DEPIGMENTATION OF HAIR IN MICE

DUE TO TUMOUR-PROMOTERS AND

OTHER CHEMICALS

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ABSTRACT

Sixty-six compounds including carcinogens, related compounds and non-carcinogens were dissolved in dimethyl sulphoxide or saline and injected intradermally into C57 Black male mice. Localised areas of depigmentation occurred with some chemicals. The effect was related to tumour-promotion with 56% of known tumour-promoters tested producing depigmentation compared with 24% of carcinogens and 17% of substances not known to be carcinogenic. Direct carcinogens such as alkylating agents which have both initiating and tumour-promoting activity produced depigmentation. Indirect carcinogens such as the polycyclic aromatic hydrocarbons and aromatic amines which require metabolic activation were not active. Some compounds known to cause occupational leukoderma were active. Amongst the tumour-promoters, tetradecanoyl phorbol acetate (TPA), carbon dioxide snow and croton oil produced the most prominent depigmentation. The dose producing the effect in half the injected sites (i.e. ED_{50}) was 0.018 μg for TPA, 0.18 μg for nitrogen mustard and 101 μg for chloroform. Other tumour-promoters causing slight or moderate depigmentation included Tween 20, saccharin and dodecane. The promoters anthralin and phenobarbitone did not produce depigmentation when injected in tolerated doses. Histological examination and electron microscopy of depigmented areas showed loss of pigment in affected hair and a decrease in melanocytes at the hair bulbs. The latent period for depigmentation was usually between three to six weeks, but ground crocidolite fibres produced depigmentation after sixteen weeks. Other forms of asbestos fibres caused no depigmentation. The depigmentation of hair might be developed as a short-term screening test for tumour-promoters.

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INTRODUCTION

The term 'cancer' (Latin for crab) means any malignant neoplasm or
new growth. It is not one specific disease entity but in reality
has to be considered as a group of different diseases with different
causative factors possessing a few features in common. The main
feature is the ability of cancer cells to exhibit excessive autono-
mous, progressive and usually irreversible growth. There is also a
tendency for such cells to spread both locally as well as via lym-
phatic and blood vessels to other parts of the body as metastases.
In the process of spreading, surrounding tissues are compressed,
invaded and destroyed and often the eventual outcome is death.
Histologically, cancer cells show abnormal mitotic figures, with
considerable variation in the cell and nuclear size and shape as well
as an increase in size and number of nucleoli per cell. The nuclear
DNA tends to be coarsely aggregated and distributed near the nuclear
membrane. The cellular cytoplasm is scanty and more deeply basophilic
staining. Cancer cells are often immature and not well differentiated.
There are a few exceptions where cancer cells do not show all of these
characteristics. Malignant rodent ulcers and argentaffinomas are
relatively slow growing and thyroid carcinomas often show well-
differentiated cells on histological examination and can continue to
function and produce thyroglobulin.

Cancer is now the second most frequent cause of death in western
countries - the leading cause of death being heart disease. In the
United States in 1900, tuberculosis was the most common cause of death,
with heart disease fourth and cancer eighth. By 1970, heart disease
had become the most frequent cause followed by cancer, cerebro-vascular

diseases and accidents (Levin et al 1974). The same trend is also apparent in mortality statistics from developing countries. Part of this is due to the diminished problems of tuberculosis and other infective conditions as a result of the advent of new, effective pharmacological agents and improvements in environmental sanitation and hygiene aiding in reducing mortality due to these diseases. An increasing expectation of life with a larger proportion of people over forty years of age in the general population also contributes to the increase in cancer incidence. People are now living long enough to be in the age group where most malignancies occur. In the past, the infective illnesses caused death before this age group was reached.

There are as yet, few effective cures for cancers. Choriocarcinomas can be treated with a 70% cure rate (Galton 1978) and Burkitt's lymphoma has a 20 - 50% successful cure rate and as high as 80% complete remission rate. Greater than 50% cure rate after a single dose of cyclophosphomide has been reported for Burkitt's lymphoma and the prognosis is especially good if detected early and the lesions are localized (Scott 1978). However, for a large majority of the malignancies, the efficacy of treatment is considered in terms of three or five year survival rates rather than cure rates. Cancers are usually treated either surgically, by radiotherapy , by the use of anti-cancer drugs or by a combination of these. Surgery has the major disadvantage of being disfiguring, as in radical mastectomy for carcinoma of the breast. Radiotherapy can cause side effects such as radiation burns, hair loss and radiation sickness. Chemotherapy is not specific enough and drugs acting against actively dividing cells do not distinguish between tumour cells and normal cells. Hence, actively dividing

normal cells such as those of the bone marrow, gonads and the gut are affected. Cancer patients on chemotherapy can suffer complications such as bleeding from the gastro-intestinal tract and loss of hair. In spite of these side effects, the cure rates are low. Hence, the emphasis should be on the prevention of cancer, as effective treatment appears to be lacking. Efforts directed towards prevention would be financially more worthwhile than those directed towards cure. However, before an attempt is made to put prevention into practice, what is first needed is knowledge of the aetiology of cancer. Prevention can only be fully effective if it can be directed at the specific aetio-logical agent or agents. Unfortunately, in the case of cancer, know-ledge of specific aetiology is inadequate. There are some identifiable causes of cancer and these include:-

(a) Genetic factors

(b) Viral factors

(c) Physical factors

(d) Chemical factors

These factors are relevant only in a small proportion of cancer cases. In the majority of cases, specific causes are unidentifiable.

1. GENETIC FACTORS

Several cancers exist where an autosomal dominant or recessive or sex-linked gene appears to be primarily responsible. Retinoblastoma is an example where autosomal dominance is the probable mode of inheritence. This malignant tumour is responsible for 1% of all cancer deaths in infants and approximately 5% of childhood blindness (Lynch et al 1976). Albinism is an example of an autosomal recessive condition where there is a related increased risk of

basal and squamous cell carcinoma of the skin.

Chromosomal abnormalities can also be linked to an increased risk of cancer (Lancet 1977). In mongolism (Down's Syndrome) trisomy of chromosome 21 exists with an increased risk of acute leukaemia. In patients with the Philadelphia chromosome there is microscopically visible loss of chromosomal material. This is related to an increased risk of developing chronic myeloid leukaemia (Nowell et al 1960; Baikie et al 1960).

2. VIRAL FACTORS

Viruses can cause warts or papillomas of the skin in humans. Burkitt's lymphoma, a malignancy of lymph nodes first described by Burkitt in 1958, is related to exposure to the Epstein-Barr virus (Epstein and Achong 1973). There is also evidence of virus related tumours in animals. Rous (1911) showed that an RNA (ribonucleic acid) virus related to the myxoviruses caused sarcomas in fowls. The papova viruses (including the papilloma, polyoma and vacuolating agent) are DNA (deoxyribonucleic acid) viruses which cause a variety of tumours in rats, rabbits, mice and hampsters.

3. PHYSICAL FACTORS

Radiation is a cause of leukaemia. It was within a few years of the discovery of x-rays that its indiscriminate use was linked to numerous superficial squamous carcinomas. Later, chronic myeloid leukaemia was noted as a major occupational risk in radiologists. X-rays, γ-rays, alpha and beta particles and radio-isotopes can all cause cancer. Besides the carcinogenic effects, radiation also has mutagenic effects as has been shown on experiments with Drosophila (Fahmy and Fahmy 1970 and 1972).

4. CHEMICAL CAUSES

Chemicals used in the workplace have been linked to various cancers and examples of these occupational cancers are well-documented. One of the earliest descriptions of such cancer is that of carcinoma of the scrotum in chimney sweeps described by Sir Percival Pott in his classic paper 'Chirurogical Observations' in 1775. The scrotal cancers were thought to be a result of the constant deposition of soot from the chimneys into the folds of the scrotal skin. Following Pott's clinical observations, Yamagiwa and Ichigawa in Japan in 1917 were able to produce tumours by repeated applications of coal tar to the ear of rabbits. Kennaway and associates in 1924, isolated the first carcinogen, 3,4-benzpyrene from coal tar and later identified other carcinogens in the polycyclic hydrocarbons group. Benzpyrene is believed to be responsible for the carcinogenic activity of tar and soot. The term carcinogen was first used by Sir James Paget in 1853 in his book 'Lectures on Surgical Pathology' to mean a cancer-producing agent. Since then, many such agents have been identified and isolated. Rehn in 1895, described the first cases of bladder cancer due to exposure to aromatic amines in German workers in the synthetic dye industry. 2-Naphthylamine and benzidine have been implicated as causative carcinogens. More recently, cases of the rare tumour angiosarcoma of the liver have been linked to exposure to vinyl chloride monomer (Creech and Johnson 1974, Tabershaw and Gaffey 1974).

There are many theories on how agents bring about the carcinogenic process e.g. the immunological theory (Green 1954) and the genetic regulating mechanisms theory (Jacob and Monod 1961). Theories on chemical carcinogenesis may be grouped into those based on a

genetic effect and those based on an epigenetic effect. The
somatic mutation theory (Boveri 1914) belongs to the former group.
It suggests that DNA modification is an important step in the caus-
ation of cancer. Chemical carcinogens act by altering the DNA to
a form characteristic of a malignant cell. Such DNA alterations
are somatic mutations which may include frame-shift mutations as
well as base-pair deletions. In studies on Drosophila (Fahmy and
Fahmy 1970) carcinogens have been shown to cause small gene dele-
tions, resulting in mutants. The hypothesis that mutation is a
critical event in carcinogenesis is also supported by statistical
studies on retinoblastoma (Knudson 1971). There is considerable
evidence that, with few exceptions, carcinogens are mutagens (Ames,
McCann and Yamasaki 1975; Sugimura et al 1976).

The epigenetic theories are concerned not with the genetic infor-
mation in the cell but with the expression of that information.
Some genetic effects may be repressed ~~de-repressed~~ by carcinogens.
This could lead to an upset in the hormonal balance or immune
capacity and thereby stimulate proliferation of the neoplastic cell.
This could also explain increases in foetal antigens and isoenzyme
patterns seen in certain cancers e.g. increased α1-foetoprotein in
primary hepatocarcinomas of the liver, teratomas and carcinomas of
the gastro-intestinal tract; human placental lactogen and placental
alkaline phosphatase in carcinoma of the bronchus; the foetal sulpho-
glycoprotein antigen in gastric carcinoma (Lowing 1977).

Several similar theories divide the carcinogenic process into
stages (Berenblum 1941, Rous and Beard 1935, Mottram 1944a and 1944b,
Kline and Rusch 1944). Rous (1941) adopted the terms 'initiation'
and 'promotion' to describe two stages in carcinogenesis. Initiation

is the first stage where normal tissue cells are converted into 'latent tumour cells' or 'sensitized cells' by initiating agents. Such agents have included a sub-threshold dose of a carcinogen such as 9,10-dimethyl-1,2-benzanthracene, 3,4-benzpyrene and β-radiation. The initiated cell then needs to be transformed into tumour cells by regular exposure to a tumour-promoter. Tumour-promoters are by themselves non-carcinogenic. They act slowly and prolonged treatment to an initiated cell is needed for effective promotion. Table 1 summarizes the differentiating features between initiators and promoters.

<p align="center">TABLE 1</p>

<p align="center">A COMPARISON OF BIOLOGIC PROPERTIES</p>

<p align="center">OF</p>

<p align="center">INITIATING AGENTS AND PROMOTING AGENTS</p>

<p align="center">(from Weinstein 1978)</p>

Initiating Agents	Promoting Agents
1. Carcinogenic by themselves 'Solitary Carcinogens' *	1. Not carcinogenic alone
2. Must be given before promoting agent	2. Must be given after the initiating agent
3. Single exposure is sufficient	3. Require prolonged exposure
4. Action is 'irreversible' and additive	4. Action is reversible (at early stage) and not additive
5. No apparent threshold	5. Probable threshold
6. Yield electrophiles - bind covalently to cell macromolecules	6. No evidence of covalent binding
7. Mutagenic	7. Not mutagenic

*   Van Duuren (1976) refers to some initiating agents that are

*   non-carcinogenic to mouse skin e.g. chrysene, urethane,
epichlorhydrin and chloromethyl methyl ether (CMME). However,
chrysene is considered a borderline carcinogen (Hartwell 1951)
and urethane has been shown to cause lung tumours in mice (Salaman
and Roe 1953). Epichlorhydrin and CMME induced sarcomas in mice
by subcutaneous injections (Van Duuren et al 1974). So, in general,
initiating agents are carcinogenic.

In addition to initiation and tumour-promotion, a third term often used
is co-carcinogen. Shear (1938) and Cabot et al (1940) introduced the
term to cover the enhancement of skin tumour formation by creosote in
conjunction with 3,4-benzpyrene. Wolf (1952) defined it as a substance
which, though not in itself carcinogenic, can enhance tumour incidence
and shorten the latent period when applied together with or shortly
after the carcinogen. Since then 'co-carcinogen' has been used to cover
all factors that in some way promote the formation of tumours. It,
therefore, includes tumour-promoters. However, it has also been used
as a term quite separate from tumour-promotion (Van Duuren 1976). A
restricted definition confines co-carcinogen to chemicals which when
applied simultaneously with initiators will increase the yield of
tumours formed. Tumour-promoters only enhance tumour-formation by
regular application after initial exposure to the initiating agent.
This latter definition has been adopted for this thesis.

Chemical carcinogens can also be divided into direct-acting carcinogens
(or primary or complete carcinogens) and pro-carcinogens (or secondary
carcinogens). Direct acting carcinogens usually act at the site of
application and do not require metabolic activation. Pro-carcinogens
must undergo metabolic activation before they can exert their carcino-
genic activity. Such activation is often the result of action by liver,
skin or other tissue enzymes converting the pro-carcinogen into an
active metabolite - the ultimate carcinogen. Pro-carcinogens do not

usually act at the point of application. Table 2 shows examples of direct-acting carcinogens and pro-carcinogens.

TABLE 2

DIRECT-ACTING CARCINOGENS AND PRO-CARCINOGENS

Direct-Acting Carcinogens	Pro-Carcinogens
1. HN2 (Methyl-bis-(2-chloroethyl)-amine). 2. Methyl Iodide 3. Benzyl Chloride 4. Benzoyl Chloride 5. Benzotrichloride 6. Dimethyl Sulphate	1. Polycyclic Aromatic Hydrocarbons (a) Dibenzanthracene (b) Dimethylbenzanthracene (c) Benzanthracene (d) Benzpyrene (e) 3-Methylcholanthrene 2. Aromatic Amines (a) 2-Naphthylamine (b) Benzidine (c) Phenanthrene 3. Dialkyl Hydrazines (a) Dimethylhydrazine

The Health, Education and Welfare Secretary of the United States has estimated that at least 20% of all cancers in the United States may be work-related (Califano 1978). This implies that in a majority of cases, chemical carcinogens may be involved. The estimate was based on studies by the National Cancer Institute and the National Institute for Environmental Health Sciences and is higher than earlier estimated figures of 1 - 5% of cancers being attributable to occupational exposure to carcinogens (Higginson and Muir 1976, Doll 1977, Wynder and Gori 1977).

Estimates may vary but the over-riding factor is that the causative agent or agents in such occupational cancers can often be identified and efforts directed to such identification would be useful before preventive measures are instituted.

Methods used for identification of carcinogens have included epidemiological studies - these are studies based on human experience. They can be either:-

(a) Descriptive, where one describes a cluster of similar rare cancers occurring under similar circumstances or a change in cancer incidence following introduction of a new chemical or process.

(b) Prospective, where a defined group of workers subject to exposure to specific chemicals is followed up and the effects noted over a given period of time.

(c) Retrospective, where cancer cases are defined and are then traced to obtain specific information regarding exposure to various agents, previous occupations and other points in the past history to try to find out whether there is an excess of such factors in these cancer cases.

The disadvantage of epidemiological studies is that they can be very time-consuming and costly. Retrospective studies have the disadvantage of relying on memory of interviewees and prospective studies have the problem of loss to follow up. They still afford the most conclusive method of deciding whether a chemical is a possible human carcinogen. In view of the many disadvantages and limitations, other methods of identifying carcinogens are needed.

One method is by examining the chemical structure of compounds and

comparing this with that of known carcinogens. Chemicals which show similarity in structure can be expected to exert similar effects. It is possible that carcinogenicity of compounds may be predicted from their structure. The following examples show chemicals with similar chemical structure all with some degree of carcinogenic potential. (see Table 3 overleaf).

TABLE 3

CARCINOGENIC CHEMICALS WITH SIMILAR CHEMICAL STRUCTURE

EXAMPLE 1 - CHLORINATED HYDROCARBONS

Chemical	Formula	Carcinogenic Status	Reference
Vinyl Chloride	$\begin{array}{c} H \quad\quad Cl \\ C = C \\ H \quad\quad H \end{array}$	Causes angiosarcomas of the liver in humans Carcinogenic in mice and rats following exposure by inhalation	Creech and Johnson 1974 Tabershaw and Gaffey 1974 Maltoni and Lefemine 1974 Viola, Bigotti and Caputo 1971
Trichloroethylene	$\begin{array}{c} H \quad\quad Cl \\ C = C \\ Cl \quad\quad Cl \end{array}$	Produces liver tumours in mice but not rats after oral administration	Lloyd, Moore and Breslin 1975
Perchloroethylene	$\begin{array}{c} Cl \quad\quad Cl \\ C = C \\ Cl \quad\quad Cl \end{array}$	Carcinogenic in mice	NIOSH Current Intelligence Bulletin 1978

TABLE 3

CARCINOGENIC CHEMICALS WITH SIMILAR CHEMICAL STRUCTURE

EXAMPLE 2 - AROMATIC AMINES
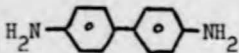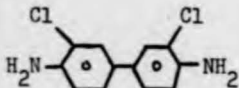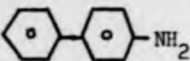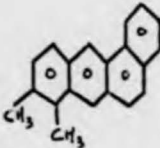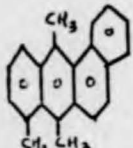
Chemical	Formula	Carcinogenic Status	Reference
Benzidine	H_2N—⬡—⬡—NH_2	Carcinogenic to mouse, rat, hamster and possibly dog	Bonser, Clayson and Jull 1956 Spitz, Maguigan and Dobriner 1950 Boyland, Harris and Horning 1954 Saffiotti, Cefis and Montesano 1967
		Evidence of carcinogenicity in humans	Case, Hosker and McDonald 1954
3,3'-Dichlorobenzidine	H_2N—⬡—⬡—NH_2 with Cl, Cl	Carcinogenic to rat after oral and and subcutaneous administration. Also to hamsters after oral administration	Pliss 1959 Safiotti, Cefis and Montesano 1967
4-Aminobiphenyl (xenylamine)	⬡—⬡—NH_2	Carcinogenic to mouse, rat and dog	Clayson, Lawson and Pringle 1967 Walpole, Williams and Roberts 1954 and 1952
		Exposure in man linked to bladder cancer	Melick et al 1955

TABLE 3

CARCINOGENIC CHEMICALS WITH SIMILAR CHEMICAL STRUCTURE

POLYCYCLIC AROMATIC HYDROCARBONS

Chemical	Formula	Carcinogenic Status	Reference
Benzanthracene		Complete carcinogen for mouse skin and carcinogenic to mouse	Kennaway 1930 Bock and King 1959
		No epidemiological evidence for carcinogenicity in man but coal tar and other materials known to cause cancer in man may contain benzanthracene	IARC Monographs n the Evaluation of Carcinogenic Risk of Chemicals to man Vol III pg 56 - 57 (1972)
5,6-Dimethyl-benzanthracene		Carcinogenic in mice	Haddow and Kon 1947
5,9,10-Trimethyl-benzanthracene			Haddow and Kon 1947

However, there are exceptions to the link between similarity in
chemical structure and carcinogenic activity. The following examples
illustrate such exceptions:-

Example 1
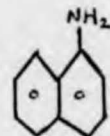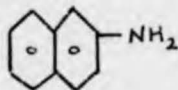
2-Naphthylamine is carcinogenic but 1-naphthylamine is not (Boyland
1978a).

Example 2
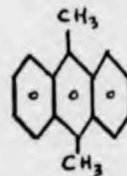
Anthracene is non-carcinogenic but 9,10 dimethylanthracene is
carcinogenic (Boyland 1978a).

Example 3
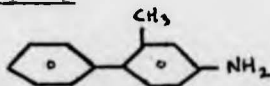
2-Methyl-4-amino-biphenyl is not carcinogenic though it is structurally
similar to the earlier examples benzidine, 4-amino biphenyl and 2-methyl
benzidine which are carcinogens (Miller et al 1956).

Example 4
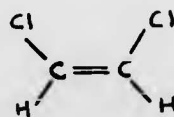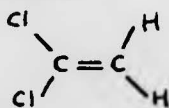
1,1-Dichloroethylene and 1,2-Dichloroethylene are both structurally
similar to vinyl chloride, trichloroethylene and perchloroethylene.
They are treated as suspect carcinogens because of this similarity in

structure but as yet there are no definite reports on their carcino-
genicity (Fairchild 1978).

Example 5
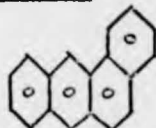
Benzanthracene and some of its dimethyl derivatives are carcinogenic
(see Table 3 page 29) but the addition of a phenolic (OH) group or
hydrogenation of individual rings results in loss of carcinogenicity
(Haddow and Kon 1947).

Hence, it is not just similarity in structure alone, but the nature,
number and position of the side-chains as well that seems to determine
carcinogenic status. It has been suggested that carcinogenic activity
depends on an optimal molecular complexity (Haddow and Kon 1947).
However, existing structure-activity studies of structural association
with carcinogenicity have not produced any generally applicable
principles common to various series of carcinogens (Ashby 1978). This
lack of inter-series consistency limits the use of structure in pre-
dicting carcinogenicity.

Long-term animal studies are widely used for predicting carcinogenicity.
Test animals under laboratory conditions are regularly dosed with sus-
pected chemicals and if they produce significantly more tumours than a
similarly treated control group, then the chemical concerned is deemed
carcinogenic. The extrapolation of these results of animal tests to
humans is, however, difficult. Chemicals can exert different effects
even amongst related species of animals. 2-Naphthylamine is a bladder
carcinogen in man, dogs and in high doses to hamsters, but not in rats

or rabbits and only weakly carcinogenic to mice. Aflatoxin B is
suspected of causing liver cancer in man but does not elicit a neo-
plastic response in mice, though newborn mice are affected. Tri-
chloroethylene causes tumours in mice but not in rats. However, in
general, there is evidence that substances which are carcinogenic
to man as shown by epidemiological studies will also produce tumours
in laboratory animals. In a recent review of eighty-two chemicals
with epidemiological evidence of human cancer effect, all were shown
to be positive in animal tests with the exception of arsenic,
(Altschuler 1978). At least six chemicals were shown to be carcino-
gens in animals before epidemiological studies confirmed their
carcinogenicity in humans. These are, 4-aminodiphenyl, diethylstil-
boestrol, mustard gas, vinyl chloride, aflatoxin and bis-chloromethyl
ether. In the absence of a better method, animal studies will need
to be used, though care must be exercised in the interpretation of
results so obtained. Another argument commonly used against animal
tests is that the dose of test compounds used in such tests is too
high and, therefore, inappropriate when extrapolated to man. There
is a misconception though that at high enough levels, virtually any
substance will be carcinogenic. Some pesticides and industrial
chemicals have been tested at high doses and found to be negative
(Maugh 1978). The use of high doses increases the proportion of
animals with tumours for carcinogens and, therefore, less animals
need to be used for the tests. Also, high doses may compensate for
unknown cummulative effects, and for the shorter life span of some
of the test animals.

One of the limitations in the use of conventional animal tests is

that existing world facilities only allow some 200 - 500 new chemicals to be tested each year (Saffiotti 1976). Such conventional tests are also costly; approximately £500,000 for a full test on any single chemical and this takes one-and-a-half to three years (the life-span of the test animals) before results are obtained. Hence, what is needed are short-term tests which are as specific and reliable in detecting chemicals carcinogenic to man. These tests should be cheap, easy to perform and produce results in a relatively short period of time. A variety of such short-term tests are already in use. Some of them are based on empirical observations and others on possible carcinogenic effects. Most of the tests are still being evaluated and many need improvements and standardisation. Some examples of such tests are:-

1. The Ames Test (Ames et al 1973 and 1975)

This test developed by Professor B N Ames of the University of California, Berkely, U.S.A. is based on the premise that mutagens and carcinogens are closely related. A histidine-dependant strain of Salmonella typhimurium is incubated with test chemicals and if such chemicals are carcinogenic, they will exert a mutagenic effect causing reversion of histidine-dependence to histidine-independence and thereby allowing the bacteria to grow in a medium lacking histidine. The chemically-induced reversion rate is compared to the spontaneous reversion rate and a two-fold increase is indicative of a positive response i.e. the test chemical is mutagenic and, therefore, likely to be a carcinogen. This test works well with direct carcinogens such as alkylating agents and nitrosamides but not with indirect carcinogens such as the polycyclic aromatic hydrocarbons and aromatic amines. Indirect carcinogens require

metabolic activation to the ultimate carcinogen before this exerts its carcinogenic effects. Hence, the addition of a supernatant from centrifuging rat or human tissue homogenates was incorporated into the test system. This supernatant known as the S-9 mix (because the rat or human tissue is minced and centrifuged at 9,000r) theoretically provides the tissue enzymes for activating the indirect carcinogens under test. The yield of such enzymes is increased by injecting rats with Aroclor before killing them and removing the liver for preparation of the S-9 mix. Aroclor induces the rat liver enzymes and is the best available inducing agent for such use. Other inducing agents include 3-methyl-cholanthrene and phenobarbitone.

2. Styles Cell Transformation Test (Styles 1977)

This is based on mammalian cell transformation in culture by carcinogens. Syrian hamster kidney cells and either human lung fibroblasts or human liver-derived cells are exposed to five doses of test compounds in vitro in a medium with S-9 mix. After incubation the cells are centrifuged and re-suspended and the growth of the transformed cultures counted. A 2.5-fold increase in colonies over the control is considered positive.

3. Rabin's Test (Williams and Rabin 1971)

Degranulation of rough endoplasmic reticulum (RER) from rat liver by carcinogens is the main feature of Rabin's test. Radio tracer techniques are used to quantitatively assay the degree of degranulation.

4. Sebaceous Gland Test (Bock and Mond 1958)

A decrease in the sebaceous gland/hair follicle ratio in mouse skin

after direct application of a carcinogen is the basis of this test.

5. Tetrazolium Reduction Test (Iversen and Evensen 1962)

Mouse skin is exposed to the test compound and incubated in solutions of tetrazolium red. Carcinogenic activity of the test compound is indicated by reduction of the colourless tetrazolium to coloured formazan. This colour change can be spectrophotometrically measured.

6. Implant Test (Oppenheimer et al 1959)

This test is based on the tissue reaction to subcutaneous implants in mice. Implants of millipore-filter discs impregnated with a gelatinous suspension of the test compound are used. Histological examination is made after three months and lesions are scored on an arbitrary scale. The presence of a fibroblast capsule, its degree of thickness, the presence of necrosis are some features taken into account in the scoring. Such features appear to be related to carcinogenicity of the test compound.

In an evaluation of these short-term tests, Purchase and associates found Ames' and Styles' tests to be the two best tests detecting over 90% of the carcinogens and non-carcinogens tested (Purchase, Longstaff and Ashby 1978).

All these short-term tests do not have cancer induction as an end point. Instead they have some feature which appears with the use of carcinogens but not with non-carcinogens. The appearance of depigmentation of hair in mice following intradermal injections of various carcinogens appears to be a similar feature apparently common to carcinogens. Boyland and Sargent (1951) ~~and 1953~~ noted that irradiation of black mice resulted in a permanent

area of depigmentation both at the site of entry and exit of the
x-rays. A similar effect was produced by single intradermal injec-
tions of nitrogen-mustard (Boyland et al 1948). Both radiation and
nitrogen-mustard produce carcinogenic effects. Searle (1970)
reported depigmentation following skin application of 8-hydroxy-
quinoline in mice and Schoental (1971) reported depigmentation
following injections of N-methyl-N-nitrosourethane in mice and rabbits
and Mitsuyama (1966) described permanent depigmentation in C57 black
mice following intradermal injections of chlorambucil, thio-TEPA and
carzinophilin - all of which are anti-cancer agents.

All these observations suggest that the production of localised areas
of depigmentation in dark-haired mice following a single exposure to
carcinogens may be a feature peculiar to carcinogens. This link
between carcinogens and depigmentation is further supported by studies
in the hamster when oral dosing with the carcinogen dibenzanthracene
had resulted in permanent depigmentation (Aubert and Bohoun 1970).
Lesker and Kaplan (1974) in a follow-up study on adult Mexicans showed
a possible association between greyness of hair and mortality. Davies
(1979) commenting on this study maintained that the possibility of an
association between early greying of hair and cancer cannot be excluded.
Hence, some evidence exists for laboratory animals as well as for
humans, suggesting a connection between carcinogens and depigmentation
of hair. This feature as a distinguishing point between carcinogens
and non-carcinogens may be useful in a screening test for carcinogens.
Hence, the main objectives of this study are:-

1. To investigate the relationship between chemical compounds, with
   particular emphasis on carcinogens, and the production of

depigmentation of hair in dark-haired mice.

2. To investigate the feasibility of using such an effect in a short-term screening test for carcinogens (or classes of carcinogens e.g. promoting agents).

3. To describe such depigmentation in terms of gross and microscopic features and to discuss the possible mechanisms underlying the production of such an effect.

