three applications of the carcinogen at intervals of one day or one month but no croton oil, or a single application of the carcinogen but no croton oil, showed no significant tumour yield.

EXPERIMENT II

Re-examination of latent periods of papillomata induced in mice by three monthly applications of 1.5% 9,10-dimethyl-1,2-benzanthracene followed by repeated applications of croton oil.

Introduction

In Experiment I it was shown that if tumours are induced in mouse skin by three applications of 1.5% 9,10-dimethyl-1,2-benzanthracene followed by repeated applications of croton oil, tumours begin to appear after the first week of croton oil treatment. The latent periods are significantly shorter than those of the group given only a single application of the carcinogen, then repeated applications of croton oil.

It was assumed that the shortening of the latent periods in the group given three applications of 9,10-dimethyl-1,2-benzanthracene, and then croton oil is due to the promoting action of the repeated applications of 9,10-dimethyl-1,2-benzanthracene. It is known that the topical application of benzpyrene or methylcholanthrene possesses a promoting action on rabbit skin (Freidewald and Rous, 1944a), and that a low concentration of 9,10-dimethyl-1,2-benzanthracene can act as a promotor when it is preceeded by a single dose of a high concentration of the carcinogen (Saffiotti & Shubik,1956b;Klein,1956:1960). On the contrary, urethane does not possess such promoting action (Salaman & Roe, 1953).

The present experiment was planned to test this hypothesis and to evaluate the shortening of the latent periods observed when the three applications of carcinogen were given at monthly intervals.

Material and Method

One hundred forty virgin Swiss mice bred in the Pathological Institute of McGill University were used. They were six to eight weeks old at the beginning of the experiment. The experiment consisted of two groups, each of seventy mice, (Table 4). Group I was given three applications of 1.5% 9,10-dimethyl-1,2-benzanthracene at intervals of one month, followed by twice weekly applications of five percent croton oil. Group II was given three applications of 1.5% 9,10-dimethyl-1,2-benzanthracene, at intervals of one month, but no croton oil treatment.

Table 4

Scheme of Treatment of Mice

Groups	Initiating Agent	Interval Between Applications	Promotion
I.GCA	3 Applications of DMBA	One Month	Croton Oil
II.GCB	3 Applications of DMBA	One Month	Nil.

Results

The results are summarized in Tables I and II and Figures I - IV, in Appendix II.

The same measures as were used in Experiment I were used to express the tumour yield.

To make the comparison easier, the tables and figures include the tumour incidence of the group given a single application of the carcinogen followed by repeated applications of croton oil in Experiment I. The findings in this group are typical of many such experiments performed at McGill University. The following table (Table 5) gives a summary of the tumour yield at the first, sixth, twelfth and the eighteenth weeks of the croton oil treatment.

Table 5

Summary of the Tumour Yields

Group	Papillomata per Survivor				Papillomata per Papilloma Bearing Mouse				Percentage of mice bearing Papillomata			
	week of CO Treatment				week of CO Treatment				week of CO Treatment			
	1	6	12	18	1	6	12	18	1	6	12	18
DMBA 1x & CO	0	0.1	2.5	4.0	0	1.5	5.0	5.5	0	7	50	72
DMBA 3x monthly & CO	0.02	2.0	8.5	11.9	1.0	3.2	9.7	11.9	1.8	62.5	87.5	100
DMBA 3x monthly	0	0	0.16	0.09	0	0	2.5	1.0	0	0	1	1

In the group given three applications of the carcinogen followed by repeated applications of croton oil, tumours began to appear in the first week of croton oil treatment and then steadily increased in number. The tumour yields were very similar to the corresponding group in Experiment I. One carcinoma was observed in this group.

The group given three applications of the carcinogen, but no croton oil, produced only a few tumours in a few mice.

The latent periods are shown in the following table (Table 6). They are of the same order as those seen in the comparable group in Experiment I.

Experiment II

Table 6

Average Latent Periods

Group	Average Latent Periods of Papilloma bearing Mice	Average Latent Periods of papillomata
(I) (DMBA 1x & CO	11.1	13.9
(I) (DMBA 3x monthly & CO)	4.5	8.9
		(Weeks)

Summary

Groups of mice were initiated with three applications of 1.5% 9,10-dimethyl-1,2-benzanthracene at intervals of one month. In one group, twice weekly applications of 5% croton oil followed, the other did not receive croton oil.

In the group with croton oil treatment, tumours began to appear in the first week of the croton oil treatment and the latent periods were considerably less than when a single application of carcinogen is given as initiator.

EXPERIMENT III

Epidermal carcinogenesis induced in mice by one or three applications of 0.1% 9,10-dimethyl-1,2-benzanthracene followed by repeated applications of croton oil.

Introduction

In Experiment I, it was shown that three applications of the carcinogen at intervals of one day, one week, and one month, followed by croton oil, produced more tumours than did a single application of carcinogen followed by croton oil. The concentration of the carcinogen, used was 1.5% in pure mineral oil. The results appeared to contradict the findings of Shubik and Ritchie (1953), who demonstrated that the number of tumours induced decreased as the number of applications of the carcinogen increased, but supports the findings of Vesselinovitch

and Gilman(1957) and Vesselinovitch(1958) who showed a significant increase in the tumour yield when two or three applications of carcinogen were given at intervals of one month. During the analysis of the result of Experiment I, it was found that there was a fairly high mortality rate in the groups in which three applications of carcinogen were given at intervals of one day, or one week. The reaction of the skin in these groups was more severe than after a single application, being manifested by a prolonged epilation and a high degree of focal ulceration or necrosis. One might consider that the high mortality rate produced the discrepancy in the results. It could be that when 1.5% 9,10-dimethyl-1,2-benzanthracene in pure mineral oil was applied three times at an interval of one day or one week, many mice which developed a refractory state died in the early stages of the experiment, and only mice which did not survived.

The present experiment was planned to clarify this point. In order to avoid the prolonged epilation, ulceration and necrosis of skin after three applications of carcinogen, the concentration of carcinogen was reduced to 0.1%.

Method

A total of 370 virgin female Swiss mice, purchased from the Rockland Farm (New City, New York) were used. They were about four to five weeks old at the time of purchase. The mice were divided into nine groups as shown in Table 7. Groups I & IV received one application of 0.1% 9,10-dimethyl-1,2-benzanthracene by dropping method. Groups II & VII received three applications of carcinogen at one day intervals. Groups III & VIII received three applications of carcinogen at intervals

Table 7

Scheme of Treatment of Mice

Group		Initiation	Interval between application of DMBA	Promotion
I	GJA	1 application of DMBA	--	croton oil
II	GJB	3 applications of DMBA	1 day	croton oil
III	GJC	3 applications of DMBA	1 week	croton oil
IV	GJD	3 applications of DMBA	1 month	croton oil
V	GJE	1 application of DMBA	--	nil
VI	GJF	nil	--	croton oil
VII	GJG	3 applications of DMBA	1 day	nil
VIII	GJH	3 applications of DMBA	1 week	nil
IX	GJI	3 applications of DMBA	1 month	nil

DMBA: 0.1% in Nujol
CO: 5 % in Nujol

of one week. Groups IV & IX received three applications of carcinogen at intervals of one month. Groups I to IV were given 5% croton oil twice weekly, starting one week after the last application of the carcinogen. Group V and Groups VII to IX received no croton oil. Groups I to IV consisted of sixty mice each and Groups V to IX of thirty mice each.

Results

The results are summarized in tables I to IX, and Figures I to IV in Appendix III. The tumour yield was expressed in the same mensuration as used in Experiment I. The average number of papillomata per survivor, average number of papillomata per papilloma bearing mouse, and the percentage of mice bearing papillomata were used.

The mortality rate (Appendix III, Fig. IV) was relatively low, and the number of survivors in the experimental groups at the 20th to 22nd week of croton oil was satisfactory. In general, the reaction of the skin after three applications of the carcinogen followed by croton oil was mild. No ulceration or necrosis of the skin occured in any of the groups.

The single application of carcinogen followed by twice weekly croton oil treatment (Group I) resulted in the usual pattern of tumour formation. There was a steady increase of papillomata from the sixth week of croton oil treatment and the flattening of the curve had become manifest by the terminal stage of the experiment.

The groups which received three applications of carcinogen at intervals of one day or one week, and then repeated applications of

croton oil (Groups II & III) showed somewhat similar curves for the appearance of tumours. The initial increase in the tumour yield in these two groups was sharper than in groups given a single application of carcinogen and then croton oil. It reached its peak after about 16 to 18 weeks of croton oil treatment and then became flattened.

The group which received three applications of carcinogen at intervals of one month, and then repeated applications of croton oil (Group IV), showed intermediate curves for the appearance of tumours.

Papilloma began to appear after six weeks of croton oil treatment and followed the usual pattern.

The following table (Table 8) gives a summary of the tumour yield at the 12th, 16th and 20th week of croton oil treatment, expressed by the three different measures.

It can be seen that the yield of papillomata was much the same in the groups given three applications of carcinogen at intervals of one day or one week, and then croton oil. It was greater in these groups, than in the group given a single application of the carcinogen and then croton oil. The ratio of the yield of tumours in these groups to that in the group given only one application of the carcinogen is 2:1 if the papillomata per survivor are considered, 1.5:1 if the papillomata per papilloma bearing mouse are considered, and 1.25:1 if the percentage of mice bearing papillomata are considered. The yield of papillomata in the group given three applications of carcinogen at intervals of one month and then croton oil is slightly lower than that in groups given three applications of carcinogen at intervals of one day or one week.

Table 8

Summary of Tumour Yields

Groups	Papillomata per Survivor			Papillomata per Papilloma Bearing Mouse			Percentage of mice bearing Papillomata		
	week of CO Treatment			week of CO Treatment			week of CO Treatment		
	12	16	20	12	16	20	12	16	20
DMBA 1x & CO	3.8	5.3	6.6	7.4	9.3	10.7	51	57	21
DMBA 3x daily & CO	10.3	12.5	11.2	14.0	14.4	13.3	74	87	85
DMBA 3x weekly & CO	7.1	10.7	13.5	11.6	12.7	16.1	62	84	84
DMBA 3x monthly & CO	6.6	7.2	8.1	11.0	11.7	11.6	60	62	69
DMBA 1x	0	0	0	0	0	0	0	0	0
CO only	0	0.3	0.4	0	1.2	1.7	0	16	24
DMBA 3x daily	0	0	0	0	0	0	0	0	0
DMBA 3x weekly	0.2	0.4	0.4	4	7	8	3	3	3
DMBA 3x monthly	0.3	0.2	0	2.6	1.6	0	12	12	0

It is, however, definitely higher than that in the groups given only a single application of carcinogen and then croton oil. The ratio of the tumour yield in this group to that in the group given one application of carcinogen is 1.5:1 if the papillomata per survivor are considered, 1.2:1 if the papillomata per papilloma bearing mouse, or the percentage of mice bearing papillomata are considered.

The average latent periods of the papilloma and of all papilloma are shown in Table 9.

The latent periods are approximately the same in all four groups. There are no particular differences between the groups given three applications of the carcinogen and then croton oil and the group given a single application of the carcinogen and then croton oil.

The group which received no carcinogen but only repeated applications of croton oil, produced 7 papillomata on four mice.

The groups which received one application of the carcinogen or three applications of the carcinogen at a one day interval, but received no croton oil, produced no tumours.

The groups which received three applications of carcinogen at intervals of one week or one month but received no croton oil produced a few papillomata on a few mice.

Summary

Groups of mice were initiated by a single application of 0.1% 9,10-dimethyl-1,2-benzanthracene in mineral oil, or three applications of the same carcinogen at intervals of one day, one week or one month. Twice weekly treatment with 5% croton oil followed. Three applications

Table 9

Average Latent Periods

Group	Average Latent period of papilloma bearing mice	Average latent period of papillomata
DMBA 1x & C.O.	10.5	13.3
DMBA 3x daily & C.O.	10.1	12.2
DMBA 3x weekly & C.O.	10.8	13.5
DMBA 3x monthly & C.O.	9.9	12.2

(weeks)

of the carcinogen followed by croton oil produced more tumours than did a single application of the carcinogen followed by croton oil. Three applications of the carcinogen at intervals of one day or one week produced more tumours than three applications at intervals of one month. There was no significant difference in the latent periods of papilloma bearing mice or of all papillomata between the groups given three applications of the carcinogen and the group given a single application of the carcinogen.

Groups given a single application of the carcinogen or three applications of the carcinogen at daily intervals but given no croton oil, produced no tumours.

Groups given three applications of the carcinogen at intervals of one week or one month, but no croton oil produced a few papillomata.

EXPERIMENT IV

Histological studies of the skin of mice given three monthly applications of 1.5% or 0.1% 9,10-dimethyl-1,2-benzanthracene followed by 5% croton oil.

Introduction

In the previous experiments (Experiments I, II & III), it was shown that when mice were initiated by three applications of 1.5% 9,10-dimethyl-1,2-benzanthracene at intervals of one month, papillomata began to appear after one week of croton oil treatment. The latent periods of papilloma bearing mice and all papillomata were significantly shorter than those in groups given a single application of the same

carcinogen followed by repeated applications of croton oil. On the other hand, when mice were initiated by three monthly applications of 0.1% 9,10-dimethyl-1,2-benzanthracene, papilloma began to appear only after five or six weeks of croton oil treatment. That is to say, the shortening of the latent periods did not occur.

It was assumed that applications of 9,10-dimethyl-1,2-benzanthracene at monthly intervals exerted a promoting action. Three applications of a 1.5% concentration at monthly intervals almost completed promotion, so that visible tumours were rapidly manifested by the croton oil. Failure to induce such a promoting action by three applications of a 0.1% solution at monthly intervals might be caused by an insufficiency of promoting action, due to the lower concentration, since, apparently, the action of promotion is related to the frequency of promotor applications (Holsti,1959) and the concentration of the promotor (Merenmies, 1959;Frei & Ritchie,1962).

The changes induced in the skin of mice by applications of promotors have been studied by many people (Salaman & Gwynn,1951;Setälä, 1961). No histological change has been found to be peculiar to the promoting agents. To study this matter further, advantage was of the observation noted, and histological changes induced by three applications of a 1.5% or a 0.1% solution of 9,10-dimethyl-1,2-benzanthracene at intervals of one month were compared.

Material and Method

A total of forty-four virgin female Swiss mice purchased from

Rockland Farms were used. They were seven weeks old at the beginning of the experiment.

Four mice in the resting phase of their hair cycle were sacrificed for the study of normal skin. The remaining forty mice were divided in two groups. One group received three applications of 1.5% 9,10-dimethyl-1,2-benzanthracene in mineral oil at intervals of one month by the dropping method. The other received three applications of 0.1% 9,10-dimethyl-1,2-benzanthracene in mineral oil at intervals of one month by the dropping method. Both groups then received twice weekly applications of 5% croton oil beginning one week after the last application of the carcinogen. At the time of the first application of the carcinogen, all mice were at the resting phase of the hair cycle.

Three mice from each group were killed four weeks after the first application of the carcinogen, three four weeks after the second application of carcinogen, and three one week after the third application of the carcinogen. Three mice from each group were also killed one week after the beginning of croton oil treatment, and three two weeks after start of the croton oil treatment.

Results

The epidermis of normal mice consists of two or three layers of small cuboidol cells of undifferentiated type (Fig. 1). Mitosis are infrequent. The dermis consists of interwoven, rather coarse bands of highly refractible collagen in which many hair follicles and sebaceous glands are seen. Underneath the layer of collagenous tissue, is a layer

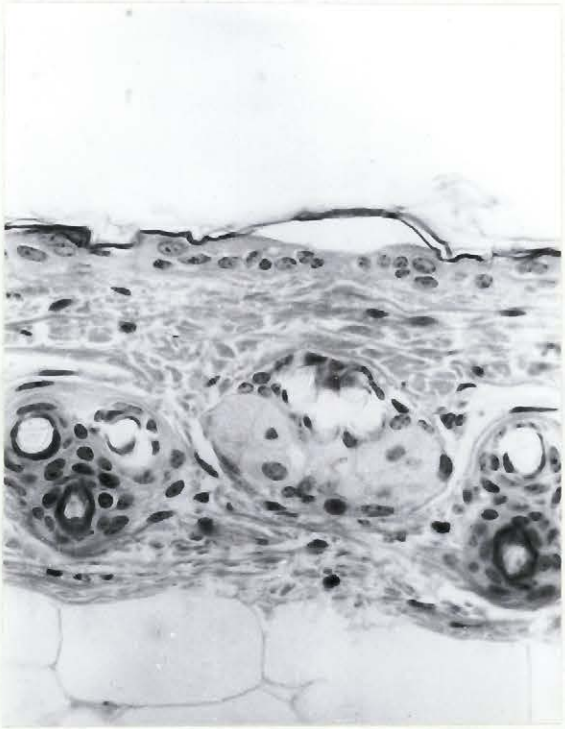
of adipose tissue. No cellular infiltration is seen in normal skin. Four weeks after a single application of 1.5% 9,10-dimethyl-1,2-benzanthracene, the epidermis became four to five cells thick and the cells assumed a typical squamous appearance (Fig. 2). It is possible to distinguish a stratum malpigii, a stratum granulosum, and a superficial keratimic layer. Signs of differentiation have appeared in the form of intracellular bridges and keratohyaline granules. Many cells and nuclei are now twice their normal diameter. There is slight hyperkeratosis. The dermis is slightly edematous and is infiltrated by a moderate number of acute inflammatory cells, as well as by chronic inflammatory cells. The hair follicle and sebaceous glands appear to be intact.

Four weeks after a single application of 0.1% 9,10-dimethyl-1,2-benzanthracene, the epidermis still remains two to three cells thick with no appreciable differentiation in stratum spinosum and stratum granulosum (Fig. 3). There is a marked hyperkeratosis. The hair follicles and sebaceous glands are intact. The cellular infiltration is less than that of the skin treated with 1.5% 9,10-dimethyl-1,2-benzanthracene.

Four weeks after two applications of 1.5% 9,10-dimethyl-1,2-benzanthracene, the epidermis becomes still thicker, consisting of 6-7 layers of cells (Fig. 4). Many cells, nuclei and nucleoli have enlarged to three times normal. Mitosis can be identified frequently in the basal layer. The hair follicles are now altered. Many of them are transformed into a short club-like extension of cells from the base of the epidermis. The number of sebaceous glands is slightly diminished. The dermis shows slight oedema and cellular infiltration, but of the same

FIGURE 1

Normal Skin



(x 430)

FIGURE 2

4 weeks after a single application of
1.5% 9,10-dimethyl-1,2-benzanthracene

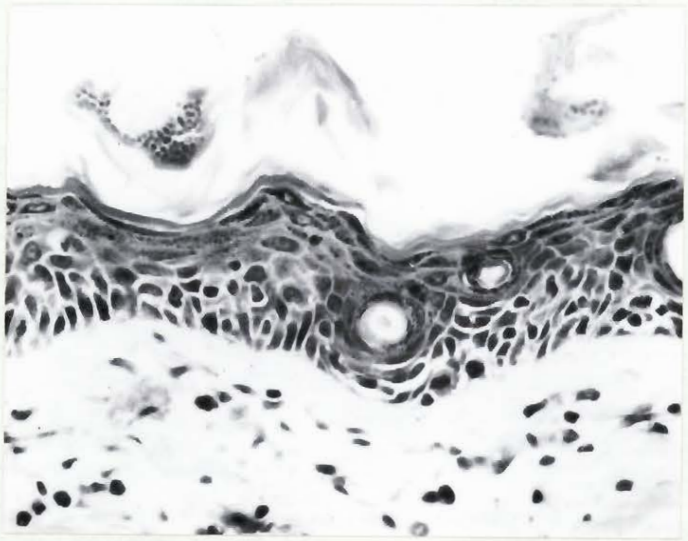


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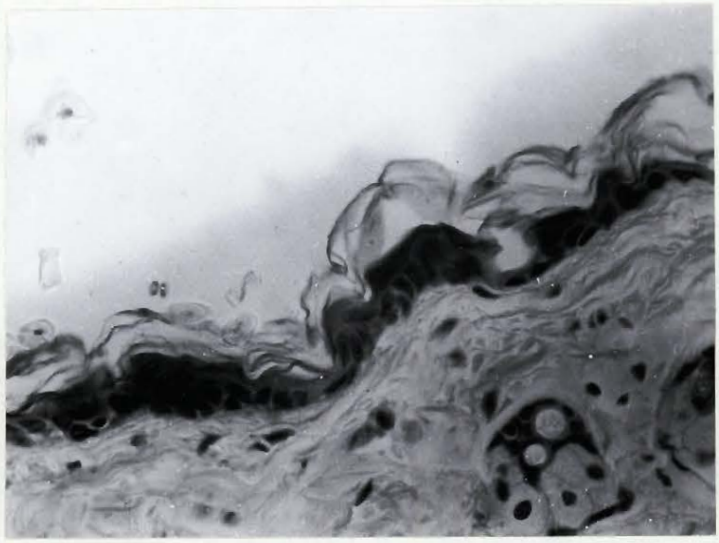
FIGURE 3

4 weeks after a single application of
0.1% 9,10-dimethyl-1,2-benzanthracene



(x 430)

degree as seen in sections taken four weeks after a single application.

The appearance of the skin four weeks after two applications of 0.1% 9,10-dimethyl-1,2-benzanthracene is very similar to that of the skin after a single application of this concentration of the carcinogen (Fig. 5). The epidermis still remains thin, consisting of only two to three layers of cells.

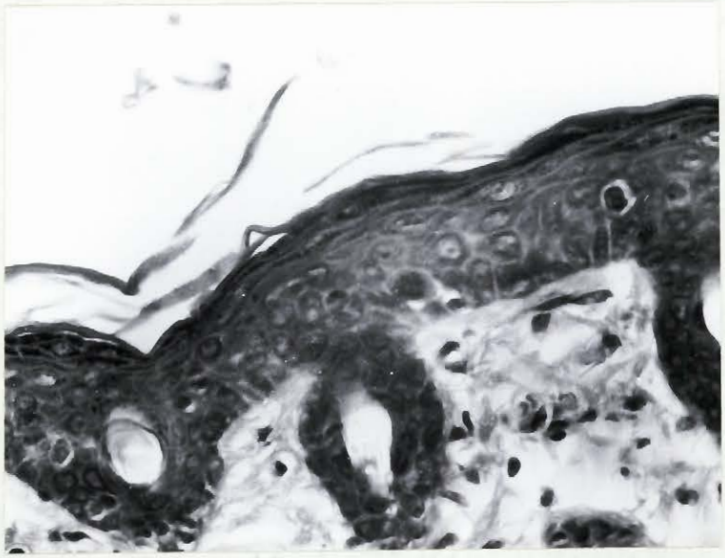
One week after three applications of 1.5% 9,10-dimethyl-1,2-benzanthracene, the epidermal hyperplasia reaches its maximum, being seven to eight layers thick (Fig. 6). Many of the cells, nuclei, and nucleoli have enlarged still more, the cells and nuclei to five to six times normal. The cells, particularly in the deeper layers, become irregular in size and arrangement. Cells near the surface are flattened and keratin has increased in amount. The dermis shows swollen collagen fibers and occasional fragmentation and wide separation of bundles. In some areas, there is proliferation of fibroblasts, and the inflammatory reaction is intense.

One week after three applications of 0.1% 9,10-dimethyl-1,2-benzanthracene the epidermis is hyperplastic being four to five layers thick (Fig. 7). The arrangement of cells is still regular. The size of cells and nuclei is about half the size of the cells treated by 1.5% 9,10-dimethyl-1,2-benzanthracene. There is marked oedema of the dermis and swollen collagen fibers.

One week after the start of application of croton oil in the group given 1.5% 9,10-dimethyl-1,2-benzanthracene, the epidermal hyperplasia remains as before. In one focal area, there was a marked

FIGURE 4

4 weeks after two applications of 1.5%
9,10-dimethyl-1,2-benzanthracene at monthly interval



(x 430)

FIGURE 5

4 weeks after two applications of 0.1%
9,10-dimethyl-1,2-benzanthracene at monthly interval

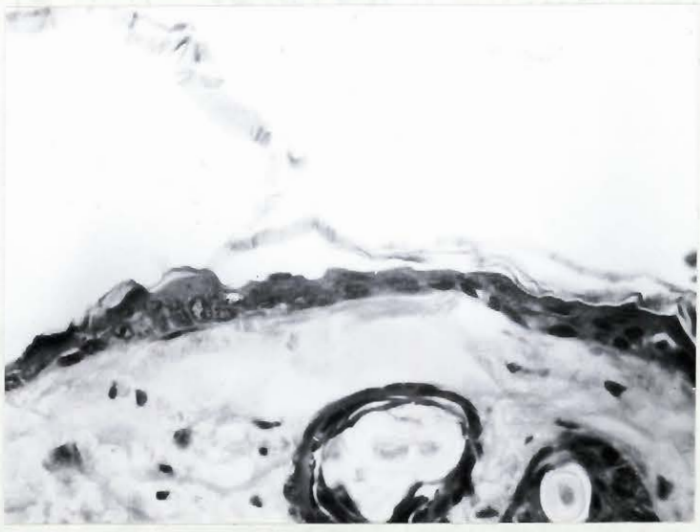


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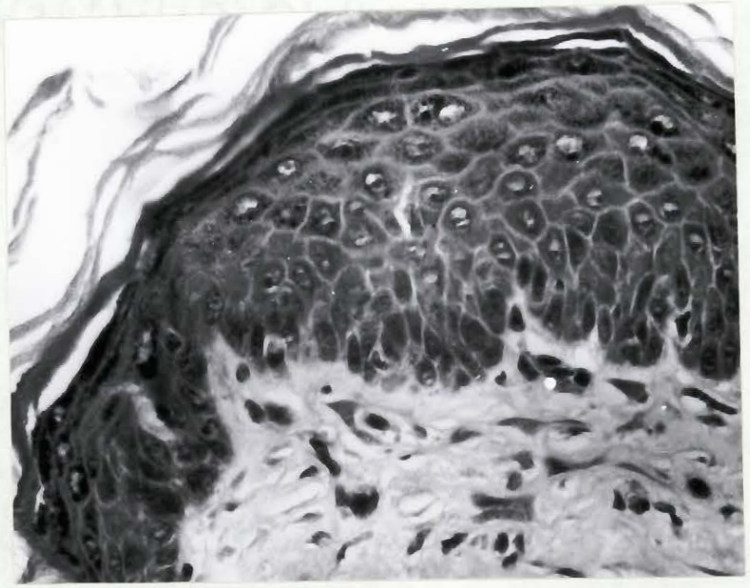
FIGURE 6

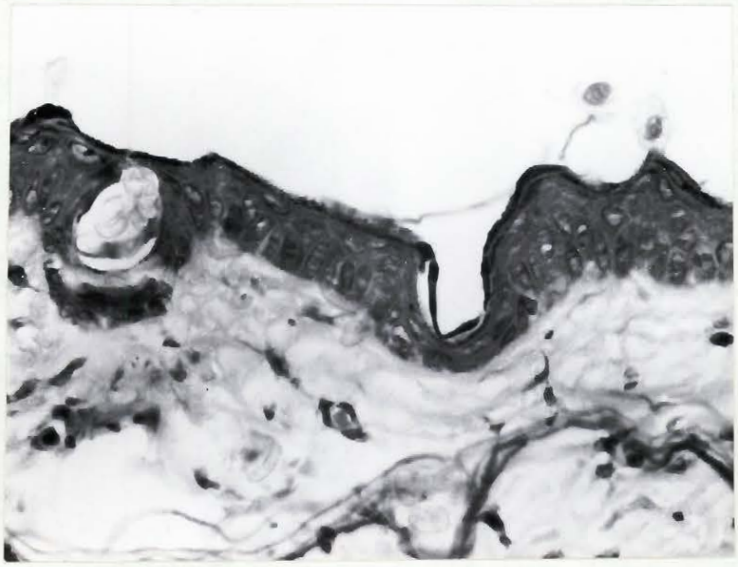
One week after three applications of 1.5%
9,10-dimethyl-1,2-benzanthracene at monthly intervals



(x 430)

FIGURE 7

one week after three applications of 0.1%
9,10-dimethyl-1,2-benzanthracene at monthly intervals



(x 430)

down growth of epidermal cells which penetrated deep into the epidermis (Fig. 8). A marked hyperkeratosis with many acute inflammatory cells is seen on the epidermal surface. The dermis shows an intense infiltration by acute inflammatory cells and capillary engorgement.

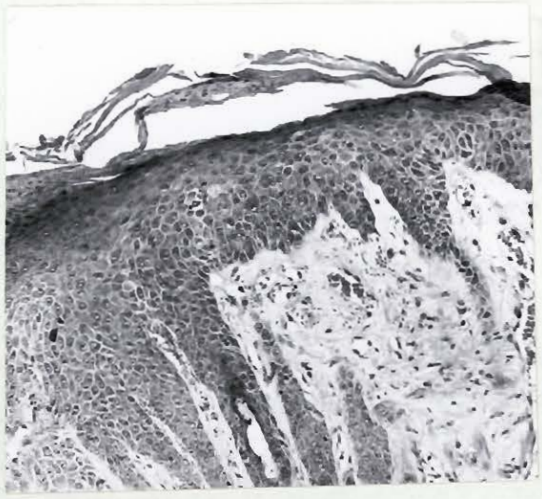
The striking feature in the skin one week after the start of applications of croton oil in the group given 0.1% 9,10-dimethyl-1,2-benzanthracene is the greatly increased degree of epidermal hyperplasia (Fig. 9). The epidermis is six to seven layers of cells thick, with a fairly regular arrangement. The differentiation of the epidermal cells into strata has become clear. Alteration occurs in the hair follicles so that solid buds of cells extend into the dermis. There is also an intense cellular infiltration, consisting of mainly acute inflammatory cells.

Two weeks after application of croton oil in the groups given 1.5% 9,10-dimethyl-1,2-benzanthracene, there were three small papillomata on one mouse. Histologically, the proliferating epithelium is raised up in a polypoid fashion (Fig. 10). The remainder of the epidermis is hyperplastic as in the previous week, and focally shows pseudoepitheliomatous proliferation. The dermis shows a similar appearance to that of the previous week.

Two weeks after the start of applications of croton oil in the group given 0.1% 9,10-dimethyl-1,2-benzanthracene, the epidermis is in its maximum hyperplastic state (Fig. 11). There are six to seven cell layers. There is an increase in the cell size and in the nuclear size. However, the arrangement of cells is still regular giving even thickness

FIGURE 8

one week after applications of croton oil
in the group given three applications of
1.5% 9,10-dimethyl-1,2-benzanthracene



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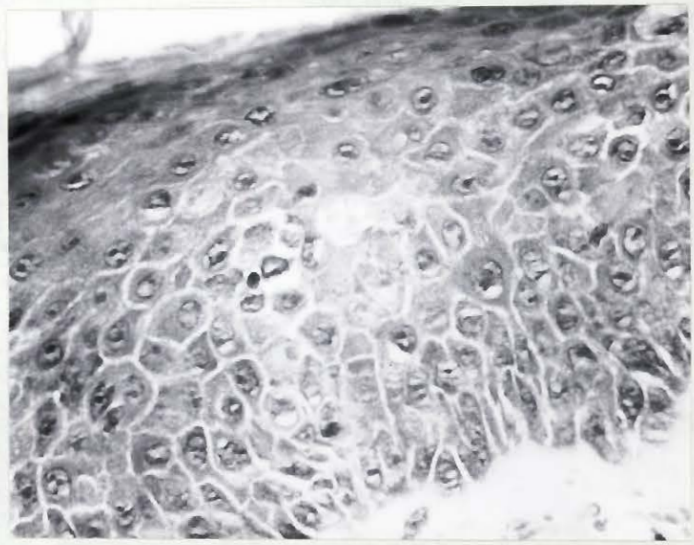

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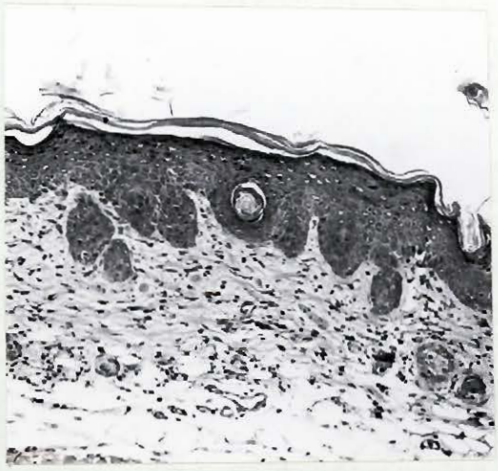
FIGURE 9

one week after applications of croton oil
in the group given three applications of
0.1% 9,10-dimethyl-1,2-benzanthracene



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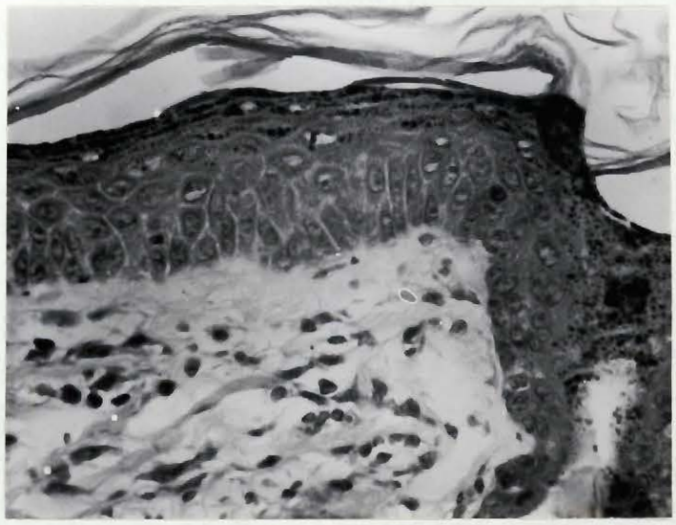

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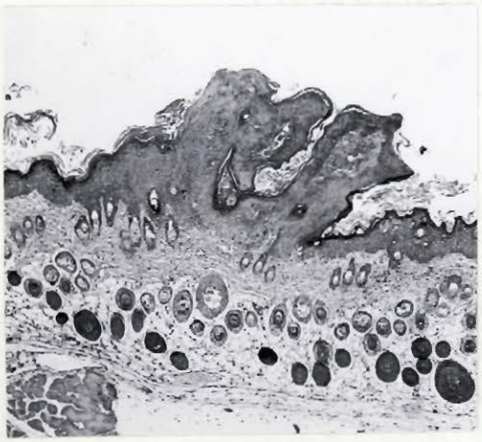
FIGURE 10

Two weeks after applications of croton oil in the group given three applications of 1.5% 9,10-dimethyl-1,2-benzanthracene



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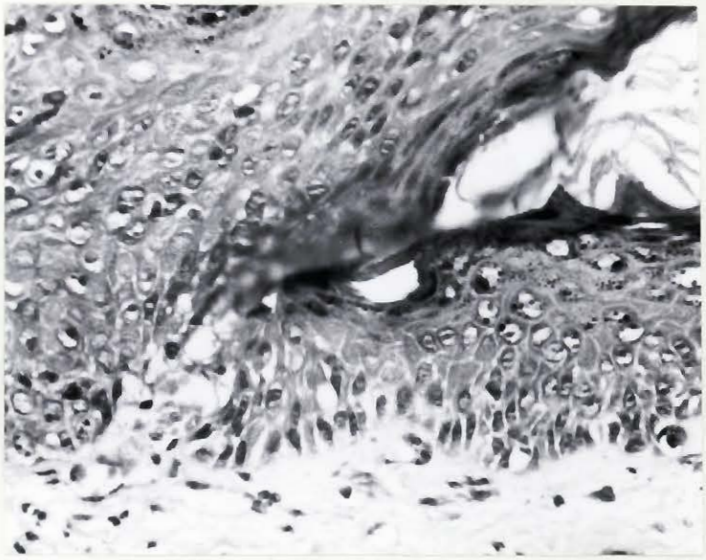

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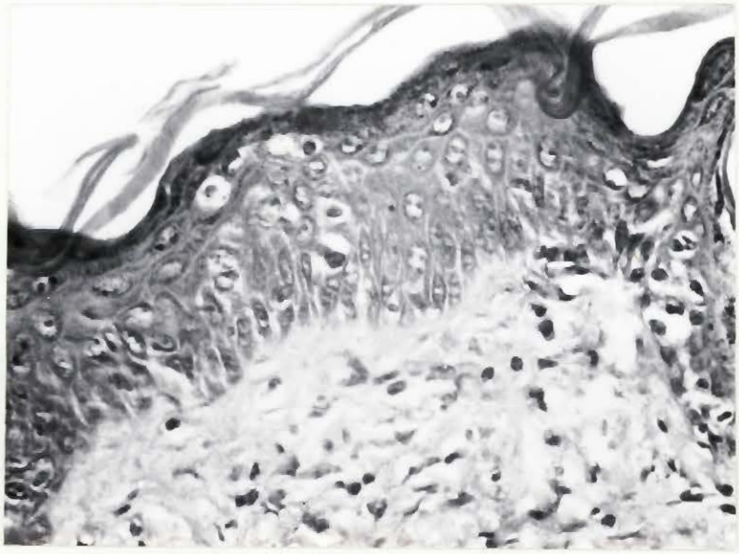
FIGURE 11

Two weeks after applications of croton oil in the group given three applications of 0.1% 9,10-dimethyl-1,2-benzanthracene



(x 430)

of epidermis. Intense cellular infiltration and engorgement of capillaries are the features of the dermis.

Summary

Groups of mice were initiated by three applications of 1.5% or 0.1% 9,10-dimethyl-1,2-benzanthracene at intervals of one month. One week after the last applications of initiator, twice weekly 5% croton oil treatments were begun in each group. Mice were killed after one, two or three applications of initiator, and after one or two weeks of croton oil treatment. Histological studies of the skin of the mice in each group were carried out. The significant changes after 1.5% 9,10-dimethyl-1,2-benzanthracene were intense epidermal hyperplasia, with slight dysplasia and a considerable degree of cellular atypia. Subsequent applications of croton oil did not change the histological appearance significantly. Papillomata appeared from hyperplastic areas of epidermis after two weeks of croton oil treatment.

The change of the skin after applications of 0.1% 9,10-dimethyl-1,2-benzanthracene showed a minor degree of epidermal hyperplasia without dysplasia or cellular atypia. Subsequent applications of croton oil induced rapid epidermal hyperplasia and an intense inflammatory cell infiltration in the dermis.

EXPERIMENT V

Epidermal carcinogenesis induced in mice by one or three injections of urethane followed by repeated applications of croton oil.

Introduction

Urethane possesses a striking initiating action on mice skin when applied topically (Salaman & Roe,1953;Graffi,Vlamynch & Schultz, 1953). Later it was shown to have initiating action on mouse skin also when administered orally (Haran-Ghera & Berenblum,1956), or by intraperitoneal injection (Ritchie,1957).

