FURTHER STUDIES ON INCOMPLETE CARCINOGENESIS: TRIETHYLENE MELAMINE (T.E.M.), 1,2-BENZANTHRACENE AND β-PROPIOLACTONE, AS INITIATORS OF SKIN TUMOUR FORMATION IN THE MOUSE.

F. J. C. ROE AND M. H. SALAMAN.

From the Cancer Research Department, London Hospital Medical College, London, E.1.

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IN previous papers (Salaman and Roe, 1953 ; Roe and Salaman, 1954) it was shown that the application of urethane (ethyl carbamate) to the dorsal skin of mice followed by repeated applications of croton oil gave rise to many epidermal tumours, some of which were malignant,* and that the number of tumours which appeared was roughly proportional to the total dose of urethane applied. Urethane alone, applied repeatedly in large doses, produced no tumours, nor any recognisable histological change in the skin. Repeated application of croton oil alone produced very few tumours. Repeated application of urethane following a single " initiating " dose of a carcinogenic hydrocarbon failed to produce any skin tumours.

The object of these experiments was to test the hypothesis that there exist " incomplete carcinogens " which act as " initiators " but not as " promoters " of carcinogenesis. The meaning of these terms was defined. It was concluded that urethane was such an incomplete carcinogen for mouse skin.

2-Acetylaminofluorene, p-dimethylaminoazobenzene (butter yellow), and NN-di-(2-chloroethyl)-β-naphthylamine (R48), were tested by a similar technique, but none of these showed any initiating power. Methyl bis (β-chloroethyl) amine hydrochloride (nitrogen mustard) gave an equivocal result. This substance has been re-examined, and the result is included among those reported here.

In an attempt to find other initiators of carcinogenesis a further 28 substances have been screened for this property by methods essentially similar to those previously used. The majority were selected from the very large number of substances which possess one or more biological activities in common with urethane, or which are related to it chemically.

Selection of substances.

A list of substances screened is given in Table I. It was found convenient to divide them into classes, but it is not implied that the class-names completely describe the biological activity of each substance (e.g. nitrogen mustard is classified as an antileukaemic agent, but could equally well be classed among the substances known to exert a specific effect on cells undergoing mitosis).

* Among 45 mice which survived until the end of treatment with various doses of urethane in conjunction with croton oil there have been 5 carcinomata and 1 sarcoma. These have appeared 6–13 months from the beginning of treatment.

The first four substances which we have examined are classed as *antileukaemic agents*. These are of particular interest because of the frequent association between inhibition of tumour growth and carcinogenesis (Haddow, 1935, 1938 ; Haddow and Robinson, 1937, 1939 ; Haddow, Scott and Scott, 1937 ; Badger *et al.*, 1942).

The antileukaemicactivity of urethane itself is well authenticated both in human and animal leukaemia (Paterson *et al.*, 1946 ; Engstrom, Kirschbaum and Mixer, 1947 ; Berman and Axelrod, 1948).

1,4-Dimethanesulphonoxybutane (Myleran) was first synthesized and shown to be an antileukaemic agent by Haddow and Timmis (1951, 1953). It is now widely used in the treatment of human leukaemia, and is especially valuable in the chronic myeloid variety (Galton, 1953 ; Petrakis *et al.*, 1954 ; Boyland, 1954). Koller (1953 ; 1954, personal communication) produced sarcomata in rats by the subcutaneous injection of Myleran, and commented on the abnormal mitotic figures in these tumours.

Triethylene melamine (T.E.M.) was synthesized simultaneously at three centres (Haddow, 1950 ; Rose, Hendry and Walpole, 1950 ; and Burchenal *et al.*, 1950), and has found a place in the treatment of lymphatic leukaemia (Paterson, Kunkler and Walpole, 1953 ; Bayrd *et al.*, 1952). Rose *et al.* (1950) commented on the cytotoxic effects of T.E.M., and compared them with those due to nitrogen mustard, as described by Gaensler *et al.* (1948). There is evidence that T.E.M. possesses carcinogenic activity under certain conditions. Walpole *et al.* (1954) report the early development of sarcomata in rats following twice-weekly subcutaneous injection of T.E.M. in arachis oil (total dosage : 0·7 to 1·1 mg./100 g.). Shimkin (1954) reports an increased incidence of pulmonary adenomata in mice treated with T.E.M. Hendry *et al.* (1951) reported an increased incidence of lung tumours in mice injected intraperitoneally with T.E.M., but failed to induce sarcomata in mice by repeated subcutaneous injection of the substance (0.025 mg. weekly for over a year). Lewis and Crossley (1950) found that T.E.M. possessed tumour-inhibitory activity against sarcomatous implants, and also reported a decreased incidence of spontaneous lung tumours in the treated mice. Graffi, in a review (1953) of his work and that of his colleagues, described briefly an experiment in which mice were painted with T.E.M. and croton oil. No tumours resulted from this treatment.

Nitrogen mustard has been known as an antileukaemic agent since the end of the last war, when a leucopenia was observed in men accidentally exposed to it (Goodman *et al.*, 1946). Its present role in the therapy of leukaemia is reviewed by Gellhorn (1953), Wilkinson, Haddow and Nabarro (1953), and Boyland (1954). The carcinogenicity of nitrogen mustard has been investigated by several workers. Boyland and Horning (1949) reported that 10 out of 14 mice injected subcutaneously with nitrogen mustard (1 mg./kg. weekly for 36 weeks or more) developed tumours (8 lung adenomata, 2 lymphosarcomata, 1 uterine fibromyoma, and 1 spindle-cell sarcoma at the injection site). Griffin, Brandt and Tatum (1951) induced similar tumours after injecting nitrogen mustard subcutaneously, intraperitoneally, or intravenously. Heston, Lorenz and Deringer (1953), and Shimkin (1954), induced pulmonary adenomata in mice with nitrogen mustard. Narpozzi (1953) tested nitrogen mustard for initiating activity on mouse skin, using croton oil as the promoting agent. To 60 mice he gave 15 applications of 0·05 per cent nitrogen mustard on alternate days, and after an interval of 10 days he began applications of croton oil (5 per cent in liquid paraffin) ; the latter were given on

alternate days for over a year. During this period 6 mice developed up to 5 warts each, but no malignant tumours were observed. In our experience this number of tumours may well have been due to the croton oil itself (see p. 185). The author did not include a group of mice painted with croton oil only.

Petering (1952), Gellhorn (1953), and Boyland (1954), review the status of aminopterin in the treatment of malignant disease, including leukaemia. Aminopterin has been shown to exert a specific effect on mitosis (Hughes, 1950). There are no reports ascribing any carcinogenic action to aminopterin, or to related folic acid analogues.

The second class of substances has been designated as *exerting a specific effect on cells undergoing mitosis.*

An effect of urethane itself on the process of mitosis was reported by Ludford (1936). He observed abnormal metaphases following treatment of tissue cultures with 2 per cent urethane. Lasnitzki (1949) studied the effect of 0·4 per cent urethane on growth and mitosis in cultures of normal and malignant tissues from the mouse. The growth and mitotic rates of normal tissues were reduced to approximately half the control levels. Cultures of C57 sarcoma and adenocarcinoma 63, however, showed enhancement of growth, and mitotic rates of 2 to 4 times the control values. An increased number of abnormal cell divisions were seen in the adenocarcinoma cultures. Dustin, P. (1947*b*), studied the effect of urethane on the epithelium of the intestinal crypts, and observed an effect similar to that caused by X-irradiation. He therefore placed urethane in the class of "*radiomimetic agents*" (Dustin, A. P., 1929). More recently Loveless and Revell (1949) have pointed out that there is little evidence that urethane gives rise to the irregular chromosome-breakage which characteristically appears 18–24 hours after exposure to X-irradiation.

The arrest of mitosis in metaphase by colchicine is well known (see Ludford (1953) for review).

Podophyllin exerts a similar action (King, 1948 ; King and Sullivan, 1946).

Cornman (1947) claimed that coumarin produced metaphase-arrest in the roots of *Allium cepa* and *Lilium longiflorum.* Ostergren (1948) found that coumarin produced chromosome bridges and breaks in *Allium cepa.* Since this substance is commonly used in some countries as a flavouring agent in tobacco (Vasic, 1953), we thought it worth while including in the present series. It is a lactone (see p. 182).

The evidence that hydroquinone belongs to this class is dependent on the findings of Zylberszac (1939), and Dustin P. (1947*a*), in the epithelium of the intestinal crypts of the mouse. The latter concluded that hydroquinone exerted a radiomimetic action. Loveless and Revell (1949), on the other hand, doubt whether hydroquinone should be regarded as exerting a specific effect on mitosis.

Sodium cacodylate was one of the organic arsenical compounds studied by Dustin and his collaborators (Piton, 1929 ; Dustin and Piton, 1929 ; Dustin and Grégoire, 1933), and was observed to give rise to metaphase-arrest indistinguishable from that due to colchicine.

None of the substances included in this category can be regarded as definitely carcinogenic for mouse skin. Harde (1939) applied colchicine to the skin of 26 mice weekly. Three mice eventually developed tumours of the skin, a further 2 mice developed tumours of other organs. Hamperl (1946) produced no tumours in mice after painting with colchicine. Berenblum (1951) tested podophyllotoxin on mouse skin, and concluded that it possessed slight anticarcinogenic, but no

carcinogenic or co-carcinogenic activity ; Gwynn and Salaman (1953) found no evidence of co-carcinogenic activity.

