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CYTOTOXIC AGENTS: III, DERIVATIVES OF ETHYLENEIMINE

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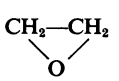
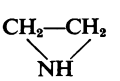
In Parts I and II of this series (Hendry, Rose, and Walpole, 1951 ; Hendry, Homer, Rose, and Walpole, 1951) we described the cytotoxic and tumour-inhibitory properties exhibited by certain polymethylolamide and bis-epoxide derivatives. Substances of these types were selected because of their use in textile technology in modifying the properties of fabrics. They are believed to bring about their peculiar effects by the formation, by virtue of their polyfunctional reactivity, of covalent linkages across the cellobiose or peptide chains of the textile fibres. It seemed not unlikely that the specific biological effects of these compounds were manifestations of a similar behaviour within the cell, and it was suggested that here the affected system was either protein or nucleoprotein, possibly of nuclear origin, since chromosome damage in dividing cells seemed to underlie the more significant features of their action.

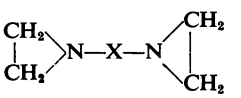
Compounds of a third chemical type were known to us to have applications similar to those of the methylolamides and epoxides in the textile field, and indeed were already under investigation in that connexion by our colleagues in these laboratories. These compounds were based on ethyleneimine (II), which in its chemical reactivities closely resembles the analogous ethylene oxide (I) molecule. The simple parent substance, like ethylene oxide, itself produces modifications in the physical properties of fibres, but the most effective agents in this series contain two or more imine residues, just as the most useful epoxides contain two epoxide groups. It is perhaps worthy of note at this point that the most convenient mode of combination of the imine residues in the molecule is usually through a linkage at the nitrogen atom (III), whereas the epoxide groups must necessarily be combined through a group attached to one of the carbon atoms (IV).



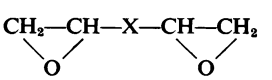
(I)



(II)

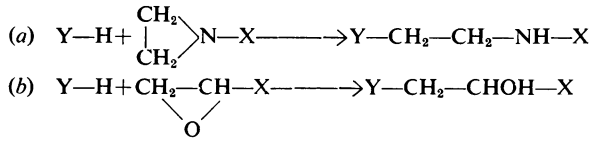


(III)

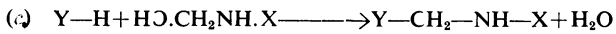


(IV)

Nevertheless it is found that N-substituted ethyleneimines and C-substituted ethylene oxides show a like reactivity towards nucleophilic reagents, leading to chemical union with the second reactant according to the schemes:



The methylolamides described in Part I behave similarly according to the scheme:



Well over a hundred derivatives of ethyleneimine have been prepared and subjected to a variety of biological tests during the past two years, so that it has become possible to relate structure with effect fairly closely. As already indicated, some of the compounds were derivatives that had been initially conceived as textile auxiliary agents. These included the early 1 : 3 : 5-triazine derivative, 2 : 4 : 6-tris-ethyleneiminotriazine (Serial No. 322), certain bis-ureas (276, *et seq.*), and bis-sulphonamides (287–294).

At an early stage it became apparent that, with a few notable exceptions, marked cytotoxic and tumour-inhibitory action in the animal was dependent upon the presence within the drug molecule of at least two ethyleneimine residues, and the chemical aspects of the research (to be recorded elsewhere) became essentially an investigation of the many possible ways in which this could be brought about. Since ethyleneimine is a dialkylamine, the preparation of amide structures was an important theme, not least because the necessary products could be obtained at temperatures below those at which secondary reactions intervened. The facility with which condensation occurs between the amine and chloroheterocyclic structures was also exploited. Thus, with cyanuric chloride as starting material, the three chlorine atoms could be replaced stepwise more or less at will by one or two ethyleneimine residues and other amine or alkoxy groupings* (compare 272, 314, 320, and 321). Similarly, in the guanamine series,* it was possible to prepare compounds such as 324 and 325. Di- and trichloropyrimidines were utilized in the same way,* although with these the necessary reactivity towards ethyleneimine could be obtained only by having present certain other substituents in the pyrimidine nucleus (compare 261, 269, 299, 304, etc.).

As the investigation developed, isolated instances were found of monoethyleneimine derivatives having tumour-inhibitory activity, for example, 2 : 4-dinitrophenylethyleneimine (257), and attempts to elucidate what seemed to be the peculiar mode of action of this compound led to the preparation of related substances such as 259, 335, 337, and 342.

During the course of this research it was learned that workers at the Sloan-Kettering Institute, New York, had independently come to examine substances based on ethyleneimine, including a number formulated in the Tables below. It is a pleasure to record here the valuable exchange of notes and discussion that has followed this coincidental discovery.

* Patent protection pending.

Experimental

Toxicity.—Toxicity tests in mice and rats were carried out on all compounds, given intraperitoneally, as described in an earlier paper (Hendry, Rose, and Walpole, 1951). The majority were either dissolved in water or, if insoluble, suspended by milling in an aqueous solution of Dispersol OG, 1.5 per cent, and Dispersol LN, 0.05 per cent. The remainder were dissolved or suspended in arachis oil. Solutions and suspensions were kept in the refrigerator; where marked instability was known or suspected they were made up freshly before injection. Ethyleneimine was kept over caustic pellets and dissolved in CO_2-free water immediately before use. The results of these tests are not recorded here, but from them an estimate was made of the highest doses that could be given to rats daily, Sundays excepted, for the first 10 to 12 days after implantation of the Walker tumour, without any of the animals dying by the fourteenth. This schedule of dosage was used in initial tests for tumour-inhibitory activity.

Tumour inhibition.—Our standard method of test for inhibitory activity, using the Walker tumour, has also been described (Walpole, 1951) and was briefly as follows.

Single subcutaneous implants were made in stock rats of about 100 g. in weight, and the rats were then separated into groups of from 10 to 15. One group was kept untreated as control and the others dosed as above with the compounds under test. On the fourteenth or, occasionally, the fifteenth day—the day of implantation being taken as day 0—all the animals surviving in the experiment were weighed and killed and the tumours dissected out and weighed. The percentage increase in gross weight ($\triangle W$) of the survivors in each group and the percentage inhibition of tumour growth (I) in each of the treated groups was then calculated, the latter according to the formula.

$$I = \frac{(M_{50}\ \text{controls} - M_{50}\ \text{treated})}{M_{50}\ \text{controls}} \times 100$$

where M_{50} was the mean weight of the n largest tumours in any group of $2n$.

Results

In Tables I to III are listed all the compounds examined. The serial and code numbers, and the name and formula of each, are shown together with the total (intraperitoneal) dose in mg. per 100 g. rat given over the first 10 to 12 days after implantation of the tumour. Compounds will be referred to in the sequel either by name or by serial number or both.

Table I includes ethyleneimine itself (Serial No. 246), polyethyleneimine (246a), and a number of monoethyleneimine derivatives (247–274). Many of these are inactive or inhibit tumour growth no more than might be expected from their general "toxic" action. A few, however, are active in the sense that with them the inhibition produced is greater than can be accounted for on that basis. Of these the dimethoxytriazine 272 has already been reported by workers at the Sloan-Kettering Institute [see Note in review by Philips (1950)] as showing appreciable tumour-inhibitory and cytotoxic activity. To this must now be added other heterocyclic monoethyleneimines, notably the corresponding diaminotriazine 271, the dichloropyrimidine 262, and the phenyldichloropyrimidine 263. With all these activity is comparatively low, being no more than one-tenth to one-hundredth that of the nearest related bis-ethyleneimine derivatives, and the methods of preparation were such that the possibility cannot be excluded that the latter were present as contaminants in amount sufficient to produce the observed biological effects. Great

TABLE I

ACTION OF SOME MONO-FUNCTIONAL ETHYLENEIMINE DERIVATIVES UPON THE WALKER TUMOUR IN RATS

a.s., aqueous solution; o.s., oily solution; a.d., aqueous dispersion; o.d., oily dispersion; $\triangle W$, 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; L, sample

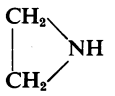
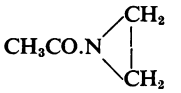
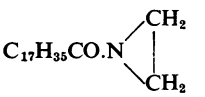
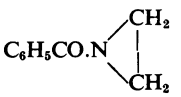
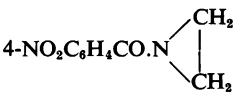
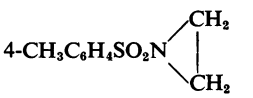
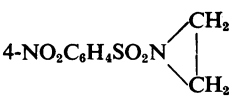
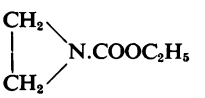
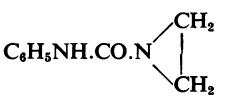
Serial No.	Code Number	Name	Formula	Form	Total dose (mg./100 g. i.p.)
246	10355	Ethyleneimine		a.s. (CO_2-free water)	0.5
246a	9162	Polyethyle n	$[-CH_2CH_2NH-]_n$	a.s.	8.5
		AMIDES			
247	9209	Acetylethyleneimine		,,	13
248	9952	Stearoylethyleneimine			Untested
249	9210	Benzoylethyleneimine		o.s.	10
250	10577	*p*-Nitrobenzoyl-ethyleneimine		a.d.	15
251	9211	*p*-Toluene sulphonyl-ethyleneimine		,,	52.5
252	10755	*p*-Nitrobenzene sulphonylethylene-imine		,,	47.5
		URETHANES AND UREAS			
253	10356	Carbethoxyethylene-imine		o.s.	4
254	9212	Phenylcarbamyl-ethyleneimine		a.d.	20

TABLE I (*continued*)

limited in amount, maximal dose not given; *A*, percentage anaphases in excess of controls showing specific chromosome effects in tumour tissue from rats bearing the Walker tumour, 24 hours after the doses shown; *B*, true chromosome bridges; *D*, degenerating cells; *E*, "exploded" nuclei; *F*, chromosome fragments; *M*, accessory micronuclei; *P*, pyknotic nuclei; *S*, "sticky" chromosome bridges

Tumour inhibition					Cytotoxic action			
$\triangle W$		M_{50}		*I*	Dose (mg. per 100 g. i.p.)	Tumour		Bone marrow
Control	Treated	Control	Treated			*A*	Remarks	
15.0	−3.2	29.1	21.8	25	0.5	4		A few *S*
27.7	31.0	31.6	26.7	16	—	—		—
35.1	13.4	36.1	27.5	24	—	—		—
					20	9		Normal
35.4	13.2	37.9	34.4	9	—	—	—	—
45.7	28.4	42.5	36.7	14	5.0	3		A few *S*
35.4	38.7	37.9	43.5	0	—	—	—	—
34.6	24.6	46.1	35.3	23	8.0	4	—	Normal
39.7	18.1	49.4	35.5	28	4	3	—	A few *S* and *P*
35.4	18.8	37.9	41.6	0	—	—	—	—

TABLE I (*continued*)

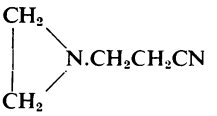
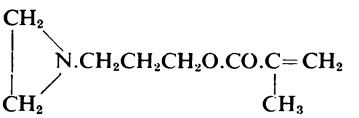
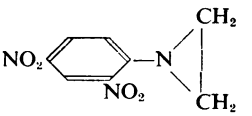
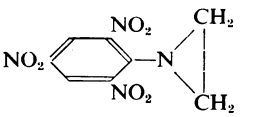
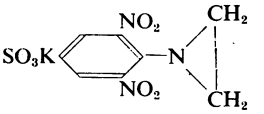
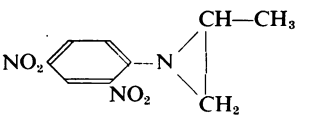
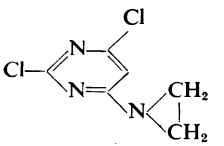
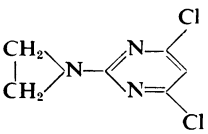
Serial No.	Code Number	Name	Formula	Form	Total dose (mg./100 g. i.p.)
		NITRILES AND ESTERS			
255	10574	β-Ethyleneimino-propionitrile		a.s.	7.2
256	9213	γ-Ethyleneimino-propyl methacrylate		o.s.	29
		NITROPHENYL DERIVATIVES			
257	10200	2:4-dinitrophenyl-ethyleneimine		a.d.	25
258	10357	2:4:6-trinitrophenyl-ethyleneimine		,,	40
259	10425	2:6-dinitrophenyl-ethyleneimine-4-sulphonic acid (K salt)		,,	375
260	10800	N-2:4-dinitrophenyl-C-methyl-ethyleneimine		,,	130
		PYRIMIDINES			
261	9605	2:4-dichloro-6-ethyleneimino-pyrimidine		o.d.	9.5
262	11154	4:6-dichloro-2-ethyleneimino-pyrimidine		a.d.	100

TABLE I (*continued*)

Tumour inhibition					Cytotoxic action			
$\triangle W$		M_{50}		I	Dose (mg. per 100 g. i.p.)	Tumour		Bone marrow
Control	Treated	Control	Treated			A	Remarks	
52.4	16.3	32.3	17.8	45	2	4	(Mean of 2 results)	A few *S*
35.1	29.9	36.1	35.0	3	—	—	—	—
18.8	−10	35.7	0.8	98	10	60–64	Some inhibition of mitosis. Many *D* and *E*	*B* and *F* and a few *D* and *P*
					5		Mitosis inhibited. *D*, *E*, and *M*	A few *B* and *F*
					2.5	52		A few *B*, *F*, and *S*; some *D* and *P*
16.6	13.0	30.5	30.4	0	20	35		A few *B*, *F*, and *S*
					5	6		Normal
24.6	19.1	23.0	9.4	59	20	53		A few *S*
43.6	12.0	46.0	24.0	48	30	3		Normal
42.6	27.1	42.3	30.7	27	2	23		,,
					1	12		,,
37.0	23.0	37.0	9.8	74	15	53		A few *B* and *F*