As mentioned earlier, Shubik and Ritchie(1953) reported that the number of tumours induced by initiation and promotion were fewer if two or three applications of 9,10-dimethyl-1,2-benzanthracene were used as initiator, than if only one was used. The need for further investigation of their results were discussed in the introduction to Experiment I of this thesis. One of the theories Shubik and Ritchie suggested to explain their results was that the second or third applications of 9,10-dimethyl-1,2-benzanthracene might damage the skin so severely that the cells which might otherwise have developed into tumours were destroyed. There is no doubt that such applications of 9,10-dimethyl-1,2-benzanthracene do damage to the skin (Pullinger,1940;1941:Glucksman,1945:Setälä,Merenmies,Stjernvall, Aho & Kajanne,1959). However, urethane, on the contrary, produces no significant histological change in the skin when administered topically or by intraperitoneal injection (Roe & Salaman,1954;Ritchie,1957). Thus the use of urethane as the initiator should avoid the necrotizing effect of 9,10-dimethyl-1,2-benzanthracene. The second explanation Shubik and Ritchie suggested was that a refractory state might be induced by the first application of the carcinogen, so that the second or third applications of the carcinogen were not able to act as the initiator. If more

papillomata were induced when three intraperitoneal injections of urethane were given as initiator, than were induced when a single injection was given, the evidence would suggest that Shubik and Ritchie's findings with 9,10-dimethyl-1,2-benzanthracene were due to the damage done to the skin by this carcinogen. If no more papillomata were induced with three injections of urethane than with one, the evidence would suggest that the initiating agent does indeed induce a refractory state. In the present experiment, an attempt was made to clarify these points.

Method

A total of 420 virgin female Swiss mice from the Rockland Farms, New City, New York, were used. They were about four to five weeks old at the time of purchase. The mice were divided into nine groups as shown in Table 10. Groups I and VI received one injection of urethane. Groups II and VII received three injections of urethane at daily intervals. Groups III and VIII received three injections of urethane at intervals of one week. Groups IV and IX received three injections of urethane at intervals of one month. Groups I to V were given croton oil twice weekly, starting one week after the last injection of urethane in each group. Group V received only croton oil, starting on the same date as in group I. Groups VI to IX received no croton oil treatment. Groups I to V consisted of seventy mice each, and Groups VI to IX of thirty mice each. Each dose of urethane was 0.03 ml. of a ten percent (weight per volume) solution given by intraperitoneal injection. The weights of the mice were recorded every week during the first six weeks and thereafter, once every two weeks.

Table 10

Scheme of Treatment of Mice

Group		Initiating Agent	Interval between Injections of Urethane	Promoting Agent
I	GBA	1 injection of Urethane IP	--	croton oil
II	GBB	3 injections of Urethane IP	1 day	croton oil
III	GBC	3 injections of Urethane IP	1 week	croton oil
IV	GBD	3 injections of Urethane IP	1 month	croton oil
V	GBE	nil	--	croton oil
VI	GBF	1 injection of Urethane IP	--	nil
VII	GBG	3 injections of Urethane IP	1 day	nil
VIII	GBH	3 injections of Urethane IP	1 week	nil
IX	GBI	3 injections of Urethane IP	1 month	nil

Result

The reaction of the mice to the injections of urethane was demonstrated by a short lived deviation of the average weight in each group. (Appendix IV, Table I). After one week, the average weight of the mice was reduced approximately 0.5 gm. by a single injection, and 2.7 gm. by three daily injections. One week after the last injection of urethane, the mice usually began to resume a normal average weight.

There was a high mortality rate in groups that were given three injections of urethane at intervals of one day or one week, followed by repeated applications of croton oil. The high mortality rate in the group given only repeated applications of croton oil was somewhat unexpected. It may be due to a severe epidemic in the very early part of the experiment. The evaluation of the mortality curve reveals that the best time for the evaluation of the tumour yield was about the thirteenth or fifteenth week of croton oil applications, when the number of survivors was not too low, but the experiment had continued long enough to give a reliable result.

No obvious skin changes were observed in the groups given urethane alone. However, the skin reaction of the groups given urethane followed by croton oil treatment, differed markedly from one another. The skin changes seen when croton oil followed a single injection of urethane were more or less similar to those seen in the mice when croton oil followed a single application of a polycyclic hydrocarbon. However, mice given three injections of urethane at one day intervals and then croton oil showed severe skin changes after three to four weeks of croton oil treatment. (Appendix IV, Figure V). The mice which showed this sort of change eventually died. Similarly, but to a lesser degree, the group given three injections of urethane at intervals of one week and then

croton oil, developed dermatitis. The group given three injections at intervals of one month and then croton oil showed skin changes similar to those of the group given a single injection and then croton oil.

The tumour yields are expressed by the same measures used in Experiment I. The results are summarized in Tables II to X, and Figures I to IV, in Appendix IV. The following table shows a summary of the tumour yield in each group at the thirteenth and fifteenth weeks of the croton oil treatment (Table 11).

Table 11

Summary of the Tumour Yields

Groups	Papillomata per survivor		Papillomata per Papilloma bearing mouse		Percentage of mice bearing Papillomata	
	week of CO Treatment		week of CO Treatment		week of CO Treatment	
	13	15	13	15	13	15
Urethane 1x	0.8	1.2	2.4	2.4	39	46
Urethane 3x daily	2.9	3.6	7.1	8.6	43	40
Urethane 3x weekly & CO	1.4	1.9	4.3	4.7	35	46
Urethane 3x monthly & CO	3.1	2.4	5.8	3.9	57	58
CO only	0	0.2	0	2.0	0	9
Urethane 1x	0	0	0	0	0	0
Urethane 3x daily	0	0	0	0	0	0
Urethane 3x weekly	0	0	0	0	0	0
Urethane 3x monthly	0	0	0	0	0	0

It can be seen that the yield of tumours was greater in the groups given three injections of urethane at daily or monthly intervals and then croton oil, than in the group given a single injection of urethane followed by croton oil, but that the yield in the group given three injections of urethane at intervals of one week and then croton oil was little more than in the group given one injection and then croton oil. The yield in the groups given three injections of urethane at intervals of one day or one month and then croton oil were much the same. The ratio of the yield of tumours in the group given three injections of urethane at intervals of one day or one month to that in the group given a single injection or three injections at intervals of one week is 3:1 if the papillomata per survivor are concerned, 2:1 if the papillomata per papilloma bearing mouse are concerned, and 1.3:1 if the percentage of mice bearing papillomata are concerned.

The tumours induced by a single injection of urethane or three injections of urethane followed by croton oil were all benign papillomata. Two papillomata turned into carcinomata, one in the group given three injections at intervals of one day, and the other in the group given three injections at intervals of one month. (Appendix IV, Figure VI).

In all experimental groups, papillomata began to appear, on the average at five to six weeks after the commencement of the croton oil treatment. The average latent periods of papilloma bearing mice and all papillomata are shown in the following table (Table 12).

Table 12

Average Latent Periods

Group	Average latent periods of papilloma bearing mice	Average latent period of papillomata
Urethane 1x & CO	12.3	15.2
Urethane 3x daily & CO	10.1	13.8
Urethane 3x weekly & CO	10.0	14.4
Urethane 3x monthly & CO	9.0	15.8
		(weeks)

It can be seen that the average latent periods in the group given three injections of urethane are slightly shorter than that in the group given a single injection of urethane. However, the differences are rather small.

In the group given only repeated applications of croton oil, papillomata began to appear at the fourteenth week of the treatment and six papillomata were observed on two mice.

No tumours were induced in the groups given a single injection or three injections of urethane, but no croton oil.

Summary

Groups of mice were initiated by one or three injections of urethane given at intervals of one day, one week or one month. Twice weekly applications of five percent croton oil followed. The tumour yields were compared. The groups given three injections at intervals of one day or one month showed a higher tumour yield than the group given a single injection of urethane when followed by repeated applications of croton oil. The group given three injections of urethane at intervals of one week showed a lower tumour yield than the groups given three injections of urethane at intervals of one day or one month, developing not a much greater yield than did the group given a single dose of urethane. The groups given one or three injections of urethane at intervals of one day, one week, or one month, but no croton oil treatment, showed no tumour formation.

EXPERIMENT VI

Estimation of urethane in the blood after a single and three daily intraperitoneal injections in mice.

Introduction

It has been suggested that the carcinogenic effect of urethane is due to urethane itself, without the participation of any metabolite (Berenblum,Kaye & Trainin,1960). Furthermore, Kaye(1960) suggested that the greater response to the carcinogenic action of urethane in young mice, is due to a greater retention of urethane in the blood, than in the blood of older mice. Urethane is known to be hydrolysed quickly after injection into the animal body, giving end products, such as ethanol, carbon dioxide and water. It is also known that after injection, urethane is distributed rapidly throughout all organs of the body in equal proportion (Archer,Chapman,Rhoden & Warren, 1948). It is almost completely excreted from the body within twenty-four hours (Skipper,Bennet,Bryan,White & Newton,1951).

In the previous experiment (Experiment V), groups of mice were initiated by one or three injections of urethane, given at intervals of one day, one week, or one month, and repeated applications of croton oil followed. The groups given three injections of urethane at intervals of one day showed a higher tumour yield than the group given a single injection of urethane. The difference of the tumour yield in these groups might be due to a difference in the retention or concentration of

urethane in the blood. The pattern of disappearance of urethane from the blood after a single injection might differ from that after three daily injections, because the first injection of urethane might modify the excretion rate of urethane from the body. The pattern of excretion of urethane from the blood was therefore studied after a single intraperitoneal injection, and after three daily intraperitoneal injections.

Materials and Method

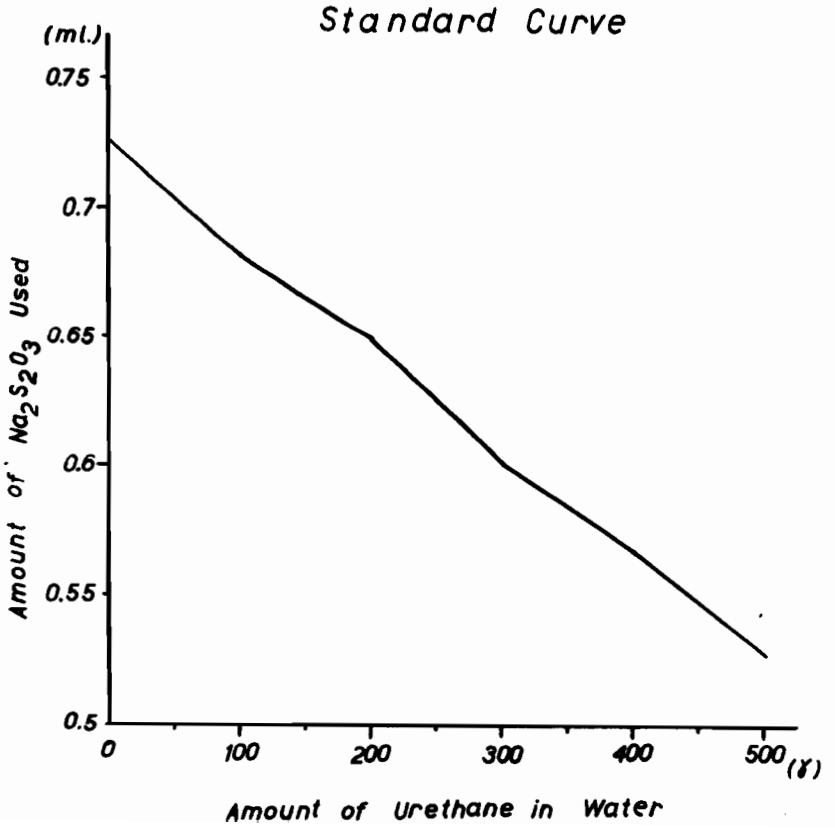
Swiss male mice bred in the Pathological Institute of McGill University were used. Each mouse was approximately nine weeks old, and weighed 24-25 grams. One group of thirty-two mice received a single injection of urethane and a second group of thirty-two, received three injections of urethane at daily intervals. The drug was given intraperitoneally as 0.3 ml. of a 10% (w/v) aquaeous solution. In the first group, mice were killed 0.5, 1, 2, 3, 4, 5, 6, & 7 hours after injection of urethane. In the second, they were killed at similar intervals, after the last injection. The mice were killed by cervical dislocation, four from each group, being killed at each interval.

The method used for the estimation of urethane in the blood was similar to that used by Boyland and Rhoden(1949). The basis of this estimation is the hydrolysis of urethane to ethanol by caustic alkalies.1ml. of 0.025N $K_2Cr_2O_7$ in 10N H_2SO_4 is placed in the central compartment of a Conway Unit. 0.1 ml. of blood which contains an unknown amount of urethane is incubated with 2 ml. of IM K_2CO_3 in the outer ring of one unit and with 2 ml. of 2N KOH in the other. The units are sealed

and incubated at 37°C for 19 hours. At the end of the incubation period, KI is added to the acidic $K_2Cr_2O_7$, and the liberated I_2 was titrated with 0.1 N $Na_2S_2O_3$. The difference in titre between the two units is proportional to the amount of urethane present. At the beginning, a standard curve (Fig. 12) was obtained by estimating the amount of $Na_2S_2O_3$ used for the titration of I_2 liberated from acid $K_2Cr_2O_7$ after incubating 0.1 ml. of distilled water, containing a known amount of urethane (0, 50, 100, 200, 400, and 500 γ) with K_2CO_3.

Experiment VI

Figure 12



Results

The results of the experiment are illustrated in Table 13 and Figure 13. In the table, the amount of urethane recovered from 0.1 ml. of blood was expressed as γ.

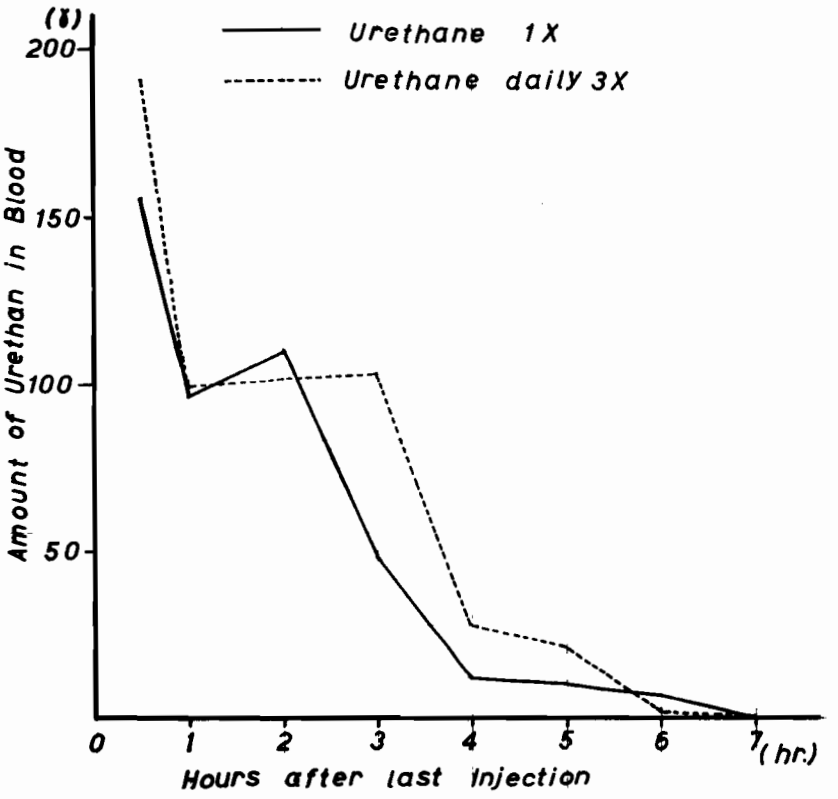
Table 13

Amount of Urethane in Blood after a single Injection and three Injections at daily intervals (γ per 0.1 ml. of blood)

Time after last injection	Urethane 1X					Urethane 3X daily				
	I	II	III	IV	Mean	I	II	III	IV	Mean
1/2	185	125	167	135	153	190	167	219	190	191
1	53	103	80	145	95	80	90	103	125	99.5
2	156	90	135	65	111	80	114	125	90	102
3	53	0	80	53	49	114	145	65	90	104
4	7	36	0	0	11	65	0	23	23	28
5	7	0	7	23	9	7	0	36	36	20
6	0	7	0	23	8	0	0	0	7	2
7	0	0	0	0	0	0	0	0	0	0

FIGURE 13

Pattern of disappearance of Urethane from Blood



It can be seen that one half hour after the last intraperitoneal injection there was about 150 to 200 urethane per 0.1 ml. of blood. The group which was given three injections of urethane shows a slightly higher concentration than the group which was given a single injection. One hour after the injection, there was about 100 of urethane per 0.1 ml. of blood, but little difference between the groups. Thus, the excretion of urethane from the blood took place quickly in both groups. Seven hours after the last injection, there was no detectable amount of urethane in either group. As can be seen in Fig. 13, the overall pattern of the disappearance of urethane from the blood is very similar in the two groups.

Summary

The groups of mice were given a single injection or three daily injections of 0.3 ml. of ten per cent urethane intraperitoneally. The amount of urethane remaining in the blood after the last injection was estimated. The pattern of disappearance of urethane from both groups was compared. It was shown that urethane starts to disappear immediately after the last injection. No detectable amount of urethane was present seven hours after the last injection. The pattern ofdisappearance was approximately the same in both groups.

EXPERIMENT VII

The Effect of Orotic acid on Initiation and Promotion During Epidermal Carcinogenesis in Mice

Introduction

The possibility that carcinogens might affect nucleic acid synthesis has been mentioned. Cowen(1949) demonstrated the inhibitory action of pentose nucleotides upon urethane induced lung tumours, and concluded that pentose nucleotide might act by directly supplementing the normal cell purine and so rendering the urethane ineffective. The changes of nucleic acid metabolism induced by urethane were also discussed by Boyland and Koller(1954). In their study of the effect of urethane on mitosis in the Walker rat carcinoma, they suggested that urethane might interfere with cellular synthesis of thymine by inhibiting the methylation of uracil to thymine, so leading to a deficiency of thymine. Roe(1955) investigated the influence of purine precursors on epidermal carcinogenesis by urethane followed by croton oil. He found that the initiating action of urethane on the skin was inhibited, if formate and glycine were both administered together with it, though neither had any effect when administered alone.

Rogers(1957a) did a series of experiments to find out to what extent the number of lung tumours initiated by a single injection of urethane might be influenced by simultaneous exposure of the animals to DNA, RNA, their chemical components, precursors, and substances influencing their rate of synthesis. He demonstrated that a single injection of DNA hydrolysate immediately prior to exposure of mice to urethane profoundly reduced the number of tumours initiated. Aminopterine, known to inhibit nucleic acid synthesis, increased the carcinogenic activity of injected urethane. This increase could be prevented by the injection of DNA hydrolysate. Of the components of nucleic acid tested, the pyrimidines

proved the most active inhibitors. Of the pyrimidine precursors, it was shown that orotic acid and dihydro-orotic acid exerted a profound inhibitory influence upon urethane carcinogenesis. Furthermore, orotic acid was also shown to inhibit the lung tumours induced in mice by painting with methylcholanthrene (Rogers,1957b). It was suggested that the mechanism through which methylcholanthrene initiates neoplastic change was also related to the synthesis of nucleic acids.

There is little experimental evidence to suggest that polycyclic hydrocarbon carcinogens may interfere with nucleic acid metabolism, though Evensen(1961;1962) has reported that both methylcholanthrene and 9,10-dimethyl-1,2-benzanthracene interfere with the synthesis of DNA in epidermal cells shortly after their application to the skin of mice, and Heidelberger and Davenport(1961) presented evidence to show that dibenzanthracene or its derivatives are bound firmly in vivo to DNA and RNA.

Orotic acid is known readily to enter the pathway of synthesis of nucleic acid pyrimidine. It is relatively non-toxic, and might be expected to antagonize the action of the carcinogen if it acts by interfering with pyrimidine synthesis. The present experiment is designed to determine if the yield of tumours induced by a single application of 9,10-dimethyl-1,2-benzanthracene followed by repeated applications of croton oil was modified by the administration of orotic acid.

Material and Method

A total of 420 virgin female Swiss mice purchased from the

Rockland Farms were used. They were divided into six groups as shown in Table 14. Groups I-IV received a single application of 1.5% 9,10-dimethyl-1,2-benzanthracene followed by twice weekly applications of croton oil. Group I received no additional treatment. Group II was given orotic acid during the period of initiation. Group III was given orotic acid during the period of promotion. Group IV was given orotic acid during both initiation and promotion. The orotic acid was given as a 0.1% solution in the drinking water when it was given during the initiation, and a 0.05% solution when it was given during promotion. When given during initiation, it was begun 2 days before the application of carcinogen, and continued for 7 days thereafter. When given during promotion, it was begun on the day of the commencement of croton oil, and continued throughout the experiment.

Table 14

Scheme of Treatment of Mice

Group		Initiation	Promotion
I	GFA	DMBA	CO
II	GFB	DMBA + Orotic Acid	CO
III	GFC	DMBA	CO + Orotic Acid
IV	GFD	DMBA + Orotic Acid	CO + Orotic Acid
V	GFE	DMBA	nil
VI	GFF	nil	CO

Results

The results are summarized in Tables I to VI, and Figures I to IV in Appendix VI.

The orotic acid was well tolerated by the mice. There was no demonstrable ill effect on animals which received orotic acid during initiation, promotion, or both. The mortality rate was low, so that number of survivors in four groups at the 20th week was satisfactory. The tumour yields were expressed in the same measures as used in the Experiment I.

The pattern of the appearance of tumours was similar in each group, and followed the usual pattern. Tumours began to appear at the 5th week of croton oil treatment, and gradually increased in number until about the 14th or 16th week of the croton oil treatment. The following table (Table 15) gives a summary of the tumour yields in each group at the 12th, 16th and 20th weeks of croton oil treatment.

If the average number of papillomata per survivor is concerned, there is no significant difference in the tumour yields between the group given a single application of carcinogen and then repeated croton oil and the group given the same treatment with supplemental orotic acid during initiation. If the papillomata per papilloma bearing mouse are considered, the group given supplemental orotic acid during initiation showed a slightly lower tumour yield than the group given no orotic acid. However, the difference is slight. The group given supplemental orotic acid during promotion showed a moderately higher tumour yield than the group given no orotic acid. This is true both when papillomata per survivor and when papillomata per papilloma bearing mouse are considered.

Table 15

Summary of Tumour Yields

Groups	Papillomata per survivor			Papillomata per Papilloma Bearing Mouse			Percentage of mice bearing Papillomata		
	week of CO Treatment			week of CO Treatment			week of CO Treatment		
	12	16	20	12	16	20	12	16	20
DMBA & CO	7.8	9.9	10.0	11.9	13.6	15.1	65	73	66
DMBA + OA & CO	7.6	10.2	10.4	10.7	13.4	13.5	71	76	77
DMBA & CO + OA	10.4	14.3	15.7	13.2	16.0	17.4	78	89	90
DMBA+OA & CO + OA	8.3	9.9	9.5	11.3	12.8	11.6	74	77	83
DMBA only	0	0	0	0	0	0	0	0	0
CO Only	0.05	0.1	0.2	2	1.3	2	0.2	1	1

The group given supplemental orotic acid both during initiation and promotion showed approximately the same tumour yield as the group given no orotic acid.

If the percentage of mice bearing papillomata is considered, there were no significant differences between the four groups.

The average latent period of papillomata bearing mice and all papillomata are shown in the following table. (Table 16).

Table 16

Average Latent Periods

Group	Average latent period of papilloma bearing mice	Average latent period of papillomata
DMBA & CO	8.6	10.9
DMBA + OA & CO	8.0	11.9
DMBA & CO + OA	7.5	11.1
DMBA + OA & CO + OA	8.5	11.6
		(weeks)

It can be seen that all four groups show approximately the same latent periods for the induction of tumours.

The group given a single application of carcinogen but no croton oil produced no papillomata.

The group given repeated application of croton oil but no initial carcinogen showed a relatively high tumour yield. The observation of this group was continued up to the 40th week of the treatment. At the 25th week, there were 44 survivors, in which six mice bore 18 papillomata. At the 30th week, there were 42 survivors, in which 16 mice bore 35 papillomata.

Summary

Several groups of mice were given a single application of 1.5% 9,10-dimethyl-1,2-benzanthracene in mineral oil followed by twice weekly 5% croton oil treatment. The first group received no additional treatment. The second group received orotic acid in the drinking water during initiation. The third group received orotic acid during the entire period of promotion. The fourth group received orotic acid both during initiation and promotion. The administration of orotic acid during the initiation did not modify the tumour yield. The administration of orotic acid during promotion slightly increased tumour yield, but the difference is probably not significant. The group given only croton oil showed an unusually large number of papillomata at the later stages of the experiment.

Experiment VIII

Effect of higher concentrations of orotic acid and of thymine on the initiation of epidermal carcinogenesis in mice.

Introduction

In the previous experiment (Experiment VII) it was shown that the supplemental administration of orotic acid in the drinking water during the period of initiation of epidermal carcinogenesis did not produce any significant alteration on the tumour yield. This is in contrast to the experiments of Rogers(1957a,1957b), who demonstrated that the supplemental orotic acid did decrease the number of tumours of the lung induced by injections of urethane or by painting with methylcholanthrene.

The failure to modify the initiating action of 9,10-dimethyl-1,2-benzanthracene by supplemental orotic acid administration can be explained in three ways. Firstly, the mechanism of action of hydrocarbons in the initiation of skin tumours might be different from that of urethane or methylcholanthrene in lung tumour formation. Secondly, the amount of orotic acid added to the drinking water might be too small to overcome the large dose of carcinogen used. Thirdly, the level of disturbance of nucleic acids synthesis by 9,10-dimethyl-1,2-benzanthracene might be beyond the level of orotic acid.

It was shown that among the pyrimidine bases only thymine reduced the number of adenomata induced by a single exposure of the animals to urethane (Rogers,1957a). Also, Fink and Fink(1955) reported

that supplemental administration of thymine diminished the incidence of spontaneous pulmonary adenomata in mice. Furthermore, the frequency of abnormal mitosis in the Walker rat carcinoma induced by urethane is reduced, and recovery is accelerated, by the simultaneous administration of thymine (Boyland & Koller,1954). Orotic acid has proved to be one of the substances most effectively and universally incorporated in nucleic acid synthesis (Carter,1956). It is incorporated both in RNA and DNA. On the other hand, thymine has been shown to be incorporated specifically into DNA (Chargaff & Davidson,1955).

In the present experiment, the effect of orotic acid on the initiation of epidermal carcinogenesis was reinvestigated, using a higher concentration of orotic acid than previously used. The investigation was also extended to determine whether the administration of thymine during the stage of initiation by 9,10-dimethyl-1,2-benzanthracene modifies the initiating effect of the initiator.

Materials and Methods

A total of 318 virgin female Swiss mice purchased from Rockland Farms were used. They were divided into five groups as shown in the Table 17. The mice were 7-8 weeks old at the beginning of the experiment. Group I received a single application of 1.5% 9,10-dimethyl-1,2-benzanthracene in mineral oil by the dropping method but no further treatment. Groups II, III and IV received a single application of 1.5% 9,10-dimethyl-1,2-benzanthracene in mineral oil by the dropping method and then by twice weekly applications of 5% croton oil. Group III was given supplemental orotic acid in drinking water as a concentration of 0.6%

during the period of initiation. In the previous experiment, the concentration of orotic acid used was 0.1%. Group IV was given three daily intraperitoneal injections of thymine in distilled water, a day before, on the day of, and the day after initiation. Group V received twice weekly applications of croton oil without receiving an initial application of the carcinogen. The experimental results are recorded up to the 16th week of croton oil treatment.

Table 17

Scheme of Treatment of Mice

Group		Initiation	Promotion
I	ACA	DMBA	nil
II	ACB	DMBA	CO
III	ACC	DMBA + Orotic Acid	CO
IV	ACD	DMBA + Thymine	CO
V	ACE	nil	CO

Results

The results are summarized in tables I to V, and figures I to IV in Appendix VII.

The administration of 0.6% orotic acid in the drinking water and intraperitoneal injections of 4% thymine were well tolerated by the mice. The mortality rate was low, so that the number of survivors at the 16th week of croton oil treatment was satisfactory.

Tumours in the experimental groups began to appear at the 5th week of croton oil treatment, and then gradually increased in number until about the 13th to the 14th week. The usual pattern of tumour production was followed. By the 15th and 16th week of croton oil treatment, the number of tumours was becoming more or less stabilized. Table 18 gives a summary of the tumour yields in each group at the 14th and the 16th weeks of croton oil treatment.

It can be seen that the tumour yields were higher in the group given an application of 9,10-dimethyl-1,2-benzanthracene and then repeated croton oil than in the groups given the same treatment with supplemental orotic acid or with injections of thymine. The tumour yields in the group given supplemental orotic acid administration were about the same in the group given supplemental thymine.

The difference in tumour yields between the groups with and without orotic acid or thymine is more apparent if the tumour yield is expressed as the average number of papillomata per survivor. The magnitude of the difference is about 1.5:1. The difference in tumour yields was less impressive if tumour yield was expressed as average

Table 18

Summary of Tumour Yields

Groups	Papillomata per Survivor		Papillomata per Papilloma bearing mouse		Percentage of mice bearing Papillomata	
	weeks of CO Treatment		weeks of CO Treatment		weeks of CO Treatment	
	14	16	14	16	14	16
DMBA 1x	0	0	0	0	0	0
DMBA & CO	12.8	13.2	18.4	17.7	66.7	74.5
DMBA + OA & CO	8.7	8.5	14.8	14.0	58.5	60.9
DMBA + Thymine & CO	7.8	7.9	13.8	13.2	56.1	60.0
CO only	0.3	0.4	2.3	1.6	1	4.2

number of papillomata per papilloma bearing mouse or percentage of mice bearing papillomata.

The average latent period of all experimental groups is shown in table 19.

Table 19

Average Latent Periods

Group	Average latent period of papillomata bearing mice	Average latent period of papillomata
DMBA & CO	7.8	9.9
DMBA + Orotic acid &CO	7.2	10.3
DMBA + Thymine &CO	7.8	9.7

As can be seen, the average latent periods are more or less the same in the three groups.

The group given an application of 9,10-dimethyl-1,2-benzanthracene but no croton oil produced no tumours.

In the group given repeated applications of croton oil but no initial application of the carcinogen, tumours appeared in a few mice after the 9th week of treatment. At the 16th week, there are

16 papillomata on 10 mice.

Summary

3 groups of mice were initiated by an application of 9,10-dimethyl-1,2-benzanthracene, twice weekly applications of 5% croton oil followed. The first group received no additional treatment. The second group received 0.6% orotic acid in drinking water during initiation. The third group received injections of 4% thymine during initiation. The group given the standard application of 9,10-dimethyl-1,2-benzanthracene and then croton oil but no supplement produced more tumours than did the group given orotic acid or that given thymine. The group given orotic acid, produced about the same yield of tumours as did the group given thymine. The group given an application of 9,10-dimethyl-1,2-benzanthracene without subsequent croton oil did not develop any tumours. The group given twice weekly croton oil without an initial treatment of carcinogen produced only a few papillomata on a few mice.

III DISCUSSION AND CONCLUSION

The results of the experiments bring up several points for discussion. The first parts of the experiments were designed to investigate the response of mice to different doses of initiator when initiation was followed by promotion. In particular, the effect of varying of the interval between the applications of initiator when more than one dose of initiator was given was studied. Other experiments dealt with the problem of whether or not an initiator, particularly a polycyclic hydrocarbon, exerts its action through the interference with nucleic acid metabolism. Several experiments were designed to promote better understanding and give further support to the major points to be discussed.

1. Toxicity of Carcinogen

There was a high mortality in the groups given three applications of 1.5% 9,10-dimethyl-1,2-benzanthracene at intervals of one day or one week and then repeated applications of croton oil. On the contrary, the mortality rate in the groups given three applications of 0.1% 9,10-dimethyl-1,2-benzanthracene at intervals of one day or one week was very low. It is known that large doses of polycyclic hydrocarbons applied on the skin have some retarding effect on the growth of mice (Haddow & Robinson,1939). It has also been shown that acute and delayed intoxication occurs in mice when they are painted with a large amount of 9,10-dimethyl-1,2-benzanthracene (Shubik & Della Porta,1957). Daily

applications of 0.08 ml. of 2% 9,10-dimethyl-1,2-benzanthracene in acetone produced more than 50% mortality within 16 days. Furthermore, a single application of 10 mg. of the same carcinogen as a 2% solution in acetone produced more than 80% mortality within 11 days. A single application of 1.5% 9,10-dimethyl-1,2-benzanthracene, as used in Experiment I, amounts to a dose of approximately 1.0 to 1.5 mg. of the carcinogen. Three daily applications equal 3 to 4.5 mg. However, with an 0.1% concentration, as used in Experiment III even three applications total less than 0.5 mg. of the carcinogen. The figures of Shubik and Della Porta can not be applied directly to the present experiments, since the solvent used was mineral oil, not acetone, and the susceptibility of the mice might be different. Nevertheless, mice given total of 3 to 4.5 mg. of 9,10-dimethyl-1,2-benzanthracene at daily or weekly intervals showed considerable degree of intoxication, and applications of a total of less than 1 mg. of 9,10-dimethyl-1,2-benzanthracene demonstrated no toxic effects. These findings are compatible with the findings of Shubik and Della Porta.

Although the number of mice used in Experiment I is small, in the group given three applications of the carcinogen at one month intervals, and then croton oil, the mortality rate was about the same as that of the group given a single application of the carcinogen and then the croton oil. This indicates that the prolongation of the interval between the carcinogen application from one day or one week to one month, lowers the degree of acute and delayed intoxication in mice, which is produced by the carcinogen, or allows for recovery between

applications. The high mortality rate in the group given three applications of the carcinogen at monthly intervals in Experiment II appeared to contradict this thought. However, the mortality rate in this group was modified greatly due to an infectious epidemic in the colony, so that no conclusion can be drawn.

The mortality rate of the group given three injections of urethane at intervals of one day or one week and then croton oil was also high, but it was again low in the group given three injections of urethane at intervals of one month and then croton oil. Again the interval of one month seemed to allow for recovery from the intoxication.

As expected, there were no appreciable skin changes in the mice given a single injection, or three injections of urethane, but no croton oil. However, a severe necrotizing reaction occurred in the skin of mice given three injections of urethane at intervals of one day or one week, and then croton oil. This might be due to a non-specific decrease of the general tolerance of the mouse to irritation, for croton oil is a strong irritant; or it might be to some specific change or changes which alter the reaction of the skin to the subsequent croton oil applications occurred. Salaman and Gwynn(1951) claimed that such specific changes are produced in the skin by 9,10-dimethyl-1,2-benzanthracene. They found that the percentage of resting cells in the epidermis rendered hyperplastic by croton oil was similar to that found after repeated applications of 9,10-dimethyl-1,2-benzanthracene, if the croton oil was applied to skin prepared by an application of 9,10-dimethyl-1,2-benzanthracene, but not if the croton oil was applied to normal skin. No such change is known to follow initiation by urethane.

2. Refractory State and Necrotizing Effect

Shubik and Ritchie(1953) found that two or three applications of 0.2% or 1.0% 9,10-dimethyl-1,2-benzanthracene in liquid paraffin, given at intervals of one week and followed by croton oil, produced less tumours in mice than did a single application of the carcinogen followed by croton oil. In contrast, Vesselinovitch and Gilman found that two or three applications of an 0.25% or an 1.5% solution of the same carcinogen in liquid paraffin given at intervals of one month produced more tumours when followed by croton oil than did a single application of the same concentration of the carcinogen followed by croton oil (Vesselinovitch & Gilman,1957;Vesselinovitch,1958).

It should be noticed in these experiments that Shubik and Ritchie allowed intervals of one week between the applications of carcinogen, while Vesselinovitch and Gilman allowed intervals of one month.

Shubik and Ritchie suggested two explanations of their failure to increase the tumour yield by giving more applications of initiator. Firstly, they suggested that the applications of carcinogen in the concentration they used might produce a necrotizing effect on mouse skin, which might destroy some of the latent tumour cells produced by the carcinogen, so that repeated applications of carcinogen would destroy more latent tumour cells than they produced, and so would not act in an additive way. Alternatively, they suggested that mice might develop a refractory state after a single application of carcinogen, so the subsequent applications of carcinogen were ineffective.

Vesselinovitch and Gilman explained their results by suggesting that the one month interval between the applications of carcinogen was longer than the refractory state produced by the previous applications of the carcinogen, and so an additive effect was produced.

The work of Ball and McCarter(1960) also suggested the existence of a refractory state. Using 9,10-dimethyl-1,2-benzanthracene as initiator, they showed that the amount of carcinogen in the skin increased when quantity of carcinogen applied was increased from 7.5 ug to 75 ug, but decreased as the amount of carcinogen applied was increased from 75 ug to 300 ug. The tumour yield in mice given 75 ug of carcinogen and then croton oil is about the same as the yield in mice given 300 ug of carcinogen and then croton oil. Terracini, Shubik and Della Porta(1960) also failed to increase the tumour yield on mice in which tumours were induced by a single application of 9,10-dimethyl-1,2-benzanthracene when amount of carcinogen applied was increased above 200 ug. These results indicate that a refractory state may develop in mice if an excessive amount of carcinogen is applied.

The problem of a necrotizing effect was investigated by Dammert (1961a). Five ulcerative doses of Zephiran after initiation by 9,10-dimethyl-1,2-benzanthracene was reported to enhance the tumour incidence by shortening the latent period. However, the tumour incidence was lower if measured by number of tumour bearing animals or the total number of tumours. The results indicate that ulceration of skin after an application of initiator might destroy some of the latent tumour cells induced by initiator.

In Experiment I, the tumour yield in the three groups given three applications of 1.5% 9,10-dimethyl-1,2-benzanthracene was about the same, whether the carcinogen was given at intervals of one day, one week or one month, but the yield was definitely higher in the groups given three applications of carcinogen and then croton oil, than in the group given a single application and then croton oil. The magnitude of the difference varies according to the measure used. If the yield is expressed as papillomata per survivor, the ratio of the tumour yield between the group given a single application of initiator and the groups given three applications is about 1:3. If the yield is expressed as papillomata per papilloma bearing mouse, the ratio is about 1:2. If the yield is expressed as the percentage of mice bearing papillomata, it is about 1:1.5.

The results in the group given three applications of initiator at intervals of one month and then croton oil are in agreement with the finding of Vesselinovitch. Vesselinovitch expressed the tumour yield as tumours per survivor, and gave the ratio of difference in tumour yield between the group given a single application of 9,10-dimethyl-1,2-benzanthracene and then croton oil and that of the group given three applications of the same carcinogen at intervals of one month and then croton oil as about 1:2.