A third class of substances with *narcotic* properties were tested for power to initiate carcinogenesis. They resemble urethane not only in their action on nervous tissue, but also in retarding or suppressing mitosis (Ludford, 1953, for review) ; and the possibility was considered that there might be some connection between these properties and that of initiation of carcinogenesis. Urethane was one of the narcotics used by Quastel and his associates in their study of the chemical mechanism of hypnotic action (Quastel and Wheatley, 1933, 1934 ; Michaelis and Quastel, 1941). They showed that these substances all have an inhibiting effect on aerobic glycolysis by brain tissue *in vitro*. The effect is reversible, i.e. when the drug is removed by washing the greater part of the glycolytic activity reappears. Indole also has a powerful inhibitory effect on aerobic glycolysis of brain *in vitro* ; but in this case activity is not restored by washing the tissue (Quastel and Wheatley, 1934).

A group of narcotics was chosen for test which included members of a wide variety of chemical types (see Table I, Class III). Indole was also tested (Class VI), for the reason given above.

Following the discovery by Nettleship and Henshaw (1943) of the lung tumour-inducing property of urethane, Larsen, Rhoads and Weed (1946) examined a series of hypnotics for the same property (including chloral hydrate, paraldehyde, and phenobarbitone). None of the substances tested increased the incidence of pulmonary tumours in mice.

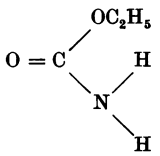
The fourth class of substances has only two members, both of which possess a *pharmacological action in some respect similar to urethane*, though they cannot be included in the preceding classes.

o-Phenanthroline inhibits the photochemical decomposition of water by chloroplasts *in vitro*, and photosynthesis *in vivo* (Warburg and Luttgens, 1944 ; Arnon and Whatley, 1949). A similar inhibitory action has been ascribed to the urethanes as a group (see Gaffron and Fager, 1951, for review).

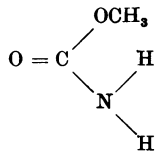
Physostigmine was included because its activity as a choline-esterase inhibitor has been attributed to the urethane group in its molecule, although urethane itself is inactive in this respect. (Stedman and Stedman, 1931, 1932 ; Eadie, 1942).

The substances in the fifth class were chosen because they could be regarded as *urethane derivatives* or in some way *structurally related to urethane*. Their structural formulae are shown in Fig. 1.

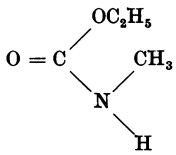
Other workers have examined urethane analogues from different standpoints. Lefèvre (1939), and Simonet and Guinochet (1939), described a colchicine-like effect in plant cells due to treatment with ethyl N-phenylcarbamate (phenylurethane). Dustin (1947*b*) found that ethyl N-phenylcarbamate was more toxic than urethane, and caused similar changes in mouse intestine, whereas methylcarbamate was of low toxicity and caused no narcosis or mitotic disturbance. Larsen (1947*a*, 1947*b*, 1948), studying the narcotic and lung tumour-inducing activities of urethane analogues, concluded that methylcarbamate was inactive in both these respects, and that increasing N-alkylation led to decreasing lung tumour-inducing activity. He considered that N-alkylated urethanes probably acted only after dealkylation to urethane in the body. Skipper *et al.* (1948) made an exhaustive study of urethane derivatives, including methylcarbamate, ethyl-



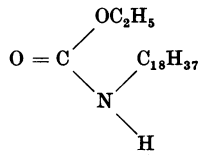
Ethyl carbamate (urethane).



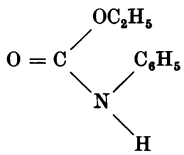
Methyl carbamate (Group 30).



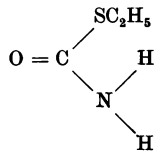
Ethyl N-methyl carbamate (Group 31).



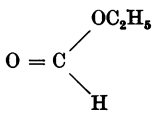
Ethyl N-octadecyl carbamate (Group 32).



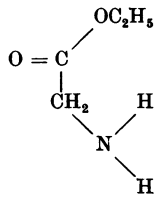
Ethyl N-phenyl carbamate (Group 33).



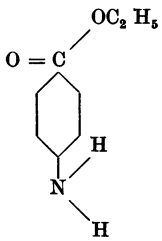
Ethyl thiolcarbamate (Group 34).



Ethyl formate (Group 30).



Glycine ethyl ester (Group 35).



Benzocaine (p-amino ethyl benzoate) (Group 29).

FIG. 1.—Structural formulae of urethane, urethane derivatives, and related compounds. (Group numbers refer to Table I, Class V.)

thiolcarbamate, ethyl N-methylcarbamate, and ethyl N-phenylcarbamate. The toxicity of these four, except methylcarbamate, if expressed on a molar/body weight basis, was greater than that of urethane. Methylcarbamate alone showed no anaesthetic properties. Ethyl N-methyl carbamate and ethyl N-phenylcarbamate both caused leucopenia when administered in non-lethal doses, but neither produced such a striking reversal of the differential leucocyte count as urethane. Rose *et al.* (1950) examined some thirty simple homologues of urethane for growth inhibitory activity against the Walker carcinoma 256 in rats. Of these only *bis*- and *tris*-carbethoxyamine even approached urethane in activity.

Benzocaine, apart from its structural relation to urethane (Fig. 1), is of interest in that it has a slight local anaesthetic action. Moreover it is an ester of p-aminobenzoic acid, and thus a potential inhibitor of the action of the sulphonamides. A similar action has been ascribed to urethane (Johnson, Eyring, and Kearns, 1943).

The subcutaneous injection of very large doses of ethyl N-octadecylcarbamate in rats has been found to produce sarcomata (Walpole, 1953, personal communication). Because of this result we thought it worth while testing as an initiator.

The sixth class consists of four *miscellaneous* substances, 1,2-benzanthracene, cantharidin, indole, and β-propiolactone.

1,2-Benzanthracene was selected mainly in order to repeat the work of Graffi *et al.* (1953). In their experiments 75 mice received alternate applications (each of 3 drops) of 0·5 per cent 1,2-benzanthracene in acetone and 5 per cent croton oil in liquid paraffin, at 3 to 4 day intervals. This treatment was continued for a year, by which time there were 18 benign skin tumours in the 9 surviving mice. At the same time 13 survivors of 160 mice, which had been treated with croton oil only, showed only one tumour. Previously Berenblum (1941) recorded a negative result from a similar experiment, in which two groups of mice were painted weekly, one with a saturated solution of 1,2-benzanthracene in acetone, and the other with a similar solution containing also 0·025 per cent croton oil resin. No tumours were present in either group after 20 weeks of continuous treatment.

Steiner and Falk (1951) reported the production of 8 sarcomata in 44 mice injected subcutaneously with 5 mg. of 1,2-benzanthracene, and in the same paper reviewed the findings of other workers on the carcinogenicity of this substance. Isolated tumours of mouse epidermis, the tissue in which we are primarily interested, have been seen following skin application of 1,2-benzanthracene (Kennaway, 1930 ; Cook, 1933 ; Barry *et al.*, 1935 ; and Hill *et al.*, 1951).

As pointed out by Steiner and Falk (1951) 1,2-benzanthracene is of interest in the study of the relationship between carcinogenic potency and molecular structure, because of its borderline position both in the scale of carcinogenicity and that of physico-chemical properties (e.g. electron density at the " K " region, photodynamic effects, and ultraviolet absorption spectrum) which have been correlated with carcinogenicity (Mottram and Doniach, 1938 ; Jones, 1940 ; Koffler and Markert, 1951 ; Badger, 1954 (review)).

The inclusion of indole has already been discussed in connection with substances of Class III (*vide supra.*)

β-propiolactone $\begin{Bmatrix} CH_2—CH_2 \\ | \quad | \\ O——CO \end{Bmatrix}$ was found to possess mutagenic activity in *Neurospora* (Smith and Srb, 1951). Walpole *et al.* (1954) found it to be carcinogenic for the subcutaneous tissue of the rat. The latter authors commented on the resemblance in chemical properties between this substance and ethyleneimine. In view of these reports, and the simple nature of the compound, we thought it worth including in the present series of tests.

Cantharidin, a powerful skin irritant, reduced the carcinogenic effect of tar when applied alternately with the latter to mouse skin ; in this respect it resembled mustard gas (Berenblum, 1935). Orr (1938) tested cantharidin for carcinogenic action on mouse skin and obtained no tumours. Gwynn and Salaman (1953)

tested it for co-carcinogenic activity, with a negative result. The possibility that this intensely toxic substance might be an initiator of carcinogenesis had thus not been excluded. In view of the properties of β-propiolactone it is worth noting that cantharidin is also a lactone.

Choice of dose and mode of application.

It was not found possible to follow a uniform scheme of dosage throughout.

In the case of the chemical analogues of urethane the object was to compare their initiating power with that of urethane itself; they were therefore used in quantities molecularly equivalent to, or greater than, a dose of urethane the effect of which was known. Fortunately this could be done without interference by toxic effects. An exception was ethyl N-octadecyl carbamate, of which an equimolecular dose would have been difficult to apply, and which was included because of the results of Walpole (1953, personal communication) already referred to.

