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CYTOTOXIC AGENTS: II, BIS-EPOXIDES AND RELATED COMPOUNDS

BY

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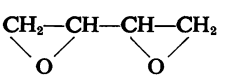
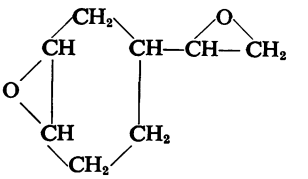
In Part I (Hendry, Rose, and Walpole, 1951) of the present series of papers, we described the discovery of "radiomimetic" activity in a series of methylolamide derivatives. Although this discovery emerged from investigations undertaken on the supposition that urethane might function as a chemically reactive grouping towards amines of cellular origin, it became apparent that the biological effects of these substances were more closely related to their use in textile technology. The "cross-linking" action, upon which their effects upon textile fibres seem to depend, might well lead to the linkage of cellular components in a manner analogous to that which has been suggested for the nitrogen mustards, and which is believed by Haddow and his co-workers to be responsible for the specific cytotoxic effects of the latter. In fact the first methylolamides which we examined were selected from a number made available to us in 1947 by our colleagues who were studying their use in the textile field. This technological interest extended to compounds of other chemical types having similar applications and similar "cross-linking" potentialities, notably certain epoxide and ethylenimine derivatives, and these by analogy were deemed worthy of biological examination. This communication concerns substances of the former type, namely, those based on the ethylene oxide ring system. Brief reference to them has been made in an earlier note (Rose, Hendry, and Walpole, 1950).

The chemistry of these substances has been intensively investigated during the past six or seven years in the laboratories of Canadian Industries Ltd., Montreal, from whom some of our earlier specimens were obtained; including the important dioxide (II) from butadiene dimer (4-vinyl *cyclo*hexene). For the butadiene dioxides (I) we are indebted to our colleague Dr. W. F. Beech.



(I) 8838 (mixed isomers) and 9137 (*meso*)



(II) 8840

The selection of compounds for examination was based upon a number of premises which were modified or added to as the work proceeded. Since our findings in the methylolamide series suggested that polyfunctionality was essential for activity, structures carrying two epoxide groups were first examined as tumour inhibitors (initially against the transplanted Walker carcinoma) and several found to be active. It was nevertheless considered desirable to check the presumed inactivity of mono-epoxides. Some 25 compounds were therefore examined in which a single epoxide radical was linked to a wide variety of groupings, including acyclic and carbocyclic structures giving substances, e.g., those with serial numbers 203, 196, 197, and 199 (Table I) related to butadiene and vinyl *cyclo*hexene dioxides respectively, and also basic groupings, as 205 and 206. In some instances, because of ease of access, the epoxide ring was introduced in the form of a derivative of glycidol; in others the oxide was obtained, indirectly, by oxidation from the corresponding olefine. In view of the possibility of *in vivo* oxidation it was considered desirable to examine the parent hydrocarbons corresponding not only to the mono-epoxides but also to the active bis-epoxides. Some monoene mono-epoxides were also examined. In addition, examples of (mono) epoxides derived from certain terpenoid compounds (limonene, safrole) were included because of the special interest which attached to these potential epoxide precursors of natural occurrence.

The bis-epoxides as a whole were similarly diverse in character. Since the specific biological effects observed were at first believed to depend upon the capacity of each of the epoxide groups to react with groupings carrying active hydrogen ($R—\underset{\backslash O /}{CH}—CH_2 + HX \rightarrow R—\underset{|}{\underset{OH}{CH}}—CH_2—X$), such factors as the steric and inductive effects of proximal parts of the molecule were regarded as important. It was hoped that the significance of these factors and in addition that of spatial considerations, such as the distance between the epoxide groups, could be deduced from a study of the results obtained with the variety of examples of the bis-functional type examined.

In Part I we drew attention to the carcinogenic potentialities of tumour inhibitory cytotoxic agents of the radiomimetic class and described our failure unequivocally to demonstrate tumour production. We have tested two active members of the present series as carcinogens and shown that one of them readily produces malignant tumours in the mouse and the rat. The mutagenic activity of the nitrogen mustards is well known, and here, too, the association with specific cytotoxic activity seems close. The one bis-epoxide tested for such activity has produced mutation in a strain of *Penicillium*.

Methods

Preliminary toxicity tests in mice and rats were carried out on the compounds in the same way as described in the previous paper for those which formed the subject of that report (Hendry, Rose, and Walpole, 1951). The majority were oil soluble and were given in solution in arachis oil. A few were dissolved in water or suspended by milling in an aqueous solution of Dispersol OG (1.5 per cent) and Dispersol LN (0.05 per cent). Many of the epoxides are decomposed in aqueous solution, and with these substances water was as far as possible avoided as a solvent. From the results of these tests an estimate was made of the highest level at which rats bearing the Walker tumour

could be dosed daily, Sundays excepted, for the first 10 to 12 days of experiment without any of the rats dying by the fourteenth. This schedule of dosage, starting the day after implantation of the tumour, was used in the initial tests for tumour inhibitory activity.

Our routine method of test for inhibitory activity against the Walker tumour has also been described (Walpole, 1951). The percentage increase in gross weight ($\triangle W$) of the survivors in each group of rats and the percentage inhibition of tumour growth (I) in each of the treated groups was 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 Table I are listed all the compounds which come under consideration. The serial and code number, name and formula of each is shown together with the total dose in mg. per 100 g. rat given over the first 10 to 12 days after implantation of the tumour. Dosing was invariably intraperitoneal. Compounds will be referred to in the sequel by their serial numbers.

