Brit. J. Pharmacol. (1954), **9**, 306.

CYTOTOXIC AGENTS: IV, THE CARCINOGENIC ACTIONS OF SOME MONOFUNCTIONAL ETHYLENEIMINE DERIVATIVES

BY

A. L. WALPOLE, D. C. ROBERTS, F. L. ROSE, J. A. HENDRY, AND R. F. HOMER

From Imperial Chemical Industries Ltd., Hexagon House, Manchester, 9

(RECEIVED MARCH 13, 1954)

In earlier papers in this series (Part I, Hendry, Rose, and Walpole, 1951; II and III, Hendry, Homer, Rose, and Walpole, 1951a and b) we described the tumour growth-inhibitory and cytotoxic activity of some methylolamides, epoxides, and ethyleneimines. We reported that a few had been found to be mutagenic and a few carcinogenic. We also suggested a possible mode of action for these compounds and for related substances of the "mustard" class.

Compounds of these functional types have been studied widely during the past few years. Interest was stimulated by the hope of finding among them agents for the treatment of neoplastic disease. Hence in most instances they were examined initially for growth-inhibitory activity against animal tumours and for the distinctive type of cytotoxic action which came to be associated with it (chromosome fragmentation and bridge formation). Testing for mutagenic and carcinogenic activity was at first restricted to a few of the active inhibitors of tumour growth. The results suggested that the inhibition of normal and malignant growth and the induction both of mutations and of tumours might be due to a single type of cytotoxic activity, and that the capacity to inhibit tumour growth and to elicit the associated cytotoxic changes was limited, in the main, to polyfunctional derivatives—compounds containing at least two chemically reactive groupings in the molecule. Since the corresponding monofunctional derivatives were found, with few exceptions, to be devoid of both these properties, the general tendency was to conclude that they would be inactive also as mutagens and carcinogens.

This conclusion was premature. A publication by Rapoport (1948) recording the mutagenic activity of ethyleneimine and some simple monoepoxides was at first overlooked, but several workers, two years later, reported similar activity in β-chloroethylamines (Stevens and Mylroie, 1950; Auerbach and Moser, 1950; Jensen, Kirk, and Westergaard, 1950). In Part III of the present series we reported the induction of sarcomata in rats with stearoylethyleneimine, and, in a footnote, mentioned that we had found myristoyl- and caproylethyleneimine to be active in the same sense. We explained our reasons for testing stearoylethyleneimine as a carcinogen. Activity was predicted on the supposition that, by the packing together of its fatty chains, this substance would tend to form micelles bearing regularly spaced reactive ethyleneimine groups. It was the first compound of the chemical type under discussion in which we were able to demonstrate carcinogenic activity in the absence of marked tumour inhibitory activity.

Since the tendency towards micelle formation in linear hydrocarbon derivatives with polar end groups falls off as the hydrocarbon chain is shortened, we have now prepared and examined lower homologues in the acylethyleneimine series. In addition, several alkylsulphonylethyleneimines have been synthesized, and β-propiolactone (Table II, 21) has been included for test. This substance was found by Smith and Srb (1951) to be mutagenic, and is, in fact, analogous to ethyleneimine in structure and chemical reactivity.

Finally, a series of compounds has been prepared, based, first, upon the carcinogenic activity of 4-dimethylaminoazobenzene (Butter Yellow), and, second, upon the unexpected activity as a tumour inhibitor of hexamethylmelamine. The suggestion has already been made (Part III, p. 405) that *in vivo* oxidation of a methyl group to labile methylol in both these compounds precedes their action in the cell. Since the replacement of the dimethylamino residues of the melamine derivative by ethyleneimino groups produces a dramatic increase in tumour growth-inhibitory activity it was concluded that a similar modification of Butter Yellow, giving 4-ethyleneiminoazobenzene, might

lead to a corresponding increase in carcinogenic potency. Unfortunately we were unable to make this substance, but a related compound (16) containing an additional ureido residue, was prepared. Compound 17 was a variation on the same theme, as were several other substances (18, 19, and 20) carrying the dimethylamino group.

METHODS

Throughout the experiments to be described, our animals were housed upon sawdust in galvanized wire mesh cages and were allowed to feed at will upon a composite diet in pellet form with tap water freely available at all times. The diet, obtainable from Scottish Agricultural Industries Ltd. has the following percentage composition by weight:

Ground barley ..	26.5	Dried skimmed milk	13.0
Ground oats ..	18.5	Meat and bone meal	8.6
Bran	18.3	White fish meal ..	4.4
Maize meal ..	8.6	Dried yeast ..	1.3
Salt	0.4	"Nuclo"	0.4

"Nuclo" is a proprietary vitamin concentrate containing 800 I.U. of vitamin A and 100 B.S.I. units of vitamin D_3 per g.

Toxicity

As a preliminary to further examination, toxicity tests were carried out on each compound in mice and rats. The compound was dissolved in arachis oil or, if insufficiently soluble, suspended in it by milling. In some cases, where solubility in water permitted, aqueous solutions also were prepared. Solutions and suspensions were stored at 4° C. and where marked instability was suspected were made up freshly before injection. Each compound was given first to stock albino mice, usually by intraperitoneal injection, in single doses of 1,000, 500 250, 125, and 50 mg./kg., four mice being injected at each dose level. The animals were observed from time to time during the first hour or so for the more obvious signs of acute pharmacological action, such as narcosis or convulsions, and deaths were recorded up to seven days. With the more toxic compounds, the range of doses was extended downward until a level was reached at which at least half the mice survived.

On the basis of the results obtained in these acute tests in mice, doses were chosen for repeated administration to rats. Stock albino rats were injected intraperitoneally or subcutaneously, and in most cases by both routes, once daily for five successive days. A group of three animals was treated at each dose level; they were weighed each day before injection and the doses adjusted in proportion to the body weight. Daily weighing and the recording of deaths was continued up to the tenth day.

The dose levels used in subsequent tests were based upon the results of these experiments in rats, the trends in body weight as well as deaths among the treated animals being taken into account. It was also borne in mind in testing for inhibitory activity against the growth of tumours that tumour-bearing animals are rather more susceptible than normal to the toxic action of many compounds.

Carcinogenic Activity

In the initial testing of compounds for carcinogenic activity, albino rats from our closed but randomly mated colony were used. Six male and six female rats, each weighing between 80 and 120 g. at the start of experiment, were treated with each compound. The latter, dissolved or suspended in sterilized arachis oil, was injected subcutaneously, twice weekly, in the right flank of the animals. The oil was sterilized by heating at 140° C. for 1 hr. The rats were weighed before each injection and the dose made proportional to the body weight. The dose level was, in most cases, near the maximum tolerated. Rats which on any occasion were found to have lost weight since the previous injection, or which were obviously ill, were not injected again until the weight loss had been made good or condition regained. The duration of treatment was variable. With some compounds, twice weekly injections were continued until after local tumours had begun to appear, with others it was discontinued earlier, in some instances when only ten doses had been given, whereas in a further series of experiments only one dose of each compound under test was given.

The volume of fluid given at each injection ranged from 0.1 to 0.5 ml./100 g. body wt., and the total volume received by the rats in different groups differed widely. In control groups rats were given arachis oil alone in total doses up to and exceeding the largest amount given with any of the compounds tested.

After the last injections had been given, the rats were inspected daily and examined at about fortnightly intervals. As will be seen from the Tables I and II, tumours developed at the injection site in a high proportion of the treated animals. These tumours could usually be distinguished at an early stage, by palpation, from the subcutaneous accumulations of oil which were often still present when the tumours first appeared. The approximate time of their first appearance is recorded in the tables.

Tumours of various kinds developed at other sites in a number of animals. According to type and location, some were found before death, others at autopsy.

A few rats died unexpectedly in the course of experiment, either while still under treatment or later, and death in some instances was not discovered until some hours after it had occurred. A few rats became very ill—usually as a result of intercurrent pulmonary infection—and were killed when death seemed imminent. The bodies of all these animals were carefully examined. Any tumours found and the liver, kidneys, adrenals, spleen, pancreas, lungs, heart, testes or ovaries and one or more lymph nodes were sectioned for study. This was omitted, however, where the bodies of animals when found were too far decomposed to make histological examination profitable. From a few rats dying unobserved, tumours known to have been present had been eaten away when their bodies were discovered.