In the case of nitrogen mustard, which had given a result of doubtful significance in a previous experiment (Salaman and Roe, 1953), a dose was used which produced most tumours in the former test.

In the other groups, whenever possible, the largest doses which just failed to produce toxic symptoms, or other signs of general effect, e.g. narcosis, were used. In some cases, however, the limit was set by the maximum attainable concentration in a suitable solvent, in others by the fact that strong solutions left heavy deposits of solid substance as the solvent evaporated, which were rubbed off without penetrating the skin.

The mode of application also varied to some extent. In the early stages of the work it was thought that repeated doses of the initiator given alternately with croton oil would probably produce more tumours than one, or perhaps two, applications of the initiator followed, after an interval, by weekly applications of croton oil. Experience has shown that initiation is either demonstrable by both methods (e.g. urethane, T.E.M.) or not demonstrable by either (e.g. nitrogen mustard, chloral hydrate). The methods are therefore to be regarded as qualitatively equivalent.

Details of dosage and methods of application are given in the following section, and in Table I.

MATERIALS AND METHODS.

Mice.

Stock albino male mice of the " S " strain (Salaman and Gwynn, 1951; Salaman and Roe, 1953), fed on cubes prepared according to the Rowett Institute formula (Thomson, 1930*a*, 1930*b*) plus fresh greenstuff twice a week, and water *ad libitum*, were used throughout. They were vaccinated on the tail with sheep lymph (kindly supplied by the Lister Institute of Preventive Medicine) as a precaution against ectromelia. Only positive reactors were used. At the beginning of experiments they were 7–9 weeks old.

Chemical substances and solvents.

Test substances.—Benzocaine (ethyl ester of p-amino benzoic acid), cantharidin, carbromal (1-bromo-ethyl-butyryl-urea), chlorbutol (1 : 1 : 1-trichloro-2-methyl-2-propanol), coumarin, ethyl carbamate (urethane), ethyl formate (ethone), ethyl

N-phenylcarbamate (phenylurethane), glycine ethyl ester hydrochloride, hydroquinone, indole, methyl carbamate, methyl sulphonal, ortho-phenanthroline (AR grade), physostigmine (eserine), and sodium cacodylate, were obtained from British Drug Houses, Ltd.

1,2-Benzanthracene, colchicine, and β-propiolactone were obtained from Messrs. L. Light and Co., Ltd.; chloral hydrate from T. H. Smith and Son, Ltd. (Edinburgh); nitrogen mustard (HN2, methyl bis (β-chloroethyl) amine-hydrochloride) from Messrs. Merck and Co.; and phenobarbitone (luminal) from Messrs. Huffer and Smith, Ltd. (Croydon, Surrey).

Paraldehyde (2,4,6-trimethyl-1,3,5-trioxane), and podophyllin resin (for preparation of podophyllin extract, see below) were obtained from Messrs. Allen and Hanburys, Ltd.

Dr. Walpole of Pharmaceutical Division, Imperial Chemical Industries, Ltd., kindly supplied ethyl N-methylcarbamate, ethyl N-octadecylcarbamate, ethyl N-thiolcarbamate (thiourethane), and triethylene melamine (T.E.M., tris ethyleneimino-s-triazine).

Mr. G. Timmis of the Chester Beatty Institute, London, kindly supplied us with Myleran (GT 2041, 1 : 4-dimethane-sulphonoxy butane).

Aminopterin (4-amino-pteroylglutamic acid) was kindly supplied to us by Cyanamid Products, Ltd., Lederle Laboratories Division.

Croton oil.—Expressed oil of the same batch used in previous experiments in this laboratory (e.g. Salaman and Gwynn, 1951; Salaman and Roe, 1953) was used. It was obtained from Messrs. Boots Pure Drug Co., retailing the product of Messrs. Stafford Allen and Sons, Ltd., 20 Wharf Rd., N.1.

Solvents.—Acetone (AR grade), and ether (Aether Puriss. grade) were obtained from British Drug Houses, Ltd., carbowax 300 (polyethylene glycol of average molecular weight 300) from General Metallurgical and Chemical Co., Ltd., and methyl alcohol from James Burrough, Ltd.

Preparation of solutions.

All solutions were prepared weight per total volume in the solvent shown (Table I) unless otherwise stated. The preparation of glycine ethyl ester from the hydrochloride is described in the experimental section.

The podophyllin extract used was prepared from the crude resin by extraction with acetone. Dosage was calculated on the dry weight of the extract after filtration and evaporation.

Technique of application.

The hair was clipped from the whole back, from forelimbs to tail, before treatment and at intervals when necessary. Solutions were delivered from calibrated pipettes, care being taken that they spread as evenly as possible over the whole clipped area. A glass spreader was used for solutions which did not otherwise spread easily (e.g. glycine ethyl ester hydrochloride).

Recording of tumours.

Mice were examined throughout the course of croton oil treatment at weekly intervals, and all tumours of 1 mm. diameter and over recorded.

The groups of mice in which tumours appeared were kept after the end of croton oil treatment and examined at intervals for the development of malignant tumours. Many of these mice are still under observation.

Examination of mice for lung adenomas at post mortem.

The majority of mice were killed one week after the end of the standard course of croton oil treatment, and examined *post mortem*. The lungs were removed and the surface of each lobe carefully examined, in the fresh state, for the presence of pulmonary adenomata.

Histological examination.

Specimens of skin from mice additional to each group were removed under ether anaesthesia three days after one and/or two applications of the test substance. Mice which died during or were killed at the end of the tests were examined *post mortem* for lung adenomata and other abnormalities. Specimens for histological examination were fixed in Zenker's fluid, embedded in paraffin wax, and stained with haematoxylin and eosin-Biebrich scarlet (Gwynn and Salaman, 1953).

Experimental.

A. *Skin Tumour Production.*

Treatment with croton oil alone.

A group of mice (Group 43) were reserved as croton oil controls. They received weekly applications of 0·3 ml. 0·5 per cent croton oil in acetone. Altogether these mice have received 72 weekly applications, but for the purposes of controlling the present experiments only the first 18 applications need be considered, and for convenience we have called these " a standard course of croton oil treatment."

Tumour incidence.—Table I shows that one week after the 18th application of croton oil one of the 20 survivors bore 3 tumours. Subsequently tumours appeared at a steady rate, reaching the levels of 1 tumour per mouse in 19 survivors after 44 applications, 2 tumours per mouse in 17 survivors after 57 applications, and 3 tumours per mouse in 12 survivors after 66 applications. So far 3 malignant tumours have been seen in this group (after 55, 67, and 72 weekly applications respectively).

Class I : Antileukaemic agents.

Four substances in this class were tested : nitrogen mustard, Myleran, triethylene melamine (T.E.M.), and aminopterin. Table I gives details of treatment.

Groups 1 and 2 received applications of nitrogen mustard weekly for 15 weeks at dose-levels indicated by the results of a previously reported experiment (Salaman and Roe, 1953).

In a preliminary test single doses of Myleran and T.E.M. were given, which subsequently proved to be too high (Groups 3 and 6 respectively). A majority of the mice in both groups died, but the survivors were given the standard course of 18 weekly applications of croton oil.

TABLE I.—*The Screening of a Range of Substances for Initiating Activity.*

Group.	Number of mice.	Treatment with the test substance. Substance.	Total dose.	Method of application.†	Interval between 1st application of test substance and beginning of croton oil treatment.‡	Tumour incidence at the end of croton oil treatment. Tumour-bearing/surviving mice.	Tumours/surviving mice.
				CLASS I.—*Antileukaemic Agents.*			
1	10	Nitrogen mustard	1·5 mg.	15 weekly applications 0·03%	3 days*	3/10	4/10
2	10	,, ,,	4·5 ,,	15 ,, ,, 0·1%	3 ,, *	1/9	1/9
3	24	Myleran	2·0 ,,	1 application, 0·67%	3 weeks	0/7	0/7
4	10	,,	3·6 ,,	6 weekly applications, 0·067% and 4 weekly applications, 0·2%	4 days*	0/8	0/8
5	10	,,	6·0 ,,	10 weekly applications, 0·2%	4 ,, *	1/18	1/18
6	24	Triethylene melamine	0·5 ,,	1 application, 0·17%	3 weeks	3/7	7/7
7	10	,, ,,	0·24 ,,	1 ,, 0·08%	3 ,,	5/10	18/10
8	10	,, ,,	1·8 ,,	15 weekly applications, 0·04%	3 days*	7/10	22/10
9	10	,, ,,	0·24 ,,	1 application, 0·08%	No croton oil treatment	0/8	0/8
10	10	,, ,,	1·8 ,,	15 weekly applications, 0·04%	,, ,, ,,	0/9	0/9
11	25	Aminopterin	0·15 to 0·21 mg.	5 to 6 weekly applications, 0·01 to 0·02% in saturated solution of sodium bicarbonate in methanol (see text)	3 days*	1/14	1/14
				CLASS II.—*Substances Exerting a Specific Effect on Mitosis.*			
12	10	Colchicine	1·0 mg.	1 application, 0·33%	3 weeks	0/10	0/10
13	10	,,	3·6 ,,	15 weekly applications, 0·08%	3 days*	1/8	1/8
14	25	Coumarin	45·0 ,,	1 application, 15%	3 weeks	1/17	2/17
15	40	,,	150·0 ,,	1 application, 10%, followed at weekly intervals by 12 applications of 3·3%	10 days*	0/13	0/13
16	24	Hydroquinone	20·0 ,,	1 application, 6·7%	3 weeks	1/22	1/22
17	20	Podophyllin	4·5 ,,	15 weekly applications, 0·1%	3 days*	0/19	0/19
18	25	Sodium cacodylate	85·0 ,,	1 application, 28%, in methyl alcohol	3 weeks	0/19	0/19
				CLASS III.—*Narcotic Agents.*			
19	20	Carbromal	30·0 mg.	2 weekly applications, 5%	4 weeks	1/13	1/13
20	20	Chloral hydrate	24·0 ,,	2 ,, ,, 4%	4 ,,	4/17	4/17
21	20	,, ,,	225·0 ,,	15 ,, ,, 5%	3 days*	4/20	4/20
22	20	Chlorbutol	96·0 ,,	2 ,, ,, 16%	4 weeks	0/18	0/18
23	20	Methylsulphonal	48·0 ,,	2 ,, ,, 8%	4 ,,	2/18	4/18
24	20	Paraldehyde	300·0 ,,	2 ,, ,, 50%	4 ,,	1/18	1/18
25	20	Phenobarbitone	12·0 ,,	2 ,, ,, 2%	4 ,,	1/19	1/19
26	20	,,	90·0 ,,	15 ,, ,, 2%	3 days*	1/20	1/20