It will be seen that the unsaturated hydrocarbons examined (serial nos. 188–195) produced little or no inhibition of tumour growth in maximal tolerated doses. Several of these compounds are potential precursors of the active di-epoxide, 4-vinyl *cyclo*hexene dioxide, and this result disposes of the suggestion that they might be oxidized to the latter *in vivo* to any appreciable extent. 1 : 5-Hexadiene (195) is also virtually inactive, so that it is unlikely that this is oxidized in the body to the corresponding bis-epoxide, reported active by Ross (1950).

The mono-epoxides which follow (serial nos. 196–220) are similarly inactive or of low activity ; the tumour inhibition produced was never clearly greater than could be accounted for by the general toxic action. These compounds include 4-vinyl *cyclo*hexene monoxide (196), and the fact that this compound has but little action at a very high dose level leads to a conclusion similar to those drawn above. The results with the mono-epoxides taken as a whole support the contention that the presence of only one epoxide group in a molecule is insufficient to give rise to activity.

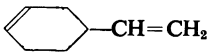
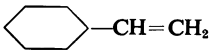
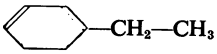
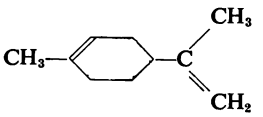
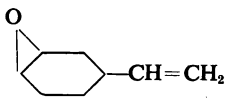
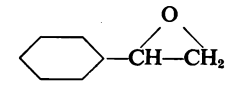
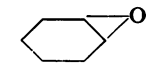
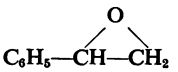
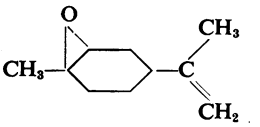
Of the bis-epoxides examined, however (serial nos. 221–240), several were found to produce very considerable inhibition of tumour growth, although even here there is a considerable variation in activity. Even with the most active, however, the margin between the lethal dose and the dose required to produce marked inhibition of tumour growth is small, and for that reason we consider it unlikely that any of this type will be found to have any therapeutic application.

Cytotoxic effects

A selection of mono- and bis-epoxides and related compounds has been examined for activity in the production of specific chromosome damage in dividing cells of the Walker tumour. Single intraperitoneal doses of the different compounds were given to tumour-bearing rats, and Feulgen-stained squash preparations were made from tumour tissue taken when the animals were killed 24 hours later. Similar preparations were made of bone marrow taken from the rats immediately after they

TABLE I

Action of some epoxides and related compounds upon the growth of the Walker tumour in rats. $\triangle W$ = mean percentage increase in gross weight of the tumour-bearing rats;

Serial No.	Code Number	Compound	
		Name	Formula
		HYDROCARBONS	
		(*a*) *Related to vinyl*cyclo*hexene*	
188	9448	4-Vinyl*cyclo*hexene*	
189	9449	Vinyl*cyclo*hexane*	
190	9510	4-Ethyl*cyclo*hexene*	
191	9254	*dl*-Limonene (dipentene)	
192	9109	Styrene	$C_6H_5—CH=CH_2$
193	9294	1-Phenylbutadiene	$C_6H_5—CH=CH—CH=CH_2$
194	9291	α-Vinylnaphthalene	$\alpha\text{-}C_{10}H_7CH=CH_2$
		(*b*) *Related to* 1 : 3-*butadien*	
195	9135	1 : 5-Hexadiene	$CH_2=CH—CH_2—CH_2—CH=CH_2$
		MONOEPOXIDES	
		(*a*) *Related to vinyl*cyclo*hexene monoxide*	
196	9446	4-Vinyl*cyclo*hexene monoxide	
197	9515	Vinyl*cyclo*hexane oxide	
198	9447	*Cyclo*hexene oxide	
199	9110	Styrene oxide	
200	9297	*dl*-Limonene monoxide	

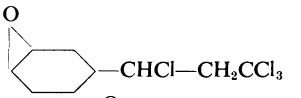
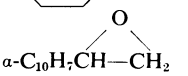
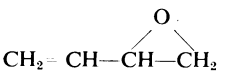
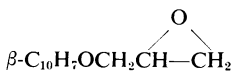
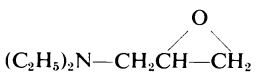
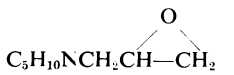
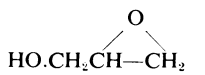
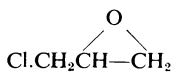
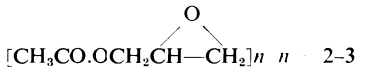
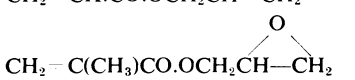
*Obtained from Canadian Industries Ltd.

TABLE I (*continued*)

M_{50} = mean weight of the n heaviest tumours in groups of $2n$; I = percentage inhibition of tumour growth; o.s. = oily solution; a.s. = aqueous solution; a.d. = aqueous dispersion

Form	Total dose (mg./100 g. i.p.)	Tumour inhibition				
		$\triangle W$		M_{50}		I
		Control	Treated	Control	Treated	
o.s.	480	48.0	36.3	42.1	38.4	9
,,	325	48.0	43.7	42.1	36.8	13
,,	300	45.3	47.6	28.5	37.0	0
,,	275	29.1	29.1	39.4	35.6	10
,,	425	38.5	35.3	35.4	28.9	18
,,	185	35.1	29.6	36.1	38.2	0
,,	325	46.2	47.1	36.9	39.0	0
,,	50	45.6	33.4	38.5	31.2	19
,,	400	48.0	20.5	42.1	26.7	37
,,	300	17.1	24.2	35.1	35.3	0
,,	170	39.8	14.0	30.3	25.7	15
,,	220	34.6	20.7	35.4	29.2	18
,,	300	36.7	23.7	37.2	23.7	36