CHAPTER 2   MATERIALS

Chemicals

Equipment

Laboratory Animals

MATERIALS

(A) CHEMICALS

    I ALKYLATING AGENTS

        1. Nitrogen mustard ⌐Methyl-bis-(2-chloroethyl)-amine⌐or HN2

        2. Dimethyl sulphate

        3. Methyl iodide

        4. Benzyl chloride

        5. Benzoyl chloride

        6. Benzotrichloride

   II POLYCYCLIC AROMATIC HYDROCARBONS

        1. 1,2:5,6-Dibenzanthracene

        2. 9,10-Dimethyl-1,2-benzanthracene

        3. 6,9,10-Trimethyl-1,2-benzanthracene

        4. Benzanthracene

        5. Benzo(a)pyrene (Benzpyrene)

        6. Phenanthrene

        7. 3-Methylcholanthrene

  III AROMATIC AMINES

        1. 2-Naphthylamine

        2. Benzidine

        3. Aniline

   IV CARBAMATES

        1. Methyl carbamate

        2. Ethyl carbamate (urethane)

        3. Propyl carbamate

        4. Sodium diethyldithiocarbamate

V PHENOLS

1. Pyrocatechol (Catechol)
2. Methyl catechol
3. 4-tertiary butyl catechol
4. p-tertiary butyl phenol
5. Dihydroxyphenylalanine (DOPA)
6. Phenol
7. Pyrogallol
8. Butylated hydroxy anisole
9. Hydroquinone monobenzyl ether
10. 8-Hydroxyquinoline
11. Adrenaline hydrogen tartrate
12. Histamine hydrochloride

VI ASBESTOS FIBRES

1. Chrysotile A and B
2. Crocidolite
3. Amosite
4. Anthophyllite

VII TUMOUR-PROMOTERS

1. 12-O-tetradecanoyl-phorbol-13-acetate (TPA)
2. Anthralin
3. Dodecane
4. Tween$^{(R)}$ 20, 40 and 80
5. Limonene
6. Croton oil
7. Phenol
8. Carbon dioxide snow

9. Iodoacetic acid

10. Sodium deoxycholate

11. Saccharin

12. Griseofulvin

13. 1,-fluoro-2,4-dinitrobenzene

14. Phenobarbitone

15. Sodium metabisulphite

VIII MISCELLANEOUS

1. Chloroform

2. 1,1,1,Trichloroethane

3. Dimethyl sulphoxide

4. Dimethyl acetamide

5. Acetone

6. Hydrochloric acid

7. Sodium hydroxide

8. Normal saline

9. Putrescine

10. Ethanol

11. Arachis oil

EQUIPMENT

Standard laboratory equipment (including fume cupboard, appropriate glassware, forceps etc) and special facilities for the handling and disposal of carcinogens. Specific equipment used included:-

(a) ULTRAMATIC weighing balance, Model UM3

     Stanton Instruments Ltd, England

(b) YALE MICROLANCE (B - D) hypodermic needles

     12G, 19G and 25G

     Becton, Dickinson & Co Ltd, Ireland

(c) PLASTIPAK (B - D) disposable syringes

     1, 5 and 20 ml

     Becton, Dickinson & Co Ltd, Ireland

(d) ISOMANTLE heater

     Isopad Ltd, Borehamwood, England

(e) VOSS STIRRER

     Voss Instruments Ltd, Maldon, Essex, England

(f) BENCHKOTE polythene-backed absorbent paper

     Whatman Ltd, England

(g) REGENT DISPO surgeons' gloves

     L.R. Industries Ltd, London, E4

(h) BUR'N'BIN metal containers for disposal of chemicals waste

     Metal Box Ltd, Clayton, Manchester, England

LABORATORY ANIMALS

(a) C57 Black mice

(b) Tyzzer Original White mice

(c) CBA Brown mice

All the mice used were pure-strain inbred mice of both sexes, age three to eight weeks, and weighing 20 - 30 gm each. The majority of the experiments were performed on male C57 Black mice. The mice were kept two to four in a cage measuring 13 cm (W) x 30 cm (L) x 13 cm (H) or 13 cm (W) x 44 cm (L) x 13 cm (H). They were fed a standard diet of Dixons 86 (Ref: Appendix Ia) before 30 August 1978 or B.P. Expanded No 1 (Ref: Appendix 1b) after 30 August 1978, and tap-water. A specially prepared cis-retinoic acid diet and placebo diet (Ref: Appendix Ic) was used for one experiment.

The cages were lined with saw-dust and kept in standard animal house conditions at a temperature of 21° - 25°C and a relative humidity of approximately 50%. These cages were placed in different racks and different parts of the animal room. The positions of the cages were changed regularly to allow mice to be randomly exposed to environmental conditions in different areas of the room in case there were any variations in such conditions. This also helps in ensuring that when the mice are examined it will be done 'blind' i.e. without the examiner knowing which cage the particular mouse had come from.

CHAPTER 3 METHOD

Preparation of test chemicals

Preparation of test animals

Intradermal injections

Periodic examination of injected mice

Microscopy

DISCUSSION ON METHODOLOGY

Choice of C57 Black mouse as the model for study

Choice of chemicals to test

Choice of number of injection sites per mouse

Choice of volume of solution per injection

Choice of concentrations of test solutions

Choice of solvents/mediums

METHOD

A standard basic procedure was adopted for testing various chemicals
on the mice. The procedure consisted of single intradermal injections
of solutions of various chemicals. The mice were then periodically
examined to see whether depigmentation appeared at the injection
site/s.

(a) Preparation of Test Chemicals

The test chemicals were selected and appropriate solutions made
using normal saline or dimethyl sulphoxide as diluent. All
solutions were prepared immediately before use so as to prevent
deterioration of unstable compounds in solutions e.g. nitrogen
mustard. The fume cupboard was used for preparation of solutions
and any transport of chemicals within the laboratory was done
using secondary containers to prevent spillage. A code of safe
practice for potentially carcinogenic materials (London School of
Hygiene and Tropical Medicine - School Code of Safe Practice for
Potentially Carcinogenic Materials) was adhered to. Extra care
was taken in the handling and disposal of such materials.

(b) Preparation of Test Animals

An appropriate number of test animals, usually male C57 black mice,
were selected and placed two to four in an adequately labelled
cage. Each mouse was then coded by recording its tail markings
and/or by marking the ears using an ear-punch e.g. for four mice
in a cage:-

Mouse No 1 - No ear-punch mark on either ear
Mouse No 2 - Ear-punch mark on right ear
Mouse No 3 - Ear-punch mark on left ear
Mouse No 4 - Ear-punch mark on both ears

(c) Intradermal Injections

Single intradermal injections of the test solutions were then
made into the ventral surface of each mouse. A small syringe
with 0.05 ml markings and a fine gauge needle (12G) was used for
the injections. Each mouse was held by gripping the skin behind
the ears between thumb and forefinger of one hand, and fixing
the tail between the thenar eminence and the remaining three
fingers of the same hand (Aw 1975). The ventral surface was
therefore exposed leaving the other hand free to manipulate the
syringe. To enable ease of penetration of the needle into the
mouse skin, the injection site was first moistened using a ball
of cotton wool soaked in absolute alcohol. The alcohol brings
the hairs together, thereby exposing areas of bare skin for
injection. The injections were prepared with the needle almost
parallel to the skin surface so that the solution was introduced
intradermally and not subcutaneously nor intraperitoneally. A
successful intradermal injection was indicated by the appearance
of a wheal. Following withdrawal of the needle, a pair of forceps
was used to grip the puncture site. This helped to prevent leak
of injected solution out through the injection puncture wound.
All injected mice were left in the laboratory for four hours
before being returned to the animal house. No form of
anaesthesia was used for the injections.

(d) Periodic Examination of Injected Mice

After the initial injections, all mice were examined two to three
times a week for any macroscopic changes at the injection sites.
All examinations were done blind i.e. without the observer knowing

from which cage the mouse had come and therefore not knowing the chemical the mouse had been injected with. This reduces observer bias.

A hand lens was used under adequate lighting for closer scrutiny of the injection sites during examination. In particular, depigmentation was the main feature looked for. The degree of depigmentation was recorded and a subjective scale used viz:-

+ + + - marked depigmentation extending 1 mm from the margin of depilation or, where there was no depilation, an area of 2 mm diameter

  + + - obvious depigmentation

    + - slight depigmentation

    ± - doubtful depigmentation

    - - no depigmentation

In all instances where doubtful or slight depigmentation was recorded, the mice concerned were then examined daily to see whether further changes occurred. Only where there was slight depigmentation confirmed on at least three consecutive daily examinations was this considered positive depigmentation. Doubtful cases were considered negative.

(e) Microscopy

(1) For examination of skin sections under the light microscope, selected mice showing obvious macroscopic changes were killed by dislocation of the neck. The skin area for histological examination was then removed using a pair of scissors. This tissue was kept in 10% formal saline before fixing, preparing of slides and staining using haematoxylin

and eosin or toluidene blue stain.   Minimal delay was
allowed between killing the mice and removal and preser-
vation of the excised skin area, to prevent excessive
post-mortem autolytic changes in the cellular structure
of the tissue concerned.

(2) Hair specimens for microscopy were obtained by using a
pair of forceps to pluck the hair out from skin areas of
interest.   These were then mounted on glass slides using
euparol to form a permanent mounting.

(3) Special sections were also taken for electron microscopy
and the procedure is detailed in Appendix 2.

DISCUSSION ON METHODOLOGY

The method as described was designed with specific reasons for the
choice of:-

1. C57 Black mouse as the model for study.

2. Chemicals to test.

3. The number of injection sites per mouse.

4. The volume of solution per injection.

5. Concentrations of test solutions to use.

6. Solvents/mediums.

1. CHOICE OF THE C57 BLACK MOUSE AS THE MODEL FOR STUDY

The mouse was chosen as the test animal because this was origin-
ally used by Boyland ₐ(1951 and 1952), Searle (1970), Schoental
(1971) and Matsuyama (1966) in their independant experiments on
depigmentation by chemicals. Schoental also showed similar
depigmentation in rabbits. The strains of mice used by the
various researchers include C57, C3H, ddCF2 and CBA. Pure strain
C57 Black mice was the test-strain chosen for this study because
being a pure black mouse allows the depigmentation to show up
easier. The size of the C57 Black mouse allows ease of handling
by one person for the purpose of intradermal injections and
regular examination. C57 Black mice also have a relatively low
spontaneous disease rate (Innes et al 1969)

2. CHOICE OF CHEMICALS TO TEST

Chemicals for testing were selected on the basis of their reported
carcinogenic activity and chemical classification with an attempt

to choose representatives from each class of chemical carcinogens
(see Table 4). A theoretical ideal way of selecting compounds
for investigating an effect such as depigmentation which appears
to be related to carcinogens, is to prepare a list of carcinogens
and one of non-carcinogens. A statistically random sample of
equal numbers of chemicals from both lists can then be selected
and tested according to the method described. The results regard-
ing the appearance of depigmentation can be statistically tested
to see whether the depigmentation effect is significantly related
to carcinogens. However, there are practical limitations to this
approach. This includes the difficulty of defining carcinogens
and non-carcinogens. Various attempts at producing a list of
carcinogens have resulted in different chemicals and different
numbers on the list depending on the criteria used. Rall (1978),
Director of the National Institute of Environmental Health Sciences,
lists 26 chemicals that have been firmly associated with cancer in
man and 56 more where the epidemiological evidence is less definite.
If a carcinogen is defined as one that has epidemiologic proof of
causing cancer in man, then a list of carcinogens will contain 26
to 82 compounds depending on how one assessed the epidemiologic
evidence. According to National Cancer Institute sources (Maugh
1978) about 7000 chemicals have been tested for carcinogenicity in
animals and a few more than 1000 have been reported to be carcino-
genic. A list of carcinogens based on such animal data will, there-
fore, include over 1000 chemicals. Based on chemicals used in U.S.
workplaces, The Occupational Safety and Health Administration
(OSHA) in 1978 (Chemical and Engineering News 1978) issued a
tentative list of carcinogens consisting of:-

(a) 269 Category I carcinogens based on human data, tests in
     two mammalian species or one species if replicated.

(b) 218 Category II carcinogens based on reports of carcino-
     genicity in only one species and with results not
     replicated.

(c) 396 Category III substances where there was no or meagre
     evidence of carcinogenicity.

The National Institute for Occupational Safety and Health (NIOSH)
has, however, been reported to have identified more than 2000
substances where there is some evidence of tumourigenicity and
carcinogenicity (Chemical and Engineering News 1978).

The NCI in its survey of compounds summarizes data from published
information relevant to chemical carcinogens. This information
is regularly updated in the light of new evidence available. How-
ever, no pronouncement on carcinogenicity is made. An assessment
has often to be made on the basis of the data provided. For
example, in the 1972 - 73 volume a study by Van Duuren (1972) was
summarized where 15 mice were given 4-nitrobenzene carboperoxic
acid (p-nitroperbenzoic acid). Two mice developed sarcomas at
the injection sites. No mention was made of any use of controls.
Similarly in the same volume, a study by Malejke-Giganti and
others (1973) reports six rats given N-hydroxy 3-fluorenamine
of which three developed tumours. Studies such as these based
on small numbers of experimental animals without the use of
controls are difficult to evaluate.

* NCI - National Cancer Institute

The International Agency for Research in Cancer also produces information on carcinogenicity of individual chemicals on the basis of data compiled, reviewed and evaluated by its working group of experts. Such data is revised from time to time. To date, eighteen volumes of the IARC Monographs on the Evaluation of Carcinogenic Risk have been published. This includes data on some 350 chemicals. However, the conclusion in many cases is that 'the results are insufficient for evaluation' or 'no evaluation can be made on the basis of available data' or 'with regards human data, no case reports on epidemiological studies are available'. Again, it would be difficult to compile a list from either the NIOSH data or the IARC data. One could attempt to produce such a list by defining specific criteria and then reviewing the data accordingly. Obviously the carcinogens with epidemiologic evidence of effects in humans would need to be included in this list. Purchase and others (1978) produced a list based on the following criteria:-

(a) Any materials shown to produce malignant tumours in any mammalian species as a result of application to the skin, i.p. or i.v. injection, or orally (including intragastrically) have been regarded as carcinogenic.

(b) Initiating and promoting agents have been classified as carcinogens.

(c) Tumours arising in the urinary bladder as a result of bladder implantation techniques have not been considered as meaningful.

(d) Tumours arising at the site of subcutaneous injection (i.e. as

sarcomas) have been ignored unless accompanied by the
appearance of tumours at other sites.

(e) Negative results after s.c. injection or bladder implant-
ation have been regarded as significant, and were considered
an indication of non-carcinogenesis.

(f) Where there is only an increase in the incidence of common
tumours in mice (e.g. of hepatomas or lung adenomas in
susceptible strains) the data have been ignored, unless there
have been concurrent appearances of other tumours at different
sites.

(g) Evidence based solely on the appearance of benign tumours has
been considered insufficient for a positive classification.

(h) Compounds which were negative in studies which have continued
for the major part of the animal's lifespan have been classi-
fied as non-carcinogens. Where there is no reason to suspect
carcinogenicity (e.g. natural products of mammalian systems)
or on theoretical grounds (e.g. by analogy with a closely
related chemical known to be positive) then the compounds
in question have been classed as negative.

The problem is even greater when one tries to compile a list of
non-carcinogens. This would be a very extensive list if it is to
include chemicals with no evidence of carcinogenicity since it has
been estimated that there are over 4.3 million known chemicals
(Culliton 1978) and those that are considered not carcinogens by
whatever criteria is used would be on a huge list of non-
carcinogens. This would obviously include some very rare chemicals

which are carcinogenic but have not been tested. Hence a statistically random sample from such a list, even if it is possible to compile one, would not necessarily be a representative sample as it could include potential carcinogens and rare chemicals which are not easily available for testing.

Even in selected pairs of compounds (one carcinogenic and another similar structurally but non-carcinogenic) which have been used for assessing short-term tests (Purchase et al 1978) there is some doubt as to whether such classification for pairing is valid (Boyland 1978a).

In view of the difficulties of this theoretical approach, the method, therefore, used for selecting chemicals for testing is based on the principle of practicality. Chemicals tested were those which were available including many which are known human carcinogens such as asbestos, benzidine, 2-naphthylamine and common compounds which appear to be obvious non-carcinogens such as HCl , NaOH, saline, and alcohol. If the depigmentation effect is to be of any use in differentiating between carcinogens and non-carcinogens, it must firstly be positive for obvious carcinogens such as 2-naphthylamine, nitrogen-mustard and the other examples mentioned. Therefore, rather than a random sample from a compiled list of carcinogens - which as discussed has practical difficulties - a selected sample from available carcinogens was used. A working list of carcinogens was compiled from available compounds, where literature reviewed showed either epidemiologic evidence of carcinogenic effect on humans or well-designed studies showed carcinogenic or tumourigenic effect in animals.

Tumour-promoters and co-carcinogens were classified as a separate group as they are by themselves non-carcinogenic, but would not strictly fall into the non-carcinogenic group since they do under appropriate conditions increase tumour yield. Asbestos fibres were placed in a separate sub-group under carcinogens since there is little evidence as yet to indicate whether they are direct or indirect carcinogens. There is some suggestion that asbestos may be a co-carcinogen (Selikoff, Hammond and Churg 1968).

TABLE 4

COMPOUNDS TESTED BY THE DEPIGMENTATION TEST

DIRECT CARCINOGENS

Alkylating Agents	References
Nitrogen mustard (Methyl-bis-(2-chloroethyl)-amine)	Boyland and Horning 1949 Schmähl and Osswald 1970
Dimethyl sulphate	Druckrey et al 1966 and 1970
Methyl iodide	Druckrey et al 1970
Benzyl chloride	Matsushita et al 1975 Sakabe, Matsushita and Koshi 1976 Sakabe and Fukuda 1977
Benzoyl chloride	Matsushita et al 1975
Benzotrichloride	Matsushita et al 1975

TABLE 4 (Continued)

INDIRECT CARCINOGENS

Polycyclic Aromatic Hydrocarbons	References
1,2:5,6-Dibenzanthracene	Kennaway 1930
9,10-Dimethyl-1,2-benzanthracene	Haddow and Kon 1947
6,9,10-Trimethyl-1,2-benzanthracene	Haddow and Kon 1947
Benzanthracene	Bock and King 1959 Kennaway 1930
Benzo(a)pyrene	IARC Monographs Vol III
3-Methylcholanthrene	Rinkus and Legator 1979 Kataoka 1976

TABLE 4 (Continued)

INDIRECT CARCINOGENS

Aromatic Amines	References
2-Naphthylamine	Case et al 1954 Goldwater, Rosso and Kleinfeld 1965 Hueper 1942 Hueper, Wiley and Wolfe 1938
Benzidine	IARC Monographs Vol I
Aniline	BIBRA Information Bulletin 1978

Carbamates	References
Ethyl carbamate (urethane)	IARC Monographs Vol XII
Propyl carbamate	Larsen 1941

TABLE 4 (Continued)

OTHER CARCINOGENS

Carcinogens	References
Chloroform	Eschenbrenner and Miller 1945
1,2-Dimethylhydrazine	Hawks, Farber and Magee 1971/72 Toth and Wilson 1971
Asbestos	Doll 1955 Glynne 1935 Wagner and Berry 1969 Reeves et al 1971 Mancuso and Coulter 1963 Selikoff, Churg and Hammond 1965
8-hydroxyquinoline	Allen et al 1957 Pliss and Volfson 1970

TABLE 4 (Continued)

TUMOUR PROMOTERS

Tumour-Promoters	References
12-O-Tetradecanoyl-phorbol-13-acetate (TPA)	Hecker 1968 Van Duuren 1969 Van Duuren and Sivak 1968
Anthralin	Bock and Burns 1963 Boutwell 1967 Segal, Katz and Van Duuren 1971
Dodecane	Safiotti and Shubik 1963
Tween 20, 40 and 80	Setälä 1956 and 1960
Limonene	Roe and Pierce 1960
Croton oil	Berenblum 1941
Phenol	Boutwell and Bosch 1959 Rusch, Bosch and Boutwell 1955
Carbon dioxide snow	Berenblum 1930
Iodoacetic acid	Gwyn and Salaman 1953

TABLE 4 (Continued)

TUMOUR PROMOTERS

Tumour-Promoters	References
Sodium desoxycholate	Marx 1978
Saccharin	Boyland 1979 Cohen et al 1978 Mondal, Brankow and Heidelberger 1978
Griseofulvin	Barich, Schwartz and Barich 1962 De Matteis, Donnelly and Runge 1966 Weston Hurst and Paget 1963
1-Fluoro-2,4-dinitrobenzene	Bock et al 1969
Pyrocatechol (Catechol)	IARC Monographs Vol XV Van Duuren, Katz and Goldschmidt 1973
Phenobarbitone	Clemmensen, Frederiksen and Plum 1974 Periano et al 1971 and 1973 Walker, Thorpe and Stevenson 1973 Weisburger et al 1975

TABLE 4 (Continued)

TUMOUR PROMOTERS

Tumour-Promoters	References
Hydroquinone	Boyland et al 1964 Roe and Salaman 1955
Sodium metabisulphite	Laskin et al 1970

TABLE 4 (Continued)

OTHERS: NON-CARCINOGENS, NON-TUMOUR-PROMOTERS,

AND COMPOUNDS NOT YET TESTED FOR CARCINOGENIC NOR TUMOUR-PROMOTING ACTIVITY

Compounds	References - Available only for tested compounds
Phenanthrene	Dipple 1976
Pyrene	Dipple 1976 Van Duuren 1976
Methyl carbamate	IARC Monographs Vol XII
Diethyldithiocarbamate	IARC Monographs Vol XII
Dimethylsulphoxide (DMSO)	-
Dimethylacetamide (DMAC)	-
Acetone	-
Normal Saline	-
1,1,1, Trichloroethane	National Cancer Institute Report 1977
Arachis oil	-

TABLE 4 (Continued)

OTHERS: NON-CARCINOGENS, NON-TUMOUR-PROMOTERS,

AND COMPOUNDS NOT YET TESTED FOR CARCINOGENIC NOR TUMOUR-PROMOTING ACTIVITY

Compounds	References - Available only for tested compounds
Hydrochloric acid	-
Sodium hydroxide	-
Ethanol	-
Pyrogallol	Van Duuren 1976
4-tertiary butyl catechol	-
p-tertiary butyl phenol	-
Methyl catechol	-
Butylated hydroxyanisole	-
Putrescine	-
Di-hydroxy phenylalanine (DOPA)	-

TABLE 4 Continued)

Compounds	References - Available only for tested compounds
Adrenaline hydrogen tartrate	-
Histamine hydrochloride	-

3. CHOICE OF NUMBER OF INJECTION SITES PER MOUSE

Theoretically, any number of injections can be performed on the ventral surface of the mouse. The practical limitations to the number of sites would be:-

(a) Difficulty in identifying the site of injection during subsequent examinations if there are more than six or eight injections.

(b) A large number of injections would mean a large total volume test solution injected per mouse. This could be lethal to the mouse.

The ideal number of injection sites per mouse appears to be one to six (see Fig 1).

FIG 1

NO AND POSITION OF INJECTION SITES PER MOUSE

No of Injection Sites

Position of Injection Sites

on Ventral Surface

One
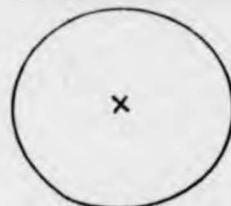
Two
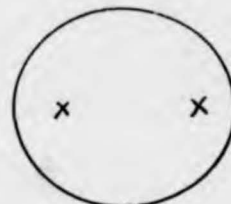
Three
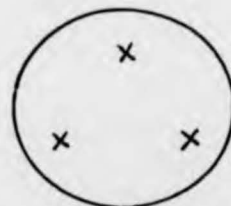
Four
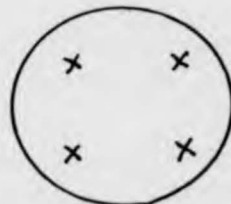
Six
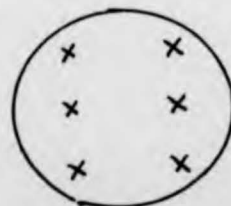
4. CHOICE OF VOLUME OF SOLUTION PER INJECTION

In making the choice of volume of test solution per injection, the principle followed was to give just sufficient a dose to pro- duce a visible wheal which would indicate a successful intradermal injection. A large volume would limit the number of injections per mouse because of the possibility of toxic or lethal effects. With large volumes, there is also an increased likelihood of leakage of injected solution out through the hypodermic needle puncture wound in the mouse skin.

The ideal volume was 0.05 mls. Volumes of less than 0.025 mls were too small and more than 0.1 ml too large to ensure certainty of successful intradermal injections.

5. CHOICE OF CONCENTRATIONS OF TEST SOLUTIONS

At least three concentrations of each test chemical were prepared. An assessment of the toxicity of the compound was first made from published data (the MERCK Index) or by toxicity testing on groups of C57 black mice. The highest concentration used was one just below the minimal lethal dose or,if the compound was not very soluble, a saturated solution was used. Ten-fold dilutions of this were then prepared. Thus a range of concentrations of the chemical was tested. The depigmentation effect may be best elicited by a particular concentration of the test chemical. Hence, using this wide range of concentrations decreases the likelihood that such an effect might be missed because of use of the wrong concentration.

6. CHOICE OF SOLVENTS/MEDIUMS

The compounds used for animal testing were available in one of
the following forms:-

(a) Powder - fine or coarse   e.g. Tetradecanoyl phorbal acetate

(TPA)

(b) Crystals   e.g. Nitrogen-mustard

(c) Liquid   e.g. Benzyl chloride

(d) Emulsion   e.g. Dimethyl benzanthracene

(e) Fine fibres   e.g. Asbestos

An appropriate solvent/medium had to be selected for preparing the
test compounds into solutions or suspensions of different concen-
trations for the intradermal injections.

The following solvents/mediums were considered:-

(a) Distilled water

(b) Normal saline

(c) Dimethyl acetamide

(d) Dimethyl sulphoxide

(e) Acetone

(f) Arachis oil

(a) Distilled Water

Distilled water was not considered suitable because a positive
result occurred with the use of 0.05 mls of distilled water
(Aw 1975). Boyland and Sargent (1951) noted a similar effect
in 19% of injected sites. This was attributed to a possible
cellular change following exposure to the hypotonic solution.

(b) Normal Saline

Only some of the substances used were soluble in normal
saline. No positives were however produced and this could
be due to its isotonicity compared with distilled water.

(c) Arachis Oil

This was useful as a solvent for hydrocarbons such as
3-methyl cholanthrene but its viscosity made it difficult
to use, especially when injecting solutions made with this
oil through a fine gauge needle (12 G) using micro-syringes.
It was difficult to accurately inject a small amount,
(0.025 mls) of the viscose solution but warming reduced the
viscosity and allowed easier injection.

(d) Acetone, Dimethyl Acetamide (DMAC) and Dimethyl Sulphoxide
(DMSO)

Most of the test compounds were soluble in these three
solvents.However, in terms of toxicity, dimethyl acetamide
is the most toxic followed by acetone, with dimethyl
sulphoxide being the least.

TABLE 5

COMPARATIVE TOXICITY OF SOLVENTS TESTED

Solvent	LD_{50} Rats (Published data in Merck Index)	Lethal dose in 2/3 C57 black mice (intradermal)
DMSO	20 mg/kg (oral)	13.2 gm/kg (2/3)
Acetone	8.45 gm/kg (oral)	6.3 gm/kg (2/3)
DMAC	3.8 gm/kg (1/p)	3.8 gm/kg (2/3)

Dimethyl sulphoxide was, therefore, used as solvent/medium for preparing most of the test solutions because of:-

(i)   The large number of compounds soluble in it.

(ii.) The low toxicity - when compared with acetone or dimethyl acetamide.

(iii) Its miscibility with water.

0.05 mls injections at four sites was the upper limit of the volumes and the number of sites for injection used in each C57 black mouse was:-

(i)   0.05 mls at two to four sites per mouse.

or

(ii) 0.025 mls at four sites per mouse.

CHAPTER 4   RESULTS

Macroscopic features

General effects

Local effects

Discussion on observed depigmentation

Latent period before depigmentation

Dose-response relationship

Overall results

The link between tumour-promoters and depigmentation

Specificity and sensitivity of the depigmentation test

RESULTS

1. MACROSCOPIC FEATURES

General effects:-

(a) Death of the Mice

This occurred when the maximum concentration of the test compound used was too high. The choice of the maximum concentration of solution used for any test chemical was based on its solubility in the solvent and its documented toxicity - as expressed by an LD_{50} (see methodology). If the reported LD_{50} pertains to rats, rabbits or guinea-pigs with no specific data for mice, then the choice of the highest concentration of test solution based on an estimated LD_{50} for mice according to proportional body weight, may cause death in the C57 Black mice. This would be so if the compound is actually more toxic for mice than for the other species of animals mentioned.

Even when the published LD_{50} refers to mice, because of strain differences the susceptibility of the C57 Black may be different. Death of the mice due to a high concentration of chemical used, usually occurred within 36 hours of injection, with the animal becoming progressively unwell after the injection. In the event of this occurring, the experiments were repeated with the lethal concentrations omitted. Surviving mice from the initial experiments were kept and regularly examined for depigmentation.

. Death of mice also occurred because of illness. In such instances, death may occur several weeks or months from the time of injection, depending on when the onset of illness

started. On regular examination of the mice, any such
animals were isolated and observed daily. Mice dying four
weeks after the last appearance of depigmentation in the
group of mice similarly injected would have the depigmentation/
non-depigmentation results included in the results for that
experiment. Death of a mouse before the last appearance of
depigmentation in any other mice in the same group or within
four weeks after the last appearance of such depigmentation
will mean exclusion of the depigmentation/non-depigmentation
results for the dead mouse from the results for that group of
mice. This is because it may be possible that had this mouse
survived it may have shown depigmentation at any injected
sites which are not depigmentated at the time of death.

Instances of death of mice in the course of the various experi-
ments are summarized in Table 6 .