TABLE I (*continued*)

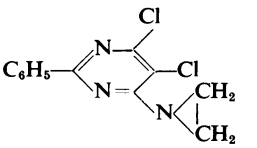
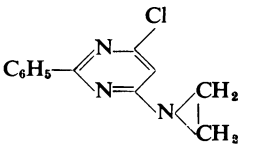
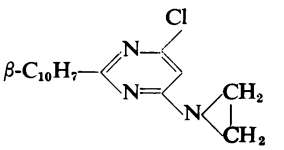
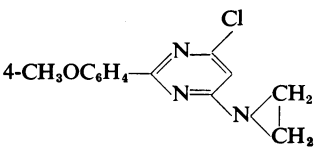
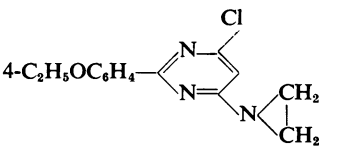
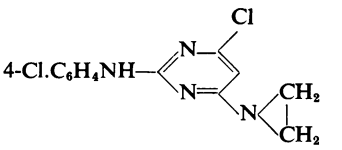
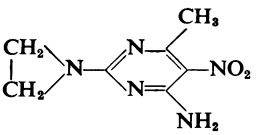
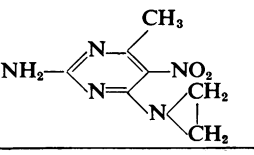
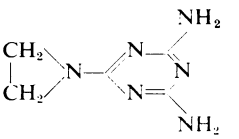
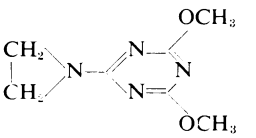
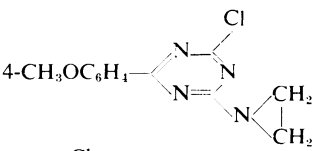
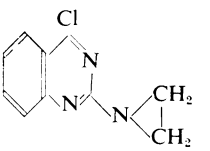
Serial No.	Code Number	Name	Formula	Form	Total dose (mg./100 g. i.p.)
263	11126	4: 5-dichloro-6-ethyleneimino-2-phenylpyrimidine		a.d.	100
264	10038	4-chloro-6-ethyleneimino-2-phenylpyrimidine		,,	26
265	10165	4-chloro-6-ethyleneimino-2-β-naphthylpyrimidine		,,	15
266	10407	4-chloro-6-ethyleneimino-2-*p*-anisylpyrimidine		,,	26
267	10166	4-chloro-6-ethyleneimino-2-*p*-phenetylpyrimidine		,,	25
268	10036	4-chloro-6-ethyleneimino-2-*p*-chloranilinopyrimidine		,,	25
269	9951	5-nitro-4-amino-2-ethyleneimino-6-methylpyrimidine		,,	10
270	10427	5-nitro-2-amino-4-ethyleneimino-6-methylpyrimidine		,,	13.5

TABLE I (*continued*)

Tumour inhibition					Cytotoxic action			
$\triangle W$		M_{50}		I	Dose (mg. per 100 g. i.p.)	Tumour		Bone marrow
Control	Treated	Control	Treated			A	Remarks	
37.0	3.6	37.0	2.0	95	10	39		A few *B* and *F*
37.1	26.7	40.8	36.1	12	8	6	A few *D*	Normal
31.8	11.5	46.7	25.3	46	2	0		A few *S*
45.6	37.7	42.9	44.0	0	5	2		Normal
18.7	8.7	35.7	27.8	23	10	0		A few *S*
18.7	−4.8	35.7	12.1	66	20	0		,,
45.7	22.8	42.3	32.1	25	0.5	5		No specimen
23.2	8.2	34.3	17.0	50	5	2–4		A few *S*

TABLE I (*continued*)

Serial No.	Code Number	Name	Formula	Form	Total dose (mg./100 g. i.p.)
		1 : 3 : 5-TRIAZINES			
271	9609	2 : 4-diamino-6-ethyleneimino-1 : 3 : 5-triazine		a.d.	9.0
272	10876	2-ethyleneimino-4 : 6-dimethoxy-1 : 3 : 5-triazine		,,	35
273	10304	2-chloro-4-ethyleneimino-6-*p*-anisyl-1 : 3 : 5-triazine		,,	6
		QUINAZOLINES			
274	10307	4-chloro-2-ethyleneiminoquinazoline		,,	40

care was exercised in the isolation and purification of these monofunctional compounds, but their instability and similarity in general physical and chemical properties to related polyfunctional derivatives raised serious difficulties, while the detection of likely impurities by elementary chemical analysis was beyond the limits of error of the methods routinely employed. Set against the likelihood of contamination is the constancy of the effects produced by successive preparations of the same substance—an observation inconsistent with the expected variability in composition. An additional possibility is that, in solution, some disproportionation of the mono-compounds to bis-ethyleneimine derivatives occurs. Chemical evidence on this point has not been sought.

These arguments apart, tumour-inhibitory activity has now been found in 2 : 4-dinitrophenyl-ethyleneimine, 257, which on account of its mode of preparation and ease of crystallization must be regarded as essentially pure. This result in particular, together with certain observations upon carcinogenic activity described below, compels acceptance of the occurrence of activity in monofunctional derivatives. Further consideration of this fact is deferred to a later section.

TABLE I (*continued*)

Tumour inhibition					Cytotoxic action			
$\triangle W$		M_{50}		I	Dose (mg. per 100 g. i.p.)	Tumour		Bone marrow
Control	Treated	Control	Treated			A	Remarks	
44.1	−5.7	44.1	6.6	85	5 2.5		Mitosis completely inhibited	Severe damage, many *D* and *P*. Moderate damage, some *D* and *P*
					1.25	52	Mitosis slightly inhibited	A few *B* and *F*
33.0	4.7	30.5	0.96	97	15 7.5		Mitosis almost completely inhibited. Many *D* and a few *F*	Some *F* and *D*
32.7	0.3	37.8	14.2	62	1.0	63	Some inhibition of mitosis and some *D* and *E*	A few *B* and *S* and some *F*
20.8	22.8	34.6	29.3	15 L	—	—	—	—

The preparation of the dinitro-sulphonic acid derivative 259 was called for in view of a possible alternative explanation of the activity of 2 : 4-dinitro-phenylethyleneimine in which the *in vivo* reduction of the *p*-nitro group to azoxy or azo with the consequent formation of a bis-ethyleneimino-azoxy- or azo-benzene was visualized. Since the *p*-sulphonic acid is active this possibility is largely ruled out.

Table II comprises compounds containing two or more ethyleneimine residues, and it will be seen that, with some few exceptions, tumour-inhibitory activity of the more specific type is consistently present throughout. Among the most effective substances are the bis- and tris-ethyleneimino derivatives from pyrimidine and from 1 : 3 : 5-triazine. Because of preparative difficulties comparison between precisely analogous members of these two series was possible in only a few instances. The most active substance of all is tris-ethyleneimino-*s*-triazine, 322, while replacement of one ethyleneimine group in this by dimethylamino gives a compound, 321, of comparable activity, suggesting that the third imine residue is superfluous. The pyrimidines analogous to these triazine compounds could not

TABLE II

ACTION ON THE WALKER TUMOUR OF SOME DERIVATIVES CONTAINING TWO OR MORE ETHYLENEIMINE GROUPS

Conventions as in Table I

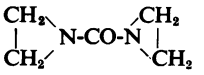
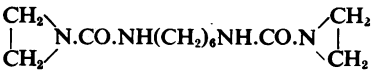
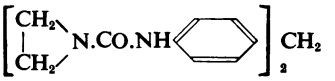
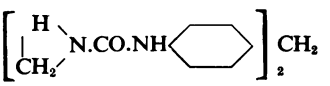
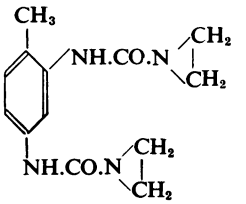
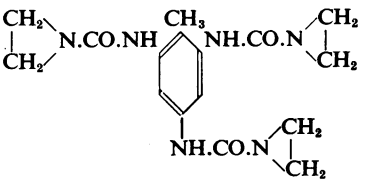
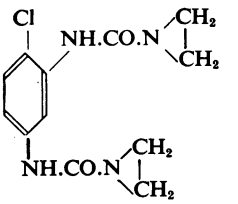
Serial No.	Code Number	Name	Formula	Form	Total dose (mg./100 g. i.p.)
		UREAS			
275	10358	N: N′-bis-*cyclo*ethylene urea		a.s.	2.4
276	9214	N: N′-bis-*cyclo*ethylenecarbamyl-hexamethylenediamine		,,	0.8
277	9215	N: N′-bis-*cyclo*ethylenecarbamyl-*p*-*p*′-diamino-diphenylmethane		a.d.	6.5
278	9608	N: N′-bis-*cyclo*ethylenecarbamyl-*p*: *p*′-diamino-di-*cyclo*hexylmethane		,,	1.3
279	9604	N: N′-bis-*cyclo*ethylenecarbamyl-2: 4-diamino-toluene		o.d.	1.2
280	9606	N: N′: N″-tris-*cyclo*ethylenecarbamyl-2: 4: 6-triamino-toluene		a.d.	5.0
281	9607	N: N′-bis-*cyclo*ethylenecarbamyl-2: 4-diamino-chlorobenzene		o.d.	8.0

TABLE II (*continued*)

Tumour inhibition					Cytotoxic action			
$\triangle W$		M_{50}		I	Dose (mg. per 100 g. i.p.)	Tumour		Bone marrow
Control	Treated	Control	Treated			A	Remarks	
44.2	0.5	31.6	3.8	88	1.0	45	Some inhibition of mitosis. A few *D* and *E*	A few *B*, *F*, and *S*
53.7	13.2	48.5	1.3	97	—	—	—	—
35.4	8.7	37.9	12.7	67	—	—	—	—
30.6	−9.9	31.7	2.0	94	0.4	88		No sample
30.6	6.4	31.7	1.2	96	0.4		Mitosis almost completely inhibited. A few *D* and *E*	*B*, *F*, and *S*, and some *D* and *P*
					0.1	53		
22.4	13.0	28.4	7.7	73	2.5		Mitosis almost completely inhibited. Some *B*, *F*, and *E*	No sample
					1.0	68	A few *D*, *E*, and *M*	
30.6	6.8	31.7	0.5	98	5		Mitosis almost completely inhibited	Divisions few. Polymorphs swollen; many D

TABLE II (*continued*)

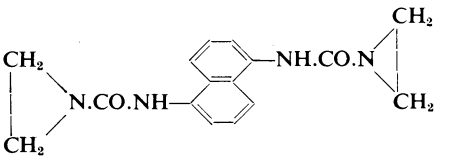
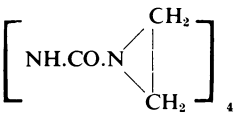
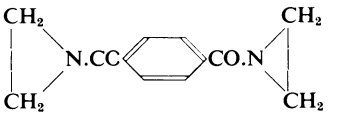
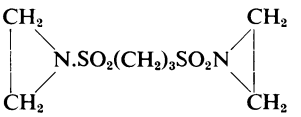
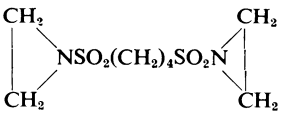
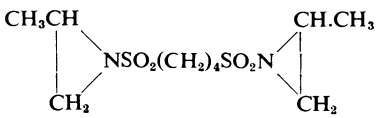
Serial No.	Code Number	Name	Formula	Form	Total dose (mg./100 g. i.p.)
282	9860	N: N′-bis-*cyclo*-ethylenecarbamyl-1 : 5-diamino-naphthalene		a.d.	8.0
283	9815	Silicon tetra-*cyclo*ethylene urea		,,	27.5
		CARBOXYLIC ACID AMIDES			
284	9959	Succinylethylene-imine		a.s.	3.0
285	9958	Adipylethyleneimine		,,	5.5
286	9732	Terephthalyl-ethyleneimine		,,	11
		SULPHONAMIDES			
287	10694	ω : ω′-bis-(ethylene-iminosulphonyl)-propane		a.d.	0.9
288	10796	ω : ω′-bis-(ethylene-iminosulphonyl)-butane		,,	1.8
289	10799	ω : ω′-bis-(C-methyl-ethyleneimino-sulphonyl)butane		,,	30.0

TABLE II (*continued*)

Tumour inhibition					Cytotoxic action			
ΔW		M_{50}		I	Dose (mg. per 100 g. i.p.)	Tumour		Bone marrow
Control	Treated	Control	Treated			A	Remarks	
33.8	9.4	39.0	5.2	87	4	65		No sample
22.0	16.2	41.8	40.1	4	—	—	—	—
22.0	14.0	41.8	44.2	0	2	1–6		Normal
20.7	13.0	34.6	20.8	40	1.0	77	Some inhibition of mitosis; some *D* and *E*	A few *B* and *F*
50.0	12.7	44.8	24.9	44	10	14	Some inhibition of mitosis	Several *D* and *P*; a few *B* and *F*
					5	13		A few *B* and *F*
24.0	−4.6	29.1	0.6	98	0.4		Almost complete inhibition of mitosis. A few *F*	Severe damage; no division; many *D*
					0.2	90	Some *D* and *E*	
32.8	−11.9	37.7	0.25	99	2.0		Complete inhibition of mitosis; many *D* and *E*	Severe damage; many *D* and *P*
					1.0	97		
32.8	−8.6	37.7	0.09	>99	10		Almost complete inhibition of mitosis; a few *F*	Many *D* and *E*