TABLE I (*continued*)

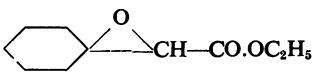
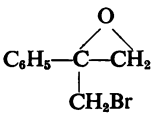
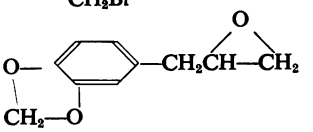
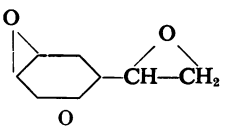
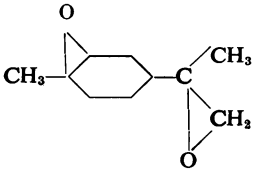
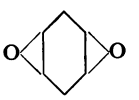
Serial No.	Code Number	Compound	
		Name	Formula
201	9560	4-(α : γ : γ : γ-tetrachloropropyl) *cyclo*hexene oxide	
202	9444	α-Vinylnaphthalene oxide	 Untested *v.* Table II
		(*b*) *Related to* 1 : 3-*butadiene monoxide*	
203	9136	1 : 3-Butadiene monoxide	
		(*c*) *Derivatives of glycidol*	
		(i) *Ethers*	
204	3928	β-Naphthylglycidyl ether	
		(ii) *Amines*	
205	9252	Diethylamino epihydrin	
206	9293	Piperidino epihydrin	
207	9621	N(2 : 3-epoxy-*n*-propyl)-*p*-chloraniline	
		(iii) *Alcohols and esters*	
208	2338	Glycidol	
209	9096	Epichlorhydrin	
210	9593	Glycidyl acetate	
211	9595	poly-Glycidyl acetate	
212	10061	Glycidyl acrylate*	
213	10062	Glycidyl methacrylate*	

*Obtained from Canadian Industries Ltd.

TABLE I (*continued*)

Form	Total dose (mg./100 g. i.p.)	Tumour inhibition				
		ΔW		M_{50}		I
		Control	Treated	Control	Treated	
o.s.	350	40.8	41.4	32.8	32.1	2
,,	135	55.1	19.5	36.5	20.8	43
,,	300	36.7	18.6	37.2	26.0	30
,,	60	45.6	26.3	38.5	29.0	25
a.s.	80	35.1	24.9	36.1	34.7	4
o.s.	275	34.4	16.6	39.7	30.6	23
a.s.	140	30.7	27.5	31.7	37.4	0
o.s.	42	33.1	27.5	35.7	26.0	27
,,	70	17.2	27.2	35.1	35.9	0
,,	325	29.9	22.2	43.1	33.8	22
,,	3.5	26.4	26.4	33.6	33.6	0
,,	150	32.6	12.5	42.6	26.0	39

TABLE I (*continued*)

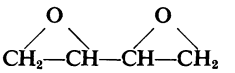
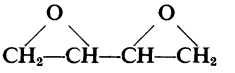
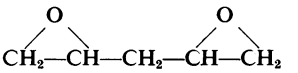
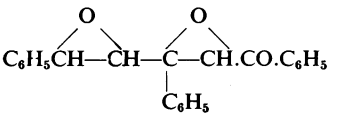
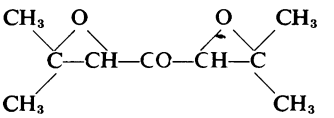
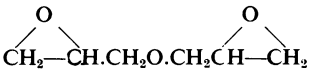
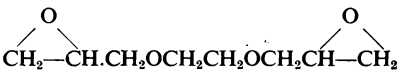
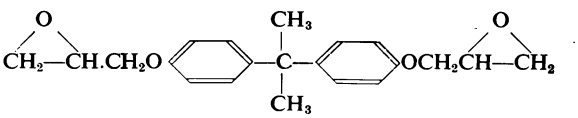
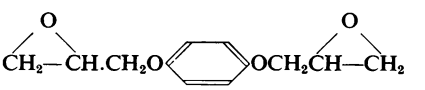
Serial No.	Code Number	Compound: Name	Compound: Formula	
214	10209	Glycidyl oleate	$C_8H_{17}CH{=}CH.C_7H_{14}CO.OCH_2CH{-}CH_2$	Untested. See Table II
215	9893	Glycidyl stearate	$C_{17}H_{35}CO.OCH_2CH{-}CH_2$	
		(*d*) *Miscellaneous*		
216	9366	α: β-Epoxymesityl oxide	$(CH_3)_2C{-}CH.CO.CH_3$	
217	9512	2-Carbethoxy-1-oxa*spiro*-2: 5-octane		
218	9513	Sodium phenylglycidate	$C_6H_5{-}CH{-}CH.CO_2Na$	
219	9615	α-Phenyl-α-bromomethyl ethylene oxide		
220	9643	Safrole oxide		
		DI-EPOXIDES		
		(*a*) *Related to vinylcyclohexene dioxide*		
221	8840	4-Vinyl*cyclo*hexene dioxide*		
222	9253	*dl*-Limonene dioxide		
223	10058	1: 4-*cyclo*hexadiene dioxide*		

*Obtained from Canadian Industries Ltd.