Most of the injection-site tumours were allowed to grow until their existence as progressively growing neoplasms appeared beyond doubt. None was observed to regress. When they began seriously to inconvenience the rats bearing them, the latter were killed and a careful post-mortem examination carried out. The local tumour, any other tumours found, and the main organs were taken for section from each animal.

In some cases the local tumour was first exposed under aseptic conditions and fragments from the periphery implanted subcutaneously, by trochar and cannula, into six to ten young adult stock rats of body wt. about 100 g. Most of the tumours failed to grow in any of the recipient animals when transplanted in this way. This is attributed to the fact that the rats used in these experiments were from a randomly mated stock. Either of the two following procedures was therefore adopted with other rats which developed tumours at the injection site. In the first, the rat was anaesthetized with ether and the tumour excised as completely as possible. A fragment was then implanted subcutaneously in the opposite flank of the animal from which it had been taken (autologous transplantation). Alternatively, fragments of tumour were implanted subcutaneously in the ventral cervical region of very young stock rats. The latter were less than 48 hrs. old and were anaesthetized for operation by placing them for 10 min. or so in the freezing compartment of a domestic refrigerator. The operative mortality in such experiments was zero.

Autologous transplantation was invariably successful and the resulting tumours were never observed to regress. The proportion of tumours which grew on transplantation into very young rats was also significantly higher than when young adults were used.

The rats not already accounted for were killed at about two years from the start of experiment. They comprised animals which had not developed externally detectable tumours by that time, together with those in which such tumours were still quite small. If a tumour was known to be present or if anything suggestive of neoplasia was found at autopsy, a full histological examination was carried out. The main organs were taken for section at autopsy also from all animals in the control groups treated with arachis oil alone.

Later experiments with acyl ethyleneimines of intermediate chain length dissolved in a polyethylene glycol, Carbowax 300, and with lower members of this series in aqueous solution, were identical in general plan with those using arachis oil. Ethyleneimine itself was also tested in aqueous solution, being freshly dissolved, on each occasion upon which it was given, in CO_2-free distilled water. Details of dosage are given in Table III. Tests upon aqueous β-propiolactone are in progress and controls with distilled water have also been set up.

In our experiments on mice we used randomly bred stock albino mice (designated W in the Tables), and inbred mice of strains C_3Hf and C, raised from animals obtained from Dr. J. G. Carr and Dr. H. B. Andervont respectively. The compounds chosen for test were dissolved in sterilized arachis oil and the solutions injected subcutaneously, twice weekly, into groups of 20 mice, each of which weighed about 20 g. The initial dose was usually near the maximum tolerated, and the same dose was given to each mouse throughout the period of treatment irrespective of body weight changes. However, mice which at any time seemed ill were not injected again until they appeared to have recovered. The volume of solution given at each injection varied from group to group, from 0.2 ml./mouse in the earlier experiments to 0.025 ml. in the later. The duration of treatment and the total quantity of compound and of oil given were also variable. In control groups, mice were given arachis oil alone at comparable levels and for comparable lengths of time.

In these experiments many mice died at a comparatively early stage as a result either of the toxic action of the substances given, of intercurrent infection, or of both. A detailed post-mortem examination was not carried out upon those which succumbed in any group before the first injection-site tumour in that group was detected. All animals which died or were killed thereafter were carefully autopsied but, as a rule, tissues were taken for histological examination only from those in which tumours were detected macroscopically.

Tumour Inhibition

Our standard procedure for testing substances for tumour growth-inhibitory activity, using the Walker carcinoma 256 in rats, has been described in detail in an earlier paper (Walpole, 1951).

Cytotoxic Action

The method for assessing the capacity of compounds to induce, in dividing cells of the Walker carcinoma growing in rats, effects of the kind regarded as characteristic of tumour growth-inhibitory substances of the "radiomimetic" class has also already been described (Part I).

Results

Carcinogenesis

The subcutaneous injection-site tumours, which constituted a majority of the neoplasms, were mostly sarcomata of spindle, round or mixed cell type. Where diagnosis was based solely upon microscopic appearances within the tumour, the time of first recognition is shown in the tables in parenthesis. Otherwise local infiltration into the skin or into the subjacent muscle, or into both, was found. The tendency for these tumours to metastasize was not marked; metastases in distant organs were found in very few animals.

Table I.—The subcutaneous injection of arachis oil alone in a total dose of 5 ml. or more per 100 g. rat gave rise to local sarcomata in a number of animals. The yield was variable, being highest in a group in which the total dose of oil was 7 ml./100 g. body wt. and in which five of the twelve animals were affected. The sarcomata in these control groups developed comparatively late in the

TABLE I

THE INCIDENCE OF NEOPLASMS IN RATS GIVEN ARACHIS OIL ALONE BY SUBCUTANEOUS INJECTION

An asterisk against the figure for the time of death of a rat indicates that the animal was found dead and in an advanced state of post-mortem decomposition and that no tissues were taken for section. The letter M indicates that metastases were found. Animals in which more than one tumour was found are identified by a number written as a superscript to the left of the figures for the time of recognition or finding of the tumours. Where the time of first recognition of a local sarcoma is shown in parenthesis the diagnosis was based solely upon microscopic appearances within the tumour

No. and Sex of Rats	Duration of Dosing (Days)	Total Volume of Oil Given (ml./100 g.)	Sarcomata at Injection Site		Other Neoplasms		Rats Dead Without Tumours	
			No. of Rats Affected	Time of First Recognition of Tumour in Days from the Start of Dosing	No. of Rats Affected	Type and Time when Found	No.	Time of Death
12♂	33	2·5	0		2	1 Anaplastic carcinoma in lung, 725 1 Interstitial cell tumour of testes, 769	10	203, 610, 689*, 696*, 702, 725, 768, 769, 769, 769
12♀	33	2·5	0		1	1 Carcinoma of uterus, 681	11	541*, 568, 570, 575, 625*, 659, 671, 725, 768, 768, 768
10♂	67	5·0	1	568	2	1 Anaplastic carcinoma of pancreas, 371 1 Islet cell adenoma of pancreas, 676	7	441 595, 621, 676, 676, 676, 676
9♀	67	5·0	0		3	1 Mammary fibroadenoma, 595 1 Fibroma at injection site, 643 1 Carcinoma of uterus, 671	6	209, 580, 582, 595, 671, 671
6♂	97	7·0	4	516, 571, 694, 722M	1	1 Tumour of the adrenal medulla, 740	1	740
6♀	97	7·0	1	694	2	2 Uterine carcinomata, 595, 740	3	375, 664, 730
6♂	103	14·2	1	^{1}649	2	1 Anaplastic tumour in lung, ^{1}649 1 Pituitary adenoma, 630	4	28*, 495, 581, 702
6♀	103	14·2	2	488, 661	1	1 Sarcoma adherent to liver, 397	3	509*, 600, 690
Animals dosed daily, Saturdays and Sundays excluded								
12♂	300	39·7	1	576	0		11	73, 253, 322, 329, 383, 435, 572, 572, 572, 575, 575
12♀	300	39·7	4	421, (537), 544, (^{2}575)	1	1 Caecal adenoma, ^{2}575	8	30, 262, 434, 501, 575, 575, 582, 582

experiment, the earliest appearing at about 421 days. Of the other miscellaneous neoplasms which occurred in control animals given arachis oil, the commonest was carcinoma of the uterus, which was encountered in four out of a total of 45 female rats. To what extent these miscellaneous tumours may be attributed to the oil is uncertain.

Table II.—Most of the acylethyleneimines tested, of the general type R.CO.N  (Nos. 1, 3, 5–10) when injected repeatedly in oil, gave a high yield of local sarcomata, most of which appeared before the first in the control rats. A crude sample of stearoylethyleneimine (2) containing unsaturated material, probably the oleoyl homologue, behaved similarly, whereas a pure sample appeared less potent. With lauroylethyleneimine (4) only one of the twelve treated rats developed a local sarcoma, and this did not appear until the 522nd day. In single dose experiments only one sarcoma was obtained, and that, strangely enough, with lauroylethyleneimine. It was detected 123 days from the start of experiment.