TABLE II (*continued*)

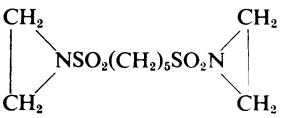
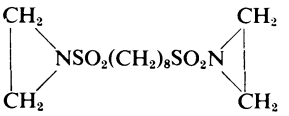
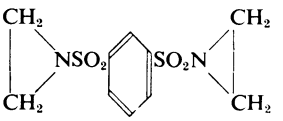
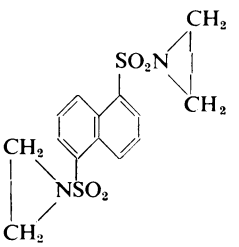
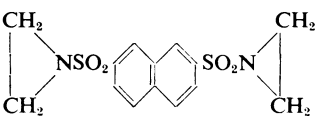
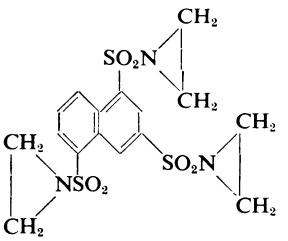
Serial No.	Code Number	Name	Formula	Form	Total dose (mg./100 g. i.p.)
290	10695	ω: ω′-bis-(ethylene-iminosulphonyl)-pentane		a.d.	1.6
291	10797	ω: ω′-bis-(ethylene-iminosulphonyl)-octane		,,	23
292	9953	*m*-bis-(ethylene-iminosulphonyl)-benzene		,,	22.5
293	9814	1 : 5-bis-(ethylene-iminosulphonyl)-naphthalene		,,	150
294	9813	2 : 7-bis-(ethylene-imino sulphonyl)-naphthalene		,,	80
295	9817	1 : 3 : 5-tris(ethylene-imino sulphonyl)-naphthalene		,,	125

TABLE II (*continued*)

Tumour inhibition					Cytotoxic action			
$\triangle W$		M_{50}		I	Dose (mg. per 100 g. i.p.)	Tumour		Bone marrow
Control	Treated	Control	Treated			A	Remarks	
24.0	−7.0	29.1	0.06	>99	0.5		Complete inhibition of mitosis	Severe damage; no divisions, many *D* and *P*
					0.25		Almost complete inhibition of mitosis; some *B* and *F*	
34.6	−4.4	46.1	0.9	98	5		Almost complete inhibition of mitosis; a few *F*	Some *D* and *P* and some *B* and *F*
27.5	13.4	20.9	12.7	40	2.5		Complete inhibition of mitosis	Some *B*, *F*, and *D*
16.8	−3.3	27.8	3.9	86	100	30	Some *E* and *M*	A few *F*
16.8	−11.6	27.8	2.6	91	25	51	A few *D*	A few *B* and *F*
16.8	−3.1	27.8	11.9	57	25	28		,,

TABLE II (*continued*)

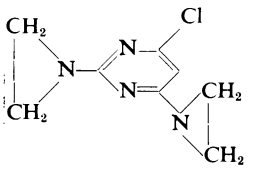
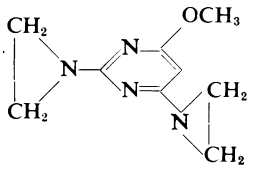
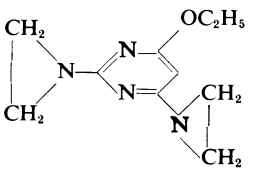
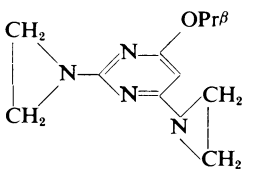
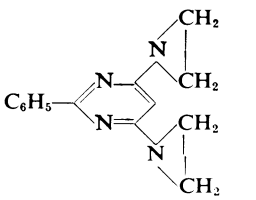
Serial No.	Code Number	Name	Formula	Form	Total dose (mg./100 g. i.p.)
		PYRIMIDINES			
296	9955	4-chloro-2: 6-bis-ethyleneimino-pyrimidine		a.s.	0.7
297	10552	2: 4-bis-ethylene-imino-6-methoxy-pyrimidine		,,	0.55
298	10553	2: 4-bis-ethylene-imino-6-ethyoxy-pyrimidine		,,	0.9
299	10575	2: 4-bis-ethylene-imino-6-*iso*propoxy pyrimidine		o.s.	0.45
300	10208	4: 6-bis-ethylene-imino-2-phenyl-pyrimidine		a.d.	8.0

TABLE II (*continued*)

Tumour inhibition					Cytotoxic action			
$\triangle W$		M_{50}		I	Dose (mg. per 100 g. i.p.)	Tumour		Bone marrow
Control	Treated	Control	Treated			A	Remarks	
33.7	9.7	37.4	0.4	99	0.25		Almost complete inhibition of mitosis. Many *E*, some *F*	Many *D*, some *B* and *F*
34.2	5.3	33.4	0.7	98	0.125		Almost complete inhibition of mitosis. Many *D* and *E*; a few *F*	No divisions. Many cells necrotic and degenerating
45.7	0.6	42.5	1.5	97	0.05	63	Some inhibition of mitosis	A few *B*, *F*, and *S*
45.7	6.9	42.5	2.2	95	0.125		Almost complete inhibition of mitosis; some *E* and *F*	No divisions; many *D*
33.0	10.4	27.5	1.75	94	1.0		Complete inhition of mitosis	A few *F* and *S*
					0.5	84	Many *D* and *E*	

TABLE II (*continued*)

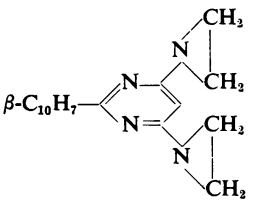
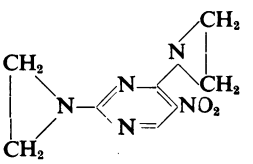
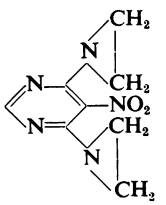
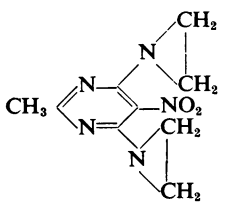
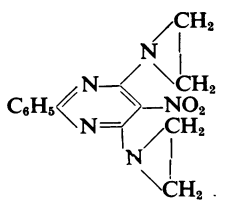
Serial No.	Code Number	Name	Formula	Form	Total dose (mg./100 g. i.p.)
301	10404	4: 6-bis-ethylene-imino-2-β-naphthyl pyrimidine		a.d.	2.5
302	9896	5-nitro-2: 4-bis-ethyleneimino-pyrimidine		,,	2.0
303	9895	5-nitro-4: 6-bis-ethyleneimino-pyrimidine		,,	20
304	9894	5-nitro-4: 6-bis-ethyleneimino-2-methylpyrimidine		,,	17
305	10037	5-nitro-4: 6-bis-ethyleneimino-2-phenylpyrimidine		,,	112.5

TABLE II (*continued*)

Tumour inhibition					Cytotoxic action			
$\triangle W$		M_{50}		I	Dose (mg. per 100 g. i.p.)	Tumour		Bone marrow
Control	Treated	Control	Treated			A	Remarks	
23.2	3.9	34.3	0.64	98	0.5		Complete inhibition of mitosis. Some *D* and *E*	A few *F* and *S*
					0.25	64	Some *D* and *E*	
31.2	12.8	39.0	2.4	94	1.0		Complete inhibition of mitosis; many *D*	No specimen
45.9	20.4	42.3	3.0	93	5.0		Complete inhibition of mitosis; many *D*	Many *D* and *P*
45.9	12.8	42.3	0.9	98	2.0		Almost complete inhibition of mitosis; some *B* and *F* and many *D* and *E*	Some *B* and *F*
					1.0	87	Some inhibition of mitosis; some *D*, *E*, and *M*	
37.0	11.2	40.8	19.1	53	75	61	Some *D* and *E*	A few *B* and *F*

TABLE II (*continued*)

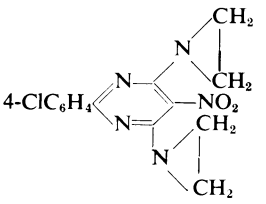
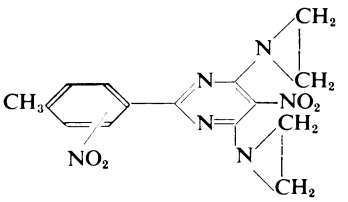
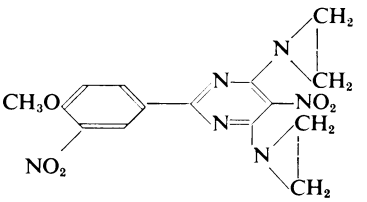
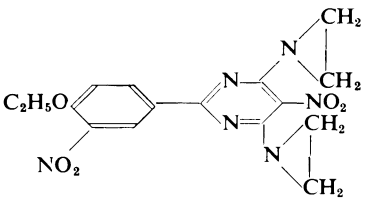
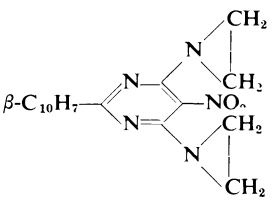
Serial No.	Code Number	Name	Formula	Form	Total dose (mg./100 g. i.p.)
306	10406	5-nitro-4: 6-bis-ethyleneimino-2-*p*-chlorophenyl-pyrimidine		a.d.	17.5
307	10550	x′: 5-dinitro-4: 6-bis-ethylene-imino-2-*p*-tolyl-pyrimidine		,,	10
308	10316	3′: 5-dinitro-4: 6-bis-ethylene-imino-2-*p*-anisyl-pyrimidine		,,	27.5
309	10167	3′: 5-dinitro-4: 6-bis-ethylene-imino-2-*p*-phenetylpyrimidine		,,	212.5
310	10382	5-nitro-4: 6-bis-ethyleneimino-2-β-naphthyl-pyrimidine		,,	35

TABLE II (*continued*)

Tumour inhibition					Cytotoxic action			
$\triangle W$		M_{50}		I	Dose (mg. per 100 g. i.p.)	Tumour		Bone marrow
Control	Treated	Control	Treated			A	Remarks	
45.5	0.8	42.9	15.9	63	20	4–7		A few *S*
16.6	6.1	27.4	22.5	17.9	1.0	1		Normal
16.6	−2.1	27.4	18.8	31	2.5	0		A few *S*
32.7	10.4	42.6	21.8	49	100	3	Some inhibition of mitosis	,,
35.2	−10.6	38.3	8.0	79	100	1		,,

TABLE II (*continued*)

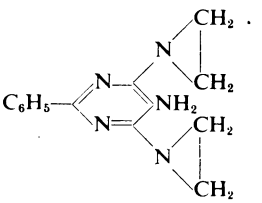
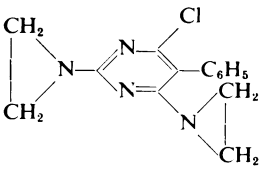
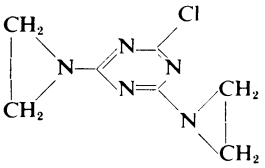
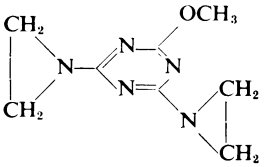
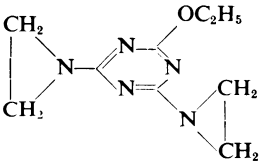
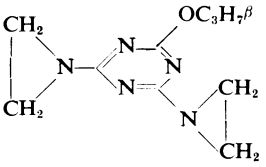
Serial No.	Code Number	Name	Formula	Form	Total dose (mg./100 g. i.p.)
311	10665	5-amino-4 : 6-bis-ethyleneimino-2-phenylpyrimidine		a.d.	5
312	10426	4-chloro-2 : 6-bis-ethyleneimino-5-phenylpyrimidine		,,	2
		1 : 3 : 5-TRIAZINES			
313	10568	2-chloro-4 : 6-bis-ethyleneimino-1 : 3 : 5-triazine		o.d.	1.3
314	10379	2 : 4-bis-ethyleneimino-6-methoxy-1 : 3 : 5-triazine		a.s.	1.0
315	10380	2 : 4-bis-ethyleneimino-6-ethoxy-1 : 3 : 5-triazine		,,	1.5
316	10381	2 : 4-bis-ethyleneimino-6-*iso*propoxy-1 : 3 : 5-triazine		,,	2.0

TABLE II (*continued*)

Tumour inhibition					Cytotoxic action			
$\triangle W$		M_{50}		I	Dose (mg. per 100 g. i.p.)	Tumour		Bone marrow
Control	Treated	Control	Treated			A	Remarks	
36.7	33.7	41.8	18.6	56	0.5		Almost complete inhibition of mitosis, many *E* and *D* and some *F*	A few *B*, *F*, and *S*
23.2	2.9	34.3	1.5	96	0.5		Complete inhibition of mitosis; many *E* and *D*	Some *D* and *P*
45.7	9.4	42.5	20.3	52	0.4	56		Many *B* and *F*
					0.2	45		Some *B* and *F*
35.2	−0.5	38.3	<0.2	>99	1.0		Complete inhibition of mitosis	Many *D* and *P*
					0.5	78	Some inhibition and some *E*	
16.6	−6.0	30.2	0.8	97	1.0		Complete inhibition of mitosis; some *E*	Many *D* and *P*; no divisions
					0.5	98	Some inhibition	Many *D* and *P* and some *F*
15.0	1.6	29.1	1.1	96	1.0	92		Many *D*, *P*, and *E* and some *F*

TABLE II (*continued*)