TABLE I (*continued*)

Form	Total dose (mg./100 g. i.p.)	Tumour inhibition				
		$\triangle W$		M_{50}		I
		Control	Treated	Control	Treated	
o.s.	300	36.7	31.8	37.2	27.5	26
,,	300	45.3	42.7	28.5	33.1	0
a.s.	300	45.6	45.2	45.5	48.0	0
o.s.	16	27.4	14.4	26.0	25.5	2
,,	150	17.3	5.7	29.8	25.7	14
,,	225	40.6	13.0	39.5	2.5	94
,,	265	51.1	35.0	44.2	34.4	22
a.s.	150	37.3	8.0	44.6	25.3	43

TABLE I (*continued*)

Serial No.	Code Number	Compound	
		Name	Formula
		(*b*) *Related to* 1 : 3-*butadiene dioxide*	
224	8838	1 : 3-Butadiene dioxide (mixed isomers)	
225	9137	Meso-1 : 3-butadiene dioxide	
226	10059	1 : 4-Pentadiene dioxide*	
227	9561	1 : 3-Diphenyl-4-benzoyl-1 : 3-butadiene dioxide	
228	9445	Phorone dioxide	
		(*c*) *Derivatives of glycidol*	
		(i) *Ethers*	
229	8839	Diallyl ether dioxide	
230	9367	Ethylene glycol diglycidyl ether	
231	10317	Diethylene glycol diglycidyl ether*	
232	10060	Butane-1 : 4-diol diglycidyl ether*	
233	9514	Diphenylolpropane diglycidyl ether	
234	9364	Hydroquinone diglycidyl ether	

*Obtained from Canadian Industries Ltd.

TABLE I (*continued*)

Form	Total dose (mg./100 g. i.p.)	Tumour inhibition				
		$\triangle W$		M_{50}		I
		Control	Treated	Control	Treated	
o.s.	16	32.4	5.9	20.6	0.6	97
,,	5	50.1	19.2	39.8	18.0	55
,,	250	26.4	16.9	33.6	23.8	29
a.d.	200	40.8	39.2	32.8	31.5	4
o.s.	80	43.3	49.3	36.0	39.9	0
,,	20	32.7	6.7	40.4	21.5	47
,,	50	48.7	5.2	45.05	16.7	63 at 15 days
,,	150	26.0	14.7	31.2	4.9	84
,,	120	31.8	2.2	46.7	12.2	74
,,	400	43.3	32.7	36.0	37.2	0
a.d.	80	26.6	−0.3	39.0	23.0	41

TABLE I (*continued*)

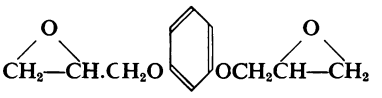
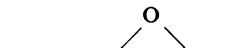
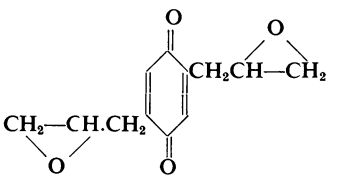
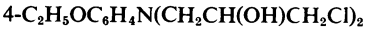
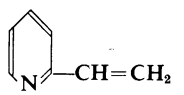
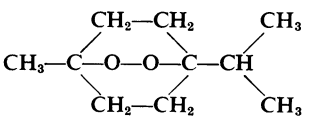
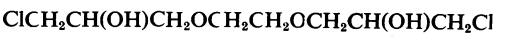
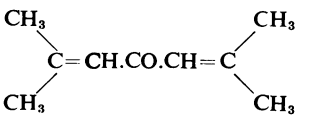
Serial No.	Code Number	Compound	
		Name	Formula
235	9511	Resorcinol diglycidyl ether	
		(ii) *Amines*	
236	9295	N: N-di-(2′: 3′-epoxy-*n*-propyl)-*p*-phenetidine	
237	9559	N: N-di-(2′: 3′-epoxy-*n*-propyl)*p*-anisidine	
238	9588	N: N-di-(2′: 3′-epoxy-*n*-propyl)-*p*-toluidine	
239	9587	N: N-di-(2′: 3′-epoxy-n-propyl)-aniline	
		(*d*) *Miscellaneous di-epoxides*	
240	9368	2: 5-di-(2′: 3′-epoxy-*n*-propyl)-benzoquinone	
		MISCELLANEOUS COMPOUNDS	
241	9296	N: N-di-(3′-chloro-2′-hydroxy-*n*-propyl)-*p*-phenetidine	
242	9365	2-Vinylpyridine	
243	9373	Ascaridole (oil of wormseed)	
244	9622	Ethyleneglycol-di-(3′-chloro-2′-hydroxy-*n*-propyl)ether	
245	8841	Phorone	

TABLE I (*continued*)

Form	Total dose (mg./100 g. i.p.)	Tumour inhibition				
		$\triangle W$		M_{50}		I
		Control	Treated	Control	Treated	
o.s.	35	45.6	17.1	45.5	33.1	27
,,	350	50.0	6.2	38.2	2.3	94
,,	170	34.4	−4.1	39.7	4.5	86
	190	24.5	10.0	32.4	28.6	12
,,	190	24.5	7.7	32.4	21.6	33
,,	275	26.6	25.2	39.0	36.8	6
a.d.	200	35.1	29.0	36.1	31.5	13
o.s.	75	26.6	28.8	39.0	33.3	15
,,	20	17.3	22.2	29.8	27.7	7
,,	450	34.4	38.3	39.7	42.8	0
,,	250	40.9	16.4	17.9	24.2	0

had been killed. A description of the chromosome aberrations regarded as characteristic of the action of true radiomimetic agents has already been given (Hendry, Rose, and Walpole, 1951). The increase over controls in the percentage of anaphases showing abnormalities of this type in tumour tissue from rats treated with the compounds in the doses shown is recorded in Table II. Mainly qualitative observations upon cytotoxic effects in the bone marrow and the results of tumour inhibition tests with the substances in question are also included.