Group.	Number of mice.	Treatment with the test substance. Substance.	Total dose.	Method of application.†	Interval between 1st application of test substance and beginning of croton oil treatment.‡	Tumour incidence at the end of croton oil treatment. Tumour-bearing / surviving mice.	Tumours / surviving mice.
		CLASS IV.—*Pharmacological Analogues of Urethane not Included in Classes I, II, and III.*					
27	20	o-Phenanthroline	300·0 mg.	10 weekly applications, 10%	3 days*	0/20	0/20
28	20	Physostigmine	2·25 ,,	15 ,, ,, 0·05%	3 ,, *	0/17	0/17
		CLASS V.—*Urethane Derivatives and Related Compounds.*					
29	20	Benzocaine	1·2 g.	15 weekly applications, 27%	3 days*	0/19	0/19
30	20	Ethyl formate	2·76 g.	10 weekly applications of 0·3 ml. of the neat agent	3 ,, *	0/14	0/14
31	18	Ethyl N-methyl carbamate	250·0 mg.	2 weekly applications, 42%	3 weeks	7/17	9/17
32	20	Ethyl N-octadecyl carbamate	180·0 ,,	9 weekly applications, 6·7%, in ether	3 days*	0/20	0/20
33	20	Ethyl N-phenyl carbamate	500·0 ,,	2 applications (with an interval of 15 mins.), 13·9% on 1st, 3rd, 5th, 8th, 10th, and 12th days	3 weeks	6/19	16/19
34	18	Ethyl thiolcarbamate	180·0 ,,	3 applications at intervals of 4 days, 20%	3 ,,	1/16	1/16
35	20	Glycine ethyl ester (for preparation see text)	0·57 g.	10 weekly applications, 19% in 20% aqueous carbowax 300	3 days*	0/18	0/18
36	20	Methyl carbamate	1·12 g.	15 weekly applications, 25%	3 ,, *	1/18	1/18
		CLASS VI.—*Miscellaneous Substances.*					
37	20	1,2-Benzanthracene	6·0 mg.	2 weekly applications, 1%	3 weeks	7/18	21/18
38	10	,,	6·0 ,,	2 ,, ,, 1%	No croton oil treatment	0/8	0/8
39	10	,,	45·0 ,,	15 ,, ,, 1%	,, ,, ,, ,,	0/9	0/9
40	20	Cantharidin	0·63 ,,	9 weekly applications, 0·01%, followed by 6 weekly applications, 0·02%	17 days*	4/17	6/17
41	20	Indole	12·0 ,,	2 weekly applications, 2%	4 weeks	2/20	2/20
42	20	β-propiolactone	155·0 ,,	2 weekly applications, 10% and 5% respectively, followed, after an interval of two weeks, by 12 weekly applications, 2·5% (see text)	24 days*	15/19	160/19
		Control Group.					
43	20	Croton oil only (standard course‡)	—	—	—	1/20	3/20

* The earlier weekly applications of croton oil alternated at 3 to 4-day intervals with those of the test substance.

† Each application was 0·3 ml. of solution in acetone (unless otherwise stated) at the concentration shown (w/v in the case of solids, and v/v in the case of liquids.

‡ Croton oil treatment was standardized throughout at 18 applications of 0·3 ml. of 0·5 per cent solution in acetone.

Groups 4 and 5 were painted weekly with Myleran for 10 weeks, receiving altogether a higher total dose than mice in Group 3. As in all groups in which the test substance was given in divided doses at weekly intervals the first applications of croton oil alternated at 3 to 4 day intervals with those of the test substance.

Group 7 was given a single application of T.E.M., and Group 8 weekly applications of T.E.M. for 15 weeks. In both groups croton oil treatment was begun 3 days after the first application of T.E.M. When the standard 18 applications had been given it was decided to continue them for a few weeks, since at that time the number of tumours was increasing rapidly.

Groups 9 and 10 were given T.E.M. treatment corresponding to that of Groups 7 and 8 respectively, but no croton oil treatment thereafter.

Considerable difficulty was encountered in preliminary solubility and toxicity tests with aminopterin. The most suitable solvent for skin application was found to be a saturated solution of sodium bicarbonate in methyl alcohol (approximately 0·05 per cent). This solution was made up immediately before use. A single dose of 0·3 ml. 0·02 per cent aminopterin in this solvent was given to 20 mice in Group 11. This dose proved to be too high, and many died. Subsequently the survivors, together with 5 hitherto untreated mice, received 5 weekly applications of 0·3 ml. 0·01 per cent aminopterin in the same solvent. All mice began a standard course of croton oil treatment 3 days after the first application of aminopterin.

Tumour incidence.—The number of tumour-bearing mice, and the incidence of tumours in mice surviving until one week after the end of croton oil treatment, are shown in Table I. One week after the 18th application of croton oil 47 tumours were present in 27 survivors (Groups 6, 7, and 8) which had received treatment with T.E.M. and croton oil. This tumour incidence increased with further croton oil treatment. Fig. 2 shows the rates of development of tumours in these groups and, for comparison, that in Group 43 which received croton oil treatment only.

A significance test for the difference between the mean numbers of tumours per mouse in Groups 6 and 7 (pooled) and in Group 43, gives $t = 2{\cdot}90$ on 35 degrees of freedom, a value which could be exceeded by chance with a probability of $P = 0{\cdot}006$. The comparison between the mean numbers of tumours per mouse in Group 8 and in Group 43 gives $t = 2{\cdot}42$ on 28 d.f.; $P = 0{\cdot}02$. No tumours were observed in 17 survivors which received T.E.M. only (Groups 9 and 10).

It is concluded that T.E.M. is an effective initiator of skin carcinogenesis in the mouse, but not carcinogenic in the doses used.

Nitrogen mustard and croton oil (Groups 1 and 2) produced 5 tumours in 19 survivors at the end of croton oil treatment. This was in accordance with our previously reported borderline result with this substance (Salaman and Roe, 1953), the mean number of tumours being slightly greater than that obtained with croton oil only, but not significantly so ($t = 0{\cdot}57$ on 37 d.f.; $P = 0{\cdot}57$).

The distributions of the number of tumours on individual mice, in all the groups reported in this paper, are markedly skew. The significance levels indicated by the t-tests will be somewhat affected by this fact. Moreover it is possible that small differences in the incidence of tumours will be more readily detected by some quantity other than the mean number of tumours per mouse. For the comparison of Groups 1 and 2 (pooled) with Group 43, for instance, it is possible to rank together the mice in both series in order of number of tumours borne, and to ask whether the mean rank for Groups 1 and 2 (pooled) differs significantly from that

for Group 43. The appropriate test (Wilcoxon's) is described by Kruskal and Wallis (1952, pp. 590–592). Formulae for the expected value and variance of $\overline{R}$, the mean rank for mice in, say, the control group (it is immaterial which group is chosen), are given by Kruskal and Wallis. The square of the difference between $\overline{R}$ and its expected value, divided by its variance, is distributed approximately as χ^2 on 1 d.f. Application of this test to the series in question gives $\chi^2 = 1{\cdot}90$ on 1 d.f.; $P = 0{\cdot}16$. The difference is still not significant. However in some other groups, as shown below, the ranking test indicates a significant difference at the customary 5 per cent level, whereas the t-test does not.