TABLE 6

COMPOUNDS LETHAL TO C57 BLACK MICE FOLLOWING INTRADERMAL INJECTIONS

No	Compound	Concentrations Injected Per Mouse (gm/100 ml)	Total Amount Injected (µg)	No of Deaths Total No of Mice
1.	Croton Oil	10, 1, 0.1, 0.01	5,555	4/4
		5, 0.5, 0.05	2,775	5/14
		1, 0.1, 0.01	555	1/4
2.	Anthralin	2, 0.2, 0.02	1,110	6/8
3.	Phenobarbitone Sodium	20, 2, 0.2,	11,100	4/4
		10, 1, 0.1	5,550	4/6
4.	Tween 80	1, 0.1	550	1/4
5.	p-tertiary Butyl Phenol	10, 1, 0.1	5,550	3/8
6.	Chloroform	10, 1, 0.1	5,550	5/8
7.	Benzyl Chloride	10, 1, 0.1	5,550	1/4
8.	Dimethyl Sulphate	10, 0.1	5,050	4/4

TABLE 6 (Continued)

No	Compound	Concentrations Injected Per Mouse (gm/100 ml)	Total Amount Injected (µg)	No of Deaths / Total No of Mice
9.	Adrenaline	2, 1, 0.1, 0.01	1,555	4/4
		1, 0.1, 0.01, 0.001	1,555	4/4
10.	Sodium Metabisulphite	10, 1, 0.1	5,550	2/4
11.	Dimethyl Sulphoxide	100% soln. (110 gm) at 6 sites	330 mg.	2/3
12.	Dimethyl Acetamide	100% soln. (94 gm) at 4 sites	188 mg.	8/8
13.	Acetone	100% soln. (79 gm) at 4 sites	158 mg.	2/3
14.	1-Fluoro-2,4-dinitrobenzene	1, 0.1, 0.01	555	4/4

(b) Anaesthetic Effect

An immediate anaesthetic effect was noted following intrader-
mal injections of phenobarbitone sodium and chloroform.
Anaesthetised mice were kept in a warm area of the laboratory
during the period of anaesthesia and were returned to the
animal house on full recovery.

Local Effects

(a) Wheal Formation

Each successful intradermal injection resulted in a wheal at
the site which gradually disappeared as the injected solution
dispersed. In previous experiments using arachis oil as
solvent the wheals persisted for more than three weeks (Aw 1975).
Compounds which are oils produce viscous solutions which do
not disperse easily and therefore result in persistence of
wheals at the injection sites. Croton oil is an example where
the wheals remained but within a week there was ulceration at
the site due to the highly irritant nature of croton oil
Injection of asbestos fibre suspension - both ground and
unground, also resulted in a persistent swelling at the site.
This is due to the insoluble fibres remaining at the site of
injection.

Disappearance of the wheal may be due to ulceration of the
skin when irritant compounds were used or it may be followed
by no further observable change as when non-irritants such as
saline and absolute alcohol were injected.

(b) Ulceration and Abscess Formation

The following compounds in the concentrations used caused
ulceration at the sites of injection.

TABLE 7

COMPOUNDS CAUSING ULCERATION AT INJECTION SITES

No	Compound	Concentration
1.	Tween 80	10 gm/100 ml
2.	9,10-Dimethyl-1,2-benzanthracene in fat emulsion	100% emulsion) refer to page for
3.	6,9,10-Trimethyl-1,2-benzanthracene in fat emulsion	100% emulsion) constituents of emulsion
4.	Acetone	100%
5.	Nitrogen mustard (HN2)	At concentration > 1 mgm /100 ml
6.	Croton oil	At concentration > 0.5 gm/100 ml
7.	1-Fluoro-2,4-dinitrobenzene	At concentration > 0.1 gm/100 ml
8.	Sodium metabisulphite	At concentration > 1 gm/100 ml
9.	12-O-Tetradecanoyl-phorbol-13-acetate (TPA)	At concentration > 0.002 gm/100 ml

Most of these substances are strong skin irritants or
vesicants and caused skin ulceration. Ulceration was
followed by either:-

(i) Healing with scarring and permanent hair loss at the
site.

(ii) Abscess formation - This was noted in two out of four
sites when sodium metabisulphite was injected at a
concentration of 10 gm/100 ml saline. The aqueous
solution injected was acidic and the abscesses led to
death of two mice on the fifty-seventh and fortieth
day. This may be due to bacterial infection.

The irritant nature of these chemicals is not related to the
effectiveness in producing depigmentation. Only four compounds
nitrogen mustard, croton oil, 1-fluoro-2,4-dinitrobenzene and
TPA caused ulceration and definite depigmentation. The remain-
ing five compounds caused ulceration but no distinct depigment-
ation.

(c) Depilation

Loss of hair at the injection site occurred with irritant
compounds and when dimethyl sulphoxide (DMSO) was used as
solvent. DMSO is locally irritative and when injected intra-
dermally caused small areas of depilation in ten to thirteen
days. This appeared to be temporary with a re-growth of nor-
mal dark hair over the area within two to three weeks. How-
ever, with a stronger irritant such as dimethyl acetamide or
acetone, permanent areas of depilation resulted. This was

also true for DMSO when irritant compounds were dissolved in
it for injection, e.g. with croton oil, 1-fluoro-2,4-
dinitrobenzene, nitrogen mustard and sodium metabisulphite,
large areas of ulceration and permanent depilation occurred.
In general, the more irritant the test solution, the larger
the area of hair loss and the more likely it is to be per-
manent.

(d) Tumour Formation

Papillomas occurred in two out of twelve mice injected with
3-methylcholanthrene. This was noted on the 110th and 125th
day after injection with a concentration of 0.2 mg/ml. 0.05 ml
of 3-methylcholanthrene in arachis oil was injected at three
concentrations in twelve mice. One papilloma was noted in
each of the two mice injected with the highest concentration
of 0.2 mg/ml. Fig 2 shows one of the tumours still attached
to the adjacent skin and cut to show the inside of the tumour.
The tumours were fleshy, hard, well vascularized and well
defined of 1 cm diameter. They were attached to the surround-
ing skin but not to the tissues below. No depigmentation was
noted at these sites injected with 3-methylcholanthrene

3-methylcholanthrene is a polycyclic aromatic hydrocarbon.
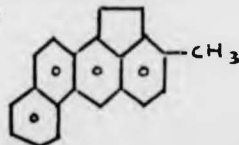
3-methylcholanthrene

It is a powerful indirect carcinogen and single doses of 10 ug
induced papillomas in two   sites. The results for 3-methyl-
cholanthrene are as summarized in table 8 .

FIG 2 Papilloma due to 3-Methylcholanthrene

FIG 2 Papilloma due to 3-Methylcholanthrene

FIG 2 Papilloma due to 3-Methylcholanthrene

TABLE 8

DEPIGMENTATION AND TUMOUR FORMATION DUE TO 3-METHYLCHOLANTHRENE

Concentration (In Arachis Oil)	Amount µg	No of Positive Sites / Total No of Sites Injected	
		Depigmentation	Tumours
0.2 mg/ml	10	0/12	2/12
0.02 mg/ml	1.0	0/12	0/12
0.002 mg/ml	0.1	0/12	0/12

(e) Depigmentation

Depigmentation occurred with twenty out of the sixty-six chemicals tested. The degree of depigmentation was graded into + + + (marked depigmentation), + + (obvious depigment- ation) and + (slight depigmentation) according to the sub- jective scale described in the methodology. This subjective grade for any chemical is based on the site showing maximum depigmentation out of all sites injected with the same compound.

Figs 3 and 4 show the comparative difference before and after injection of nitrogen mustard (HN2). Two areas of marked depigmentation (+ + +) are present in Fig 4 - one near the left fore-leg and the other near the right hind-leg. The latter also shows a small central area of depilation.

Fig 5 shows a Tyzzer Original White mouse injected with HN2 Only a small area of depilation is present on the ventral surface near the fore-legs. No change in hair pigmentation occurred.

FIG 3 Before Intradermal Injection of Nitrogen Mustard (HN2)

FIG 4 After Intradermal Injection of Nitrogen Mustard (HN2)

FIG 3   Before Intradermal Injection of Nitrogen Mustard (HN2)

FIG 4   After Intradermal Injection of Nitrogen Mustard (HN2)

FIG 3 Before Intradermal Injection of Nitrogen Mustard (HN2)

FIG 4 After Intradermal Injection of Nitrogen Mustard (HN2)

FIG 5 Tyzzer Original White Mouse Injected with Nitrogen Mustard (HN2)

FIG 6 CBA Brown Mouse Injected with Nitrogen Mustard (HN2)

FIG 5 Tyzzer Original White Mouse Injected with Nitrogen Mustard (HN2)

FIG 6 CBA Brown Mouse Injected with Nitrogen Mustard (HN2)

FIG 5 Tyzzer Original White Mouse Injected with Nitrogen Mustard (HN2)

FIG 6 CBA Brown Mouse Injected with Nitrogen Mustard (HN2)

FIG 7   C57 Black Mouse Injected with Pyrocatechol

FIG 8   C57 Black Mouse Injected with 12-O-Tetradecanoyl-Phorbol-
13-Acetate (TPA)

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FIG 7   C57 Black Mouse Injected with Pyrocatechol

FIG 8   C57 Black Mouse Injected with 12-O-Tetradecanoyl-Phorbol-13-Acetate (TPA)

FIG 7   C57 Black Mouse Injected with Pyrocatechol
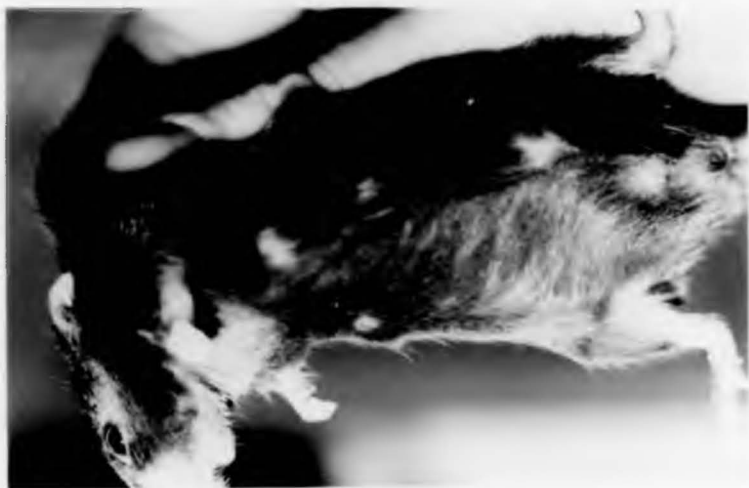
FIG 8   C57 Black Mouse Injected with 12-O-Tetradecanoyl-Phorbol-
13-Acetate (TPA)

Fig 6 shows a CBA Brown mouse also injected with HN2. In
this case there was slight depigmentation (+) surrounding a
central area of depilation (near the right hind-leg). The
depigmentation was less obvious than for the C57 Black mice
similarly injected.

Fig 7 shows a C57 Black mouse injected with pyrocatechol.
Obvious depigmentation (+ +) is present at two sites - one
near the left fore-leg and the other near the left hind-leg.
Two other sites - one between the previous two and the other
on the right side show slight depigmentation (+).

Fig 8 shows a C57 Black mouse injected with TPA. Marked
depigmentation (+ + +) is present stretching from one
injected site anterior to the right hind-leg to another site
anterior to the left hind-leg.

Table 9 summarizes the maximum degree of depigmentation
caused by the twenty positive chemicals.

TABLE 9
CHEMICALS CAUSING DEPIGMENTATION
(ACCORDING TO MAXIMUM DEGREE OF DEPIGMENTATION)

Maximum Degree of Depigmentation	No	Chemicals
+ + +	1.	TPA
	2.	HN2
	3.	CO_2 Snow
+ +	1.	Croton Oil
	2.	Pyrocatechol
	3.	p-tertiary butyl phenol
	4.	4-tertiary butyl catechol
	5.	Chloroform
	6.	Dimethyl sulphate
+	1.	Methyl iodide
	2.	Benzyl chloride
	3.	Saccharin
	4.	Tween 20
	5.	Dodecane
	6.	Phenol
	7.	Griseofulvin
	8.	Hydroquinone monobenzyl ether
	9.	Butylated hydroxyanisole
	10.	Ground Crocidolite
	11.	1-Fluoro-2,4-dinitrobenzene

Discussion on Observed Depigmentation

Latent Period Before Depigmentation

For most of the chemicals injected where positive depigmentation occurred, the latent period before the appearance of depigmentation varied from three weeks (as for TPA) to sixteen weeks (in mice pretreated with urethane and injected with crocidolite). The average latent period was from three to six weeks. This latent period would be expected to vary considerably between chemicals and even for the same chemical injected in a number of mice. The reason for this is that the growth of hair in the mouse undergoes cyclical changes and synchronous groups of hair follicles alternate between a phase of active growth and an inactive resting phase. This causes successive growth waves over the coat of the mouse (Eaton 1976, Borum 1954). Dry (1976) divided the hair cycle into three stages:-

(a) Anagen - the growth phase.

(b) Catagen - the short transitional phase when the follicle decreases in size.

(c) Telogen - the resting phase when the hair is retained as club hair.

Anagen to telogen takes about nineteen days and telogen may last for several months. The latent period before depigmentation therefore depends on the stage that the injected site hairs are at during the time of injection. In telogen, this will lead to a prolonged latent period of depigmentation and it will be shorter if the hairs are in anagen.

Dose-Response Relationship

In the instances where depigmentation was marked (+ + +) e.g.
nitrogen mustard (HN2), TPA, carbon dioxide snow, or obvious (+ +)e.g.
croton oil, catechol, 4-tertiary butyl catechol and p-tertiary butyl
phenol, chloroform and dimethyl sulphate, a sigmoidal relationship
was observed between dose and proportion of sites showing depigment-
ation.

FIG 9

SIGMOIDAL DOSE-RESPONSE CURVE

Percentage of sites showing positive depigmentation
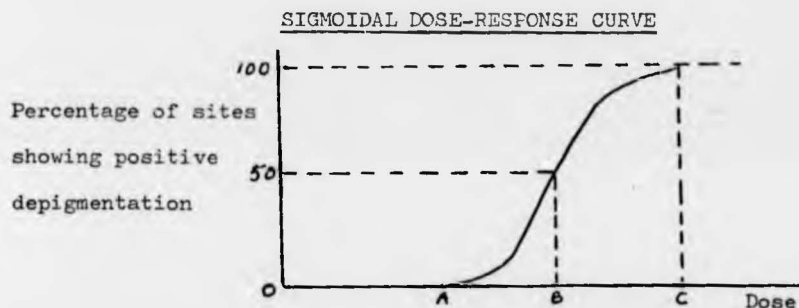
At low doses no effect is noted until the minimum effective dose is
reached (A). At doses greated than this,some proportion of sites will
show positive depigmentation. At point (C) 100% effect is noted; all
sites show depigmentation and this occurrs for all doses higher than
(C), except when the lethal dose is reacted,when death of the test
animals will result. Point (B) represents the ED_{50} i.e. the effective
dose at which 50% of the sites injected show depigmentation.

This effect is best demonstrated by the tests using TPA (See page 130).
For the doses tested, the minimum effective dose was 0.008 μg TPA and
the minimum dose needed to produce 100% depigmentation (corresponding
to point (C) was 5.0 μg with the ED_{50} estimated at 0.18 μg using a logit
transformation analysis (Cox 1970). This is a statistical technique
for converting a sigmoidal relationship into a linear relationship and
the latter is easier to use inferentially, especially in the estimation
of ED_{50}.

For the chemicals producing only slight depigmentation (+) most of
them appeared to have a sigmoidal relationship. The range of doses
used is limited and this relationship is not always obvious. However,
for three tumour-promoters i.e. 1,fluoro-2,4-dinitrobenzene, saccharin
and dodecane, a different dose response was obtained.

FIG 10

DOSE-RESPONSE CURVE WITH 'OPTIMAL DOSE EFFECT'

Percentage of Sites
showing positive
depigmentation
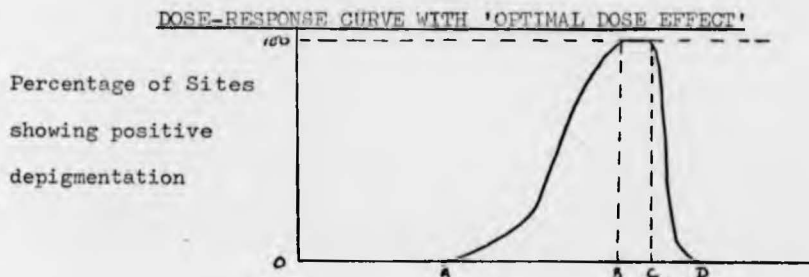
These three compounds showed an 'optimal dose effect'. This is shown in
Fig 10 where dose A represents the minimum effective dose and B repre-
sents the dose which produced 100% effect. However, the interesting
feature is dose C to D where as the dose is increased the effect
decreases. Therefore, at a higher dose a lower proportion of sites
showing positive depigmentation is noted. The graph could theoretically
be extended so that beyond dose D no depigmentation occurs up to the
lethal dose. This relationship could represent a toxic effect where
beyond dose D, all melanocytes and hair cells in the area are destroyed,
and therefore an area of depilation is noted without any depigmentation.
At the lower doses A to D, some hair cells survive to produce depigment-
ation. The importance of this relationship is that a tested chemical
may be recorded as not producing depigmentation even if it is capable
of doing so. This will result if the doses selected for testing are
inappropriate (i.e. if it does not fall within the A to D range as shown
in Fig 10. This range could possibly be very narrow for some chemicals).
The 'optimal dose effect' relationship was noted for two weak tumour-

promoters; the depigmentation observed was also slight. It is for this group of tumour-promoters where the depigmentation test may be limited in its ability to detect them as tumour-promoters.

A third dose-response relationship is when the minimum effective dose exceeds the lethal dose. With such chemicals, tests done using any dose less than the lethal dose would produce negative results. Doses close to the effective dose will kill the test animals. Such instances will lead to negative depigmentation being recorded even if the theoretical possibility of producing depigmentation exists. Chemicals which are very toxic could come into this group.

2. OVERALL RESULTS

   The link between tumour-promoters and depigmentation

   The sixty-six chemicals tested by the depigmentation test
   included carcinogens, tumour-promoters and compounds not known
   to be carcinogenic. The results are as shown in Table 10.

   TABLE 10

   SUMMARY OF RESULTS FOR ALL COMPOUNDS TESTED

No	Categories	No of Chemicals Causing Depigmentation	Total No Of Chemicals Tested	%
1.	Carcinogens ·	6	25	24
2.	Tumour-Promoters	10	18	56
3.	Compounds Not Known to be Carcinogenic	4	23	17
	Total	20	66	

Twenty (30%) of the chemicals tested were positive. The effect
occurred more frequently with tumour-promoters than for the other
two groups i.e. 56% compared with 24% for carcinogens and 17% for
compounds not known to be carcinogenic. The specific chemicals
causing depigmentation in each category are as listed in Table II.

LIST OF CHEMICALS CAUSING DEPIGMENTATION

Carcinogens	Tumour-Promoters	Compounds Not Known to be Carcinogenic
(a) Direct Carcinogens	1. Croton oil	1. Catechol (Pyrocatechol)
1. HN2	2. TPA	2. 4-tertiary butyl catechol
2. Dimethyl sulphate	3. CO_2 snow	3. p-tertiary butyl phenol
3. Methyl iodide	4. Dodecane	4. Butylated hydroxyanisole
4. Benzyl chloride	5. Tween 20	
(b) Indirect Carcinogens	6. Phenol	
None	7. Saccharin	
(c) Undetermined Mode of Carcinogenic Action	8. 1-Fluoro-2,4-dinitro benzene	
	9. Griseofulvin	
1. Chloroform	10. Hydroquinone monobenzyl ether	
2. Ground Crocidolite		

TABLE 11

TABLE 12

LIST OF CHEMICALS NOT CAUSING DEPIGMENTATION

Carcinogens	Tumour-Promoters	Non-Carcinogens

(a) Direct Carcinogens
 	
   1. Benzoyl chloride
 	
   2. Benzotrichloride
 	
(b) Indirect Carcinogens
 	
   1. Dibenz(a,h)anthracene
 	
   2. 7,12-Dimethylbenzanthracene
 	
   3. 5,7,12-Trimethylbenzanthracene
 	
   4. Benzanthracene
 	
   5. Benzpyrene
 	
   6. 3-Methylcholanthrene
 	
   7. 2-naphthylamine
 	
   8. Benzidine
	1. Anthralin
	
	2. Tween 40
	
	3. Tween 80
	
	4. Limonene
	
	5. Iodoacetic acid
	
	6. Desoxycholic acid
	
	7. Phenobarbitone
	
	8. Sodium metabisulphite
		1. Phenanthrene
		
		2. Pyrene
		
		3. Methyl carbamate
		
		4. Sodium diethyl dithio carbamate
		
		5. Dimethylsulphoxide
		
		6. Dimethyl acetamide
		
		7. Acetone
		
		8. Saline
		
		9. Arachis oil
		
		10. Hydrochloric acid
		
		11. Sodium hydroxide

TABLE 12 (Continued)

Carcinogens	Tumour-Promoters	Non-Carcinogens
(b) Indirect Carcinogens		12. 1,1,1, Trichloroethane
9. Aniline		13. Ethanol
10. Ethyl carbamate		14. DOPA
11. Propyl carbamate		15. Pyrogallol
(c) Undetermined Mode of Carcinogenic Action		16. Methyl catechol
1. 1,2-Dimethylhydrazine		17. Putrescine
2. Chrysotile A		18. Adrenaline hydrogen tartrate
3. Chrysotile B		19. Histamine hydro-chloride
4. Anthophyllite		
5. Amosite		
6. 8-Hydroxyquinoline		

Four of the carcinogens causing depigmentation are direct-acting carcinogens and would, therefore, have both initiating and tumour-promoting activity. Thus, the positive depigmentation obtained with these four chemicals could be attributed to their tumour-promoting activity. Hence, out of the twenty chemicals producing depigmentation, fourteen have tumour-promoting activity (70%). A total of twenty-four chemicals with tumour-promoting activity (eighteen tumour-promoters and six direct-acting carcinogens) were tested altogether and depigmentation was positive for sixteen of these (67%). This reflects the sensitivity of the depigmentation test for tumour-promoters i.e. its ability to identify tumour-promoters correctly. The specificity of the test is its ability to detect non-tumour-promoters correctly. Forty-two chemicals without known tumour-promoting activity were tested and six showed positive depigmentation. Two of these were carcinogens with undetermined mode of carcinogenic action i.e. chloroform and ground crocidolite. Their status as direct or indirect carcinogens is not known. If they are direct-acting carcinogens with tumour-promoting activity, sixteen of the compounds producing depigmentation would have tumour-promoting activity (i.e. 80%).

Ground crocidolite is a form of asbestos. The mode of carcinogenic action of asbestos fibres is unknown but there is a possibility of it being a tumour-promoter (see page 164). The four non-carcinogens producing positive depigmentation belong to a group of chemicals known to cause occupational leukoderma in exposed workers. Further discussion of these compounds follows on page 175.

Table 13 summarizes the sensitivity and specificity indices
calculated according to different criteria used for including
chemicals as tumour-promoters.

TABLE 13

SPECIFICITY AND SENSITIVITY OF DEPIGMENTATION TEST

FOR TUMOUR-PROMOTERS

(USING DATA FROM INITIAL AND FOLLOW-UP EXPERIMENTS)

No	Criteria	Sensitivity %	Specificity %
1.	Tumour-promoters per se	10/18 = 56	38/48 = 79
2.	Tumour-promoters + direct-acting carcinogens	14/24 = 58	36/42 = 86
3.	Tumour-promoters + direct-acting carcinogens + chloroform	15/25 = 60	36/41 = 88
4.	Tumour-promoters + direct-acting carcinogens + chloroform + asbestos	16/30 = 53	33/36 = 89

Hence, the sensitivity of the depigmentation test for tumour-
promoters, based on the results of all tests done on all the
compounds used, varies from 53% to 60% and the specificity varies
from 79% to 89% depending upon the criteria used.

The depigmentation test, therefore, is a specific and reasonably
sensitive test for tumour-promoting activity.

CHAPTER 4   RESULTS (Continued)

Specific Experiments

(a)   Alkylating agents

(b)   Polycyclic hydrocarbons

(c)   Aromatic amines and the carbamates

(d)   Other carcinogens

(e)   Tumour-promoters

(f)   Non-carcinogens and non-tumour-promoters

3. SPECIFIC EXPERIMENTS

(a) Alkylating Agents

An alkylating agent is one which can readily introduce alkyl
groups at reactive sites in proteins and nucleic acids under
physiological conditions. An alkyl group is a monovalent radical
obtained by substituting one hydrogen atom from an aliphatic or
aromatic hydrocarbon.

Table 14 compares the efficacy of six alkylating agents in
producing depigmentation. All six alkylating agents have pub-
lished evidence of carcinogenicity. Of these, nitrogen mustard
and dimethyl sulphate produced very prominent depigmentation and
methyl iodide and benzyl chloride only slight depigmentation.
Benzoyl chloride and benzotrichloride were negative. Alkylating
agents probably exert their carcinogenic effect by being absorbed
into cell surfaces and then by slow diffusion into the aqueous
phase of the cell. This has been shown to occur for mustard gas
in Ehrlich ascites cells (Brookes and Lawley 1960). The agents
then alkylate proteins and nucleic acid. The guanine bases of
deoxyribonucleic acid (DNA) are most susceptible to alkylation
and an alkylated guanine moeity may be replaced by an adenine-
thymine pair during replication. This alters the DNA code and
produces a mutagenic effect which by the somatic mutation theory
of carcinogenesis could lead to a carcinogenic effect. It is
possible that the production of depigmentation by such agents is
due to a mutagenic effect on the genes responsible for melanin
synthesis or that the proteins involved in melanogenesis are
altered by alkylation resulting in defective, reduced or absent
melanin production. This lack of normal melanine in the hair
cells causes the depigmentation effect.

TABLE 14

COMPARISON OF ALKYLATING AGENTS IN PRODUCING DEPIGMENTATION

Alkylating Agent	Amount Injected µg	Proportion of Sites with Depigmentation / Total No of Sites Injected
Nitrogen Mustard (HN2)	3 0.3 0.03 0.003 Control (DMSO alone)	4/4 4/4 4/4 0/4 0/4
Dimethyl Sulphate	500 50 Control (DMSO alone)	4/4 3/4 0/4
Methyl Iodide	50 5 0.5 Control (DMSO alone)	4/4 0/4 0/4 0/4
Benzyl Chloride	5000 500 50 Control (DMSO alone)	4/4 3/4 1/4 0/4
Benzoyl Chloride	5000 500 50 Control (DMSO alone)	0/4 0/4 0/4 0/4
Benzotrichloride	5000 500 50 Control (DMSO alone)	0/4 0/4 0/4 0/4

However, no depigmentation was seen with benzoyl chloride and benzo-
trichloride. Both are carcinogens with a chemical structure similar
to benzyl chloride which was positive. The reason for this may be
that benzoyl chloride is hydrolysed in aqueous medium to benzoic acid,
benzaldehyde and benzyl alcohol. Benzyl chloride is hydrolysed at a
much slower rate and there is therefore more of the parent compound
available to produce the depigmentation effect seen. Benzotrichloride
is a strong mutagen but requires metabolic activation before it exerts
such effects. This may account for the negative result in the depigmen-
tation test where benzotrichloride was injected without consideration
of metabolic activation. Yasuo et al (1978) tested benzotrichloride,
benzoyl chloride and benzyl chloride for mutagenicity by using rec-
assay with Bacillus Subtilis and reversion assay with E.coli and
Salmonella typhimurium. Benzyl chloride was positive, benzoyl chloride
negative and benzotrichloride was positive only after metabloic activ-
ation. These results are comparable to those obtained by the depigment-
ation test.

Comparison of the efficacy of solutions of nitrogen mustard (HN2) in different diluents (viz saline and DMSO) in producing depigmentation

Saline and dimethyl sulphoxide were used as solvents for HN2 to see
whether the depigmentation effect was altered by the use of different
diluents. Three concentrations of HN2 were prepared with each diluent
and 0.05 ml of each solution injected into C57 Black male mice under
similar conditions.

TABLE 15

COMPARISON OF DIFFERENT DILUENTS

ON THE DEPIGMENTATION EFFECTS DUE TO NITROGEN MUSTARD (HN2)

Solution	Amount μg	Depigmentation	Proportion of Sites with Depigmentation Total No of Sites Injected
HN2 in Saline	3.0	+ +	4/4
	0.3	+	4/4
	0.03	-	0/4
	0.003	-	0/4
HN2 in Dimethyl sulphoxide (DMSO)	3.0	+ +	4/4
	0.3	+	4/4
	0.03	+	4/4
	0.003	-	0/4

With 0.3 and 3.0 μg of HN2, depigmentation occurred in all injected

sites whether saline or DMSO was used as diluent.   With 0.03 μg of

HN2 in DMSO, depigmentation again occurred in all ~~three~~ four sites injected,

but no depigmentation was observed with the same amount of HN2 in

saline.   This suggests that DMSO may increase the efficacy of HN2 in

producing depigmentation.   DMSO is a very hygroscopic liquid and is

a primary skin irritant.   These properties, absent for saline, may

account for its enhancing the depigmentation effect of HN2.   DMSO used

by itself as a control did not produce any depigmentation whether used

at 100%, 50%, 20%, or 1% solutions.   There was also no difference in

the latent period before initial depigmentation appeared, whether DMSO

or saline was used.   This period was between three to five weeks for

both.