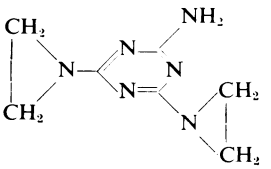
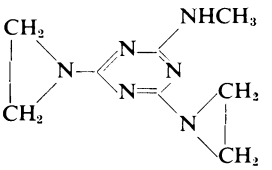
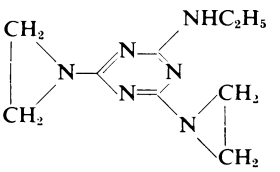
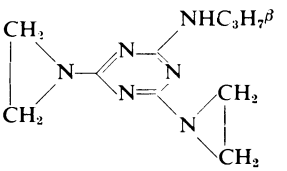
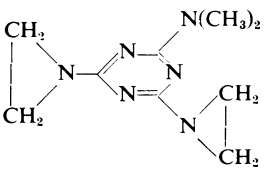
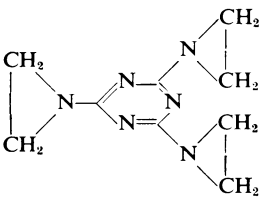
Serial No.	Code Number	Name	Formula	Form	Total dose (mg./100 i.p.)
317	9733	2-amino-4: 6-bis-ethyleneimino-1: 3: 5-triazine		a.d.	0.18
318	9858	2-methylamino-4: 6-bis-ethyleneimino-1: 3: 5-triazine		a.s.	0.4
319	9912	2-ethylamino-4: 6-bis-ethyleneimino-1: 3: 5-triazine		,,	0.35
320	9859	2-*iso*propylamino-4: 6-bis-ethyleneimino-1: 3: 5-triazine		,,	0.4
321	9960	2-dimethylamino-4: 6-bis-ethylene-imino-1: 3: 5-triazine		,,	0.13
322	9500	2: 4: 6-tris-ethylene-imino-1: 3: 5-triazine		,,	0.11

TABLE II (*continued*)

Tumour inhibition					Cytotoxic action			
$\angle W$		M_{50}		I	Dose (mg. per 100 g. i.p.)	Tumour		Bone marrow
Control	Treated	Control	Treated			A	Remarks	
32.4	25.9	31.6	0.7	98	—	—	—	—
28.5	11.5	37.2	1.6	96	0.01		Almost complete inhibition of mitosis. A few *M*	No specimen
28.5	20.5	37.2	1.2	97	—	—	—	—
28.5	13.6	37.2	1.2	97	0.01	74	A few *M*	No specimen
26.7	18.3	30.3	0.1	>99	—	—	—	—
17.0	7.0	39.6	0.7	98	See Table V			

TABLE II (*continued*)

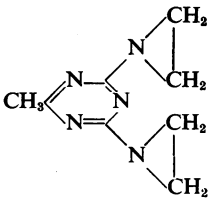
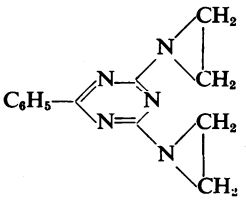
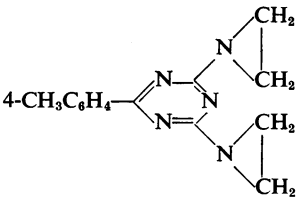
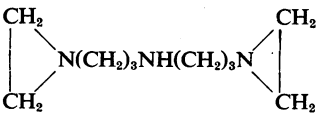
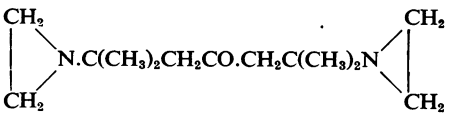
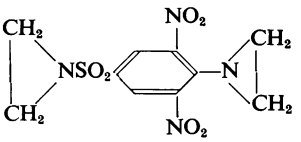
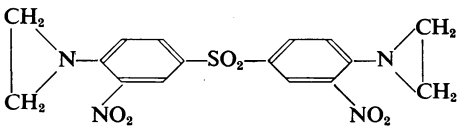
Serial No.	Code Number	Name	Formula	Form	Total dose (mg./100 g. i.p.)
323	10306	2: 4-bis-ethyleneimino-6-methyl-1: 3: 5-triazine		a.s.	0.6
324	10042	2: 4-bis-ethyleneimino-6-phenyl-1: 3: 5-triazine		a.d.	6
325	10305	2: 4-bis-ethyleneimino-6-*p*-tolyl-1: 3: 5-triazine		,,	9
		MISCELLANEOUS			
326	9816	N: N′-di-(β-ethyleneimino-ethyl)-oxamide		,,	0.6
327	10573	di-(γ-ethyleneiminopropyl)amine		a.s.	0.21
328	10549	β: β′-bis-ethyleneimino-di-*iso*-butyl ketone		,,	12

TABLE II (*continued*)

Tumour inhibition					Cytotoxic action			
$\triangle W$		M_{50}		I	Dose (mg. per 100 g. i.p.)	Tumour		Bone marrow
Control	Treated	Control	Treated			A	Remarks	
16.6	6.7	30.2	1.5	95	0.5		Almost complete inhibition of mitosis. Many *D* and *E*	Many *D* and *P* and extensive disorganization ; no divisions
					0.25	91		No specimen
14.0	2.5	26.5	1.1	96	2.0		Almost complete inhibition of mitosis; many *D* and *E*	Some *D* and *P*; a few *S*
					1.0	76	Many *D* and *E*	Some *B*, *F*, and *S*
38.9	9.8	39.3	2.7	93	2.0		Some inhibition of mitosis; many *F* and some *E*	Some *B*, *F*, and *S* and *D* and *P*
					1.0	12		A few *S*
16.8	16.4	27.8	6.7	76	1.0	93	Some inhibition of mitosis; many D	*B* and *F* and many *P* and *D*
44.1	6.8	31.6	15.8	50	0.4		Complete inhibition of mitosis; many *D* and *E*	Many *D* and *P*
					0.2	22	A few *D* and *E*	A few *S*
44.1	4.1	31.6	17.2	46	4.0		Complete inhibition of mitosis; some *F* and many *D* and *E*	A few *B* and *S* and some *F*

TABLE II (*continued*)

Serial No.	Code Number	Name	Formula	Form	Total dose (mg./100 i.p.)
329	10754	2: 6-dinitro-4-ethyleneimino-sulphonyl-*N*-phenylethyleneimine		a.d.	15
330	10666	*m*: *m'*-dinitro-*p*: *p'*-bis-ethyleneimino-diphenylsulphone		,,	125
331	9954	Polymer from 2-chloro-4-ethylene-iminopyrimidine		,,	110
332	10359	Polymer from N: N'-bis-*cyclo*ethylene urea		a.s.	15

be made, but comparison of the bis-ethyleneimino-pyrimidines 296–299 with the corresponding triazines 313–316 shows that the former nucleus consistently provides the more active compound. The introduction of a bulky substituent such as phenyl into either the pyrimidine or triazine rings in these structures markedly reduces activity.

Because of the asymmetry of the pyrimidine nucleus the bis-ethyleneimino compounds derived therefrom are of two isomeric forms, 2 : 4- and 4 : 6- respectively. The latter would appear to be the less active, but in order to prepare them additional substituents (phenyl or nitro) had to be introduced into the nucleus, and this may account, in part at least, for their relatively lower activity.

Activity is high also in some compounds where the ethyleneimine residues are linked by other conjunctive groups. Thus, for example, the bis-urea, 276, derived from 1 : 6-hexamethylene diamine, is almost as effective as tris-ethyleneimino-triazine, while other bis-ureas, including some in which the link is part cyclic and part acyclic, are also strongly inhibitory of tumour growth. Of a small series of related dicarboxylamides, adipyl-ethyleneimine, 285, and terephthalyl-ethyleneimine, 286, are slightly active, while succinyl-ethyleneimine, 284, produces no inhibition in maximal tolerated doses. The low activity of this class is possibly due to

TABLE II (*continued*)

Tumour inhibition					Cytotoxic action			
ΔW		M_{50}		I	Dose (mg. per 100 g. i.p.)	Tumour		Bone marrow
Control	Treated	Control	Treated			A	Remarks	
48.2	11.7	38.4	4.8	88	20		Complete inhibition of mitosis; many *D*	*D* and *P*; also some *B*, *F*, and *S*
					10	71	—	As above, but not so marked
31.6	−1.7	40.4	1.4	97	40		Complete inhibition of mitosis	A few *S*
					10	12		,,
14.0	18.0	26.5	30.7	0	20	6		Normal
39.7	28.9	49.4	40.3	18	1.0	14		,,

secondary intramolecular condensations leading to unreactive oxazoline derivatives. With the sulphonamides, 287–295, where such auto-condensation cannot occur, higher activity is found. With aliphatic derivatives such as $\omega : \omega'$-bis-(ethyleneimino-sulphonyl) propane, 287, total doses of the order of 1 mg. per 100 g. are strikingly effective, whereas in the bis- and tris-sulphonamido derivatives of naphthalene, e.g., compounds 293–295, the maximum tolerated doses are of the order of one hundred times greater, and even with these large doses full tumour inhibition is difficult to achieve. It is suggested that this result is attributable to the lower solubility and consequent poorer absorption of the latter substances. It will be noted that considerably larger doses of the bis-C-methylethyleneimine 289 than of the unmethylated 288 are tolerated, but that with a comparable effect upon tumour growth.

Table III contains miscellaneous compounds related to active and inactive ethyleneimines and examined in that connexion. The two trimethyleneimine derivatives, 333 and 334, corresponding to active ethyleneimines, have little action in high doses, as indeed might be expected from the relatively greater stability of the four membered ring. The four compounds following, 335–338, are related to the active dinitrophenyl-ethyleneimine 257, from which they differ in having in

TABLE III

ACTION ON THE WALKER TUMOUR OF MISCELLANEOUS COMPOUNDS RELATED TO THE ETHYLENEIMINES

Conventions as in Tables I and II

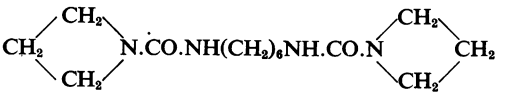
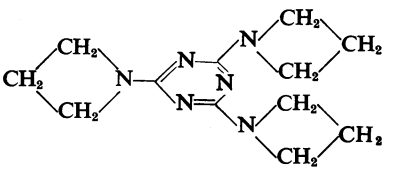
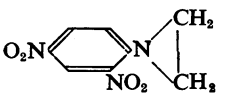
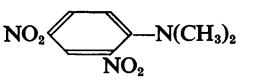
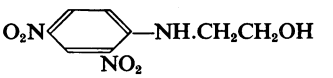
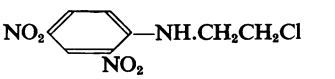
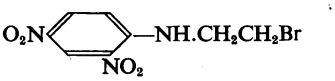
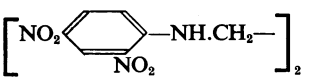
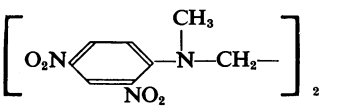
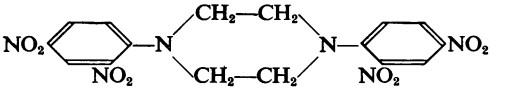
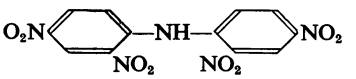
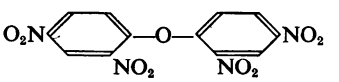
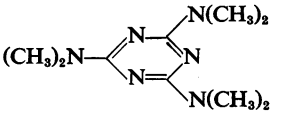
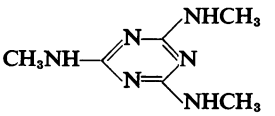
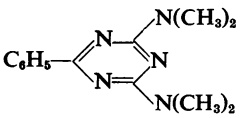
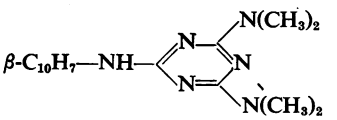
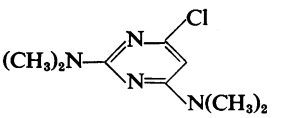
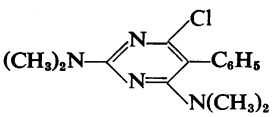
Serial No.	Code Number	Name	Formula	Form
		TRIMETHYLENEIMINES		
333	10202	N: N′-bis-*cyclo*-trimethylene carbamyl hexamethylene diamine		a.d.
334	10071	2: 4: 6-tris-trimethyleneimino-1: 3: 5-triazine		,,
		COMPOUNDS BEARING ON ACTIVITY OF		
335	10424	2: 4-dinitrophenyldimethylamine		,,
336	10560	N-2: 4-dinitrophenylethanolamine		,,
337	10561	N-β-chloroethyl-2: 4-dinitroaniline		,,
338	10562	N-β-bromoethyl-2: 4-dinitroaniline		,,
339	10677	N: N′-bis-(2: 4-dinitrophenyl)-ethylenediamine		,,
340	10676	N: N′-dimethyl-N: N′-bis-(2: 4-dinitrophenyl) ethylenediamine		,,
341	10675	N: N′-bis-(2: 4-dinitro-phenyl)-piperazine		,,

TABLE III (*continued*)

Total dose (mg./100 g. i.p.)	Tumour inhibition					Cytotoxic action		
	$\triangle W$		M_{50}		I	Dose (mg. per 100 g. i.p.)	Tumour	Bone marrow
	Control	Treated	Control	Treated			A	
200	32.7	33.3	42.6	38.9	9 L	—	—	—
75	32.7	27.4	42.6	30.0	30 L	40	4	Normal
120	24.6	8.4	23.0	25.0	0	40	2	,,
160	25.0	16.8	24.5	22.1	10	10	1	A few *S*
60	31.6	27.2	40.4	37.0	8	10	1	,,
30	32.8	24.7	37.8	34.5	9 L	20	0	,,
90	34.2	21.9	33.4	20.9	37	12	0	Normal
150	36.7	37.6	41.8	38.3	8 L	75	0	,,
75	36.7	31.1	41.8	34.7	17 L	40	0	,,

TABLE III (*continued*)

Serial No.	Code Number	Name	Formula	Form
342	10753	bis-(2: 4-dinitro-phenyl)amine		a.d.
343	10793	bis-(2: 4-dinitro-phenyl) ether		,,
		COMPOUNDS BEARING ON ACTIVITY OF HEXAMETHYL MELAMINE		
344	10567	2: 4: 6-tris-dimethyl-amino-1: 3: 5-triazine		,,
345	8758	2: 4: 6-tris-methyl-amino-1: 3: 5-triazine		a.s.
346	10953	2: 4-bis-dimethyl-amino-6-phenyl-1: 3: 5-triazine		a.d.
347	10954	2: 4-bis-dimethyl-amino-6-β-naphthyl-amino-1: 3: 5-triazine		,,
348	10794	4-chloro-2: 6-bis-dimethylamino-pyrimidine		,,
349	10795	4-chloro-2: 6-bis-dimethylamino-5-phenyl-pyrimidine		,,

place of the ethyleneimine residue a grouping which might be expected to behave in an analogous manner. All four compounds were inactive. In the next five, 339–343, two 2 : 4-dinitrophenyl residues are linked together by various conjunctive groups. These too were inactive.