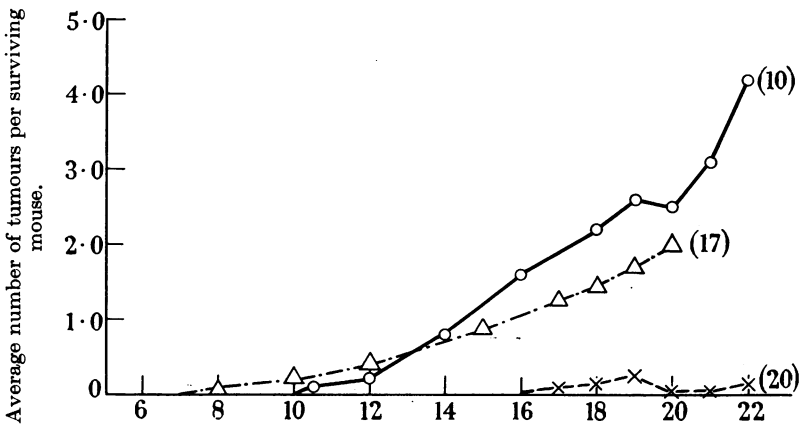


FIG. 2.—Initiating effect of triethylene melamine (T.E.M.).

△— - — - —△ Groups 6 and 7 (combined): 0·5 mg., and 0·24 mg. T.E.M. respectively (given as a single application in each case) followed by 20 weekly applications of of 0·5 per cent croton oil (beginning 3 weeks after treatment with T.E.M.).

○————○ Group 8: 1·8 mg. T.E.M. (15 weekly applications of 0·04 per cent) and 22 weekly applications of 0·5 per cent croton oil, alternating at first with the former.

× - - - - - - - - - × Group 43: Control group: weekly croton oil treatment only.

Acetone was the solvent throughout. The numbers of mice in each group at the beginning of the experiment and further details of treatment are shown in Table I. Numbers of survivors are shown in brackets.

Combinations of Myleran and croton oil (Groups 3 to 5) produced no more tumours than croton oil alone.

An attempt was made to give a higher total dose of Myleran. As in all experiments involving skin applications it is difficult to say how much of the apparent toxic effect of a substance is due to absorption from the gastro-intestinal tract following licking, and how much to absorption through the skin. To ascertain these proportions in the case of Myleran a group of 10 mice were prevented from licking themselves by means of plaster of Paris collars; 5 were then painted with 10 mg. Myleran to the clipped dorsal skin, and the remaining 5 mice similarly with 5 mg. Thereafter mice were kept in separate compartments to prevent their licking one another. The collars were removed after one week. All the mice which received 10 mg. Myleran died within 3 weeks, but those which received 5 mg. remained healthy. The latter were again put in plaster collars and given a

further application of 5 mg. each. Within 3 weeks of this second treatment all 5 were very sick or dead.

It was concluded that Myleran in the maximum tolerated dose was not an initiator of carcinogenesis in mouse skin, and that toleration could not be increased by the prevention of licking.

Mice treated with aminopterin and croton oil (Group 11) developed no tumours.

Class II : Substances exerting a specific effect on mitosis.

Five substances in this class were tested : colchicine, coumarin, hydroquinone, podophyllin, and sodium cacodylate. Details of treatment are given in Table I.

Tumour incidence.—In the doses employed none of these substances showed any evidence of initiating activity.

Class III : Narcotic agents.

Six substances were included in this class : carbromal, chloral hydrate, chlorbutol, methyl sulphonal, paraldehyde, and phenobarbitone. Details of treatment are given in Table I.

Tumour incidence.—In Group 20, which received two applications of 4 per cent chloral hydrate with a week's interval between them, followed by a standard course of croton oil, 4 tumours were present on 17 survivors one week after the end of croton oil treatment. The mean number of tumours does not differ significantly from that in Group 43 treated with croton oil only ($t = 0{\cdot}93$ on 35 d.f. ; $P = 0{\cdot}36$). The ranking test gives $\chi^2 = 2{\cdot}30$ on 1 d.f. ; $P = 0{\cdot}13$. The substance was re-tested at a higher dose (Group 21 : 15 weekly applications of 5 per cent). However in this group also the yield of tumours (4 in the 20 survivors) did not differ significantly from that in mice receiving croton oil only (Group 43).

In Group 25 which received 2 applications of 2 per cent phenobarbitone with an interval of one week between them, followed by a standard course of croton oil treatment, only one papilloma was present a week after the final application of croton oil. However a week later an ulcerated tumour appeared which was found, on histological examination, to be a fairly well differentiated squamous carcinoma which had penetrated the panniculus. Although an isolated finding this was, in our view, a remarkable one. We have never seen a malignant skin tumour arising spontaneously in mice of the strain used for these experiments. However, as described above, in Group 43, which received croton oil weekly for up to 72 weeks, 3 carcinomata have appeared, though none before the 55th week.

Because of this isolated finding phenobarbitone was re-tested at a higher dose level, in Group 26. However, no malignant tumours appeared in the group. It may be profitable to explore this part of the field again when we have learnt more of the process of initiation, and of the development of malignancy.

Class IV : Pharmacological analogues of urethane not included in Classes I, II and III.

Two substances in this class were tested : o-phenanthroline and physostigmine. Details of treatment are given in Table I.

Tumour incidence.—No tumours were seen in either group at the end of the standard course of croton oil treatment.

Class V : Urethane derivatives and related compounds.

Eight substances were included in this class : benzocaine (p-amino ethyl benzoate), ethyl formate, ethyl N-methyl carbamate, ethyl N-octadecyl carbamate, ethyl N-phenylcarbamate, ethyl thiolcarbamate, glycine ethyl ester, and methyl carbamate. Details of treatment are given in Table I.

The solution of glycine ethyl ester (Group 35) was made freshly each week immediately before use, by the following method. The solvent was first prepared by adding 1 volume of carbowax 300 to 4 volumes of distilled water. A 30 per cent w/v solution of glycine ethyl ester hydrochloride was then made up in this solvent. To this solution aqueous sodium hydroxide was added drop-wise until it was neutral to phenol red. Finally the volume was adjusted, by adding more of the prepared solvent, to make a 19 per cent w/v solution of glycine ethyl ester.

Tumour incidence.—Of 17 survivors which received 250 mg. ethyl N-methyl carbamate followed by a standard course of croton oil (Group 31) 7 bore a total of 9 tumours at the end of treatment. The difference between the mean number of tumours in Group 31 and that in Group 43, is not significant by the *t*-test ($t = 1{\cdot}57$ on 35 d.f. ; $P = 0{\cdot}12$). The ranking test, however, gives $\chi^2 = 6{\cdot}2$ on 1 d.f. ; $P = 0{\cdot}013$. In Group 33, which received 500 mg. of ethyl N-phenyl carbamate, of 19 mice surviving until the end of croton oil treatment 6 bore a total of 16 tumours. The difference between the mean number of tumours in Group 33 and that in Group 43 does not quite reach the customary level of significance by the *t*-test ($t = 1{\cdot}87$ on 37 d.f. ; $P = 0{\cdot}07$), but the ranking test gives $\chi^2 = 4{\cdot}3$ on 1 d.f. ; $P = 0{\cdot}04$. It must be emphasized that the ranking test was chosen after the data had been examined, and perhaps exaggerates the significance.

Fig. 3 shows the rates of development of tumours in Groups 31, 33, and 43, together with that in a group from a previous experiment (Salaman and Roe, 1953) which received a dose of urethane (240 mg.) approximately equivalent on a molar basis to those of the test substances in Groups 31 and 33.

We conclude that ethyl N-methyl carbamate and ethyl N-phenyl carbamate probably both possess some initiating activity, but of a lesser degree than that of urethane itself. Two further groups of mice are at present under test, both receiving ethyl N-phenyl carbamate at higher dosage than Group 33. One of these groups will receive no croton oil treatment, in order to determine whether the test substance is itself carcinogenic for mouse skin. (See Addendum II).

Ethyl thiolcarbamate (Group 34), given as three applications of 20 per cent w/v in acetone at intervals of 4 days, followed by croton oil treatment, gave rise to a solitary tumour in the 16 surviving mice. In a previously reported experiment (Roe and Salaman, 1954) a group of mice received three applications of 20 per cent w/v urethane in acetone at intervals of 4 days, followed by croton oil treatment ; in this group all of the 18 survivors bore tumours, and the total tumour yield was 70. We conclude that the substitution of a sulphur atom for the ester-linkage oxygen atom deprives the urethane molecule of its initiating activity.

Benzocaine, ethyl formate, glycine ethyl ester, and methyl carbamate, all applied in relatively high doses, showed no evidence of initiating activity.

Ethyl N-octadecyl carbamate in the dose given (see introduction) was without initiating effect.

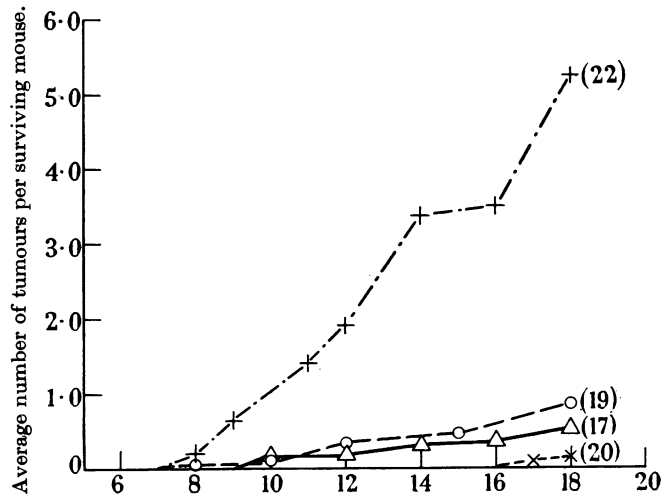


FIG. 3.—Initiating effect of urethane derivatives compared with that of urethane itself.