As with compounds tested earlier and reported upon in Part I, there is an obvious correlation between tumour inhibitory activity and the capacity to induce in the tumour chromosome changes of the radiomimetic type. Only one compound in the present selection, namely, 1 : 4-pentadiene dioxide (226), which proved active in the latter sense, failed to produce a significant inhibition of tumour growth. The reason for this failure is not clear, but it may be that in the tumour growth test dosage was not pushed to the limit. Lack of material has so far prevented a repetition of the test.

Carcinogenic activity

Two of the more active inhibitors of tumour growth, namely, 1 : 3-butadiene dioxide (mixed isomers) and 4-vinyl *cyclo*hexene dioxide, have been tested for carcinogenic activity in rats and mice.

In rats, butadiene dioxide (0.4 per cent) dissolved in arachis oil was injected intraperitoneally in a dose of 2 mg. per 100 g. body weight twice weekly for six to seven weeks (13 doses) and in a dose of 1 mg. per 100 g. for the following six weeks (12 doses). Ten male and four female stock albino rats of average weight 125 g. were used and all were dead 18 (calendar) months from the start of treatment. In one male which died at 13 months (15–16 months old) large masses of a mixed-cell sarcoma were found in the peritoneal cavity, adherent to and infiltrating the stomach and body walls, and nodules of tumour tissue throughout the mesentery. A chondrosarcomatous mass was found enveloping the left lower ribs. Mixed cell sarcomata were seen in one testis and an unidentified epithelial tumour in the other. No tumour was found in any other rat in this group.

In mice the compound, dissolved in arachis oil or in acetone (A.R.), was applied repeatedly to the interscapular region of the skin. In one experiment 20 male stock mice received 1 drop daily (Saturdays and Sundays excepted) of a 10 per cent solution by volume in arachis oil for three months (66 doses). All the mice, which were mature at the beginning of the experiment, died within eight months thereof. No tumour was found in any animal, but loss of spermatogenesis was observed in the tubules of the testes in one. In a further experiment 20 male stock mice received two drops twice weekly of a 25 per cent solution of the compound in acetone for three weeks, followed by two drops of a 12.5 per cent solution twice during the week following. All died within 11 months of the commencement of the experiment. No tumours were observed in these mice, although areas of very pronounced infiltration with lymphoid cells, which may have been a leukaemic change, were found in the liver of one. Pyknosis of lymphoid cells was observed in the lymph nodes of several others.

In all these experiments a rather large proportion of the animals died during the early stages and further tests are being carried out with lower levels of dosage.

TABLE II

Growth-inhibitory and cytotoxic effects on the Walker tumour in rats of some epoxides and related compounds. *I* = percentage inhibition of tumour growth; A = percentage anaphases in excess of controls showing specific chromosome damage in tumour tissue from rats bearing the Walker tumour, twenty-four hours after the doses shown; B = true chromosome bridges; D = degenerating nuclei; E = "exploded" metaphases; F = chromosome fragments; P = pyknotic nuclei; S = "sticky" chromosome bridges

Serial No.	Compound	Tumour inhibition		Cytotoxic action			
		Total dose: mg. per 100 g., i.p.	*I*	Dose: mg. per 100 g., i.p.	Tumour A	Tumour	Bone marrow
221	4-Vinyl*cyclo*hexene dioxide	225	94	25 12.5	53 43	Some E and D	Some B, F, and S Some F and S
237	N: N-di-(2′: 3′-epoxy-*n*-propyl)-*p*-anisidine	170	86	30	76	Mostly showers of fragments. Many E	A few B, F, and S
231	Diethylene glycol diglycidyl ether	150	84	40 20 10	43 — 39	Some inhibition of mitosis. A few E Almost complete inhibition of mitosis. A few F	Some inhibition of mitosis. Some F and P Some inhibition of mitosis. Some P A few P
232	Butane-1: 4-diol diglycidyl ether	120	74	20 10	62 38	Some E	A few F and B A few F and B
223	1: 4-Cyclohexadiene dioxide	150	43	50	0		Normal
213	Glycidyl methacrylate	150	40	30	1		A few P and S
226	1: 4-Pentadiene dioxide	250	29	100 50 25	41 42 2		B, F, and S A few P A few S
207	N-(2: 3-epoxy-*n*-propyl)-*p*-chloro-aniline	275	24	50	10		A few B
220	Safrole oxide	150	14	25	6		A few S
243	Ascaridole	20	7	4	1		A very few S
212	Glycidyl acrylate	3.5	0	0.5	2		A few S
219	α-Phenyl-α-bromomethyl-ethylene oxide	16	0	2 5	7 0	(Mean of 2 early tests)	A few S Normal
244	Ethylene glycol di-(3′-chloro-2′-hydroxy-*n*-propyl)ether	450	0	50	0		A few S
214	Glycidyl oleate	Untested		100	3		A few S
215	Glycidyl stearate—Crude Pure	,,		50 75	2 6		A few S A few S
202	α-Vinylnaphthalene oxide	,,		100	0		A few P

Vinyl *cyclo*hexene dioxide (5 per cent) dissolved in arachis oil was given intraperitoneally to ten male and four female stock albino rats in a dose of 25 mg. per 100 g., twice weekly for ten weeks. Seven months from the start of treatment mixed-cell sarcoma tissue was found widely disseminated in the peritoneal cavity of one male rat. One large area of the lung of this animal was infiltrated with tumour tissue. The sarcoma has been maintained for over 20 successive subcutaneous transplantations in stock rats. No other tumour has been seen in animals in this group, 6 of which are still alive 21 (calendar) months after the commencement of dosing. Loss of spermatogenesis has been observed in several of the males which have died and that at an age before the testes normally atrophy. Experiments in which the compound is being given subcutaneously to rats are in progress.