The results of the group given three applications of 1.5% 9,10-dimethyl-1,2-benzanthracene at intervals of one week and then croton oil are, however, in contrast with the findings of Shubik and Ritchie. The results of the present Experiment I do not show that either a

refractory state developed or that the skin was excessively damaged. It is difficult to explain this discrepancy. There is some difference between the experiments of Shubik and Ritchie and those now being discussed. The dose applied was slightly higher in the present experiment than in the experiments of Shubik and Ritchie. Again, there might be a difference in the susceptibility of the mice to carcinogen between the present experiment and the experiment of Shubik and Ritchie. These differences might produce different reactions on the mice, and so lead to the discrepancy of the results.

One might consider the high mortality rate of the present experiment as evidence of such a difference in the mice, for no such mortality occurred in Shubik and Ritchie's experiment. It could be that when 1.5% 9,10-dimethyl-1,2-benzanthracene was applied three times at intervals of one week, for instance, many mice which developed a refractory state died in the early stage of experiment, and only mice which did not survived. In Experiment III, this high mortality was avoided by using a low concentration of 9,10-dimethyl-1,2-benzanthracene as initiator. In this experiment, even three applications of 0.1% 9,10-dimethyl-1,2-benzanthracene at intervals of one day, produced no apparent toxic effect on the mice. The tumour yield was again higher in the groups given three applications of initiator and then croton oil, than in the group given a single application of initiator and then croton oil. However, the tumour yield in the group given three applications of initiator at intervals of one month and then croton oil was lower than in the groups given three applications of initiator at intervals of one day or one week and then croton oil. The ratio of the tumour yields between

the group given a single application of initiator and croton oil, and the groups given three applications of initiator at intervals of one day or one week and then croton oil was about 1:2 if the tumour incidence is measured as tumours per survivor. The ratio of the tumour yields between the group given a single application of initiator and then croton oil and the group given three applications of initiator at intervals of one month and then croton oil was about 1:1.5. The reason for the low tumour yield in the group given three applications of carcinogen at intervals of one month then croton oil is not clear. Nevertheless, the results do not support the theory that many mice which developed a refractory state died in the early stages of the experiment in which 1.5% 9,10-dimethyl-1,2-benzanthracene was applied three times at intervals of one day or one week.

It can be concluded that three applications of carcinogen produced more tumours than a single application when followed by croton oil, whether carcinogen is given at intervals of one day, one week or one month. Moreover, in spite of the minor differences, the findings are compatible with the theory that the yield of papillomata on mice varies directly with the dose of carcinogen, as proposed by McCarter, Szerb and Thompson(1956) and Ball and McCarter(1960).

The findings with urethane in Experiment V were somewhat different. Three injections of urethane at intervals of one day or one month produced more tumours when followed by croton oil than did a single injection of urethane followed by croton oil. However, three injections of urethane at intervals of one week followed by croton oil

produced no more tumours than did a single injection of urethane followed by croton oil.

With topically applied urethane as initiator followed by croton oil as promotor, Roe and Salaman(1954) found that three applications of urethane gave the same number of tumours per mouse when given at intervals of four days as when given at intervals of two hours. These periods are too short to be comparable with those of the present experiment. Haran-Ghera and Berenblum(1956) gave urethane by mouth and followed with croton oil. They found that the tumour yield was the same, when ten doses of urethane were given, as when a single dose of the same total quantity was given in the ten doses.

The results of unpublished experiments performed at Pathological Institute McGill University (Ritchie,1958-1959) should be considered at this point. The experiments were designed for the same purpose as was the present experiment. In one experiment, one injection or three injections of 0.3 ml. of 10% urethane were given intraperitoneally at intervals of one day, one week or one month as initiator and followed by croton oil as promotor. In a second experiment, three injections of 0.1 ml. of 10% urethane were given intraperitoneally at intervals of one day, one week, or one month and followed by croton oil. The results are shown in Tables I to V in Appendix V. The tumour yields at the 150th day and the 175th day of croton oil treatment in the first experiment, and 168th and 196th day of croton oil treatment in the second experiment, are summarized in the following table (Table 1).

Table 1

Summary of Tumour Yields
(Experiment I and II - Ritchie,1958-1959)

Experiment I

	Average number of papillomata per survivor		Average number of papillomata per papilloma bearing mice		Percentage of mice bearing papillomata	
Days after CO	150	175	150	175	150	175
Urethane 1x and CO	0.1	0.3	1.0	1.4	20	20
Urethane 3x daily and CO	1.9	2.0	3.5	3.0	54	59
Urethane 3x weekly and CO	0.5	0.6	3.3	3.5	17	17
Urethane 3x monthly and CO	1.2	1.6	3.8	3.7	31	43
CO only	0.2	0.2	1.4	1.4	13	13

Experiment II

Days after CO	168	196	168	196	168	196
Urethane 3x daily and CO	0.3	0.4	2.7	2.2	11	18
Urethane 3x weekly and CO	0.3	0.6	2.0	2.1	17	27
Urethane 3x monthly and CO	0	0.1	1.0	2.3	14	21
CO only	0	0	0	1.0	0	3

As can be seen, the number of tumours induced in the second experiment was very small. This may be due to the reduction of the dose of urethane given, or to the different susceptibility of the mice used. It is, however, evident that differences between the groups are small. Because of the small number of tumours induced, the result of the second experiment is unreliable.

The first experiment, however, is comparable to the present one. It shows that the group given a single injection of urethane and then croton oil developed few more tumours than did the group given only croton oil, but that the group given three injections of urethane at daily intervals and then croton oil developed a great many tumours. The group given three injections of urethane at weekly intervals and then croton oil developed few more tumours than did the group given only one injection of urethane and then croton oil, and the group given three injections of urethane at intervals of one month and then croton oil developed an intermediate number of tumours. This is a pattern of response similar to that which was seen in the present experiment.

Thus when three injections of urethane are used as initiator, varying the interval between injections results in different tumour yields. If the interval between injections is one week, three injections of urethane produce about the same tumour yield as does a single injection. If the interval is one day or one month, the yield is about 2 times that observed when a single injection is given. In order to explain this finding, the possible role of a refractory state and of a necrotizing

reaction should be considered.

The high yield of tumours found when three injections of urethane were given at daily intervals can be explained by assuming that three injections of urethane served as a single injection, coming so close together that no refractory state developed. The high yield with three injections of urethane at monthly intervals followed by croton oil could be because after a month the refractory state was passing. The poor tumour yield found when three injections of urethane were given at weekly intervals and followed by croton oil could be because a refractory state developed after the first injection, so that the second or third injections were ineffective. However, the failure to demonstrate such refractory state when three applications of 1.5% or 0.1% 9,10-dimethyl-1,2-benzanthracene were given at weekly intervals stands against this theory of a refractory state.

The severe necrotizing reactions which occurred in the skin of mice given three injections of urethane at intervals of one day or one week and then repeated applications of croton oil should be also considered. Such severe necrotizing reaction might destroy the latent tumour cells induced by the injections of urethane, and so reduce the ultimate tumour development. However, in spite of the severe necrotizing reaction which occurred in the skin of mice given three injections of urethane at daily intervals and then croton oil, they developed many tumours. As the necrosis occurring in this group was at least as severe as that occurring in the group given weekly injections and then croton oil, it seems unlikely that the necrotizing reaction can explain the lower tumour yield in the group given three injections of urethane at

weekly intervals and then croton oil.

Thus on balance, the results of Experiment V suggest that the theory that three injections of urethane given at weekly intervals induce a refractory state in mouse skin, is more satisfactory than the assumption that the results observed are due to a necrotizing reaction.

In Experiment VI, direct chemical measurement of the concentration of urethane in blood after a single and three daily intraperitoneal injections was determined. It was assumed that there might be some modification in the blood level of urethane when more than one injection of urethane was given. Earlier, Berenblum, Kaye and Trainin(1960) suggested that no metabolite is involved in urethane carcinogenesis, and studies by Kaye(1960b) suggested that the greater retention of urethane in blood might result in greater response of mice to the carcinogenic action of urethane. Archer, Chapman, Rhoden and Warren(1948) and Boyland and Rhoden(1949) estimated by chemical methods the amount of urethane in various organs of rats after subcutaneous injection. They found that the urethane was distributed rapidly throughout all organs of the body in equal proportion, and was excreted from the body within 24 hours. The detection of radioactivity after the injection of radioactive urethane by Bryan, Skipper and White(1949) and Skipper, Bennett, Bryan, White, Newton and Simpson(1951) confirmed these observations.

In the present experiment, the estimation was based on the measurement of ethanol yielded from hydrolysis of urethane by caustic alkali, a method which was used by Boyland and Rhoden(1949). The theoretical

disadvantage of this method is that the blood may contain compounds other than urethane which on hydrolysis with alkali may yield volatile oxidizable substances. However, the estimation by Boyland and Rhoden showed no indication of any substances which react as does urethane in human blood or the blood of normal rats or rabbits.

As previous investigators observed, the present study showed that the catabolism of urethane took place shortly after the injection. The concentration of urethane in blood was largest one half hour after a single injection of urethane, and there was already a slight decrease one hour after the injection. This is in accord with the findings of Boyland and Rhoden, who found the largest amount of urethane in the blood of rats one hour after subcutaneous injection. More than half of the urethane had disappeared from the blood three hours after the injection. 7 hours after a single injection of urethane, there was no detectable urethane left in the blood. In this experiment, mice were anaesthetized within 5-10 minutes, and remained anaesthetized for 5-6 hours. The recovery from anaesthesia occurred about the time free urethane disappeared from the blood. The studies with radioactive urethane showed that some radioactivity remained in the blood of mice 24 and 48 hours after an intraperitoneal injection of urethane (Bryan, Skipper & White, 1949). However, the presence of radioactivity need not indicate the presence of free urethane. The activity might be the result of fixation of $C^{14}O_2$ which was produced from the catabolism of carbonyl labelled urethane.

The pattern of disappearance of urethane from blood in the group given three daily injections of urethane was very similar to that

observed after a single injection. The amount of urethane recovered from the blood one half hour after the third injection of urethane was slightly higher than one half hour after a single injection, though the values observed overlapped. One hour after the injection, the amount of urethane in the blood in the two groups was approximately the same.

The repeated injections of urethane, thus, do not induce any change in the capacity of the mice to excrete urethane from blood. Since urethane is completely excreted from the blood 7 hours after a single injection, no urethane was present in the blood at the time of the second and the third injection.

It follows that the tumour yield was greater when three injections of urethane were given than with only one, not because the concentration of urethane was greater, but because the tissue was exposed to it for a longer time.

To summarize, neither a refractory state or a necrotizing reaction could be demonstrated in the experiments with three applications of 9,10-dimethyl-1,2-benzanthracene followed by croton oil. When followed by croton oil, three applications of carcinogen consistently produced more tumours than did a single application, whether the carcinogen was given at intervals of one day, one week or one month.

On the other hand, the experiment with three injections of urethane followed by croton oil did demonstrate what could be considered a refractory state in mouse skin. When three injections of urethane were followed by croton oil, the tumour yield was relatively high, when the injections were given at daily or monthly intervals, but low when the

injections were given at weekly intervals.

3. Latent Period

The shortening of the time interval between the commencement of croton oil treatment and the appearance of tumours in the groups given three applications of 1.5% 9,10-dimethyl-1,2-benzanthracene and then croton oil in Experiment I was not expected. Papillomata began to appear in the first week of croton oil treatment in the group given three applications of the carcinogen at intervals of one month, in the second week of croton oil treatment in the group given three applications at one week intervals, and in the fourth week in the group given three applications at intervals of one day. When a single application of carcinogen is given, papillomata began to appear in the fifth or sixth week. The shortening of this time interval was shown both when the latent period was calculated as the latent period of papilloma bearing mice and when the latent period of all papillomata was calculated. This finding was confirmed in Experiment II in which mice were initiated by three applications of 1.5% 9,10-dimethyl-1,2-benzanthracene at monthly intervals and croton oil followed. Papillomata again appeared in the first week of croton oil treatment. When the concentration of the initiator was reduced to 0.1% this shortening of the latent periods was not observed. Papillomata began to appear about five to seven weeks after the commencement of croton oil treatment, even when initiator was given three times at monthly intervals. Similarly, the latent periods were not shortened when three injections of urethane were used as initiator (Experiment V).

The shortening of the latent periods in the group given three applications of 1.5% 9,10-dimethyl-1,2-benzanthracene and then croton oil was interpreted as being due to a promoting action exerted by the carcinogen. It is known that carcinogens, such as benzpyrene or methylcholanthrene possess promoting action on rabbit skin (Friedewald & Rous,1944a), and that repeated applications of a low concentration of 9,10-dimethyl-1,2-benzanthracene can act as a promotor when preceeded by a single application of a high concentration of the carcinogen (Saffiotti & Shubik,1956b;Klein,1956;Klein,1960). On the contrary, it has been claimed that urethane does not possess such promoting action (Salaman & Roe,1953).

The failure to demonstrate any promoting action when three applications of 0.1% 9,10-dimethyl-1,2-benzanthracene were given might be explained by assuming that the lower concentration of this carcinogen lacked promoting power. This assumption is supported by the observation that the promoting power of Tween 60 and croton oil is directly related to their concentration (Merenmies,1959;Frei & Ritchie,1962). A similar explanation would be applicable to the experiments of Vesselinovitch (1958) in which three applications of 0.25% 9,10-dimethyl-1,2-benzanthracene were given at monthly intervals as an initiator, but did not cause shortening of the latent period. Once again, the concentration of carcinogen might be too low.

In Experiment I, the three applications of carcinogen given at intervals of one month had almost completed promotion, so that visible tumours were rapidly manifested by the croton oil. No tumours were

induced in the group given three applications of the same carcinogen at monthly intervals but no croton oil. Thus, in order to complete the process of promotion, and to elicit visible tumours, the applications of croton oil were necessary.

The rapidity with which croton oil completed the promotion suggests that the mechanism of promotion was the same with croton oil and 9,10-dimethyl-1,2-benzanthracene. This conclusion is important, for it in turn suggests that the mechanism by which the carcinogen induces tumours is also staged, and that the stages may be the same as those of the carcinogen-croton oil sequence.

The strong promoting power exerted by 1.5% 9,10-dimethyl-1,2-benzanthracene was in striking contrast to the negligible promoting power exerted by an 0.1% concentration of the same carcinogen when they were given three times at intervals of one month. In order to elucidate this difference more clearly, the histological investigation of mouse skin treated with 1.5% or 0.1% 9,10-dimethyl-1,2-benzanthracene was performed (Experiment IV). Many investigators studied the histological changes of mouse skin treated with various carcinogenic hydrocarbons (Pullinger,1940;Cramer & Stowell,1942;Setälä,Merenmies,Stjernvall,Aho & Kajanne,1959). Their main object was to find any change or changes in skin specific for the carcinogenic or initiating action of hydrocarbons, and their descriptions were focused on the skin reaction immediately after or up to 2 weeks after the application of the carcinogen. The first and the most striking difference between the skin treated with 1.5% and 0.1% 9,10-dimethyl-1,2-benzanthracene was the degree of epidermal hyperplasia. Epidermal hyperplasia was apparent and intense 4 weeks after a single application of a 1.5% solution of the carcinogen, and increased

progressively to reach its peak after the third application of the carcinogen. On the contrary, the skin of mice treated with an 0.1% concentration of the carcinogen was close to normal in appearance 4 weeks after a single application, and 4 weeks after the second application of the carcinogen. One week after the third application of the carcinogen, the epidermis was slightly hyperplastic, but the degree was much less than that noted with 1.5% carcinogen.

The second striking feature of the skin treated with 1.5% 9,10-dimethyl-1,2-benzanthracene was the presence of cellular atypia and an irregular cell arrangement in the hyperplastic epidermis. These changes were not observed in the skin treated with an 0.1% solution of the same carcinogen. The dermal changes in both groups were very similar, exhibiting some alteration of collagen fibers, hair follicles, sebaceous glands and an intense inflammatory infiltration.

Whether the difference in the degree of epidermal hyperplasia is responsible for the difference in promoting power between the 1.5% and the 0.1% solution is not known. However, the lack of any epidermal hyperplasia after injections of urethane (Ritchie,1957), and lack of promoting power of urethane suggest that lack of epidermal hyperplasia after treatment with 0.1% carcinogen may well be responsible for the absense of promoting power. The rapid development of epidermal hyperplasia after the commencement of croton oil treatment in the group given three applications of 0.1% carcinogen demonstrates that one of the striking actions of croton oil was its hyperplasia inducing power. This is not,

of course, to say that promoting power and the ability to induce hyperplasia are the same. The evidence suggests that they are not, for not all agents inducing hyperplasia can promote (Shubik,1950a). It may well be, however, that the ability to induce hyperplasia is a necessary part of promoting power.

The findings also suggest that 9,10-dimethyl-1,2-benzanthracene is an efficient initiator, even when given in low dosage, but is a relatively poor promotor, being efficient only when given in high concentration.

Both in Experiment I and Experiment II, a few malignant tumours were produced in the groups given three applications of 9,10-dimethyl-1,2-benzanthracene and then croton oil. In experiments with a single application of the carcinogen followed by repeated applications of croton oil, the tumours are almost all benign papillomata. It might be that this difference arose because the promoting power of croton oil is able to induce only benign tumours, while that of carcinogen can induce also malignant ones. Papillomata induced by a single high concentration of carcinogen and repeated applications of a low concentration of carcinogen as promotor show a high rate of malignant transformation (Saffiotti & Shubik,1956b). Alternatively, the croton oil might inhibit the power of 9,10-dimethyl-1,2-benzanthracene to induce malignant tumours as suggested by Allsopp(1951).

4. Tumours induced by 9,10-dimethyl-1,2-benzanthracene alone or by croton oil alone.

There have been reports that a single application of

9,10-dimethyl-1,2-benzanthracene is able to elicit many tumours on mouse skin. (Law,1941;Salaman & Roe,1956b;Terracini,Shubik & Della Porta,1960). In Experiments I and III, the groups given a single application of 1.5% or 0.1% 9,10-dimethyl-1,2-benzanthracene in pure mineral oil developed no tumours until twenty-five weeks after the application. The use of different strains of mice, or different solvents and different doses may account for this discrepancy.

Three applications of 0.1% 9,10-dimethyl-1,2-benzanthracene (Experiment III) induced no papillomata when given at daily intervals, a few papillomata when given at weekly intervals, and a few papillomata when given at monthly intervals.

It was therefore perhaps surprising to see that a relatively large number of tumours were produced in the group given three applications of 1.5% 9,10-dimethyl-1,2-benzanthracene at intervals of one week, particularly as practically no tumours were produced in the group given three applications of the same concentration of the carcinogen at intervals of one month or one day.

Cramer and Stowell, (1941;1943b), stated that the total dose necessary to induce carcinomata in one hundred percent of animals diminished as the applications became increasingly infrequent, and Salaman and Roe(1956b) who compared the incidence of malignant tumours induced in mice by two applications of carcinogen given at different intervals concluded that more carcinomata appeared if the two applications of carcinogen were given at intervals of twenty-two days

or more. The general conclusion may not, however, be always true. It may be that there is a certain optimal time interval between applications of carcinogen when several applications are used. This may differ according to the strain or the concentration of carcinogen used.

It is also possible that the failure to induce many tumours with three applications of 1.5% 9,10-dimethyl-1,2-benzanthracene given at daily or monthly intervals was because the promoting activity of the applications were inadequate. It is known that croton oil must be applied continuously for at least five or six weeks if it is to promote, and that if applications of croton oil are interrupted the promoting effect is lost, so that on resumption of croton oil treatment it is as if the croton oil were being given for the first time (Salaman,1952; Klein,1950a;1953b). It could be that when the carcinogen was applied at daily intervals it was not present long enough to promote, and that when it was applied at monthly intervals the promoting effect was lost between applications. Only when it was given at weekly intervals was it given long enough and often enough to promote. As for the failure to produce more than an occasional tumour when three applications of an 0.1% solution of the carcinogen were used, this may well be because, as stated earlier, the weak concentration of carcinogen lacks promoting power.

A high rate of malignant transformation was seen in the group given three applications of 1.5% 9,10-dimethyl-1,2-benzanthracene at intervals of one week. This difference of the nature of the tumours induced by carcinogen alone and the tumours induced by applications of

carcinogen and promotor is well documented by Shubik(1950b and Shubik, Baserga and Ritchie(1953).

In earlier experiments on two stage carcinogenesis repeated applications of croton oil alone produced no tumours or only an occasional one (Berenblum,1941a;1941b;Shubik,1950b). This was significant in the formulation of two stage theory of epidermal carcinogenesis, because it suggested that the promoting action of croton oil was specific, and not due to a weak carcinogenic action. Later, several investigators found that croton oil could induce a few tumours alone, usually after a long period of treatment (Roe & Salaman,1955;Roe,1956b;Ritchie,1957). This can be explained by the different susceptibility of the mice used by various investigators. Also, since croton oil is not a pure chemical, its constituents vary accordingly in different batches.

In Experiment I, twice weekly applications of 5% croton oil produced no tumours during 25 weeks of treatment. In Experiment III, similar treatment resulted in an occasional papilloma. At the 20th week of treatment, there were 4 papilloma bearing mice with a total of 7 papillomata. In Experiment VII, the group given only croton oil had 5 papilloma bearing mice, with a total of 10 papillomata at the 20th week. Treatment was continued, and there was an increase in the number of papilloma bearing mice. The highest incidence was at the 30th week of treatment, with 16 papillomata bearing mice with a total of 35 papillomata. None of the papillomata induced in these experiments became malignant.

The discovery that croton oil has some carcinogenic power does not weaken the two stage theory of epidermal carcinogenesis. The important thing is that the action of the carcinogen and croton oil are different. As has been mentioned earlier, a weak solution of carcinogen is an efficient initiator but a poor promotor. Croton oil, conversely, a poor initiator but a good promotor. Initiation and promotion remain clearly different, and can be easily distinguished.

5. Effect of Orotic Acid and of Thymine on Initiation

There is some evidence which suggests that carcinogenic substances may act on the DNA of living cells. The disturbance or inhibition of DNA synthesis induced by ionizing radiation (Kelly,1957; Beltz & Applegate,1959), and by nitrogen mustard (Goldthwait,1952; Davidson & Freemann,1955) favour the view that neoplasia might result from a disturbance in the replication and function of nucleic acid. Although direct evidence for such an action of polycyclic hydrocarbons in vivo is lacking (Goldthwait,1960), it is known that carcinogenic hydrocarbons can be bound to DNA (Boyland & Green,1962).

Experiments by Heidelberger and Davenport(1961) suggest that there is a considerable binding of 1,2,5,6-dibenzanthracene to DNA and RNA in mouse skin after its topical applications. Boyland and Green (1962) demonstrated the binding of benzpyrene to DNA in vitro. The significance of this to epidermal carcinogenesis is not clear. Evensen (1961;1962) showed a decrease in DNA synthesis in epidermal cells shortly

after the application of methylcholanthrene, benzpyrene or 9,10-dimethyl-1,2-benzanthracene to mouse skin. He proposed that the initial action of carcinogen might be an interference in the epidermal cells with the synthesis of DNA.

Rogers(1957a) postulated that the carcinogenic action of urethane on the lung might be exerted through a disturbance of DNA synthesis. At the time of urethane injection, he administered known precursors of nucleic acids, and found an inhibitory effect on lung tumour formation when pyrimidine or pyrimidine precursors were given. In particular, orotic acid had a striking inhibitory effect on urethane carcinogenesis in the lung. Orotic acid is known to enter both the pathway of synthesis of DNA and RNA pyrimidine. Thymine which is known to be incorporated only in DNA, also showed a striking inhibitory effect.

Earlier, Boyland and Koller(1954) showed that the frequency of nuclear abnormalities produced by urethane was reduced and recovery was accelerated by the simultaneous administration of thymine. This suggests that urethane might interfere with cellular synthesis of thymine by inhibiting the methylation of uracil to thymine, so leading to a deficiency of thymine.

The observation by Fink and Fink(1955) suggests that not only urethane induced pulmonary adenomata, but also the development of spontaneous lung adenomata in mice may be significantly inhibited by the administration of thymine. Furthermore, the incidence of lung adenomata induced by methylcholanthrene was also reduced by supplemental administration of orotic acid (Rogers,1957b). Since both urethane

and methylcholanthrene are potent initiators in mouse skin, it was asked whether the action of these agents on epidermal cells might not be similar to their action on the lung.

In Experiment VII, the effect of orotic acid on the incidence of skin tumours induced by an application of 9,10-dimethyl-1,2-benzanthracene followed by repeated applications of croton oil was investigated. Orotic acid was added to the drinking water during the stage of initiation, promotion, or both. The results were not consistent. Mice given orotic acid during promotion produced more tumours than did control mice given no orotic acid. This might indicate that orotic acid accelerates the action of the promotor. However, the group given orotic acid during both initiation and promotion have virtually the same tumour yield as did the control. Probably the administration of orotic acid during promotion is without effect. Orotic acid given during initiation, in the concentration used in the first experiment, did not influence the incidence of tumours. It was remarked earlier that this could be interpreted in several ways. Firstly, the initiating action of urethane or methylcholanthrene on mouse skin might be different from the carcinogenic action of these substances on the lung. Secondly, the failure to modify tumour incidence by supplemental orotic acid administration might be because the amount of orotic acid given during initiation was too small. Thirdly, the initiating action of 9,10-dimethyl-1,2-benzanthracene might disturb DNA synthesis at a level of disturbance beyond that of orotic acid.

These possibilities were investigated in Experiment VIII. Tumours were induced by a single application of 9,10-dimethyl-1,2-benzanthracene followed by repeated applications of croton oil. In one group, a concentration of orotic acid 6 times that used before was given during initiation. In another, thymine was given by intraperitoneal injections during initiation. Tumour yield in the groups given orotic acid or thymine was considerably lower than that in the group given neither. The inhibitory effect of thymine was apparently a little stronger than that of orotic acid. Thus, the results of Experiment VIII are in accord with the finding of Rogers on pulmonary adenomata. Both thymine and orotic acid had an inhibitory effect on the initiation by 9,10-dimethyl-1,2-benzanthracene.

The results suggest that initiating action of 9,10-dimethyl-1,2-benzanthracene on mouse skin is similar to the carcinogenic action of urethane or methylcholanthrene on lung. This is reasonable since both urethane and methylcholanthrene are also able to act as an initiator on mouse skin. Both thymine and orotic acid are known to be precursors for the synthesis of DNA, and their inhibitory effect on initiation by 9,10-dimethyl-1,2-benzanthracene suggests that the action of the initiator might be related to DNA synthesis. What sort of an effect 9,10-dimethyl-1,2-benzanthracene is exerting on the DNA is more difficult to assess. The speculations proposed by Rogers(1957a) to explain the inhibitory action of orotic acid and of thymine on urethane induced pulmonary adenomata can also be applied to the present experiment.

The initiating action of 9,10-dimethyl-1,2-benzanthracene might be due to a nonspecific reduction of the quantity of thymine available at the time of DNA synthesis. However, there are substances which act to reduce the synthesis of thymine, and which show no initiating activity. For example, aminopterine is known to interfere with the synthesis of thymine (Chargaff & Davidson,1955), but it has not been known as an initiator or carcinogen. Alternatively, the initiating action of 9,10-dimethyl-1,2-benzanthracene might be the result of temporary interference in the synthesis of certain nucleic acid components, which in turn might ultimately lead to a modification of the final structure of the nucleic acid molecule.

The fact that the inhibitory effect of thymine is slightly stronger than that of orotic acid might indicate that the nucleic acid affected in initiation is DNA rather RNA. Thymine is incorporated into DNA only, while orotic acid is incorporated into both DNA and RNA. The difference between the two inhibitory powers is, however, too slight to deserve much weight.

In conclusion, the present experiments suggest that the initiating action of 9,10-dimethyl-1,2-benzanthracene on mouse skin is similar to the carcinogenic action of urethane or methylcholanthrene on the lung. Its action might be related to metabolic interference in the synthesis of nucleic acid, probably DNA.

SUMMARY

1. The literature concerned with the development of our knowledge of epidermal carcinogenesis and the theory of the two stage carcinogenesis is reviewed. Carcinogenesis with urethane and current speculations on the mechanism of carcinogenesis, particularly as related to a disturbance in nucleic acid metabolism, are discussed. The dose response of animals to the carcinogen used as initiator in the two stage carcinogenesis is reviewed.

2. The dose response of mice to the initiator, 9,10-dimethyl-1,2-benzanthracene, in two different concentrations, was studied in the two stage process of epidermal carcinogenesis. One or three applications of carcinogen given at intervals of one day, one week or one month were followed by repeated applications of croton oil. It was found that mice given three applications of the carcinogen at intervals of one day, one week or one month and then croton oil developed more tumours than did mice given a single application of the carcinogen and then croton oil.

3. A similar experiment using an intraperitoneal injection or injections of urethane as initiator was performed. It was found that mice given three injections of urethane at daily or monthly intervals and then croton oil developed more tumours than did mice given a single

injection of urethane and then croton oil. Mice given three injections of urethane at intervals of one week and then croton oil developed few more tumours than did the mice given a single injection of urethane and then croton oil.

4. It was shown that three applications of 1.5% 9,10-dimethyl-1,2-benzanthracene given at intervals of one day, one week or one month and followed by croton oil shortened the latent period, the shortening increasing as the intervals between the applications of carcinogen lengthened. Three applications of 0.1% 9,10-dimethyl-1,2-benzanthracene or three injections of urethane did not induce this effect.

5. Histological alterations in mouse skin treated by 1.5% or 0.1% 9,10-dimethyl-1,2-benzanthracene were compared when the carcinogen was given three times at monthly intervals. Intense epidermal hyperplasia with atypicality of epidermal cells was observed in the mouse skin treated by the higher concentration of carcinogen.

6. The effect of nucleic acid precursors, particularly orotic acid and thymine, on the initiation of epidermal carcinogenesis was investigated. It was found that administration of orotic acid in high concentration and of thymine induced a significant inhibitory effect on tumour formation.

7. The findings are discussed in the light of the theory of the two stage mechanism of carcinogenesis.

8. It is concluded that three applications of 1.5% or 0.1% 9,10-dimethyl-1,2-benzanthracene at intervals of one day, one week or one month do not induce a refractory state or a necrotizing effect in the skin, but that three injections of urethane at intervals of one week may induce a refractory state.

9. It is suggested that the promoting action of 1.5% 9,10-dimethyl-1,2-benzanthracene is similar to that of croton oil, and that a low concentration of the same carcinogen and urethane has little promoting action.

10. It is suggested that the promoting action of 1.5% 9,10-dimethyl-1,2-benzanthracene is partly related to the marked epidermal hyperplasia induced.

11. It is noted that though three injections of urethane given at daily or weekly intervals give rise to no obvious change in the skin of mice, the occurrence of extensive necrosis when croton oil is applied subsequently to the skin so treated show that the urethane did indeed produce some change in the host, which is manifested in the altered reaction of the skin.

12. It is suggested that the initiating action of 9,10-dimethyl-1,2-benzanthracene on mouse skin is similar to the carcinogenic action of urethane on lung, and its action might be related to the disturbance of desoxyribonucleic acid metabolism.

CLAIM OF ORIGINALITY

1. The experiments clarify the conflict as to whether initiation induces a refractory state in mouse skin or a necrotizing reaction. Several new observations have been made.

2. Mice given three applications of 9,10-dimethyl-1,2-benzanthracene at various intervals and then croton oil produced consistently more tumours than those given a single application of the same carcinogen and then croton oil. These findings show that 9,10-dimethyl-1,2-benzanthracene did not induce a refractory state and did not produce a serious necrotizing reaction.

3. Mice given three injections of urethane at weekly intervals and then croton oil produced about the same number of tumours as those given a single injection and then croton oil. This finding perhaps suggests that the injections of urethane induced a refractory state, but other explanations are possible.

4. Three injections of urethane given at daily or weekly intervals induce no obvious change in the skin, but extensive necrosis develops when croton oil is applied subsequently to the skin so treated. The results indicate that urethane is responsible for a changed sensitivity in the host.

5. The shortening of the latent period of papillomata induced by three applications of 1.5% 9,10-dimethyl-1,2-benzanthracene at intervals of one day, one week, or one month followed by croton oil has not been reported. The theory that the shortening of the latent period is due to the promoting power of the carcinogen and that failure to induce the shortening of the latent period by three injections of urethane or three applications of 0.1% 9,10-dimethyl-1,2-benzanthracene is due to poor promoting power, is put forward.

6. The theory that promoting action of 9,10-dimethyl-1,2-benzanthracene is partly related to its power to induce epidermal hyperplasia is proposed.

7. An estimation by a chemical method of the concentration of urethane in the blood after intraperitoneal injection has not previously been done on mice. The finding that urethane disappears from the blood about seven hours after intraperitoneal injection, and that the pattern of disappearance after a single or three injections was similar is original.

8. The supplemental administration of orotic acid or thymine during initiation by 9,10-dimethyl-1,2-benzanthracene reduced the tumour yield when croton oil followed. It is proposed that the initiating action of 9,10-dimethyl-1,2-benzanthracene is similar to the carcinogenic action

of urethane or methylcholanthrene on the lung. The results also give further evidence for the theory that the action of 9,10-dimethyl-1,2-benzanthracene is related to the disturbance of the synthesis or the metabolism of desoxyribonucleic acid in the epidermal cells.

BIBLIOGRAPHY

Allison,A.C. and Lightbown,J.W.: Inhibition of cellular respiration by co-carcinogenic fractions of croton oil.
Nature, 189: 892-895, 1961

Allsopp,C.B.: Effects of ultra-violet radiation on 3,4-benzpyrene. The carcinogenic activity of water-soluble photo-derivatives when used with croton oil as co-carcinogen.
Brit. J. Cancer, 5: 273 - 279, 1951

Altenburg,E.: The production of mutation by ultra-violet light.
Science, 78: 587, 1933

Archer,H.E., Chapman,L., Rhoden,E. and Warren,F.L.: The estimation of urethane in blood.
Biochem. J., 42: 58-59, 1948

Armitage,P. and Doll,R.: The age distribution of cancer and a multistage theory of carcinogenesis.
Brit. J. Cancer, 8: 1-12, 1954

Armitage,P. and Doll,R.: A two stage theory of carcinogenesis in relation to the age distribution of human cancer.
Brit. J. Cancer, 11: 161-169, 1957

Ashley,C.A.: Epithelial and stromal roles in carcinogenesis. I. Histological changes and ultimate fate of internally transplanted skin and component parts separated by trypsin digestion.
Arch. Path., 72: 86-97, 1961

Auerbach,C. and Robson,J.M.: Chemical production of mutation.
Nature, 157: 302, 1946

Avery,O.T., Macleod,C.M. and McCarthy,M.: Nature of the substance inducing transformation of pneumococcal types: Induction of transformation by desoxyribonucleic acid fraction isolated from Pneumococcus type III.
J. Exp. Med., 79: 137-157, 1944

Badger,C.M., Cook,J.W., Hewett,C.L., Kennaway,E.L., Kennawa,N.M. and Martin,R.H. The production of cancer by pure hydrocarbons VI.
Proc. Roy. Soc., 131B: 170-182, 1942

Ball,J.K. & McCarter,J.A.: A study of dose and effect in initiation of skin tumours by a carcinogenic hydrocarbon.
Brit. J. Cancer, 14: 577-590, 1960

Bauer,K.H.: Mutationstheorie der Geshwulst-Entstehung.
Springer-Verlag, Berlin, 1928

Bayon,H.: Epithelial proliferation induced by the injection of gas works tar.
Lancet, 2: 1579, 1912

Beck,S. and Peacock,P.: The latent carcinogenic action of 3,4-benzpyrene; Results of intermittent applications to the skin of mice.
Brit. J. Exper. Path., 21: 227 - 230, 1940

Beltz,R.E. and Applegate,R.L.: X-ray inhibition of enzyme changes associated with the DNA synthesis of liver regeneration.
Bioch. Biophys. Res. Comm. 1: 298-301, 1959

Bendich,A., Pahl,H.B. and Beiser,S.M.: Chromatographic fractionation of the deoxyribonucleic acids with special emphasis on the transforming factor of pneumococcus.
Cold Spring Harbor Symp. Quant. Biol., 21: 31-48, 1956

Berenblum,I.: Tumour formation following freezing with carbon dioxide snow.
Brit. J. Exper. Path., 10: 179 - 184, 1929

Berenblum,I.: Further investigations on the induction of tumours with carbon dioxide snow.
Brit. J. Exper. Path., 11: 208 - 211, 1930

Berenblum,I.: The carcinogenic action of croton resin.
Cancer Res., 1: 44-48, 1941a

Berenblum,I.: The mechanism of carcinogenesis. A study of the significance of carcinogenic action and related phenomena.
Cancer Res., 1: 807 - 814, 1941b

Berenblum,I.: 9,10-dimethyl-1,2-benzanthracene as a highly potent carcinogen for rabbit skin.
Cancer Res., 5: 265 - 268, 1945

Berenblum,I.: The carcinogenic action of 9,10-dimethyl-1,2-benzanthracene on the skin and subcutaneous tissue of the mouse, rabbit, rat and guinea pig.
J. Nat. Cancer Inst., 10: 167-174, 1949

Berenblum,I.: A speculative review: the probable nature of promoting action and its significance in the understanding of carcinogenesis.
Cancer Res., 14: 471 - 477, 1954.

Berenblum,I.: Circumstantial evidence pointing to differences between cancers in terms of etiologic factors.
Cancer Res., 16: 675 - 680, 1956

Berenblum,I.: Some new implications of the two stage mechanism in the study of skin carcinogenesis.
Ciba Foundation - Carcinogenesis
Churchill Ltd., 1959, 55 - 65.