Tumours appeared particularly early and in all but one of the rats given 4-chloro-6-ethyleneimino-2-phenyl-pyrimidine (12). *N-cyclo*Ethyleneureidoazobenzene (16) produced local sarcomata in seven of the twelve rats treated with it, but only three of these had appeared by the 400th day and the total volume of oil given with this compound was large (7–8 ml.). Ethyleneimine itself (11) gave tumours in six of the group of twelve rats treated; but of these also only three appeared before the 400th day.

Three ethyleneiminosulphonyl alkanes of the type R.SO$_2$N were tested, the alkyl radicals being heptyl (13), pentyl (14), and propyl (15) respectively. One mammary fibroadenoma was the only tumour obtained with these substances.

Of the four substances bearing the dimethylamino group, none gave a significant yield of early local sarcomata. Most of the rats injected with *p* - *N* : *N* - dimethylureidoazobenzene (17) showed liver abnormalities. Sclerosis of portal tracts and bile-duct hyperplasia were common. In two animals bile-duct "cystadenomata" were present, and, in another, a hepatoma. No study of the early liver changes produced by this compound has been made, but these observations are of interest in view of its relationship to Butter Yellow.

TABLE II

THE INCIDENCE OF NEOPLASMS IN RATS GIVEN SOME ETHYLENEIMINE DERIVATIVES AND RELATED SUBSTANCES, DISSOLVED OR SUSPENDED IN ARACHIS OIL AND INJECTED SUBCUTANEOUSLY

The conventions are the same as in Table I. In addition the following symbols are used: ○ indicates homologous transplantation failed, ● succeeded. △ indicates autologous transplantation failed, ▲ succeeded. □ indicates transplantation into newborn rats failed, ■ succeeded. + indicates tumour known to have been present eaten away when body of animal found. E indicates the presence of direct extension of the local tumour into the thoracic cavity.

No.	Substance: Formula	No. and Sex of Rats	Duration of Dosing (Days)	Maximum Total Dose (mg./100 g.)	Maximum Total Vol. of Oil (ml./100 g.)	Sarcomata at Injection Site: No. of Rats Affected	Sarcomata at Injection Site: Time of First Recognition of Tumour in Days from the Start of Dosing	Other Neoplasms: No. of Rats Affected	Other Neoplasms: Type and Time when Found	Rats Dead Without Tumours: No.	Rats Dead Without Tumours: Time of Death	Duration of Experiment (Days)
1	$CH_3(CH_2)_7CH{=}CH(CH_2)_7CON\langle^{CH_2}_{CH_2}$	6♂	49	170	1·7	6	138, 138, 138, 147 (154), 155	0		0		
		6♀	49	170	1·7	5	(154), 154, 154, 166, 178	0		1	136*	
2	$CH_3(CH_2)_{16}CON\langle^{CH_2}_{CH_2}$ (*a*) Crude	5♂	32	100	5·0	4	(111), 158○, 158, 448	1	1 Unidentified tumour in adrenal, 562	0		
		5♀	32	100	5·0	4	130○, [3]130, 130, 130	2	1 Carcinoma at injection site, [3]120 1 Sarcoma in thorax, 340	0		
	(*b*) Pure	6♂	35	110	2·75	5	385▲, 410▲, (410+), 427▲, 509	0		1	280	
		6♀	35	110	2·75	2	254, 293△	2	1 Mammary ca. at injection site, 382▲. 1 Anaplastic ca. probably of uterine origin, 472	2	293, 441	
3	$CH_3(CH_2)_{12}CON\langle^{CH_2}_{CH_2}$	6♂	141	332·5	6·65	5	(150), [4]153, 153, 168, (201)	1	1 Lymphosarcoma, [4]161	1	116	
		6♀	141	332·5	6·65	3	168, (168), 229	1	1 Mammary fibroadenoma, 168	2	15, 130	
		6♂	1	12·5	0·2	0		0		6	489 (6)	
		6♀	1	12·5	0·2	0		1	1 Mammary fibroadenoma at injection site, 450	5	224, 489 (4)	
4	$CH_3(CH_2)_{10}CON\langle^{CH_2}_{CH_2}$	6♂	32	100	2·5	1	(522)	1	1 Sarcoma in neck invading salivary gland, 647	4	232, 401*, 540, 663	
		6♀	32	100	2·5	0		3	1 Mammary ca. at injection site, 387. 1 Sarcoma invading stomach wall, 458○. 1 Mammary fibroadenoma, 585	3	98, 388, 741	
		6♂	1	12·5	0·1	1	123	0		0		531
		6♀	1	12·5	0·1	0		0		0		
5	$CH_3(CH_2)_7CON\langle^{CH_2}_{CH_2}$	6♂	191	76	7·6	5	(157), 191, 203, 213, 238	1	1 Adenocarcinoma of large intestine, 171	0		
		6♀	191	86	8·6	6	121, ([5]203), [6]203, 255M, 255, 296	2	1 Lymphoid leukaemia, [6]213 1 Mammary carcinoma [5]203	0		
		6♂	1	2	0·1	0		0		6	431, 489(5)	
		6♀	1	2	0·1	0		0		6	489(6)	

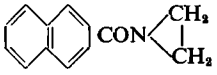
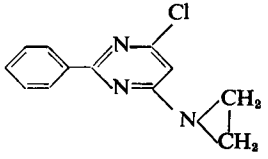
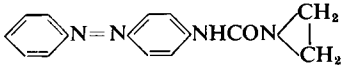
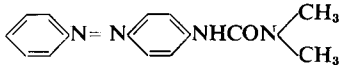
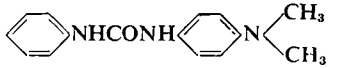
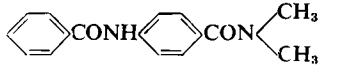
6	$CH_3(CH_2)_4CON\langle^{CH_2}_{CH_2}$	6♂	133	49·5	8·25	6	196, 229, (229), 239, 257, 314	0		0		
		6♀	133	51	8·5	6	196, (210), 259, 259, 277, 243○▲	0		0		
		6♂	1	2	0·1	0		0		6	123, 446(5)	
		4♀	1	2	0·1	0		0		4	429(4)	
7	$(C_2H_5)_2CH.CON\langle^{CH_2}_{CH_2}$	6♂	198	42	4·2	6	169E, 198, 261▲, 275, 301, 301	0		0		
		6♀	198	44	4·4	3	198○, 210, [7]220	5	3 Mammary carcinomata at injection sites, [7]220▲, 220, 245 1 Uterine sarcoma [7]314 1 Lymphoid leukaemia, 311	0		
		6♂	1	2	0·1	0		0		6	427, 489(5)	
		6♀	1	2	0·1	0		1	1 Mammary fibroadenoma, 489	5	489(5)	
8	$CH_3(CH_2)_2CO.N\langle^{CH_2}_{CH_2}$	6♂	198	22	4·4	6	191, 191▲, [8]211▲, [9]231▲, 262, 344	2	1 Mediastinal sarcoma, [8]237 1 Sarcoma in thorax, [9]263	0		
		6♀	198	22	4·4	5	177M, 245, 262M, [10]314, 333▲	2	1 Mammary carcinoma, 303 1 Uterine sarcoma, [10]314	0		
		6♂	1	1	0·1	0		0		6	489(6)	
		6♀	1	1	0·1	0		0		6	489(6)	
9	$CH_3CON\langle^{CH_2}_{CH_2}$	3♂	101	21	2·1	2	173, 180	1	1 Carcinoma of auditory sebaceous gland, 177	0		
		3♀	101	9	0·9	1	180	1	1 Anaplastic carcinoma in forestomach, 595	1	697	
		6♂	184	15	3·0	4	196▲, 196M, 206, 393	1	1 Anaplastic tumour at injection site, 231	1	517	
		6♀	184	7·75	1·55	6	(247), 260▲, 275○▲, 288, 288▲, 482	0		0		
		6♂	1	0·5	0·1	0		0		1	58	515
		6♀	1	0·5	0·1	0		0		1	352	
10		6♂	141	125	5·0	6	[11]183, [12]200, 200, 250, 213 358	2	2 Carcinomata of auditory sebaceous glands, [11]183, [12]200	0		
		6♀	141	97·5	3·9	6	169, [13]170, [14]170, 183, [15]299, [16]319	4	2 Mammary carcinomata at injection sites [13]184, [15]332 1 Mammary carcinoma, [16]319 1 Sarcoma in thorax, [14]204	0		
11	$HN\langle^{CH_2}_{CH_2}$	6♂	67	2	2·0	5	355, 355, 367, 426, 511	0		0		546
		6♀	67	2	2·0	1	(426)	0		0		
12		6♂	114	96	6·4	6	154, 154, 154, 154, 154, 154	0		0		
		6♀	114	96	6·4	5	111, 134, 154, 154, 154	0		1	62	
13	$CH_3(CH_2)_6SO_2N\langle^{CH_2}_{CH_2}$	6♂	54	150	1·2	0		0		3	14, 522, 593	601
		6♀	54	150	1·2	0		0		2	416, 551	