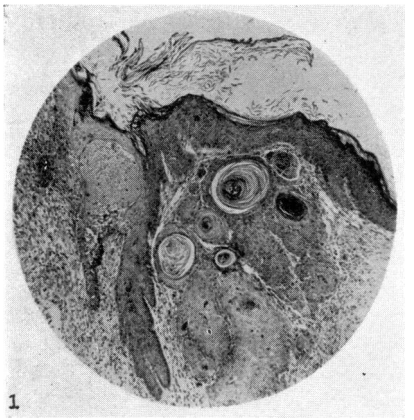
A much higher yield of tumours is obtained when the compound is applied to the skin of mice. Twenty stock albino males were painted in the interscapular region with one drop (ca. 16 mg.) of the compound, five times weekly for a total of twelve months. Skin papillomata began to appear within a few months, and many of these subsequently became malignant. The last of the mice died some 21 months after the start of treatment. Nine died without tumours, and, in two, papillomata which had developed during painting regressed after its cessation. Malignant tumours developed in the remainder as follows: Four had squamous cell carcinoma at the site of painting ; in one of these metastases were present in the lung, while in two lung adenomata showing no signs of malignancy were found. Three had mixed cell sarcomata in the tissues underlying the painted area. These sarcomata had ulcerated through at the surface, the epithelium being completely lost. In two mice both types of tumour were found together—a sarcoma ulcerating through at the surface bordered by carcinoma of the skin. In the lung of one of these animals a tumour of a third type, probably malignant, was found. Figs. 1 to 3 illustrate the appearance of the several tumours in this mouse.

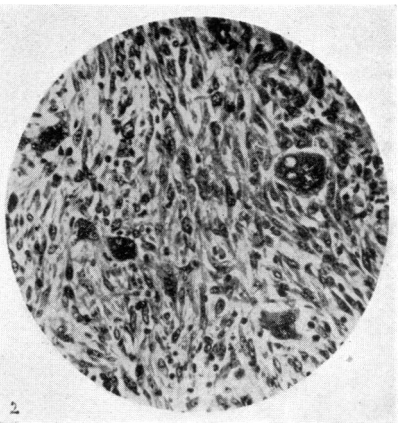
The vinyl *cyclo*hexene dioxide used in the foregoing experiments was the commercial product and was contaminated with water insoluble material. In a further experiment a highly purified sample, completely soluble in water, was used. This sample was rather more toxic and rather more active in the production of specific cytotoxic effects in the Walker tumour than the original sample. A solution in acetone (3 parts) is being applied to the skin of mice at the rate of 1 drop per animal twice weekly. Loss of fur from the painted region was noted about one month after the start of treatment and the first papilloma made its appearance at two months.

Mutagenic activity (with Miss M. C. Frank)

Butadiene dioxide (mixed isomers) was tested for mutagenic activity upon the mould *Penicillium chrysogenum*. The latter was grown on agar slopes and aqueous suspensions of the spores, containing $3–6 \times 10^6$ per ml., were treated with the compound in aqueous solution. It proved to be rather toxic. At concentrations of 3 to 4 per cent it killed all the spores within 10 minutes at 24° C. ; at 0.5 per cent, the percentage survival after 10 to 60 minutes' exposure was 0.5 to 1. Concentrations of 0.5 and 0.25 per cent were used in the tests for mutagenic activity.

After treatment with the compound, the spore suspensions were plated on agar medium and the plates incubated at 24° C. The colonies which developed within





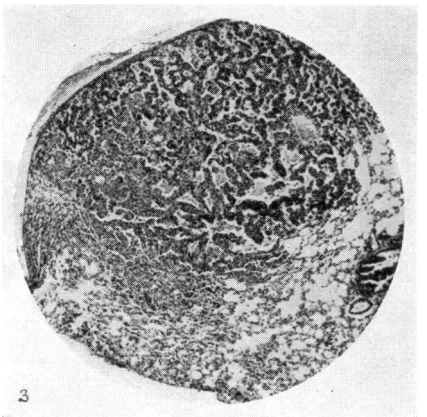


FIG. 1.—Section of tumours produced in mouse by painting the skin with 4-vinyl *cyclo*hexene dioxide. Field shows squamous-cell carcinoma of the skin at the periphery of a mixed-cell sarcoma (on left). H.E. × 77.

FIG. 2.—High-power view of sarcoma shown in Fig. 1. H.E. × 310.