Berenblum,I., Ben-Ishai,D., Haran-Ghera,N., Lapidot,A., Simon,E. & Trainin,N.: Skin initiating action and lung carcinogenesis by derivatives of urethane (ethyl carbamate) and related compounds.
Biochem. Pharmacol., 2: 168-176, 1959

Berenblum,I. and Haran-Ghera,N.: Significance of the sequence of initiating and promoting actions in the process of skin carcinogenesis in the mouse.
Brit. J. Cancer, 9: 268 - 271, 1955a

Berenblum,I. and Haran-Ghera,N.: The initiating action of ethyl carbamate (urethane) on mouse skin.
Brit. J. Cancer, 9: 453 - 456, 1955b

Berenblum,I. and Haran-Ghera,N.: A quantitative study of the systemic initiating action of urethane in mouse skin carcinogenesis.
Brit. J. Cancer, 11: 77 - 84, 1957a

Berenblum,I. and Haran-Ghera,N.: The induction of the initiating phase of skin carcinogenesis in the mouse by oral administration of 9,10-dimethyl-1,2-benzanthracene, 20-methylcholanthrene, 3,4-benzpyrene & 1,2,5,6-Dibenzanthracene.
Brit. J. Cancer, 11: 85 - 87, 1957b

Berenblum,I. and Haran-Ghera,N.: Papilloma formation in the forestomach of the mouse following oral administration of urethane (ethyl carbamate).
Cancer Res., 17: 329-331, 1957c

Berenblum,I., Kaye,A.M. and Trainin,N.: In vivo test for a carcinogenic metabolite in plasma of animals given injections of ethyl carbamate (urethane).
Cancer Res., 20: 38-43, 1960

Berenblum,I. and Schoental,R.: The apparent anticarcinogenic action of lanolin.
Cancer Res., 7: 390-392, 1947

Berenblum,I. and Shubik,P.: The role of croton oil applications, associated with a single painting of a carcinogen, in tumour induction of the mouse's skin.
Brit. J. Cancer, 1: 379 - 382, 1947a

Berenblum,I. and Shubik,P.: A new quantitative approach to the study of the stages of chemical carcinogenesis in the mouse's skin.
Brit. J. Cancer, 1: 383 - 391, 1947b

Berenblum,I. and Shubik,P.: An experimental study of the initiating stage of carcinogenesis, and a re-examination of the somatic cell mutation theory of cancer.
Brit. J. Cancer, 3: 109-117, 1949a

Berenblum,I. and Shubik,P.: The persistence of latent tumour cells induced in the mouse's skin by a single application of 9,10-dimethyl-1,2-benzanthracene.
Brit. J. Cancer, 3: 384-386, 1949b

Berenblum,I. and Trainin,N.: Possible two-stage mechanism in experimental leukaemogenesis.
Science, 132: 40-41, 1960

Bielka,H. and Graffi,A.: Untersuchen uber die leukemogene Wirkung von nucleinsauren aus Virus induziertem Leukamiegewebe.
Acta Biol. Med. Germanica, 3: 515-517, 1959

Bielschowsky,F.: Tumours of the thyroid produced by 2-acetyl-aminofluorene and allyl-thiourea.
Brit. J. Exper. Path., 25: 90-95, 1944

Bielschowsky,F.: Experimental nodular goiter.
Brit. J. Exper. Path., 26: 270-275, 1945

Bielschowsky,F.: The role of thyroxine deficiency in the formation of experimental tumours of the thyroid.
Brit. J. Cancer, 3: 547-549, 1949

Biesele,J.J.: Mitotic Poisons and the Cancer Problem, Amsterdam, 1958
Elsevier Publishing Co.

Billingham,R.E., Orr,J.W. and Woodhouse,D.L.: Transplantation of skin components during chemical carcinogenesis with 20-methylcholanthrene.
Brit. J. Cancer, 5: 417-432, 1951

Bloch,B.: Die experimentelle Erzeugung von Röntgen-Carcinomen beim Kaninchen, nebst allgemeinen Bermerkungen über die Genese der experimentellen Carcinome.
Schweiz. Med. Wschr., 57: 857-865, 1924

Bloch,B. and Dreifuss,W.: Uber die experimentelle Erzeugung von Carcinomen mit Lymphdrüsen und Lungenmetastasen durch Teer Bestandteile
Schweiz. Med. Wschr., 51: 1033-1037, 1921

Blum,H.F.: Carcinogenesis by Ultraviolet Light. Princeton University Press, 1959

Booth,J., Boyland,E. and Orr,S.F.D.: A spectroscopic study of the nature of the complexes of purines with aromatic compounds.
J. Chem. Soc., 598-603, 1954

Borrel,P.A.: Epithélioses infectueses et Epithéliomas.
Ann. Inst. Pasteur, 17: 81-118, 1903

Borrel,A.: Le problème étiologique du cancer.
Z. Krebsforsch., 7: 265-275, 1909

Bosc,F.J.: Les Epithéliomas parasitaires. La clavelée et l'Epithélioma claveleux.
Zbl. Bact. Parasit. Infek., (Orig.) 34: 413-420, 517-526, 666-674, 1903

Boveri,T.: The Origin of Malignant Tumours.
Jena, Fischer, 119, 1914
Transl. by Boveri,M.
Ballière, Tindall & Cox, London, 1929

Boyland, E.: Different types of carcinogens and their possible modes of action: Review
Cancer Res., 12: 77-84, 1952

Boyland,E.: Recent progress in carcinogenesis. Biochemical aspects.
Prog. Exp. Tumor Res., 1: 162-178, 1960

Boyland,E. and Green,B.: The interaction of polycyclic hydrocarbons and nucleic acids.
Brit. J. Cancer, 16: 507-517, 1962.

Boyland,E. and Horning,E.S.: The induction of tumours with nitrogen mustards.
Brit. J. Cancer, 3: 118-123, 1949

Boyland,E. and Koller,P.C.: Effects of urethane on mitosis in the Walker rat carcinoma.
Brit. J. Cancer, 8: 677-684, 1954

Boyland,E. and Rhoden,E.: The distribution of urethane in animal tissue, as determined by a microdiffusion method and the effect of urethane treatment on enzymes.
Biochem. J., 44: 528-531, 1949

Bresnick,E.: The effect of urethane upon the pyrimidine biosynthesis.
Fed. Proc., 19: 313, 1960

Brooks,S.C. and Baumann,C.A.: Skin sterol. X. Studies with certain hyperplasia inducing hydrocarbons
Cancer Res., 16: 357-363, 1956

Bryan,C.E., Skipper,H.E. and White,L.: Carbamates in the chemotherapy of leucaemia.
The distribution of radioactivity in tissues of mice following injection of carbonyl labelled urethane.
J. Biol. Chemist., 177: 941-950, 1949

Bryan,W.R. and Shimkin,M.B.: Quantative analysis of dose response data obtained with three carcinogenic hydrocarbons in strain C3H male mice.
J. Nat. Cancer Inst., 3: 503-531, 1943

Bryson,V.: Carbamate induced phage resistant mutants of E. Coli,
Proc. 8th Internat. Cong. Genet. Hereditas,
Suppl. 545, 1949

Burckhardt,H. and Müller,W.: Versuche zur Krebserzeugung durch lange fortgesetzte äusere Einwirkung auf das Gewebe.
Beit. Klin. Chir., 130: 364-373, 1923

Burdette,W.L.: The significance of mutation in relation to the origin of tumour: A review.
Cancer Res., 15: 201-226, 1955

Burrows,E., Hieger,I. and Kennaway,E.L.: The experimental production of tumours of connective tissue.
Amer. J. Cancer, 16: 57-67, 1932

Burrows,H., Hieger,I. and Kennaway,L.: Experiments in carcinogenesis; the effect of the subcutaneous and intraperitoneal injection of lard, olive oil and other fatty materials in rats and mice.
J. Path. Bact., 43: 419-426, 1936

Butler,J.A.V. and Davison,P.F.: Deoxyribonucleic protein, a genetic material.
Adv. Enzymol., 18: 161-190, 1957

Cabot,S., Shear,N., Shear,M.J., and Perrault,A.,: Studies in carcinogenesis. XI. Development of skin tumours in mice painted with 3,4-benzpyrene and creosote oil fraction.
Amer. J. Path., 16: 301-312, 1940

Calcutt,G.: Sulphydryl groups and tumour induction by chemical agents.
Brit. J. Cancer, 15: 673-681, 1961a

Calcutt,G.: A further study of the effects of polycyclic hydrocarbons and croton oil on mouse skin - SH levels
Brit. J. Cancer, 15: 855-859, 1961b

Calcutt,G. and Coates,J.: The sulphydryl levels of mouse skin during treatment with polycyclic hydrocarbons and croton oil.
Brit. J. Cancer, 15: 360-366, 1961

Carter,C.: Metabolism of purines and pyrimidines.
Ann. Rev. Biochem., 25: 123-246, 1956

Carr,J.G.: Production of mutations in mice by 1,2,5,6-dibenzanthracene.
Brit. J. Cancer, 1: 152-156, 1947

Carruthers,C.: Influence of heptaldehyde on carcinogenic action of methylcholanthrene.
Arch. Path., 30: 1184-1191, 1940

Carruthers,C.: Chemical studies on the transformation of mouse epidermis to squamous-cell carcinoma: A review.
Cancer Res., 10: 255-265, 1950

Chargaff,E. and Davidson, J.M.: The Nucleic Acids. Vol. I.
Academic Press, New York, 1955

Cherbuliez,E. and Bernhard,K.: Recherches sur la graine de croton. XI Sur le crotonoside (2-oxy-6 amino-purine-d-riboside)
Helv. Chim. Acta., 15: 467-471, 1932

Cohnheim,J.: Lectures on General Pathology. Inflammation.
pp. 242-382 London, 1889, The New Sydenham Society.

Conway,B.E., Gilbert,L. and Butler,J.A.V.: The action of ionizing radiation and of radiomimetic substances on deoxyribonucleic acid. III. The molecular weights of deoxyribonucleic acid degraded by X-ray and by treatment with a "nitrogen mustard".
J. Chem. Soc., 3421-3425, 1950

Cook,J.W., Hewett,C.L. and Hieger,I.: The isolation of a cancer producing hydrocarbon from coal tar.
J. Chem. Soc., 395-405, 1933

Cook,J.W. and Haslewood,G.A.D.: The synthesis of 5,6-dimethyl-1,2-benzanthraquinone, a degradation product of deoxycholic acid.
J. Chem. Soc. 428-433, 1934

Cook,J.W., Robinson,A.M. and Goulden,F.: Polycyclic aromatic hydrocarbons. Part XV. New homologues of 1,2-benzanthracene.
J. Chem. Soc., 393-396, 1937

Cooper,Z.K. and Reller,H.C.: Mitotic frequency in methylcholanthrene epidermal carcinogenesis in mice.
J. Nat. Cancer Inst., 2: 335-344, 1942

Court-Brown,W.M.: Role of genetic change in neoplasia
Brit. Med. J., 1: 961-964, 1962

Cowdry,E.V.: Experimental epidermal methylcholanthrene carcinogenesis in mice.
J. Invest. Dermat., 6: 15-40, 1945

Cowdry,E.V.: Epidermal Carcinogenesis.
Advances in Cancer Res., I: 57-101, 1951

Cowdry,E.V.: Cancer Cells.
W.B. Saunders Co., Philadelphia & London, 1955

Cowen,P.N.: Inhibition of the carcinogenic properties of urethane by pentose nucleotides.
Brit. J. Cancer, 3: 94-97, 1949

Cowen,P.N.: Skin grafting in the study of carcinogenesis.
Brit. J. Cancer, 13: 228-235, 1959

Cramer,W. and Stowell,R.E.: Carcinogenesis in the mouse's skin by the infrequent application at long intervals of methylcholanthrene.
Cancer Res., 1: 849-852, 1941

Cramer,W. and Stowell,R.E.: The early stages of carcinogenesis by 20-methylcholanthrene in the skin of mice. II Microscopic tissue change.
J. Nat. Cancer Inst., 2: 379-402, 1942

Cramer,W. and Stowell,R.E.: Skin carcinogenesis by a single application of 20-methylcholanthrene.
Cancer Res., 3: 36-42, 1943a

Cramer,W. and Stowell,R.E.: On the quantitative evaluation of experimental skin carcinogenesis by methylcholanthrene, the factors of dosage, time, spacing of applications and the multiplicity of the carcinogenic response.
Cancer Res. 3: 668-681, 1943b

Daels,F.: Contribution a l'étude du cancer expérimental.
Compt. Rend. Cong. Cancer 54 Strasburg, 1923

Daels,F. and Biltris,R.: Contribution a l'étude de la provacation de tumeurs malignes expérimentales au moyen de substances radio-actives.
Bull. Assoc. Franc. l'étude du cancer 20: 32, 1931

Dammert,K.: The effect of five ulcerating doses of alkyldimethylbenzylammoniumchloride (Zephiran) on the tumour promoting action of polyoxyethylene sorbitan monostearate (Tween 60).
Acta Path. Microbiol. Scand., 53: 22-32, 1961a

Dammert,K.: A histological and cytological study of different methods of skin tumorigenesis in mice.
Acta Path. Microbiol. Scand., 53: 33-49, 1961b

Danielli,J.F.: Separate nuclear and cytoplasmic actions of drugs
Nature, 170: 1042-1044, 1952

Dao,T.L.: Mammary carcinogenesis by 3-methylcholanthrene. I. Hormone aspects in tumour induction and growth.
J. Nat. Cancer Inst., 23: 567-585, 1959

Daoust,R.: Mitotic activity in mouse epidermis during the induction periods of papilloma.
Acta Path. Microbiol. Scand., 45: 159-166, 1959

Darlington,C.D.: The plasmagene theory of the origin of cancer.
Brit. J. Cancer, 2: 118-126, 1948

Darlington,C.D.: Plasmagene theory and cancer genesis. in Genetics and Cancer, pp. 9-24, The University of Texas M.D. Anderson Hospital and Tumor Institute, Austin, 1959, University Texas Press.

Davibhadhana,B.: Numerical proportions of different cells in mouse epidermis during early methylcholanthrene carcinogenesis.
Cancer Res., 12: 165-169, 1952

Davidson,J.D. and Freeman,B.B.: Effect of anti-tumour drugs upon P^{131} incorporation into nucleic acids of mouse tumours.
Cancer Res., 15: 31-37, 1955

Deelman,H.T.: Uber experimentelle maligne Geschwülste durch Teereinwirkung bei Mäusen.
Z. Krebsforsch., 18: 261-284, 1922

Deelman,H.T.: Uber die Histogenese des Teerkrebses.
Z. Krebsforsch., 19: 125-170, 1923

Deelman,H.T.: Beobachtungen an experimentellen Tumorwachstum. I. Uber den Zusammenhang zwischen Regeneration und Tumor-bildung.
Z. Krebsforsch., 21: 220-226, 1924

Deelman,H.T. and Van Erp, J.P.: Die Entstehung des experimentellen Teerkrebbes und Bedeutung der Zellenregeneration.
Z. Krebsforsch., 24: 86-98, 1926

Della Porta,G., Shubik,P., Dammert,K. and Terracini,B.: Role of polyoxyethylene sorbitan monostearate in skin carcinogenesis in mice.
J. Nat. Cancer Inst., 25: 607-625, 1960

Demerc,M.: Mutations induced by carcinogens.
Brit. J. Cancer, 2: 114-117, 1948

Deringer,M.K.: Response of Strain HR/De mice painting with urethane.
J. Nat. Cancer Inst., 29: 1107-1121, 1962

Derom,E.: Influence de la température sur la production du cancer expérimental du goudron chez la souris.
Bull. Ass. Franc. Cancer, 13: 422-464, 1924

DiMayorca,G.A., Eddy,B.E., Stewart,S.E., Hunter,W.S., Friend,C. and Bendich,A.: Isolation of infectious desoxyribose nucleic acid from SE polyoma-infected tissue culture.
Proc. Nat. Acad. Sci., 45: 1805-1808, 1959

Dipaolo,J.A.: Influence of altered atmospheric oxygen on urethane induced pulmonary tumours in mice.
J. Nat. Cancer Inst., 23: 535-540, 1959

Doell,R.G.: Urethane induction of thymic lymphoma in C 57bI mice.
Nature, 194: 588-589, 1962

Doniach,I. and Mottram,J.C.: On the effect of light upon the incidence of tumours in painted mice.
Amer. J. Cancer, 39: 234-240, 1940

Dormanns,E.: Uber die Einflüsse sehr schwacher Bestrahlungen mit ultravioletten Licht in Kombination mit Teerungen.
Z. Krebsforsch., 40: 577-584, 1934

Drysdale,R.B., Hopkins,A., Thomson,R.Y., Smellie,R.M. and Davidson,J.N.: Some effects of nitrogen and sulphur mustards on the metabolism of nucleic acids in mammalian cells.
Brit. J. Cancer, 12: 137-148, 1958

Duran-Reynolds,M.L.: Carcinogenesis in cortisone treated mice following vaccina dermal infection and application of methylcholanthrene.
J. Nat. Cancer Inst., 29: 635-651, 1962

DuRuisseau,J.P. and Quastel,J.H.: Effects of croton oil on sugar absorption by isolated surviving guinea pig intestine.
Proc. Soc. Exp. Biol. Med., 100: 711-714, 1959

Dustin,P.: The cytological action of ethyl carbamate (urethane) and other carbamic esters in normal and leukaemic mice and in rabbits.
Brit. J. Cancer, 1: 48-59, 1947

Elion,G.B., Bieber,S., Nathan,H. and Hitchings,G.H.: Uracil antagonism and inhibition of mammary adenocarcinoma
Cancer Res., 18: 802-817, 1958

Ellerman,V. and Bang,O.: Experimentellen Leukamie bei Hühnern.
Zbl. Bakt. Parasit. Infek., (Orig.) 46 595-609, 1908

Evensen,A.: Changes in the synthesis of desoxyribonucleic acid (DNA) and in mitotic count in the epidermis of hairless mice after a single application of one per cent 3-methylcholanthrene. Acta Path. Microbiol. Scand. Suppl. 148: 43-51, 1961

Evensen,A.: Effects of carcinogens on cell proliferation. Acta Path. Microbiol. Scand. Suppl. 156: 95-143, 1962

Ewing,J.: Neoplastic Diseases; A Treatise on Tumors. Philadelphia, 1940, W.B. Saunders Co.

Falks,H.L., Steiner,P.E., Goldfein,S., Breslow,A. & Hyks,R.: Carcinogenic hydrocarbons and related compounds in processed rubber. Cancer Res., 11: 318-324, 1951

Falks,H.L., and Steiner,P.E.: The identification of aromatic polycyclic hydrocarbons in carbon blacks. Cancer Res., 12: 30-39, 1952

Feulgen,R. and Rossenbeck,H.: Mikroskopisch, chemischer Nachweis einer Nucleinsäure von Typus der Thymonuclein-Zellkeren in mikroskopischen Präparaten. Z. Physiol. Chem., 135: 203-243, 1924

Fiala,S., Fiala,A. and Glinsmann,W.: Mechanism of carcinogenesis and proliferation of tumour cells in rat liver. Pathologie Biologie, 9: 613-616, 1961

Fibiger,J. and Bang,F.: Production expérimentale du cancer du goudron chez la souris blanche. Compt. Rend. Soc. Biol., 83: 1157-1160, 1920

Fieser,L.F.: Chemistry of Natural Products related to Phenanthrene. New York, 1936

Fieser,L.F.: Carcinogenic activity, structure, and chemical reactivity of polynuclear aromatic hydrocarbons. Amer. J. Cancer, 34: 37 - 124, 1938

Fieser,L.F., Fieser,M., Hershberg,E.B., Newman,M.S., Seligman,A.M. and Shear,M.J.: Carcinogenic activity of the cholanthrenes and of other 1,2-benzanthracene derivatives. Amer. J. Cancer, 29: 260-268, 1937

Findlay,G.M.: The experimental production of cancer by one application of tar. Lancet, i: 714-715, 1925

Findlay,G.M.: Ultraviolet light and skin cancer. Lancet, 215: 1070-1073, 1928

Findlay,G.M.: Cutaneous papillomata in the rat following exposure to ultraviolet light.
Lancet, 218: 1229-1231, 1930

Fink,K. and Fink,R.M.: Effect of pyrimidines on growth of spontaneous lung tumours.
Proc. Amer. Ass. Cancer Res., 2: 16, 1955

Fisher,J.C. and Holloman,J.H.: A hypothesis for the origin of cancer.
Cancer, 4: 916-918, 1951

Fraenkel-Conrat,H., Singer,B. and Williams,R.C.: Infectivity of viral nucleic acid.
Biochem. Biophys. Acta, 25: 87-96, 1957

Frie,J.V. and Ritchie,A.C.: Epidermal carcinogenesis by carcinogen followed by various concentrations of croton oil.
J. Nat. Cancer Inst., 29: 687-697, 1962

Frieben: In Vereine und Kongresse, Arzl. Verein Hamburg, 21 X
Fortschr. Geb. Röntgenst., 6: 106, 1902

Friedewald,W.F. and Rous,P.: The initiating and promoting elements in tumour production. An analysis of the effect of tar, benzpyrene and methylcholanthrene on rabbit skin.
J. Exper. Med., 80: 101-125, 1944a

Friedewald,W.F. and Rous,P.: The determining influence of tar, benzpyrene and methylcholanthrene on the character of the benign tumours induced therewith in rabbit skin.
J. Exper. Med., 80: 127-144, 1944b

Friedewald,W.F. and Rous,P.: The pathogenesis of deferred cancer. A study of the after effects of methylcholanthrene upon rabbit skin.
J. Exper. Med., 91: 457-484, 1950

Fujinami,A. and Inamoto,K.: Uber Geschwülste bei japanischen Haushühnern, inbesondere über einen transplantablen Tumor.
Z. Krebsforsch., 14: 94-119, 1914

Gellhorn,A.: The cocarcinogenic activity of cigarette tobacco tar.
Cancer Res., 18: 510-517, 1958

Gillman,T., Hathorn,M. and Penn,J.: Apparent paradoxical effect of lanolin on induction of skin and lung tumours by topically applied methylcholanthrene.
Brit. J. Cancer, 10: 384-393, 1956

Glinos,A.D., Bucher,N.L.R. and Aub, J.C.: The effect of liver regeneration on tumour formation in rats fed 4-dimethyl-aminoazo-benzene.
J. Exper. Med., 93: 313-324, 1951

Glücksmann,A.: The histogenesis of benzpyrene-induced epidermal tumours in the mouse
Cancer Res., 5: 385-400, 1945

Gödel,A.: Kritisches Sammelreferat uber das experimentelle Terrkarcinom.
Deutsch. Med. Wschr., 49: 1284-1285, 1315-1316, 1923

Goldthwait,D.A.: Effect of nitrogen mustard on nucleic acid metabolism.
Proc. Soc. Exp. Biol. Med., 80: 503-504, 1952

Goldthwait,D.A.: Nucleic acid and cancer.
Amer. J. Med., 29: 1034-1059, 1960

Goulden,F. and Tipler,M.M.: Experiments on the identification of 3,4-benzpyrene in domestic soot by means of the fluorescene spectrum.
Brit. J. Cancer, 3: 157-160, 1949

Graffi,A.: Versuche über den Wirkungsmechanismus der cancerogenen Kohlenwasserstoffe. Unterschiedliche Reaktionsweise topographisch verschiedener Felder der Kaninchenhaut auf cancerogene Kohlenwasserstoffe.
Arch. Geschwulstforsch., 3: 217-221, 1951

Graffi,A., Scharsach,F. and Heyer,E.: Zur Frage der Initialwirkung cancerogener Kohlenwasserstoffe auf die Mäusehaut nach intravenoser, intraperitonealer und oraler Applikation.
Naturwissenshaften, 42: 181, 1955

Graffi,A., Vlamynch,E., Hoffman,F. and Schulz,I.: Untersuchungen über die geschwulstauslösend Wirkung verschiedener chemischer Stoffe in der Kombination mit Crotonöl.
Arch. Geschwulstforch., 5: 110-126, 1953

Green,H.N.: An immunological concept of cancer: A preliminary report.
Brit. Med. J., 2: 1374-1380, 1954

Green,H.N.: The immunological theory of cancer.
Acta Unio. Internat. Contra Cancrum, 17: 215-232, 1961

Greenstein,J.P.: Biochemistry of Cancer.
Academic Press, 2nd ED., New York, 1954

Guyer,M.F. and Claus,P.E.: Effects of urethane (Ethyl carbamate) on mitosis.
Proc. Soc. Exper. Biol. Med., 64: 3 - 5, 1947

Gwynn,R.H.: Tumour promoting action of croton oil fractions.
Brit. J. Cancer, 9: 445 - 452, 1955

Gwynn,R.H.: Combined effect of initiating dose of DMBA and urethane.
Ann. R. Brit. Emp. Cancer Campgn., 34: 192, 1956

Gwynn,R.H., and Salaman,M.H.: Studies on co-carcinogenesis. SH reactors and other substances tested for co-carcinogenic action in mouse skin.
Brit. J. Cancer, 7: 482-489, 1953

Haddow,A.: Transformation of cells and viruses.
Nature, 154: 194-199, 1944

Haddow,A. and Robinson,A.M.: The association of carcinogenicity and growth inhibitory power in the polycyclic hydrocarbons and other substances.
Proc. Roy. Soc. (Biol.), 127: 277-287, 1939

Haddow,A. and Saxton,W.A.: Influence of carbamic esters (urethane) on experimental animal tumours.
Nature, 157: 500 - 503, 1946

Hadler,H.I., Darchun,V. and Lee,K.: The interaction of polynuclear hydrocarbons with mouse skin protein.
J. Nat. Cancer Inst., 22: 661-668, 1959

Haenszel,W., Loveland,D.B. and Sirken,M.G.: Lung cancer mortality as related to residence and smoking histories. I. White males.
J. Nat. Cancer Inst., 28: 947-1001, 1962

Haga,I.: Experimentelle Untersuchungen über die Erzeugung atypischer Epithel und Schleimhautwucherungen.
Z. Krebsforsch., 12: 525-576, 1913

Hall,W.H.: The role of initiating and promoting factors in the pathogenesis of tumours of the thyroid.
Brit. J. Cancer, 2: 273 - 280, 1948

Hall,W.H. and Bielschowsky,F.: The development of malignancy in experimentally induced adenomata of thyroid.
Brit. J. Cancer, 3: 534-541, 1949

Hanau,A.: Erfolgreiche experimentelle Uebertragung von Carcinom.
Fortschr. Med., 7: 321-339, 1889

Haran-Ghera,N.: The role of mammotropin in mammary tumour induction in mice.
Cancer Res., 21: 790-795, 1961

Haran-Ghera,N. and Berenblum,I.: The induction of the initiating phase of skin carcinogenesis in the mouse by oral administration of urethane (ethyl carbamate).
Brit. J. Cancer, 10: 57-60, 1956

Hartwell,J.L.: Survey of Compounds Which Have Been Tested for Carcinogenic Activity.
Nat. Cancer Inst., Bethesda, Md., 1941

Hartwell,J.L.: Survey of Compounds Which Have Been Tested for Carcinogenic Activity.
Nat. Cancer Inst., Bethesda, Md., 2nd ED., 1951

Hawkins,J.A. and Murphy,J.B.: The effect of ethyl urethane anaesthesia on the acid-base equilibrium and cell contents of the blood.
J. Exper. Med., 42: 609-618, 1925

Heidelberger,C.: The relation of protein binding to hydrocarbon carcinogenesis. Ciba Foundation Symposium on Carcinogenesis. pp. 179-192, J. & A. Churchill Ltd. 1959

Heidelberger,C. and Davenport,R.: Local functional component of carcinogenesis.
Acta Unio. Internat. Contra Cancrum, 17: 55-62, 1961

Heidelberger,C. and Moldenhauer,M.: Quantitative studies on the binding of various hydrocarbons to the protein of the skin of mice.
Proc. Amer. Ass. Cancer Res., 2: 24, 1955

Henshaw,P.S. and Meyer,H.L.: Minimal number of anaesthetic treatments with urethane required to induce pulmonary tumours.
J. Nat. Cancer Inst., 4: 523-525, 1944

Henshaw,P.S. and Meyer,H.L.: Further studies on urethane induced pulmonary tumours.
J. Nat. Cancer Inst., 5: 415-417, 1945

Hieger,I.: The spectra of cancer-producing tars and oils and of related substances.
Biochem. J., 24: 505-511, 1930

Hieger,I.: On the mechanism of carcinogenesis by chemical compounds
Amer. J. Cancer, 28: 522-529, 1936

Hieger,I.: Carcinogenesis. New York, 1961, Academic Press.

Holsti,P.: Tumour promoting effects of some long chain fatty acids in experimental skin carcinogenesis in mouse. Acta Path. Microbiol. Scand., 46: 51-58, 1959

Holsti,P.: Influence of frequency of application on the tumour promoting effect of croton oil. Acta Path. Microbiol. Scand., 48: 316-318, 1960

Homburger,F. and Fishman,W.H.: The Physiopathology of Cancer. Hoeber-Harper, New York, 1959

Horton,A.W., Denman,D.T. and Trosset,R.P.: Carcinogenesis of the skin. II accelerating properties of aliphatic and related hydrocarbons. Cancer Res., 17: 758-766, 1957

Houck,J.C., Jacob,R.A. and Vickero,R.: Dermal chemical changes with local inflammation and necrosis. Amer. J. Path., 40: 531-541, 1962

Huxley,J.: Biological Aspects of Cancer. George Allen & Unwin, Ltd., London, 1958

Ito,Y. and Evans,C.A.: Induction of tumours in domestic rabbits with nucleic acid preparation from partially purified Shope papilloma virus and from extracts of the papillomas of domestic and cottontail rabbits. J. Exp. Med., 114: 485-500, 1961

Iversen,O.H.: Cell metabolism in experimental skin carcinogenesis. I. Effects of benzene and some polycyclic hydrocarbons on the endogenous dehydrogenese activity in the epidermis and the stripped skin in hairless mice studied by means of a tetrazolium reduction method. Acta Path. Microbiol. 50: 17-24, 1960

Iversen,O.H.: Effects of carcinogens on mitochondrial function. in Experimental skin carcinogenesis in mice. Acta Path. Microbiol. Scand. Suppl. 156: 29-91, 1962.

Iversen,S. and Edelstein,J.M.: On the mechanism of experimental carcinogenesis, the early mitotic effect of 9,10-dimethyl-1,2-benzanthracene and of 1,2,5,6- dibenzanthracene on epidermal cells of the mouse ear. Acta Path. Microbiol. Scand., 30: 213-222, 1952

Jaffe,W.G.: Carcinogenic action of ethyl urethane on rats. Cancer Res., 7: 107-112, 1947

Jonkhoff,A.R.: Röntgen Carcinom bei Mäusen. Z. Krebsforsch., 26: 32-41, 1927

Kandutsch,A.A. and Baumann,C.A.: Skin sterol, VIII. Effect of carcinogens, co-carcinogen and of certain hyperplastic agents.
Cancer Res., 15: 128-132, 1955

Kaplan,H.S.: Possible mechanisms of virus carcinogenesis.
Fed. Proc., 21: 1-4, 1962

Kawamoto,S., Kirshbaum,A., Ibanez,M.L., Trentin,J.J., and Taylor,H.E.: Influence of urethane on the development of spontaneous leukaemia and on the induction of hemangiomas in the AKR and the C58 strains of mice.
Cancer Res., 21: 71-74, 1961

Kawamoto,S., Kirschbaum,I.A. and Taylor,G.: Urethane and leukaemogenesis in mice.
Cancer Res., 18: 725-729, 1958

Kaye,A.M.: Chromatographic studies in relation to ethyl carbamate (urethane) carcinogenesis using C labelled compounds.
Cancer Res., 20: 44-49, 1960a

Kaye,A.M.: A study of the relationship between the rate of ethylcarbamate (urethane) catabolism and urethane carcinogenesis.
Cancer Res., 20: 237-241, 1960b

Kelly,L.S.: Effect of radiation on DNA synthesis in mammalian cells.
Prog. Biophys. Biophysic. Chem., 8: 144-163, 1957

Kennaway,E.L.: Experiments on cancer producing substances.
Brit. Med. J., II: 1-4, 1925

Kim,U. and Furth,J.: Relation of mammary tumours to mammotropes. I. Induction of mammary tumours in rats.
Proc. Soc. Exp. Biol. Med., 103: 640-642, 1960

Kit,S.: Nucleic acid synthesis in the neoplastic cell and impact of nuclear changes on the biochemistry of tumour tissue.
Cancer Res., 20: 1121-1148, 1960

Klärner,P. and Gieseking,R.: Zur Ultrastruktur des Lungentumors der Maus.
Z. Krebsforsch., 64: 7-21, 1960

Klein,G., Sjörgren,H.O., Klein,E. and Hellstrom,K.E.: Demonstration of resistance against methylcholanthrene induced sarcoma in the primary autochtonous host.
Cancer Res., 20: 1561-1572, 1960

Klein,M.: Influence of continued and intermittent painting with croton oil on skin tumourigenesis in mice.
J. Nat. Cancer Inst., 14: 83-90, 1953a

Klein,M.: The promoting action of croton oil in skin tumourigenesis.
Cancer Res., 13: 427-430, 1953b

Klein,M.: Induction of skin tumours in the mouse with minute dose of 9,10-dimethyl-1,2-benzanthracene alone or with croton oil.
Cancer Res., 16: 123-127, 1956

Klein,M.: A comparison of the initiating and promoting action of 9,10-dimethyl-1,2-benzanthracene and 1,2,5,6-dibenzanthracene in skin tumourigenesis.
Cancer Res., 20: 1179-1183, 1960

Kline,B.E. and Rusch,H.P.: Some factors that influence the growth of neoplastic cells.
Cancer Res., 4: 762-767, 1944

Knox,L.C.: Trauma and tumours.
Arch. Path., 7: 274-309, 1929

Kohn-Speyer,A.C.: Effect of ultraviolet radiation on the incidence of tar cancer in mice.
Lancet, 217: 1305-1306, 1929

Koller,P.C.: The movements of chromosomes within the cell and their dynamic interpretation.
Genetica, 16: 447-464, 1934

Koller,P.C.: Chromosome behaviour in tumours: Readjustment of Boveri's theory. in Cell Physiology of Neoplasia. pp. 9-37 University of Texas M.D. Anderson Hospital and Tumor Institute, Austin, 1960, Univ. Texas Press.

Kossel,A.: Uber die chemischer Zusammensetzung der Zelle.
Arch. Anat. Physiol., 49: 181-186, 1891

Lacassagne,A.: Apparition de cancers de la mammille chez la soris male, soumis a des injections de folliculine.
Compt. Rend. Acad. Sci., 195: 630-632, 1932

Lacour,F., Lacour,J., Havel,J. and Huppert,J.: Transplantable malignant tumours in mice induced by preparations containing ribonucleic acid extracted from human and mouse tumours.
J. Nat. Cancer Inst., 24: 301-327, 1960

Larsen,C.D.: Evaluation of the carcinogenicity of a series of esters of carbamic acid.
J. Nat. Cancer Inst., 8: 99-101, 1947

Lasnitzki,I.: Some effects of urethane on the growth and mitosis of normal and malignant cells in vitro.
Brit. J. Cancer, 3: 501 - 509, 1949

Latarjet,R.: Production d'une mutation bactérienne par des substances cancérigènes ou non.
Compt. Rend. Soc. Biol., 142: 453-455, 1948

Latarjet,R., Rebeyrotte,N. and Moustacchi,E.: Production de cancer multiples chez des sourisayant recu de l'acide nucléique extrait de tissus leucémiques isologues ou homologues.
Compt. Rend. Acad. Sci., 264: 853-855, 1958

Lathrop,A.E. and Loeb,L.: The influence of pregnancies on the incidence of cancer in mice.
Proc. Soc. Exp. Biol. Med., 11: 38-40, 1913

Law,J.O.: Regeneration and carcinogenesis in the liver.
Ann. R. Brit. Emp. Cancer Campgn., 34: 301-303, 1956

Law,L.W.: Multiple skin tumours in mice following a single painting with 9,10-dimethyl-1,2-benzanthracene.
Amer. J. Path., 17: 827 - 831, 1941

Leitch,A.: Paraffin cancer and its experimental production.
Brit. Med. J., 2: 1104-1106, 1922a

Leitch,A.: Experimental production of cancer by arsenic.
Brit. Med. J., 2: 1107-1108, 1922a

Leitch,A.: The experimental inquiry into cause of cancer.
Brit. Med. J., 2: 1-7, 1923

Liang,H.M.: Localized changes in methylcholanthrene treated epidermis.
Cancer Res., 8: 211-214, 1948

Lijinsky,W.: A tumour promoting principle from croton oil.
Biochem. J., 70: 5, 1958

Lindsay,D.: The action of urethane and of Tween 60 on the skin of mice.
Ann. R. Brit. Emp. Cancer Campgn., 34: 372, 1956

Lockhart-Mummery,J.P.: The Origin of Cancer.
J. & A. Churchill, London, 1934

Löwenthal,C.: Uber die traumatische Entstehung der Geschwülste.
Arch. Klin. Chir., 49: 1-100, 1895

Ludford,R.J.: Skin regeneration and cancer
Brit. J. Exper. Path., 10: 193 - 196, 1929

Luria,S.E.: Viruses, cancer cells and genetic concept of virus infection.
Cancer Res., 20: 677-688, 1960

Lwoff,A.: Lysogeny.
Bact. Rev., 17: 269-337, 1953

MacKenzie,I. and Rous,P.: The experimental disclosure of latent neoplastic changes in tarred skin.
J. Exper. Med., 73: 391 - 415, 1941

Maini,M.M. and Stich,H.F.: Chromosomes of tumour cells II. Effect of various liver carcinogens on mitosis of hepatic cells.
J. Nat. Cancer Inst., 26: 1413-1427, 1961

Maltoni,C. and Prodi,G.: Extension of two stage carcinogenesis to various tissues other than the skin: carcinogenesis in the rat liver. in Recent Contributions to Cancer Research in Italy. edited by P. Bucalossi and U. Veronesi, pp. 96-100, 1960. Casa Editrice Ambrosina, Milano.

Marchant,J. and Orr,J.W.: Further attempts to analyse the roles of epidermis and deeper tissue in experimental chemical carcinogenesis by transplantation and other methods.
Brit. J. Cancer, 7: 329 - 341, 1953

Marie,P., Clunet,J. and Raulot-Lapointe,G.: Nouveau cas de tumeur maligne provoquée par une radiodermite expérementale dans le rat blanc.
Bull. Ass. Franc. Cancer, 5: 125-135, 1912

McCarter,J.A.: The control of certain factors of dosage in epidermal carcinogenesis.
J. Nat. Cancer Inst., 17: 399 - 403, 1956

McCarter,J.A.: Tumour production in relation to benzpyrene in skin.
Acta Unio. Internat. Contra Cancrum, 15: 178-180, 1959

McCarter,J.A. Szerb,J.C. and Thompsom,G.E.: The influence of area of skin exposed, duration of exposure, and concentration in determining tumour yield in the skin of the mouse after a single application of carcinogenic hydrocarbon.
J. Nat. Cancer Inst., 17: 405 - 419, 1956

Meacham,G.G., Tillotson,F.W. and Heinle,R.W.: Liver damage after prolonged urethane therapy.
Amer. J. Clin. Path. 22: 22-27, 1952

Merenmies,L.: Zum Mechanismus der Hauttumobildung bei Mäusen untersucht mit Helfe einiger nichtionosierbaren oberflachenaktiven Tumorauslosersubstanzen. Acta
Acta Path. Microbiol. Scand. Suppl. 130: 1-107, 1959

Mider,G.B. and Morton,J.J.: Skin tumours following a single application of methylcholanthrene in C57 brown mice.
Amer. J. Path., 15: 299 - 302, 1939

Miescher,F.: Histochemische und Physiologische Arbeiten.
Leipzig, Germany, 1897

Miller,E.C.: Studies on the formation of protein bound derivatives of 3,4-benzpyrene in epidermal fraction of mouse skin.
Cancer Res. 11: 100-108, 1951

Miller,E.C. and Miller,J.A.: In vivo combination between carcinogens and tissue constituents and their possible role in carcinogenesis.
Cancer Res. 12: 547-556, 1952

Miller,J.A. and Miller,E.C.: The carcinogenic azo dyes.
Advances in Cancer Res. I: 339-396, 1953

Moodie,M.M., Reid,C. and Wallick,C.A.: Spectrometric studies of the persistence of fluorescent derivatives of carcinogens in mice.
Cancer Res. 14: 367-371, 1954

Morgan,T.H.: The Physical Basis of Heredity. Philadelphia, 1919
J.B. Lippincott Co.