<u>Comparison of HN2-induced depigmentation on different strains of mice</u>

<u>of both sexes</u>

Four concentrations of HN2 in DMSO were injected under identical
conditions into the following groups of mice:-

 (i) Four C57 Black male mice

 (ii) Four C57 Black female mice

(iii) Four CBA Brown male mice

 (iv) Four CBA Brown female mice

 (v) Four Tyzzer Original White male mice

 (vi) Four Tyzzer Original White female mice

TABLE 16

COMPARISON OF THE DEPIGMENTATION EFFECT DUE TO NITROGEN MUSTARD (HN2)

ON DIFFERENT STRAINS OF MICE OF BOTH SEXES

Mice	Amount of HN2 (µg)	Depigmentation	Proportion of Sites with Depigmentation / Total No of Sites Injected
C57 Black ♂	5.0	+ +	4/4
	0.5	+ +	4/4
	0.05	+	3/4
	0.005	+	0/4
C57 Black ♀	5.0	+ +	4/4
	0.5	+ +	3/4
	0.05	+	4/4
	0.005	+	1/4
CBA Brown ♂	5.0	+	4/4
	0.5	+	3/4
	0.05	−	0/4
	0.005	−	0/4
CBA Brown ♀	5.0	+	4/4
	0.5	+	2/4
	0.05	−	0/4
	0.005	−	0/4
Tyzzer Original ♂	5.0	−	0/4
	0.5	−	0/4
	0.05	−	0/4
	0.005	−	0/4
Tyzzer Original ♀	5.0	−	0/4
	0.5	−	0/4
	0.05	−	0/4
	0.005	−	0/4

The results show no difference in effect whether male or female mice were used. However, with different strains of mice, there was an obvious difference. Tyzzer Original mice are white and only depilation was observed following the intradermal injections with no change in pigmentation. The Brown CBA mice showed some depigmentation which was not as distinct as that due to equal amounts of HN2 in C57 Black mice. Hence, the depigmentation effect is best seen in C57 Black mice of either sex. Figs 4, 5, and 6 (see pages 83 and 84) show the effect of HN2 on different strains of mice.

Effective dose of HN2 in producing depigmentation

Multiple experiments were performed in C57 Black male mice using different concentrations of HN2 in dimethyl sulphoxide. The results produced a dose-effect relationship from which the ED_{50} (effective dose at which 50% of the test sites would produce positive depigmentation) was statistically estimated.

TABLE 17

DEPIGMENTATION DUE TO NITROGEN MUSTARD (HN2)

Amount of HN2 μg	Proportion of Sites with Depigmentation / Total No of Sites Injected
5.0	4/4
3.0	4/4
0.5	4/4
0.3	4/4
0.05	3/4
0.03	4/4
0.005	0/4
0.003	0/4

The ED_{50} for nitrogen mustard $\underline{/}$methyl-bis-(2-chloroethyl)-amine$\underline{7}$ was estimated at 0.018 μg using a logit transformation analysis (Cox 1970, Nelder 1975). The approximate 95% confidence interval was 0.008 μg to 0.04 μg. This ED_{50} is ten times less than that for the tumour-promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (see page 130).

(b) Polycyclic Hydrocarbons

The hydrocarbons listed in Table 18 were similarly tested by the
depigmentation test.

TABLE 18

COMPARISON OF HYDROCARBONS

Carcinogenic		Non-Carcinogenic	
	Depigmentation		Depigmentation
1,2:5,6-Dibenzanthracene (DBA)	−	Phenanthrene	−
		Pyrene	−
9,10-Dimethyl-1,2-benzanthracene (DMBA)	±		
6,9,10-Trimethyl-1,2-benzanthracene (TMBA)	−		
1,2-Benzanthracene	−		
Benzo(a)pyrene	−		
3-Methyl-cholanthrene	−		

All produced no depigmentation at various concentrations, with the
exception of DMBA. Experiments using an old bottle of fat emulsion
preparation of DMBA (containing 15% DMBA) produced the results
shown in Table 19 . The diluent used was normal saline.

TABLE 19

INJECTION OF 9,10-DIMETHYL-1,2-BENZANTHRACENE (DMBA) EMULSION

Amount of DMBA Injected μg	Degree of Depigmentation	Proportion of Sites with Depigmentation / Total No of Sites Injected
18.75	+	2/2
3.75	±	1/2
0.75	-	0/2
0.15	-	0/2

In view of DMBA being the only polycyclic hydrocarbon producing positive depigmentation, it was decided to investigate this further. Analysis of the constituents in the fat emulsion preparation showed the following:-

1. Poloxalkol   0.3%)   Special 15% fat emulsion with
)
2. Lecithin   1.2%)   9,10-Dimethyl-1,2-benzanthracene
)
3. Cottonseed oil 15.0%)   (5mg/gm)
)
4. Water   )   Upjohn Co, Kalamazoo, Michigan, USA
)
5. DMBA   )

Repeat experiments were performed using DMBA in DMSO i.e. without the other constituents of the emulsion. This produced the results in Table 20.

TABLE 20

INJECTION OF 9,10-DIMETHYL-1,2-BENZANTHRACENE IN DMSO

Amount μg	Volume Injected ml	Proportion of Sites with Depigmentation / Total No of Sites Injected
500	)	0/8
100	)	0/4
37.50	)	0/4
18.75	) 0.05	0/4
10.00	)	0/4
3.75	)	0/4
0.75	)	0/4
0.15	)	0/4
0.03	)	0/4

In addition to the four concentrations of DMBA previously used, five additional concentrations were used to widen the range of amounts of DMBA tested. These included three concentrations greater than the maximum of 18.75 μg previously injected (37.50 μg, 100 μg and 500 μg). 10 μg of DMBA was also tested as an intermediate concentration between 18.75 and 3.75 μg, the two concentrations which previously produced a positive depigmentation. A lower concentration of 0.03 μg was also tested.

In all cases, no depigmentation was observed. It was concluded that the depigmentation noted with the first emulsion preparation is due not to the DMBA but to the other constitutents present. To confirm this a preparation of the fat emulsion without DMBA but with the other constituents in the same proportions was obtained and tested. Different

dilutions were prepared with normal saline.

TABLE 21

INJECTION OF FAT EMULSION ALONE

Emulsion in Normal Saline	Volume Injected ml	Proportion of Sites with Depigmentation / Total No of Sites Injected
100%	)	0/12
20%	) 0.05	0/12
4%	)	0/12
0.8%	)	0/12

Suprisingly, the emulsion without the presence of DMBA also produced no depigmentation. Repeat experiments were then performed using the old bottle of emulsion with DMBA and a new bottle of the same preparation (See Table 22 overleaf).

TABLE 22

INJECTION OF OLD AND NEW 9,10-DIMETHYL-1,2-BENZANTHRACENE

Emulsion	Amount µg	Volume Injected ml	Degree of Depigmentation	Proportion of Sites with Depigmentation / Total No of Sites Injected
Old DMBA Emulsion	100% emulsion containing 18.75 µg DMBA	)))	+	1/8
	20% emulsion containing 3.75 µg DMBA	)))) 0.05	+	2/8
	4% emulsion containing 0.75 µg DMBA	)))	-	0/8
	0.8% emulsion containing 0.15 µg DMBA	))	-	0/8
New DMBA Emulsion	100% emulsion containing 18.75 µg DMBA	)))	-	0/8
	20% emulsion containing 3.75 µg DMBA	)))) 0.05	-	0/8
	4% emulsion containing 0.75 µg DMBA	)))	-	0/8
	0.8% emulsion containing 0.15 µg DMBA	))	-	0/8

The old emulsion produced slight depigmentation when used at 100%
and 20% concentrations. Depigmentation was only noted in one and two
out of eight sites injected respectively. The new emulsion was
negative when similar concentrations were tested.

It is likely that the interaction of DMBA together with the poloxalkol,
lecithin, cottonseed oil and water was responsible for the positive
depigmentation seen. DMBA by itself or the other substances without
DMBA will not produce depigmentation. It was noted that the depigment-
ation, though present, was only slight and only positive in a small
proportion of the sites injected. The results will have to be treated
with care, because of the age of the original emulsion which pro-
duced positive depigmentation.

A similar emulsion containing the carcinogenic 6,9,10-trimethyl-1,2-
benzanthracene was tested using 0.05 ml of emulsion per injection.

TABLE 23

INJECTION OF 6,9,10-TRIMETHYL-1,2-BENZANTHRACENE (TMBA)

TMBA in Fat Emulsion	Proportion of Sites with Depigmentation / Total No of Sites Injected
100% containing 18.75 µg TMBA	0/12
20% containing 3.75 µg TMBA	0/12
4% containing 0.75 µg TMBA	0/12
0.8% containing 0.15 µg TMBA	0/12

The results showed that TMBA in a similar emulsion did not produce any depigmentation. Hence, the effect noted with old DMBA in emulsion doed not hold for fresh preparations of other similar polycyclic aromatic hydrocarbons in emulsion form.

Two non-carcinogenic polycyclic hydrocarbons, phenanthrene and pyrene tested at the following concentrations produced negative results.

TABLE 24

INJECTION OF NON-CARCINOGENIC POLYCYCLIC AROMATIC HYDROCARBONS IN DMSO

Hydrocarbon	Amount μg	Volume Injected ml	Proportion of Sites with Depigmentation / Total No of Sites Injected
Phenanthrene	5000 500 50 Control (DMSO alone)	))) 0.05))	0/4 0/4 0/4 0/4
Pyrene	5000 500 50 Control (DMSO alone)	))) 0.05))	0/4 0/4 0/4 0/4

Four carcinogenic polycyclic aromatic hydrocarbons, 1,2-benzanthracene,

3,4-benzpyrene, 3-methylcholanthrene and 1,2:5,6-dibenzanthracene

were also similarly tested.

TABLE 25

INJECTION OF CARCINOGENIC POLYCYCLIC AROMATIC HYDROCARBONS IN DMSO

Hydrocarbon	Amount µg	Volume Injected ml	Proportion of Sites with Depigmentation / Total No of Sites Injected
Benzanthracene	114 60	) 0.05	0/4 0/4
Benzo(a)pyrene	126 60	) 0.05	0/4 0/4
3-Methylcholanthrene	10 2	) 0.05	0/16 0/16
1,2:5,6- Dibenzanthracene	140 14 10 7	))) 0.05)	0/12 0/12 0/12 0/12

Conclusion

Polycyclic aromatic hydrocarbons - whether carcinogenic or non-
carcinogenic - produced no depigmentation. The carcinogenic aromatic
hydrocarbons are indirect carcinogens and need to be converted to the
ultimate carcinogen to be active. Such conversion may also be needed
for positive depigmentation to be produced.

(c) Aromatic Amines and the Carbamates

The negative results obtained for the polycyclic aromatic
hydrocarbons as compared to the alkylating agents may be due to
the former being indirect carcinogens whereas the latter are
direct carcinogens. Indirect carcinogens require metabolic
activation before they are converted to the ultimate carcinogen.
In the case of 3-methyl cholanthrene, 1,2:5,6-dibenzanthracene,
benzanthracene and 7-methyl benzanthracene, epoxide derivatives
are ultimate carcinogens and these epoxides act as alkylating
agents (Lawley 1976). Aromatic amines and carbamates are two
different groups of indirect carcinogens and some examples from
each group were tested.

TABLE 26

INJECTION OF CARCINOGENIC AROMATIC AMINES

	Concentration (W/V) %	Amount µg	Volume Injected (ml)	Proportion of Sites with Depigmentation / Total No of Sites Injected
2-Naphthylamine in DMSO	5.0	2500	)	0/8
	0.5	250	) 0.05	0/8
	0.05	25	)	0/8
	0.005	2.5	)	0/8
Benzidine in DMSO	2.5	1250	)	0/8
	0.5	250	) 0.05	0/8
	0.05	25	)	0/8
	0.005	2.5	)	0/8
Aniline in DMSO	5.0	2500	)	0/8
	0.5	250	) 0.05	0/8
	0.05	25	)	0/8
	0.005	2.5	)	0/8

TABLE 27

INJECTION OF CARCINOGENIC CARBAMATES

	Concentration (W/V) %	Amount µg	Volume Injected (ml)	Proportion of Sites with Depigmentation / Total No of Sites Injected
Ethyl Carbamate (Urethane) in DMSO	4.0	2000	)	0/8
	0.4	200	)	0/8
	0.04	20	) 0.05	0/8
	0.004	2	)	0/8
Propyl Carbamate in DMSO	4.0	2000	)	0/8
	0.4	200	)	0/8
	0.04	20	) 0.05	0/8
	0.004	2	)	0/8

TABLE 28

INJECTION OF NON-CARCINOGENIC CARBAMATES

	Concentration (W/V) %	Amount μg	Volume Injected (ml)	Proportion of Sites with Depigmentation / Total No of Sites Injected
Methyl Carbamate in DMSO	4.0	2000	)	0/8
	0.4	200	) 0.05	0/8
	0.04	20	)	0/8
	0.004	2	)	0/8
Diethyl Dithiocarbamate in DMSO	1.0	500	)	0/8
	0.1	50	) 0.05	0/8
	0.01	5	)	0/8
	Control (DMSO alone)	0	)	0/8

The results show that all the aromatic amines and carcinogenic and non-carcinogenic carbamates chosen for testing did not produce depigmentation. 2-Naphthylamine, benzidine, ethyl carbamate and propyl carbamate are all indirect carcinogens. While ethyl carbamate has produced lung tumours in mice (Salaman and Roe 1953) it is not carcinogenic for mouse skin (Van Duuren 1976). There is no definite animal or human data showing methyl carbamate to be carcinogenic (IARC Monographs Vol 12). Diethyldithiocarbamate is also non-carcinogenic but is a chelating agent (Merck Index 1976). It has been suggested that metal chelation plays a role in chemical carcinogenesis (Weisburger et al 1963, Williams 1971 and 1972).

Enzyme-inducing agents can increase the synthesis of some microsomal metabolising enzymes (Conney 1967). Such agents include 3-methyl cholanthrene, barbiturates, steroids, polycyclic aromatic hydrocarbons and quinones. By increasing the microsomal enzymes the conversion of pro-carcinogens to ultimate carcinogens may be enhanced. 2-Naphthylamine and benzidine and three ₿ polycyclic aromatic hydrocarbons were tested after pretreatment with phenobarbitone and 3-methyl cholanthrene as inducing agents.

Phenobarbitone Pretreatment

Phenobarbitone sodium (0.5%) was given in place of drinking water to four C57 Black male mice two days before the intradermal injections of the polycyclic hydrocarbons and the aromatic amines and continued for a week after the injections.

On the basis of each mouse drinking approximately 2.5 ml of water

a day, this would give 1.25 mg of phenobarbitone per mouse per day= 50 mg per kg body weight. This was the dose previously used for mice in similar experiments (Alberts and Van Daalen Wettes 1976).

3-Methylcholanthrene Pretreatment

1 mg of 3-methylcholanthrene was dissolved in 0.2 ml of arachis oil and this was injected intra-peritoneally into each C57 Black male mouse twenty-four hours before the intradermal injections of carcinogenic hydrocarbons or aromatic amines.

TABLE 29

INJECTION OF INDIRECT CARCINOGENS IN MICE PRE-TREATED WITH PHENOBARBITONE

Indirect Carcinogen	Amount μg	Proportion of Sites with Depigmentation / Total No of Sites Injected	
		Pretreatment with Phenobarbitone	No Pretreatment
Polycyclic Hydrocarbons			
1,2:5,6-Dibenzanthracene	139	0/4	0/4
	70	0/4	0/4
	25	0/12	0/12
	5	0/12	0/12
9,10-Dimethyl-1,2-Benzanthracene emulsion	250	0/4	0/4
	125	0/4	0/4
	50	0/4	0/4
	25	0/4	0/4
	5	0/4	0/4
Benzanthracene	114	0/4	0/4
Aromatic Amines			
2-Naphthylamine	450	0/4	0/4
Benzidine	5000	0/4	0/4
	1000	0/4	0/4
	500	0/4	0/4
	100	0/4	0/4

TABLE 30

INJECTION OF INDIRECT CARCINOGENS IN MICE PRE-TREATED WITH 3-METHYLCHOLANTHRENE

Indirect Carcinogen	Amount µg	Proportion of Sites with Depigmentation / Total No of Sites Injected	
		Pretreatment with 3-Methylchloanthrene	No Pretreatment
Polycyclic Hydrocarbons			
1,2:5,6-Dibenzanthracene	139	0/12	0/12
	125	0/12	0/12
	70	0/12	0/12
	50	0/12	0/12
	25	0/12	0/12
	5	0/12	0/12
9,10-Dimethyl-1,2-Benzanthracene emulsion	250	0/4	0/4
	125	0/4	0/4
	50	0/4	0/4
	25	0/4	0/4
	5	0/4	0/4
Benzanthracene	114	0/4	0/4
Aromatic Amines			
2-Naphthylamine	450	0/4	0/4
Benzidine	5000	0/4	0/4
	1000	0/4	0/4
	500	0/4	0/4
	100	0/4	0/4

The use of phenobarbitone or 3-methylcholanthrene as enzyme
inducing agents did not alter the results of the depigmentation
with regards to 1,2;5,6-dibenzanthracene, 9,10-dimethyl-1,2-
benzanthracene, 2-naphthylamine and benzidine. All were negative
at a range of concentrations used.

Several reasons may exist for the negative results obtained.

(a)   Phenobarbitone and 3-methylcholanthrene are ineffective as
      enzyme inducing agents for the skin of C57 Black mice.
      Phenobarbitone increases liver microsomal enzymes but not
      skin enzymes.

(b)   The amounts of phenobarbitone and 3-methylcholanthrene were
      either too small or not given for an adequate period of
      time. Increasing the period for which phenobarbitone was
      administered or repeated intra-peritoneal administrations
      of 3-methylcholanthrene may produce different results.

(d)   Other Carcinogens

Chloroform (Trichloromethane)
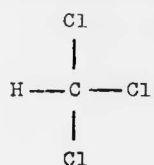
```
        Cl
        |
H ── C ── Cl                    Molecular weight 110.39
        |
        Cl
```

This is a volatile, non-flammable heavy liquid with a sweet odour.
It has been used as an anaesthetic, as a solvent and cleansing
agent, in pharmaceuticals and toiletries and as a refrigerant and
aerosol propellant.   Chloroform is hepatocarcinogenic in mice
(Eshenbrenner and Miller 1945).   No long term studies on similar
effects in man have been reported.

Chloroform dissolved in dimethyl sulphoxide (DMSO) was tested for
depigmentation by intradermal injections in C57 Black mice.   The
results show positive depigmentation induced by chloroform.   The
dose-response relationship was sigmoidal and the ED_{50} was estimated
by a logit transformation analysis (Cox 1979, Nelder 1975) as equal
to 101 µg (Approximate 95% confidence interval being 48 µg to
215 µg).

TABLE 31

DEPIGMENTATION DUE TO CHLOROFORM IN DMSO

Concentration (W/V) %	Amount µg	Volume Injected (ml)	No of Mice Used	No of Sites Injected	No of Sites With Positive Depigmentation
10	5000	0.05	)	3	3
1.0	500	0.05	)	11	10
0.1	50	0.05	) 19	11	2
0.05	25	0.05	)	8	2
0.005	2.5	0.05	)	8	0
Control (DMSO)	0	0.05	)	19	0

1,2-Dimethylhydrazine (Symmetrical dimethylhydrazine or SDMH)

```
     H    H    H    H
     |    |    |    |
H — C — N — N — C — H          Molecular weight 60.1
     |              |
     H              H
```

This is a clear colourless hygroscopic liquid which is corrosive
to the skin. It is carcinogenic to mice - producing colonic and
rectal tumours when given subcutaneously (Hawks et al 1971/1972)
and angiosarcomas of the muscle, liver and pararenal tissues when
given orally (Toth and Wilson 1971). No data is available on the
carcinogenic risk to man. SDMH probably acts as a pro-carcinogen,
requiring metabolic activation to the ultimate carcinogen before
exerting its carcinogenic effect (IARC Monographs).

Solutions of SDMH (Aldrich Chemical Co) in dimethyl sulphoxide
were injected intradermally in C57 Black male mice. No depigment-
ation was noted with SDMH and this is consistent with the results
for other pro-carcinogens e.g. the polycyclic aromatic hydrocarbons,
aromatic amines and carbamates.

TABLE 32

INJECTION OF 1,2-DIMETHYLHYDRAZINE IN DMSO

Concentration (W/V) %	Amount μg	Volume Injected (ml)	No of Mice Used	No of Sites Injected	No of Sites With Positive Depigmentation
1.0	500	0.05	)	10	0
0.1	50	0.05	) 10	10	0
0.01	5	0.05	)	10	0
Control (DMSO)	0	0.05	)	10	0

(e) Tumour-Promoters

Tumour-promoters are agents which accelerate and increase tumour
incidence on repeated application following exposure to an
initiating agent. The biological properties of tumour-promoters
have been summarised (see page 22). Croton oil was the first
tumour-promoter to be extensively studied (Berenblum 1941). This
is an extract from the seeds of croton tiglium L. (Euphorbiacea).
In the 1960's the active principle in croton oil was isolated
by Van Duuren and Hecker independently. It was termed TPA
(tetradecanoyl phorbol acetate) or PMA (phorbol myristate acetate).
This is the most potent tumour-promoter known for mouse skin
(Furstenberger and Hecker 1972, Hecker 1971 and Boutwell 1974).
After the phorbol esters, anthralin is the next most active
tumour-promoter (Van Duuren 1976) but close analogues of anthralin
such as anthrone, 1,8,dihydroxyanthraquinone and 18 dihydroxy-
anthracene are inactive. Setälä (1956 and 1960) showed Tweens
and Spans as weak tumour-promoters. Other weak tumour-promoters
are phenol (Boutwell and Bosch 1959), limonene, and iodoacetic
acid. Berenblum (1930) showed that carbon dioxide snow, when
applied repeatedly to mouse skin after initial application of tar,
was a tumour-promoter. The list of suspected tumour-promoters
continues to grow and included in such a list are the drug
phenobarbitone, the artificial sweeteners - saccharin (Boyland
1979) and sodium cyclamate, the bile acids (Aries et al 1969)
and cholesterol (Cruse,Lewin and Clark 1979) which are thought to
play a role in human colonic cancer.

Several tumour-promoters were tested for their efficiency in
producing depigmentation.

TABLE 33

DEPIGMENTATION BY TUMOUR-PROMOTERS

Tumour-Promoter	Depigmentation
1. Croton oil	+ +
2. TPA	+ + +
3. Anthralin	-
4. Tween 20	+
5. Tween 40	-
6. Tween 80	-
7. Dodecane	+
8. Limonene	$\pm$
9. Phenol	+
10. CO_2 snow	+ + +
11. Iodoacetic acid	-
12. Deoxycholic acid	-
13. Saccharin	+
14. 1-Fluoro-2,4-dinitrobenzene	+
15. Griseofulvin	+
16. Phenobarbitone	-
17. Hydroquinone monobenzyl ether	+
18. Sodium metabisulphite	-

Ten out of the eighteen (56%) tumour-promoters tested produced positive depigmentation. This is most marked for TPA and CO_2 snow and croton oil.

12-0-Tetradecanoyl phorbol-13-acetate (TPA)

For TPA, different concentrations injected in 0.05 ml volumes
produced the following results:-

TABLE 34

PROPORTION OF DEPIGMENTED SITES DUE TO TPA

Amount of TPA Injected	No of Positive Sites of Depigmentation / Total Number of Sites Injected
50 µg	10/10
5 µg	19/19
1 µg	8/9
0.2 µg	3/9
0.04 µg	2/10
0.008 µg	1/10
0.002 µg	0/10
Control (Dimethyl Sulphoxide alone)	0/19

TPA-induced depigmentation was dose-related and the ED_{50} estimated at
0.18 µg using a logit transformation analysis (Cox 1979). At doses of
0.002 µg and below, no depigmentation was noted. Sites injected with
dimethylsulphoxide alone, as a control showed only localised depilation
without any depigmentation.

Croton Oil

Croton oil is an irritant, clear, yellow oil which is soluble in dimethylsulphoxide (DMSO). Testing for depigmentation with different amounts of croton oil in DMSO produced the following results:-

TABLE 35

DEPIGMENTATION DUE TO CROTON OIL IN DMSO

Amount of Croton Oil	Proportion of Sites Showing Depigmentation
250 µg	4/8
50 µg	0/8
25 µg	0/8
5 µg	0/8
Control (DMSO alone)	0/8

Doses of 500 µg and above killed the mice used. Doses of 50 µg and less produced no depigmentation. Only when 250 µg of croton oil was used did depigmentation appear in four out of eight sites. The effective dose for producing depigmentation is therefore close to the lethal dose in C57 Black mice. The ED_{50} of 250 µg is more than 1300 times that of TPA where the estimated ED_{50} was 0.18 µg. Van Duuren et al (1973) obtained 1.0 g of TPA from 453 g of croton oil. Hence, one would expect the ED_{50} of croton oil to be at least 453 times that of TPA if the depigmentation effect is due solely to the TPA part of the croton oil. The fact that the ED_{50} is more than 1300 times that of TPA indicates that much more croton oil is required to produce depigmentation than its equivalent content of pure TPA.

The depigmentation noted with croton oil was not as prominent (+ +)
as that produced by TPA (+ + +). There was also a delay in the latent
period before depigmentation appeared. This was an average of fifty-
three days (Range: thirty-two to seventy-one days) compared to twenty-
four days (Range: twenty to thirty-one days) for TPA. The latent
period did not differ much for different concentrations of the same
compound.

Anthralin
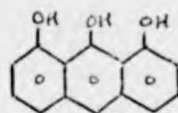
Anthralin produced no depigmentation when used in the following
concentrations (ten sites injected for each concentration).

TABLE 36

INJECTION OF ANTHRALIN IN DMSO

Amount of Anthralin	Proportion of Sites Showing Depigmentation
500 µg	0/10
250 µg	0/10
50 µg	0/10
25 µg	0/10
5 µg	0/10
2.5 µg	0/10
Control (DMSO alone)	0/20

When injected at a maximum concentration of 2 gm/100 ml of DMSO
(i.e. 1000 µg of anthralin) this killed four out of five mice used.
The surviving mouse showed no depigmentation when observed regularly
for thirty weeks. Anthralin is a fairly active tumour-promoter and
while the results of the test showed 56 % of tumour-promoters tested
produced positive depigmentation, anthralin was not amongst these.
Anthralin is unstable in a solution and a dimer is spontaneously formed
which is inactive as a tumour-promoter (Segal et al 1971, Van Duuren
1976). Such dimerization occurs in acetone and could occur in dimethyl-
sulphoxide. The loss of tumour-promoting activity would then correlate

with the negative depigmentation result. A further possible explanation is that the effective dose of anthralin for producing depigmentation and/or tumour promotion is higher than the lethal dose and, therefore, in the concentrations used, anthralin produced no effect and at higher concentrations death of the mice resulted. In experiments on tumour promotion using dimethylbenzanthracene (DMBA) for the first treatment and anthralin in acetone for the second treatment of female Swiss mice, Segal and others (1971) noted a tumour promotion effect with 80 µg of anthralin. However, this dose had to be applied three times a week for fifty-nine days before the first squamous papillomas appeared. In a similar experiment using TPA instead of anthralin only 2.5 µg applied three times weekly used under similar conditions produced tumours in fifty-four days.

Anthralin was also used as a pharmaceutical agent in the treatment of psoriasis. It does not cause depigmentation nor has there been a reported increase in the malignancies in patients using this agent. However, it is a tumour-promoter in Swiss mice. The negative depigmentation in C57 Black mice may be due to a difference in species sensitivity and that perhaps it is not an effective tumour-promoter for C57 mice.

Tween

The Tweens are commercially-prepared surface active agents which
together with surfactant detergents and bile acids can be grouped as
dipolic agents. These are compounds which in an electric field
behave like molecular rods with two opposite electric charges separ-
ated by a definite distance. Tweens are esters of various sorbitols
with ethylene oxide units introduced into the free hydroxyl groups.

    e.g. Tween 20 - Poly oxyethylene sorbitan monolaureate

            Tween 40 - Poly oxyethylene sorbitan monopalmitate

            Tween 80 - Poly oxyethylene sorbitan

Setälä (1960) showed that these Tweens(and related Spans) exhibit
tumour-promoting activity. Of the three examples mentioned, he found
Tween 40 to be the most active followed by Tween 80 and Tween 20.

These three dipolic agents were tested by the depigmentation test.

TABLE 37

DEPIGMENTATION DUE TO TWEENS

Solution	Amount of Tween Used	Proportion of Sites Showing Depigmentation
Tween 20 in DMSO	5000 μg 500 μg 50 μg	2/4 1/4 0/4
Tween 40 in DMSO	5000 μg 500 μg 50 μg	0/4 0/4 0/4
Tween 80 in DMSO	5000 μg 500 μg 50 μg	0/4 0/4 0/4

Only Tween 20 produced slight depigmentation but neither Tween 40 nor Tween 80 did. This fits in with Setala's finding that all products of the Tween group do <u>not</u> under similar experimental conditions possess obvious tumour-enhancing properties. However what does not fit is that Setala found Tween 20 to be the least active of the three tumour-properties, yet this was the only Tween to produce positive depigmentation. This difference may be due to different strains of mice used for Setala's studies as opposed to the present study. It has been argued (Van Duuren 1976) that the Tween compounds tested by Setala were not pure and were active only in massive doses and the Tween compounds used for the depigmentation test may have a better standard of purity but it still required very large doses to produce an effect. The degree of depigmentation produced by Tween 20 was slight and a very high concentration of 5000 μg in 0.05 ml of solution produced depigmentation in only 50% of test sites.