TABLE III (*continued*)

Total dose (mg./100 g. i.p.)	Tumour inhibition					Cytotoxic action		
	$\triangle W$		M_{50}		I	Dose (mg. per i.p.)	Tumour	Bone marrow
	Control	Treated	Control	Treated			A	
97.5	33.0	31.2	30.5	40.2	0	30	2	A few *S*
65	34.6	16.1	46.1	37.8	18	20	0	Normal
80	25.0	−5.4	24.5	4.8	79	10	31	A few *S* and *P*
147.5	32.8	12.4	37.7	14.9	60	20	18	A few *F* and *S*
205	27.4	7.5	32.9	19.7	40 L	50	7	Normal
220	27.4	2.3	32.9	21.4	35	50	0–7	,,
67.5	34.6	25.7	46.1	36.1	22	10	0	,,
300	34.6	10.1	46.1	24.1	48	50	0	,,

The demonstration that tris-(dimethylamino)-triazine, 344, is active confirms a finding of Buckley, Stock, Crossley, and Rhoads (1950) that the substance produces slight but definite inhibition of the Crocker mouse sarcoma 180. This result is considered further in the general discussion.

Cytotoxic action (with J. M. Gates)

The majority of the compounds listed in Tables I to III have been examined for the capacity to induce chromosome effects of the type regarded as characteristic of the action of true "radiomimetic" agents in dividing cells of the Walker tumour. Their effect upon the bone marrow of rats was studied at the same time. Single intraperitoneal doses of the compounds were given to rats bearing tumour implants of several days' growth and Feulgen-stained squash preparations were made from tumour tissue and bone marrow taken when the animals were killed 24 hours later. The distinguishing features of these specific chromosome effects have already been described (Hendry, Rose, and Walpole, 1951). They consist essentially of chromosome fragmentation, accompanied by the reunion of fragments in various ways and to a variable extent and leading to the appearance, among other manifestations, of dicentric chromosome bridges and of chromosome fragments at anaphase. These effects are to be distinguished from non-specific "toxic" changes, e.g., chromosome "stickiness" and its sequelae, nuclear pyknosis, etc. The increase over controls in the percentage of anaphases showing abnormalities of the former type in tumour tissue from rats treated with the compounds in the doses shown is recorded in the column headed *A* in Tables I to III, with supplementary remarks in the next column. Qualitative observations upon cytotoxic effects in the bone marrow are included in the same Tables.

It will be seen that a high proportion of the compounds examined in this way caused pronounced cytotoxic effects in the tumour cells. Typical of the more active in this respect—for details with one such, 322, see Table V—was the production, with comparatively low doses, of specific chromosome damage and in addition, with higher doses, an inhibition of mitosis which might be complete. A full range of doses was not always examined and both these distinct but presumably related phenomena were not always observed. Thus with some few compounds, e.g., 302 and 303, complete mitotic inhibition only was seen with the doses tested, but although this action is not restricted to "radiomimetic" agents the general picture here is such that it seems reasonable to assume that lower doses of the compounds in question would have caused the appearance of specific chromosome effects. The converse, however, appears not to be true in that with some compounds, e.g., 282, the highest doses which could be given failed to produce marked inhibition of mitosis.

The above compounds as a whole, where *A* is greater than 15 per cent, were active inhibitors of tumour growth. To this generalization only five exceptions have been found, namely, 258, 261, 292, 327, 328. It should be pointed out, however, that the doses which can be given in an acute (24-hour) test for cytotoxic activity often greatly exceed those tolerated in chronic experiments upon the inhibition of tumour growth, where some toxic action of a compound unrelated to its specific effect upon the chromosomes may be the factor limiting dosage. This may account for the failure to obtain pronounced tumour inhibition with these few compounds.

With those compounds which appeared completely devoid of specific cytotoxic activity or with which the proportion of anaphases in treated tumours showing

specific chromosome effects was small (*A* less than 15 per cent) tumour-inhibitory activity was, in general, negligible. With some few of these compounds, e.g., 268, the inhibition of tumour growth appeared to be rather greater than could be accounted for by non-specific " toxicity " as judged by their effect on gross weight gain (Walpole, 1951), but with none of these compounds did it exceed 66 per cent.

Carcinogenic activity

Of the compounds listed in Tables I, II, and III, only two, namely, stearoyl-ethyleneimine, 248, and tris-ethyleneimino-1 : 3 : 5-triazine, 322, have been examined in such a way that carcinogenic activity, if present, might be detected. The results with the former are described below, and those with the latter in the section (page 398) devoted to that compound. Other ethyleneimine derivatives are on test in connexion with theories developed in the Discussion (page 408).

Stearoyl-ethyleneimine, 248

A 2 per cent solution of the compound in arachis oil was injected subcutaneously in the right flank of stock albino rats, about 100 g. in body weight. Five male and five female rats were each given 10 mg. per 100 g. twice weekly for five weeks, while a similar group were given 10 doses of arachis oil alone (0.5 ml. per 100 g.) and served as controls. Twelve (calendar) months after the first injection no tumour had developed among the controls. In the rats given stearoyl-ethyleneimine progressively growing tumours appeared at the injection site in three of the males and two of the females. Four of these were diagnosed histologically as mixed-cell sarcomata. The first of these appeared between three and four months from the beginning of the experiment, and when the animal was killed at four months was a roughly spherical mass some 2 in. in diameter. The other three were first noted at about six, six, and seven months respectively. Two were successfully transplanted into stock rats; transplantation of the other two was not attempted. The fifth subcutaneous tumour, which appeared at about eight months, was a spindle-cell sarcoma. One other of the females lost condition rapidly at about 11 months and was killed. A mixed-cell sarcoma was found filling the thoracic cavity and infiltrating the heart muscle and lung.

The stearic acid from which the stearoyl-ethyleneimine used in this experiment was prepared was the commercial product and undoubtedly contained some unsaturated material together with higher and lower homologues. The experiment is now being repeated with a product obtained from specially purified stearic acid containing only traces of other acids.

Tris-ethyleneimino-1 : 3 : 5-triazine, 322

On account of the profound inhibition of the growth of the Walker tumour produced by this substance in minute doses its properties have been studied in detail with results which are summarized below. These findings in no way contradict those already published by workers at the Sloan-Kettering Institute (see, e.g., Buckley, Stock, Crossley, and Rhoads, 1950; Burchenal, Johnston, Stock, Crossley, and Rhoads, 1950).

The compound is a colourless crystalline solid, readily soluble in water and in many organic solvents. It is stable at low temperatures but readily polymerizes

on being heated. Aqueous solutions have been kept for some months at 4° C. without appreciable change in composition.

Action on Tumour.—The inhibition of growth of the Walker tumour produced when it is given by various routes is shown in Table IV. In these experiments it was administered in approximately equal doses daily or on alternate days for the first 10 to 12 days after implantation of the tumour. The percentage inhibition of tumour growth, *I*, was calculated as usual from the tumour weights at 14 days. Deaths occurring among the treated animals before the end of experiment are recorded.

It will be seen that the compound is active by mouth as well as parenterally, but that 50 to 100 times the intravenous dose is required by mouth to produce a comparable effect.

TABLE IV

Effect of route of administration and dosage of tris-ethyleneimino-1 : 3 : 5-triazine (322) upon its growth-inhibitory action on the Walker tumour. The compound was given, except where otherwise indicated, over the first ten to twelve days after implantation of the tumour

Route of administration	Total dose mg./100 g. rat	*I* (% inhibition of tumour growth)	Rats dead by the 14th day
Intraperitoneal	0.11	98	0/11
,, ...	0.25	99	2/22
,, ...	0.2 (0.1 on days 1 and 2)	96	5/11
Intravenous	0.055	83	0/11
,, ...	0.11	96	0/11
Oral	0.5	18	0/11
,, ...	2.5	84	1/11
,, ...	5.0	93	0/11

In experiments in which tumour inhibition was pronounced it was accompanied by a fairly marked reduction in the increase in gross weight of the treated tumour-bearing rats as compared with that of untreated controls. If dosing was delayed until the implants had been growing for several days, inhibition of their growth was less pronounced and in these circumstances no actual regressions were obtained.

Single intraperitoneal doses given to rats bearing Walker tumours implants of several days' growth produce cytotoxic changes in the tumour cells which vary with the dose. Some results of experiments of this kind are summarized in Table V. Apart from specific "radiomimetic" effects, inhibition of mitosis, also a feature though not a distinguishing feature of "radiomimetic" cell poisons, was observed with doses down to 0.025 mg. per 100 g. Qualitatively similar changes were seen in the cells of the crypts of Lieberkühn in the gut of these animals, but owing to technical difficulties a quantitative comparison was not carried out. In this tissue also mitotic inhibition was in evidence 24 hours after doses down to 0.025 mg. per 100 g., and for 45 hours after doses down to 0.05 mg. Similar changes were seen also in the bone marrow. With higher doses there was an obvious reduction in the cellularity of the marrow, which to the naked eye was a dark red viscous fluid. Degenerating and pyknotic cells were abundant and the

TABLE V

Details of the cytotoxic action of tris-ethyleneimino-1 : 3 : 5-triazine (322) upon the Walker tumour in rats. *B*, true chromosome bridges; *F*, chromosome fragments

Dose (mg./ 100 g. rat i.p.)	Time after dosing (hr.)	% Anaphases abnormal		Other effects
		B	*F*	
0	—	1–3	1–2	($B + F = 2 - 4$)
0.0125	24	5–16	75–78	Some "sticky" bridges; some degenerating cells. Some cells with shattered chromosomes, and some with accessory micronuclei
	48	10–20	55–60	Many degenerating cells, "exploded" nuclei and cells with micronuclei
	72	16	40	As at 48 hours
0.025	24	16	66	Perhaps some slight inhibition of mitosis
	48			Most cells shattered, the few anaphases visible have chromosome bridges or fragments
	72			As at 48 hours
0.05	24			Some inhibition of mitosis; all anaphases abnormal; some degenerating cells
	48			As at 24 hours
	72			Slight inhibition of mitosis. Some normal, some shattered divisions; many degenerating cells
0.1	24			Almost complete inhibition of mitosis; all divisions abnormal
	48			Mitosis still largely inhibited; many degenerating cells and cells with accessory micronuclei
	72			No specimen

megakaryocytes were enormously swollen. With doses in the range covered in Table V, the effects were more comparable with those seen in the tumour and gut.

The specific cytotoxic action of the compound is not limited to mammalian tissues, but as with the agents studied by Loveless and Revell (1949) is seen also, for example, in the dividing cells of the root tips of the broad bean. Growing roots were immersed for an hour in aqueous solutions of 322 at various concentrations and then replaced in water. The tips of lateral roots were taken at intervals thereafter and from them Feulgen-stained squash preparations were made. Vicia chromosomes being fewer in number and much larger than those in the rat, changes in them are much more easily seen and characterized. The results of one such series of observations are shown in Table VI.

Toxicology of 322

The toxicology of the compound has been studied in several animal species with results which may be summarized as follows.

Mice.—Male stock albino mice, about 20 g. in body weight, were given single intraperitoneal doses of the compound, freshly dissolved in distilled water. All the mice in groups of six given doses of 1,000, 500, 250, and 100 mg. per kg. respectively died within 24 hours, of 50 mg. within three days, and of 25, 10, and 5 mg. within six days. With doses down to 500 mg. per kg. the mice suffered from convulsions ; an attack could be precipitated by sound or mechanical stimulus. On smaller doses they displayed ruffled fur, weakness, splayed hind limbs, coarse tremor, and diarrhoea. The median lethal dose for these mice was 2.5–5 mg. per kg., deaths being recorded up to seven days.