Mori-Chavez,P.: Lung tumours induced at high altitude by urethane (ethyl carbamate) in strain A mice.
J. Nat. Cancer Inst. 29: 945-961, 1962

Mottram,J.C.: Production of epithelial tumours by a combination of beta radiation and painting with benzpyrene.
Amer. J. Cancer, 32: 76 - 79, 1938

Mottram,J.C.: A developing factor in experimental blastogenesis.
J. Path. Bact., 56: 181 - 187, 1944a

Mottram,J.C.: A sensitizing factor in experimental blastogenesis.
J. Path. Bact., 56: 391 - 402, 1944b

Müller,H.J.: Artificial transmutation of the gene.
Science, 66: 84 - 87, 1927

Murphy,E.D.: Promoting effect of estrogen in cervical carcinogenesis after limited exposure of endocervical epithelium of the mouse to methylcholanthrene.
Proc. Amer. Ass. Cancer Res., 3: 254, 1961

Murphy,J.B. and Strum,E.: The effect of urethane on lymphatic leukaemia in rats.
Science, 104: 427, 1946

Narat,J.K.: Experimental production of malignant growths by simple chemicals.
J. Cancer Res. 9: 135-147, 1925

Nettleship,A. and Henshaw,P.S.: Induction of pulmonary tumours in mice with ethyl carbamate (urethane).
J. Nat. Cancer Inst., 4: 309 - 319, 1943

Nordling,C.O.: A new theory on the cancer inducing mechanism.
Brit. J. Cancer, 7: 68 - 72, 1953

Oberling,C.: The Riddle of Cancer. translated by W.H. Woglom, New Haven, 1944, Yale University Press.

Odashima,S.: Development of liver cancers in the rat by 20-methylcholanthrene painting following initial 4-dimethylaminoazobenzene feeding.
Gann, 50: 321-345, 1959

Orr,J.W.: The influence of ischemia on the development of tumours.
Brit. J. Exper. Path., 15: 73 - 79, 1934

Orr,J.W.: The effect of interference with the vascular supply on the induction of dibenzanthracene tumours.
Brit. J. Exp. Path. 16: 121-126, 1935

Orr,J.W.: The changes antecedent to tumour formation during the treatment of mouse skin with carcinogenic hydrocarbons.
J. Path. Bact., 46: 495 - 515, 1938

Orr,J.W.: The histology and histogenesis of pulmonary adenomata of mice.
Brit. J. Cancer, 1: 316-322, 1947

Orr,J.W.: The early effects of 9,10-dimethyl-1,2-benzanthracene on mouse skin, and their significance in relation to the mechanism of chemical carcinogenesis.
Brit. J. Cancer, 9: 623 - 630, 1955

Orr,J.W.: The mechanism of chemical carcinogenesis, with particular reference to the time of development of irreversible changes in the epithelial cells.
Brit. Med. Bull., 14: 99-100, 1958

Orr,J.W.: Permutation of the stromal tissue during carcinogenesis.
Acta Unio. Internat. Cancr. 17: 64-70, 1961

Parodi,U.: Sulla produzione sperimentale del carcinoma da catrame.
Pathologica 15: 569-581 & 604-610, 1923

Paterson,E., ApThomas,I., Haddow,A. and Watkinson,J.M.: Leukaemia treated with urethane, compared with deep X-Ray therapy
Lancet, 250: 677-682, 1946

Peirce,W.E.H.: Tumour promotion by lime oil in the mouse forestomach
Nature 189: 497-498, 1961

Pietra,G., Rappaport,H. and Shubik,P.: The effects of carcinogenic chemicals in new-born mice
Cancer, 14: 308-317, 1961

Plaut,A. and Sobel,H.: Human sebum as vehicle for methylcholanthrene
Cancer Res., 9: 294-296, 1949

Potter,V.R.: Biochemical approach to cancer problem.
Fed. Proc. 17: 691-697, 1958

Pound,A.W.: The initiation of tumour formation by urethane in the mice.
Brit. J. Cancer 16: 246-256, 1962

Prehn,R.T.: Immunity to methylcholanthrene carcinogenesis.
Proc. Am. Ass. Cancer Res. 3: 53, 1959

Prehn,R.T.: Tumour-specific immunity to transplanted dibenz(ah)anthracene induced sarcoma.
Cancer Res. 20: 1614-1617, 1960

Prehn,R.T. and Main,J.M.: Immunity to methylcholanthrene-induced sarcoma.
J. Nat. Cancer Inst. 18: 769-778, 1957

Puck,T.: Action of radiation on mammalian cells. III. Relationship between reproductive death and induction of chromosome anomalies by X-irradiation of euploid human cells in vitro.
Proc. Nat. Acad. Sci. 44: 772-780, 1958

Pullinger,B.D.: The first effects on mouse skin of some polycyclic hydrocarbons.
J. Path. Bact., 50: 463-471, 1940

Pullinger,B.D.: Correlation between carcinogenic potency and the first skin reaction to certain hydrocarbons.
J. Path. Bact., 53: 287 - 288, 1941

Reller,H.C. and Cooper,Z.K.: Mitotic incidence in the first 48 hours of methylcholanthrene epidermal carcinogenesis.
Cancer Res. 4: 236-240, 1944

Révész,L.: Detection of antigenic differences in isologous host-tumour system by pretreatment with heavy irradiated tumour cells.
Cancer Res. 20: 443-451, 1960

Ritchie,A.C.: The effect of local injections of adrenalin on epidermal carcinogenesis in the mouse.
J. Nat. Cancer Inst., 12: 839 - 846, 1952

Ritchie,A.C.: Epidermal carcinogenesis in the mouse by intra-peritoneally administered urethane followed by repeated applications of croton oil.
Brit. J. Cancer, 11: 206 - 211, 1957

Ritchie,A.C., and Saffiotti,U.: Orally administered 2 acethylaminofluorene as an initiator and as a promotor in epidermal carcinogenesis in the mouse.
Cancer Res., 15: 84 - 88, 1955

Roe,F.J.C.: Tumour-initiating action of urethane and its inhibition by purine precursors.
Nature 175: 636-637, 1955

Roe,F.J.C.: The development of malignant tumours of mouse skin after initiating and promoting stimuli. I. The effect of a single application of 9,10-dimethyl-1,2-benzanthracene (DMBA), with and without subsequent treatment with croton oil.
Brit. J. Cancer, 10: 61 - 69, 1956a

Roe,F.J.C.: The development of malignant tumours of mouse skin after initiating and promoting stimuli. III. The carcinogenic action of croton oil.
Brit. J. Cancer, 10: 72 - 78, 1956b

Roe,F.J.C.: Tests of tumour promoting effect of croton oil on rabbit skin.
Ann. R. Brit. Emp. Cancer Campgn. 34: 191, 1956c

Roe,F.J.C.: Further investigation of the mechanisms of tumour initiation and tumour promotion.
Ann. R. Brit. Emp. Cancer Campgn. 36: part 2, 160 - 161, 1958

Roe,F.J.C. and Glendenning,O.M.: The carcinogenicity of beta-propiolactone for mouse skin.
Brit. J. Cancer, 10: 357 - 362, 1956

Roe,F.J.C. and Peirce,W.E.H.: Tumour promotion by citrus oils: Tumours of the skin and urethral orifice in mice.
J. Nat. Cancer Inst., 24: 1389 - 1403, 1960

Roe,F.J.C. and Peirce,W.E.H.: Tumour promotion by euphorbia latices.
Cancer Res., 21: 338 - 344, 1961

Roe,F.J.C. and Salaman,M.H.: A quantitative study of the power and persistency of the tumour initiating effect of ethyl carbamate on mouse skin.
Brit. J. Cancer, 8: 666 - 676, 1954

Roe,F.J.C. and Salaman,M.H.: Further studies on incomplete carcinogenesis; Triethylene melamine (T.E.M.), 1,2-benzanthracene and B propiolactone, as initiators of skin tumour formation in the mouse.
Brit. J. Cancer, 9: 177 - 203, 1955

Roe,F.J.C., Salaman,M.H. and Cohen,J.: Incomplete carcinogens in cigarette smoke condensate: Tumour promotion by phenolic fraction.
Brit. J. Cancer, 13: 623 - 633, 1959

Roffo,A.H.: Krebs und Sarcom durch Ultraviolet und Sonnenstrahlen.
Z. Krebsforsch., 41: 448-467, 1935

Rogers,S.: Studies of the mechanism of action of urethane in initiating pulmonary adenomas in mice.
II Its relation to nucleic acid synthesis.
J. Exper. Med. 105: 279 - 307, 1957a

Rogers,S.: Inhibitory influence of a normally occurring pyrimidine precursor upon methylcholanthrene carcinogenesis.
Proc. Soc. Exp. Biol. Med., 96: 464-465, 1957b

Rogers,S.: Comparative carcinogenesis.
Arch. Path., 70: 661 - 674, 1960

Rondoni,P. and Carbellini,A.: Lazione cancerogena delle dibenzo acridine e lustione come fattore realizzante.
Tumori 10: 106-118, 1936

Rous,P.: A transmissible avin neoplasm (Sarcoma of the common fowl)
J. Exper. Med., 12: 696 - 705, 1910

Rous,P.: A sarcoma of the fowl transmissible by an agent separable from the tumour cells.
J. Exper. Med., 13: 397 - 411, 1911

Rous,P. and Beard,J.W.: A virus induced mammalian growth with the characters of a tumour. (The shope rabbit papilloma.)
J. Exper. Med., 60: 741 - 764, 1934

Rous,P. and Beard,J.W.: The progression to carcinoma of virus induced rabbit papillomas, (shope).
J. Exper. Med., 62: 523 - 546, 1935

Rous,P. and Kidd,J.G.: Conditional neoplasm and subthreshold neoplastic state.
J. Exper. Med., 73: 365 - 390, 1941

Rundles,R.W. and Coonrad,E.V.: Treatment of multiple myeloma.
Proc. third Nat. Cancer Conf. Detroit, 1956 pp. 389-392
Philadelphia, Lippincott Co., 1957

Rusch,H.P.: Carcinogenesis: A facet of living process
Cancer Res., 14: 407 - 417, 1954

Rusch,H.P., Bosch,D. and Boutwell,R.K.: Influence of irritants on mitotic activity and tumour formation in mouse epidermis.
Acta Unio Internat. Cancrum, 11: 699-703, 1955

Rusch,H.P. and Kline,B.E.: Further evidence for successive stages in the formation of neoplasms.
Arch. Path. 42: 445-454, 1946

Rusch,H.P., Kline,B.E. and Bauman,C.A.: The nonadditive effect of ultraviolet light and other carcinogenic procedures.
Cancer Res., 2: 183-188, 1942.

Saffiotti,U. and Shubik,P.: The role of burning in carcinogenesis.
Brit. J. Cancer, 10: 54 - 56, 1956a

Saffiotti,U. and Shubik,P.: The effect of low concentration of carcinogen in epidermal carcinogenesis. A comparison with promoting agent.
J. Nat. Cancer Inst., 16: 961 - 969, 1956b

Salaman,M.H.: The latent period of co-carcinogenesis
Brit. J. Cancer, 6: 155 - 159, 1952

Salaman,M.H.: The use of co-carcinogens in the study of carcinogenesis
Ciba Foundation, Carcinogenesis,
Churchill Ltd., p. 70 - 78, 1959

Salaman,M.H.: Components of local carcinogenesis.
Acta Unio Internat. Contra Cancrum, 17: 12-19, 1961

Salaman,M.H. and Glendenning,O.M.: Tumour promotion in mouse skin by sclerosing agents.
Brit. J. Cancer, 11: 434-444, 1957

Salaman,M.H. and Gwynn,R.H.: The histology of co-carcinogenesis in mouse skin.
Brit. J. Cancer, 5: 252-263, 1951

Salaman,M.H. and Roe,F.J.C.: Incomplete carcinogens: Ethyl carbamate, (urethane) as an initiator of skin tumour formation in the mouse.
Brit. J. Cancer, 7: 472-481, 1953

Salaman,M.H. and Roe,F.J.C.: The development of malignant tumours of mouse skin after "initiating" and "promoting stimuli". II The influence of alternate applications of croton oil on malignant tumour production by repeated applications of diluted 9,10-dimethyl-1,2-benzanthracene (DMBA)
Brit. J. Cancer, 10: 70-71, 1956a

Salaman,M.H. and Roe,F.J.C.: The development of malignant tumours of mouse skin after initiating and promoting stimuli. IV Comparison of the effects of single and divided initiating doses of 9,10-dimethyl-1,2-benzanthracene
Brit. J. Cancer, 10: 79-88, 1956b

Salaman,M.H. and Roe,F.J.C.: Further test for tumour initiating activity; N,N-DI(2 chloroethyl)-P-aminophenylbutric acid (CB1348) as an initiator of skin tumour formation in the mouse.
Brit. J. Cancer, 10: 363-378, 1956c

Schmiedeberg,O.: Uber die pharmakolischen Wirkungen und die therapeutische Anwedung einiger Carbaminsauer-Ester
Arch. Exper. Path. Parmak., 20: 203-216, 1885

Schultz,J.: The role of somatic mutation in neoplastic growth, in Genetics and Cancer. pp. 25-42, The University of Texas M.D. Anderson Hospital and Tumor Institute, University Texas Press, Austin, 1959

Seelig,M.G. and Cooper,Z.K.: A review of the recent literature of tar cancer (1927 - 1931 inclusive).
Amer. J. Cancer, 17: 589-667, 1933

Setälä,H.: Tumour promoting and co-carcinogenic effects of some non-ionic lipohilic hydrophilic (surface active) agents.
Acta Path. Microbiol. Scand. Suppl. 115, 1956

Setälä,K.: Tumour production using polyethylene glycols as carriers for carcinogenic hydrocarbons.
Acta Path. Microbiol. Scand., 26: 280 - 295, 1949

Setälä,K.: Nature and mechanism of tumour promotion in skin carcinogenesis in mice.
Acta Unio. Internat. Contra Cancrum, 17: 32-41, 1961a

Setälä,K.: Die gegensätzliche Wirkung von Karzinogen und Tumorauslöser auf Keratinisierendes Gewebe (Mäuseepidermis).
Verhand Deut. Gesell. Path. 45Tag pp. 223-226, 1961b

Setälä,K., Merenmies,L., Stjernvall,L., Aho,Y., and Kajanne,P.: Mechanism of experimental tumorigenesis.
1. Epidermal hyperplasia in mouse caused by locally applied tumour initiator and dipole type tumour promotor.
J. Nat. Cancer Inst., 23: 925 - 951, 1959

Setälä,K., Merenmies,L., Niskanen,E.E., Nyholm,M. and Stjernvall,L.: Mechanism of experimental tumourigenesis. VI. Ultrastructural alterations in mouse epidermis caused by locally applied carcinogen and dipole-type tumour promotor.
J. Nat. Cancer Inst. 25: 1155 - 1166, 1960

Setälä,K., Merenmies,L., Stjernvall,L., Nyholm,M. and Aho,Y.: Mechanism of experimental tumourigenesis. V. Ultrastructural alterations in mouse epidermis caused by Span 60 and Tween 60 type agents.
J. Nat. Cancer Inst. 24: 355-385, 1960

Setälä,K., Niskanen,E.E., Merenmies,L., Nyholm,M., and Stjernvall,L.: Mechanism of experimental tumourigenesis.
8. Ultra-structural alterations in mouse epidermis caused by carcinogen administered orally.
J. Nat. Cancer Inst., 26: 985 - 1009, 1961

Setälä,K., Setälä,H., and Holsti,P.: A new and physicochemically well-defined group of tumour promoting (co-carcinogenic) agents for mouse skin.
Science, 120: 1075 - 1076, 1954

Shear,M.J.: Studies in carcinogenesis.
V Methyl derivatives of 1,2-benzanthracene.
Amer. J. Cancer, 33: 499 - 537, 1938

Shooter,K.V.: The physical chemistry of deoxyribonucleic acid.
Prog. Biophy. Biophys. Chem., 8: 309-346, 1957

Shope, R.E.: Infectious papillomatosis of rabbits.
J. Exper. Med., 58: 607 - 624, 1933

Shubik,P.: Studies on the promoting phase in the stages of carcinogenesis in mice, rats, rabbits and guinea pigs.
Cancer Res., 10: 13-17, 1950a

Shubik,P.: The growth potentialities of induced skin tumours in mice. The effect of different methods of chemical carcinogenesis.
Cancer Res., 10: 713-717, 1950b

Shubik,P., Baserga,R., and Ritchie,A.C.: The life and progression of induced skin tumours in mice.
Brit. J. Cancer, 7: 342-351, 1953

Shubik,P. and Della Porta,G.: Carcinogenesis and acute intoxication with large doses of polycyclic hydrocarbons.
Arch. Path., 64: 691-703, 1957

Shubik,P., Della Porta,G., Spencer,K. and Pietra,G.: The inhibition of 9,10-dimethyl-1,2-benzanthracene skin carcinogenesis by simultaneous application of urethane.
Proc. Amri. Ass. Cancer Res., 2: 345-346, 1958

Shubik,P., Goldfarb,A.R., Ritchie,A.C. and Lisco,H.: Latent carcinogenic action of beta irradiation on mouse epidermis.
Nature, 171: 934-935, 1953

Shubik,P. and Hartwell,J.L.: Survey of Compounds Which Have Been Tested for Carcinogenic Activity, Suppl. I.
Nat. Cancer Inst., Bethesda,Md., 1957

Shubik,P. and Ritchie,A.C.: Epidermal carcinogenesis in the mouse, induced by one, two or three applications of 9,10-dimethyl-1,2-benzanthracene, followed by repeated applications of croton oil.
Cancer Res., 13: 343-346, 1953

Shubik,P., Saffiotti,U., Feldman,R., and Ritchie,A.C.: Studies on promoting action in skin carcinogenesis.
Proc. Amer. Assoc. Cancer Res., 2: 146, 1956

Sicé,J.: Tumour promoting principles in seeds of croton tiglium L.
Arch. Int. Pharmacodyn., 65: 408-415, 1958

Simpson,W.L., Carruthers,C. and Cramer,W.: Loss of carcinogenic activity when methylcholanthrene is dissolved in anhydrous lanolin
Cancer Res., 5: 1-4, 1945

Simpson,W.L. and Cramer,W.: Sebaceous glands and experimental skin carcinogenesis in mice.
Cancer Res., 3: 515-518, 1943

Skipper,H.E., Bennet,L.L., Bryan,C.E., White,L., Newton,M.N. and Simpson,L.: Carbamate in the chemotherapy of leukaemia. VIII. Over all tracer studies on carbonyl labelled urethane, methyl labelled urethane, and methyl labelled ethyl alcohol.
Cancer Res., 11: 46-51, 1951

Spielmann,P.E.: The Constituents of Coal Tar. London, 1924

Stamer,S.: Further experimental studies on intravenous injection of 9,10-dimethyl-1,2-benzanthracene in mice (tolerance excretion effect on normal blood cells).
Acta Path. Microbiol. Scand., 22: 65-72, 1945

Stocks,P.: A study of the age curve for cancer of the stomach in connection with a theory of the cancer producing mechanism
Brit. J. Cancer, 7: 407-417, 1953

Strong,L.C.A.: Genetic analysis of the induction of mutation by methylcholanthrene. XI. Germinal mutations and other sudden biological changes following subcutaneous injection of methylcholanthrene.
Proc. Nat. Acad. Sci. 31: 290-293, 1945

Strong,L.C.: The induction of mutations by a carcinogen.
Brit. J. Cancer, 3: 97-108, 1949

Stowell,R.E. and Cramer,W.: The effects of solvents in methylcholanthrene epidermal carcinogenesis. A comparison of benzene and acetone.
Cancer Res. 2: 193-197, 1942

Svoboda,D.: Ultrastructure of pulmonary adenomas in mice.
Cancer Res., 22: 1197-1201, 1962

Takayama,S.: Effect of 4-nitroquinoline N-oxide painting on azo dye hepatocarcinogenesis in rats, with note on induction of skin fibrosarcoma.
Gann 52: 165-171, 1961

Tanaka,S. and Southam,C.M.: Joint action of West Nile virus and chemical carcinogens in production of papillomas in mice.
J. Nat. Cancer Inst., 29: 711-722, 1962

Tannenbaum,A.: The initiation and growth of tumours
I. Effects of underfeeding.
Amer. J. Cancer, 38: 335-350, 1940

Tannenbaum,A.: The genesis and growth of tumours. Effect of caloric restriction per se.
Cancer Res., 2: 460-467, 1942.

Tannenbaum,A.: The dependence of the genesis of induced skin tumours on the caloric intake during different stages of carcinogenesis.
Cancer Res. 4: 673-677, 1944a

Tannenbaum,A.: The importance of differential consideration of the stages of carcinogenesis in the evaluation of co-carcinogenic and anti-carcinogenic effects.
Cancer Res. 4: 678-682, 1944b

Tannenbaum,A.: Studies on urethane carcinogenesis.
Acta Un. Int. Cancer, 17: 72-87, 1961

Tannenbaum,A. and Silverstone,H.: Urethane as a multi-potential carcinogen.
Cancer Res. 18: 1225-1231, 1958

Tatum,E.L.: Chemically induced mutations and their bearing on carcinogenesis.
Ann. N.Y. Acad. Sci. 49: 87-97, 1947

Terracini,B., Shubik,P. and Della Porta,G.: A study of skin carcinogenesis in the mouse with single applications of 9,10-dimethyl-1,2-benzanthracene at different dosages.
Cancer Res. 20: 1538-1541, 1960

Tomatis, L., Terracini,B. and Shubik,P.: Effect of a single application of croton oil in skin carcinogenesis.
Proc. Soc. Exp. Biol. Med., 109: 18-20, 1962

Toth,B., Tomatis,L. and Shubik,P.: Multipotential carcinogenesis with urethane in the Syrian golden hamster.
Cancer Res., 21: 1537-1541, 1961

Taussig,J., Cooper,Z.K. and Seelig,M.G.: The effect of light on benzpyrene cancer in mice.
Surg. Gyn. Obst., 66: 989-993, 1937

Trams,E.G., Nadkarni,M.V. and Smith,P.K.: On the mechanism of the alkylating agents. I. Interaction of alkylating agents with nucleic acids.
Cancer Res., 21: 560-566, 1961a

Trams,E.G., Nadkarni,M.V. and Smith,P.K.: The mechanism of action of the alkylating agents. II. Effects of nitrogen mustard, myleran, and X-radiation on nucleic acid biosynthesis.
Cancer Res., 21: 567-570, 1961b

Tsutsui,H.: Uber das kunstlich erzeugte Cancroid bei der Maus.
Gann, 12: 17-21, 1918

Twort,J.M. and Twort,C.C.: Comparitive activity of some carcinogenic hydrocarbons.
Amer. J. Cancer, 35: 80-85, 1939

Vesselinovitch,S.D.: The effect of the time interval and sequence of the initiating and promoting stimuli on the tumour yield.
Cancer Res., 18: 1314 - 1318, 1958

Vesselinovitch,S.D. and Gilman,J.P.W.: Factors modifying experimental epidermal carcinogenesis in mice.
Cancer Res., 17: 52 - 55, 1957

Vogt,M.: Mutations auslassung bei Drosophila durch Aethylurethane
Experimentia, 4: 68 - 69, 1948

von Euler,H. and Hevesy,G.: Wirkung der Röntgenstrahlen auf den Umsatz der Nukleinsäure im Lensen Sarkom.
Kgl. Danske Vindenskab. Selskab. Biol. Medd.
17: 3-38, 1942.

von Hansemann,D.: Uber asymmetrische Zellteilung in Epithelialkrebsen underen biologische Bedeutung.
Virchows Arch., 119: 299-326, 1890.

Wacker,L. and Schmincke,A.: Experimentelle Untersuchungen zur kausalen Genese atypischer Epithelwucherungen.
München Med. Wscht., 58: 1607 - 1613, 1911

Waller,R.E.: The benzpyrene content of town air.
Brit. J. Cancer, 6: 8 - 21, 1952

Warburg,O.: Uber die Oxydationen in labenden Zellen nach Versuchen am Seeigelie.
Z. Physiol. Chem., 66: 305 - 342, 1910

Warburg,O.: On the origin of cancer cells.
Science 123: 309-314, 1956

Watson,A.F.: Recent researches in experimental carcinogenesis.
Cancer Rev., 7: 445 - 462, 1932

Watson,J.D. and Crick,F.H.C.: The structure of DNA
Cold Spring Harbor Symp. Quant. Biol. 18:
123-131, 1953

Weiss,W.G. and Heidelberger,C.: The interaction of carcinogenic hydrocarbons with tissue constituents. II. 1,2,5,6-dibenzanthracene 9,10-C in skin.
Cancer Res., 13: 250-254, 1953

Weiland,H. and Dane,E.: Untersuchungen über die Konstitution der Gallensäuren III. Uber die Haftstelle der Seitenkette.
Z. Physiol. Chem., 219: 240-244, 1933

Willis,R.A.: Pathology of Tumours, III ed.
Butterworth & Co., London, 1960

Woglom,W.H.: The Study of Experimental Cancer, A Review.
New York, 1913 Columbia Univ. Press.

Woglom,W.H.: Experimental tar cancer.
Arch. Path., 2: 533-576 & 709-752, 1926

Wolff,J.: Die Lehre von der Krebskrankheit von den Alsten Zeiten bis zur Gegenwart. Teil I. Jena Fischer, 1907

Woodhouse,D.L.: Experiments on interaction of polycyclic hydrocarbons with epidermal constituents.
Brit. J. Cancer 8: 346-352, 1954

Woodhouse,D.L.: Further investigations on interaction of polycyclic hydrocarbons with epidermal constituents.
Brit. J. Cancer 9: 418-425, 1955

Wynder,E.L. and Hoffmann,D.: A study of tobacco carcinogenesis. VIII. The role of acidic fractions as promotor.
Cancer 14: 1306-1315, 1961

Yamagiwa,K. and Ichikawa,K.: Experimental studies on the pathogenesis of epithelial tumours. The first report.
Tokyo Igakkai Zassi, 30: 1-43, 1916

Yamagiwa,K. and Ichikawa,K.: Uber die Künstliche Erzeugung von carcinom (IVte Mitteilung)
Gann, 11: 19-26, 1917

Yokoro,K. and Furth,J.: Relation of mammotrope to mammary tumour. V. Role of mammotropes in radiation carcinogenesis.
Proc. Soc. Exp. Biol. Med. 107: 921-924, 1961

Yokoro,K. and Furth,J.: Determining role of "mammotropins" in induction of mammary tumours in mice by virus.
J. Nat. Cancer Inst. 29: 887-909, 1962

Zilber,L.A.: Specific tumour antigen
Advances in Cancer Res. 5: 291-329, 1958

APPENDIX I

SUMMARY OF THE RESULTS OF EXPERIMENT I

APPENDIX I

TABLE I

SUMMARY OF RESULTS OF GROUP I (GAA)

Weeks of C.O. Treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors	Papillomata per survivor	Papillomata per Papilloma bearing survivor
0	60	0	0	0	0
1	60	0	0	0	0
2	59	0	0	0	0
3	59	0	0	0	0
4	59	0	0	0	0
5	58	1	1	0.02	1.0
6	58	6	4	0.10	1.50
7	56	19	8	0.34	2.38
8	56	44	13	0.79	3.46
9	51	79	19	1.55	4.16
10	50	91	21	1.82	4.33
11	47	114	23	2.43	4.95
12	44	109	22	2.48	4.95
13	43	117	26	2.71	4.50
14	41	134	23	3.27	5.84
15	39	133	24	3.41	5.54
16	36	128	24	3.56	5.33
17	36	132	23	3.67	5.74
18	35	137	25	3.97	5.48
19	34	145	24	4.26	6.04
20	32	157	25	4.90	6.28
21	31	145	25	4.68	5.80
22	28	128	21	4.54	6.10
23	27	123	20	4.56	6.15
24	24	120	19	5.00	6.32
25	19	99	15	5.21	6.60

APPENDIX I

TABLE I

SUMMARY OF RESULTS OF GROUP I (GAA)

Percentage of survivors bearing Papillomata	Number of new Papillomata appearing	Number of regressing Papillomata	Papillomata per survivor by increment	Papillomata per Papilloma bearing by increment
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
1.7	1	0	0.02	1.00
6.9	5	0	0.11	2.25
14.3	12	0	0.33	3.75
23.2	26	0	0.79	5.75
37.2	36	0	1.50	7.64
42.0	17	5	1.84	8.45
48.9	30	2	2.48	9.75
50.0	25	11	3.05	10.89
60.5	18	7	3.47	11.58
56.1	28	8	4.15	12.80
61.6	13	14	4.48	13.34
66.7	14	1	4.87	13.92
63.7	14	11	5.26	14.53
71.5	8	3	5.49	14.85
70.6	10	1	5.78	15.38
78.1	18	6	6.34	16.10
80.6	10	16	6.66	16.50
75.0	9	6	6.98	16.93
74.1	5	6	7.17	17.18
79.2	12	4	7.67	17.81
78.9	3	3	7.83	18.01

APPENDIX I

TABLE II

SUMMARY OF RESULTS OF GROUP II (GAB)

Weeks of Croton Oil Treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing Survivors	Papillomata per survivor	Papillomata per Papilloma bearing survivor
0	60	0	0	0	0
1	58	0	0	0	0
2	55	0	0	0	0
3	52	0	0	0	0
4	49	8	6	0.16	1.33
5	44	26	10	0.59	2.60
6	42	49	16	1.17	3.06
7	41	83	21	2.02	3.95
8	40	134	27	3.35	4.97
9	35	170	26	4.86	6.54
10	33	196	26	6.00	7.53
11	30	207	23	6.90	9.00
12	28	196	22	7.00	8.91
13	27	225	22	8.33	10.20
14	26	210	21	8.08	10.00
15	23	165	18	7.18	9.17
16	19	129	14	6.79	9.21
17	13	86	8	6.61	10.67
18	9	59	6	6.55	9.83
19	8	55	6	6.88	9.17
20	7	51	5	7.28	10.02
21	7	52	5	7.44	10.50
22	7	48	5	6.86	9.60
23	6	46	5	7.67	9.20
24	6	46	6	7.67	7.67
25	6	45	6	7.30	7.30

APPENDIX I

TABLE II

SUMMARY OF RESULTS OF GROUP II (GAB)

Percentage of survivors bearing Papillomata	Number of new Papillomata appearing	Number of Regressing Papillomata	Papillomata per survivor by increment	Papillomata per Papilloma bearing by increment
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
12.2	8	0	0.16	1.33
22.7	18	0	0.57	3.13
38.1	24	1	1.14	4.63
51.2	34	0	1.97	6.25
67.5	52	0	3.27	8.18
74.3	46	0	4.58	9.95
78.7	26	4	5.37	10.95
76.7	19	2	6.00	11.78
78.5	19	12	6.68	12.64
81.5	25	5	7.61	13.78
81.0	8	15	7.92	14.16
78.2	7	13	8.22	14.55
73.7	9	6	8.78	15.19
61.5	4	11	9.09	15.69
66.6	5	6	9.65	16.52
75.0	1	5	9.78	16.69
71.4	0	2	9.78	16.69
71.4	2	1	10.07	17.09
71.4	0	4	10.07	17.09
83.3	0	2	10.07	17.09
100.0	2	3	10.40	17.49
100.0	0	3	10.40	17.49

APPENDIX I

TABLE III

SUMMARY OF RESULTS OF GROUP III (GAC)

Percentage of survivors bearing Papillomata	Number of new Papillomata appearing	Number of regressing Papillomata	Papillomata per survivor by increment	Papillomata per Papilloma bearing by increment
0	0	0	0	0
0	0	0	0	0
9.3	8	0	0.15	1.60
21.7	11	0	0.39	2.70
36.6	25	0	0.98	4.37
51.4	51	1	2.44	7.20
63.6	54	2	4.08	9.77
68.8	30	0	5.02	11.13
75.0	35	6	6.11	12.58
75.8	32	7	7.21	14.03
76.0	18	5	7.93	14.97
76.0	20	5	8.73	16.02
76.0	17	9	9.41	16.91
79.1	13	5	9.95	17.59
87.0	18	2	10.73	18.49
76.5	6	13	11.16	18.95
78.5	4	17	11.45	19.31
77.7	1	6	11.56	19.45
71.4	0	2	11.56	19.45
71.4	0	7	11.56	19.45
71.4	0	6	11.56	19.45
83.3	1	6	11.73	19.65
83.3	1	4	11.90	19.85
80.0	0	2	11.90	19.85
80.0	0	3	11.90	19.85
80.0	0	0	11.90	19.85

APPENDIX I

TABLE IV

SUMMARY OF RESULTS OF GROUP IV (GAD)

Weeks of croton oil treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors	Papillomata per survivor	Papillomata per Papilloma bearing survivor
0	26	0	0	0	0
1	23	5	3	0.22	1.67
2	23	8	5	0.35	1.60
3	22	19	8	0.86	2.38
4	21	42	11	2.00	3.82
5	21	69	14	3.29	4.93
6	21	86	15	4.10	5.73
7	21	113	16	5.38	7.06
8	21	137	15	6.52	9.13
9	20	131	13	6.55	10.08
10	19	133	12	7.00	11.09
11	18	130	12	7.22	10.83
12	17	137	12	8.06	11.41
13	13	108	10	8.22	10.80
14	12	102	9	8.50	11.33
15	11	92	9	8.36	10.22
16	10	80	9	8.00	8.89
17	9	68	8	7.56	8.50
18	7	50	5	7.14	10.00
19	5	49	4	9.80	12.25
20	5	46	4	9.04	11.50
21	3	33	3	11.00	11.00
22	2	18	2	9.00	9.00
23	1	11	1	11.00	11.00
24	1	15	1	15.00	15.00
25	1	15	1	15.00	15.00

APPENDIX I

TABLE IV

SUMMARY OF RESULTS OF GROUP IV (GAD)

Percentage of survivors bearing Papillomata	Number of new Papillomata appearing	Number of regressing Papillomata	Papillomata per survivor by increment	Papillomata per Papilloma bearing by increment
0	0	0	0	0
13.0	5	0	0.22	1.67
21.7	3	0	0.35	2.27
36.0	13	0	0.94	3.90
52.4	21	0	1.94	5.81
67.0	28	1	3.27	7.81
71.5	21	4	4.27	9.21
76.2	37	0	6.03	11.52
71.5	26	1	7.27	13.25
65.0	5	10	7.52	13.63
63.1	12	9	8.15	14.63
66.6	13	1	8.87	15.71
70.5	11	4	9.52	16.62
76.9	6	2	9.98	17.22
75.0	13	2	11.06	18.66
81.8	2	12	11.24	18.88
90.0	0	12	11.24	18.88
88.9	1	6	11.35	19.01
71.3	1	2	11.49	19.21
80.0	2	2	11.89	19.71
80.0	2	5	12.29	20.21
100.0	0	4	12.29	20.21
100.0	0	3	12.29	20.21
100.0	1	0	13.29	21.21
100.0	4	0	17.29	25.21
100.0	0	0	17.29	25.21

APPENDIX I

TABLE V

SUMMARY OF RESULTS OF GROUP V (GAE)

Weeks of croton oil Treatment	Survivors	Total number Papillomata of survivors	Papillomata bearing survivors
0	60	0	0
1	60	0	0
2	59	0	0
3	59	0	0
4	58	0	0
5	58	0	0
6	57	0	0
7	54	0	0
8	53	0	0
9	51	0	0
10	49	0	0
11	48	0	0
12	47	0	0
13	43	0	0
14	41	0	0
15	40	0	0
16	39	0	0
17	38	0	0
18	35	0	0
19	31	1	1
20	30	1	1
21	26	1	1
22	25	1	1
23	22	0	0
24	22	0	0
25	21	0	0

APPENDIX I

TABLE VI

SUMMARY OF RESULTS OF GROUP VI (GAF)

Weeks of croton oil Treatment	Survivors	Total Number Papillomata of survivors	Papillomata bearing survivors
0	29	0	0
1	29	0	0
2	29	0	0
3	29	0	0
4	29	0	0
5	29	0	0
6	29	0	0
7	29	0	0
8	29	0	0
9	29	0	0
10	25	0	0
11	24	0	0
12	23	0	0
13	21	0	0
14	18	0	0
15	17	0	0
16	16	0	0
17	16	0	0
18	15	0	0
19	14	0	0
20	14	0	0
21	10	0	0
22	9	0	0
23	9	0	0
24	7	0	0
25	7	0	0

APPENDIX I

TABLE VII

SUMMARY OF RESULTS OF GROUP VII (GAG)

Weeks of Croton Oil Treatment	Survivors	Total number Papillomata of survivors	Papillomata bearing survivors
0	30	0	0
1	30	0	0
2	30	0	0
3	28	0	0
4	28	1	1
5	22	1	1
6	22	3	3
7	20	2	2
8	20	2	2
9	20	2	2
10	20	2	2
11	20	2	2
12	19	2	2
13	17	2	2
14	17	2	2
15	13	1	1
16	11	1	1
17	11	1	1
18	11	1	1
19	10	1	1
20	10	1	1
21	9	1	1
22	9	1	1
23	9	1	1
24	9	1	1
25	9	1	1

APPENDIX I

TABLE VIII

SUMMARY OF RESULTS OF GROUP VIII (GAH)

Weeks of croton oil Treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors	Papillomata per survivor
0	30	0	0	0
1	28	0	0	0
2	27	7	3	0.27
3	27	19	7	0.70
4	27	22	9	0.71
5	26	27	9	1.04
6	24	27	9	1.12
7	23	18	8	0.78
8	23	18	7	0.78
9	23	17	6	0.74
10	23	16	6	0.69
11	21	15	5	0.71
12	19	5	3	0.26
13	18	6	3	0.33
14	16	6	3	0.37
15	16	6	3	0.37
16	15	6	3	0.40
17	10	6	3	0.60
18	9	6	3	0.67
19	8	6	3	0.67
20	7	6	3	0.71
21	6	6	3	1.00
22	6	6	3	1.00
23	6	5	3	0.83
24	5	5	3	1.00
25	4	4	3	1.00

APPENDIX I

TABLE VIII

SUMMARY OF RESULTS OF GROUP VIII (GAH)

Papillomata per Papilloma bearing survivor	Percentage of survivors bearing Papillomata	Number of new Papillomata appearing	Number of regressing Papillomata
0	0	0	0
0	0	0	0
2.33	11.1	7	0
2.71	26.9	12	0
2.44	29.6	3	0
3.00	34.6	6	1
3.00	37.5	0	0
2.25	34.7	0	1
2.57	30.4	1	0
2.83	26.0	0	0
2.67	26.1	0	1
3.00	23.8	0	0
1.67	15.8	0	0
2.00	16.7	0	0
2.00	18.7	0	0
2.00	18.7	0	0
2.00	20.0	0	0
2.00	33.0	0	0
2.00	30.0	0	0
2.00	37.5	0	0
2.00	42.9	0	0
2.00	50.0	0	0
2.00	50.0	0	0
2.00	50.0	0	0
1.67	60.0	0	0
1.33	75.0	0	0