TABLE II—*continued*

THE INCIDENCE OF NEOPLASMS IN RATS GIVEN SOME ETHYLENEIMINE DERIVATIVES AND RELATED SUBSTANCES, DISSOLVED OR SUSPENDED IN ARACHIS OIL AND INJECTED SUBCUTANEOUSLY

The conventions are the same as in Table I. In addition the following symbols are used: ○ indicates homologous transplantation failed, ● succeeded. △ indicates autologous transplantation failed, ▲ succeeded. □ indicates transplantation into newborn rats failed, ■ succeeded. + indicates tumour known to have been present eaten away when body of animal found. E indicates the presence of direct extension of the local tumour into the thoracic cavity.

No.	Substance: Formula	No. and Sex of Rats	Duration of Dosing (Days)	Maximum Total Dose (mg./100 g.)	Maximum Total Vol. of Oil (ml./100 g.)	Sarcomata at Injection Site: No. of Rats Affected	Sarcomata at Injection Site: Time of First Recognition of Tumour in Days from the Start of Dosing	Other Neoplasms: No. of Rats Affected	Other Neoplasms: Type and Time when Found	Rats Dead Without Tumours: No.	Rats Dead Without Tumours: Time of Death	Duration of Experiment (Days)
14	$CH_3(CH_2)_4SO_2N$ CH_2 CH_2	6♂	49	90	0·9	0		0		0		601
		6♀	49	90	0·9	0		0		3	424, 474, 504	
15	$CH_3(CH_2)_2SO_2N$ CH_2 CH_2	6♂	49	125	1·0	0		0		0		601
		6♀	49	125	1·0	0		1	1 Mammary fibroadenoma 425	0		
16		6♂	108	111	7·4	3	([17]418), 418, (488)	1	1 Adenoma of small intestine, [17]418	3	307, 549*, 570	
		6♀	108	114	7·6	4	277, 362, 400, 515	0		2	347, 375	
17		6♂	83	260	5·2	0		2	2 Bile duct cystadenomata 470, 686	4	25*, 375, 580, 656	
		6♀	83	260	5·2	0		5	1 Leukaemia, 374. 1 Intra-abdominal carcinomatosis, 470. 1 Hepatoma, 474○□. 1 Tumour of unknown origin in lung, 446. 1 Mammary fibroadenoma, 611	1	692	
18		6♂	14	1,000	5	0		2	1 Carcinoma of jaw, 688. 1 Pituitary tumour, 697	4	512, 565, 686*, 703	
		6♀	14	1,000	5	2	(362), 521M	1	1 Carcinoma of uterus, 536M	3	699, 703, 703	
19		6♂	63	400	3·2	0		0		0		508
		6♀	63	400	3·2	1	(497)	0		0		
20	$CH_3(CH_2)_{16}CON$ CH_3 CH_3	6♂	75	800	5	2	(517), 578	2	1 Carcinoma of auditory sebaceous gland, 390. 1 Sarcoma of optic orbit, 637	2	733, 733	
		6♀	75	800	5	2	489▲, 501▲	1	1 Early carcinoma of uterus, 614	3	643, 733, 733	
21		6♂	90	44	2·2	5	192, 192M, 266●□, 266, 386	0		1	59*	508
		6♀	90	48	2·4	4	192, 249●, 285, 321	0		1	234	

β-Propiolactone (21) behaved like the more active acylethyleneimines.

A few neoplasms of types not encountered in the arachis oil controls were seen in rats treated with one or other of the ethyleneimine derivatives. They included carcinoma at the injection site, carcinoma of the auditory sebaceous gland, and leukaemia. Two of the rats given stearoylethyleneimine (2, (a) and (b)) developed a carcinoma at the injection site; one was of mammary origin, but the origin of the other was not determined. Two of the six female rats given diethylacetylethyleneimine (7) had a mammary carcinoma in this region, and, in a third, the mass which developed at the injection site was found to consist of two tumours, a sarcoma, and, contiguous with it, a carcinoma arising apparently from mammary tissue. This compound gave similar results when administered in Carbowax 300 (v. infra). In two of the rats given β-naphthoylethyleneimine (10), a sarcoma and a mammary carcinoma were found, side by side, at the injection site. Mammary fibroadenomata and carcinomata, more or less remote from the injection residue, were found in a few rats given other ethyleneimine derivatives, but it should be noted that mammary fibroadenoma was seen in an arachis oil treated control, and such tumours have, in fact, been encountered in untreated rats of our stock.

Carcinoma of the auditory sebaceous gland was found in a male rat given acetylethyleneimine (9), in two males given β-naphthoylethyleneimine (10), and in one which received *N*:*N*-dimethylstearamide (20). Tumours of this type, as is well known, are readily induced in rats of certain strains by the oral administration of 2-acetylamidofluorene and have been encountered also in animals of this species following treatment with a variety of aromatic amines (v. e.g., Haddow, Harris, Kon, and Roe, 1948; Walpole, Williams, and Roberts, 1952). Their histogenesis has been described by Skoryna, Ross, and Rudis (1951). They have never been observed in untreated rats in Skoryna's colony or in ours, or in rats of our stock treated with arachis oil alone.

Two leukaemias, both lymphoid in type, and one lymphosarcoma were found in animals dosed respectively with diethylacetyl- (7), nonanoyl- (5), and myristoyl-ethyleneimine (3). A third leukaemia, probably of the same type, developed in a female rat given *p*-*N*:*N*-dimethylureidoazobenzene (17). The incidence of "spontaneous" lymphoid tumours in our stock is negligible.

Mediastinal or intrathoracic sarcomata found in several rats carrying sarcomata at the injection site may have been direct extensions or secondary deposits from the latter. In some instances clear evidence of direct extension was found.

Table III.—The results listed in Table II are such as to suggest that ethyleneimine, certain mono-functional derivatives thereof, and β-propiolactone may be regarded as carcinogenic in the rat in their own right. In view, however, of the uncertainty as to the precise part played by the oil in the genesis of tumours in animals given these substances, other vehicles were used for their injection, and Table III records the results obtained employing Carbowax 300 and water. A high yield of local sarcomata is evident in the rats given each of three acylethyleneimines in the Carbowax. These tumours appeared between 142 and 378 days after the first injections. In addition, mammary carcinoma developed at the injection site in one female rat given caproylethyleneimine (6), in two females and one male given nonanoylethyleneimine (5), and in no less than five of the six females given diethylacetylethyleneimine (7). Four of these five animals had subcutaneous sarcomata also at the injection site.

In rats given Carbowax 300 alone, no injection-site tumours have appeared. In the group given the higher dose of this solvent (6.5 ml./100 g. body wt.) a rat which died on day 448 was found to have a poorly differentiated carcinoma of the prostate, and another, which died on day 565, a mammary fibroadenoma and an intra-abdominal carcinoma of undetermined origin. It is unlikely that any of these tumours was due to the Carbowax.

In the series in which water was used as the vehicle for injection local sarcomata appeared in ten of the twelve rats given butyrylethyleneimine (8), in four of the group given acetylethyleneimine (9), and in one of those given ethyleneimine itself (11). No other tumours have been encountered to date in the animals in this series, the experiments having now been running for 450–550 days.

The yield of local tumours obtained with butyrylethyleneimine given in water was comparable with that obtained with the same substance in arachis oil. There are a number of factors which might account for the lower incidence with acetylethyleneimine when given in the former solvent. Among these may be mentioned the irritant action of the lower members of the acylethyleneimine series, which is most apparent when they are given in water. Aqueous acetylethyleneimine injected subcutaneously causes local necrosis and ulceration, making repeated injection at the same site difficult and the dose uncertain.