△————△ Group 31 : 250 mg. ethyl N-methyl carbamate (2 applications of 42 per cent with an interval of one week) followed by a standard course of croton oil treatment (18 applications of 0·5 per cent).

○— — — —○ Group 33 : 500 mg. ethyl N-phenyl carbamate (2 applications 13·9 per cent on 1st, 3rd, 5th, 8th, 10th, and 12th days) followed by a standard course of croton oil treatment.

+— · — · —+ A group from a previous experiment : 240 mg. urethane (2 applications 20 per cent w/v with an interval of 15 minutes, on 1st and 8th days) followed, 4 weeks after the 1st application of urethane, by a standard course of croton oil treatment.

×· · · · · · · · · · × Group 43 : Control group : weekly croton oil treatment only.

Acetone was the solvent throughout. The numbers of mice in Groups 31, 33, and 43 at the beginning of the experiment are shown in Table I. The group receiving urethane and croton oil consisted of 26 mice. Numbers of survivors at the end of the experiment are shown in brackets.

Class VI : Miscellaneous substances.

The four substances included in this class were 1,2-benzanthracene, cantharidin, indole, and β-propiolactone. Details of treatment are given in Table I. In the case of Group 37, painted with 1,2-benzanthracene and croton oil, treatment with the latter was continued for two weeks beyond the standard course, since at that time the number of tumours was increasing rapidly.

Tumour incidence.—In mice which received 1,2-benzanthracene and croton oil (Group 37), one week after the 18th application of the latter there were 7 tumour-bearing mice and a total of 21 tumours among the 18 survivors. At this stage the mean number of tumours differed significantly from that in Group 43 ($t = 2·29$ on 36 d.f. ; $P = 0·03$). When two further applications of croton oil were given the incidence rose to 43 tumours in 13 mice out of 18 survivors. The rate of development of tumours is shown in Fig. 4. A control group which received similar doses of 1,2-benzanthracene without croton oil treatment developed no tumours (Group 38). Moreover no tumours appeared in a further group which were painted weekly with 1 per cent 1,2-benzanthracene for 15 weeks

(Group 39). We conclude that 1,2-benzanthracene is an initiator of carcinogenesis for mouse skin, but not carcinogenic in the doses given.

Of the 20 mice receiving applications of cantharidin together with a standard course of croton oil (Group 40) 17 survived until the end of treatment, and of these 4 bore a total of 6 tumours. The mean number of tumours does not differ significantly from that in Group 43 either by the t-test ($t = 0{\cdot}97$ on 37 d.f. ; $P = 0{\cdot}34$), or by the ranking test ($\chi^2 = 2{\cdot}45$ on 1 d.f. ; $P = 0{\cdot}12$).

Indole (Group 41) showed no evidence of initiating activity.

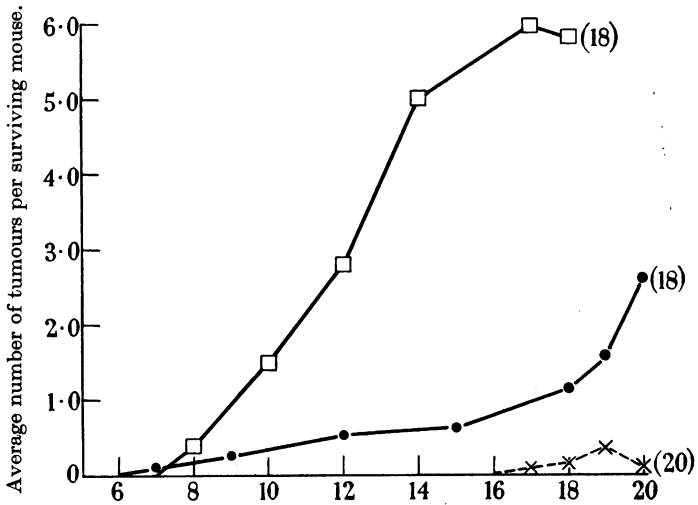


FIG. 4.—The initiating effect of 1,2-benzanthracene compared with that of 9,10-dimethyl-1,2-benzanthracene (DMBA).

●——● Group 37 : 6 mg. 1,2-benzanthracene (2 applications of 1 per cent with an interval of one week) followed by a standard course of croton oil treatment (18 applications of 0·5 per cent).

□——□ A group from a previous experiment : 0·3 mg. DMBA (single application 0·2 ml. 0·15 per cent) followed, after an interval of 4 weeks, by a standard course of croton oil treatment.

×- - - - - - - - - × Group 43 : Control group : weekly croton oil treatment only.

Acetone was the solvent throughout. There were 20 mice in each group at the beginning of the experiment. Numbers of survivors are shown in brackets.

After the first application of 10 per cent β-propiolactone mice of Group 42 showed considerable ulceration and scabbing of the skin. The beginning of croton oil treatment was therefore postponed, and the concentration of β-propiolactone in the second application reduced to 5 per cent. However the inflammatory skin reaction persisted, and all treatment was withheld for a further fortnight. By then the skin lesions had healed, and weekly applications of 2·5 per cent β-propiolactone were begun. The skin reaction to this concentration was minimal. Weekly croton oil applications were begun 3 days later. Of 19 survivors one week after the final application of croton oil 15 bore a total of 160 tumours. The mean number of tumours differs significantly from that in Group 43 ($t = 3{\cdot}55$ on 37 d.f. ; $P = 0{\cdot}001$). The rate of development of tumours is shown in Fig. 5. In 3 mice

tumours appeared before the 6th application of croton oil, which was earlier than tumours normally appear in mice painted weekly with croton oil following treatment with an initiator. We are therefore led to suspect either that β-propiolactone is carcinogenic for mouse skin, or that the scarring resulting from the first two applications of β-propiolactone acted as an additional promoter of tumour formation. The substance is being tested again, both by itself and together with croton

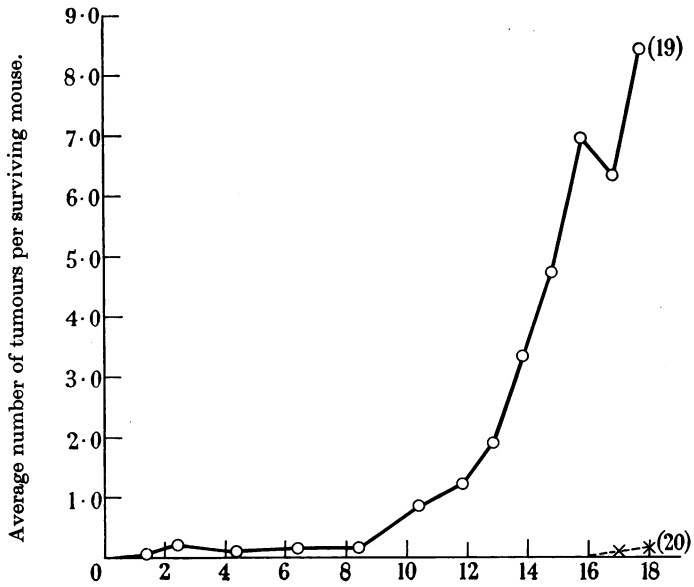


FIG. 5.—Tumour incidence in mice treated with β-propiolactone and croton oil.

○————○ Group 42: 155 mg. β-propiolactone (14 weekly applications, beginning at 10 per cent and falling to 2·5 per cent, see text) and 18 weekly applications of 0·5 per cent croton oil alternating at first with the former.

×- - - - - - - - -× Group 43: Control group: weekly croton oil treatment only.

Acetone was the solvent throughout. Both groups consisted of 20 mice at the beginning of the experiment. Numbers of survivors are shown in brackets.

Note.—In Group 42 tumours began to appear after only one week of croton oil treatment (i.e. 6 weeks earlier than in any other group).

oil. There is no doubt that β-propiolactone possesses initiating activity, but it is not yet possible to say whether in non-ulcerating doses it has promoting power as well. (See Addendum I.)

B. *Histological Findings in the Skin.*

The presence or absence of hyperplasia 3 days after the first and/or second applications of the test substances is shown in Table II.