APPENDIX I

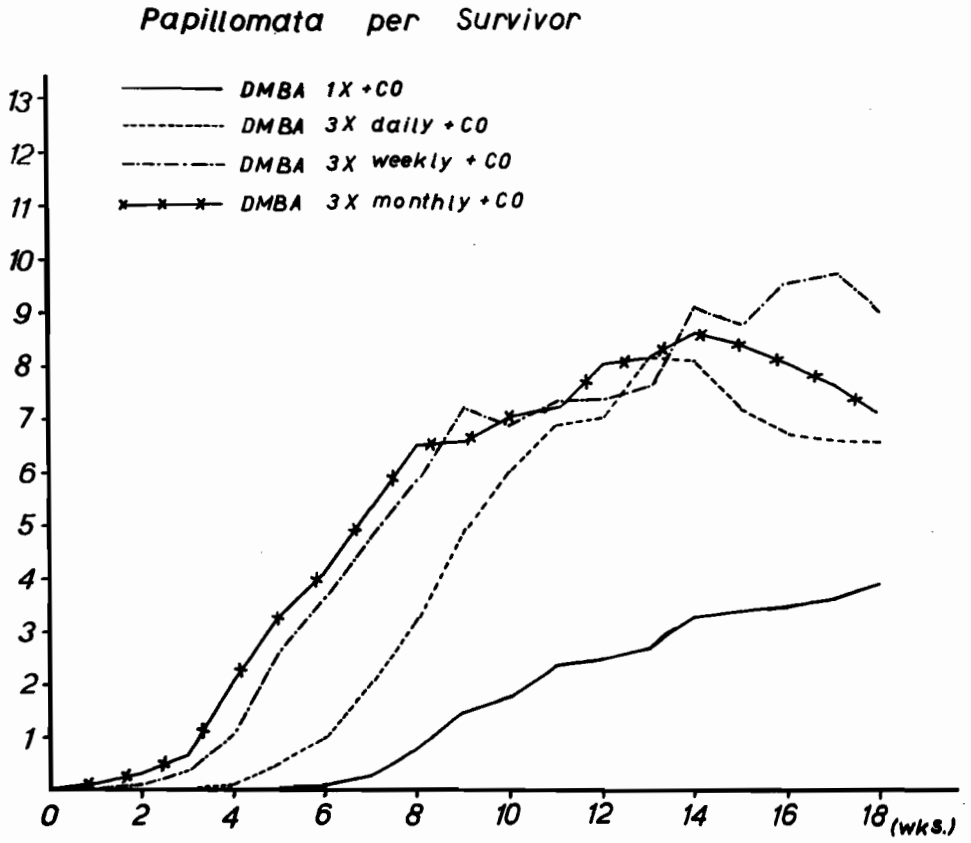
TABLE IX

SUMMARY OF RESULTS OF GROUP IX (GAI)

Weeks of croton oil Treatment	Survivors	Total number Papillomata of survivors	Papillomata bearing survivors
0	29	0	0
1	29	0	0
2	29	0	0
3	29	0	0
4	28	0	0
5	28	0	0
6	26	0	0
7	20	0	0
8	19	0	0
9	18	0	0
10	16	0	0
11	14	0	0
12	13	0	0
13	13	1	1
14	13	1	1
15	13	1	1
16	10	1	1
17	8	1	1
18	8	1	1
19	8	1	1
20	6	1	1
21	6	1	1
22	6	1	1
23	5	1	1
24	5	1	1
25	4	1	1

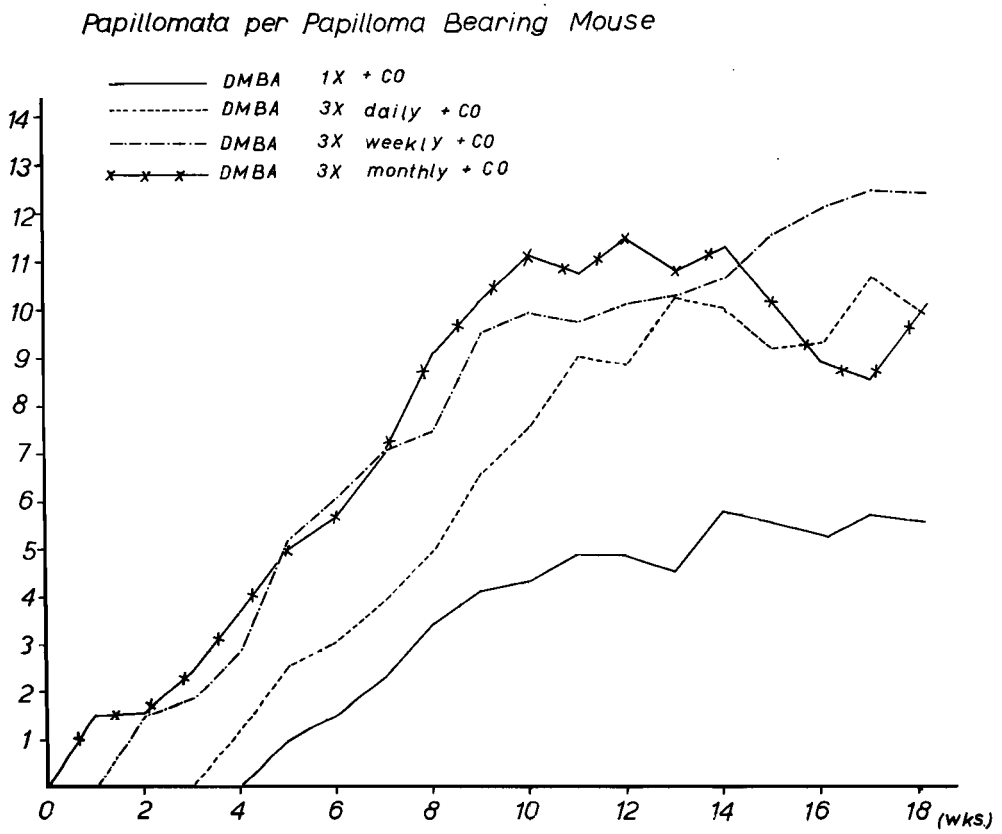
APPENDIX I

FIGURE I



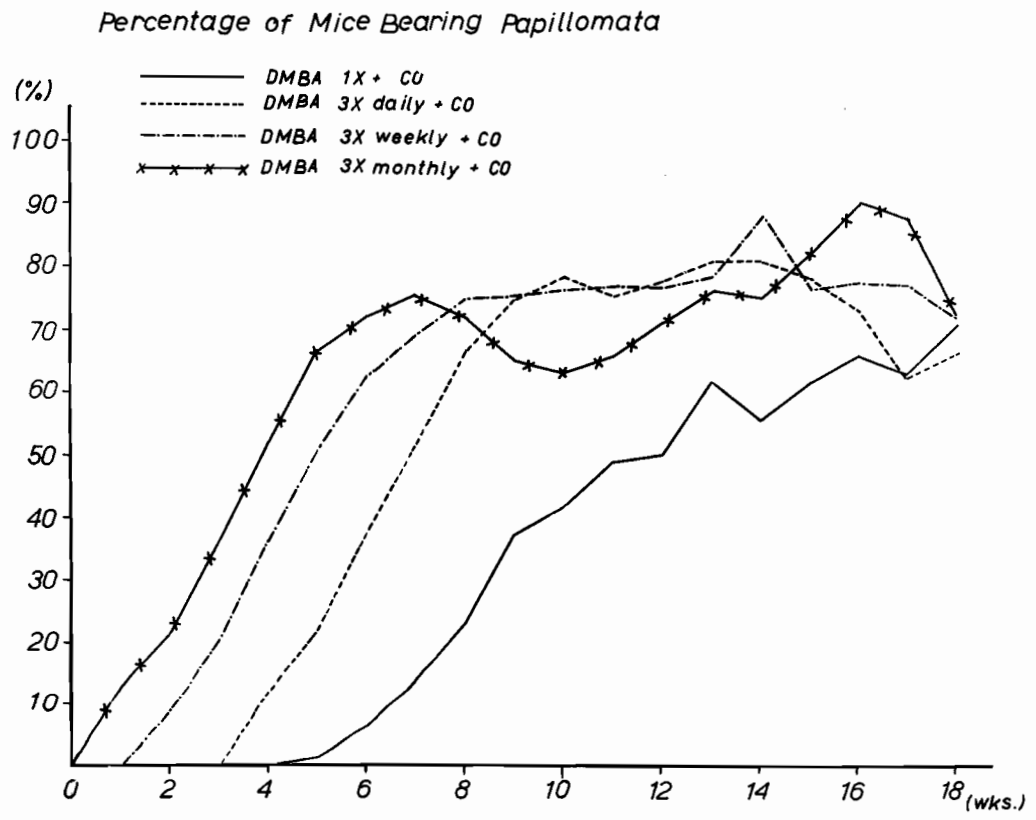
APPENDIX I

FIGURE II



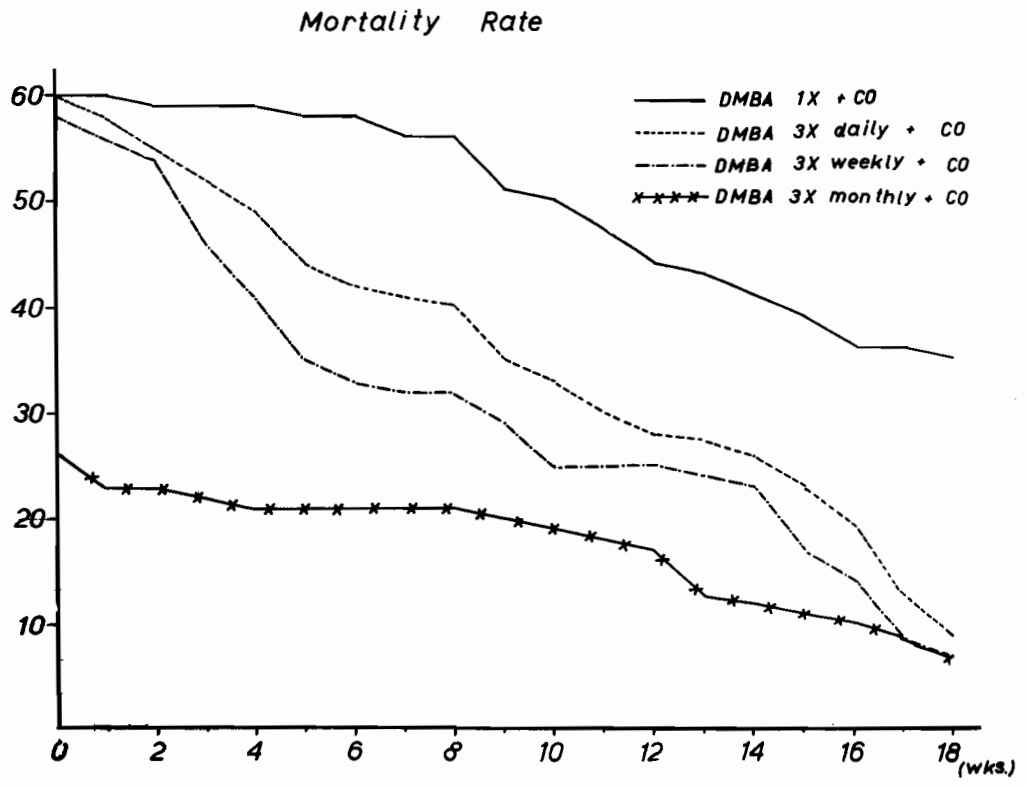
APPENDIX I

FIGURE III



APPENDIX I

FIGURE IV



APPENDIX I

FIGURE V

REACTION OF MOUSE SKIN TO CARCINOGEN

GROUP I

DMBA once and CO

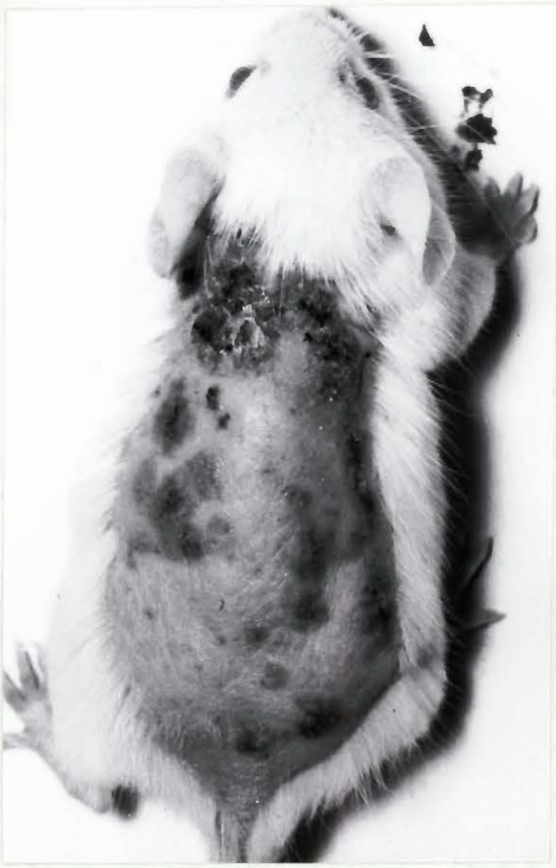
One week after
CO Treatment

GROUP II

DMBA 3x daily

and CO

One week after
CO Treatment



APPENDIX I

FIGURE VI

CARCINOMATA ON MOUSE SKIN

GROUP VII

DMBA 3x weekly

no CO

GROUP VII

DMBA 3x weekly

No CO

APPENDIX II

SUMMARY OF THE RESULTS OF EXPERIMENT II

APPENDIX II

TABLE I

SUMMARY OF RESULTS OF GROUP I (GCA)

Weeks of croton oil treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors	Papillomata per survivor	Papillomata per Papilloma bearing survivor
0	59	0	0	0	0
1	54	1	1	0.02	1.0
2	44	4	4	0.1	1.0
3	37	6	6	0.2	1.0
4	33	17	11	0.5	1.6
5	33	46	18	1.4	2.8
6	32	64	20	2.0	3.2
7	31	93	21	3.0	4.4
8	28	120	22	4.3	5.5
9	28	146	24	5.2	6.1
10	27	179	22	6.6	8.2
11	26	199	20	7.7	10.0
12	24	203	21	8.5	9.7
13	22	182	18	8.3	10.1
14	19	181	16	9.5	11.3
15	14	140	12	10.0	11.7
16	13	138	11	10.6	12.6
17	11	125	10	11.4	12.5
18	10	119	10	11.9	11.9
19	8	58	8	7.3	7.3
20	8	58	7	7.3	8.3

APPENDIX II

TABLE I

SUMMARY OF RESULTS OF GROUP I (GCA)

Percentage of survivors bearing Papillomata	Number of new Papillomata appearing	Number of regressing Papillomata	Papillomata per survivor by increment	Papillomata per Papilloma bearing by increment
0	0	0	0	0
1.8	1	0	0.02	1
9.0	3	0	0.09	1.8
16.2	2	0	0.14	2.1
33.3	11	0	0.5	3.1
54.5	31	0	1.4	4.8
62.5	16	0	1.9	5.6
67.7	29	0	2.8	7.0
78.5	29	3	3.9	8.3
85.7	29	3	4.9	9.5
81.5	34	0	6.2	11.0
76.9	25	5	7.1	12.3
87.5	13	11	7.7	12.9
81.8	19	11	8.5	14.0
84.3	11	1	9.1	14.7
85.7	12	2	10.0	15.7
84.6	11	8	10.8	16.7
90.9	2	11	11.0	16.9
100.0	1	7	11.1	17.0
100.0	0	2	11.1	17.0
87.5	2	3	11.4	17.2

APPENDIX II

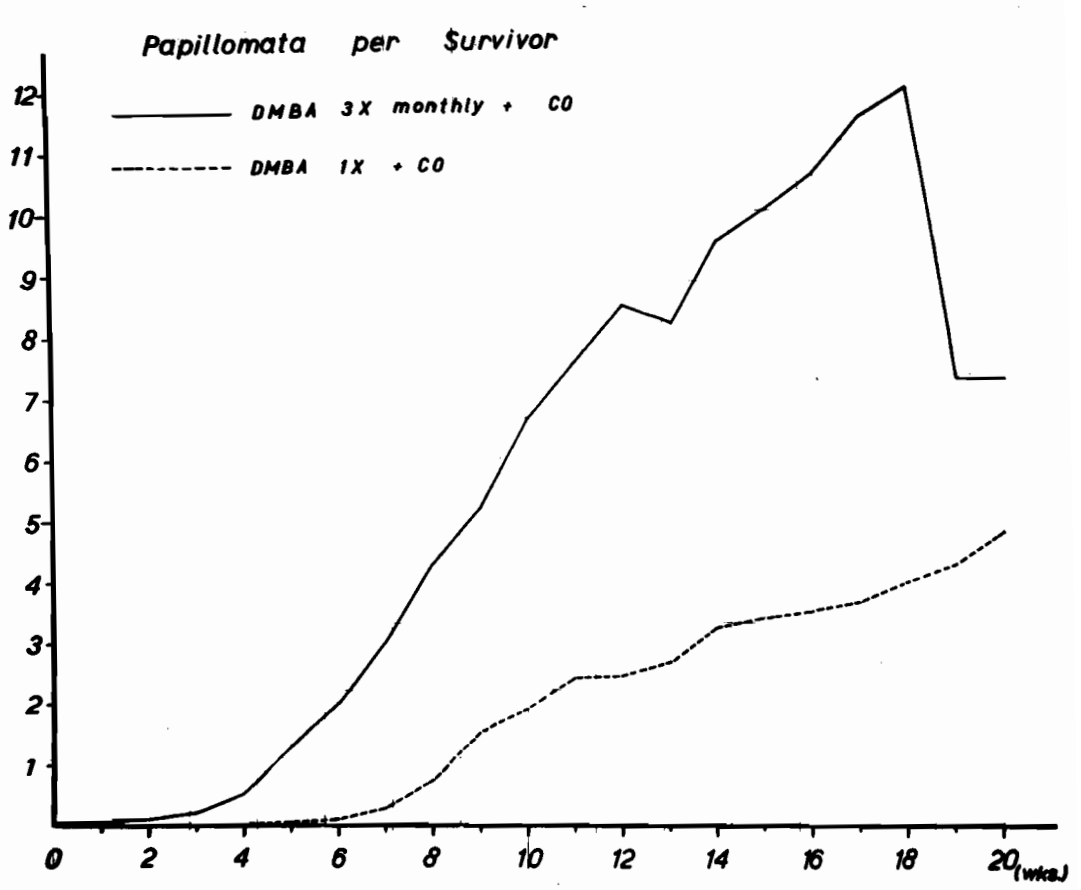
TABLE II

SUMMARY OF RESULTS OF GROUP II (GCB)

Weeks of croton oil treatment	Survivors	Total number of Papillomata on survivor	Papillomata bearing survivors
0	60	0	0
1	59	0	0
2	54	0	0
3	46	0	0
4	43	0	0
5	38	0	0
6	36	0	0
7	35	1	1
8	33	2	1
9	30	3	1
10	28	4	1
11	28	5	2
12	28	5	2
13	27	5	2
14	26	4	1
15	23	3	2
16	22	3	2
17	22	2	2
18	21	2	2
19	21	2	2
20	21	2	2

APPENDIX II

FIGURE I



APPENDIX III

SUMMARY OF THE RESULTS OF EXPERIMENT III

APPENDIX III

TABLE I

SUMMARY OF RESULTS OF GROUP I (GJA)

Weeks of croton oil treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors	Papillomata per survivor	Papillomata per Papilloma bearing survivor
0	52	0	0	0	0
1	51	0	0	0	0
2	50	0	0	0	0
3	50	0	0	0	0
4	50	0	0	0	0
5	50	0	0	0	0
6	50	3	1	0.06	3.0
7	50	9	3	0.18	3.0
8	50	31	10	0.62	3.1
9	50	62	16	1.24	3.9
10	49	99	19	2.02	5.2
11	49	133	23	2.92	5.8
12	49	186	25	3.79	7.4
13	48	201	27	4.19	7.4
14	48	236	27	4.92	8.7
15	46	235	25	5.11	9.4
16	44	233	25	5.30	9.3
17	44	239	25	5.43	9.6
18	44	235	24	5.34	9.8
19	44	273	26	6.20	10.5
20	44	290	27	6.59	10.7
21	44	287	26	6.52	11.0
22	42	259	25	6.16	10.4

APPENDIX III

TABLE I

SUMMARY OF RESULTS OF GROUP I (GJA)

Percentage of survivors bearing Papillomata	Number of new Papillomata appearing	Number of regressing Papillomata	Papillomata per survivor by increment	Papillomata per Papilloma bearing by increment
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
2.0	3	0	0.06	3.0
6.0	6	0	0.18	5.0
20.0	22	0	0.62	7.2
32.0	31	0	1.24	9.1
38.8	37	0	2.00	11.0
46.9	36	2	2.73	12.6
51.0	54	1	3.83	14.8
56.2	29	1	4.43	15.9
56.2	37	2	5.20	17.3
54.3	15	4	5.53	17.6
56.8	10	9	5.76	18.0
56.8	15	9	6.10	18.6
54.5	11	15	6.35	19.1
59.1	45	7	7.37	20.8
61.4	19	2	7.80	21.5
59.1	6	9	7.94	21.7
59.5	0	11	7.94	21.7

APPENDIX III

TABLE II

SUMMARY OF RESULTS OF GROUP II (GJB)

Weeks of croton oil treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors	Papillomata per survivor	Papillomata per Papilloma bearing survivor
0	49	0	0	0	0
1	48	0	0	0	0
2	48	0	0	0	0
3	48	0	0	0	0
4	48	0	0	0	0
5	48	14	2	0.29	7.0
6	48	39	5	0.81	7.8
7	48	61	9	1.27	6.8
8	47	108	14	2.30	7.7
9	47	205	25	4.36	8.2
10	46	216	24	4.70	9.0
11	46	355	30	7.72	11.5
12	46	475	34	10.33	14.0
13	46	523	35	11.37	14.9
14	46	546	37	11.87	14.8
15	46	554	39	12.04	14.2
16	46	574	40	12.48	14.4
17	46	551	40	11.98	13.8
18	46	523	38	11.37	13.8
19	46	545	40	11.85	13.6
20	46	517	39	11.24	13.3
21	46	477	39	10.37	12.2
22	46	440	38	9.57	11.6

APPENDIX III

TABLE II

SUMMARY OF RESULTS OF GROUP II (GJB)

Percentage of survivors bearing Papillomata	Number of new Papillomata appearing	Number of regressing Papillomata	Papillomata per survivor by increment	Papillomata per Papilloma bearing by increment
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
4.2	14	0	0.29	7.0
10.4	25	0	0.81	12.0
18.8	22	0	1.27	14.4
29.8	39	0	2.10	17.2
53.2	97	0	4.16	21.1
52.2	20	0	4.59	21.9
65.2	109	0	6.96	25.5
73.9	140	0	10.39	29.6
76.1	47	3	11.41	30.9
80.4	44	11	12.37	32.1
84.8	24	16	12.89	32.7
87.0	37	17	13.69	33.6
87.0	5	28	13.80	33.7
82.5	5	33	13.91	33.8
87.0	75	53	15.54	35.4
84.8	14	42	15.84	35.8
84.8	0	40	15.84	35.8
82.5	3	40	15.91	35.9

APPENDIX III

TABLE III

SUMMARY OF RESULTS OF GROUP III (GJC)

Weeks of croton oil treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors	Papillomata per survivor	Papillomata per Papilloma bearing survivor
0	48	0	0	0	0
1	45	0	0	0	0
2	43	0	0	0	0
3	42	0	0	0	0
4	42	0	0	0	0
5	41	0	0	0	0
6	40	0	0	0	0
7	40	0	0	0	0
8	39	17	7	0.44	2.4
9	39	62	15	1.59	4.1
10	39	145	20	2.13	7.3
11	39	195	23	5.00	8.5
12	39	278	24	7.13	11.6
13	38	334	27	8.79	12.4
14	38	356	28	9.37	12.7
15	38	374	31	9.84	12.1
16	38	405	32	10.66	12.7
17	38	504	32	13.26	15.8
18	37	522	32	14.11	16.3
19	37	508	32	13.73	15.9
20	37	498	31	13.46	16.1
21	35	474	30	13.54	15.8
22	34	444	29	13.06	15.31

APPENDIX III

TABLE III

SUMMARY OF RESULTS OF GROUP III (GJC)

Percentage of survivors bearing Papillomata	Number of new Papillomata appearing	Number of regressing Papillomata	Papillomata per survivor by increment	Papillomata per Papilloma bearing by increment
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
17.9	17	0	0.44	2.4
38.5	45	0	2.59	5.4
51.3	83	0	4.72	9.6
59.9	50	0	6.00	11.8
61.5	83	0	8.13	15.3
71.1	57	1	9.63	17.4
73.7	24	2	10.26	18.3
81.6	31	3	11.08	19.3
84.2	38	17	12.08	20.5
84.2	123	24	15.32	24.3
89.1	33	8	16.21	25.3
89.1	4	18	16.32	25.4
83.8	8	18	16.54	25.7
85.7	6	27	16.71	25.9
85.3	3	21	16.80	26.0

APPENDIX III

TABLE IV

SUMMARY OF RESULTS OF GROUP IV (GJD)

Weeks of croton oil treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors	Papillomata per survivor	Papillomata per Papilloma bearing survivor
0	52	0	0	0	0
1	44	0	0	0	0
2	44	0	0	0	0
3	44	0	0	0	0
4	44	0	0	0	0
5	44	0	0	0	0
6	42	9	4	0.21	2.3
7	42	21	8	0.5	2.6
8	41	46	9	1.12	5.1
9	41	94	13	2.3	7.2
10	40	119	15	2.98	7.7
11	40	194	20	4.85	9.7
12	40	263	24	6.58	11.0
13	40	267	24	6.67	11.1
14	40	273	24	6.83	11.4
15	40	280	23	7.00	12.2
16	39	280	24	7.18	11.7
17	38	276	25	7.26	11.0
18	37	282	24	7.62	11.8
19	36	291	25	8.08	11.6
20	36	290	25	8.06	11.6
21	36	284	24	7.89	11.8
22	33	263	20	7.97	11.4

APPENDIX III

TABLE IV

SUMMARY OF RESULTS OF GROUP IV (GJD)

Percentage of survivors bearing Papillomata	Number of new Papillomata appearing	Number of regressing Papillomata	Papillomata per survivor by increment	Papillomata per Papilloma bearing by increment
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
9.5	9	0	0.21	2.3
19.0	12	0	0.50	3.8
21.9	25	0	1.11	6.6
31.7	48	0	2.28	10.3
37.5	25	0	2.91	12.0
50.0	75	0	4.79	15.8
60.0	69	0	6.52	18.7
60.0	6	2	6.67	19.0
60.0	8	2	6.87	19.3
57.5	16	9	7.27	20.0
61.5	19	9	7.76	20.8
65.8	8	12	8.08	21.1
64.9	16	7	8.27	21.8
69.4	19	10	8.55	22.6
69.4	4	5	8.66	22.8
66.7	12	18	8.99	23.3
69.7	0	20	8.99	23.3

APPENDIX III

TABLE V

SUMMARY OF RESULTS OF GROUP V (GJE)

Weeks of croton oil treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors
0	27	0	0
1	27	0	0
2	27	0	0
3	27	0	0
4	26	0	0
5	26	0	0
6	25	0	0
7	25	0	0
8	25	0	0
9	25	0	0
10	25	0	0
11	24	0	0
12	24	0	0
13	24	0	0
14	24	0	0
15	24	0	0
16	24	0	0
17	24	0	0
18	24	0	0
19	23	0	0
20	23	0	0
21	23	0	0
22	23	0	0

APPENDIX III

TABLE VI

SUMMARY OF RESULTS OF GROUP VI (GJF)

Weeks of croton oil treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors
0	26	0	0
1	26	0	0
2	22	0	0
3	21	0	0
4	21	0	0
5	21	0	0
6	20	0	0
7	19	0	0
8	19	0	0
9	19	0	0
10	18	0	0
11	18	0	0
12	18	0	0
13	18	0	0
14	18	4	3
15	18	4	3
16	18	5	4
17	18	6	5
18	18	6	5
19	18	7	5
20	17	7	4
21	17	6	4
22	17	7	5

APPENDIX III

TABLE VII

SUMMARY OF RESULTS OF GROUP VII (GJG)

Weeks of croton oil treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors
0	24	0	0
1	24	0	0
2	24	0	0
3	24	0	0
4	24	0	0
5	24	0	0
6	24	0	0
7	24	0	0
8	24	0	0
9	24	0	0
10	24	0	0
11	24	0	0
12	24	0	0
13	22	0	0
14	22	0	0
15	22	0	0
16	21	0	0
17	20	0	0
18	20	0	0
19	20	0	0
20	19	0	0
21	19	0	0
22	19	0	0

APPENDIX III

TABLE VIII

SUMMARY OF RESULTS OF GROUP VIII (GJH)

Weeks of croton oil treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors
0	30	0	0
1	29	0	0
2	29	0	0
3	29	0	0
4	29	0	0
5	29	0	0
6	28	0	0
7	28	0	0
8	28	0	0
9	28	3	1
10	28	4	1
11	28	4	1
12	28	4	1
13	28	7	1
14	28	8	1
15	28	8	1
16	28	8	1
17	28	9	1
18	28	9	1
19	28	9	1
20	28	8	1
21	28	8	1
22	28	7	1

APPENDIX III

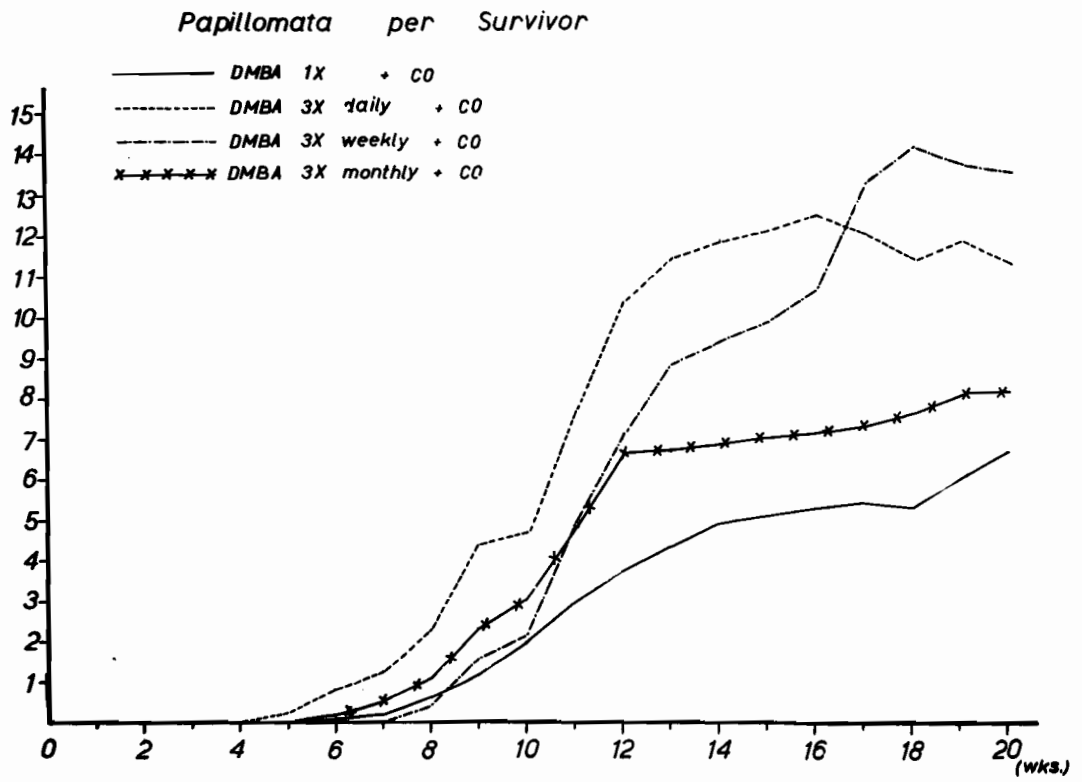
TABLE IX

SUMMARY OF RESULTS OF GROUP IX (GJI)

Weeks of croton oil treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors
0	26	0	0
1	25	0	0
2	25	0	0
3	24	0	0
4	24	0	0
5	24	0	0
6	24	0	0
7	24	8	3
8	24	8	3
9	24	8	3
10	24	8	3
11	24	8	3
12	24	7	3
13	24	5	3
14	24	5	3
15	24	5	3
16	24	4	2
17	24	1	1
18	24	0	0
19	24	0	0
20	23	0	0
21	22	1	1
22	22	1	1

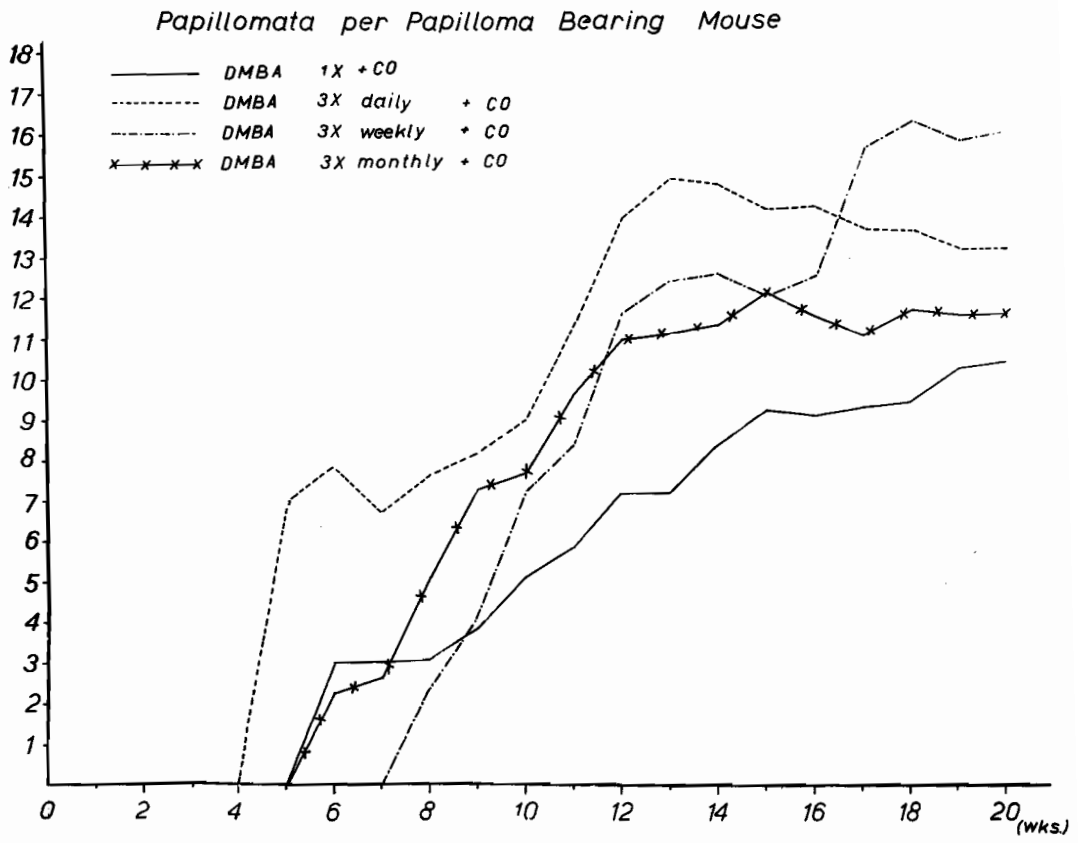
APPENDIX III

FIGURE I



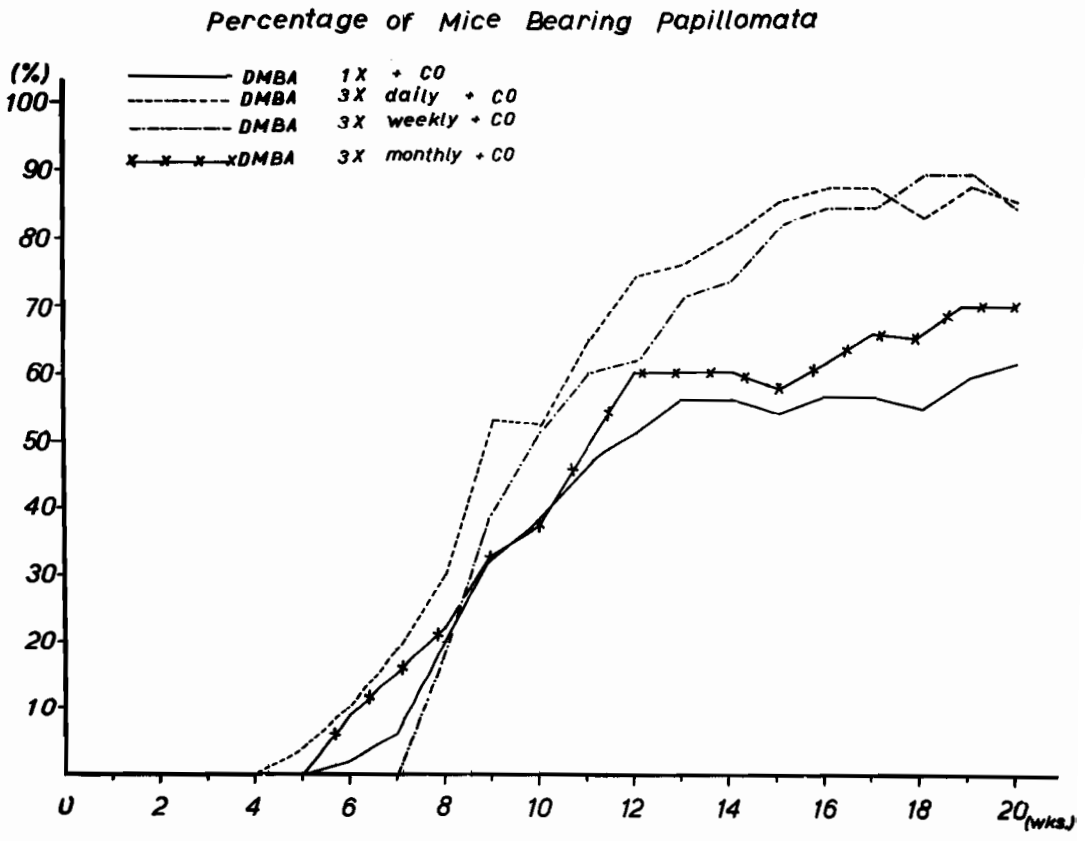
APPENDIX III

FIGURE II



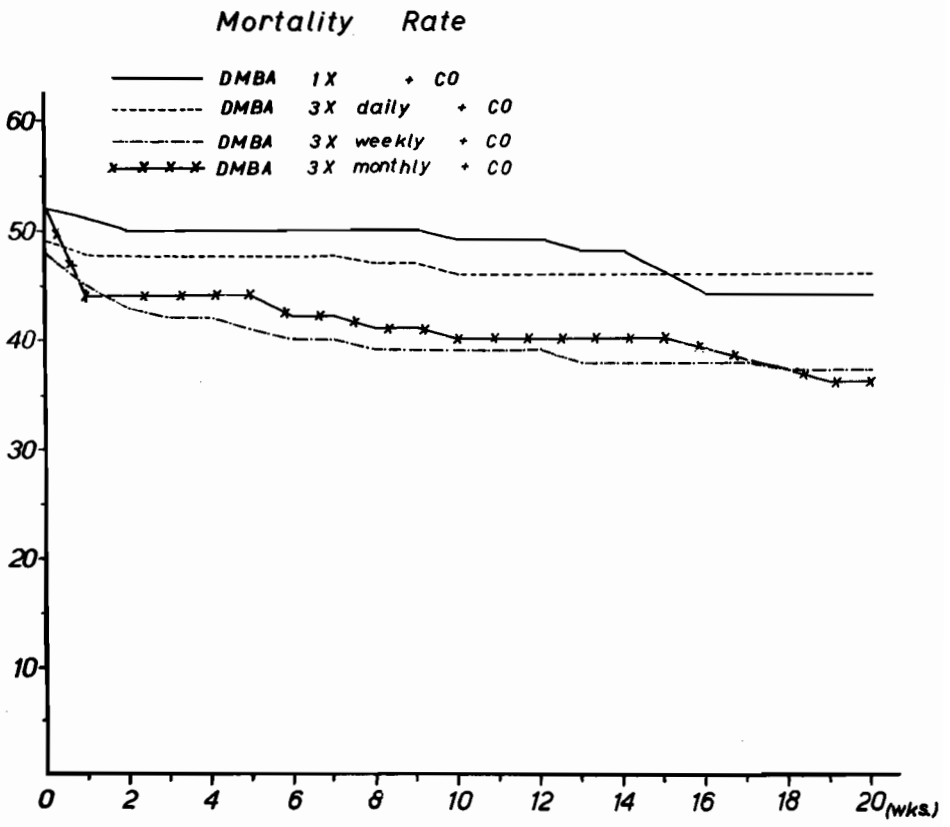
APPENDIX III

FIGURE III



APPENDIX III

FIGURE IV



APPENDIX IV

SUMMARY OF THE RESULTS OF EXPERIMENT V

APPENDIX IV

TABLE I

AVERAGE WEIGHT OF MOUSE IN EACH GROUP

Weeks after the beginning of experiment	0	1	2	3	4	5	6	8	10	12
Group I Urethane 1x & C.O.	21.4	21.1	21.3	22.2	24.2	24.9	25.2	26.7	27.4	27.3
Group II Urethane 3x daily & C.O.	21.4	18.7	19.5	21.8	23.1	24.6	25.5	26.3	27.8	29.7
Group III Urethane 3x weekly & C.O.	21.3	20.6	19.8	21.6	22.9	24.7	25.7	27.8	29.2	30.4
Group IV Urethane 3x monthly & C.O.	21.6	21.0	21.3	23.9	24.3	23.8	24.8	26.1	25.7	27.2
Group V C.O. only	20.6	21.2	20.7	22.3	23.2	23.8	24.6	26.3	26.9	27.6
Group VI Urethane 1x	20.3	20.9	21.1	23.1	23.4	24.0	24.5	25.0	25.9	27.3
Group VII Urethane 3x daily	20.9	18.8	19.9	22.4	23.9	24.1	24.8	25.9	26.4	27.4
Group VIII Urethane 3x weekly	20.6	20.5	21.2	22.3	24.6	25.5	27.0	27.9	27.8	29.0
Group IX Urethane 3x monthly	20.3	20.6	21.4	23.4	24.1	23.2	23.8	25.3	24.8	25.1