TABLE III

THE INCIDENCE OF NEOPLASMS IN RATS GIVEN SOME ETHYLENEIMINE DERIVATIVES DISSOLVED IN CARBOWAX 300 OR WATER AND INJECTED SUBCUTANEOUSLY

The conventions are the same as in Tables I and II

No.	Substance: Formula	No. and Sex of Rats	Duration of Dosing (Days)	Maximum Total Dose (mg./100 g.)	Maximum Total Vol. of Solvent (ml./100 g.)	Sarcomata at Injection Site: No. of Rats Affected	Sarcomata at Injection Site: Time of First Recognition of Tumour in Days from the Start of Dosing	Other Neoplasms: No. of Rats Affected	Other Neoplasms: Type and Time when Found	Rats Dead Without Tumours: No.	Rats Dead Without Tumours: Time of Death	Duration of Experiment (Days)
	In "Carbowax 300"											
5	$CH_3(CH_2)_7CON\langle^{CH_2}_{CH_2}$	6♂	137	74	3·7	4	158, 168, [12]263, 324	3	1 Reticulosis, 124. 1 Adenoma of rectum, 144. 1 Mammary ca. at injection site, [12]263	0		
		6♀	137	72	3·6	5	[13]142, 151, 158, 168E, 263M	2	2 Mammary carcinomata at injection sites, [13]142, 196	0		
6	$CH_3(CH_2)_4CON\langle^{CH_2}_{CH_2}$	6♂	119	52·5	3·5	4	256, (256+), 378, 378	0		1	328	450
		6♀	119	52·5	3·5	4	144M■, 219●■, (287+), 287	1	1 Mammary ca. at injection site, 154	0		
7	$(C_2H_5)_2CH.CON\langle^{CH_2}_{CH_2}$	6♂	245	40	4·0	6	154, 168M, 236○■, 259, 288M, 327	0		0		
		6♀	245	46	4·6	5	[14]211○■, [18]248, [18]266, [17]266, 346	5	5 Mammary carcinomata at injection sites, 196, [14]211, [18]248, [18]266, [17]266	0		
	Controls ("Carbowax 300" alone)	6♂	180	—	5·0	0		0		0		448
		6♀	180	—	5·0	0		0		0		
		A6♂	150	—	6·5	0		1	1 Carcinoma of prostate, 448	5	669*, 669*, 737*, 744, 744	
		A6♀	150	—	6·5	0		1	1 Mammary fibroadenoma with intra-abdominal squamous cell carcinoma of unknown origin, 565	5	744, 744, 744, 744, 744	
	In Water											
8	$CH_3(CH_2)_2CON\langle^{CH_2}_{CH_2}$	6♂	179	22·5	4·5	6	83, 219●■, 237M, 252○, (328+), 328○	0		0		
		6♀	179	22·5	4·5	4	154■, [18]266, 288, 312	1	1 Mammary fibroadenoma, [18]266	2	28, 428	
9	$CH_3CON\langle^{CH_2}_{CH_2}$	6♂	110	16	3·2	3	245●■, (245), 252M○	0		0		449
		6♀	110	8	1·6	1	266	0		0		
11	$HN\langle^{CH_2}_{CH_2}$	6♂	59	1·2	1·2	0		1	1 Transitional cell carcinoma in kidney, 456	1	517	540
		6♀	59	1·0	1·0	2	166, 447	0		3	361*, 448, 537	

A, Injected daily, Saturdays and Sundays excluded.

TABLE IV

THE INCIDENCE OF LOCAL SARCOMATA IN MICE GIVEN SOME ETHYLENEIMINE DERIVATIVES, DISSOLVED IN ARACHIS OIL AND INJECTED SUBCUTANEOUSLY

Where the time of first recognition of a tumour is shown in parenthesis, the diagnosis was based solely upon microscopic appearances within the tumour. The symbol ● indicates that the tumour was transplanted into mice of the same strain as that in which it arose. The transplants invariably grew.

Compound		Mice		Duration of Dosing (Days)	Maximum Total Dose (mg./20 g.)	Maximum Total Vol. of Oil (ml./20 g.)	Sarcomata at Injection Site		Mice Dead Without Local Sarcomata. Time of Death in Days from the First Injection	Duration of Experiment (Days)
No.	Formula	No. and Sex	Strain				No.	Time When First Recognized (Days)		
3	$CH_3(CH_2)_{12}CON\langle^{CH_2}_{CH_2}$	20♀	W	40	33	2·2	1	(223)	44 (2), 45 (2), 46, 49 (2), 50, 51, 55 (2), 57 (3), 112, 113, 154, 224, 262	
		20♀	W	1	3	0·2	1	315	44, 45 (4), 46, 47, 51 (2), 53, 55, 64, 223, 300, 364, 444 (2), 465, 473	
		20♂	C_3Hf	222	80	2·0	13	228●, 228●, 243, 243, (243), 272, 309, 316, 316, 359●, 373, 395, 395	35 (2), 93, 343, 361	395
5	$CH_3(CH_2)_7CON\langle^{CH_2}_{CH_2}$	20♂	C_3Hf	137	10	3·8	4	210●, 250, 501, 501	36, 78, 83, 159, 270, 307, 312, 322, 344, 347, 412, 483, 506	504
		10♀	C_3Hf	1	0·8	0·1	0		119, 431 (2), 345, 494, 537. 545 (2), 569 (2)	
6	$CH_3(CH_2)_4CON\langle^{CH_2}_{CH_2}$	16♂	C_3Hf	179	15·3	2·55	5	228, 272, 283●, 311●, 460	41, 44, 45, 52, 53, 56, 104, 110, 221, 500, 531	385
		20♀	C_3Hf	284	7·2	2·4	2	373, 373	49, 80 (2), 94, 113, 130 (2), 193 (2)	
		17♀	C	1	0·5	0·1	0		29 (2), 72, 224, 227 (2), 234, 245, 314, 317, 375, 384 (2), 414, 420, 421, 422	
7	$(C_2H_5)_2CHCON\langle^{CH_2}_{CH_2}$	16♀	C_3Hf	137	9·75	3·8	10	158, 201, 201, 210●, 242, 272●, 300, 313●, 313●, 313●	28, 95, 425, 433, 443, 443	
8	$CH_3(CH_2)_2CON\langle^{CH_2}_{CH_2}$	20♀	C_3Hf	137	9·75	3·8	3	225, 242●, 328●	11 (2), 13, 17. 18 (2), 20 (2), 27, 207, 213, 283, 497, 500	504
		10♂	C	1	0·3	0·1	0		39, 46, 57, 258, 320 (6)	
		10♂	C_3Hf	1	0·3	0·1	0		12, 57, 66 (2), 89 (3), 435 (3)	
9	$CH_3.CON\langle^{CH_2}_{CH_2}$	20♂	C	59	4·0	0·8	6	185, 201, 224●, 259, 272, 375●	46 (2), 110, 138, 158, 334, 366, 375, 387, 392, 415, 420 (2), 482	
		20♀	C_3Hf	229	3·6	1·8	5	275, 319●, (293), 347, 347	25, 28, 35 (2), 42, 286, 310, 319, 347, 369	371

TABLE V

THE ACTION OF SOME ETHYLENEIMINE DERIVATIVES AND RELATED COMPOUNDS UPON THE WALKER TUMOUR

A, percentage anaphases in excess of controls showing specific chromosome effects, in tumour tissue from dosed rats; ΔW, mean percentage increase in gross weight of tumour-bearing rats; M_{50}, mean weight of n heaviest tumours in groups of 2n; I, percentage inhibition of tumour growth