Carbon Dioxide Snow

Berenblum (1930) produced tumours by applying tar to the skin of mice followed by repeated application of carbon dioxide snow. Such tumours did not occur when tar and carbon dioxide snow were applied together. Hence, carbon dioxide snow acts as a tumour-promoter but not as a co-carcinogen. Local application of pieces of carbon dioxide snow (about 4 mm diameter in size) to the skin of C57 Black mice produced the following results:-

TABLE 38

DEPIGMENTATION DUE TO CARBON DIOXIDE SNOW

Time of Application	Proportion of Sites Showing Depigmentation	Mean Latent Period
$\frac{1}{2}$ minute	0/3	-
1 minute	3/3	32 days
2 minutes	3/3	29 days

The degree of depigmentation was more prominent with the two minute application compared with the one minute application. There was no depigmentation with the half minute application. There was little difference in the latent period before appearance of depigmentation whether the application was for one minute or two minutes. This suggests that a minimum time of application is required before visually detectable depigmentation appears. This could be termed the 'threshold application time'. Once the threshold application time is exceeded, it takes about the same length of time for the change from pigmented hair to depigmented hair. This could be related to the rate of hair growth in the mice.

Similar results were produced by Boyland and Sargent (1951) using carbon dioxide snow for two minutes in mice. The latent period before appearance of depigmentation was nineteen days. This is shorter than the twenty-nine days mentioned above and may be due to a difference in:-

  (i)   The strain of mice used.
 (ii)   The size of the pieces of carbon dioxide snow.
(iii)   The definition of what constitutes positive depigmentation.

In this study, where there is doubtful depigmentation this is considered negative. Slight depigmentation has to be observed as such on three consecutive occasions before being considered positive.

Boyland also applied carbon dioxide snow directly to the fur of mice and no depigmentation occurred. This important observation indicates that it is an effect on the skin which manifests as depigmentated hair and not a direct effect on the hair itself.

To test whether the depigmentation was due to some chemical reaction between the carbon dioxide snow and the skin or merely an effect of the decreased temperature produced by the carbon dioxide snow, the experiment was repeated using carbon dioxide snow in aluminium containers. This shielded the carbon dioxide snow from direct contact with the skin but conducted the lowered temperature through to cause cooling of the area of skin in contact with the container. The results obtained are as follows:-

TABLE 39

DEPIGMENTATION DUE TO CARBON DIOXIDE SNOW IN METAL CONTAINERS

Time of Application	Proportion of Sites Showing Depigmentation	Mean Latent Period
$\frac{1}{2}$ minute	0/4	—
1 minute	2/4	34 days
2 minutes	4/4	34 days

The results show that the depigmentation due to carbon dioxide snow is a temperature effect and not due to any chemical reaction between carbon dioxide snow and the skin. The extent of depigmentation was less pronounced compared with that produced by direct application of carbon dioxide snow.

For the one minute applications, half of the sites produced no depigmentation even after observation for one hundred days. The use of the containers probably reduced the cooling effect on the skin so that it is less cold compared with direct application. Hence, even though it was applied above the apparent threshold application time, some of the sites did not produce depigmentation because of the slightly higher temperature acting on the skin. The threshold for obvious depigmentation, therefore, depends both on the application time as well as on the effective temperature. The latent period was not much different; it was thirty-four days compared with thirty-two and twenty-nine days for direct application.

Taylor (1949) produced similar depigmentation in rat hair by freezing. It was suggested that the effect may be a result of the lowered

temperature producing ischaemia and that depigmentation resulted
because of susceptibility of the melanocytes to aschaemia. This
view is supported by Selye's experiments (1967) where experimental
ischaemia produced by clamping a skin fold of a rat for eight hours
also resulted in depigmentation. It is possible that sudden greying
of hair following severe stress may be the result of vasoconstriction
of superficial vessels causing ischaemic changes to pigment cells
(Riley 1971).

Adrenaline which is a vasoconstrictor was tested for this effect by
single intradermal injections into C57 Black mice.

Results

Injection at four sites with 0.05 ml of adrenaline hydrogen tartrate
(0.01%, 0.1%, 1.0% and 2.0% wt/vol) resulted in death of the test
animals (four animals used) within twenty-four hours. Adrenaline is
the principal sympathomimetic hormone produced by the adrenal medulla.
It is a cardiac stimulant and in excessive amounts can cause cardiac
arrhythmias and death.

A repeat experiment was performed with reduced concentrations of
adrenaline (0.001%, 0.01%, 0.1% and 1.0%) per mouse. This was also
lethal to four mice used. A third experiment performed with only two
sites per mouse injected with:-

(i) 0.01%)
) adrenaline hydrogen tartrate in distilled water.
(ii) 0.001%)

did not produce any depigmentation when observed weekly for more than
six months. Since higher concentrations were lethal to C57 mice,
smaller concentrations had to be used and the negative depigmentation

may be due to the dose used being ineffective in producing vasocon-
striction or that the vasoconstriction produced was not of sufficient
duration or extent to result in damage to the melanocytes. This
result does not confirm the suggestion that ischaemia may cause
depigmentation.

Freezing results in tissue injury and histamine is released from most
cells locally following tissue injury. It may be possible that the
depigmentation effect is mediated by histamine release. This may be
the common link between tumour-promotion and depigmentation. Freezing
by carbon dioxide snow acts as a tumour-promoter (Berenblum 1930).
Cutaneous wounding also has a tumour-promoting effect as shown by
experiments by Deelman and Van Erp (1927). They applied carcinogenic
tars to mice and produced an initiating effect and then made cutaneous
wounds which increased the incidence of tumours. Croton oil, TPA
and 1-fluoro-2,4-dinitrobenzene are all skin irritants and are effec-
tive in producing depigmentation. Nitrogen mustard, dimethylsulphate
and methyl iodide are all skin vesicants and also produced depigment-
ation. These three compounds are complete carcinogens and would, there-
fore, be expected to have both initiating and tumour-promoting activity.
All of these compounds with tumour-promoting activity are capable of
causing tissue injury. Freezing cutaneous wounding and chemicals
cause tissue injury and histamine release. Solutions of histamine
hydrochloride in normal saline were therefore tested by intradermal
injections into C57 Black mice.

Solutions of histamine hydrochloride in normal saline were, therefore, tested by intradermal injections into C57 Black mice.

TABLE 40

INJECTION OF HISTAMINE HYDROCHLORIDE

Concentration (W/V) %	No of Sites per mouse	No of Mice	Proportion of Sites with Depigmentation / Total No of Sites Injected
1.0	4	4	0/16
0.1	4	4	0/16
0.01	4	4	0/16

Histamine hydrochloride did not cause depigmentation when single doses of 0.01%, 0.1% and 1% solutions were injected intradermally so histamine is unlikely to be the mediating cause of depigmentation.
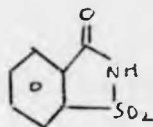
Saccharin

Saccharin is an artificial sweetener five hundred times as sweet as
sugar and its sweet taste is detectable in dilutions as low as
1/100,000. The first indication that it was carcinogenic was in 1951
when Fitzhugh and co-workers described 'an increased incidence of the
ordinarily uncommon condition of abdominal lymphosarcoma in rats fed
saccharin'. Bladder implantation experiments with cholesterol pellets
impregnated with saccharin in mice also cause bladder tumours (Allen
et al 1957). Together with previous reports on its initiating action
on the skin (Salaman and Roe 1956) it was then thought that saccharin
was a direct carcinogen. However, later experiments indicate it
functions as a tumour-promoter (Hicks et al 1975, Cohen et al 1978).
This is supported by the negative results obtained in the Ames test
and with the Styles cell transformation test. There is also no evi-
dence that it is metabolised or that it reacts with DNA. Experiments
indicate that as a tumour-promoter it is one thousand times less active
than TPA (Mondal et al 1978). Boyland (1979) suggests that the dose
required to induce bladder cancer in rats is of the order of 1 kg/kg
body weight and therefore of very low risk in so far as carcinogenesis
in man is concerned. This is supported by epidemiological evidence
that diabetics who use saccharin as a sweetener in place of sugar
much more than average do not show an increase in bladder cancer
(Armstrong and Doll 1975, Armstrong et al 1976, and Kessler 1976).
Saccharin was dissolved in sodium hydroxide and diluted with distilled
water into solutions for testing by the depigmentation test.

TABLE 41

DEPIGMENTATION DUE TO SACCHARIN IN DISTILLED WATER

Concentration (W/V) %	Amount of Saccharin µg	Depigmentation		
		Degree	Proportion of Positive Sites	Minimum Latent Period
15.0	7,500	+	1/6	36 days
3.0	1,500	+	3/6	36 days
0.6	300	-	0/6	-
0.12	60	-	0/6	-

Only high concentrations of saccharin produced depigmentation.
Saccharin is a weak tumour-promoter and if depigmentation in C57
Black mice can be caused by chemicals with tumour-promoting activity,
then the results obtained support this. It is interesting that
saccharin at a concentration of 3 gms per 100 ml produced depigment-
ation in 50% of sites and yet at a higher concentration only one site
out of six showed depigmentation. This may be due to the very high
concentration destroying the hair cells and pigment cells, thereby
suppressing the expression of depigmentation. At the appropriate
optimal dose, the pigment cells are affected by the chemicals injected
and the hair cells continue growing but are not pigmented.

Dodecane and Phenol

$$C_{12}H_{26}$$

DODECANE

OH

O PHENOL
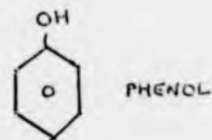
N-dodecane is a compound with both tumour-promoting and co-carcinogenic activity (Van Duuren 1976). When tested by the depigmentation test it produced the following results:-

TABLE 42

DEPIGMENTATION DUE TO DODECANE IN DMSO

Amount	Degree of Depigmentation	Proportion of Sites with Depigmentation / Total No of Sites Injected
5000 µg	+	1/4
500 µg	+	4/4
50 µg	−	0/4
Control (DMSO alone)	−	0/4

Very high concentrations produced depilation but depigmentation occurred in only one out of four sites injected. At 500 µg, this appeared to be the optimal concentration for depigmentation. 100% depigmentation occurred in the four sites injected. No depigmentation was seen for 50 µg or where DMSO was injected by itself as a control. The results with dodecane show an optimal dose type dose-response relationship (see page 90).

Phenol is a compound with tumour-promoting activity but no
co-carcinogenic activity. Boutwell and Bosch (1959) showed tumour-
promoting activity on young adult albino mice by using 7,12-dimethyl
benzanthracene (DMBA) as initiator followed by repeated applications
of phenol. In the depigmentation test the following results were
obtained:-

TABLE 43

DEPIGMENTATION DUE TO PHENOL IN DMSO

Amount	Degree of Depigmentation	Proportion of Sites with Depigmentation / Total No of Sites Injected
5000 μg	+	2/4
500 μg	+	1/4
50 μg	-	0/4
Control (DMSO alone)	-	0/4

Phenol only produced slight depigmentation in two out of four sites
with 5 mg doses. At lower concentrations, only one out of four sites
gave a positive result (i.e. 500 μg) and at 50 μg the results were all
negative. Phenol is a weak tumour-promoter (Van Duuren 1976) and this
weak positive depigmentation effect correlates with its tumour-
promoting potential. The latent period before the appearance of
depigmentation was fifty-six to eighty-four days which was longer than
for TPA or croton oil.

Boutwell and Bosch (1959) showed phenol to have one twentieth of the
potency of croton oil. The ED_{50} for depigmentation is 5000 μg for
phenol; that for croton oil using the same technique is 250 μg which

is one twentieth as effective. Hence, the efficacy of phenol as
a tumour-promoter and for producing depigmentation in mice appears
to correlate well.

Tumour-promoters with negative depigmentation test results: limonene, iodoacetic acid and deoxycholic acid

D-limonene is present in oils from lemons and oranges. It is a skin
irritant and a sensitizer. It has been shown by Roe and Pierce (1960)
to have tumour-promotion activity in mice. Iodoacetic acid has also
been shown to have tumour-promoting action in mouse skin (Gwyn and
Salaman 1953). Deoxycholic acid does not appear to have been tested
but lithocholic acid is a promoter of colon cancer in rats. Aries et al
(1969) suggested that bile acids may have a role in colon carcino-
genesis. Cruse et al (1978) considered that cholesterol may be a
more likely candidate as a colon tumour-promoter than the bile-salt
derivatives.

All three compounds with possible tumour-promoting activity were
tested by the depigmentation test and all produced negative results
in the concentrations shown.

Limonene

This produced no depigmentation when used at five sites for each con-
centration of 5µg, 50 µg and 500 µg and 0 µg (Control using DMSO alone)

Iodoacetic Acid

This was also negative when used at eight sites for each concentration
of 5 µg, 50 µg 500 µg and 0µg (Control using saline).

Sodium Deoxycholate
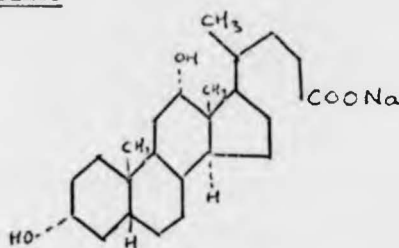
Deoxycholic acid is a secondary bile acid which has been shown to act as a colon tumour-promoter in rats (Reddy et al 1976). Studies on patients with colonic cancer have also shown high fecal levels of acids – including lithocholic acid and cholesterol metabolites (Reddy et al 1977).

TABLE 44

INJECTION OF SODIUM DEOXYCHOLATE

Concentration (W/V) %	Amount μg	Volume Injected (ml)	No of Sites With Depigmentation / Total No of Sites Injected
10	5000	)	0/8
1.0	500	) 0.05	0/8
0.1	50	)	0/8
Control (Normal Saline)	0	)	0/8

The reason for the negative results could be that while deoxycholic acid is a tumour-promoter for rat and human colon, it may not be effective for mouse skin.

Griseofulvin
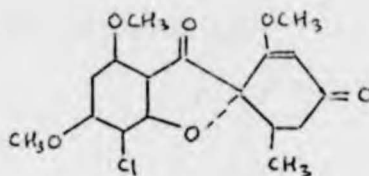
This is an antifungal agent isolated from a mould <u>Penicillium</u>

<u>griseofulvum</u>. Its tumour-promoting effect in mice was described by

Barich and co-workers in 1960 and 1962. It has also induced hepatomas

in mice when given parenterally (Epstein <u>et al</u> 1967) and orally

(Weston Hurst and Paget 1963).

Griseofulvin was dissolved in dimethylsulphoxide (DMSO) to prepare

1%, 0.1% and 0.01% (W/V) solutions for testing for depigmentation in

C57 Black male mice. The results are as shown in table 45.

<u>TABLE 45</u>

<u>DEPIGMENTATION DUE TO GRISEOFULVIN IN DMSO</u>

Concentration (W/V) %	Amount µg	Volume Injected (ml)	Proportion of Sites with Depigmentation Total No of Sites Injected
1.0	500	)	6/8
0.1	50	) 0.05	6/8
0.01	5	)	3/8
Control (DMSO alone)	0	)	0/8

The degree of depigmentation produced was slight (+) with the mean latent

period before depigmentation equal to 62 days.

1-Fluoro-2,4-dinitrobenzene (DNFB)
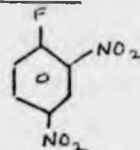
This is a vesicant and potent tumour-promoter. In skin painting experiments using 7,12-dimethyl benzanthracene as initiator, DNFB produced skin tumours in Swiss mice (Bock et al 1969)

When tested in the depigmentation test, 0.05 ml (1%) of DNFB in dimethylsulphoxide (DMSO) killed all four C57 Black male mice. The mice were injected at three sites each using 1%, 0.1% and 0.01% concentrations of DNFB. Omitting the highest concentration (i.e. 1%) resulted in survival of mice in a repeat experiment. These results are as in table 46 .

TABLE 46

DEPIGMENTATION DUE TO DNFB IN DMSO

Concentration (W/V) %	Amount µg	Volume Injected (ml)	Proportion of Sites with Depigmentation / Total No of Sites Injected
0.1	50	)	1/8
0.01	5	)	3/8
0.001	0.5	) 0.05	0/8
Control (DMSO alone)	0	)	0/8

The optimal concentration was 0.01% producing slight depigmentation (+) in three out of eight sites. The proportion of positive sites was reduced at higher and lower concentrations. This optimal dose effect was described on page 90. The mean latent period for depigmentation was fifty-six days.

Phenobarbitone
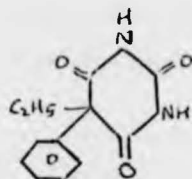
This is a long-acting barbiturate with hypnotic and sedative proper-
ties and is used pharmacologically in the management of epilepsy. It
causes enzyme induction in the mouse liver (Conney 1967) and has been
used to increase enzyme yield in the preparation of S9 mix (rat liver
homogenate microsomal mix) for the Ames test (see page 33). It causes
liver tumours in mice (Walker, Thorpe and Stevenson 1973) but was not
shown to be hepatocarcinogenic in epidemiological studies on man
(Clemmensen,Frederiksen and Plum 1974). The repeated administration of
phenobarbitone sodium after treatment with 2-acetylaminofluorene (AAF)
increased liver tumours in rats but not when the two chemicals were
administered simultaneously (Periano, Fry and Staffeldt 1971; Periano
et al 1973; Weisburger,Grantham and Weisburger 1963). Phenobarbitone,
therefore, has a tumour-promoting effect but is not co-carcinogenic.

Phenobarbitone was tested for its ability to induce depigmentation in
C57 Black male mice. Solutions of phenobarbitone were prepared for
testing by dissolving 100 mg phenobarbitone in 0.05 m N sodium
hydroxide and diluting with distilled water.

Concentrations of 20% (W/V), 2%, 0.2% and 0% (control) killed four
mice injected at four sites with 0.05 ml of each of these concen-
trations. A repeat experiment using lower concentrations of 10%, 1%,
0.1% and 0% (control) killed four out of six test mice. The two
surviving mice did not show any depigmentation. A further repeat
experiment using 0.1% and 0.01% concentrations at two sites per mouse

produced no depigmentation in eight mice injected. Table 47
summarizes the results obtained.

<u>TABLE 47</u>

<u>INJECTION OF PHENOBARBITONE IN DISTILLED WATER</u>

Concentration (W/V) %	Amount μg	Volume Injected (ml)	Proportion of Sites with Depigmentation Total No of Sites Injected
10	5000	)	0/2
1.0	500	)	0/2
0.1	50	) 0.05	0/8
0.01	5	)	0/8
Control (Distilled Water)	0	)	0/10

It is possible that the effective dose for producing depigmentation
is higher than the lethal dose for phenobarbitone in C57 Black male
mice.

(f)   Non-Carcinogens and Non-Tumour-Promoters

Twenty-three compounds with no evidence of carcinogenic nor tumour-promoting activity were tested. Some of these were compounds with published evidence of a lack of carcinogenic activity e.g. phenanthrene and pyrene. Others have not previously been tested for carcinogenic or tumour-promoting activity and therefore have no published evidence of such activity. These compounds included those with chemical structures similar to known carcinogens. These have been dealt with earlier such as two aromatic hydrocarbons (phenanthrene and pyrene) and two carbamates (methyl carbamate and diethyl dithiocarbamate). Others were commonly used solvents such as dimethyl sulphoxide (DMSO), dimethyl acetamide (DMAC), acetone and ethanol. Hydrochloric acid and sodium hydroxide were selected as examples of an acid and alkali. Saline and arachis oil were also tested. In addition, 1,1,1, trichloroethane was chosen as a compound tested for carcinogenicity and found to be inactive (National Cancer Institute Report 1977). Dihydroxy phenylalanine (DOPA) was included as an amino-acid essential for melanin formation. Pyrogallol was selected as a co-carcinogen without tumour-promoting properties (Van Duuren 1976).

Nineteen of these selected compounds produced no depigmentation when tested in different concentrations. Table 48 summarizes the tests on these compounds. Three compounds i.e. 4-tertiary butyl catechol, p-tertiary butyl phenol and pyrocatechol produced obvious depigmentation (+ +). Butylated hydroxyanisole produced slight depigmentation (+). These are dealt with in the section on further experiments (see page 175). All three compounds are chemicals known to cause occupational leukoderma.

TABLE 48

NON-CARCINOGENS PRODUCING NO DEPIGMENTATION

No	Compound	Solvent/ Medium	Concentration Tested (W/V) %	Amount µg	Volume Injected (ml)	No of Sites Injected	No of Mice
1.	Phenanthrene	DMSO	10 1.0 0.1 Control (DMSO alone)	5,000 500 50 0	))) 0.05)	4 4 4 4	))) 4)
2.	Pyrene	DMSO	10 1.0 0.1 Control (DMSO alone)	5,000 500 50 0	))) 0.05)	4 4 4 4	))) 4)
3.	Methyl Carbamate CH_3COONH_2	DMSO	5 0.5 0.05 0.005 Control (DMSO alone)	2,500 250 25 2.5 0	))) 0.05))	8 8 8 8 16	))) 16))

TABLE 48 (Continued)
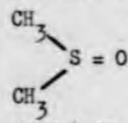
No	Compound	Solvent/ Medium	Concentration Tested (W/V) %	Amount µg	Volume Injected (ml)	No of Sites	No of Mice
4.	Sodium Diethyldithio-carbamate $(C_2H_5)_2N\ CS_2Na$	DMSO	1.0 0.1 0.01 Control (DMSO alone)	500 50 5 0	)) 0.05))	8 8 8 8	))) 8)
5.	Dimethyl Sulphoxide CH_3 $S = 0$ CH_3 $(C_2H_3)_2SO$	Distilled Water	100 50 25 10 Control (Distilled Water)	55,000 27,500 13,750 5,500 0	))) 0.05))	32 4 4 4 4	8)) 4))
6.	Dimethyl Acetamide $CH_3CON(CH_3)_2$	Distilled Water	100 50 25 10 1 Control (Distilled Water	47,000 23,500 11,750 4,700 470 0	)))))) 0.05))))	32 12 4 4 4 4	8 (All dead within 24 Hours) 3 (Death in 2 out of 3 mice))) 4)

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TABLE 48 (Continued)

No	Compound	Solvent/Medium	Concentration Tested (W/V) %	Amount μg	Volume Injected (ml)	No of Sites	No of Mice
7.	Acetone $CH_3CO\ CH_3$	Distilled Water	100	39,500	))	12	3 (Death in 2 mice)
			50	19,750	)) 0.05	4	)
			25	9,875	)	4	) 4
			10	3,950	)	4	)
			Control (Distilled Water	0	))	4	)
8.	Saline NaCl	Distilled Water	2N Saline N 0.5N 0.25N	850 425 213 106	)) 0.05))	4 4 4 4	)) 4))
9.	Arachis Oil	—	100	45,500	0.1 0.05 0.025	16 16 16	)) 4)
10.	Hydrochloric Acid HCl	Distilled Water	2M M 0.5M 0.25M	365 183 91 46	)) 0.05))	4 4 4 4	)) 4)
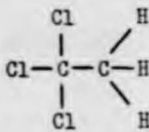
TABLE 48 (Continued)

No	Compound	Solvent/ Medium	Concentration Tested (W/V) %	Amount μg	Volume Injected (ml)	No of Sites	No of Mice
11.	Sodium Hydroxide NaOH	Distilled Water	2M M 0.5M 0.25M	400 200 100 50	))) 0.05)	4 4 4 4	))) 4)
12.	1,1,1, Trichloroethane Cl—C—C—H structure	DMSO	1.0 0.1 0.02 Control (DMSO alone)	670 67 13 0	)) 0.05))	6 6 6 6	)) 6))
13.	Ethanol C_2H_5OH	Distilled Water	100 50 25 10	39,500 19,750 9,875 3,950	)) 0.05))	4 4 4 4	)) 4))
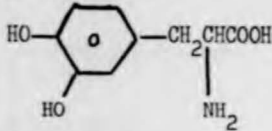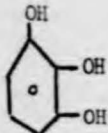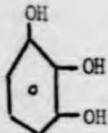
TABLE 48 (Continued)

No	Compound	Solvent/ Medium	Concentration Tested (W/V) %	Amount μg	Volume Injected (ml)	No of Sites	No of Mice
14.	3-(3,4-Dihydroxy Phenyl Alanine), DOPA HO—⬡—CH$_2$CHCOOH HO NH$_2$	Saline	0.6 0.06 0.006 Control	300 30 3 0	))) 0.05))	4 4 4 4	))) 4))
15.	Pyrogallol OH ⬡ OH OH	DMSO	10 1 0.1 0.01	5,000 500 50 5	))) 0.05)	4 4 4 4	))) 4)
16.	Methyl Catechol	DMSO	1.0 0.1 0.01 Control	500 50 5 0	))) 0.05)	8 8 8 8	))) 8)

TABLE 48 (Continued)

No	. Compound	Solvent/ Medium	Concentration Tested (W/V) %	Amount µg	Volume Injected (ml)	No of Sites	No of Mice
17.	Putrescine	Saline	10.0 1.0 0.1 0.01	5,000 500 50 5	))) 0.05)	8 8 8 8	))) 8)
18.	Adrenaline Hydrogen Tartrate	Saline	0.01 0.001	5 0.5	)) 0.05	8 8	)) 8
19.	Histamine Hydrochloride	Saline	1.0 0.1 0.01	500 50 5	)) 0.05)	16 16 16	)) 12)

The maximum concentration of methyl carbamate used was 5% (i.e. 2,500 ug per injection site. With diethyl dithiocarbamate the maximum concentration used was 1% because it is a chelating agent and may be lethal to the mice.

100% dimethylacetamide injected at four sites in 0.05 ml volumes per site killed eight out of eight mice injected (four sites per mouse). This is equivalent to a total dose of 7.5 g/kg dimethylacetamide per mouse which is about twice the LD_{50} for rats. Using a 50% solution injected again in 0.05 ml volumes at four sites, two out of three mice died (equivalent dose of 3.75 g/kg). With 25% solutions and more dilute solutions, no death resulted.

In comparison 100% dimethyl sulphoxide was not lethal when given at a total volume of 0.2 ml per mouse (i.e. 0.05 ml at four sites). 100% acetone given similarly killed two out of three mice. At 50% and lower concentrations acetone did not result in any deaths of the mice.

Physiological saline (N saline = 0.85% NaCL in distilled water) produced no depigmentation. Neither did 2N, 0.5N and 0.25N saline. However, Boyland and Sargent in 1951 produced depigmentation in stock mice by using hypertonic saline. Depigmentation was also produced by sterile distilled water in one out of four C57 mice injected intra-dermally. This observation was explained by greying resulting from cellular changes as a result of exposure to hypertonic and hypotonic solutions. It is interesting that Tatematsu et al (1975) in their studies on experimental gastric cancers in rats, mentioned sodium chloride as a possible promoter and this was not supported by their data obtained.

Arachis oil was not diluted, but injected intradermally in different
volumes i.e. 0.1, 0.05 and 0.025 ml. No depigmentation resulted.
There was practical difficulty in injecting intradermally accurate
volumes of arachis oil in the small amounts needed.

Pyrogallol was tested as there is no evidence of a carcinogenic effect
nor tumour-promoting activity but has been shown to be co-carcinogenic
(Van Duuren 1976) It also produced no depigmentation in the con-
centrations tested.

Four compounds which are not known carcinogens nor tumour-promoters
produced positive depigmentation.

These were:-

1. Pyrocatechol

2. 4-Tertiary butyl catechol

3. p-Tertiary butyl phenol

4. Butylated hydroxyanisole

Further details on these are discussed on page 175. All four are sub-
stances known to cause occupational leukoderma. This may be another
group of compounds in addition to the tumour-promoters which will
produce the depigmentation effect. If so, it will reduce the specif-
icity of the test for tumour-promoters. However, these compounds have
not been tested for tumour-promoting activity. It may be possible
that, if tested, they may show complete carcinogenic or tumour-
promoting activity.

CHAPTER 4   RESULTS (Continued)

Follow-up experiments

1.   Is the relationship valid for
     chemicals in solid form?

2.   Is the relationship affected
     by retinoids?

3.   Would depigmentation appear
     with the injection of putrescine?

4.   Would depigmentation appear with
     chemicals which are known to cause
     occupational leukoderma?

5.   Is the relationship valid for tumour-
     promoters in gaseous form?

6.   Does the effect apply to co-
     carcinogens?

4. FOLLOW-UP EXPERIMENTS

From the results obtained of tests performed on the chemicals
selected, a link between tumour-promotion and depigmentation is
indicated. Several questions may be posed regarding this observ-
ation and the following experiments have been designed to answer
them.

I Is the relationship valid for chemicals in solid form?

Asbestos exists in the form of insoluble solid fibres and in this
physical state, exposure is linked to an increased risk of
bronchogenic carcinoma (Doll 1955, Glynne 1935) and mesothelioma
(Selikoff et al 1965, Mancuso and Coulter 1965) in exposed workers.
Animal studies have also shown it to be carcinogenic (Reeves et al
1971, Wagner and Berry 1969) but little is known about the exact
mechanism of carcinogenic action. It is possible that asbestos
fibres act as tumour-promoters. Some support for this is provided
by the fact that malignancies in asbestos workers develop twenty
to thirty years after repeated exposure to asbestos and some
asbestos workers do not develop malignancies in spite of exposure.
It may be that this group of workers are exposed only to the tumour-
promoting action of asbestos without previous exposure to an appro-
priate initiator. Tobacco smoke could be a source of initiation
and this could explain the increased risk of lung carcinoma amongst
asbestos workers who smoke compared with those who do not (Doll
1971, Berry, Newhouse and Turok 1972).

Experiment I

Comparison of different types of asbestos fibres

Asbestos fibres are complex silicates and are classified essentially
on the basis of physical characteristics into two groups:-

(a)   The Serpentines - Chrysotile (white asbestos) is the most
      abundant form. Its fibres are coil-like and springy in texture.

(b)   The Amphiboles - which include crocidolite (blue asbestos), amosite
      (brown asbestos), anthophyllite, actinolite and tremolite. These
      fibres are short, sharp and rigid with pointed ends.