APPENDIX IV

TABLE II

SUMMARY OF RESULTS OF GROUP I (GBA)

Percentage of survivors bearing Papillomata	Number of new Papillomata appearing	Number of regressing Papillomata	Papillomata per survivor by increment	Papillomata per Papilloma bearing by increment
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
1.7	2	0	0.03	2.0
13.6	13	0	0.25	2.87
22.8	14	0	0.50	3.35
32.1	9	0	0.67	3.52
38.8	6	0	0.79	3.64
34.7	3	7	0.85	3.70
45.7	6	1	0.98	3.83
48.9	7	1	1.14	3.99
51.2	2	2	1.19	4.04
55.0	10	2	1.44	4.29
59.5	5	0	1.58	4.41
65.7	7	0	1.78	4.61
71.9	11	1	2.12	4.95
74.2	3	5	2.22	5.05
70.0	4	4	2.35	5.18
75.0	4	5	2.49	5.32
79.1	3	5	2.62	5.45
77.3	6	3	2.89	5.72

APPENDIX IV

TABLE II

SUMMARY OF RESULTS OF GROUP I (GBA)

Weeks of croton oil treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors	Papillomata per survivor	Papillomata per Papilloma bearing survivor
0	69	0	0	0	0
1	67	0	0	0	0
2	65	0	0	0	0
3	63	0	0	0	0
4	61	0	0	0	0
5	59	0	0	0	0
6	59	0	0	0	0
7	59	0	0	0	0
8	59	2	1	0.03	2.0
9	59	15	8	0.25	1.88
10	57	29	13	0.51	2.23
11	53	38	17	0.72	2.24
12	49	44	19	0.90	2.32
13	49	40	17	0.82	2.35
14	46	44	21	0.95	2.09
15	45	52	22	1.16	2.36
16	41	53	21	1.29	2.52
17	40	61	22	1.53	2.77
18	37	66	22	1.78	3.00
19	35	73	23	2.09	3.27
20	32	82	23	2.56	3.57
21	31	80	23	2.58	3.45
22	30	78	21	2.60	3.71
23	29	77	21	2.65	3.67
24	24	72	19	3.00	3.79
25	22	71	17	3.23	4.17

APPENDIX IV

TABLE III

SUMMARY OF RESULTS OF GROUP II (GBB)

Percentage of survivors bearing Papillomata	Number of new Papillomata appearing	Number of regressing Papillomata	Papillomata per survivor by increment	Papillomata per Papilloma bearing by increment
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
5.7	3	0	0.09	1.50
11.1	7	0	0.35	3.88
20.0	13	0	0.87	6.48
36.4	13	0	1.46	8.10
36.4	7	0	1.78	8.98
42.9	10	0	2.26	10.09
40.0	6	1	2.56	10.84
40.0	6	2	2.86	11.59
42.1	10	2	3.39	12.84
44.4	6	1	3.72	13.59
41.2	4	3	3.96	14.16
40.0	3	3	4.16	14.66
42.9	3	6	4.38	15.16
38.5	3	0	4.61	15.76
41.7	2	2	4.79	16.16
41.7	2	0	4.97	16.56
41.7	2	2	5.15	16.96
33.3	0	5	5.15	16.96
36.4	2	0	5.33	17.46

APPENDIX IV

TABLE III

SUMMARY OF RESULTS OF GROUP II (GBB)

Weeks of croton oil treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors	Papillomata per survivor	Papillomata per Papilloma bearing survivor
0	64	0	0	0	0
1	52	0	0	0	0
2	46	0	0	0	0
3	39	0	0	0	0
4	37	0	0	0	0
5	37	0	0	0	0
6	36	0	0	0	0
7	35	3	2	0.09	1.50
8	27	10	3	0.37	3.33
9	25	23	5	0.92	4.60
10	22	36	8	1.64	4.50
11	22	43	8	1.95	5.37
12	21	53	9	2.52	5.78
13	20	57	8	2.85	7.13
14	20	61	8	3.05	7.63
15	19	69	8	3.63	8.63
16	18	73	8	4.06	9.13
17	17	72	7	4.24	10.29
18	15	52	6	3.47	8.67
19	14	44	6	3.14	7.33
20	13	30	5	2.31	6.00
21	12	30	5	2.50	6.00
22	12	32	5	2.67	6.40
23	12	32	5	2.67	6.40
24	12	27	4	2.25	6.75
25	11	32	4	2.91	8.00

APPENDIX IV

TABLE IV

SUMMARY OF RESULTS OF GROUP III (GBC)

Weeks of croton oil treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors	Papillomata per survivor	Papillomata per Papilloma bearing survivor
0	50	0	0	0	0
1	46	0	0	0	0
2	43	0	0	0	0
3	42	0	0	0	0
4	41	0	0	0	0
5	41	1	1	0.02	1.00
6	34	1	1	0.03	1.00
7	34	2	2	0.06	1.00
8	33	5	4	0.15	1.25
9	30	10	8	0.33	1.25
10	29	16	8	0.55	2.00
11	29	23	8	0.79	2.88
12	26	22	8	0.85	2.75
13	24	34	8	1.42	4.25
14	23	37	8	1.61	4.63
15	23	42	9	1.87	4.67
16	22	43	10	1.95	4.30
17	19	50	10	2.63	5.00
18	18	55	10	3.06	5.50
19	16	50	8	3.13	6.25
20	16	48	7	3.00	6.86
21	13	38	6	2.92	6.33
22	12	34	6	2.83	5.67
23	11	31	6	2.82	5.17
24	10	31	5	3.10	6.20
25	9	26	4	2.89	6.50

APPENDIX IV

TABLE IV

SUMMARY OF RESULTS OF GROUP III (GBC)

Percentage of survivors bearing Papillomata	Number of new Papillomata appearing	Number of regressing Papillomata	Papillomata per survivor by increment	Papillomata per Papilloma bearing by increment
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
2.4	1	0	0.02	1.00
2.9	0	0	0.02	1.00
5.9	1	0	0.05	1.50
12.2	3	0	0.14	2.25
26.7	7	0	0.37	3.13
27.6	4	0	0.51	3.53
27.6	7	0	0.75	4.41
30.8	2	3	0.83	4.66
33.3	12	0	1.33	6.16
34.8	5	2	1.55	6.71
39.1	8	2	1.90	7.60
45.5	4	3	2.08	8.00
52.6	10	1	2.61	9.00
55.6	6	1	2.94	9.60
50.0	5	3	3.25	10.23
43.7	2	3	3.38	10.52
46.2	1	3	3.46	10.69
50.0	2	4	3.63	11.02
54.5	1	4	3.72	11.19
50.0	3	0	4.02	11.79
44.4	0	4	4.02	11.79

APPENDIX IV

TABLE V

SUMMARY OF RESULTS OF GROUP IV (GBD)

Weeks of croton oil treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors	Papillomata per survivor	Papillomata per Papilloma bearing survivor
0	63	0	0	0	0
1	62	0	0	0	0
2	62	0	0	0	0
3	62	0	0	0	0
4	62	0	0	0	0
5	61	0	0	0	0
6	60	7	4	0.12	1.75
7	59	12	6	0.20	2.00
8	58	32	14	0.55	2.28
9	56	80	24	1.48	3.45
10	51	93	26	1.82	3.58
11	42	104	24	2.48	4.33
12	38	102	21	2.68	4.86
13	32	99	17	3.09	5.82
14	31	80	18	2.58	4.44
15	26	62	16	2.38	3.88
16	23	48	14	2.09	3.43
17	20	40	12	2.00	3.38
18	15	34	11	2.27	3.09
19	14	33	10	2.36	3.30
20	14	32	10	2.29	3.20
21	14	31	10	2.21	3.10
22	13	25	9	1.92	2.78
23	12	23	9	1.92	2.56
24	12	21	7	1.91	3.00
25	12	19	7	1.73	2.88

APPENDIX IV

TABLE V

SUMMARY OF RESULTS OF GROUP IV (GBD)

Percentage of survivors bearing Papillomata	Number of new Papillomata appearing	Number of regressing Papillomata	Papillomata per survivor by increment	Papillomata per Papilloma bearing by increment
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
6.7	7	0	0.12	1.75
10.2	5	0	0.20	2.58
24.1	28	0	0.68	4.58
42.8	41	0	1.41	6.29
51.0	15	2	1.70	6.87
57.1	20	0	2.28	7.70
57.0	21	1	2.83	8.70
53.1	13	4	3.24	9.46
58.1	6	17	3.43	9.79
61.5	4	8	3.58	10.04
60.9	1	7	3.62	10.11
60.0	5	5	3.87	10.53
73.3	2	3	4.00	10.71
71.4	1	3	4.07	10.81
71.4	0	1	4.07	10.81
71.4	2	3	4.21	11.01
69.2	0	5	4.21	11.01
58.3	0	2	4.21	11.01
63.6	1	2	4.31	11.15
63.6	0	2	4.31	11.15

APPENDIX IV

TABLE VI

SUMMARY OF RESULTS OF GROUP V (GBE)

Papillomata per survivor	Papillomata per Papilloma bearing survivor	Percentage of survivors bearing Papillomata
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0.17	2.00	8.7
0.18	2.00	9.1
0.18	2.00	9.5
0.24	2.50	10.0
0.30	3.00	10.0
0.30	3.00	10.5
0.32	3.00	11.1
0.33	3.00	12.9
0.43	3.00	16.9
0.50	3.00	16.7
0.50	3.00	16.7
0.50	3.00	18.2

APPENDIX IV

TABLE VI

SUMMARY OF RESULTS OF GROUP V (GBE)

Weeks of croton oil treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors
0	68	0	0
1	62	0	0
2	56	0	0
3	49	0	0
4	47	0	0
5	38	0	0
6	31	0	0
7	31	0	0
8	27	0	0
9	27	0	0
10	24	0	0
11	24	0	0
12	24	0	0
13	23	0	0
14	23	4	2
15	22	4	2
16	22	4	2
17	21	5	2
18	20	6	2
19	20	6	2
20	19	6	2
21	18	6	2
22	14	6	2
23	12	6	2
24	12	6	2
25	11	6	2

APPENDIX IV

TABLE VII

SUMMARY OF RESULTS OF GROUP VI (GBF)

Weeks of croton oil treatment	Survivors	Total number Papillomata of survivors	Papillomata bearing survivors
0	27	0	0
1	26	0	0
2	26	0	0
3	26	0	0
4	26	0	0
5	26	0	0
6	26	0	0
7	25	0	0
8	23	0	0
9	22	0	0
10	22	0	0
11	22	0	0
12	21	0	0
13	20	0	0
14	20	0	0
15	12	0	0
16	10	0	0
17	10	0	0
18	10	0	0
19	10	0	0
20	10	0	0
21	10	0	0
22	10	0	0
23	10	0	0
24	10	0	0
25	10	0	0

APPENDIX IV

TABLE VIII

SUMMARY OF RESULTS OF GROUP VII (GBG)

Weeks of croton oil treatment	Survivors	Total number Papillomata of survivors	Papillomata bearing survivors
0	26	0	0
1	24	0	0
2	23	0	0
3	23	0	0
4	23	0	0
5	23	0	0
6	23	0	0
7	23	0	0
8	23	0	0
9	22	0	0
10	22	0	0
11	22	0	0
12	22	0	0
13	22	0	0
14	22	0	0
15	21	0	0
16	17	0	0
17	16	0	0
18	16	0	0
19	16	0	0
20	13	0	0
21	12	0	0
22	12	0	0
23	12	0	0
24	10	0	0
25	10	0	0

APPENDIX IV

TABLE IX

SUMMARY OF RESULTS OF GROUP VIII (GBH)

Weeks of croton oil treatment	Survivors	Total number Papillomata of survivors	Papillomata bearing survivors
0	22	0	0
1	20	0	0
2	19	0	0
3	18	0	0
4	18	0	0
5	18	0	0
6	18	0	0
7	18	0	0
8	18	0	0
9	18	0	0
10	18	0	0
11	17	0	0
12	13	0	0
13	10	0	0
14	10	0	0
15	9	0	0
16	9	0	0
17	9	0	0
18	8	0	0
19	7	0	0
20	7	0	0
21	7	0	0
22	7	0	0
23	7	0	0
24	7	0	0
25	7	0	0

APPENDIX IV

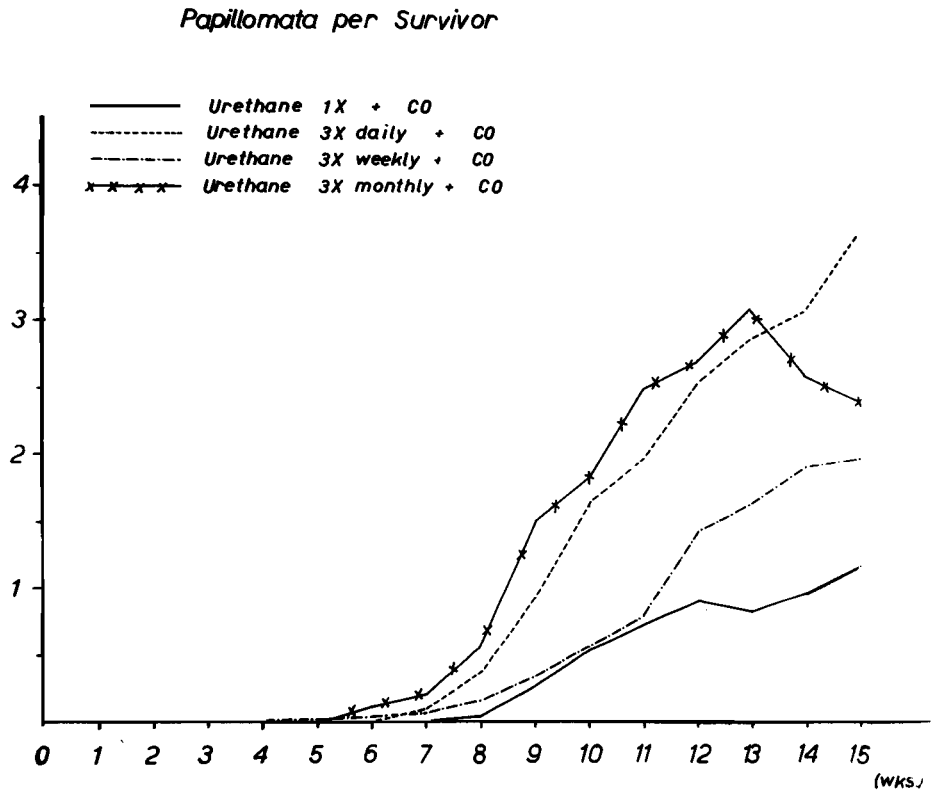
TABLE X

SUMMARY OF RESULTS OF GROUP IX (GBI)

Weeks of croton oil treatment	Survivors	Total number Papillomata of Survivors	Papillomata bearing Survivors
0	26	0	0
1	26	0	0
2	26	0	0
3	24	0	0
4	23	0	0
5	21	0	0
6	20	0	0
7	19	0	0
8	19	0	0
9	19	0	0
10	19	0	0
11	19	0	0
12	17	0	0
13	17	0	0
14	17	0	0
15	17	0	0
16	17	0	0
17	17	0	0
18	17	0	0
19	17	0	0
20	17	0	0
21	17	0	0
22	17	0	0
23	17	0	0
24	16	0	0
25	15	0	0

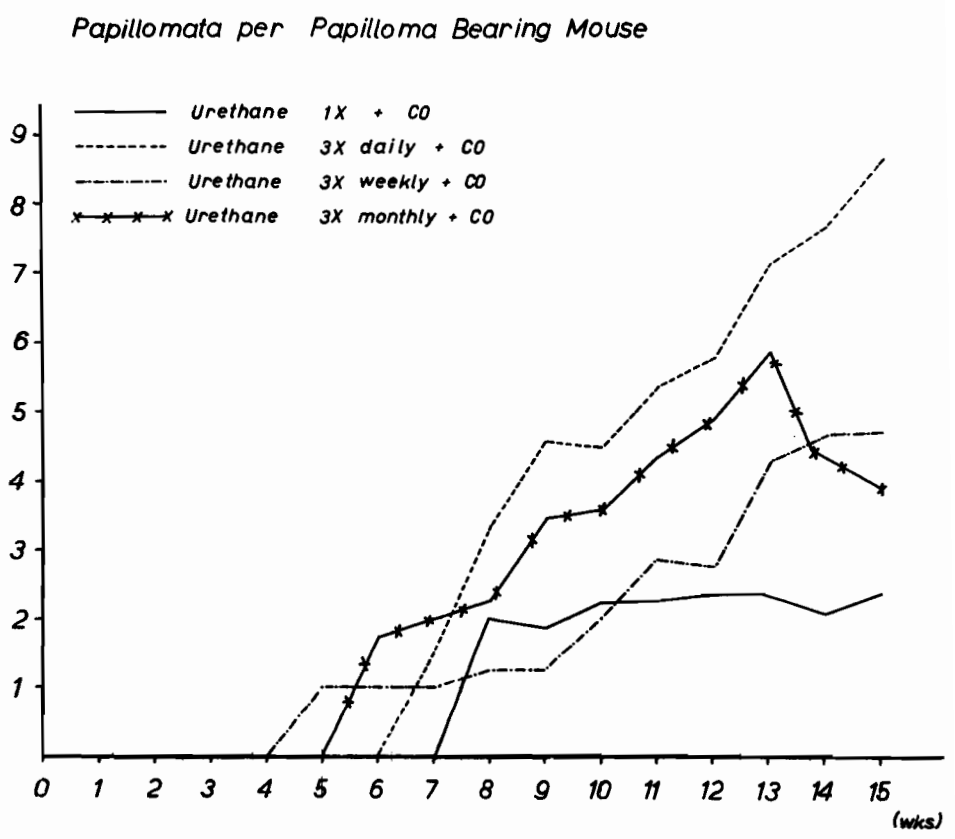
APPENDIX IV

FIGURE I



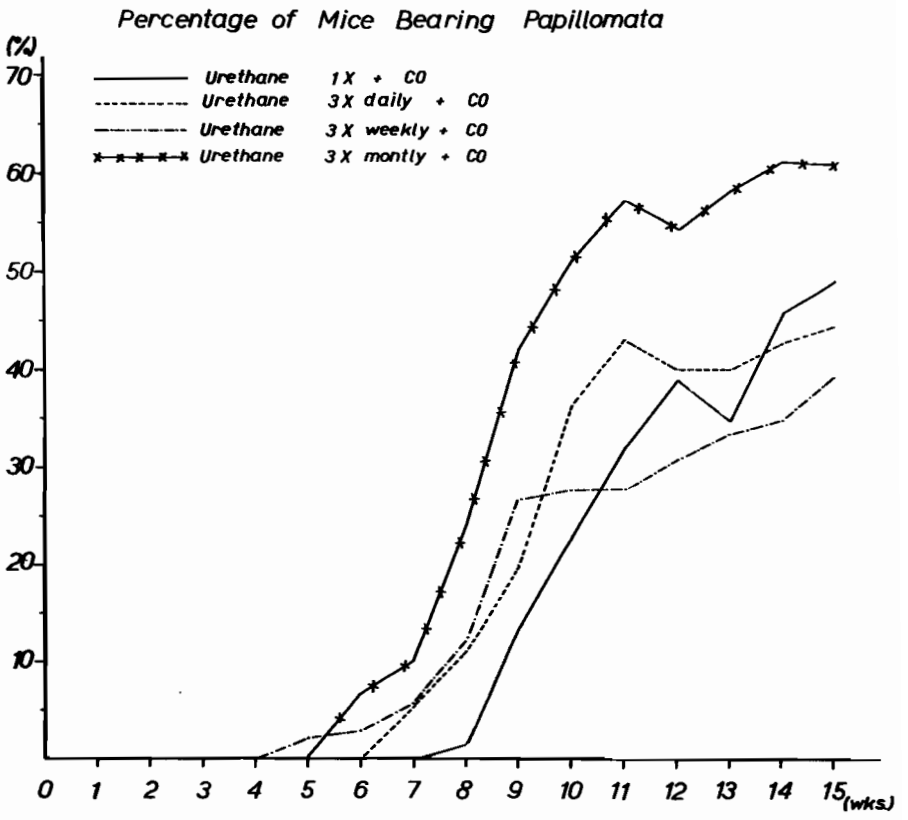
APPENDIX IV

FIGURE II



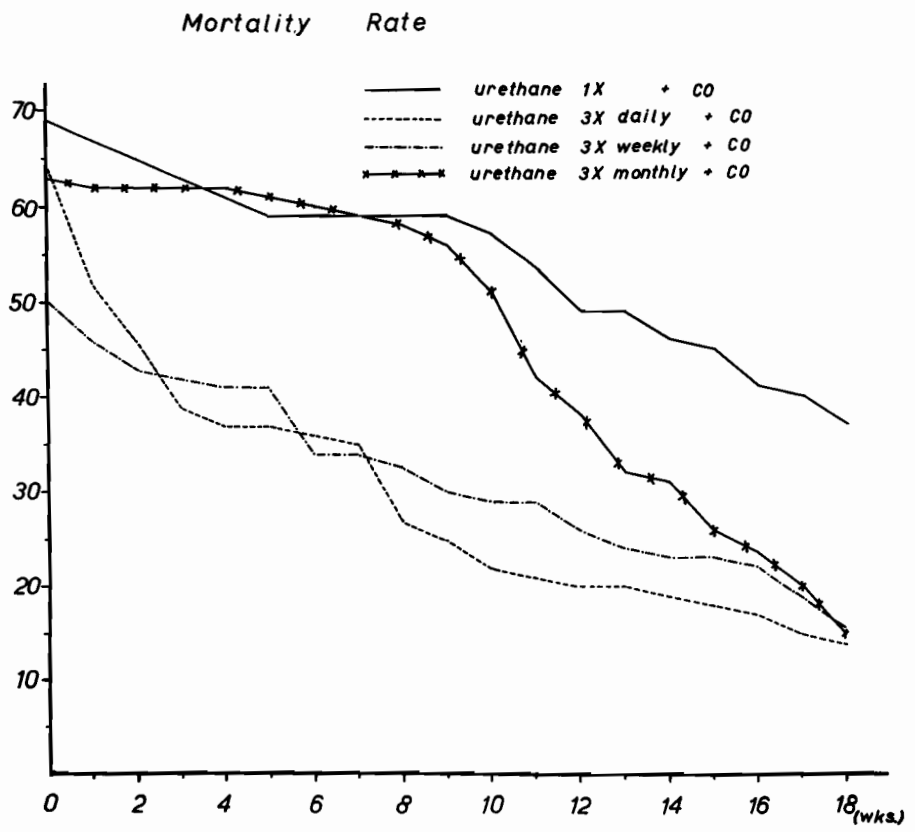
APPENDIX IV

FIGURE III



APPENDIX IV

FIGURE IV



APPENDIX IV

FIGURE V

SKIN REACTION AFTER THREE DAILY
URETHANE INJECTIONS FOLLOWED BY CROTON OIL

GROUP II

Urethane 3x daily and CO

5 weeks of CO

GROUP II

Urethane 3x daily and CO

7 weeks of CO

APPENDIX IV

FIGURE VI

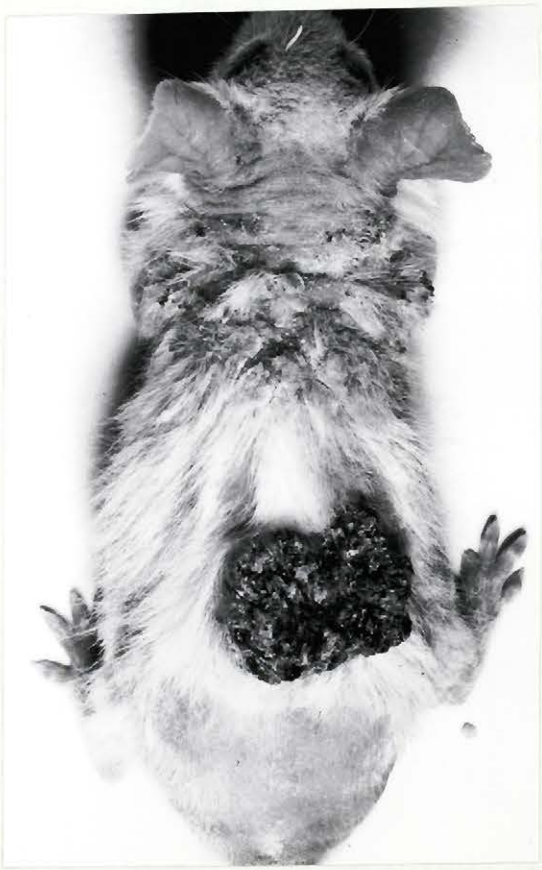
CARCINOMATA INDUCED BY URETHANE INJECTIONS AND THEN CROTON OIL APPLICATIONS

GROUP II

Urethane 3x daily and CO

GROUP IV

Urethane 3x monthly and CO



APPENDIX V

SUMMARY OF THE RESULTS OF EXPERIMENTS I, II (RITCHIE 1958-1959)

APPENDIX V

TABLE I

SUMMARY OF THE RESULTS OF EXPERIMENT I (RITCHIE 1958-1959)

Days of croton oil treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors
Urethane once followed by Croton Oil.			
0	41	0	0
25	40	0	0
50	38	0	0
75	36	0	0
100	36	0	0
125	36	3	3
150	36	5	5
175	35	9	7
200	34	15	7
225	31	22	8
250	30	23	9
275	27	40	14
300	17	35	12
Urethane three times daily followed by Croton Oil.			
0	37	0	0
25	34	0	0
50	30	0	0
75	28	0	0
100	25	9	3
125	24	26	9
150	24	45	13
175	22	43	13
200	21	45	13
225	20	69	14
250	17	69	14
275	15	86	15
300	14	85	14

APPENDIX V

TABLE I

SUMMARY OF THE RESULTS OF EXPERIMENT I (RITCHIE 1958-1959)

Average number Papillomata per survivor	Average number Papillomata per Papilloma bearing survivor	Percentage of survivors bearing Papillomata
Urethane once followed by Croton Oil.		
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0.08	1	8.3
0.14	1	19.9
0.25	1.39	20.0
0.44	2.14	20.6
0.71	2.75	25.8
0.77	2.55	30.0
1.48	2.86	51.5
2.06	2.92	70.5
Urethane three times daily followed by Croton Oil.		
0	0	0
0	0	0
0	0	0
0	0	0
0.36	3	12.0
1.08	2.89	37.5
1.87	3.46	54.2
1.95	3.91	59.1
2.14	3.46	61.9
3.45	4.93	70.0
4.06	4.93	82.4
5.73	5.73	100.0
6.07	6.07	100.0

APPENDIX V

TABLE II

SUMMARY OF THE RESULTS OF EXPERIMENT I (RITCHIE 1958-1959)

Days of croton oil treatment	survivors	Total number Papillomata on survivors	Papillomata bearing survivors
Urethane three times weekly followed by Croton Oil.			
0	32	0	0
25	32	0	0
50	28	0	0
75	27	2	1
100	25	5	4
125	25	11	4
150	24	13	4
175	24	14	4
200	24	17	5
225	20	21	4
250	17	21	4
275	17	26	5
300	16	26	5
Urethane three times monthly followed by Croton Oil.			
0	43	0	0
25	43	0	0
50	41	0	0
75	38	11	4
100	38	19	7
125	36	34	10
150	35	42	11
175	33	52	14
200	32	52	15
225	30	54	16
250	23	40	11
275	22	44	11
300	20	43	11

APPENDIX V

TABLE II

SUMMARY OF THE RESULTS OF EXPERIMENT I (RITCHIE 1958-1959)

Average number Papillomata per survivor	Average number Papillomata per Papilloma bearing survivor	Percentage of survivors bearing Papillomata
Urethane once followed by Croton Oil.		
0	0	0
0	0	0
0	0	0
0.07	2	3.6
0.20	1.25	16.0
0.44	2.75	16.0
0.54	3.25	16.7
0.58	3.50	16.7
0.71	3.40	20.8
1.05	5.25	20.0
1.18	5.25	23.5
1.53	5.20	29.4
1.62	5.20	31.2
Urethane three times monthly followed by Croton Oil.		
0	0	0
0	0	0
0	0	0
0.29	2.75	10.5
0.50	2.72	18.4
0.94	3.40	27.8
1.20	3.82	31.4
1.56	3.71	42.5
1.65	3.46	46.9
1.80	3.38	53.2
1.74	3.69	47.8
2.00	4.00	50.0
2.15	3.91	55.0

APPENDIX V

TABLE III

SUMMARY OF THE RESULTS OF EXPERIMENT I (RITCHIE 1958-1959)

Days of croton oil treatment	survivors	Total number Papillomata on survivors	Papillomata bearing survivors
Croton Oil Only.			
0	43	0	0
25	43	0	0
50	43	0	0
75	43	0	0
100	42	0	0
125	41	2	1
150	40	7	5
175	40	7	5
200	35	13	10
225	34	24	12
250	31	34	13
275	30	41	14
300	29	51	15

APPENDIX V

TABLE III

SUMMARY OF THE RESULTS OF EXPERIMENT I (RITCHIE 1958-1959)

Average number Papillomata per survivor	Average number Papillomata per Papilloma bearing survivor	Percentage of survivors bearing Papillomata
Croton Oil only.		
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0.04	2.00	2.4
0.17	1.40	12.5
0.17	1.40	12.5
0.37	1.30	28.6
0.71	2.00	35.4
1.10	2.62	42.0
1.37	2.93	46.7
1.76	3.40	51.7

APPENDIX V

TABLE IV

SUMMARY OF THE RESULTS OF EXPERIMENT II (RITCHIE 1958-1959)

Days of croton oil treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors
Urethane three times daily followed by Croton Oil.			
0	29	0	0
28	29	0	0
56	29	0	0
84	29	3	1
112	29	7	3
140	28	8	3
168	28	9	3
196	28	11	5
224	28	16	7
252	27	15	7
280	25	29	11
Urethane three times weekly followed by Croton Oil.			
0	30	0	0
28	30	0	0
56	30	0	0
84	30	2	2
112	30	2	2
140	30	10	5
168	30	10	5
196	30	24	10
224	30	24	10
252	29	34	12
280	28	43	14

APPENDIX V

TABLE IV

SUMMARY OF THE RESULTS OF EXPERIMENT II (RITCHIE 1958-1959)

Average number Papillomata per survivor	Average number Papillomata per Papilloma bearing survivor	Percentage of survivors bearing Papillomata
Urethane three times daily followed by Croton Oil.		
0	0	0
0	0	0
0	0	0
0.10	3.0	3.5
0.24	2.3	10.4
0.29	2.7	10.7
0.32	3.0	10.7
0.39	2.2	17.9
0.57	2.3	25.0
0.57	2.1	25.9
1.16	2.6	44.0
Urethane three times weekly followed by Croton Oil.		
0	0	0
0	0	0
0	0	0
0.07	1.0	6.7
0.07	1.0	6.7
0.33	2.0	16.7
0.33	2.0	16.7
0.57	2.1	26.7
0.80	2.4	33.3
1.17	2.8	41.4
1.54	3.1	50.0

APPENDIX V

TABLE V

SUMMARY OF THE RESULTS OF EXPERIMENT II (RITCHIE 1958-1959)

Days of croton oil treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors
Urethane three times monthly followed by Croton Oil.			
0	30	0	0
28	30	0	0
56	30	0	0
84	30	0	0
112	29	0	0
140	29	0	0
168	29	1	1
196	28	4	4
224	27	14	6
252	27	23	7
Croton Oil only.			
0	29	0	0
28	29	0	0
56	29	0	0
84	29	0	0
112	29	0	0
140	29	0	0
168	29	0	0
196	29	1	1
224	29	3	3
252	29	7	3
280	28	8	2

APPENDIX V

TABLE V

SUMMARY OF THE RESULTS OF EXPERIMENT II (RITCHIE 1958-1959)

Average number Papillomata per survivor	Average number Papillomata per Papilloma bearing survivor	Percentage of survivors bearing Papillomata
Urethane three times monthly followed by Croton Oil.		
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0.03	1.0	3.4
0.14	1.0	14.3
0.52	2.3	21.2
0.85	3.3	25.9
Croton Oil only.		
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0.03	1.0	3.4
0.10	1.0	10.3
0.24	2.3	10.3
0.29	4.0	7.1

APPENDIX VI

SUMMARY OF THE RESULTS OF EXPERIMENT VII

APPENDIX VI

TABLE I

SUMMARY OF RESULTS OF GROUP I (GFA)

Weeks of croton oil treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors	Papillomata per survivor	Papillomata per Papilloma bearing survivor
0	80	0	0	0	0
1	79	0	0	0	0
2	78	0	0	0	0
3	78	0	0	0	0
4	78	0	0	0	0
5	78	15	3	0.19	5.0
6	78	153	22	1.96	7.0
7	76	216	34	2.84	6.4
8	76	254	37	3.34	6.9
9	76	347	41	4.57	8.5
10	73	431	44	5.92	9.8
11	72	528	48	7.36	11.0
12	69	537	45	7.78	11.9
13	56	477	38	8.52	12.6
14	55	506	37	9.20	13.4
15	52	523	36	10.06	14.5
16	49	485	36	9.90	13.6
17	49	481	36	9.82	13.4
18	49	463	35	9.45	13.2
19	47	450	32	9.57	14.1
20	47	469	31	9.98	15.1
21	46	466	33	10.01	14.1
22	45	457	34	10.01	13.4
23	44	442	34	10.00	13.0
24	42	407	32	9.69	12.7
25	40	364	30	9.10	12.1

APPENDIX VI

TABLE I

SUMMARY OF RESULTS OF GROUP I (GFA)

Percentage of survivors bearing Papillomata	Number of new Papillomata appearing	Number of regressing Papillomata	Papillomata per survivor by increment	Papillomata per Papilloma bearing by increment
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
3.8	15	0	0.19	5.0
28.2	138	0	1.96	11.3
44.7	63	0	2.77	13.1
48.7	39	1	3.28	14.2
53.9	96	0	4.54	16.5
60.3	95	1	5.84	18.7
65.3	100	3	7.24	20.8
63.8	76	3	8.34	22.5
67.9	61	6	9.43	24.1
67.3	35	6	10.07	25.0
69.2	46	13	10.95	26.3
71.4	33	16	11.62	27.2
73.5	19	23	12.0	27.8
71.4	16	34	12.34	28.2
68.9	19	17	12.74	28.8
65.9	24	5	13.25	29.6
70.5	10	13	13.47	29.9
75.5	6	15	13.60	30.1
77.3	6	15	13.74	30.2
76.2	4	24	13.84	30.3
75.0	3	18	13.91	30.4

APPENDIX VI

TABLE II

SUMMARY OF RESULTS OF GROUP II (GFB)