No.	Substance: Formula	Cytotoxic Action: Dose: mg./100 g. i.p.	Cytotoxic Action: Effect on Tumour, A%	Cytotoxic Action: Effect on Bone Marrow	Growth Inhibition: Total Dose: mg./100 g. i.p.	Growth Inhibition: ΔW Controls	Growth Inhibition: ΔW Treated	Growth Inhibition: M_{50} Controls	Growth Inhibition: M_{50} Treated	Growth Inhibition: I
2	$CH_3(CH_2)_{16}CON\langle^{CH_2}_{CH_2}$ (*a*) Crude	20	5	A few "sticky" chromosome bridges						
		10	7	Normal						
		5	4							
	(*b*) Pure	20	9	A few "sticky" chromosome bridges						
		10	2	Normal						
		5	2							
3	$CH_3(CH_2)_{12}CON\langle^{CH_2}_{CH_2}$	20	2	Normal						
		10	0							
4	$CH_3(CH_2)_{10}CON\langle^{CH_2}_{CH_2}$	25	16	A few true chromosome bridges and fragments						
		10	6							
5	$CH_3(CH_2)_7CON\langle^{CH_2}_{CH_2}$	2·5	6	Normal						
		1·25	5							
6	$CH_3(CH_2)_4CON\langle^{CH_2}_{CH_2}$	5	12	Some nuclear pyknosis and cell degeneration						
7	$(C_2H_5)_2CH.CON\langle^{CH_2}_{CH_2}$	5·0	5	Traces of nuclear pyknosis, etc.	5	22	1	46	40	14
		2·5	0	Normal						
8	$CH_3(CH_2)_2CON\langle^{CH_2}_{CH_2}$	5·0	11	Traces of nuclear pyknosis	5·5	22	8	46	43	6
		2·5	4	Normal						

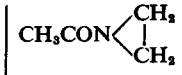
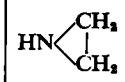
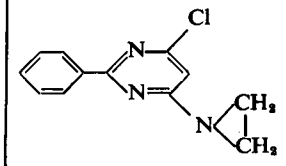
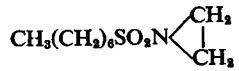
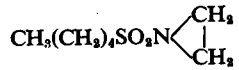
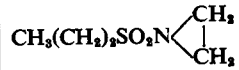
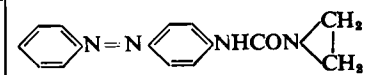
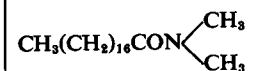
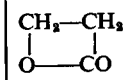
9		1·0 0·5	12 4	Some chromosome fragmentation; traces of nuclear pyknosis, etc. Some fragments and a few bridges	13	35	13	36	28	24
11		0·25 0·125	7 2	A few " sticky " chromosome bridges	0·5	15	−3·2	29	22	25
12		8·0 4·0	6 7	Normal	26	37	27	41	36	11
13		10 5	3 3	Normal						
14		15 10	10 6	A few bridges and fragments Normal						
15		10 5	0 2	Normal						
16		15 10	5 4	Normal	40	27	4	38	24	36
20		50	0	Normal						
21		5 2·5	4 3	Normal	36	34	27	36	36	0

Table IV.—While the results recorded in Table III left little doubt that the tumours which developed with the ethyleneimine derivatives at the site of injection could be attributed to the direct action of these agents themselves, it was desirable to explore their carcinogenic potentialities further by giving selected compounds subcutaneously to mice. There is evidence that the connective tissues in this species are less sensitive to the action of low grade carcinogens than are those in the rat. Thus Burrows, Hieger, and Kennaway (1936) obtained subcutaneous sarcomata in 6% of rats injected with lard alone, whereas neither these workers nor Andervont (1934) obtained any such tumours with this substance in mice. Burrows (1932) studied the effect of repeated subcutaneous injection of various fats and oils (not, however, including arachis oil) in mice, and found that, although the tissues showed inflammatory changes, no sarcomata developed.

The results of our experiments are recorded in Table IV, together with the time of death of animals in which no tumours were detected. No tumours appeared in control experiments (not tabulated) in which a group of 20 males of strain C_3Hf was given arachis oil alone twice weekly. The survivors had each received 3.9 ml. when treatment was stopped on the 137th day. Nine died before the 52nd day, and three others on days 370, 382, and 454 respectively. The rest were alive and well at the time of writing (504 days). In a repeat test, 14 out of 18 male mice of this strain have survived for more than 267 days, each having been given a total of 3.7 ml. of oil. In a further similar experiment, started with 20 male mice of strain C, eleven died by the 59th day and the rest looked ill. Treatment was discontinued, each of the survivors having had 0.85 ml. of oil. Another mouse in this group died on day 230, and the rest between the 312th and 391st day of experiment. In other tests in mice of this strain, 7 out of 15 males have survived for upwards of 342 days, and 4 out of 14 females for 232 days or more, the total volume of oil received by the mice surviving at these times being 1.1 and 1.3 ml. respectively, and 8 out of a further 13 females have survived for over 300 days, each having had 2.95 ml. of oil.

Subcutaneous sarcomata have appeared at the injection site in mice with each of the six acylethyleneimines. They appeared between 158 and 501 days after the first injection. None of these tumours was observed to regress. Many were seen to infiltrate into the skin or subjacent tissues, but no distant metastases were found. One or more of the sarcomata induced with each of the substances mentioned was transplanted subcutaneously into mice of the same strain. Transplantation was successful in every case (compare experience with randomly bred rats) and the resulting tumours grew progressively until they killed their hosts. Serial transplantation was attempted with a few of the tumours. This again invariably succeeded, and has been carried with one tumour to the nineteenth generation.

Mammary carcinoma arising at or near the injection site was seen in three, and uterine carcinoma in one, of the randomly bred mice given myristoylethyleneimine (3). Pulmonary adenomata were found in C_3Hf mice in several groups, and one mouse of this strain given diethylacetylethyleneimine (7) developed leukaemia. In the absence of precise knowledge of the incidence of such neoplasms in our untreated mice, it is not possible to say what part, if any, was played by the compounds in their genesis.

Tumour Inhibition

Table V.—This table shows the results obtained when seven of the substances listed in Table II were examined for tumour inhibition. None produced a greater inhibitory effect upon tumour growth than might be expected from its general "toxic" action, reflected in a reduction in the gain in gross weight of the tumour-bearing animals over the experimental period (cf. Walpole, 1951).

Cytotoxic Action

Table V.—The distinguishing features of the pertinent chromosome effects have already been described (Part I), and the abnormalities obtained with many of the substances now under test are recorded in column A of Table V. Qualitative observations upon the effects seen in the bone marrow are also included.* It will be seen that the extent of the specific changes produced by the most active of the present series must be regarded as slight compared with that obtained with tumour-inhibitory polyfunctional ethyleneimine derivatives (Part III).

Discussion

Our earlier study of the ethyleneimines (Part III) was mainly concerned with tumour inhibition. When numerous examples had been tested the conclusion was reached, as already mentioned, that tumour growth-inhibitory activity and the capacity

* The cytological evaluation of these preparations was carried out by Miss J. M. Gates.

to elicit the distinctive cytotoxic effects associated with it was limited in this series to polyfunctional derivatives. Some exceptions to this generalization were found, however. While a majority of the monofunctional compounds examined showed trace activity at the most, at least two, namely, 2:4-dinitrophenylethyleneimine (Part III, Serial No. 257) and 2-ethyleneimino-4:6-dimethoxy-1:3:5-triazine (Part III, S.N.272), were more active. Even with these, very much higher doses were needed than with comparable bifunctional derivatives to produce a similar degree of inhibition of tumour growth. It seems possible that the exceptional activity of these compounds is due to some special property of the system to which the single ethyleneimine residue is attached, by virtue of which that system, or some part of it, acts as a second functional group. It is perhaps significant that in each case highly polar substituents are present.

At the same time we found that, while a pronounced inhibition of tumour growth was demonstrable with a majority of the polyfunctional ethyleneimines examined, a much less marked effect could be obtained with some few, and in particular with bifunctional carboxyethyleneamides such as bis-cycloethyleneadipamide (Part III, Table VI). This substance (Serial No. 285) produced only 40% inhibition of the growth of the Walker tumour, but a 77% increase in mitoses showing specific abnormalities. Some doubt must now be attached to these figures, as we have since found that such bis-carboxyethyleneamides are less stable than was earlier supposed, and it is possible that at the time of test some decomposition had already occurred. Their instability is thought to be due either to isomerization of a carboxyethyleneamide group to an oxazoline ring system, or to partial polymerization, neither of which processes can be readily detected by elementary analysis. The high activity demonstrated with closely related bifunctional compounds containing the urea linkage (Part III, Serial Nos. 275 and 277) is consistent with the greater stability which they are known to have. Similar considerations apply to derivatives of the sulphonethyleneimine series, which are unable to isomerize, and of which several were shown to be highly active (Part III, Table VI).