In view of their possible tumour-promoting activity, the different types
of asbestos fibres were compared for ability to produce depigmentation.
Suspensions of different asbestos fibres in normal saline were injected
intradermally into C57 Black mice using a similar procedure to that
for chemicals in solution (see Methodology, page 46).

TABLE 49

EFFECTS OF DIFFERENT ASBESTOS FIBRES

No	Type of Asbestos	Injection Volume (ml)	Fibre Wt (mg)	No of Sites	Depigmentation:- No of Positive Sites
1.	Crocidolite	0.05	2.5	12	0
2.	Chrysotile A	0.05	2.5	12	0
3.	Chrysotile B	0.05	2.5	12	0
4.	Anthophyllite	0.05	2.5	12	0
5.	Amosite	0.05	2.5	12	0

No depigmentation resulted from injection of the five types of asbestos
fibres shown above.

Experiment II

Comparison of ground and unground fibres

The difficulty in producing even suspensions of some asbestos fibres
decreased the accuracy of the amount of fibres injected. The exper-
ment with chrysotile A was therefore repeated using ground chrysotile
A fibres to form an even suspension containing 3.3% chrysotile A. The
results are as shown in Table 50 with no depigmentation noted even after
thirty weeks observation.

TABLE 50

COMPARISON OF GROUND AND UNGROUND CHRYSOTILE A FIBRES

No	Type of Asbestos	Injection Volume (ml)	Fibre Wt (mg)	No of Sites	Depigmentation:- No of Positive Sites
1.	Chrysotile A	0.05	2.5	12	0
2.	Chrysotile A (ground)	0.05	1.7	12	0

Neither coarse unground chrysotile A fibres nor finely ground chrysotile
A fibres in saline suspensions produced depigmentation. It has been
suggested that the carcinogenic activity of asbestos fibres is related
to the physical characteristics of the fibres (Timbrell 1973). Grinding
up the fibres allows the preparation of an even suspension for injection
and increases the accuracy of the actual amount by weight of asbestos
injected. However, the process of grinding may alter the physical
characteristics of the fibres (Stanton and Wrench 1972) and this may
alter the carcinogenic potential and the potential to produce depigment-
ation, if present.

Experiment III

Pretreatment with Urethane

Urethane (ethyl carbamate) has been used as an initiator in two-stage carcinogenesis (Salaman and Roe 1953). It was used to pretreat C57 Black mice before intradermal injections of asbestos fibres were made. Urethane was given at a dose of 1 gm/kg body weight in distilled water to make a 10% solution and injected intraperitoneally on the first, second and fourth day of the experiment. Suspensions of ground asbestos fibres in saline were injected intradermally on the fourth day of the experiment. This allowed sufficient time for the urethane to exert its initiating effects on the mouse skin before the asbestos fibres were injected.

TABLE 51

THE EFFECTS OF PRE-TREATMENT WITH URETHANE

No	Total Dose of Urethane mg/Mouse	Type of Asbestos	Injection Volume (ml)	Fibre Wt (mg)	Total No of Sites	Depigmentation	
						No of Positive Sites	Latent Period
1.	0	Chrysotile A (Ground)	0.1	2.5	32	0	-
2.	75	Chrysotile A (Ground)	0.1	2.5	48	0	-
3.	0	Crocidolite (Ground)	0.05	2.5	32	2	)
							) 16
4.	75	Crocidolite (Ground)	0.05	2.5	48	9	) Weeks

Chrysotile A (ground) as a 5% suspension was too concentrated for injection. A 2.5% suspension was used at double the injection volume i.e. 0.1 ml to introduce 2.5 mg of fibre at each site.

The results show that no depigmentation occurred when chrysotile A was used, whether or not the mice were pretreated with urethane. However, in mice pretreated with urethane and injected with ground crocidolite, slight depigmentation was seen in nine out of forty-eight sites. Similar depigmentation was noted in two out of thirty-two sites in mice not pretreated and injected with ground crocidolite. The degree of depigmentation was minimal and the latent period before its appearance prolonged when compared with chemicals causing depigmentation e.g. TPA and nitrogen mustard. The significance of this slight depigmentation is not clear. As depigmentation appears related to tumour-promoting activity, this suggests that ground crocidolite (blue asbestos) acts as a tumour-promoter. However, unground crocidolite when tested at twelve sites was negative. It is possible that the change in physical characteristics of the fibres as a result of grinding may affect its carcinogenic potential by increasing tumour-promoting activity. This can be confirmed by following up the urethane pretreated mice injected with ground crocidolite.

The injected mice were followed up for more than fifty weeks, to see whether any skin tumours developed at the site of injection. The mice pretreated with urethane have initiating action of the mouse skin. The asbestos fibres are retained at the site of injection and a constant contact with the area of skin concerned should provide constant tumour-promoting action if asbestos is a tumour-promoter. The situation is akin to the two-stage carcinogenesis experiments on mouse skin and local tumours would be expected. However, no skin tumours were noted

and the possible explanation is that asbestos is a weak tumour-
promoter or that the dose of urethane was ineffective as initiator.

II Is the relationship affected by retinoids?

Vitamin A and its natural and synthetic analogues prevent chemical
carcinogenesis (Becci et al 1978). The absence of these retinoids
has led to squamous metaplasia in trachea and bronchial epithelium
(Harris, Sporn and Kaufman 1972, Wolbach and Howe 1925). Hypoplastic
and anaplastic changes induced by carcinogens in cultured prostate
gland tissue have been reversed by retinoids (Chopra and Wilkoff 1975).
If retinoids can prevent chemical carcinogenesis, it is possible that
other effects relevant to carcinogenesis may be inhibited e.g. tumour-
promotion and its induced depigmentation in C57 Black mice.

Experiment I
Cis-retinoic acid diet and its effect on chemically-induced
depigmentation

The addition of cis-retinoic acid to the diet of C57 Black mice was
investigated to see whether it would inhibit the development of depig-
mentation due to croton oil and TPA and para-tertiary butyl phenol
(PTBP) which causes occupational leukoderma but whose tumour-promoting
activity has not been investigated. Groups of male C57 Black mice were
allocated to one of six treatment procedures as shown in Table 52.

TABLE 52

ALLOCATION OF DIET AND NUMBERS OF MICE USED

Diet	Substances for Intra-dermal Injections		
	Croton Oil	TPA	P.T.B.P.
(a) Cis-retinoic acid diet	8	8	8
(b) Placebo diet	8	8	8

The mice were fed on the standard diet until the start of the experiment when this was replaced by the cis-retinoic acid (240 mg cis-retinoic acid in 1 kg of diet) or the placebo diet. The new diet was continued for a week before intradermal injections were performed on all the mice. Different dilutions of either croton oil. TPA or PTBP dissolved in methyl sulphoxide were injected at four sites on the ventral surface of each mouse. One of the four sites was injected with dimethylsulphoxide alone as control. The mice were then examined twice weekly for ten weeks for any macroscopic changes at the injection sites without the assessor knowing from which cage the animals had come. The cis-retinoic acid diet and the placebo diet were discontinued four weeks after the injections were performed and the animals were given the original standard diet. Previous experiments had shown that depigmentation due to such injections usually occurred within four weeks after injection (Aw and Boyland 1978). Hence, the different diets would be able to exert their effect, if any, during the latent period before depigmentation appeared.

TABLE 53

PROPORTION OF SITES OF DEPIGMENTATION AFTER TEN WEEKS

FOLLOWING INTRADERMAL INJECTIONS

Substance Injected	Amount/Conc	Proportion of Sites with Depigmentation	
		Cis-Retinoic Acid Diet	Placebo Diet
TPA	0.008 µg	1/8	0/8
	0.04 µg	3/8	5/8
	0.2 µg	7/8	7/8
	1.0 µg	8/8	8/8
Croton Oil	Control	0/8	0/7
	0.02%	1/8	1/7
	0.1%	4/8	3/7
	0.5%	7/8	7/7
PTBP	Control	0/3	1/4
	0.2%	0/3	2/4
	1.0%	1/3	3/4
	5.0%	0/3	2/4

TABLE 54

SHORTEST LATENT PERIOD BEFORE APPEARANCE OF DEPIGMENTATION

Substance Injected	Shortest Latent Period	
	Cis-Retinoic Acid Diet	Placebo Diet
TPA	18 days	21 days
Croton Oil	28 days	28 days
PTBP	28 days	39 days

Nine mice injected with PTBP and one mouse injected with croton oil died within one day of injection.

There was no difference in the proportion of positive depigmentation noted in mice fed with cis-retinoic acid diet compared with the placebo diet when croton oil or TPA was injected. There was also no significant difference in the length of the minimum latent period before depigmentation occurred. Reservations about the significance of differences in latent periods before depigmentation have been discussed (see page 88).

Cis-retinoic acid inhibited the appearance of depigmentation by PTBP. Only one site showed depigmentation out of twelve sites in three surviving mice injected with PTBP and fed on the cis-retinoic acid diet. For the placebo diet eight sites showed positive depigmentation out of sixteen sites injected. Statistical analysis using the method of Cox for combining information from several two by two contingency tables showed this difference to be statistically significant ($p < 0.05$, two-tailed test).

Discussion

Cis-retinoic acid given as described does not inhibit depigmentation induced by the known tumour-promoters, croton oil or TPA. Depigmentation due to PTBP was inhibited by cis-retinoic acid. It would be of interest and may be of practical use to see whether retinoids may be effective in inhibiting other substances known to cause occupational leukoderma (e.g. other alkyl phenols and catechols).

III Would depigmentation appear with the intradermal injection

    of putrescine?

Putrescine is a product of ornithine decarboxylase (ODC) action and
ODC is increased by tumour-promoters. Hence, putrescine levels
could be increased by tumour-promoters which might exert their depig-
menting effect by means of increased putrescine levels. This hypothesis
was tested by injecting putrescine intradermally into C57 Black mice.

Experiment I

Intradermal injection of putrescine

A 10% solution of putrescine was first prepared by reacting 1 gm of
putrescine with 10 ml of 2N HCl to neutralize it as putrescine is
strongly alkaline. Some of this solution was then diluted with saline
to produce a 1% 0.1% and 0.01% solution respectively. Intradermal
injections of 0.05 ml of these solutions produced the following
results.

TABLE 55

INJECTION OF PUTRESCINE IN SALINE

Concentration (W/V)	Amount µg	Volume Injected (ml)	No of Sites Injected	No of Sites Showing Positive Depigmentation
10%	5000	0.05	8	0
1%	500	0.05	8	0
0.1%	50	0.05	8	0
0.01%	5	0.05	8	0

Putrescine in the concentrations used produced no depigmentation. Hence raised putrescine levels are unlikely to be the mechanism for depigmentation caused by tumour-promoters in C57 Black mice.

IV   Would depigmentation occur with chemicals which are known to cause occupational leukoderma?

Occupational leukoderma or vitiligo occurs in workers exposed to chemicals causing depigmented patches on the skin.   This usually occurs following contact with the chemicals although similar effects have been described following inhalation (Chumakov, Babanov and Smirnov 1962; Ito, Nishitani and Hara 1968).   Oliver, Schwarz and Warren (1939) described leukoderma in tannery workers caused by hydroquinone monobenzyl ether. This is an antioxidant and was present in the rubber gloves of these workers.

Para-tertiary butyl phenol (PTBP) is present in resin adhesives used in the boot and shoe industry.   Malten et al (1971) described leukoderma due to this compound in plant operatives and Calnan and Cooke (1974) noted similar cases in automobile workers using a neoprene adhesive containing PTBP.   4-tertiary butyl catechol (TBC) is an anticorrosive agent which caused leukoderma in tappet assembly workers (Gellin,Possick and Perone 1970).   Other phenols and catechols have been known to cause occupational leukoderma (Hara and Nakajima 1969; Horio, Tanaka and Komura 1977). ·Studies have also shown that such chemicals can cause depigmentation in laboratory animals (Riley 1969 and 1971).

Solutions of the following catechols and phenols were prepared in dimethyl sulphoxide (DMSO) and tested in C57 Black male mice by the procedure outlined on page 45.

TABLE 56

DEPIGMENTATION DUE TO CATECHOL (PYROCATECHOL) IN DMSO

Concentration (W/V) %	Amount µg	Volume Injected (ml)	No of Sites with Positive Depigmentation / Total No of Sites Injected
0.3	167	)	7/8
0.03	16.7	)) 0.05	5/8
0.003	1.67	)	3/8
Control (DMSO alone)	0	)	0/8

TABLE 57

DEPIGMENTATION DUE TO 4-TERTIARY BUTYL CATECHOL IN DMSO

Concentration (W/V) %	Amount µg	Volume Injected (ml)	No of Sites with Positive Depigmentation / Total No of Sites Injected
0.75	375	)	8/8
0.075	37.5	)) 0.05	2/8
0.0075	3.75	)	1/8
Control (DMSO alone)	0	)	0/8

TABLE 58

INJECTION OF METHYL CATECHOL IN DMSO

Concentration (W/V) %	Amount μg	Volume Injected (ml)	No of Sites with Positive Depigmentation / Total No of Sites Injected
1.0	500	)	0/8
0.1	50	) 0.05	0/8
0.01	5	)	0/8
Control (DMSO alone)	0	)	0/8

TABLE 59

DEPIGMENTATION DUE TO P-TERTIARY BUTYL PHENOL IN DMSO

Concentration (W/V) %	Amount μg	Volume Injected (ml)	No of Sites with Positive Depigmentation / Total No of Sites Injected
10.0	5000	)	2/5
1.0	500	) 0.05	3/5
0.1	50	)	0/5
Control (DMSO alone)	0	)	0/5

TABLE 60

DEPIGMENTATION DUE TO BUTYLATED HYDROXYANISOLE (BHT) DMSO

Concentration (W/V) %	Amount μg	Volume Injected (ml)	No of Sites with Positive Depigmentation Total No of Sites Injected
1.0	500	))	7/8
0.1	50	)) 0.05	6/8
0.01	5	))	6/8
Control (DMSO alone)	0	)	0/8

TABLE 61

DEPIGMENTATION DUE TO HYDROQUINONE MONOBENZYL ETHER IN DMSO

Concentration (W/V) %	Amount μg	Volume Injected (ml)	No of Sites with Positive Depigmentation Total No of Sites Injected
10.0	5000	))	3/8
1.0	500	)) 0.05	1/8
0.1	50	))	1/8
Control (DMSO alone)	0	)	0/8

TABLE 62

INJECTION OF 8-HYDROXY QUINOLINE IN DMSO

Concentration (W/V) %	Amount µg	Volume Injected (ml)	No of Sites with Positive Depigmentation / Total No of Sites Injected
1.0	500	)	0/8
0.1	50	)) 0.05	0/8
0.01	5	)	0/8
Control (DMSO alone)	0	)	0/8

Five out of the seven compounds tested produced depigmentation. The degree of depigmentation is summarized in table 63.

TABLE 63

DEPIGMENTATION DUE TO CATECHOLS AND PHENOLS

No	Compound	Degree of Depigmentation
1.	Catechol	+ +
2.	4-Tertiary butyl catechol (TBC)	+ +
3.	p-Tertiary butyl phenol (PTBP)	+ +
4.	Butylated hydroxy-anisole (BHA)	+
5.	Hydroquinone monobenzyl ether	+
6.	8-Hydroxy quinoline	-
7.	Methyl catechol	-

Only 8-hydroxyquinoline and methyl catechol were negative. Searle
(1972) produced depigmentation in C57 Black mice by topical applic-
ation of 8-hydroxyquinoline. Single applications resulted in isolated
bands of depigmented hair. However, the effect was seen in female
mice but not in male mice; the above results were obtained with male
C57 Black mice. 8-hydroxyquinoline is a chelating agent and it has
been shown (Allen et al 1957) to increase cancer of the urinary bladder
in mice. Pliss and Volfson (1970) tested it in both rats and mice
and concluded that it was a weak carcinogen. It may be an indirect
carcinogen and only active after metabolic activation. Hence, the
negative result in the depigmentation test. There is no evidence
that methyl catechol is carcinogenic.

Catechol was shown by Van Duuren et al (1973) to increase the carcin-
genic effects of benzpyrene on mouse skin. They concluded that
catechol was co-carcinogenic but not a tumour-promoter. By producing
positive results in the depigmentation test, this reduced the specif-
icity of the test for tumour-promoters.

Hydroquinone alone was tested in bladder implantation experiments in
mice (Boyland et al 1964). Using cholesterol pellets and
hydroquinone increased the incidence of bladder tumours. However,
skin-painting experiments in mice using hydroquinone and croton oil
showed that hydroquinone was inactive as an initiator of skin
carcinogenesis (Roe and Salaman 1955). It may act as a tumour-promoter
and/or co-carcinogen rather than as an initiator. Van Duuren (1976)
however found hydroquinone to have neither tumour-promoter nor co-
carcinogenic properties. It is possible that hydroquinone has no
carcinogenic effects nor tumour-promoting carcinogenic activity on the
skin of mice but is a carcinogen to mouse bladder.

Only 8-hydroxyquinoline and methyl catechol were negative. Searle (1972) produced depigmentation in C57 Black mice by topical application of 8-hydroxyquinoline. Single applications resulted in isolated bands of depigmented hair. However, the effect was seen in female mice but not in male mice; the above results were obtained with _male_ C57 Black mice. 8-hydroxyquinoline is a chelating agent and it has been shown (Allen _et al_ 1957) to increase cancer of the urinary bladder in mice. Pliss and Volfson (1970) tested it in both rats and mice and concluded that it was a weak carcinogen. It may be an indirect carcinogen and only active after metabolic activation. Hence, the negative result in the depigmentation test. There is no evidence that methyl catechol is carcinogenic.

Catechol was shown by Van Duuren _et al_ (1973) to increase the carcinogenic effects of benzpyrene on mouse skin. They concluded that catechol was co-carcinogenic but not a tumour-promoter. By producing positive results in the depigmentation test, this reduced the specificity of the test for tumour-promoters.

Hydroquinone alone was tested in bladder implantation experiments in mice (Boyland _et al_ 1964). Using cholesterol pellets and hydroquinone increased the incidence of bladder tumours. However, skin-painting experiments in mice using hydroquinone and croton oil showed that hydroquinone was inactive as an initiator of skin carcinogenesis (Roe and Salaman 1955). It may act as a tumour-promoter and/or co-carcinogen rather than as an initiator. Van Duuren (1976) however found hydroquinone to have neither tumour-promoter nor co-carcinogenic properties. It is possible that hydroquinone has no carcinogenic effects nor tumour-promoting carcinogenic activity on the skin of mice but is a carcinogen to mouse bladder.

Acheson et al (1970) found a high incidence of nasal tumours in the boot and shoe industry. The carcinogen concerned could possibly be PTBP and, if so, then the presence of tumour-promoting activity would fit in well with the positive depigmentation obtained. However, Boutwell and Bosch (1959) tested PTBP on mouse skin and found it not to be a tumour-promoter.

There is no evidence that 4-tertiary butyl catechol and butylated hydroxy-anisole are carcinogenic. However, because of the similarity in chemical structure to hydroquinone, hydroquinone monobenzyl ether and catechol, it is possible that they may be carcinogenic or possess co-carcinogenic and/or tumour-promoting activity. Butylated hydroxy-anisole has been shown to cause epidermal micro-invasion similar to early epithelial tumour of natural or chemically-induced origin (Riley and Seal 1974).

TABLE 64

CHEMICAL FORMULAE OF AGENTS CAUSING OCCUPATIONAL LEUKODERMA
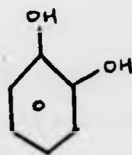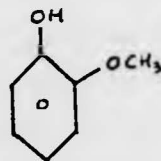
| Catechol | Methyl catechol (O-hydroxy-anisole) | Butylated hydroxy-anisole |
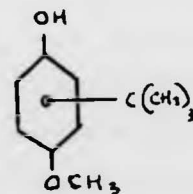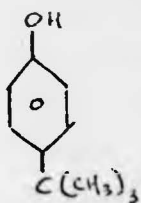
p-tertiary
butyl phenol
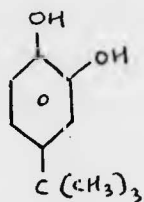
4-tertiary butyl
catechol

Hydroquinone
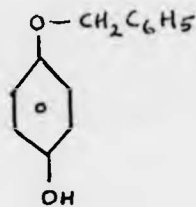
Hydroquinone
monobenzyl ether

Thus, these compounds may produce depigmentation because of possible
tumour-promoting activity. It is more likely that depigmentation as
induced by single intradermal injections of chemicals into C57 Black
male mice is not confined to tumour-promoters alone. Other groups of
compounds may also produce positive depigmentation by a similar melano-
toxic action or by some other mechanism. Agents that cause occupational
leukoderma would be one such group of compounds. Boyland and Sargent
(1951) suggested that compounds that liberate free radicals and hyper-
or hypo-tonic solutions are two other groups of chemicals which may
produce a similar depigmentation effect.

Free radicals are capable of direct combination with cell constituents such as nucleic acid. This is a feature akin to carcinogens - which may have tumour-promoting activity as in complete carcinogens. Also hypertonic solutions have been shown to enhance tumours in rats (Tatematsu 1975). Hence, there is still the possibility of tumour-promoting activity being the common link between chemicals and depigmentation in C57 Black male mice.

V Is the relationship valid for tumour-promoters in gaseous form?

In inhalation studies on rats, Laskin et al (1970) showed that initial exposure to benzo(a)pyrene followed by repeated exposure to sulphur dioxide (SO_2) resulted in squamous cell carcinomas in five out of twenty-one rats. The evidence suggests that SO_2 acts as a tumour-promoter.

In order to test SO_2 for its ability to induce depigmentation in C57 Black mice, solutions of sodium metabisulphite ($Na_2O_5S_2$) were used. This produces sulphurous acid ($SO_2 + H_2O \longrightarrow H_2SO_3$) in solution.

<div align="center">

sulphur   sulphurous
dioxide   acid

</div>

TABLE 65

INJECTION OF SODIUM METABISULPHITE IN DISTILLED WATER

Concentration (W/V) %	Amount μg	Volume Injected (ml)	Proportion of Sites with Positive Depigmentation / Total No of Sites Injected
10	5000	)	0/6
1.0	500	) 0.05	0/6
0.1	50	)	0/6
Control	0	)	0/6

No depigmentation occurred in the concentrations tested. This suggests that the relationship may not be valid for tumour-promoters in gaseous form. A total of eight mice were injected altogether. Two died following formation of abscesses at the injection site where 10% sodium metabisulphite was injected.

VI Does the effect apply to co-carcinogens?

Co-carcinogens increase tumour-yield when regularly applied simult-aneously with initiators. They are closely related to tumour-promoters and some compounds have both tumour-promoting and carcinogenic activity. There is probably also a difference in the mode of action since not all co-carcinogens are tumour-promoters and vice-versa. Table 66 (modified from Van Duuren 1976 and incorporating some of the depigment-ation test results) compares tumour-promoting and co-carcinogenic activities of some tested compounds.

TABLE 66

COMPARISON OF TUMOUR-PROMOTING AND CO-CARCINOGENIC ACTIVITIES

No	Compound	Tumour-Promoting Activity	Co-Carcinogenic Activity	Depigmentation
1.	TPA	+	+	+
2.	Anthralin	+	+	-
3.	N-dodecane	+	+	+
4.	Phenol	+	-	+
5.	Catechol	-	+	+
6.	Hydroquinone	-	-	+
7.	Pyrogallol	-	+	-
8.	Pyrene	-	+	-
9.	Benzo(a)Pyrene	-	+	-

For the above nine compounds:-

(a) Three of the four tumour-promoters showed positive depigmentation, therefore, sensitivity for tumour-promoters = 75%.

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6.	Hydroquinone	-	-	+
7.	Pyrogallol	-	+	-
8.	Pyrene	-	+	-
9.	Benzo(a)Pyrene	-	+	-

For the above nine compounds:-

(a) Three of the four tumour-promoters showed positive depigmentation,
      therefore, sensitivity for tumour-promoters = 75%.

Two of the five non-tumour-promoters showed positive depigmenta-
tion, therefore specificity for tumour-promoters = 60%

(b) Three of the seven co-carcinogens showed positive depigmentation,
therefore, sensitivity for co-carcinogens = 43%.

Two out of two non-co-carcinogens showed positive depigmentation,
therefore specificity for co-carcinogens = 0%

Using the above list of compounds which have been specifically tested
for tumour-promoting and co-carcinogenic activity and which were tested
for depigmentation, the depigmentation effect is more specific and
sensitive for tumour-promoting activity than for co-carcinogenic act-
ivity.

CHAPTER 4   RESULTS (Continued)

Microscopic features

Microscopy of hair

Microscopy of skin

5. <u>MICROSCOPIC FEATURES</u>

The skin of mammals consists of epidermis and with hairs arising
from hair bulbs in the dermis.   Fig 11 shows a diagrammatic
representation.

<u>FIG 11</u>

<u>MAMMALIAN SKIN</u>
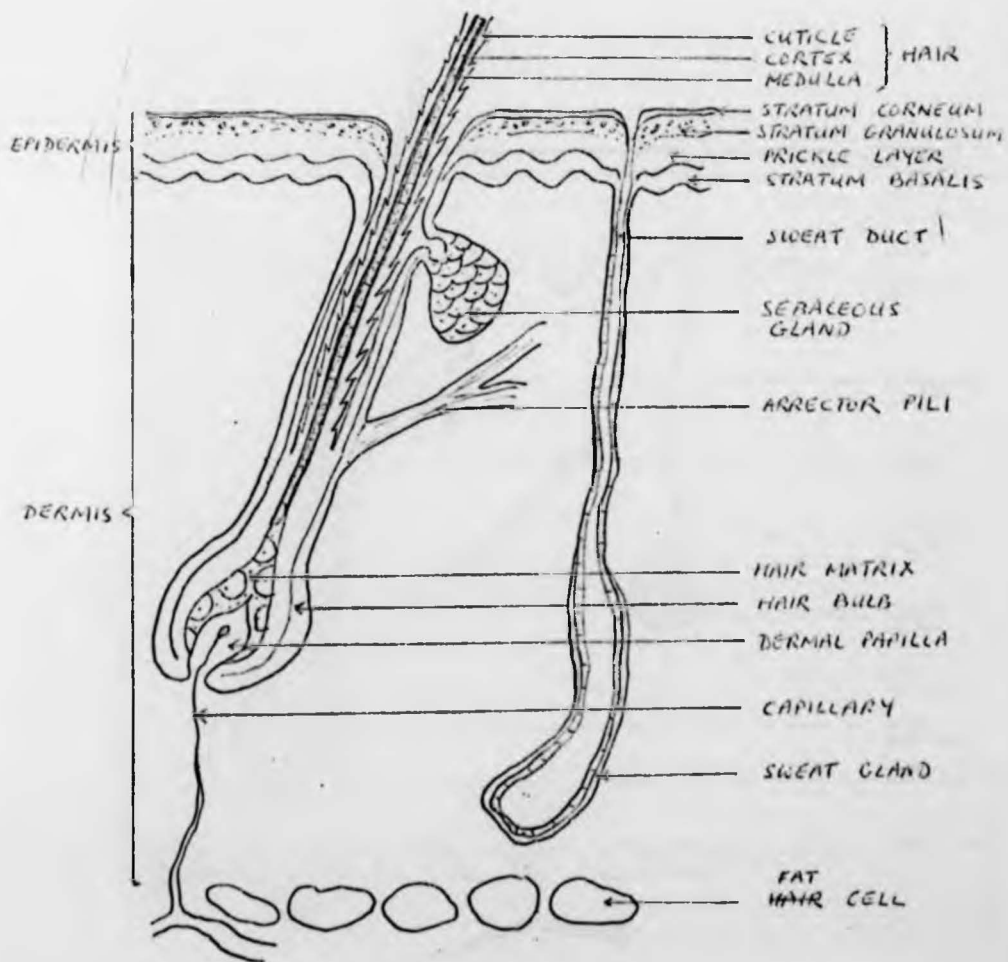
The specific features of the mouse skin have been described by
Setala (1960) and Tarin (1967) and reviewed by Raick in 1973. The
essential features of note are:-

(a) The epidermis of mouse skin is very thin with a high per-
centage of undifferentiated basal cells.

(b) The stratum corneum is clearly demarcated from the stratum
granulosum.

(c) The stratum basalis consists of basal cells with large
nuclei containing single, small, compact, round or oval
nucleoli lying close to the centre of the nucleus. The cyto-
plasm of these cells is relatively sparse. There are no
intercellular spaces or bridges between adjacent basal cells.

(d) The melanocytes are interspersed between the basal cells and
are also present in the hair bulbs. They give colour to the
skin and hair. Melanocytes have numerous cytoplasmic proces-
ses and contain specific pigment granules called melanosomes.
These are membrane-bound ovoid bodies containing melanin.

(e) The interfollicular epidermis is one to two cells thick with
the long axis of the nuclei of the first cell layer aligned
perpendicular to the epidermal surface, and the second more
superficial layer, if present, with the nuclei aligned
parallel to the epidermal surface.

Melanin is the high molecular weight pigment present in hair
and skin. It is produced by the enzymatic oxidation of
phenolic compounds. Tyrosine, a colourless amino-acid which
is first converted to dihydroxy phenylalanine (DOPA)

Fig 12 shows the steps in melanin synthesis.