FIG. 3.—Adenoma, possibly in early stage of malignancy, in an area of the lung of the same mouse. H.E. × 77.

a few days were examined macroscopically and mutants detected by changes in colour and habit of growth. Untreated spores produced green colonies having a characteristically raised centre. The mutants were of various hues, some being white or yellow and the majority various shades of yellow-green. As a matter of convenience the spore suspensions were sampled several times during treatment with the compound and colonies counted on all the plates showing a high degree of killing. It was impossible to forecast exactly what length of treatment would give suitable plates, i.e., plates containing 300 colonies or less. Control plates were also put up, as this strain of *P. chrysogenum* produces spontaneously a small number of mutants. The results may be summarized as follows:

Concentration of compound	Percentage of mutant colonies produced by 10–90 minutes' exposure		
%(w/v)	Exp. *a*	Exp. *b*	Mean
0.5	8.0	12.8	10.4
0.25		8.6	8.6
0 (control)	1.3	1.5	1.4

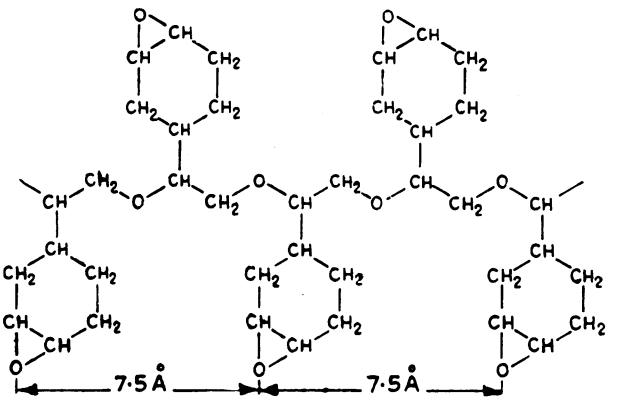
Some of the mutants were tested to see whether any change had occurred in penicillin productivity. This was found to vary over a wide range. The majority of the mutants gave poor yields, and it was evident from the types of growth obtained that many grew more slowly and less efficiently than the culture treated.

The proportion of mutants induced in *P. chrysogenum* by treatment with butadiene dioxide was rather lower than has usually been obtained with ultra-violet or *x* rays. Killing was about the same as that with ultra-violet rays but much more than with *x* rays. Similar results have been recorded for the sulphur and nitrogen mustards (cf. Bryson, 1948).

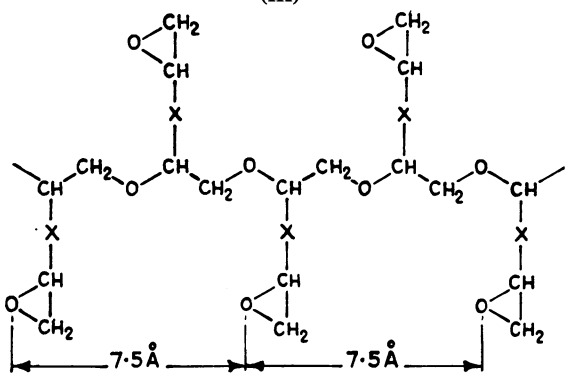
Discussion

At an early stage of this work it became clear that several considerations applicable to the methylolamides were equally relevant to the epoxides. In the first place, for significant inhibition of tumour growth in the rat, two epoxide groups in the same molecule were found to be necessary, and this suggested a biochemical equivalence of epoxide and methylolamide groups in accord with what is known of the actual chemical reactivity of the two types. Both, for example, show reactivity towards nucleophilic centres, the significance of which in the epoxide group was earlier pointed out by Rapoport (1948), who related it to the mutagenic activity shown by ethylene oxide and several derivatives in *Drosophila*. More recently, Ross (1950) has discussed the kinetics of this reaction in connexion with a range of epoxides also examined against the Walker tumour in rats, and it would appear that a certain minimal reactivity, measured *in vitro* with respect to the thiosulphate ion, must be exceeded before a compound of this type is growth-inhibitory. In agreement with our own findings, Ross observed that two such centres were required in the molecule (for a positive effect in the animal), and further suggested that an unequal reactivity was desirable, the more reactive grouping serving to anchor the molecule to a receptor, the second group then having more time to combine with another receptor. The studies of our Canadian colleagues, however, suggest an alternative explanation for the requirement of two functional groups, and one which takes into account an additional important chemical property of substances carrying the oxiran system. This property is ignored by Loveless and Revell (1949) in the simple cross-linkage hypothesis adduced by them to explain the cytotoxic effect of bis-epoxides.

It has been known for some time that ethylene oxide readily polymerizes to produce linear structures having the repeating unit [-O-CH_2-CH_2-], and this property persists in the more complex derivatives of this substance, including those carrying two oxide units. The tendency to polymerize is strongly conditioned by steric considerations, so that in 4-vinyl *cyclo*hexene dioxide (II), for example, it is possible to isolate a polymer (III) in which the second epoxide ring remains unaffected. This second oxide system is, however, the more reactive towards nucleophilic systems, so that the behaviour of (II), which is one of the more active (221 ; Table I) of the 24 *bis*-epoxides examined for inhibition of the Walker tumour, clearly suggests a separate function for each oxiran radical in these structures, the one being primarily concerned with polymerization and the other with combination with centres carrying active hydrogens such as occur in abundance in protein and, in particular, nucleoprotein systems. In the latter connexion, it may also be of



(III)



(IV)

importance to note that the dimensions of the -C-C-O- unit of the polyethylene glycol structure corresponds closely with those of the -C-C-N- unit of polypeptides, so that in a polymer of type (III) a reactive epoxide group occurs along each side of the chain at intervals of every two α-amino-acid residues. It will be recalled that a similar spacing was evident between the reactive groups in the polymeric structures proposed for the methylolamide series of tumour-inhibitors (Hendry, Rose, and Walpole, 1951), and a corollary of some importance follows from this observation in relation to the variation in structure permissible in the epoxide series. Reference to the Tables will show that tumour-inhibitory action was found in substances in which the two reactive centres are in close juxtaposition to one another as in the butadiene dioxides, and, at the other extreme, when they are some 15Å apart as in the diglycidyl ether of diethylene glycol (231). Examples of intermediate spacing occur in Table I. If the biological effects produced were due solely to bridges formed by individual molecules either along or between protein or nucleoprotein units, then it would be necessary to admit a wide range of permissible distances between the two points of attachment of each molecule to the latter. If, on the other hand, these cytotoxic agents function, not as individual molecules, but ultimately as polyreactive polymers of the generalized form (IV), then the distance between adjacent reactive groups is constant at about 7.5Å and theoretically independent of the dimension of the conjunctive group X.