Unfortunately, little information is as yet available upon the carcinogenic potentialities of polyfunctional ethyleneimine derivatives. We have already reported that triethylenemelamine (T.E.M.), given intravenously to Strong A mice, causes an increase in the incidence of pulmonary adenomata, and essentially similar results were obtained by Shimkin (1951). We failed, however, to induce neoplasia in stock albino mice by the repeated subcutaneous injection of this substance in aqueous solution (Part III, p. 398). It is, therefore, of particular interest that in a recent and as yet unfinished experiment, we have obtained sarcomata in rats at the site of subcutaneous injection of the compound in oil. Six male and six female, stock, albino rats were given twice-weekly injections of 0.01 mg. of triethylenemelamine in 0.05 ml. of arachis oil per 100 g. body wt. in total doses of from 0.7 to 1.1 mg./100 g. Tumours have so far appeared in eight of these animals, five males and four females, at times ranging from 241 to 322 days from the start of experiment. On histological examination these proved to be mixed cell or spindle cell sarcomata, infiltrating adjacent muscle. The substance is, therefore, more active as a carcinogen than any of the monofunctional ethyleneimines so far examined.

In the absence of more extensive information upon the carcinogenicity of polyfunctional ethyleneimines, it is of interest to note that Haddow and his associates have obtained tumours in several animal species with analogous polyfunctional derivatives in the "mustard," epoxide, and methane sulphonoxyalkane series. The full details of this work have not yet been published, but frequent reference to it has been made (vide, e.g., Haddow, 1951), and Koller (1953) has recorded briefly the production of sarcomata in rats with several aromatic nitrogen mustards, with butadiene dioxide and with 1:4-dimethanesulphonoxybutane ("Myleran"). We have already reported the production of carcinoma of the skin in mice by painting with vinyl*cyclo*hexene dioxide in acetone (Part II, p. 250), but repetition of this experiment with a more highly purified sample of the diepoxide has failed to yield tumours, and this finding must be regarded for the time being as *sub judice*.

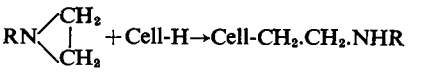
We have now obtained unequivocal evidence of carcinogenic activity with several monofunctional ethyleneimine derivatives. The substances in question were mostly *N*-acylethyleneimines, but included also 4-chloro-6-ethyleneimino-2-phenylpyrimidine (12). Testing of the free imine has been hampered by its local irritant action, but some tumours have resulted from the use of small, carefully spaced doses. On the other hand, no activity was detected with any of the three ethyleneimino-sulphonylalkanes examined, and with laurolethyleneimine (4) the yield of tumours was negligible. From what was known of mono-

functional derivatives, none of the above substances was expected to cause marked inhibition of the growth of the Walker tumour or to be highly active in our test for specific cytotoxic activity. It is evident from Table V that these expectations were amply justified.

Our knowledge of the mutagenic potentialities of the ethyleneimines is also deficient. Rapoport (1948) has recorded mutagenic activity in *Drosophila* for the free imine, and Kölmark and Westergaard (1953) have induced back mutation in an adenine dependent strain of *Neurospora crassa* with this substance. The latter authors state that triethylenemelamine proved to be very toxic to *Neurospora* and no effects on the adenine locus could be demonstrated. Earlier work in these laboratories demonstrated that triethylenemelamine caused mutations in a strain of *Penicillium chrysogenum* (Part III, p. 398), and Bird (1952) has since shown that it is active in a modified ClB test in *Drosophila.*

Some experiments of our colleague, J. M. Pryce, are of interest in this connection. Pryce has submitted suspensions of the pigmented organism *Chromobacterium prodigiosum* (*Serratia marcescens*) to the action of a wide range of mono- and polyfunctional ethyleneimine derivatives. His results will be reported and discussed in detail elsewhere, but, briefly, it was found that the substances mentioned fell into two groups with respect to their action on the bacterium. With those of one group, applied in concentrations which killed a high proportion of the bacteria, a great majority of the survivors yielded normal colonies which on subculture grew at the same rate as those from untreated controls. When only 1 in 10^8 to 1 in 10^{10} of the bacterial cells survived, however, a high proportion of mutant colonies (type A) were obtained. These developed slowly and never reached large size. They were white or pale pink in contrast to the deep red colour of the parent strain, failed to ferment many of the carbohydrates affected by the latter, and for the most part retained these characteristics on serial subculture. With substances of the second group, a high percentage of abnormal colonies (type B) was obtained when the proportion of bacteria surviving in the treated suspensions was still above ca. 1 in 10^4. These colonies showed a marked lag in pigment production (a variation which occurs to a slight extent in untreated cultures of the parent strain), and on repeated subculture gave rise to populations showing continuous variation in the amount of pigment formed. When employed in concentrations permitting the survival of only a very small proportion of the bacteria, substances of the second group also yielded mutants of the type (A) first described. From the results so far obtained it appears that polyfunctional ethyleneimines belong exclusively to the first group of substances, while monofunctional derivatives, including the free imine, fall into the second category. It is noteworthy that the polyfunctional derivatives, applied in concentrations below those required to give an appreciable yield of mutants of the first type (A) did not give rise to variants of the kind (B) which were encountered typically, when the killing effect was low, with monofunctional derivatives of the second group. However, mutants of type A were produced by substances of both groups when used in concentrations at which very few of the bacteria survived. This is taken as evidence for a qualitative difference in the mode of action of the mono- and polyfunctional derivatives respectively when applied in these lower concentrations, while at higher, more lethal concentrations, their effects are more comparable.

The discovery of carcinogenic activity in the simpler *N*-acyl ethyleneimines invalidates the hypothesis, for this series of compounds at least, that carcinogenicity depends in any way upon a tendency of the hydrocarbon chains of the agent molecules to pack together in micelles (see Part III). With hydrocarbon radicals as simple as the acetyl no such tendency exists. As an alternative, we may regard ethyleneimine as the ultimate carcinogen, the function of the acyl group being merely to modulate chemical reactivity and provide an electrically neutral derivative capable of diffusing readily into accessible cells. Such results as have been obtainable with the free imine are in keeping with this view. The most facile chemical reaction both of the free imine and of its acyl derivatives is that with nucleophilic groups, accompanied by the opening of the strained, three-membered, ethyleneimine ring. Hence we may represent the most likely form of interaction with cell components by the general scheme :



in which Cell-H is a cell component containing nucleophilic groupings such as hydroxyl, thiol, amino, etc. Further reaction may give a polyethyleneimine type of condensate, Cell-$(CH_2.CH_2.NR)_x CH_2.CH_2.NHR$.

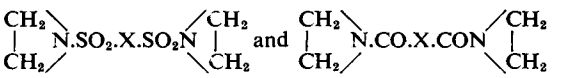
Sulphur and nitrogen " mustards," epoxides, and methylolamides have a similar type of chemical

reactivity (Part III, p. 358), while molecules containing two or three such reactive groups may be presumed to combine at more than one nucleophilic centre. It was this possibility with the nitrogen mustards in particular that led Goldacre, Loveless, and Ross (1949) to suggest "cross-linkage" as the mechanism by which these agents exert their characteristic action on dividing cells. In their hypothesis nucleophilic centres in sister chromatids were regarded as the respective sites of attachment of each polyfunctional molecule of the agent. Conceptions of this kind led Haddow and Timmis (1951) to investigate a series of bis-methanesulphonoxy alkanes, and these were indeed found to produce biological effects similar to those of the polyfunctional "mustards."

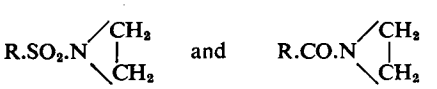
Our attention was directed to β-propiolactone by the report of Smith and Srb (1951) that they had produced mutations in *Neurospora* with this *substance*. β-propiolactone resembles ethyleneimine in several respects. It contains a similar strained ring, here four-membered, reacts readily with nucleophilic reagents, and equally readily polymerizes. These considerations, and the results obtained by Pryce with this substance in tests upon *Chromobacterium prodigiosum*, prompted us to examine it as a carcinogen, and, as shown in Table II, it was found to produce local sarcomata in rats. Although no corresponding bifunctional compound has so far been available for study, such a substance, containing two β-lactone rings suitably linked, might well prove to be a potent inhibitor of tumour growth.

These examples illustrate the fruitfulness of the concept of direct chemical interaction with cell components as the initial stage in the production of the biological effects under consideration here.