Of the substances which, in conjunction with croton oil, gave rise to skin tumours, T.E.M., ethyl N-methyl carbamate, and ethyl N-phenyl carbamate, caused no hyperplasia of the mouse epidermis when applied alone. The reaction to β-propiolactone varied remarkably from mouse to mouse: one mouse three days

TABLE II.—*Histological Appearances in Mouse Skin after One or Two Weekly Applications of the Test Substances.*

Substance.	Concentration in acetone (per cent).	Day of biopsy.	Epidermal hyperplasia.
Nitrogen mustard	0·1	3rd and 10th	++
Myleran	0·67	,, ,, ,,	0
T.E.M.	0·17	,, ,, ,,	0
Aminopterin	0·02 (in saturated solution of $NaHCO_3$ in methyl alcohol)	,, ,, ,,	0
Colchicine	0·33	,, ,, ,,	+++
Coumarin	15·0	3rd	0
Hydroquinone	6·7	,,	0
Podophyllin	0·1	3rd and 10th	++
Sodium cacodylate	28·0 (in methyl alcohol)	3rd	±
Carbromal	5·0	10th	0
Chloral hydrate	4·0	,,	0
Chlorbutol	16·0	,,	0
Methyl sulphonal	8·0	,,	0
Paraldehyde	50·0	,,	0
Phenobarbitone	2·0	,,	0
o-Phenanthroline	10·0	3rd and 10th	+
Physostigmine	0·05	3rd	0
Benzocaine	27·0	3rd and 10th	0
Ethyl formate	100·0	,, ,, ,,	0
,, N-methyl carbamate	42·0	,, ,, ,,	0
,, N-octadecyl carbamate	6·7 (in ether)	3rd	0
,, N-phenyl carbamate	20·0	3rd and 10th	0
,, thiolcarbamate	20·0	3rd	0
Glycine ethyl ester	19·0 (in 20 per cent aqueous carbowax 300)	3rd and 10th	0
Methyl carbamate	25·0	,, ,, ,,	0
1,2-benzanthracene	1·0	,, ,, ,,	++
Cantharidin	0·01	,, ,, ,,	++
Indole	2·0	10th	0
β-propiolactone	2·5	3rd and 10th	0 to ++
Croton oil	0·5	,, ,, ,,	+++

after the 2nd application had an apparently normal epidermis, whilst others similarly treated showed quite marked hyperplasia. 1,2-Benzanthracene consistently gave rise to a moderate epidermal hyperplasia.

Of the substances which gave borderline results in the tests for initiation, cantharidin caused moderate epidermal hyperplasia. The reaction of the skin to nitrogen mustard has been described in a previous report (Salaman and Roe, 1953).

Both colchicine and podophyllin gave rise to striking histological appearances in mouse skin. In both cases the epidermis was thickened and many of the cells were greatly enlarged. Many clumped metaphases were seen, as well as aberrant chromosomes, fragmentation of chromatin, multinucleate cells, and a considerable number of pyknotic nuclei. These appearances have been fully described by other authors (King, 1948 ; King and Sullivan, 1946).

c. *Induction of Lung-adenomata.*

The experiments were not designed for the study of the induction of lung adenomata. Skin application would not have been the method of choice for that purpose. However, it is worth recording that the incidence of lung adenomata, as seen at *post mortem* one week after the end of croton oil treatment, was much greater in a few of the groups than in the rest.

Ethyl N-methyl carbamate followed by weekly croton oil gave rise to 26 adenomas in 9 mice out of 17 survivors, a result which confirms that of Larsen (1948) obtained by intraperitoneal injection of the substance alone. Methyl carbamate and croton oil gave rise to 12 adenomata in 7 mice out of 18 survivors ; Larsen (1947*b*) failed to induce adenomata by intraperitoneal injection of this substance alone.

Ethyl formate and croton oil gave rise to 15 adenomata in 6 mice out of 13 survivors ; the induction of lung adenomata by this substance has not been previously reported.

Ten mice treated with T.E.M. and croton oil (Groups 6, 7, and 8) have so far been examined *post mortem* at periods of 22 to 35 weeks after the end of croton oil treatment ; the remainder are still under observation for the appearance of malignant skin tumours. Seven of the former bore a total of 63 lung adenomata. Although this result is not strictly comparable with those in the other groups, it suggests that treatment with T.E.M. followed by weekly croton oil produces a significant number of adenomata.

Of the mice painted with croton oil weekly for up to 72 weeks (i.e. 4 times the duration of croton oil treatment received by other groups), some of which are still under observation, 15 mice have so far been examined *post mortem*. Ten of these bore no adenomata, the remaining 5 bore 20, 3, 2, 1, and 1 respectively. It therefore appears that croton oil is capable of producing some lung adenomata, but at present the evidence depends mainly on the findings in a single mouse. It should be emphasised that this result cannot be used as a control for other groups because of the much longer period of croton oil treatment and greater age of the mice at the time of examination in this group.

Among the other groups still under observation for the development of malignancy (9, 10, 37, 38, 39, and 42) data on the incidence of lung adenomata is at present inadequate.

In all the remaining groups the mice were killed one week after the end of croton oil treatment. The total incidence was 45 adenomata in 394 mice examined, and did not exceed 1 adenoma per 4 survivors in any one group.

These results cannot be satisfactorily assessed until the rest of the mice still under observation have been examined *post mortem*, and until the incidence of lung adenomata in mice treated with the standard course of croton oil alone is known.

DISCUSSION.

As a sequel to the finding that urethane acts as an initiator of carcinogenesis for mouse skin (Salaman and Roe, 1953) the results of the screening of 29 substances for similar activity have been recorded. Many of these substances were selected for test because they possessed some other biological activity in

common with urethane, or could be regarded as structurally related to it. The reasons for selecting each particular substance are given in the introduction, together with a brief survey of the findings of other authors.

Three of the 29 substances tested in conjunction with croton oil by the methods described gave definite evidence of initiating activity, namely T.E.M., 1,2-benzanthracene, and β-propiolactone. Two of the derivatives of urethane tested, ethyl N-methyl carbamate and ethyl N-phenyl carbamate, each produced several tumours, but the differences between the mean number of tumours in either of these groups and that in the group treated with croton oil alone did not reach significance at the customary 5 per cent level when judged by the *t*-test. By a ranking test (Kruskal and Wallis, 1952),however, these differences were judged significant. The reason for using the latter test and the need for caution in accepting the results of its application have been given above. Three other substances, nitrogen mustard, chloral hydrate, and cantharidin, gave rise to a few more tumours than were seen in mice treated with croton oil alone, but the differences were not statistically significant by either the *t*-test or the ranking test. The remaining substances, which included Myleran, aminopterin, colchicine, podophyllin, and six narcotic agents, showed no evidence of initiating activity.

The base line against which initiating activity was measured was the tumour incidence in mice treated with croton oil only, for the same period as the test mice. Three papillomas developed in a group of 20 croton oil treated mice during this period (i.e. 18 weeks). But when applications were continued the incidence of tumours steadily increased until, at 72 weeks, an average of over three tumours per surviving mouse was reached. Moreover three malignant tumours appeared between the 13th and 17th months of treatment. Although precautions against contamination of the test mice with traces of carcinogenic hydrocarbons, or other initiators, from cages or fomites, or in the process of handling, were always taken, the possibility of such contamination cannot be excluded. Alternatively, it may be that croton oil, besides being the most powerful known promoting agent for mouse skin, has also initiating power of a low order. It must be remembered, however, that spontaneous skin tumours do occur in mice (Slye, Holmes and Wells, 1921) although we have not encountered them in the " S " strain used for these experiments ; and it is possible that latent tumour foci are present in normal mouse skin, which do not develop into tumours in the life-time of untreated mice, but may be induced to do so by prolonged treatment with croton oil.

The incidence of malignant tumours of the skin in mice treated with initiators and croton oil, and in mice treated with croton oil alone, is under study at present.

In the five groups in which initiating action is considered to have been demonstrated, not only did tumour incidence at the end of croton oil treatment exceed that of the croton oil controls, but tumours appeared much earlier. Perhaps the most interesting findings is the initiating action of T.E.M. If total dosage is calculated on either a weight or a molar basis T.E.M. is far more effective than urethane. It is also far more toxic. Like urethane it does not give rise to skin tumours when applied repeatedly in maximum sublethal doses without croton oil, nor to any epidermal hyperplasia or other recognisable histological change. Moreover, like urethane it possesses antileukaemic, antimitotic, and lung adenoma-inducing action. The only striking differences in biological activity between the two substances are the hypnotic action of urethane, and the production of subcutaneous sarcomata by T.E.M. (Walpole *et al.*, 1954).

The findings in the case of Myleran are of interest since, though an anti-leukaemic agent, and a carcinogen in rats, it failed to initiate skin tumour formation or to produce lung adenomas in the mouse. In the last respect our findings confirm those of Shimkin (1954). In view of these negative results it seems desirable to confirm the carcinogenicity of this substance for the subcutis of the rat (Koller, 1953 ; 1954, personal communication).

Cornman (1954), discussing the activity of a series of carbamates, concludes that " almost any radical added to urethane increases its effectiveness as a mitotic poison and decreases or removes its carcinogenic action " (i.e. lung adenoma induction). Our results suggest that almost any modification of the urethane molecule is likely to decrease or remove the initiating activity for mouse skin. Moreover, none of the antimitotic substances in Class II (see Table I) of the present paper showed evidence of initiating activity.

Our findings in respect of lung adenoma induction by urethane derivatives agree in the main with those of Larsen (1947*a*, 1947*b*, 1948) who tested a wide range of these substances. However the rather high incidence in the group treated with methyl carbamate and croton oil is at variance with his findings. The fairly high incidence in mice treated with ethyl formate and croton oil is also of interest. Both these results require confirmation.

Among the test substances unrelated to urethane two showed initiating action. The finding that 1,2-benzanthracene is an initiator confirms the result of Graffi (1953). In view of the relatively small dose (6 mg.) required to initiate skin tumour formation it is possible that the low carcinogenicity of this substance when applied alone (see introduction) is due to its relative lack of promoting activity. This result suggests that it would be worthwhile testing other substances on the borderline of carcinogenicity for initiating action.