TABLE VI

The cytotoxic action of tris-ethyleneimino-1 : 3 : 5-triazine (322) upon the root tip of the broad bean (Vicia). The growing bean roots were immersed for one hour in aqueous solutions of the compound in the concentrations shown and sampled at the times shown thereafter

Concentration	Time (hr.)	Appearances
10^{-3}	24	Complete inhibition of mitosis. Nuclear pyknosis widespread and many nuclei darkly stained and coarsely granular appearance (non-specific " toxic " changes)
	48	Similar to above
	72	Mitosis still suppressed, but a few prophase nuclei visible. " Toxic " changes in a few cells
10^{-4}	24	Almost complete inhibition of mitosis with " toxic " changes in some cells
	48	Divisions few in number. Breaks in some metaphase chromosomes; micronuclei, some multiple, in many cells; slight " toxic " changes
	72	Divisions more numerous and many normal. Breaks in some metaphase chromosomes; chromosome fragments, numerous in some cells, at anaphase; micronuclei in a few cells
10^{-5}	24	Marked inhibition of mitosis, a few prophase nuclei being the only evidence of that process. No " toxic " changes
	48	Divisions still few in number but all stages visible, prophases being the most numerous. Some breaks, both single and double, in metaphase chromosomes; fragments at anaphase. One or more micronuclei in many cells; some nuclei abnormal in shape.
	72	Mitotic frequency virtually normal. Breaks in some metaphase chromosomes; fragments and a few chromosome bridges at anaphase. Micronuclei in many cells
In a second preparation made from a root tip taken twenty-four hours after treatment with the compound at this concentration (10^{-5}), chromosome breaks and fragments and/or bridges were seen in about half the dividing cells.		
10^{-6}	24	No signs of " toxic " changes. Breaks, both single and double, in metaphase chromosomes with evidence of reconstitution in some cases, one triradiate chromosome being found. Fragments but no bridges at anaphase. Many normal divisions
	48	Mitotic frequency virtually normal. Some breaks in metaphase chromosomes; some fragments at anaphase; micronuclei in a few cells. Many normal divisions
	72	As at 48 hours but with a few chromosome bridges at anaphase

Ten mice dosed once daily for 12 days with intraperitoneal injections of 0.5 mg. 322 per kg. lost weight and died between the tenth and nineteenth days. In a similar group similarly dosed with 0.25 mg. per kg. one died on the tenth and one on the seventeenth day, while the remainder showed some retardation of growth.

Single intraperitoneal doses of 25 mg. per kg. or more produced rapid and widespread damage in the lymphoid tissues and the intestinal mucosa. The blood and bone marrow were not studied in this species. In the spleen of mice killed 24 to 48 hours after being dosed the changes ranged from nuclear fragmentation and marked depletion to complete destruction of the lymphoid cells in both the pulp and the Malpighian corpuscles, with replacement by proliferated reticulo-endothelial cells. In the lymph nodes pyknosis and degeneration of the lymphoid cells were seen. Cessation of mitosis was evident in the crypts of Lieberkühn in the intestine and many degenerating cells were seen there. The liver showed slight vacuolation and a few scattered necrotic cells.

With repeated dosing more extensive and varied changes were produced. The following were noted in addition to those of the types mentioned above: areas of necrosis in the liver and foci of necrosis in the adrenal cortex, in the fibro-fatty tissue associated with the pancreas, and in the glandular tissue of the thyroid. Changes in the tubules of the testes were most striking and were consistently observed; they varied in severity and included nuclear pyknosis and cellular desquamation, the development of multinucleate giant cells, and loss of spermatogenesis.

Rats.—In stock rats of both sexes and about 100 g. in body weight the median lethal dose of the compound by single intraperitoneal injection was 1.5–2 mg. per kg., deaths being recorded up to seven days.

In this species many of the pathological changes were indistinguishable from those seen in mice and as in the latter varied with the level and duration of dosage and the time elapsing before the animals died or were killed. By the third day after a single intraperitoneal injection of 5 mg. per kg. all three rats were moribund, with marked diarrhoea. When killed at this time counts on the heart blood showed a slight elevation of the erythrocyte, and a fall, almost to zero, in the total leucocyte count. The bone marrow was fluid and dark red in colour and in Feulgen-stained smears only a few normal cells could be seen. Pyknotic and degenerating cells were very numerous and the megakaryocytes were swollen. The blood vessels were stuffed with red corpuscles—a change presumably due to haemoconcentration. In the testes the effects seen varied from loss of spermatogenesis with cell desquamation in a few tubules only to complete atrophy. The spleen and abdominal lymph nodes showed acute depletion of lymphoid elements and proliferation of reticulo-endothelial cells. In the adrenal medulla abnormal vacuolation and a few necrotic cells were seen.

Guinea-pigs.—Guinea-pigs survived the intraperitoneal injection of 0.1 mg. of 322 per kg. twice daily (Saturdays and Sundays excepted) for three weeks, but during treatment the mean weight of ten pigs fell from 586 to 571 g. In guinea-pigs given a single intraperitoneal dose of 1 mg. per kg. the total leucocyte count fell rapidly, reaching a minimum in about four days. Both polymorphs and lymphocytes were affected.

Cats.—The compound was given intravenously in acute experiments with chloralosed cats. In one a dose of 1 mg. per kg. caused no alteration in the blood pressure or respiration; in another the injection of 10 mg. per kg. produced apnoea and a fall in blood pressure, but when artificial respiration was applied spontaneous breathing restarted and the blood pressure returned to normal levels. In both animals the total leucocyte count fell to a fraction of its original value during the first hour or two and then rose steadily during the next three to four hours to a figure well above the original. At this point the animals were killed.

Rabbits.—In rabbits given a single intravenous dose of 1 mg. per kg. the behaviour of the leucocyte count during the first three hours was similar to that in cats. Thereafter the total count fell, reaching a minimum in three to four days and then returned to normal over the next 20 to 30 days. (The fall in three rabbits was from an initial mean value of 6,900 leucocytes per cu.mm. to a mean of 700.) With this treatment the initial rise during the first few hours was due to a polymorphonuclear leucocytosis; in the subsequent fall to subnormal levels both polymorphs and lymphocytes were affected. The erythrocyte count remained substantially normal throughout.

Dogs.—Intravenous doses of 0.05 mg. per kg. were given on successive days to three dogs up to totals of 0.35, 0.45, and 0.45 mg. respectively. The dogs appeared to be quite unaffected by this treatment, but examination of the blood revealed a marked fall in the leucocyte count which reached a minimum a few days after the last dose and returned to normal values over 20 to 30 days. The lymphocytes appeared to be affected sooner and to recover more rapidly than did the polymorphs. The erythrocyte count

did not fall below normal limits. The dogs were killed on days 43, 30, and 30 respectively. The most marked pathological change was that seen in the testis of the one mature male animal, where complete loss of spermatogenesis was evident with cell desquamation, pyknosis, and the development of small multinucleate giant cells. Other changes included cell desquamation and casts in the renal tubules, some congestion of the alveolar capillaries with haemorrhage into the alveoli in the lungs, congestion of the vessels of the duodenal mucosa with denudation of the surface epithelium of the villi, slight vacuolation of parenchymal cells in the liver, and foci of degeneration with polymorph infiltration in the lymphoid tissues.

Mutagenic activity of 322 (with M. C. Frank)

The compound was tested for mutagenic activity upon spores of the mould *Penicillium chrysogenum*, by the method described in Part II. It was added in concentrations of 0.3 and 0.7 per cent to suspensions containing 3 to 6×10^6 spores per ml. Samples were taken at intervals between 10 and 90 minutes thereafter, plated on agar medium, and the plates incubated at 24° C. The colonies which developed within a few days were examined and mutants detected by the colour and habit of growth. The results were as follows:

Concentration of compound	Percentage of mutant colonies produced by 10–90 minutes' exposure		
%	Exp. *a*	Exp. *b*	Mean
0.7	11.7	8.0	9.85
0.3		2.7	
0.0	3.6	1.5	2.55

The effect of the higher concentration only is significant. In general the mutants grew more slowly and produced less penicillin than the culture from which they arose.

Carcinogenic activity of 322

Ten male and ten female stock albino mice were given the compound subcutaneously once a week in a dose of 0.025 mg. per 20 g. Dosing continued until the animals died or were killed. Ten died during the first twelve months and the remainder were killed about a month later. No signs of malignant disease were found in any of these mice.

Ten male strain A mice, three months old, were given ten intraperitoneal injections of 0.0075 mg. of the compound on alternate days and killed 100 days after the first dose. Pulmonary adenomata were found in eight of the mice, the number in individual animals being 5, 2, 2, 2, 2, 1, 1, 1. In an untreated control group of 15 animals killed at the same age, one had three adenomata in the lungs and one other only one. Essentially similar results have been obtained by Shimkin (1951) and are taken as evidence for low-grade carcinogenic activity.

Clinical trials of 322

Clinical trial of the compound in human malignant disease is in progress at various centres in this country and America. Preliminary reports were presented at the Fifth International Cancer Congress in Paris in July, 1950 (to appear in the Unio Internationalis Contro Cancrum Acta), and it is understood that more detailed papers are now in the press (see Note (1) added in proof, page 410).

Discussion

Tumour inhibition

From the results with ethyleneimine derivatives presented above and from the essentially similar findings with poly-methylolamides and bis-epoxides recorded in Parts I and II, it is not unreasonable to conclude that tumour inhibition with these substances (as well as with *x* rays and the sulphur and nitrogen mustards) results from their cytotoxic action, which appears to be associated with primary changes in the chromosomes and is directed preferentially, if not exclusively, towards dividing cells. This action is not restricted to malignant cells but extends to proliferating cells of all types. It is unnecessary, however, to postulate any special affinity of these compounds for malignant cells to account for the inhibition of tumour growth observed in our experiments. It may well be that the damage initially sustained by mitotically active cells of normal and malignant tissues is quantitatively as well as qualitatively identical and that the apparent preferential effect upon tumour growth as compared with body growth results from a difference in the subsequent process of proliferative repair. In this connexion it is noteworthy that once the tumour implants have become established and developed a blood supply comparable with that of normal tissues it is more difficult to inhibit tumour growth with sub-lethal doses of these agents. Karnovsky and his collaborators (1947), studying the effects of the standard nitrogen mustards upon tumours in mice and in tissue culture, could find nothing to suggest that these substances had a substantially selective effect upon neoplastic cells. We have compared the action of a single dose of trimethylol melamine upon mitotic activity in the tumour tissue and in the crypts of Lieberkühn in rats bearing the Walker tumour. In both tissues the mitotic frequency fell to a minimum value, at 12 hours, of about one half of its original value, but thereafter recovery was rather more rapid in the gut than in the tumour. Again, Elson (1949), from his study of the effect of *x* rays and of some radiomimetic bis-epoxides upon the Walker tumour, inclines to the opinion that these agents have little if any *selective* inhibitory action upon tumour growth, that their main action is one of growth inhibition both of the animal and of the tumour, but that in favourable circumstances the animal can recover from this toxic action more readily than can the tumour.

Thus it would appear that the special merit of "radiomimetic" agents is that their action is limited in the first instance to the chromosomes of actively dividing cells, as a result of which the mitotic sequence of such cells can be interrupted and lethal chromosomal aberrations induced with the minimum coincidental disturbance of structure or function in fully differentiated and non-dividing cells. Their growth-inhibitory action is therefore accompanied by a minimum of toxic side-effects.

On the other hand, there is evidence that the response in different tissues to a radiomimetic agent does not always run parallel with the mitotic activity of their constituent cells. Thus intense nuclear pyknosis and degeneration such as affects the lymphoid cells throughout the body after the administration of these agents are not seen, for example, in the parenchymal cells of the liver, even when that organ is stimulated to increased proliferative activity by partial hepatectomy. This has been demonstrated by Landing, Seed, and Banfield (1949) in the case of HN2 and by ourselves for trisethyleneimino-1 : 3 : 5-triazine, 322 (unpublished). But this

rapid and widespread destruction of lymphoid elements can scarcely be accounted for by damage to dividing cells only, and here attack upon cells at all stages is almost certainly involved. It is probably more than coincidental that such pronounced lymphotoxic activity is common to many radiomimetic agents, but the reason for it is not yet clear.

Mutagenic and carcinogenic activity

The cytotoxic action of "radiomimetic" agents, to which has been attributed the inhibition of tumour and body growth which they produce, involves, as has been seen, permanent structural changes in the chromosomes. Since permanent modifications in chromosome structure of one kind or another in fact constitute the material basis of mutation it is not surprising that mutagenic activity has been encountered with agents of this kind. In seeking to explain their cytotoxic action we postulate a chemical interaction between these substances and cell components of a protein or nucleoprotein nature, which are probably the chromosomes themselves. The requirements, in terms of reactivity and structure, for specific cytotoxic activity are discussed in detail below. The requirements for mutagenic activity are likely to be similar but not identical with them. Since the chromosome changes involved in mutation may be slight, for example, in gene or "point" mutations, whereas for growth-inhibitory effects more drastic damage to chromosome structure and, at least, a preponderance of lethal mutations is required, it is to be expected that in this respect the demands for tumour-inhibitory action will be more exacting than for mutagenicity. This conception finds support in the fact, for example, that simple monofunctional epoxides (Rapoport, 1948) and chloroethylamines (Stevens and Mylroie, 1950; Auerbach and Moser, 1950; Jensen, Kirk, and Westergaard, 1950), which are certainly not tumour inhibitors, have been shown to have mutagenic properties.

The material basis of malignancy is as yet unknown, but there is considerable support for the view that the transition of a cell from the normal to the malignant state results from a modification in some determinative cell component which is transmitted to the cell progeny at division. There are reasons why this component should not be identified with any part of the genic system of the chromosomes, and a cytoplasmic location as a "plasmagene" has been suggested by Darlington (1948) and others. Whether nuclear or cytoplasmic, such an autoreproductive factor is likely to be protein or nucleoprotein and as such susceptible to attack and modification by agents of the "radiomimetic" class. The observation of carcinogenic activity in such agents is consistent with this conception. As with mutagenic activity the requirements for carcinogenicity are not likely to be identical with those for the cytocidal activity which leads to growth inhibition and again a modification is concerned in which cell viability must needs be preserved. In this connexion it is perhaps noteworthy that stearoyl-ethyleneimine, 248, here shown to be carcinogenic, was among those compounds with which only a small though definite increase could be produced in the percentage of anaphases in the Walker tumour showing specific chromosome changes.