Weeks of croton oil treatment	survivors	Total number Papillomata on survivors	Papilloma bearing survivors	Papillomata per survivor	Papillomata per Papilloma bearing survivor
0	80	0	0	0	0
1	77	0	0	0	0
2	74	0	0	0	0
3	73	0	0	0	0
4	72	0	0	0	0
5	71	9	4	0.13	2.3
6	71	81	22	1.14	3.7
7	71	131	31	1.84	4.2
8	70	163	34	2.33	4.8
9	70	231	38	3.30	6.1
10	70	310	40	4.43	7.8
11	70	417	46	5.96	9.0
12	68	514	48	7.57	10.7
13	67	573	48	8.55	11.9
14	67	625	49	9.33	12.8
15	63	646	48	10.25	13.5
16	59	601	48	10.19	13.4
17	59	586	45	9.93	13.0
18	58	579	45	9.98	12.9
19	58	581	45	10.02	12.9
20	57	592	44	10.35	13.5
21	57	583	44	10.23	13.3
22	55	556	43	10.10	12.9
23	52	543	40	10.44	13.6
24	51	527	40	10.33	13.2
25	48	440	37	9.17	11.9

APPENDIX VI

TABLE II

SUMMARY OF RESULTS OF GROUP II (GFB)

Percentage of survivors bearing Papillomata	Number of new Papillomata appearing	Number of regressing Papillomata	Papillomata per survivor by increment	Papillomata per Papilloma bearing by increment
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
5.6	9	0	0.13	2.3
30.6	72	0	1.14	5.5
43.7	50	0	1.84	7.1
48.6	37	0	2.37	8.2
54.3	69	1	3.36	10.4
57.1	79	0	4.49	12.4
65.7	107	0	6.02	14.7
70.6	99	0	7.18	16.8
71.7	60	1	8.07	18.0
73.1	52	0	8.85	19.1
76.2	33	10	9.38	19.8
76.3	22	40	9.77	20.3
76.3	18	33	10.08	20.7
77.6	14	21	10.32	21.0
77.6	32	30	10.87	21.7
77.2	31	16	11.41	22.4
77.2	20	30	11.76	22.9
79.1	9	35	11.92	23.1
76.9	12	31	12.14	23.4
78.5	8	15	12.30	23.6
77.1	0	40	12.30	23.6

APPENDIX VI

TABLE III

SUMMARY OF RESULTS OF GROUP III (GFC)

Weeks of croton oil treatment	Survivors	Total number Papillomata on survivor	Papillomata bearing survivors	Papillomata per survivor	Papillomata per Papilloma bearing survivor
0	80	0	0	0	0
1	80	0	0	0	0
2	79	0	0	0	0
3	78	0	0	0	0
4	76	0	0	0	0
5	74	22	6	0.30	3.7
6	72	149	31	2.07	4.8
7	71	205	39	2.89	5.3
8	67	250	37	3.72	6.8
9	66	340	41	5.15	8.3
10	61	422	43	6.92	9.8
11	56	441	42	7.87	10.5
12	51	528	40	10.35	13.2
13	50	576	40	11.52	14.4
14	49	627	42	12.84	14.9
15	47	673	39	14.32	17.3
16	46	656	41	14.26	16.0
17	46	672	41	14.60	16.5
18	46	680	41	14.80	16.6
19	45	683	41	15.17	16.7
20	42	660	38	15.71	17.4
21	40	616	36	15.40	17.1
22	38	534	34	14.05	15.7
23	36	486	31	13.50	15.7
24	35	483	30	13.51	16.1
25	34	441	27	13.26	16.3

APPENDIX VI

TABLE III

SUMMARY OF RESULTS OF GROUP III (GFC)

Percentage of survivors bearing Papillomata	Number of new Papillomata appearing	Number of regressing Papillomata	Papillomata per survivor by increment	Papillomata per Papilloma bearing by increment
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
8.1	22	0	0.30	3.7
43.1	127	0	2.06	7.8
54.9	56	0	2.85	9.2
53.7	62	0	3.78	10.9
62.1	85	0	5.07	13.0
70.7	82	0	6.41	14.9
75.0	59	0	7.46	16.3
78.4	108	0	9.58	19.0
80.0	57	0	10.72	20.4
85.7	58	7	11.90	21.8
82.9	57	4	13.11	23.2
89.1	32	27	13.81	24.0
89.1	29	10	14.44	24.8
89.1	18	13	14.83	25.3
91.1	23	20	15.34	25.8
90.5	16	8	15.72	26.2
90.0	8	20	15.92	26.5
89.5	0	43	15.92	26.5
86.1	13	15	16.28	26.9
85.7	7	10	16.48	27.1
79.4	2	36	16.54	27.2

APPENDIX VI

TABLE IV

SUMMARY OF RESULTS OF GROUP IV (GFD)

Weeks of croton oil treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors	Papillomata per survivor	Papillomata per Papilloma bearing survivor
0	80	0	0	0	0
1	80	0	0	0	0
2	80	0	0	0	0
3	80	0	0	0	0
4	79	0	0	0	0
5	79	10	5	0.13	2.0
6	79	112	25	1.43	4.4
7	78	146	30	1.88	4.9
8	77	238	37	3.09	6.4
9	77	318	44	4.13	7.2
10	77	433	46	5.62	9.0
11	77	520	51	6.75	10.2
12	77	642	57	8.31	11.3
13	77	711	56	9.10	12.7
14	76	748	59	9.84	12.7
15	73	736	56	10.08	13.1
16	70	692	54	9.88	12.8
17	67	638	55	9.53	11.6
18	65	617	55	9.34	11.2
19	63	584	53	9.27	11.0
20	63	600	52	9.52	11.6
21	62	568	51	9.16	11.1
22	62	571	52	9.21	11.0
23	59	531	48	9.0	11.1
24	56	496	46	9.04	10.8
25	53	416	42	7.85	9.9

APPENDIX VI

TABLE IV

SUMMARY OF RESULTS OF GROUP IV (GFD)

Percentage of survivors bearing Papillomata	Number of new Papillomata appearing	Number of regressing Papillomata	Papillomata per survivor by increment	Papillomata per Papilloma bearing by increment
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
6.3	10	0	0.13	2.0
31.6	102	0	1.42	6.1
38.5	34	0	1.86	7.2
48.1	92	0	3.05	9.7
57.1	80	0	4.09	11.5
59.7	115	0	5.58	13.0
66.2	87	0	6.71	14.7
74.0	123	1	7.31	16.9
72.7	70	1	8.22	18.1
77.6	57	10	8.97	19.1
76.7	58	12	9.76	20.2
77.1	37	39	10.29	20.8
82.1	11	25	10.45	21.0
84.6	15	35	10.68	21.3
84.1	28	37	11.02	21.8
82.5	34	18	11.56	22.5
82.3	15	22	11.80	22.8
83.9	15	12	12.04	23.1
81.4	6	30	12.14	23.2
82.1	9	49	12.30	23.4
80.8	7	30	12.43	23.6

APPENDIX VI

TABLE V

SUMMARY OF RESULTS OF GROUP VI (GFE)

Weeks of croton oil treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors
0	50	0	0
1	49	0	0
2	49	0	0
3	49	0	0
4	49	0	0
5	49	0	0
6	49	0	0
7	49	0	0
8	49	0	0
9	48	0	0
10	47	0	0
11	46	0	0
12	45	0	0
13	44	0	0
14	44	0	0
15	44	0	0
16	43	0	0
17	42	0	0
18	40	0	0
19	40	0	0
20	38	0	0
21	38	0	0
22	34	0	0
23	33	0	0
24	32	0	0
25	31	0	0

APPENDIX VI

TABLE VI

SUMMARY OF RESULTS OF GROUP VI (GFF)

Weeks of croton oil treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors
0	50	0	0
1	50	0	0
2	50	0	0
3	50	0	0
4	50	0	0
5	50	0	0
6	50	0	0
7	50	0	0
8	50	0	0
9	50	0	0
10	49	2	2
11	48	2	2
12	48	2	1
13	48	2	1
14	48	2	1
15	48	5	4
16	47	5	4
17	47	5	4
18	47	10	5
19	46	10	5

APPENDIX VI

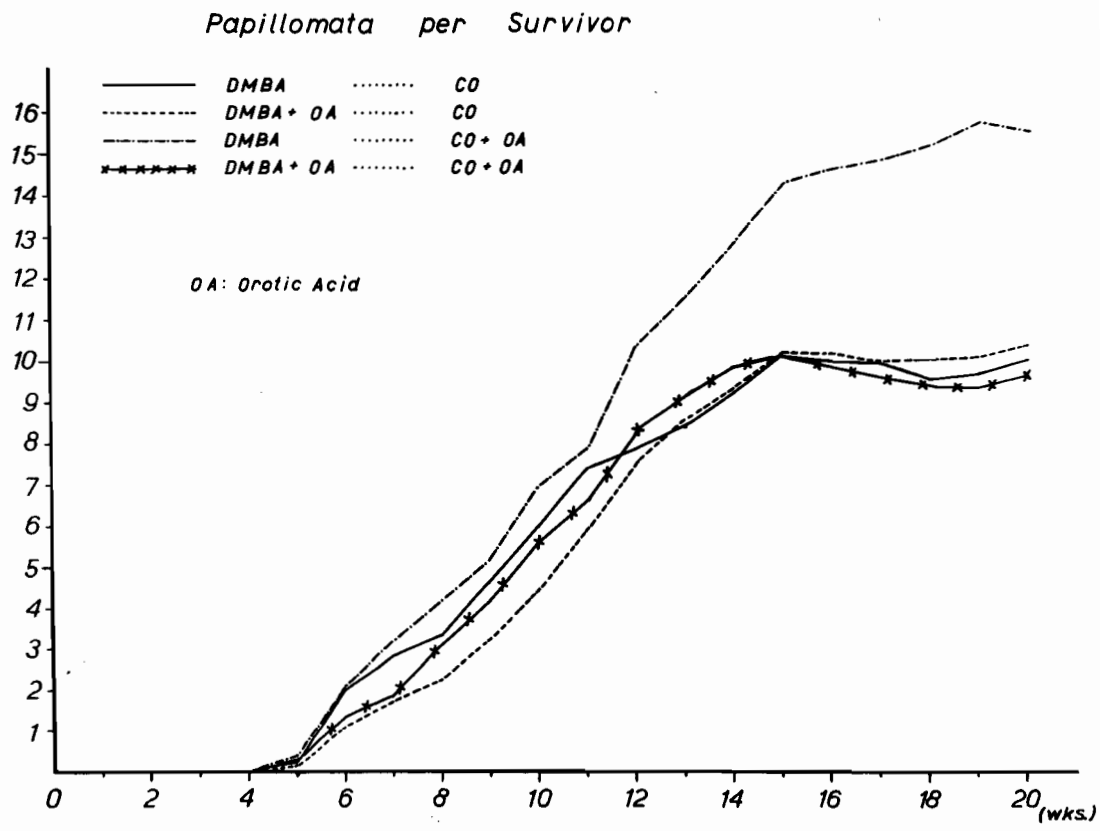
TABLE VI

SUMMARY OF RESULTS OF GROUP VI (GFF)

Weeks of croton oil treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors
20	46	10	5
21	46	10	5
22	46	17	6
23	46	17	6
24	44	17	6
25	44	18	6
26	44	22	7
27	42	26	13
28	42	29	14
29	42	30	14
30	42	35	16
31	41	33	15
32	40	32	15
33	40	28	13
34	34	17	9
35	28	19	10
36	23	15	9
37	21	17	9
38	20	17	9
39	18	18	8
40	18	16	7

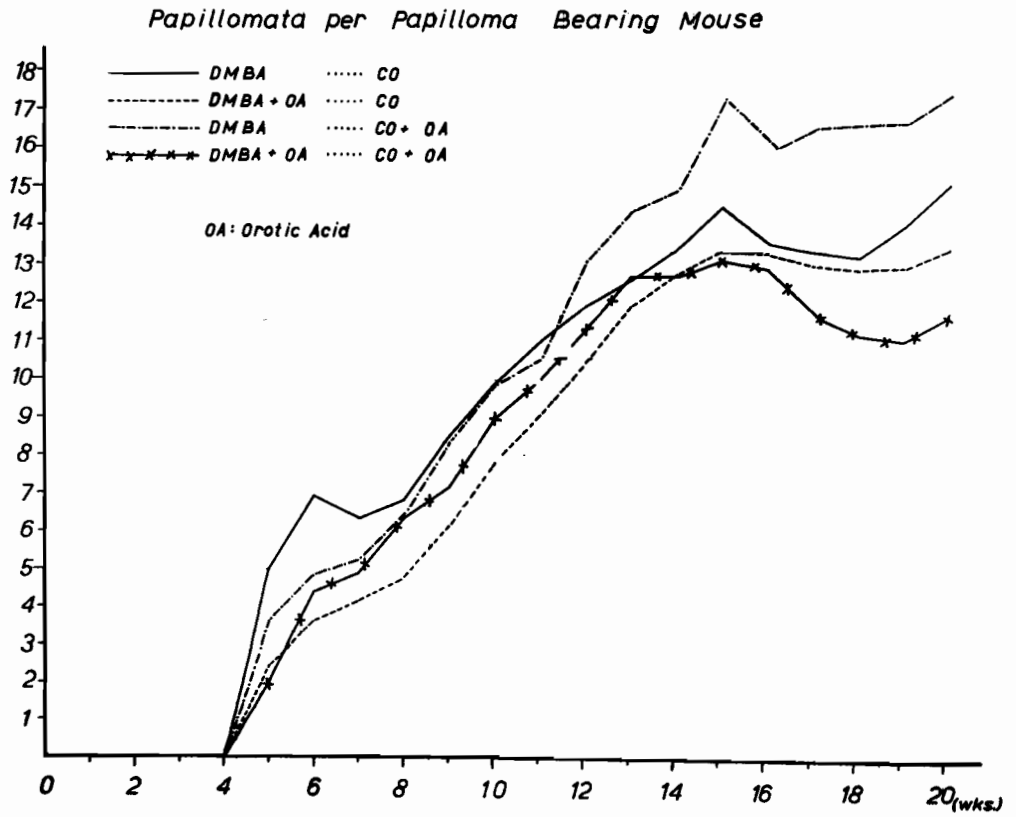
APPENDIX VI

FIGURE I



APPENDIX VI

FIGURE II



APPENDIX VI

FIGURE III

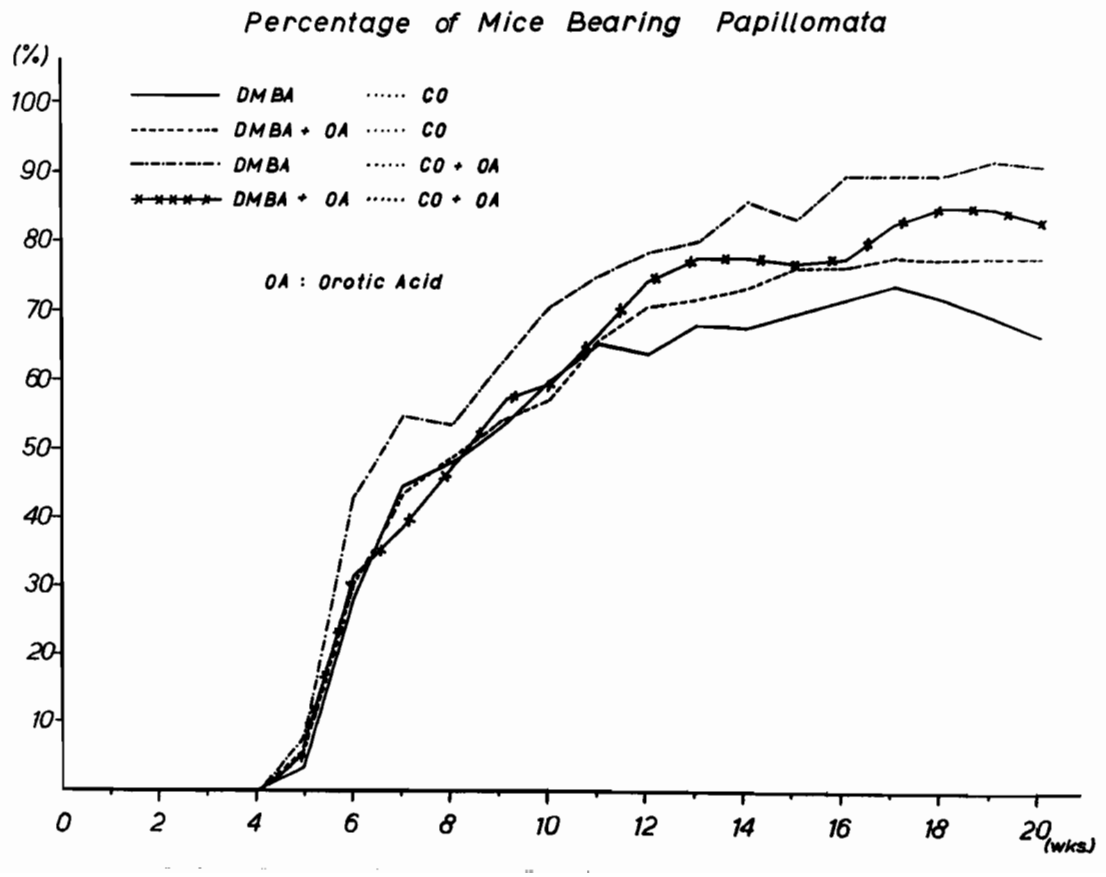


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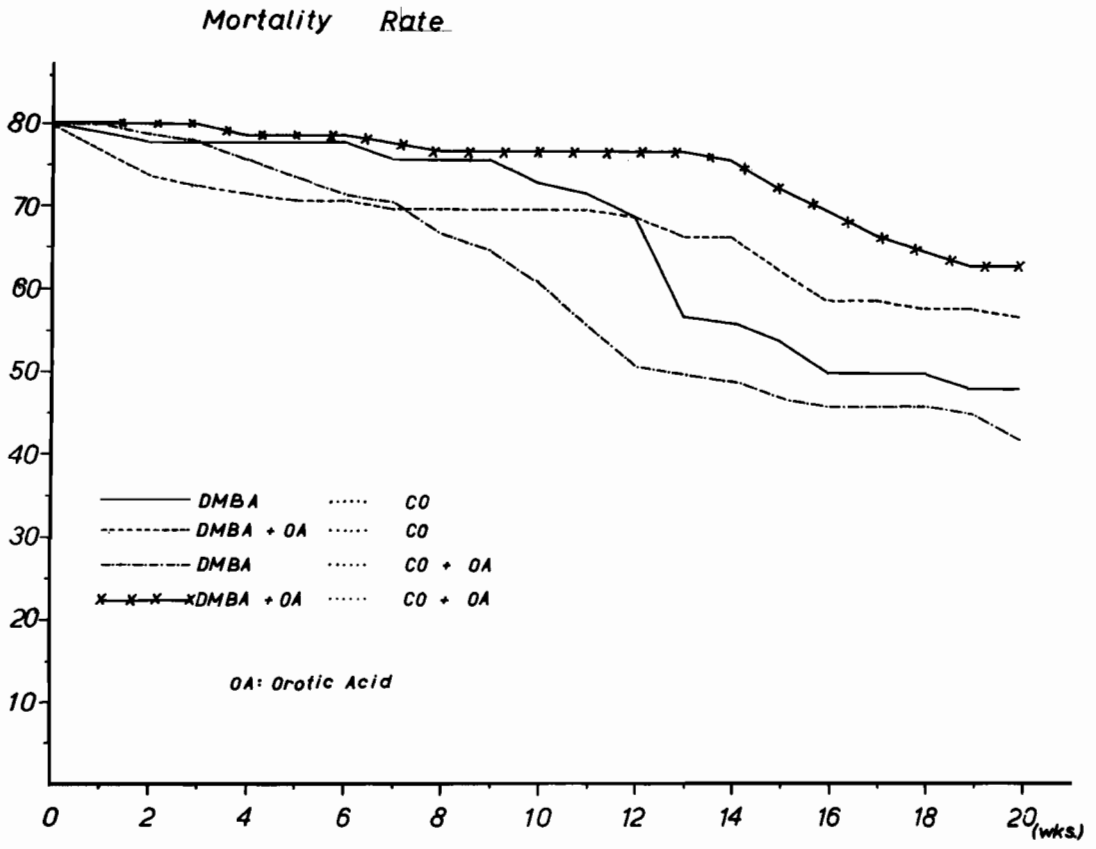
FIGURE IV



APPENDIX VII

SUMMARY OF THE RESULTS OF EXPERIMENT VIII

APPENDIX VII

TABLE I

SUMMARY OF RESULTS OF GROUP I (ACA)

Weeks of C.O. Treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors
0	54	0	0
1	54	0	0
2	54	0	0
3	54	0	0
4	54	0	0
5	54	0	0
6	54	0	0
7	54	0	0
8	54	0	0
9	54	0	0
10	54	0	0
11	53	0	0
12	53	0	0
13	53	0	0
14	53	0	0
15	53	0	0
16	53	0	0

APPENDIX VII

TABLE II

SUMMARY OF RESULTS OF GROUP II (ACB)

Weeks of C.O. Treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors	Papillomata per survivor	Papillomata per Papilloma bearing survivor
0	66	0	0	0	0
1	66	0	0	0	0
2	66	0	0	0	0
3	64	0	0	0	0
4	63	0	0	0	0
5	63	10	4	0.16	2.5
6	63	62	18	0.98	3.4
7	63	179	27	2.84	6.6
8	63	280	31	4.44	9.0
9	62	436	34	7.03	12.8
10	61	531	38	8.70	14.0
11	60	647	39	10.78	16.6
12	58	689	38	11.87	18.1
13	57	714	38	12.53	18.8
14	56	717	39	12.80	18.4
15	55	728	41	13.24	17.8
16	55	724	41	13.16	17.7

APPENDIX VII

TABLE II

SUMMARY OF RESULTS OF GROUP II (ACB)

Percentage of survivors bearing Papillomata	Number of new Papillomata appearing	Number of regressing Papillomata	Papillomata per survivor by increment	Papillomata per Papilloma bearing by increment
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
6.4	10	0	0.16	2.5
28.6	52	0	0.99	5.4
42.9	117	0	2.85	9.7
49.2	101	0	4.45	13.0
54.8	158	2	7.00	17.6
62.3	97	2	8.59	20.2
65.0	121	5	10.61	23.2
65.5	58	16	11.61	24.7
66.7	45	19	12.40	25.9
69.6	24	21	12.83	26.5
74.5	45	23	13.65	27.6
74.5	30	34	14.20	28.3

APPENDIX VII

TABLE III

SUMMARY OF RESULTS OF GROUP III (ACC)

Weeks of C.O. Treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors	Papillomata per survivor	Papillomata per Papilloma bearing survivor
0	65	0	0	0	0
1	62	0	0	0	0
2	58	0	0	0	0
3	58	0	0	0	0
4	58	0	0	0	0
5	58	2	2	0.03	1.0
6	58	42	14	0.72	3.0
7	57	135	23	2.37	5.9
8	57	212	25	3.71	8.5
9	57	324	29	5.68	11.2
10	57	394	29	6.91	13.6
11	56	457	29	8.16	15.8
12	56	476	30	8.50	15.9
13	56	476	31	8.50	15.4
14	53	459	31	8.66	14.8
15	51	457	31	8.96	14.7
16	46	393	28	8.54	14.0

APPENDIX VII

TABLE III

SUMMARY OF RESULTS OF GROUP III (ACC)

Percentage of survivors bearing Papillomata	Number of new Papillomata appearing	Number of regressing Papillomata	Papillomata per survivor by increment	Papillomata per Papilloma bearing by increment
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
3.5	2	0	0.03	1.0
24.1	40	0	0.72	3.9
40.4	93	0	2.35	7.9
43.9	77	0	3.70	11.0
50.9	115	3	5.72	14.9
50.9	71	1	6.96	17.4
51.8	70	7	8.21	19.8
53.6	33	14	8.80	20.9
55.3	20	20	9.16	21.6
58.5	7	24	9.29	21.8
60.8	23	26	9.74	22.5
60.9	19	32	10.15	23.2

APPENDIX VII

TABLE IV

SUMMARY OF RESULTS OF GROUP IV (ACD)

Weeks of C.O. Treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors	Papillomata per survivor	Papillomata per Papilloma bearing survivor
0	67	0	0	0	0
1	66	0	0	0	0
2	65	0	0	0	0
3	64	0	0	0	0
4	64	0	0	0	0
5	62	7	4	0.11	1.8
6	62	34	14	0.55	2.4
7	59	118	25	2.00	4.7
8	59	215	30	3.64	7.2
9	59	306	31	5.19	9.9
10	58	360	33	6.20	10.9
11	58	418	33	7.21	12.7
12	57	449	32	7.88	14.0
13	57	453	32	7.95	14.2
14	57	443	32	7.77	13.8
15	56	435	33	7.77	13.2
16	55	432	33	7.85	13.1

APPENDIX VII

TABLE IV

SUMMARY OF RESULTS OF GROUP IV (ACD)

Percentage of survivors bearing Papillomata	Number of new Papillomata appearing	Number of regressing Papillomata	Papillomata per survivor by increment	Papillomata per Papilloma bearing by increment
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
6.5	7	0	0.11	1.8
22.6	27	0	0.55	3.7
42.4	84	0	1.97	7.0
50.8	97	0	3.61	10.3
52.5	91	0	5.14	13.2
56.9	57	3	6.12	15.0
56.9	66	8	7.26	17.0
56.1	49	8	8.12	18.5
56.1	23	19	8.52	19.2
56.1	12	22	8.73	19.6
58.9	19	18	9.07	20.2
60.0	20	23	9.43	20.8

APPENDIX VII

TABLE V

SUMMARY OF RESULTS OF GROUP V (ACE)

Weeks of C.O. Treatment	Survivors	Total number Papillomata on survivors	Papillomata bearing survivors
0	54	0	0
1	53	0	0
2	52	0	0
3	52	0	0
4	52	0	0
5	52	0	0
6	52	0	0
7	51	0	0
8	51	0	0
9	51	2	2
10	46	3	2
11	45	3	2
12	44	5	3
13	43	5	3
14	43	7	3
15	42	11	6
16	42	16	10

APPENDIX VII

FIGURE I

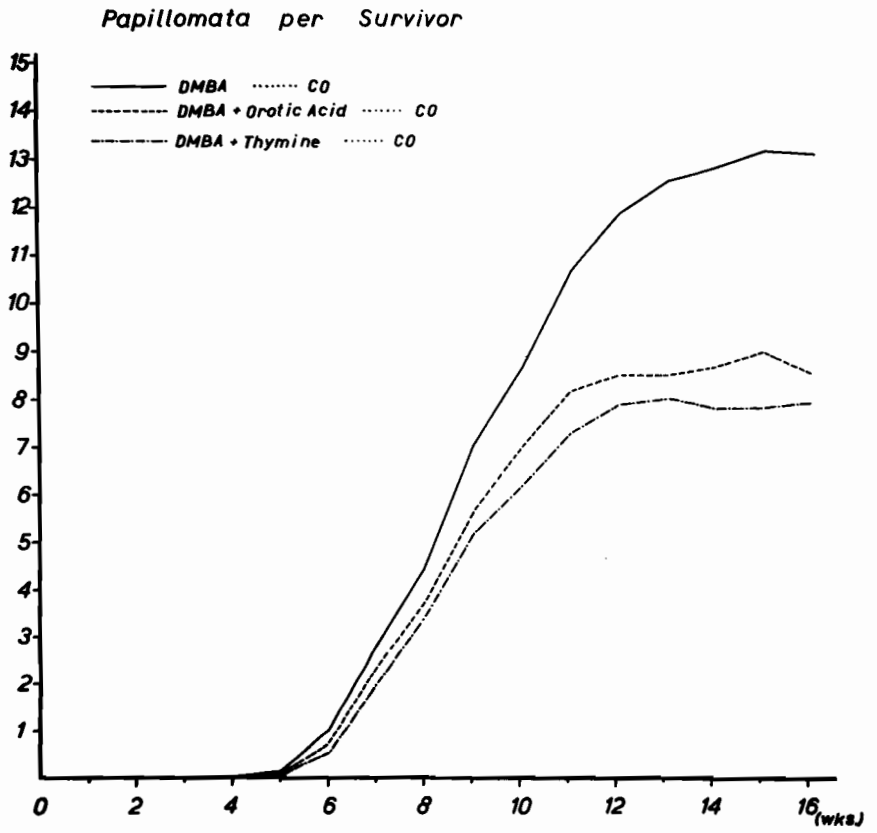


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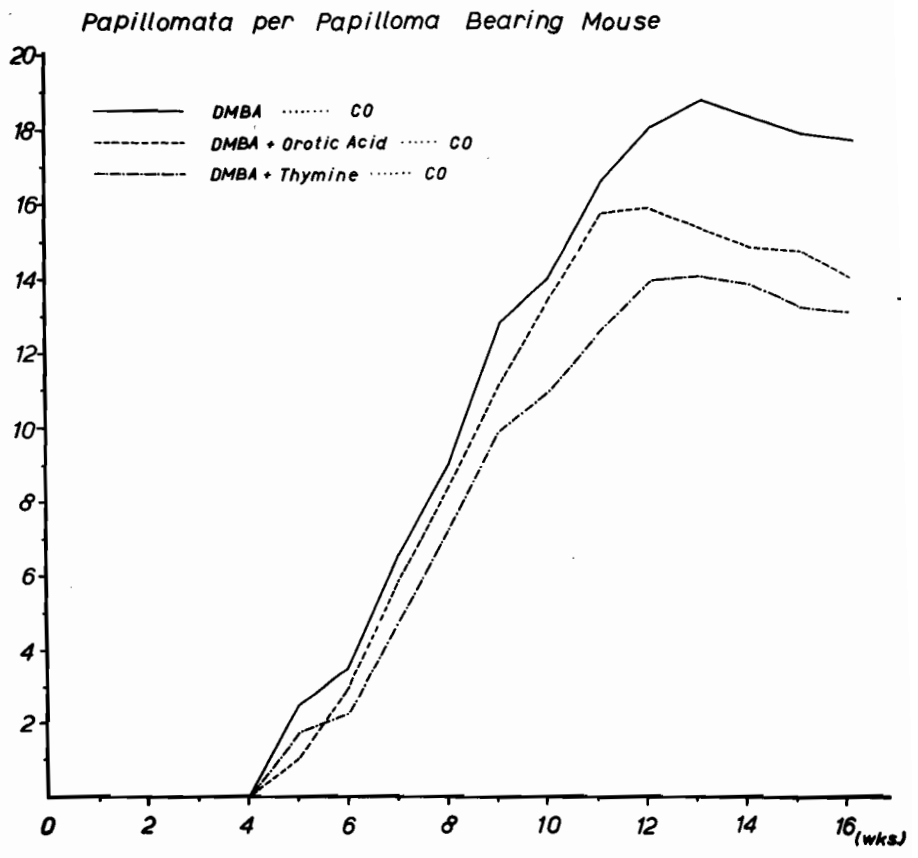
FIGURE II



APPENDIX VII

FIGURE III

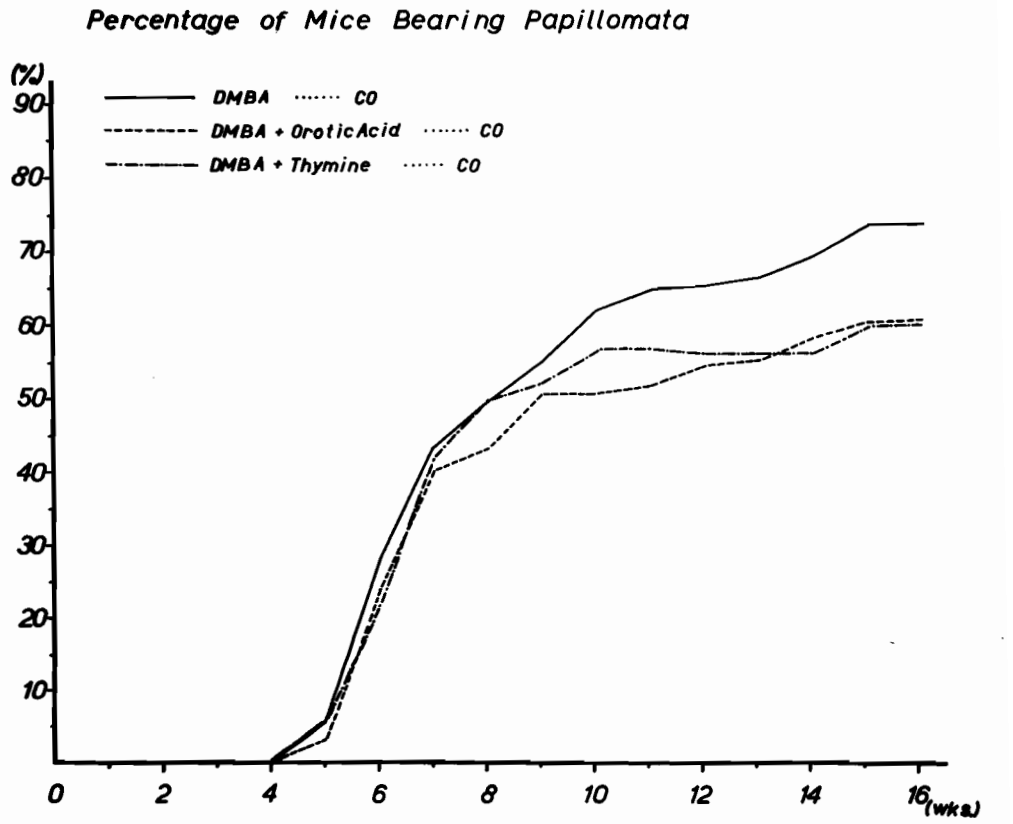


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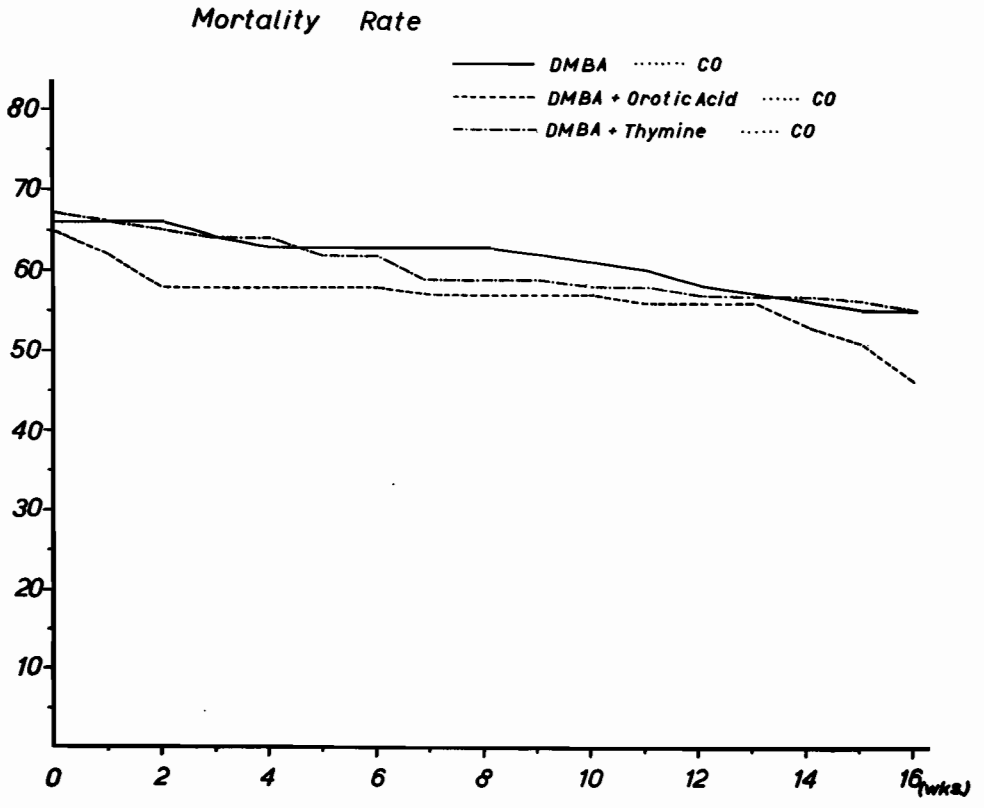
FIGURE IV



APPENDIX VIII

ILLUSTRATION OF PAPER BY YAMAGIWA AND ICHIKAWA

APPENDIX VIII

TABLE I

The First Page of The Report on Experimentally Induced Papillomata on Rabbit Ear by Yamagiwa and Ichikawa

東京醫學會雜誌 第參拾卷

大正五年一月五日發行

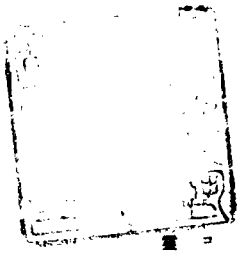
原著

上皮性腫瘍ノ發生ニ關スル實驗的研究 第一報告（附第一乃至第二十一圖）

東京醫科大學病理學教室ニ於テ

醫學博士 山極勝三郎

農學士 市川厚一



恭ク我等ノ偉大ナル恩師故 Rudolf Virchow 先生ノ靈前ニ捧ゲ報恩ノ微意ヲ表セントテ。

目次

APPENDIX VIII

TABLE II

Translation of TABLE I

Tokyo Medical Association Journal Volume 30 January 5, 1916

Experimental Studies on the Pathogenesis of Epithelial Tumours.

The First Report (With 21 Illustrations).

We wish to dedicate this work to our teacher,

Rudolph Virchow.

Pathological Institute
Tokyo University School of Medicine

Katsusaburo Yamagiwa, M.D.
Koichi Ichikawa, B.Sc.

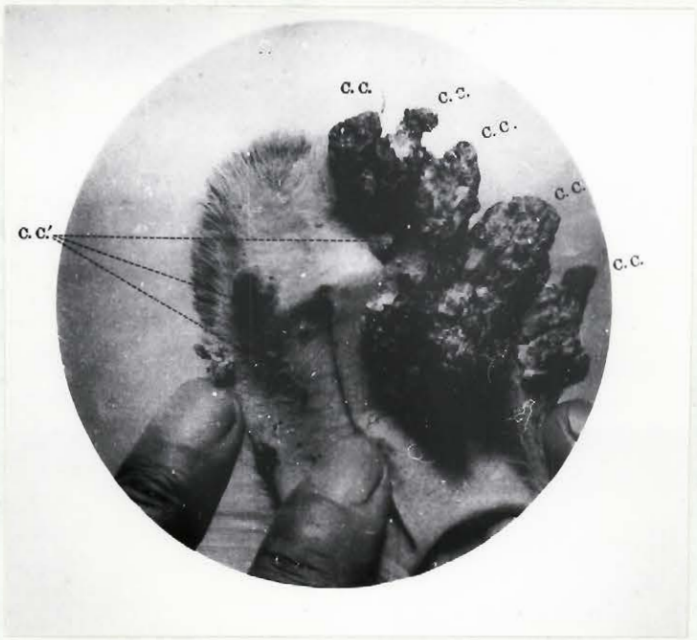
Contents:

APPENDIX VIII

FIGURE I

Photographic Illustrations of Papillomata on Rabbit Ear by Yamagiwa and Ichikawa

A: Macroscopic Appearance



B: Microscopic Appearance