β-propiolactone is undoubtedly an initiator of carcinogenesis in mouse skin. However the fact that tumours appeared as early as the second week of croton oil treatment suggests that it may prove to be a carcinogen for mouse skin, as it is for the subcutaneous tissue of the rat (Walpole *et al.*, 1954). A further suggestion that this is so is given by the fact that 4 malignant tumours have already appeared among 19 surviving mice only 25 weeks after the beginning of treatment with β-propiolactone and croton oil. Malignant tumours have only appeared in other groups much later than this. It will not be possible to decide upon the status of this substance until further tests, now under way, are complete. (See Addendum I).

Table III shows the relation between the various biological activities (as far as they are known) of a number of the substances tested. There are many gaps, and some of the negative findings refer to experiments too limited to exclude a weak positive action. However it is perhaps permissible to suggest that there is some correlation between tumour-initiating activity for mouse skin, adenoma-inducing activity for mouse lung, and carcinogenicity for other species or tissues. But there seems to be no correlation between initiating activity and antimitotic or hypnotic activities, or between initiating activity and the ability to cause hyperplastic changes in the epidermis.

It is perhaps not surprising that substances which are capable of inducing tumours of the lung or other tissues in the mouse, or in other animals, act as initiators of carcinogenesis in mouse skin. Indeed the likelihood of this was the reason for selecting several of the substances. However the range of substances

TABLE III.—*The Relation Between the Ability to Initiate Tumour Formation in Mouse Skin and Certain Other Properties.*

Substance.	Initiation of skin tumour formation (mouse).	Induction of lung adenoma-formation (mouse).	Carcinogenicity for tissues other than lung.	Anti-leukaemic action.	Specific effect on mitosis.[1]	Hypnotic action.	Production of epidermal hyperplasia (mouse).
Urethane	++	+++	—	+	+	+	0
T.E.M.	+++	++[2]	Sarcomas (S.C. rat)[3]	+	+	0	0
Nitrogen mustard (HN2)	±	±[4]	Various sites (mouse)[5]	+	+	0	++
R48	0[6]	0[6]	Various sites(rat)[7]	+	+	0	++[6]
Aminopterin	0	0[8]	—	+	+	0	0
Ethyl N-phenyl carbamate (phenylurethane)	+	0	—	0[9]	+	+[10]	0
Ethyl N-methyl carbamate (methylurethane)	+	+[11]	—	0[9]	—	+[10]	0
1,2-Benzanthracene	+	0 or ±[12]	Sarcomas (S.C. mouse)[13] and skin tumours (mouse)[14]	—	—	0	++
β-propiolactone	+++	—	Sarcomas (S.C. rat)[3] and ? skin tumours (mouse)[15]	—	—	0	0 to ++
Colchicine	0	0	Skin tumours (mouse)[16]	+[19]	+	0	+++
Myleran	0	0[17]	Sarcomas (S.C. rat)[18]	+	+	0	0

Notes.—Except where specified data are from this report, or unpublished observations in this laboratory.

(1) For references see " Selection of substances," p. 177, *et seq.*

(2) Shimkin (1954) also reported induction of lung adenomas by T.E.M.

(3) Walpole *et al.* (1954).

(4) Shimkin (1954), Heston *et al.* (1953), and Boyland and Horning (1949) all report induction of lung adenomata following injection of nitrogen mustard. We failed to do so by application of this substance to the skin.

(5) Boyland and Horning (1949), and Griffin *et al.* (1951).

(6) In mice treated with R48 and croton oil (Salaman and Roe, 1953) there was no evidence of initiation of skin tumours nor of induction of lung adenomas.

(7) Haddow and Horning (1950) reported an adrenal ganglioneuroma and a gastric carcinoma in a rat treated with R48. Koller (1953) refers to sarcomata induced in rats by subcutaneous injection of R48.

(8) Shimkin (1954) also failed to produce lung adenomata with aminopterin.

(9) Skipper and Bryan (1949) observed no depression of the white cell count in mice treated with either ethyl N-methyl carbamate or ethyl N-phenyl carbamate.

(10) Skipper *et al.* (1948).

(11) Our result confirms that of Larsen (1948).

(12) Shear and Leiter (1941) reported an increased incidence of lung adenomata in two mice 1 year after the subcutaneous injection of 5 mg. 1,2-benzanthracene but Andervont and Shimkin (1940) failed to induce adenomata by the intravenous injection of the substance. Our data is insufficient.

(13) Steiner and Falk (1951).

(14) Kennaway, 1930; Cook, 1933; Barry *et al.*, 1935; and Hill *et al.*, 1951.

(15) It is possible that β-propiolactone is carcinogenic for mouse skin (see p. 196).

(16) Harde (1939).

(17) Shimkin (1954) also failed to induce adenomata with Myleran.

(18) Koller (1953; 1954, personal communication).

(19) Dixon and Malden (1908) reported a transient leucopenia in several species following injection of colchicine. Kneedler (1945) induced a remission in a single case of acute myelogenous leukaemia, but a fatal relapse followed 13 months later.

so far tested is small, and the possibility is not excluded that initiators may be found which by themselves do not give rise to tumours in any tissue. Certainly the fact is established, and has, we think, practical as well as theoretical importance, that there exist substances which under certain conditions produce no tumours, nor any other recognised change, in a tissue, yet alter it in such a way that when a different stimulus is applied, perhaps much later (Roe and Salaman, 1954), benign and malignant tumours appear.

SUMMARY.

1. As a sequel to the demonstration that urethane applied to mouse skin followed by repeated applications of a *promoting agent* (croton oil) acts as an *initiator* of carcinogenesis, 29 substances, most of them related pharmacologically or chemically to urethane, have been screened for similar activity.

2. Of four *antileukaemic agents* tested, triethylene melamine (T.E.M.) was found to be an effective initiator of carcinogenesis but not carcinogenic, for mouse skin, in the doses tested ; nitrogen mustard, Myleran, and aminopterin, in maximum sublethal doses, showed no initiating activity.

3. Of five *substances exerting a specific effect on mitosis* (in addition to those included in the category of anti-leukaemic agents), none showed initiating activity.

4. Of six *narcotic agents* tested none showed unequivocal evidence of initiating activity.

5. Of eight *urethane derivatives and related compounds*, ethyl N-methyl carbamate and ethyl N-phenyl carbamate showed weak initiating activity. In neither case was this as strong as that of a molecularly equivalent dose of urethane. (See Addendum II).

6. Four *miscellaneous substances* were tested. Of these 1,2-benzanthracene was found to be an effective initiator of carcinogenesis but not carcinogenic, in the doses used ; and β-propiolactone was found to be an initiator of carcinogenesis. The latter is being tested for carcinogenicity at present. (See Addendum I.)

7. The use of the " t " test of statistical significance for skew distributions, such as those encountered in these results, is discussed. As an alternative, a ranking test is proposed.

8. The histological appearance of the skin following one, or two, applications of the test substances is described. Of those with initiating activity triethylene melamine, ethyl N-methyl carbamate and ethyl N-phenyl carbamate gave rise to no recognisable changes in the skin. 1,2-Benzanthracene consistently produced a moderate epidermal hyperplasia. The response to β-propiolactone varied from slight to marked epidermal hyperplasia.

9. The results are discussed, and an attempt is made to correlate initiating activity with other properties.

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ADDENDUM

Since this paper was submitted for publication some of the experiments refered to in the text as still in progress have been completed.

I: *Further observations on β-propiolactone.* (cf. pp. 194, 198, 200.)

a. A group of 10 mice painted weekly for more than 20 weeks with a sub-ulcerative concentration (0·3 ml. 2·5 per cent in acetone) of β-propiolactone has so far exhibited no skin tumours. In another group which received 10 similar applications of β-propiolactone together with a standard course of croton oil treatment, 108 papillomas were present on 7 of the 8 survivors at the end of treatment. A further group received a single application of 2·5 per cent β-propiolactone followed after an interval of 3 weeks by a standard course of croton oil. Five of the 9 survivors in this group had a total of 22 papillomas at the end of treatment.

b. At *post mortem* only 1 lung adenoma was seen in 18 mice treated with β-propiolactone and croton oil.

Conclusion: There is no evidence that β-propiolactone applied in sub-ulcerative concentration is carcinogenic for mouse skin, but its initiating action has been confirmed.

II: *Further observations on ethyl N-phenylcarbamate (phenylurethane).* (cf. pp. 191, 200.)

Twenty mice received 120 mg. phenylurethane (2 applications 20 per cent w/v in acetone with 15 minutes interval) weekly for 7 weeks, and 60 mg. weekly for a further 8 weeks. Three days after the first application of phenylurethane a standard course of croton oil was begun. At the end of treatment only 1 of the 16 survivors bore tumours (5 papillomas), an incidence not differing significantly from that in mice receiving croton oil only.

A further group which received similar treatment with phenylurethane but no croton oil developed no skin tumours.

Conclusion: This test has failed to confirm the suggestion that phenylurethane possesses weak initiating activity, and has shown that it is not carcinogenic for mouse skin in the doses tested.

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