General structural considerations

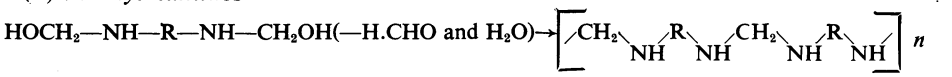
Derivatives of ethyleneimine came to be examined in this series of researches because they were known to bring about physical modifications of cellulosic and

protein fibres having much in common with those produced by certain methylolamides and epoxides. In our earlier communications, compounds of the last-named types were shown to induce characteristic mitotic disturbances in somatic cells, and it is now clear that qualitatively similar abnormalities are produced by the ethyleneimines. The association of these two properties in compounds of a single type might be coincidental, but its occurrence in all three chemical classes and its dependence in each case upon the same general structural requirements is probably significant and merits further consideration.

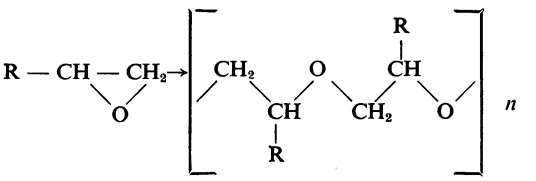
Unfortunately, precise scientific knowledge of the way in which these substances bring about their effect upon textile fibres is scanty at the present time, but it is widely accepted amongst competent technologists that one, or possibly both, of their two main reactive tendencies come into play. The first of these, the tendency to combine with nucleophilic centres, has already been mentioned (see schemes (*a*), (*b*), and (*c*) of the introduction). Except in a few cases which require separate consideration, at least two groups with reactivity of this kind must be present in each molecule of the agent if maximal effects either upon inanimate textile fibres or upon dividing cells are to be produced. From this the conclusion has been drawn that these agents produce their effect upon both systems by cross-linkage, the linkage being between polycellobiose or polypeptide units in textile fibres and, in the cell, between the protein or nucleoprotein of the chromosomes or chromatids, after the manner first proposed by Haddow and his group (Goldacre, Loveless, and Ross, 1949 ; Loveless and Revell, 1949) for the nitrogen mustards and epoxides.

The second characteristic of compounds of these three classes, to which also reference has already been made (Parts I and II) in connexion with the methylolamides and epoxides, is the facility with which they polymerize *in vitro* to give (potentially) linear structures of the following types:

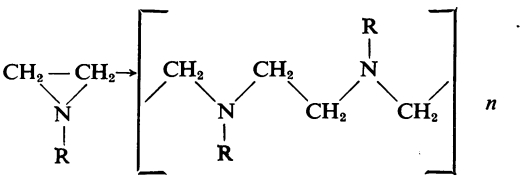
(*d*) *Methylolamides*



(*e*) *Epoxides*



(*f*) *Ethyleneimines*



Only one reactive group per molecule, or two with the methylolamides which eliminate formaldehyde, is required for polymer formation, and any other such

groups as may be present in the molecule will be accommodated initially in these polymeric units in the side chains R. Although the latter may become involved in further polymerization effects, it is known (Part II) that, in some bis-epoxides at least, the two reactive groups can behave differentially. With these compounds one epoxide group goes to form the basic repeating unit of the polymer "backbone" while the second remains in the side chain where it can then exhibit reactivity of the type shown in scheme (*b*) (page 358) above. It seems reasonable to postulate that compounds of all three types yield similar intermediate forms, with pendent groupings spaced at regular intervals along the polymer chains and capable of reacting with the nucleophilic centres of fibres or of cellular proteins or nucleoproteins. The resultant attachment of such polymer units, by numerous regularly spaced covalent bonds, either along individual polycellobiose or polypeptide chains (adlineation) or between collateral chains (cross-linkage) would be expected to bring about a much greater change in the physical and chemical properties of the substrate than would combination with unassociated molecules of the agent, as envisaged in the simpler cross-linkage hypothesis. It is suggested that a process of this nature underlies the specific biological activity common to the methylolamides, epoxides, and ethyleneimines.

Direct evidence of the occurrence of cross-linkage with or without polymer formation between cellular components or of the adlineation of polymer units thereto is inaccessible at the present time, but an appraisal of these main postulates may be made from indirect evidence obtained in the biological examination of a large number of carefully selected agents.

In so far as concerns the possibility of the formation of a polymer–cell-component complex, considerations urged in Part II in respect of the epoxides are equally applicable to the ethyleneimines. It is not inconceivable that the latter diffuse in monomeric form into cells and that there one imine radical becomes involved in the formation of a polyethyleneimine chain, while the second (and, if present, a third such radical) combines, for example, with protein or nucleoprotein. Alternatively, chemical union of the monomer with the cell-component may first occur, followed later by polymer formation through the intermolecular condensation of the remaining pendent imine groups.

In Tables I to III the activity of a number of ethyleneimine derivatives is shown in terms of both specific chromosome damage and of growth inhibition, produced in the Walker tumour. From these it has been seen that by far the most effective compounds are those containing two or three ethyleneimine radicals, and in particular derivatives of this type from pyrimidine and from 1 : 3 : 5-triazine.

In all the latter compounds, the ethyleneimino radicals are attached directly to a six-membered ring system, so that the ultimate polymer–cell-component complex, if formed, would be of the type shown in Fig. 1, with the possibility that further combination might occur between cell-components and the ethyleneimine groups attached to the ring systems shown in the upper part of the diagram. While the presence of pyrimidine or triazine nuclei would give rigidity to such a system there is no reason why ring systems *per se* should be essential, and in fact, as we have seen, certain other conjunctive groups give rise to compounds of high activity.

In Part II, it was pointed out that, within limits, the nature or dimensions of the conjunctive group linking the two essential epoxide residues had little influence

on either cytotoxic or tumour-inhibitory activity. It was suggested that this observation provided evidence against the view that these agents formed simple unimolecular cross-links and favoured the polymer hypothesis, in which the distance between the reacting groups was a function of the polyethylenoxy backbone and hence largely independent of the distance separating epoxide groups in individual molecules.

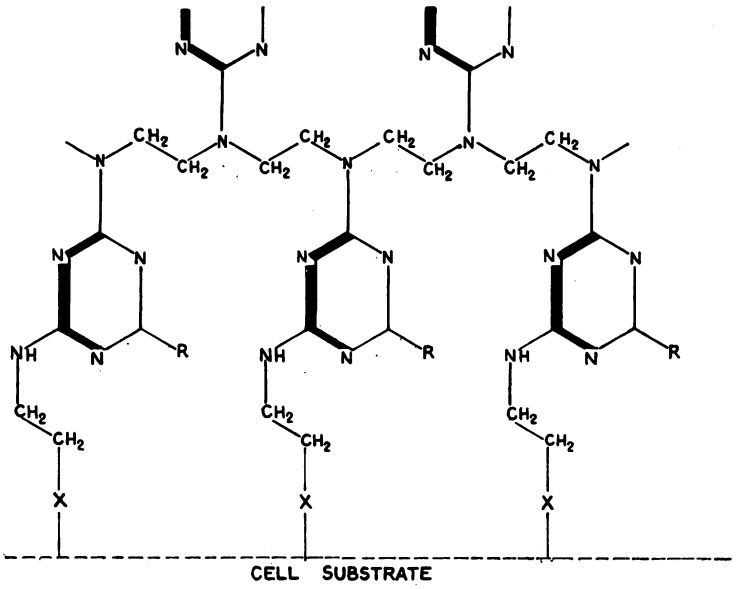


FIG. 1

Similar arguments apply in the ethyleneimine series, which provides examples of even greater permissible latitude in respect of the conjunctive group. Here active compounds range from the simple urea derivative, 275, to the bis-urea, 276, based on 4 :4′-diaminodiphenylmethane, and the octamethylene bis-sulphonamide, 291.

Special structural considerations

In Table I, several examples occur of mono-ethyleneimine derivatives showing appreciable tumour-inhibitory (and cytotoxic) activity. Similar observations have already been reported by workers at the Sloan-Kettering Institute (Philips, 1950), more especially with reference to 2-ethyleneimino-4 : 6-dimethoxytriazine, 272. In all compounds of this type activity is low and in many contamination with related bis-ethyleneimine derivatives in amounts sufficient to account for the observed biological effects cannot be excluded. But, as has been pointed out, the finding that 2 : 4-dinitrophenylethyleneimine, an essentially pure compound, also produces these effects compels acceptance of the occurrence of activity in monofunctional derivatives.

Biesele *et al.* (1950), in drawing attention to the activity of 2-ethyleneimino-4 : 6-dimethoxytriazine, mention the production of cytotoxic effects *in vitro* with ethyleneimine and glycidol. In addition, when administered intraperitoneally to rats, ethyleneimine was found to give rise to pancytopenia in a manner resembling the well-known effects of mustards. The failure in our laboratories and elsewhere to obtain

comparable growth-inhibiting effects in animal tumours *in situ* may perhaps be regarded as due to unfavourable properties on the part of these simple and labile molecules. Significantly these substances are well known to modify the chemical and physical properties of textile fibres. When they are used for this purpose the conditions of application are such that a large measure of polymerization must occur and the polymer chains can be visualized as lying alongside the fibre micelles, attached by end groups and attracted by residual valencies to them. A similar arrangement can be envisaged within living cells, but because of the feebler nature of the linking forces a greater concentration of agent would be needed for comparable interference with vital processes than that demanded of those, already described, which form polyreactive polymers. The residual valencies would be most effective in agents bearing the more highly polar groups in the side chain, and in this connexion attention is drawn to the notable tendency of polynitroaromatic systems to associate with various unsaturated structures to form relatively stable molecular complexes, which are now considered to be essentially ionic in character (Weiss, 1942). The effective cytotoxic unit from dinitrophenyl-ethyleneimine may well be of the form shown in Fig. 2, and linkage to cell components such as protein or nucleoprotein

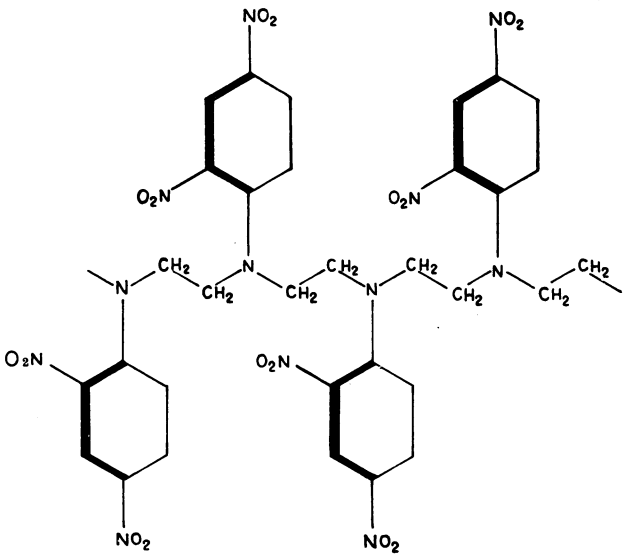


FIG. 2

may conceivably be due to association of the pendent dinitrophenyl residues with, for example, phenyl, pyrimidine, or purine nuclei in these cellular constituents. Similar considerations would apply also to the active monoethyleneimine derivatives of pyrimidine and 1 : 3 : 5-triazine mentioned above.

Miscellaneous aspects

The Tables of biological results show many other observations which have a bearing on the foregoing speculations, but which have not yet been discussed.

Confirmation with respect to the Walker tumour in rats of the slight but definite tumour-inhibition (Crocker mouse sarcoma 180) claimed for hexamethylmelamine (344) (Buckley, Stock, Crossley, and Rhoads, 1950) has already been mentioned. Following this, several dimethylamino analogues of other active ethyleneimino derivatives were made in order to determine the extent of this phenomenon (335, 346, 348, and 349), but only 344 and possibly 345 displayed any activity. On a purely chemical basis, two explanations appear possible. The first is that one or more of the dimethylamino groups become dehydrogenated *in vivo* to ethyleneimino, and the second that oxidation occurs leading to the intermediate formation of methylolamino or methylmethylolamino side-chains. The latter seems the more probable, particularly since it is known that *sym*-trimethyltrimethylolmelamine is a potent tumour-inhibitor (Part I, Hendry, Rose, and Walpole, 1951), and the work of the Madison group on 4-dimethylaminoazobenzene (*vide infra*) supports the metabolic course of events postulated for the dimethylamino residues.

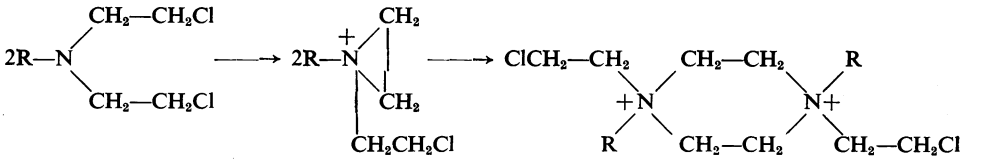
Arising from the activity shown by 2 : 4-dinitrophenylethyleneimine, a series of compounds was made carrying the same aryl residue attached to a variety of other groups (335–343). Several examples were included of bis-dinitrophenyl compounds, to see whether in these molecules the proposed ability of the polar groups to provide some form of bonding with protein or nucleoprotein would lead to a cross-linkage effect. No tumour-inhibition was observed. Similarly, the association of the dinitrophenyl radical with reactive groups such as β-bromo- and -chloro-ethylamine did not lead to active compounds. These results could all be accounted for by the absence of polymerizing properties.

Finally, attention is drawn to the urethane ethyleneimine analogue (253) which was inactive.