Another striking feature of our results is our failure to obtain tumours with ethyleneiminosulphonylalkanes, in spite of the fact that in earlier tests a number of corresponding bis (ethyleneiminosulphonyl) alkanes were all shown to have tumour growth inhibitory and specific cytotoxic activity (Part III, pp. 370, 372). Whereas compounds of the two series



(the latter with some structural reservations) are active tumour inhibitors, in only the second of the two related series of monofunctional derivatives,



have carcinogens been found. It may be that this difference in behaviour between monofunctional ethyleneimines of the two series is due to differences in absorption, tissue distribution, and metabolism. We incline rather to interpret these results as an indication that the site within the cell involved in carcinogenesis is not identical with that concerned in tumour inhibition.

It is not unreasonable to regard the inhibition of tumour growth produced by polyfunctional ethyleneimine derivatives as being dependent upon their action on the chromosomes; to view it, that is, as a manifestation of that action related to their specific cytotoxic and mutagenic activity. Ethyleneimine itself causes mutations in *Drosophila* and *Neurospora* and several of its monofunctional derivatives produce variants in *Chromobacterium prodigiosum*, although it should be remembered that there is a qualitative difference between the action of mono- and polyfunctional derivatives, at low concentrations, upon the latter organism, and that only at higher, more lethal concentrations, is their action comparable. Monofunctional ethyleneimines, with the exceptions noted above, have little visible effect upon the chromosomes of dividing cells of the Walker carcinoma in the highest doses tolerated by rats bearing this tumour. It has been shown in these laboratories, however, that when applied in aqueous solution in very high concentrations (M/100–M/50) to *Vicia* root tips, acetylethyleneimine causes chromosome fragmentation and bridge formation comparable in extent with that produced by polyfunctional ethyleneimines in very much lower concentrations (C. H. Ockey, unpublished). It thus appears that, in respect of the production of these chromosome effects, the difference between the action of mono- and polyfunctional ethyleneimines is quantitative rather than qualitative. These findings, in part, support the view that the carcinogenic effect of the agents described here is also a chromosome effect; that carcinogenesis is due to some form of gene or chromosome mutation. This, however, is not the only conceivable mechanism by which transformations in cell type may be reproduced in successive generations. Several examples are now recognized of the implication of cytoplasmic self-reproducing elements (plasmagenes) in cell heredity (v., e.g. Ephrussi, 1953), while Hinshelwood (1952), as a result of his study of bacterial adaptation, has concluded that certain effects of this nature may arise from differential action on intracellular enzymes. Our observations may equally well be taken as evidence for the view that ethyleneimines, and other substances having similar chemical reac-

tivity, owe such carcinogenic activity as they possess to chemical processes analogous to or identical with those proposed hitherto for nuclear material, but involving cytoplasmic cell components, and not necessarily those that are essentially nucleoprotein in nature. In any event, it appears from our results with ethyleneiminosulphonyl alkanes that the presence of the ethyleneimino-group in a molecule is not alone sufficient for carcinogenic activity and that a degree of specificity dependent upon other features of the molecule obtains. In the example cited this is probably referable to the steric and polar characteristics of the groups immediately attached to the nitrogen of the ethyleneimine ring (sulphonyl as compared with carbonyl).

The results obtained with the small group of Butter Yellow analogues (Table II, Nos. 16–20) require separate comment. Some of the experimental findings support the hypothesis previously advanced (Mueller and Miller, 1950; see also Part III, p. 408) that the oxidation *in vivo* of methyl to methylol, a group related in chemical reactivity to ethyleneimino, may be involved in the carcinogenic action of certain compounds containing dimethylamine residues. Thus compound 17 (*p-N*:*N*-dimethylureidoazobenzene) gave tumours remote from the site of injection only, suggesting that intermediary metabolism here precedes carcinogenesis. On the other hand, compound 16 (*p-N*-cycloethyleneureidoazobenzene), differing from the last only in that the reactive ethyleneimino- replaces the dimethylamino-group, gave several tumours at the injection site. In view of the behaviour of the arachis oil controls, the few very late tumours obtained with compounds 18, 19, and 20 cannot be regarded as significant.

Conclusion

An outstanding feature of many of the carcinogens here described is structural simplicity. The majority are aliphatic and represent the first extensive series of such compounds shown to be carcinogenic. Their activity in this direction appears to depend mainly upon the presence in their molecules of the ethyleneimine radical, acting, we suggest, as a prosthetic group by means of which they are able to combine with certain cell constituents. They can be regarded as the prototypes of a new range of carcinogens built up by introducing the ethyleneimine ring into organic chemical systems of diverse types (v. Table II, Nos. 9, 10, 12, and 16). The carcinogenic activity of β-propiolactone suggests that the β-lactone ring system might be an effective substitute for ethyleneimine in this respect.

Interpreted in this way our findings lend additional weight to the view, for which there is already much evidence, that the initial step in chemical carcinogenesis proper consists in the attachment of "foreign" residues to cell components, whereby those components become inactivated or deleted and their reproduction "blocked" (vide, e.g., Miller and Miller, 1952). It may well be that all chemical carcinogens are either already equipped with reactive centres through which such attachment may be effected—as, for example, the reactive, so-called "K" region in the carcinogenic polycyclic hydrocarbons—or acquire reactive groupings serving this function by preliminary metabolism, as with Butter Yellow (Mueller and Miller, 1950).

The study of chemical carcinogenesis has often been handicapped in the past by uncertainty whether a particular carcinogen is, of itself, the effective agent initiating malignant transformation or whether it has first to be activated by metabolic change. Such activation is known to occur with Butter Yellow, as noted above, and with β-naphthylamine (Bonser, Clayson, Jull, and Pyrah, 1952), and is suspected with 4-aminodiphenyl (Walpole *et al.*, 1952). It may be assumed with some confidence that the carcinogens described in this paper need undergo no such preliminary changes and are the initiatory agents *per se*. By their use a more direct approach should be possible to the problem of identifying the site or sites of action of carcinogens. Moreover, with the ethyleneimines it should be possible to select a "carrier" molecule suited to the detection of the agent within cells or in cellular fractions by either chemical methods or those dependent upon fluorescence or the use of radioisotopes. In this connection it is pointed out that the question of the integrity of the C-N bond attaching the acyl residue in the ethyleneamides has been left open, but it should not be too difficult to devise linkages less liable to fission than these. That present in the pyrimidine derivative (Table II, 12) might conform to this requirement.

An additional advantage of the present substances is the distinctive nature of their chemical affinities. While it is perhaps unlikely that all carcinogens act in precisely the same way, it is nevertheless possible that at some point between the first exposure of the cell to the agent and the end-result of malignancy, a wide conformity of behaviour obtains. It is considered that further study of the types of cell components most likely

to be involved in interaction with ethyleneimines and analogous structures is more likely to lead to the ultimate explanation of carcinogenic action than the mere extension of the already imposing array of carcinogens. It is hoped that others working in this field will take advantage of these observations in their own researches.

SUMMARY

1. Evidence is presented that several simple *N*-acylethyleneimines, some other monofunctional ethyleneimine derivatives, ethyleneimine itself, and β-propiolactone are carcinogenic in rats and mice.

2. No tumours were obtained with three ethyleneiminosulphonyl alkanes tested in arachis oil in rats.

3. None of the above substances, given in the highest tolerated doses to rats bearing the Walker carcinoma, produced extensive chromosome changes in the tumour, of the type associated with the inhibition of its growth. None of those tested produced a significant inhibition of the growth of the tumour. In both respects these substances differ in behaviour from typical polyfunctional ethyleneimines.

4. The information available upon the tumour-inhibitory, cytotoxic, carcinogenic, and mutagenic activity of ethyleneimine derivatives is reviewed. It is concluded that the carcinogenic action of monofunctional ethyleneimines may be due to direct chemical attack either upon the chromosomes (leading to gene or chromosome mutation) or upon cytoplasmic cell components. The information is as yet too fragmentary to enable a decision to be made between these alternative possibilities.

5. The advantages offered by ethyleneimine derivatives in the study of chemical carcinogenesis are discussed.

The authors are indebted to Mrs. B. Hubbert, Miss A. E. Berry, and Miss D. Bonson for their assistance in the laboratory, and to Miss J. J. Matthews and Miss M. H. Irving for their help with the histopathology.

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