FIG 12

MELANIN SYNTHESIS

Tyrosine ⟶ DOPA ⟶ Dopaquinone
↓
Leukodopachrome
↓
Dopachrome
↓
5,6-dihydroxyindole
↓
Melanin ⟵ Melanochrome ⟵ Indole.5,6-quinone (yellow)
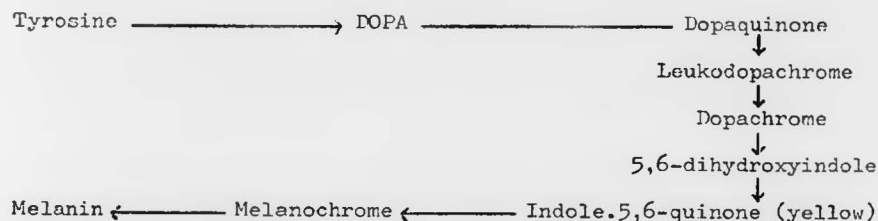
(From Fitzpatrick, Brunel and Kukita 1958)

Tyrosinase and other copper-containing enzymes are involved.
Melanin occurs as simple, uncombined melanin or in combination with
proteins to form melanoproteins. It is present in melanoblasts,
melanocytes and pigment cells in hair. Melanoblasts are the parent
pigment cells derived from the neural crest and give rise to
melanocytes at the hair bulb. These melanocytes form pigment cells
in the hair. The pigment cells are usually longitudinally arranged
in the hair cortex and medulla. Air spaces or fusi present in
between cells of the hair give sheen and colour tone to the hair
by influencing the reflection of light (Montagna and Van Scott
1958). These fusi are filled with fluid at the living portion of
the hair near and at the bulb. As the hair grows, the fusi dry
out and air replaces the fluid. Thus, the gross appearance of hair
colour depends on the melanin and fusi.

Melanoblasts also give rise to melanocytes which lie on the base-
ment membrane of the epidermis. Melanocytes have dopa oxidase
activity (DOPA positive). The activity of melanocytes rather
than actual numbers present determine skin colour.

Microscopy of Hair

Fig 13 shows a high power view (x 100 magnification) of a normal black hair from a C57 Black male mouse. The translucent cuticle consists of a single layer of pigment-free scales that form the outer part of the hair shaft. The inner cortex consists of the pigment-containing cells with lighter air spaces in between.
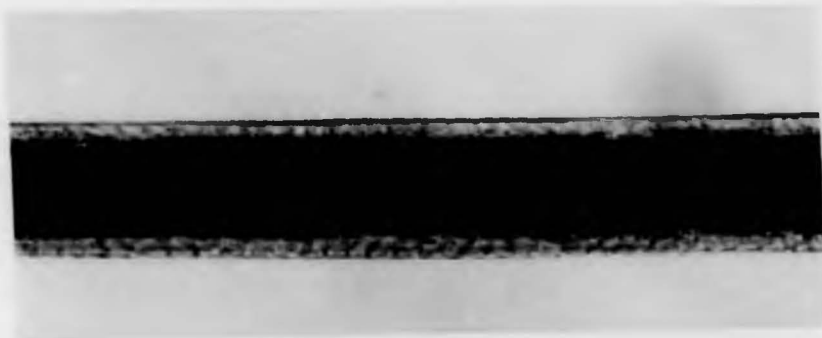
FIG 13 Normal Black Hair from C57 Black Mouse (x 100)

FIG 14 Normal White Hair from Tyzzer Original White Mouse (x 100)

Microscopy of Hair

Fig 13 shows a high power view (x 100 magnification) of a normal black hair from a C57 Black male mouse. The translucent cuticle consists of a single layer of pigment-free scales that form the outer part of the hair shaft. The inner cortex consists of the pigment-containing cells with lighter air spaces in between.

FIG 13 Normal Black Hair from C57 Black Mouse (x 100)
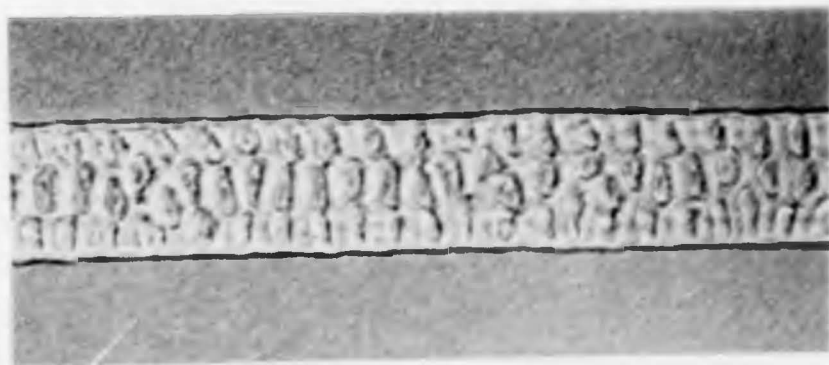
FIG 14 Normal White Hair from Tyacer Original White Mouse (x 100)

Fig 14 shows a similar view of a white hair from a Tyzzer original mouse. Beneath the cuticle of scales is the cortex with pigment-free cortical cells and air spaces in between. Some hairs in this white mouse possess a few pigment cells scattered in the hair cortex. The hairs are, therefore, not completely void of melanin.

Figs 15 and 16 show human black and white scalp hair for comparison. In the human black hair the cuticle is very thin and there are many more pigment cells in the hair cortex with hardly any air spaces seen. The white scalp hair shows the hair cortex being relatively free of pigment while the medulla retains the pigment to form a central black core throughout the hair.

Fig 17 shows a hair from a C57 Black mouse which had been injected with dimethyl sulphate. There is a distinct margin separating the well-pigmented part of the hair from the completely depigmented portion which is nearer the hair physically. This indicates an abrupt effect on pigmentation produced by the chemical injected. A similar picture is seen with chemicals causing obvious (+ +) and marked (+ + +) depigmentation e.g. nitrogen mustard (HN2) TPA and carbon dioxide snow. There appears to be no difference in the histological picture of hair taken from C57 Black mice whether they are injected with dimethyl sulphate, HN2, TPA or CO_2 snow. In any sample of hair taken from the sites of depigmentation, most of the hair will be completely depigmented or with the tips of the hair pigmented and the base depigmented (as in Fig 17). There will also be some hairs with normal pigmentation and hairs with scattered patches of pigment loss, (as in Fig 18). This may represent a difference in susceptibility and response of the pigment cells in different hair papillae to the injected chemicals.
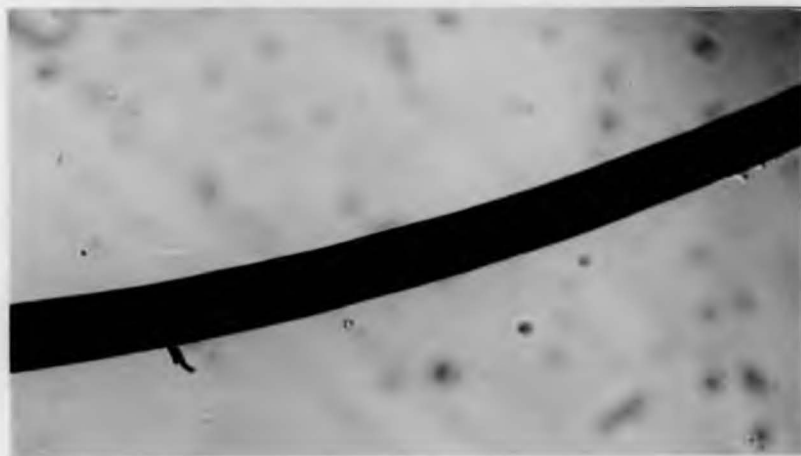
FIG 15 Normal Human Black Scalp Hair (x 400)
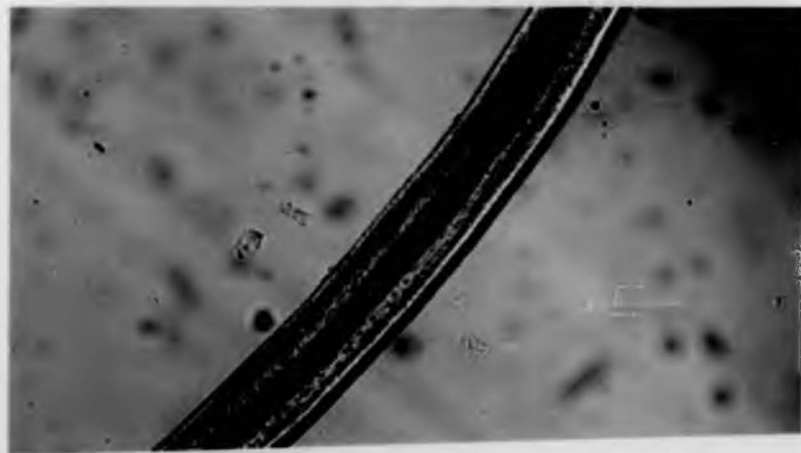
FIG 16 Normal Human White Scalp Hair (x 400)
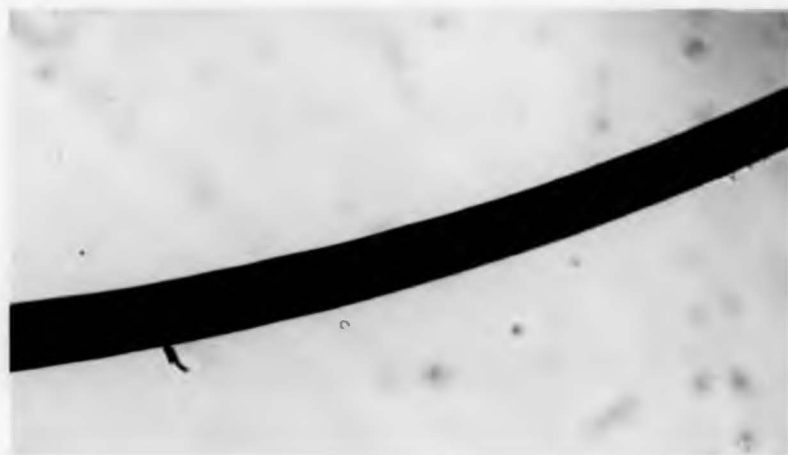
FIG 15 Normal Human Black Scalp Hair (x 400)
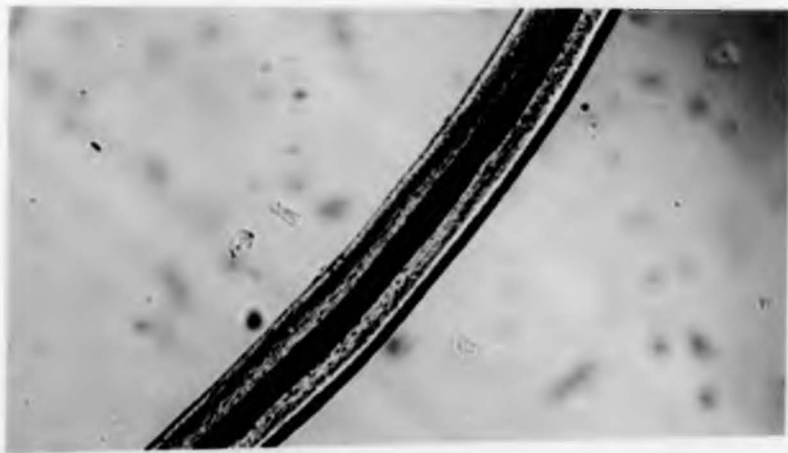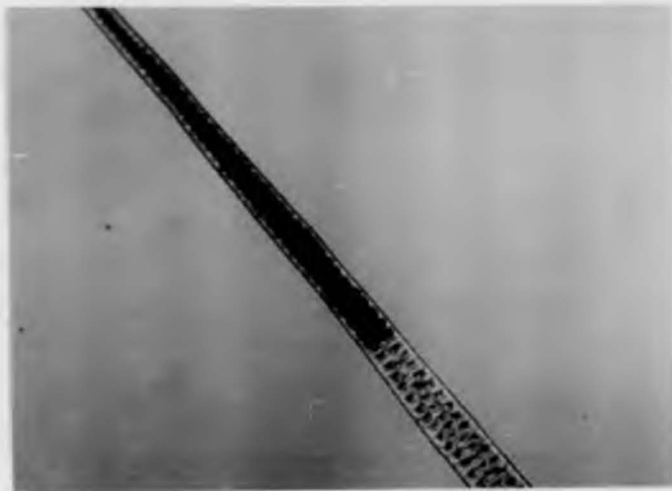
FIG 16 Normal Human White Scalp Hair (x 400)

FIG 17 Depigmented Hair from C57 Black Mouse treated with
Dimethyl Sulphate (x 50)
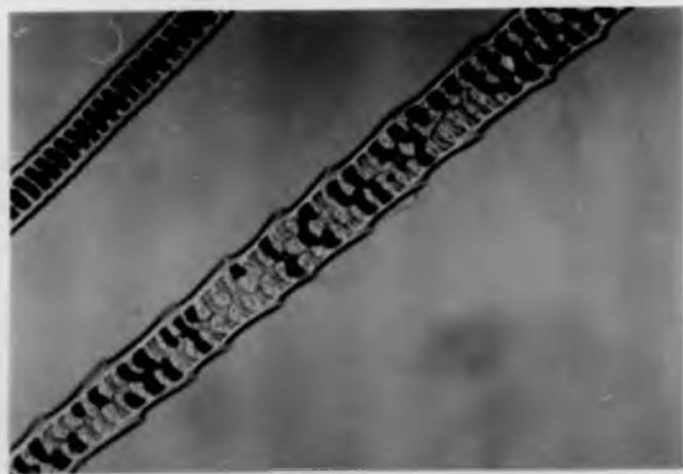
FIG 18 Depigmented Hair from C57 Black Mouse treated with
Phenol (x 50)
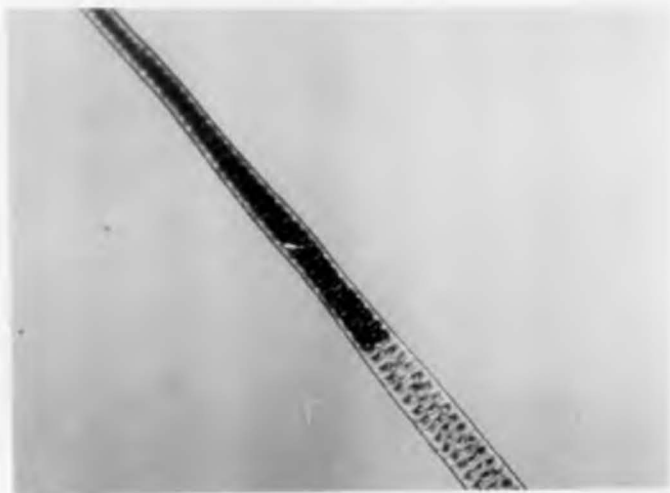
FIG 17 Depigmented Hair from C57 Black Mouse treated with
Dimethyl Sulphate (x 50)

FIG 18 Depigmented Hair from C57 Black Mouse treated with
Phenol (x 50)

Fig 18 shows a hair from a C57 Black mouse which had been injected with phenol, alongside a normal black hair. Here, the depigment-ation is patchy with retention of some pigment in scattered areas along the hair shaft. There is no abrupt loss of pigment. Such hairs are seen with the chemicals causing only slight depigmentation (+) e.g. phenol, saccharin, dodecane and Tween 20. While a majority of hairs from depigmented sites due to these chemicals show this picture, a few hairs will be normal and there will also be some with complete loss of pigment. The reason again is likely to be a difference in pigment cell susceptibility. Searle (1972) noted similar depigmented hair together with normal hair in depig-mented sites in C57 Black female mice treated with δ-hydroxy-quinoline by local skin application.

No banding of hair was observed, as might be expected if melano-genesis was only transiently affected. The loss of pigment in all the experiments producing positive depigmentation was permanent and the effect was still present even after a year from the initial intradermal injection.

Transverse sections of hair follicles were also examined by light and electron microscopy.

Fig 19 shows a normal hair follicle from a C57 Black mouse. The lighter multicellular outer sheath surrounds the darker inner sheath. The hair is seen within the inner sheath with its cuticle and pigment within cells of the hair cortex clearly visible.

Fig 20 is a similar section taken from a C57 Black mouse injected with TPA. There is loss of pigment from the inner sheath and only very little pigment in the hair cortex.
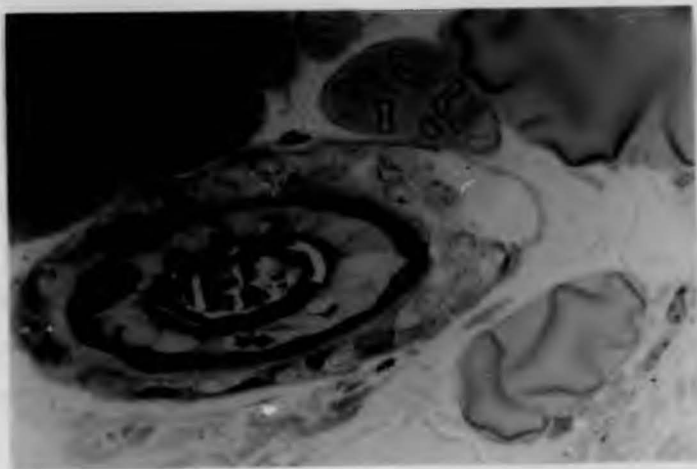
FIG 19 Transverse Section of Hair Follicle from Normal C57
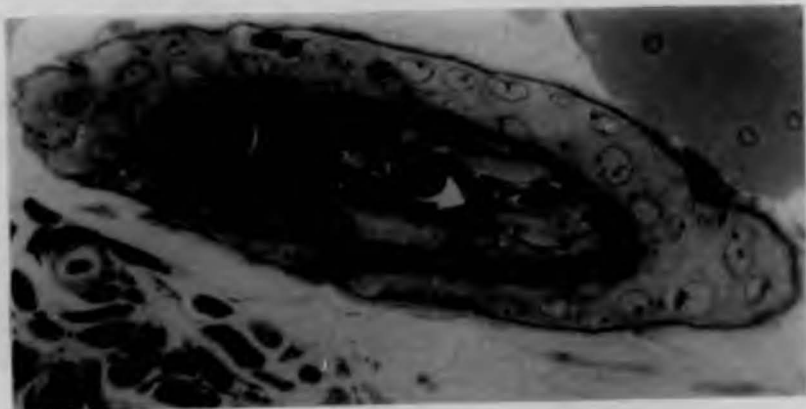
Black Mouse (x 600)

FIG 20 Transverse Section of Hair Follicle from C57 Mouse which

had been injected with TPA (x 600)
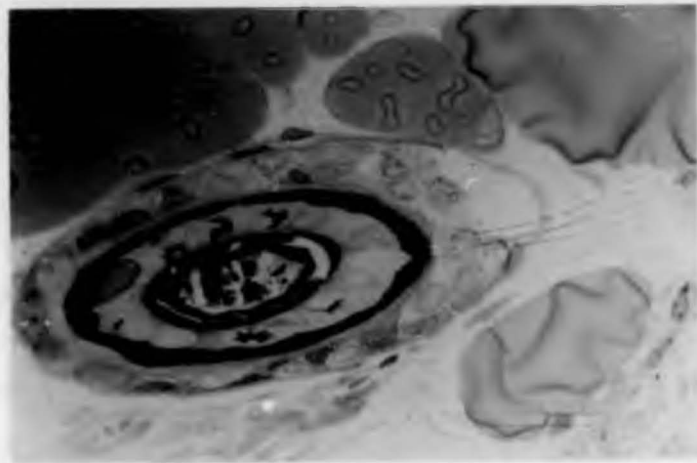
FIG 19 Transverse Section of Hair Follicle from Normal C57
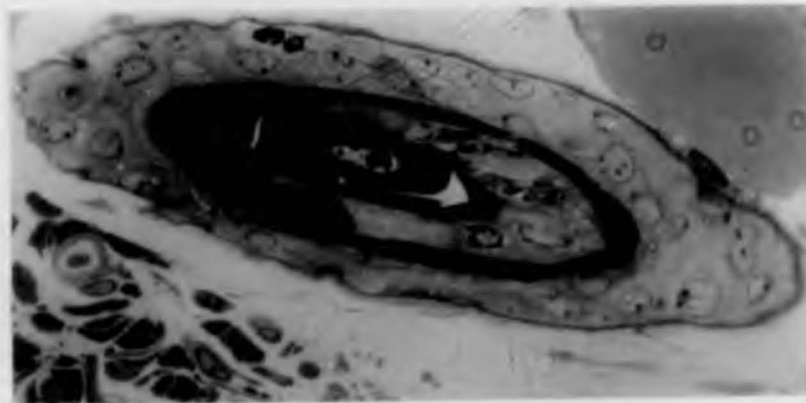
Black Mouse (x 600)

FIG 20 Transverse Section of Hair Follicle from C57 Mouse which

had been injected with TPA (x 600)

Microscopy of Skin

Examination of skin sections under the light microscope and electron microscope showed a decrease in melanocytes in the epidermis and around the hair follicles.

Fig 21 shows an electron micrograph of an area of skin where TPA was injected. The stratum corneum is clearly seen as a separate layer of compressed desquamated cells. There are a number of basal cells with large nuclei and single small nucleoli. No melanocytes are seen.

FIG 21 Area of Skin from TPA-Injected C57 Black Mouse
Electron Micrograph (x 5000)

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FIG 21 Area of Skin from TPA-Injected C57 Black Mouse
Electron Micrograph (x 5000)

FIG 22 Normal Melanocyte from C57 Black Mouse

Electron Micrograph (x 12,600)

FIG 23 Area of Skin Around Hair Papilla in Normal C57 Black Mouse

Electron Micrograph (x 1600)

FIG 22 Normal Melanocyte from C57 Black Mouse
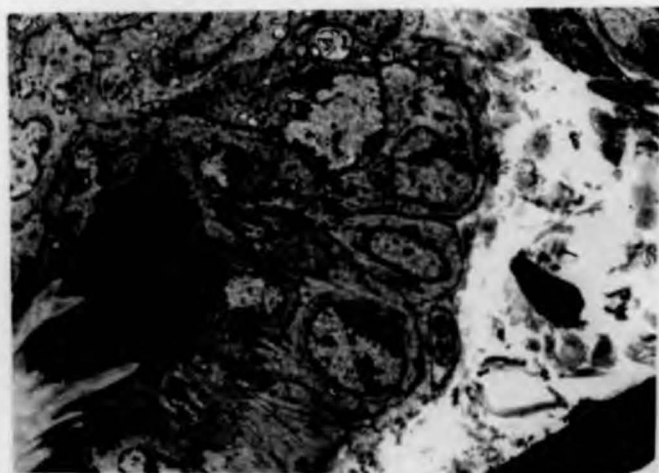
Electron Micrograph (x 12,600)

FIG 23 Area of Skin Around Hair Papilla in Normal C57 Black Mouse

Electron Micrograph (x 1600)

Fig 22 shows an electron micrograph of a melanocyte taken from an area of normal skin. This melanocyte has an irregular cell margin due to cytoplasmic processes. Several ovoid melanin granules are present in the cytoplasm. The nucleus is large and irregular and contains more than one nucleoli.

Fig 23 shows an electron micrograph of the area of skin around a hair papilla in a normal C57 Black mouse. Melanocytes incorporating melanin pigment into the growing hair are present. In mice injected with depigmenting agents, there is a lack of melanocytes in the area of the papillae.

CHAPTER 5 GENERAL DISCUSSION

Mechanism of depigmentation by tumour-promoters

Uses and limitations

Suggestions for further studies

DISCUSSION

1. Mechanism of Depigmentation by Tumour-Promoters

The reason for the observed relationship between injected tumour-promoters and depigmentation of hair is not known. Indeed, for a large proportion of depigmenting agents in general, the actual mode of action is obscure, (Searle and Riley 1978).

The exact mechanism by which tumour-promoters exert their effect is also not known. There is an abundance of theories though on how tumour-promoters act.

Boutwell (1974) suggested that tumour-promoters may act as gene activators to transform initiated cells to dormant tumour cells. Repeated exposure to tumour-promoters will then stimulate cell division by the dormant cells to produce a neoplasm. An alternative mechanism proposed by Boutwell is that tumour-promoters inhibit DNA repair. Initiators react with DNA and inhibition of proper repair leads to expression of a damaged or altered gene. However, this alternative is unlikely because it implies that tumour-promoters need to be applied soon after initiation for them to be effective. Yet, tumour-promoters can be effective in producing tumours when applied several months after an initiator (Weinstein, Wigler and Pietropaolo 1977).

Frankfurt and Raitcheva (1972) suggested that tumour-promoters act on initiated epidermis to give certain cells a selective advantage. The basal cells in particular were noted to have a shorter mitotic cycle and an increased proliferation rate. It is possible that melanocytes are amongst the cells not given this selective advantage by tumour-promoters. Their activity may be inhibited or reduced and

this could explain the depigmentation effect.

Other theories include the epidermal chalone theory (Marks et al 1972) which maintains that a mitosis-inhibiting substance termed chalone is inactivated by tumour-promoters. Depressed immunological activity is another theory of tumour-promotion (National Cancer Institute Monograph 1922). However, these theories are not well supported and do not appear relevant to the depigmentation effect.

Most of these theories are based on a variety of effects produced by tumour-promoters. The difficulty is in distinguishing between those effects essential for tumour-promotion and those that are not. Amongst those observed effects are:-

(a) The inhibition of differentiation of cells in culture (Raick 1974, Weinstein and Wigler 1977). Dedifferentiated cells remain inactive and retain their ability to divide. A continued alteration in the proportion of these cells to differentiated cells present can lead to an increase in cell division of immature cells typical of a neoplastic effect. This observation supports Frankfurt and Raitcheva's suggestion that tumour-promoters give some cells a selective advantage. A single exposure to tumour-promoters may cause a reversible effect but repeated exposures can maintain the increase in immature cells. Inhibition of differentiation has been shown in chick embryo myoblasts (Cohen et al 1977), Friend erythroleukaemic cells (Yamasaki et al 1977) and neuroblastoma cells (Ishii et al in press). The effect on neuroblastoma cells is of interest because neuroblastoma cells originate embryologically from the neural crest and this is also the source of melanocytes. The depigmentation effect due to tumour-promoters may be an inhibition effect on differentiation of melanocytes.

(b) An increase in the enzyme ornithine decarboxylase (ODC) which
correlates well with tumour-promoting activity (Boutwell 1977).
High levels of ODC have also been noted in skin carcinomas, hepatomas
and other malignant cells (Boutwell 1978, Russell 1973, Williams-
Ashman, Coppoc and Weber 1972) and lower levels in papillomas.
Raised ODC levels lead to an increase in cellular polyamines and
these are known to stimulate cellular division. The increase in
polyamines may be responsible for the growth advantage of trans-
formed cells exposed to tumour-promoters compared with non-exposed
cells. Putrescine (tetra-methylene diamine) is a produce of ODC
action and intracellular levels of putrescine are increased in
mouse skin on exposure to TPA (Weinstein and Troll 1977). To
investigate whether putrescine may be responsible for the depigment-
ation effect caused by tumour-promoters, solutions of putrescine
were injected intradermally into C57 mice (see page 174 for details).
No depigmentation was observed and this suggests that raised putre-
scine levels caused by tumour-promoters is not the cause of the
observed depigmentation.

(c) An increase in the enzyme plasminogen activator (Wigler and Weinstein
1976). This is a protease which is also secreted by neoplastic cells
(Reich 1975). It has been suggested that plasminogen activator may
break down proteins and unblock genes altered by initiation. It,
therefore, allows the expression of these genes which may lead to
tumour formation. It is possible that depigmentation due to tumour-
promoters is a result of plasminogen activator acting on proteins
responsible for melanin synthesis. However, this appears unlikely
because increased plasminogen activator seems to be confined to
phorbol esters and not other tumour-promoters (Marx 1978).

(d) Other enzymes are also increased by tumour-promoters e.g.
S-adenosyl methionine decarboxylase (O'Brien, Simsiman and
Boutwell 1975), cyclic AMP (adenosine mono-phosphate (Mufson,
Simsiman and Boutwell 1979) cyclic GMP(guanosine mono-phosphate)
(Estensen et al 1973). However, the correlation with tumour-
promoting activity is poor and they have not been as well studied
as ODC. It is unlikely that any of these enzymes form the link
between tumour-promoters and depigmentation.

Depigmentation results from a reduction in the amount or absence of
melanin present. This could occur because of inhibition of one or more
enzymes involved in melanin production. Inhibition of tyrosinase results
in decreased melanin pigmentation of hair, and this together with mental
deficiency occurs in phenylketonuria. This is a Mendelian recessive
disease which primarily causes a decrease in L-phenylalanine oxidase
activity in the liver. The enzyme lack causes a decrease in the con-
version of phenylalanine to tyrosine and the build-up of phenylalanine
inhibits tyrosinase activity (Fitzpatrick et al 1958). Tumour-promoters
may, therefore, cause depigmentation by a similar mechanism.

Boyland and Sargent (1951) investigated possible tyrosinase inhibition by
nitrogen mustard (HN2). Nitrogen mustard is a complete carcinogen and,
therefore, would possess initiating and tumour-promoting properties. It
caused a strongly positive result in the depigmentation test. Depigmen-
tated hair in Black mice was first produced by local injection of nitrogen
mustard (HN2). Dihydroxy phenylalanine (DOPA) was then injected intra-
dermally at the same site. If HN2 acts by inhibiting tyrosinase and
therefore decreasing the conversion of tyrosine to DOPA, then the sub-
sequently introduced DOPA should reverse the depigmentation. This did

not occur. Hence, for HN2-induced depigmentation, tyrosinase inhibition is not the mechanism.

Searle (1972) investigated the possibility of direct interference with copper-dependent enzymes e.g. tyrosinase as a possible mechanism of chemically-induced depigmentation. Local application of copper chelating agents viz α-benzoin oxime, 2,2-biquinolyl and 2,9-dimethyl 1,10-phenanthroline did not cause depigmentation. Inhibition of such enzymes is, therefore, unlikely to be a mechanism for depigmentation.