Further theoretical and practical implications

It is now desirable to see how far other substances known to have biological properties of the types under discussion can be fitted into the framework of the polymerization hypothesis, and how far such speculations are supported by experimental observation. In this connexion it is proposed to discuss only the "nitrogen mustards," the aromatic polycyclic hydrocarbons, and derivatives of azobenzene and stilbene.

The nitrogen mustards.—The cytotoxic, mutagenic, and carcinogenic properties of these compounds, together with their basic chemical reactions, are well established (*vide* Philips, 1950). As with the sulphur analogues, biological activity is considered to be dependent upon alkylation, mediated by the transformation of these substances into reactive cyclic ethylene-onium ions. This realization enables these compounds, and the nitrogen mustards in particular, to be discussed as special cases of the ethyleneimine type, and the close parallelism in the biological effects elicited by agents of these series lends support to this conception. The cross-linking potentialities of the nitrogen mustards as bifunctional alkylating agents in relation to these phenomena has already received consideration (Goldacre, Loveless, and Ross, 1949 ; Loveless and Revell, 1949), but the possibility of polymer formation as an intermediary stage has not so far been mentioned. Chemical studies have revealed the incidence of a certain degree of dimerization leading to (V)



(V)

(Hanby and Rydon, 1947), presumably according to the reaction sequence indicated, but polymerization to give, for example, the structure shown in Fig. 3 was not apparent, and even if it had occurred under the particular experimental conditions employed, it might not have been readily detectable. Failure to observe a polymer as a major transformation product *in vitro* does not preclude its forma-

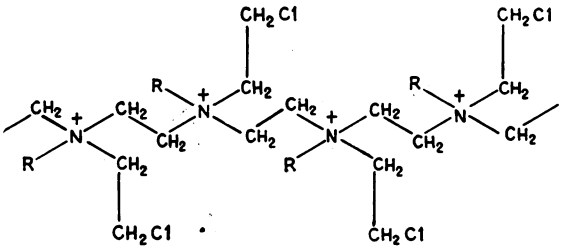


FIG. 3

tion *in vivo*, however, and the alternative possibility already proposed for the methylolamides, the epoxides, and ethyleneimines might still be applicable, namely, that the production of the drug-cell-component complex involves first the attack of single molecules of the agent upon the cell substrate, followed by intermolecular union between pendent 2-chloroethylamine residues to give the polymeric ethyleneammonium chain (Fig. 4). This view of the nitrogen mustards has the advantage

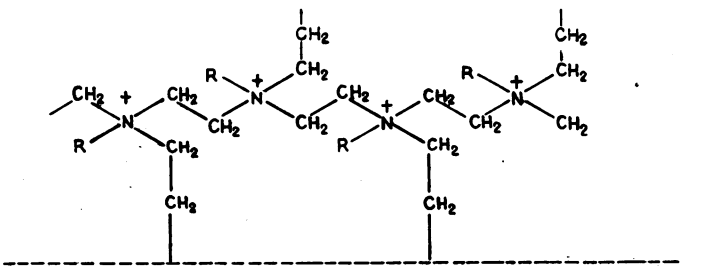


FIG. 4

that, as with the epoxides and ethyleneimines, it accommodates those members of the series in which the β-chloroethyl groups are bonded to different N atoms, even when the latter are some distance apart in the same molecule (Burchenal and Riley, 1949).

The aromatic polycyclic hydrocarbons.—This class of compound has been subjected to intensive chemical and biological investigation over a comparatively

long period of time, so that it is now possible to predict in empirical fashion the approximate structural requirements for carcinogenic activity. So far, the many attempts to analyse these requirements in terms of fine molecular structure have not been conspicuously successful (see Badger, 1948), but it was thought that the present hypothesis might be relevant, and indeed it has been possible to support this view by more or less direct experiment. Two characteristics of the carcinogenic cyclic hydrocarbons were selected as significant. The first was the need for an essentially planar structure of certain broadly optimum molecular dimensions. The second was the presence of a degree of chemical reactivity sufficient to provide facile conjugation with protein, for example, either directly or via the initial formation of a conjugate such as a mercapturic acid. The synthesis was then visualized, within the cell, of a protein or nucleoprotein unit carrying the hydrocarbon moieties as side-chains. The latter, by reason of their lipophilic nature, might then tend to associate into micelles, with the component flat molecules held together, perhaps in lamellar-like form, and possibly additionally interleaved by other hydrocarbon residues conjugated to a second peptide chain. The ultimate effect is shown schematically in Fig. 5. This arrangement clearly resembles that provided by the

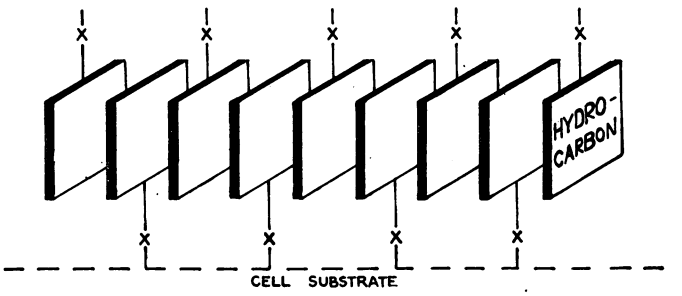


FIG. 5

reactive polymers derived from the methylolamides, epoxides, and ethyleneimines, the essential difference being the substitution of the covalent linkages between the repeating groups of the latter by residual valencies. In essence then, the carcinogenic character of the polycyclic hydrocarbons is regarded as a function of requisite chemical reactivity together with the capacity of the molecules to pack into micellar units, and it might well be that some of the differences in biological effects produced by substitution, etc., could be explained by the influence of these changes on one or both of these factors. At the moment, however, little information is available regarding the relevant physical properties of the polycyclic hydrocarbons, but acyclic hydrocarbons and their derivatives have been more intensively studied. For example, it is known that the presence of straight chain hydrocarbon groups in polar molecules (fatty acids, quaternary ammonium salts, sulphuric esters, etc.) confers micelle-forming properties. Aliphatic hydrocarbons, unlike the aromatic hydrocarbons, lack in themselves the necessary degree of chemical reactivity to combine with polypeptides and their α-amino-acid components, a lack which on the basis of the present hypotheses would make them incapable of inducing malignancy in cells. The introduction of a single group having the necessary reactivity, however, should lead to biologically active structures. This prediction has been

tested by preparing stearoyl-ethyleneimine (248), and, as recorded above, the compound was found to exhibit marked carcinogenic properties. Clearly this result is of some theoretical importance, and current researches are directed in the first place towards a study of biological effect in relation to the known influence of chain length on the micelle-forming characteristics of polymethylene systems.*

This observation also directs attention to the possibility that similar effects might be shown by the analogous epoxide, glycidyl stearate (Part II, Serial No. 215), now under test. At the time of its preparation we considered this to be a possible product of the pyrolysis of fat according to the equation shown, along with carbon dioxide and the higher aliphatic ketones found by Peacock (1949) in his studies of the induction of tumours in the stomach of the mouse by feeding with superheated fats:

$$RCO.O.CH_2—CH(O.COR)—CH_2O.COR \longrightarrow RCO.O.CH_2—\underset{\diagdown\,O\,\diagup}{CH—CH_2} + R.CO.R + CO_2$$

Derivatives of aminoazobenzene and stilbene

The carcinogenic properties of nuclear and N-methylated derivatives of 4-aminoazobenzene are well known. 4-Dimethylaminoazobenzene (Butter Yellow) in particular has been subjected to intensive study by the group of workers at the McArdle Memorial Laboratory, Madison, U.S.A., and is notable for the high yield of malignant hepatomata it produces when fed to rats (Miller, Miller, and Baumann, 1945). It has also been found to combine chemically with protein of the liver. Recently Mueller and Miller (1950), in the course of an investigation of the possible significance of enzymic demethylation in this type of compound, and as a result of the isolation of formaldehyde from the reaction products of liver homogenate experiments, have suggested that the linkage to protein is through

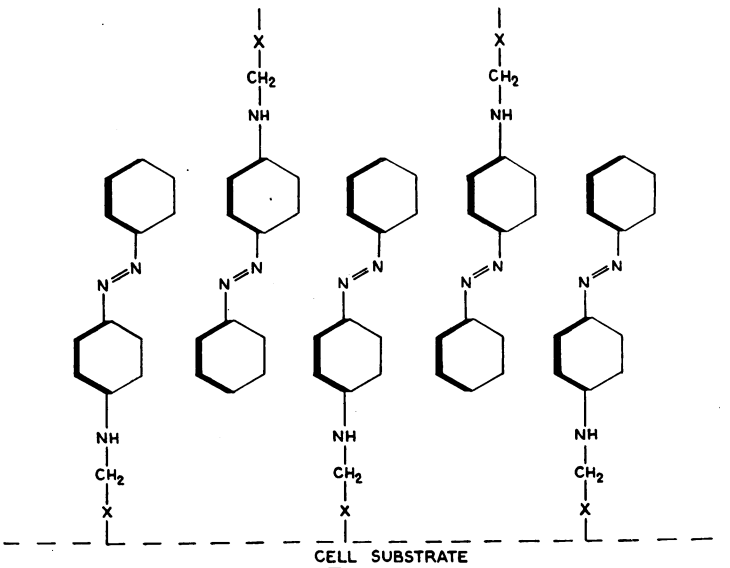


FIG. 6

*See Note (2) added in proof, p. 410.

an intermediate N-methylolaminoazobenzene. It has already been shown (Part I of this series) that an N-methylol group is capable of eliciting cytotoxic effects when suitably linked to other chemical systems, and this, together with the occurrence of the azobenzene group in a number of typical micelle-forming dyestuffs (Vickerstaff, 1950), points to a mode of action consistent with the hypothetical views now being developed. It is suggested that the attachment of the azobenzene residues to protein is followed by the orientation of the former into micelles analogous to those proposed for the polycyclic hydrocarbons (Fig. 6).

Similar arguments can be applied to the carcinogenic (and tumour-inhibitory) 4-alkylaminostilbenes of Haddow, Harris, Kon, and Roe (1948), the stilbene group again being associated in dyestuff technology with specific colloidal properties. In this connexion it is worthy of note that Koller (quoted by Boyland, 1950) has shown that 4-dimethylaminostilbene produces specific chromosome effects in the Walker tumour in rats, and we have shown that the same is true of 2′-chloro-4-dimethylaminostilbene (unpublished). So far, the analogous combination of these compounds with the protein of affected tissues has not been investigated.

Summary

1. Preceding papers in this series have described the tumour-inhibitory and cytotoxic action of certain methylolamides and epoxides. Similar observations are here recorded in respect of over a hundred N-substituted ethyleneimine derivatives. Many of those carrying two or more ethyleneimine residues have been shown to have outstanding activity against the Walker rat carcinoma 256. The most effective compound examined has been 2 : 4 : 6-tris-ethyleneimino-*s*-triazine (322), but other triazine and pyrimidine derivatives have shown activity of a like order. Where direct comparison has been possible the pyrimidines have proved more active than their triazine analogues. Other highly active compounds include $\omega : \omega'$-bis(ethyleneimino-sulphonyl) propane (287).

2. Cytotoxic effects have been studied in Feulgen-stained preparations of tumour tissue and bone marrow from treated rats and the specific chromosome damage (fragmentation and bridge formation) produced in dividing cells evaluated quantitatively in the tumour. A correlation between the capacity to produce such changes and tumour growth inhibitory activity has been demonstrated in many instances. It is concluded, on the evidence of these and other experiments, that the tumour inhibition is a manifestation of an action directed against dividing cells in general rather than against tumour cells in particular.

3. Several pyrimidine and triazine derivatives containing only one ethyleneimine residue have been found to produce definite tumour inhibition and specific chromosome damage but only at much higher dose levels than with related bi- and tri-functional compounds. While the possibility of contamination with the latter could not be ruled out in most cases, it was unlikely in that of the active 2 : 4-dinitrophenyl-ethyleneimine (257). Ethyleneimine itself produces no such effects in the living animal.

4. A hypothesis was developed in earlier papers to explain the biological properties of the methylolamides and epoxides. The initial intermolecular condensation of these agents within the affected cells was postulated, with the formation of

polymer units with chemically reactive side chains which then combine with cell components (protein or nucleoprotein). This hypothesis is shown to be applicable to the ethyleneimines, and the relationships between structure and activity are discussed in this light. The nitrogen mustards appear as a special case of the ethyleneimine type.

5. Attempts have been made to adapt the same hypothesis to explain the carcinogenic and/or tumour-inhibitory activity of compounds of other chemical types, namely, polycyclic hydrocarbons, aminostilbenes, and aminoazobenzenes. It is suggested that conjugation of the nuclear residues of these substances with cell components (e.g., protein) occurs, followed by the association of these units into micelles simulating polymers in form. Direct evidence in favour of such a mechanism has been sought by preparing and examining stearoylethyleneimine (248) which may be regarded as another micelle-forming substance capable of reacting with cell components. This substance has been shown to be a carcinogen.

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Notes added in proof

(1) The following clinical papers on 2:4:6-tris-ethyleneimino-*s*-triazine (322) have recently appeared: Karnovsky, D. A., Burchenal, J. H., Armistead, G. C., Jr., Southam, C. M., Bernstein, J. L., Craver, L. F., and Rhoads, C. P. (1951). *Arch. int. Med.*, **87**, 477. Paterson, E., and Boland, F. (1951). *Brit. J. Cancer*, **5**, (1), 28.

(2) It has now been demonstrated that, in addition to stearoyl ethyleneimine (248), myristoyl ethyleneimine ($C_{13}H_{27}CO.N(CH_2)_2$) and caproyl ethyleneimine ($C_5H_{11}CO.N(CH_2)_2$) are markedly carcinogenic.