The precise significance of the possible formation of these structures in relation to cytotoxic effects must necessarily be almost entirely a matter of speculation, but we suggest that, as in the methylolamide series discussed in the previous communication, the *bis*-epoxides diffuse in monomeric form into susceptible cells and there behave in one, or perhaps both, of two ways. One possibility is that they first polymerize to give structures corresponding to (IV), the side chains of which then react with protein or nucleoprotein of chromosomal origin to produce, by cross-linkage with multipoint attachment, the observed aberrations of mitosis and resultant effects upon cell proliferation. The second possibility involves initial reaction of one of the epoxide groups of each (monomeric) molecule with the cell component, followed by self-condensation of the free epoxide groups in the units so formed into a final polyethenoxy structure similar to that envisaged in the first suggestion. Either sequence of events would lead to a cross-linkage effect of unusual stability between, for example, two polypeptide units, one on either side of, and each joined to, the polymeric unit at a number of points. This would seem to be a significant elaboration of the concept mentioned by Loveless and Revell (1949) as a mechanistic explanation of cytotoxic effects of the nitrogen mustards and a bis-epoxide, namely, the formation of simple monomolecular link bonds between sister chromatids.

Postulation of cross-linkage in any form may, however, not be necessary, since it is certain that the function of protein or nucleoprotein within the dividing cell would be seriously impaired by the presence even of a single collateral polymer unit formed from the cytotoxic agent and held in position by numerous regularly spaced covalent bonds.

While the capacity of an agent to act in one or other of these ways may be necessary as a first condition for a specific disturbance of cell proliferation, reference to those sections of the Tables relating to the bis-epoxides indicates that tumour-inhibitory activity, which here must be referred to the sensitivity of the cells of the neoplasm consequent upon their characteristic high rate of proliferation, is dependent upon other structural requirements also. Thus, while no significant action on tumour growth in the rat was observed in substances other than those containing two oxiran radicals, not every example of the latter type was inhibitory. Some of the apparent anomalies could no doubt be explained on the basis of steric interference and rate of reaction, others on metabolic grounds, but such data are not yet sufficient for rational discussion. It is of interest, however, to note, for instance, the effect of the substituent in the benzene ring of the aniline derivatives 236, 237, 238, and 239, of which only the first pair, carrying *para* ethoxyl and methoxyl substituents respectively, were active. Similarly, although union of the oxiran groups through wholly aliphatic ether systems provides examples with high tumour-inhibitory activity (230, 231, and 232), the interposition of an arylether group (233, 234, and 235) in each case inactivates. The steric impediments, especially to polymerization, consequent upon poly-substitution of the epoxide ring are possibly responsible for the inactivity of limonene dioxide (222) and the dioxides, 227, 228, and probably 223.

An additional factor, which would condition access of the agent, is the extent to which polymerization may occur immediately after test animals have been dosed, since it might be expected that cell permeability to partially or wholly polymerized structures would be markedly reduced. Experience during the preparation of the

chemical specimens showed how widely this property would vary, since, while certain compounds appeared to have almost indefinite stability in the absence of catalysts, others passed into polymeric form before biological assays could be made.

The remaining compounds in the Tables call for little comment. As pointed out earlier, many of the simpler substances were selected as actual or potential precursors of active *bis*-epoxides, but were uniformly devoid of tumour-inhibitory action. Others, such as some of the *mono*-epoxides, were expressly included for comparison with closely related bifunctional derivatives (compare, for example, the active compound 221 (II) with 196, 197, 198, and 199).

SUMMARY

1. Following upon the discovery of radiomimetic cytotoxic activity in a series of methylolamides a range of *bis*-epoxides with similar application as cross-linking agents in textile technology has been examined both for tumour-inhibitory and cytotoxic activity against the Walker carcinoma in rats. Some allied mono-epoxides have also been tested and the series of compounds investigated has been extended to include the parent olefins and dienes as possible epoxide precursors.

2. Marked activity in these tests has been shown only by certain members of the *bis*-epoxide class, in particular butadiene dioxide, vinyl *cyclo*hexene dioxide, certain N: N-diglycidyl derivatives of primary aromatic amines, and diglycidyl ethers of aliphatic diols. In every case, however, the margin between the lethal dose and that required to produce marked inhibition of tumour growth was too small to justify therapeutic application.

3. Intraperitoneal injection of vinyl *cyclo*hexene dioxide in arachis oil to stock albino rats in a dose of 25 mg. per 100 g. twice weekly for ten weeks gave at seven months a mixed cell sarcoma which has been maintained for over twenty successive subcutaneous transplantations in stock rats. A high yield of tumours has also been obtained in mice by skin painting. Evidence for carcinogenicity was not so strong with butadiene dioxide.

4. Butadiene dioxide produced mutations in a strain of *Penicillium*.

5. The theoretical implications of these findings are discussed. Evidence is adduced that the active agents may be linear polymers based on the repeating unit [-O-CH_2-CH-], in which the second epoxide ring in the residue R thus occurs along
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R
the polymer chain at a constant step-distance. The reaction of this second epoxide system with cellular components, for example, protein or nucleoprotein, would result in a multipoint attachment which, it is suggested, might account for the cytotoxic effects observed.

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