Searle (1972) also suggested that toxic complexes formed may be responsible for chemical depigmentation. He produced local depigmentation by using 8-hydroxyquinoline which is a chelating agent, but was negative in our depigmentation test. Diethyl dithiocarbamate which is also a chelating agent was tested by the depigmentation test and this also produced no depigmentation (see page 156). Both 8-hydroxyquinoline and diethyl-dithiocarbamate form stable metal complexes with copper. Diethyldithio-carbamate has been used experimentally to treat Wilson's disease which is a genetically recessive metabolic disease where there is a progressive increase in total body copper with consequent damage to the tissues of the brain, liver and renal tubules. Some limited studies suggest that 8-hydroxyquinoline may be carcinogenic but the International Agency for Research on Cancer concluded on review of the available data that such data was insufficient to suggest definite carcinogenicity (IARC Monographs). Diethyldithiocarbamate is not known to be carcinogenic. Both these chelating agents do not produce depigmentation in our test, and this suggests that metal chelation to form toxic complexes is unlikely to be the mechanism for depigmentation.

It is possible that tumour-promoters exert their effect by virtue of

their irritant nature and that this is also responsible for damage to melanocytes therefore causing depigmentation. However, irritant chemicals tend to cause hair loss and depilation does not correlate with depigmentation. Compounds that can cause excessive skin damage are not necessarily tumour-promoters e.g. styrene, dimethylhydrazine and Lewisite. There are also compounds which are tumour-promoters which do not cause gross signs of tissue damage nor hair loss e.g. 5% phenol and some surface-active agents (Boutwell and Bosch 1959). These tumour-promoters, however, cause depigmentation. Hence the link between tumour-promoters and depigmentation is not because of a common ability to cause tissue damage.

It is possible that tumour-promoters cause depigmentation by several different mechanisms. Boyland and Sargent (1951) showed that substances which might liberate free radicals e. g. ascorbic acid, perbenzoic acid and benzene diazinium hydroxide cause depigmentation when injected intra-dermally into mice. Ascorbic acid was positive in only three out of sixteen injected sites. There is no data on whether these may be tumour-promoters. The authors also suggest that solutions which are either hypotonic or hypertonic may cause cellular changes which can result in depigmentation. Distilled water caused depigmentation in 25% of sites in C57 Black mice. Concentrated (3 molar) sodium chloride caused depig-mentation in 40% of sites injected but there was no depigmentation with 1 molar sodium chloride. Attempts have been made to induce gastric cancers in animals by salted foods in order to investigate the high incidence of stomach cancer in Japan where salted foods are frequently consumed (Sato et al 1959). The results have been negative. However, Tatematsu et al (1975) found that using N-methyl-N'-nitro-N-nitroso-guanidine or 4-nitroguinoline-1-oxide as initiator, repeated applications of hypertonic sodium chloride increased the incidence of gastric tumours

in rats. This demonstrates the tumour-promoting action of hypertonic saline and since hypertonic saline has caused depigmentation this is again evidence in support of a relationship between tumour-promotion and depigmentation.

Boyland (1949) suggested that the greying of hair induced by radiation and chemicals may be a somatic mutation effect. This is because it represented a discontinuous permanent change in part of the soma. Theories on the possible action of tumour-promoters on genes have been mentioned earlier (page 201). Davidson and Dawson in 1976 produced depigmented patches in the offspring of female mice fed with the carcinogen benzpyrene. This could be due to a mutation effect on colour genes. Fahrig (1975) noted the alteration of pigment cells in mouse embryo due to x-rays and chemicals and proposed the effect as a spot mammalian test for mutagens. Hence, it is possible that the mechanism of depigmentation by tumour-promoters is by a somatic mutagenic effect on pigment cells.

The most likely explanation for the link between tumour-promoters and the depigmentation effect is that tumour-promoters have a selective toxic action on melanocytes. This results in a reduction or absence of melanin in the skin and hair. The skin in C57 Black mice is white and therefore the effect is more prominent on the hairs which are black. This effect is selective because other cells do not seem to be permanently altered. The main ultrastructural change in the mouse skin treated with tumour-promoters is hypertrophy of the epidermal layers especially in the basal cells (Raick 1973a and 1973b). Similar features were noted with croton oil (a tumour-promoter) and 3-methylcholanthrene (an indirect carcinogen) (Burki ahd Bresnick 1975). These features appear to be transient and non-specific. A single application of TPA resulted in reversion to the normal

histological features after 120 hours. Lack of melanin in the hair was not described by Raick (1973b) who concentrated on the features seen only up to four weeks after application of TPA to female Swiss-Webster mice. Lack of melanin was noted in this present study at least three weeks after single injections in cases where definite depigmentation occurred.

Depigmentation in C57 Black male mice starts three weeks after injection with TPA and remains as such even after six months. Phorbol esters have produced a delay in the expression of melanogenesis by melanoma cells in tissue culture (Mufson et al 1978) Other phorbol derivatives which are tumour-promoters also had the same effect but non-tumour-promoters did not. The effect on the melanoma cells is abolished by dexamethasone which also abolishes skin tumour-promotion. It has therefore been suggested that phorbol esters which are tumour-promoters interfere with cellular differentiation. Depigmentation would then result if cellular differentiation of melanocytes is affected. It could be possible that all other tumour-promoters interfere with cellular differentiation and therefore cause depigmentation in the same way as the phorbol esters. There has however been a suggestion that non-phorbol tumour-promoters e.g. anthralin, limonene and iodoacetic acid, may have a different mode of action because they do not mimic the effects of TPA on chick embryo fibroblasts (CEF) (Driedger and Blumberg 1978). Anthralin, limonene and iodoacetic acid when tested by the depigmentation test were all negative. In summary, many possible mechanisms may exist for chemically-induced depigmentation in dark-haired mice but the most likely link between tumour-promoters and depigmentation is the selective toxic action of tumour-promoters on melanocytes.

2. Uses and Limitations

The Use of the Depigmentation Test as a Screening Test for Tumour-Promoters and its limitations

The practical implication of the observations on the depigmentation effect is in the screening of substances that are possibly carcinogenic. The need for a rapid short-term screening test for carcinogens has been discussed. The aim of screening in chemical carcinogenesis should be not only to separate carcinogens from non-carcinogens but to try and develop tests for detecting special classes of compounds involved in the carcinogenic process e.g. the initiators, promoters, direct carcinogens and indirect carcinogens. The depigmentation effect was investigated and found not to be related to all carcinogens but there was a good correlation with tumour-promotion. It may thus be applied as a short-term screening test for tumour-promoters.

Any short-term screening test should be cheap, easy to perform and produce rapid results. The depigmentation test is relatively inexpensive. The main costs come from obtaining the pure-strain C57 Black mice (£1 - £2 each) and from feeding and keeping them for the duration of the test. Other expenses are in the use of disposable laboratory equipment such as gloves, syringes and needles. The current cost for a test on one compound using fifty mice would be about £200.

The test is also easy to carry out, needing only one person to prepare the compounds and animals, and to inject the mice on one occasion only and then to regularly examine them. Depigmentation

appears in three to six weeks from initial injection. The only
exception is ground crocidolite where minimal depigmentation
was noted after sixteen weeks. This period is still much shorter
than the time needed for results to appear in conventional long-
term animal tests for carcinogens.

As for screening tests used in the detection of disease in human
popluations, screening tests for chemical tumour-promoters should
have a high index of validity and reliability.

The validity of a screening test is the accuracy with which it
indicates positives and negatives. It has two components:-

(a) Sensitivity - which is the ability to detect true positives
correctly.

(b) Specificity - which is the ability to detect true negatives
correctly.

The sensitivity of the test was calculated at 53% to 60% and the
specificity at 79% to 89% (see Table 13).

The reliability of a screening test is the precision with which it
gives consistent results. This depends on the variation inherent
in the method and an observer variation. For the depigmentation
test, the variation in the method includes difference between the
mice used such as age, source, diet and weight. This could all be
standardized as much as possible.

Differences also arise during the preparation of small concentra-
tions of the test compounds and in the injecting of a precise
0.05 ml of test solution intradermally. Precision in the preparation

of solutions depends on the accuracy of apparatus used. Injection
techniques may be difficult to standardize and a device which delivers
a precise volume and specific distance into the skin would be useful.
However, variability in skin thickness and movement of the mice would
make such a device impractical. Intra-observer and inter-observer
variation exists especially in the subjective decision of the absence
or presence of depigmentation and its extent. An attempt to minimize
this was made by using a single 'blind' method of examining the mice
and be requiring slight depigmentation to be confirmed on three con-
secutive daily examinations before it is recorded as positive depigmen-
tation. An improvement in this would be to use an objective method
to assess depigmentation. Instruments for such objective assessment
are available (Ferroro and Basile 1979) and one such instrument uses
diffuse reflectance spectrometry with an incorporated interferometer
(Fuller and Griffith 1978). Other limitations of the present study
include:-

(a) The small numbers of mice used to test each compound

This ranged from four to fourteen mice injected at two to six sites
per mouse. The mice were also obtained from two different sources.
The main reasons for this were the cost and availability of the
test animals and the limited financial resources for this study.
Ideally, at least twenty mice should be used to test each compound
with as much standardization as possible in the mice and procedure
used. Four sites can be used per mouse with one site as control.
Hence, the total number of test sites would be $3 \times 20 = $ sixty
sites per compound.

(b) The selection of chemicals for testing

A random method as discussed in the methodology was not practical. Chemicals for testing were selected according to their known and suspected properties with regards carcinogenesis. Some degree of bias may have been introduced in the selection of these chemicals. However, this may in fact be the preferred method of selecting tumour-promoters for testing. Selection based on published reports of possible tumour-promoting activity would appear more useful than random selection of chemicals, especially since the number of known and suspected tumour-promoters are limited at the present moment.

(c) The interpretation of the results.

The observation of the link between tumour-promoters and depigmentation is of use. However, what is not known is whether this is an essential property of all tumour-promoters or if this effect is also produced by many other groups of compounds which are not tumour-promoters. Some agents which cause occupational leukoderma, such as methyl catechol, 4-tertiary butyl catechol (TBC) and para-tertiary butyl phenol (PTBP) have produced a positive depigmentation result. These have not been tested for tumour-promoting activity and there are no published reports on a possible tumour-promoting effect. ~~However, the related compound pyrocatechol has been shown to have tumour-promoting activity (Van Duuren, Katz and Goldschmidt 1973) and therefore~~ Further tests on methyl catechol, TBC and PTPB may also show such activity.

One of the main uses of the depigmentation test would be to indicate those chemicals that need to be subjected to further tests to confirm tumour-promoting activity. Such further confirmatory tests

could be based on a skin painting technique using mouse skin pre-
treated with an initiator, such as 7,12 dimethyl benzanthracene
or urethane (ethyl carbamate) and then repeated painting with the
test chemical to see whether tumours develop significantly more
than an appropriate similar control system.

(d) The relevance to human populations

A test for tumour-promoters based on intradermal injections in
mouse skin may be relevant only to chemicals that have tumour-
promoting action on mouse skin. Most of the studies on tumour-
promotion have been performed using mouse skin because it provides
the best model for studies on initiation - and promotion. Mice
are the most widely used animals for cancer research (Mitruka,
Rawnsley and Vadhera 1976) and the mouse skin is also susceptible
to extrinsic tumourogenic agents (Setala 1960). Studies on the
mouse skin allow gross changes to be easily observed and followed
and carcinogens, co-carcinogens, tumour-promoters and any other
test chemicals can be applied directly to the site of study and
tissue specimens for histological and other analytical studies
are easily obtained. However, a chemical with tumour-promoting
action on mouse skin may cause positive depigmentation by the
technique described, but it may not be a relevant tumour-
promoter for human populations or other animal species. The same
arguement holds true though for any other test for carcinogens
or tumour-promoters and co-carcinogens based on animal studies.
The relevance to man must be considered with some reservation.
Studies on human populations are only limited to a few time-
consuming epidemiological studies and therefore for practical
purposes animal studies must be used to indicate substances which

may have tumour-promoting action in humans. The depigmentation test
would be one such test to be used with awareness of the limitations
discussed.

Other Uses of the Depigmentation Effect

(a) Tumour-promoters, because of the observed melanotoxic effect,
     could be used in the treatment of melanomas. It may seem para-
     doxical to suggest the possible treatment of a malignant tumour
     with a tumour-promoter but anti-cancer drugs such as chlorambucil,
     myeleran (Busulphan) and other alkylating agents are by
     themselves carcinogenic. The use of radiation in the treatment
     of lung cancer, carcinoma of the cervix and lymphomas is similar.

(b) The depigmentation effect may also be used to screen chemicals
     that are capable of causing occupational leukoderma. Some of
     these agents have been shown to produce positive results by the
     depigmentation test. A few of these agents may, in addition, have
     tumour-promoting activity. The use of the depigmentation test
     for industrial chemicals would indicate chemicals which are
     leukodermogenic and/or tumour-promoters and hence a possible
     chemical hazard may be identified and appropriate measures
     instituted for their safe handling before irreversible harmful
     effects are caused.

3. Suggestions For Further Studies

The results of the experiments performed indicate that several areas may be usefully investigated in further studies.

Studies on Other Compounds

With increasing evidence of more compounds having tumour-promoting activity and some with possible tumour-promoting activity, studies directed towards such compounds and their effect on hair pigmentation may provide further confirmation of the link between tumour-promoting activity and depigmentation. Such compounds include:-

(a) Some dyes which have produced local sarcomata when injected into mice may do so by means of a tumour-promoting effect. Such dyes include Patent Blue V, Blue VRS (2%) (Grasso and Golberg 1966).

(b) Substances which have a tumourogenic effect but do not seem to react with DNA. Such substances include saccharin, phenobarbitone, chloroform, carbon tetrachloride, dieldrin, DDT, thioacetamide, thiourea and 3-aminotriazole. These were termed epigenetic carcinogens by Ashby et al (1978). It is possible that these epigenetic carcinogens act by tumour-promotion. Saccharin, phenobarbitone and chloroform were tested and chloroform was positive (+ +), saccharin weakly positive (+) and phenobarbitone negative (-) for depigmentation. The remaining compounds mentioned above could be tested.

(c) Steroids (Belman and Troll 1972), retinoids (Becci et al 1978) and prostaglandin inhibitors have all been shown to inhibit tumour-promoting activity for carcinogenesis. 13-cis retinoic acid was investigated for its ability to inhibit depigmentation

It did not prevent depigmentation due to TPA or croton oil. Steroids, prostaglandin inhibitors and any other substances which may inhibit tumourogenesis may be similarly investigated to see whether depigmentation by tumour-promoters in inhibited.

Repeat Experiments Using Improved Techniques

Some of the experiments performed may be repeated using improved techniques such as an objective method of assessing depigmentation and an increased number of test animals for each test chemical. The limitation again is in the finances available.

Tests Using Unknown Compounds

One way of assessing the usefulness of the depigmentation effect to distinguish tumour-promoters from non-promoters is to use pairs of compounds unknown to the investigator. These pairs should ideally consist of a known tumour-promoter and a chemically similar non-promoter. An effective test should identify the tumour-promoter correctly from the pairs tested. The problem about such a procedure is in the selection of appropriate pairs. The lack of information on what are definite tumour-promoters also makes this approach difficult. Even for carcinogen testing where much more information is available there are difficulties in the selection of appropriate pairs for testing (Boyland 1978a and 1978b).

A simpler procedure would be not to use paired compounds but to test a group of compounds which contain tumour-promoters and non-promoters to see whether the depigmentation test picks out the promoters correctly. This should be done without the investigator knowing the identity of the compounds tested. The depigmentation test has been included as one of the non-genetic tests in an international programme for the evaluation of short-term tests for carcinogenicity. The programme compares

twenty-five assay systems for their efficacy in distinguishing forty-
two reference carcinogens and non-carcinogens (Mutation Research
Announcement 1978). When completed, this would indicate the relative
usefulness of the depigmentation test, when compared with other tests
for screening carcinogens. Preliminary tests on five of the forty-
two reference chemicals produced the results in Table 67.

These preliminary results suggest that the test may not be useful to
distinguish carcinogen from non-carcinogen. However, 4-dimethylamino-
azobenzene-4-sulphuric acid (Na salt) may be positive because it
possesses tumour-promoting activity. Compound No 1 (4-dimethyl-
aminoazobenzene) gave a doubtful positive result in two out of six
sites injected. The results may be better assessed when all tests are
completed.

TABLE 67

TESTS ON REFERENCE CHEMICALS

Code No	Chemical	Amount Injected µg	Degree of Depigmentation	Proportion of Sites With Depigmentation / Total No of Sites Injected
1	4-Dimethylaminoazobenzene (Butter yellow)	1000	±	2/6
10	4-Dimethylaminoazobenzene-4-sulphonic acid (Sodium salt)	1000	+	4/6
8	4-Nitroquinoline-N-oxide	1000	-	0/6
15	3-Methyl-4-nitroquinoline-N-oxide	1000	-	0/6
14	Hydrazine sulphate	500	-	0/6

Skin Painting Tests on Compounds Showing Positive Depigmentation

Another way of confirming the relationship between tumour-promoters
and positive depigmentation is to test those compounds which cause
depigmentation for tumour-promoting activity, where such tests have
not been done before e.g. positive depigmentation was obtained with
p-teritary butyl phenol, methyl catechol and p-teriary butyl catechol.
All these phenols have not been adequately tested for tumour-
promoting activity. Tests for tumour-promoting activity could be
performed using mouse skin (C57 Black male mice would be appropriate
in this instance) and an initiator such as 7, 12-dimethylbenzanthracene
or ethyl carbamate (urethane)

Tests Related to Enzyme Levels

Ornithine decarboxylase (ODC) and plasminogen activator (PA) are two
enzymes which seem to correlate with tumour-promoting activity. Com-
pounds which cause depigmentation may be tested for their ability to
increase levels of these enzymes in mouse skin. The finding of a
common link between tumour-promoters, depigmentation and raised ODC
and/or PA levels would be of interest.

CHAPTER 6   CONCLUSIONS

CONCLUSIONS

The following conclusions can be made from the results of all the experiments performed.

1. Permanent localized areas of depigmentation of hair may be produced by single intradermal injections of 0.05 ml solutions of different chemicals into C57 Black male mice. Dimethyl sulphoxide and saline were mainly used as solvent/medium.

2. Depigmentation appeared after a latent period of three to six weeks. This would be expected to vary depending upon the stage of the hair cycle at the time of intradermal injections. Ground crocidolite was an exception producing minimal depigmentation after a long latent period of more than sixteen weeks.

3. Of the sixty-six chemicals tested, depigmentation occurred more with tumour-promoters than carcinogens, co-carcinogens or non-carcinogens. 56% of tumour-promoters tested were positive compared with 24% of carcinogens and 17% of substances not known to be carcinogenic or tumour-promoters.

4. Prominent depigmentation (+ + +) occurred with the promoters 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and carbon dioxide snow and the complete carcinogen - nitrogen mustard (HN2) which would have tumour-promoting activity. The promoters saccharin, dodecane and Tween 20 produced slight depigmentation (+). Weak tumour-promoters as Tween 40, Tween 80, limonene and iodoacetic acid produced no depigmentation.

5. Indirect carcinogens such as polycyclic aromatic hydrocarbons and aromatic amines were negative. Direct-acting carcinogens which

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would possess both initiating and tumour-promoting activity
such as nitrogen mustard (HN2), methyl iodide and benzyl chloride
were positive.

6. Four compounds not known to have tumour-promoting activity were
positive. These were pyrocatechol, 4-tertiary butyl catechol,
para-tertiary butyl phenol and butylated hydroxyanisole. All
belong to a group of chemicals known to cause occupational
leukoderma. Seven such compounds were tested and five were
positive. The fifth compound is hydroquinone monobenzyl ether
which may have tumour-promoting activity.

7. The ED_{50} was statistically estimated by logit transformation
analysis for three compounds - nitrogen mustard (HN2), TPA and
chloroform - and was 0.018 µg, 0.18 µg and 101 µg respectively.

8. The relationship between dose and effect for positive compounds
showed two patterns. One, with increasing dose causing a greater
proportion of injected sites being depigmented. This occurred
in a sigmoidal dose-response relationship with HN2, TPA, carbon
dioxide snow, croton oil, chloroform, dimethylsulphate and phenol.
The other relationship was a dose-response curve with an optimal
dose (which was not the maximum dose used) effect as occurred with
1-fluoro-2,4-dinitrobenzene, saccharin and dodecane.

9. The specificity of the test was calculated at 53% to 60%, and the
sensitivity at 79% to 89% depending on different criteria used for
the classification of tumour-promoters, carcinogens and non-
carcinogens.

10. Depigmentation due to similar doses of nitrogen mustard was most prominent for C57 Black mice, less so for CBA Brown mice, and no depigmentation was observed with Tyzzer Original White mice. No obvious difference in depigmentation was noted between male and female mice tested.

11. The use of enzyme-inducers as phenobarbitone and 3-methyl-cholanthrene to pre-treat mice before intradermal injections of indirect carcinogens such as 1,2:5,6-dibenzanthracene, 9,10-dimethyl-1,2-benzanthracene, benzanthracene, 2-naphthylamine and benzidene did not result in any depigmentation.

12. Cis-retinoic acid did not inhibit the depigmentation due to the known tumour-promoters, croton oil and TPA. It inhibited depigmentation due to para-tertiary butyl phenol which is not a known promoter but has been known to cause occupational leukoderma. The practical implications of this is that Vitamin A analogues such as cis-retinoic acid may prevent occupational leukoderma.

13. Asbestos fibres injected as chrysotile A and B, crocidolite, amosite and anthophyllite in saline suspensions produced no depigmentation. Grinding up the fibres produced minimal depigmentation with crocidolite, but not with chrysotile A. The use of an initiator urethane (ethyl carbamate) to pre-treat mice before intradermal injections with asbestos did not alter the depigmentation effect nor result in the production of any tumours.

14. Light and electron microscopy of depigmented hair showed a loss
of pigment with an otherwise intact hair structure. This was
similar for all positive chemicals injected - the only variation
being in the extent and abruptness of pigment loss. This ranged
from a sudden complete loss of pigment as with dimethyl sulphate
to a more gradual partial scattered areas of lack of pigment
along the hair shaft as for phenol. Microscopy of skin showed
a relative decrease in melanocytes around the hair bulbs and in
the epidermis. The skin of C57 Black mice is white (only the
hairs are black) and few melanocytes are normally present.

15. The observed depigmentation is not due to an irritant effect nor
due to a direct effect of an increase in dermal putrescine,
histamine or adrenaline. It has been suggested that it may be
a somatic mutagenic effect. A direct toxic action on suseptible
melanocytes is a likely mechanism. Whatever the reason for the
observed depigmentation effect, the relationship with tumour-
promoting activity may allow it to be used as a screening test
for tumour-promoters.

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CHAPTER 8   APPENDIX

APPENDIX 1

Composition of Diets used for Test Animals

(a) Diet 86 (Dixons) - Used for all mice up to 30th August 1978.

Manufacturer: E Dixon and Sons (Ware)Ltd

Crane Mead Mills, Ware Herts

Crude Oil	%	2.03
Crude Protein	%	19.29
Crude Fibre	%	3.01
Digestible Crude Oil	%	1.44
Digestible Crude Protein	%	15.73
Digestible Crude Fibre	%	1.90
Digestible Carbohydrate	%	50.83
Gross Energy	Cals/kg	3,942
Metabolizable Energy	Cals/kg	3,548
Saturated Fatty Acids	%	0.42
Linoleic Acid	%	0.72
Other Unsaturated Acids	%	0.88
Calcium	%	1.06
Phosphorus	%	0.91
Sodium Chloride	%	1.26
Magnesium	%	0.26
Potassium	%	0.76
Sulphur	%	0.23
Iron	mg/kg	129
Copper	mg/kg	9.60
Manganese	mg/kg	29.76
Cobalt	ug/kg	75.84
Zinc	mg/kg	21.12
Iodine	ug/kg	457

Arginine	%	1.05
Lysine	%	1.06
Methionine	%	0.37
Cystine	%	0.33
Tryptophan	%	0.24
Glycine	%	1.19
Histidine	%	0.39
Threonine	%	0.70
Isoleucine	%	0.85
Leucine	%	1.33
Phenylalanine	%	0.85
Valine	%	0.92
Tyrosine	%	0.61
Aspartic Acid	%	1.29
Glutamic Acid	%	3.54
Proline	%	1.25
Serine	%	0.79
Vitamin A	IU/Kg	23,953
Vitamin D3	IU/Kg	2,937
Carotene	mg/kg	12.19
Vitamin B1 (Thiamine)	mg/kg	9.14
Vitamin B2 (Riboflavine)	mg/kg	5.13
Vitamin B6 (Pyridoxin)	mg/kg	6.07
Vitamin B12	ug/kg	15.24
Vitamin E	mg/kg	34.78
Vitamin K	mg/kg	15.28
Folic Acid	mg/kg	2.23
Nicotinic Acid	mg/kg	73.17

Pantothenic Acid	mg/kg	16.87
Choline Chloride	gm/kg	1.41
Eiotin	mg/kg	0.21
Inositol	gm/kg	2.24

(b) BP (Expanded) No 1 - Used for all mice after 30th August 1978.

Manufacturer: P Nutrition (UK) Limited

Stepfield, Wiltham, Essex CM8 3AB

Crude Oil	%	3.0
Crude Protein	%	14.8
Crude Fibre	%	4.0
Ash	%	4.8
Crude Carbohydrate	%	63.6
Digestible Crude Oil	%	2.1
Digestible Crude Protein	%	12.0
Digestible Crude Fibre	%	0.8
Digestible Crude Carbohydrate	%	56.5
Gross Energy	Cal/kg	3,390
Metabolizable Energy	Cal/kg	3,120
Digestible Energy	Cal/kg	2,810
Myristoleic Acid	%	0.02
Palmitoleic Acid	%	0.13
Oleic Acid	%	0.92
Linoleic Acid	%	0.92
Linolenic Acid	%	0.10
Arachidonic Acid	%	0.19
Lauric Acid	%	0.02
Myristic Acid	%	0.16
Palmitic Acid	%	0.47
Stearic Acid	%	0.10
Arginine	%	0.74
Lysine	%	0.75
Methionine	%	0.28

Cystine	%	0.25
Tryptophan	%	0.19
Histidine	%	0.33
Threonine	%	0.54
Isoloucine	%	0.61
Leucine	%	1.10
Phenylalanine	%	0.70
Valine	%	0.75
Tyrosine	%	0.50
Glycine	%	0.92
Aspartic Acid	%	0.81
Glutamic Acid	%	3.00
Proline	%	1.15
Serine	%	0.57
Alanine	%	0.13
Calcium	%	0.90
Phosphorus	%	0.50
Sodium	%	0.25
Chlorine	%	0.38
Magnesium	%	0.14
Potassium	%	0.72
Sulphur	%	0.19
Iron	mg/kg	70
Copper	mg/kg	9
Manganese	mg/kg	74
Zinc	mg/kg	76
Cobalt	mcg/kg	57
Iodine	mcg/kg	811

Selenium	mcg/kg	117
Fluorine	mg/kg	2
Vitamin A	IU/kg	6,610
Vitamin D	IU/kg	603
Vitamin B1	mg/kg	7.2
Vitamin B2	mg/kg	11.3
Vitamin B6	mg/kg	5.5
Vitamin B12	mcg/kg	7.7
Vitamin E	mg/kg	66.2
Vitamin K	mg/kg	10.5
Folic Acid	mg/kg	0.8
Nicotinic Acid	mg/kg	57.0
Pantothenic Acid	mg/kg	23.8
Choline	g/kg	1.60
Inositol	g/kg	2.55
Biotin	mcg/kg	265.0
Carotene	mg/kg	0.36
Xanthophyll	mg/kg	1.20

(c) Placebo diet for cis-retinoic acid experiment

   R & M No 1 Expanded

   Manufacturer: B P Nutrition (UK) Limited

                  Stepfield, Wiltham, Essex CM8 3AB

Moisture	%	11.9
Crude Fat	%	3.5
Crude Protein	%	13.6
Crude Fibre	%	2.8
Ash	%	4.34
Calcium	%	0.52
Phosphorus	%	0.68
Sodium	%	0.24
Chlorine	%	0.64
Potassium	%	0.87
Magnesium	%	0.20
Iron	mg/kg	168
Copper	mg/kg	22
Manganese	mg/kg	68
Zinc	mg/kg	68
Selenium	mg/kg	<0.02
Fluorine	mg/kg	10.0
Nitrite	mg/kg	<1.0
Vitamin A	IU/kg	3500
Vitamin E	mg/kg	133
Lead	mg/kg	<2.0
Arsenic	mg/kg	<0.2
Cadmium	mg/kg	0.25
Mercury	mg/kg	<0.01

Total Aflatoxins		None detected
Total P.C.B.		None detected
Total D.D.T.	mg/kg	0.001
Dieldrin		None detected
Lindane	mg/kg	0.003
Heptachlor		None detected
Malathion		None detected
Total Viable Organism	per gm	25×10^3
Mesophilic Spores	per gm	$< 1 \times 10^2$
Salmonellae Species		None detected
Presumptive E Coli		None detected
E Coli Type 1		None detected

(d) Cis-retinoic Acid Diet - as for (c) above with the addition of 240 mg of cis-retinoic acid per kg of diet.

APPENDIX 2

Procedure for Preparing Specimens for Electron Microscopy

1. Fix in 3% glutaraldehyde in 0.066M cacodylate for two hours.

2. Wash with 0.066M cacodylate three times (ten minutes each time) and overnight.

3. Post-fix with 1% osmium tetroxide in 0.066M cacodylate for thirty minutes.

4. Wash with 0.066M cacodylate buffer three times (ten minutes each time).

5. Wash with 30% methanol twice (five minutes each time).

6. Stain with 0.05% uranyl acetate in 30% methanol for thirty minutes.

7. Dehydrate with 30%, 60%, 70%, 80%, 90% and then 100% methanol twice (approximately five minutes with each concentration).

8. Infiltrate with:-
    (a) propylene oxide twice (ten minutes each time).

    (b) 50:50 propylene oxide/araldite for thirty minutes.

    (c) Araldite for thirty minutes and then overnight.

9. Embed in flat moulds in fresh araldite.