MUTAGENIC ACTIVITY OF COMPOUNDS RELATED TO MUSTARD GAS*

by

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Mustard gas ($ClCH_2CH_2SCH_2CH_2Cl$) and the bifunctional nitrogen mustards ($RN(CH_2CH_2Cl)_2$), originally of interest as vesicants, have recently received renewed attention as mutagens[1] and possible anticarcinogens[2]. The mechanism of action of these compounds has been the subject of several investigations[2]. DU VIGNEAUD *et al.*[3], in a study of the mode of action of mustard gas as a vesicant, attempted to simplify the problem in a chemical sense by employing the vesicant, monofunctional 2-chloroethyl-sulfides, $RSCH_2CH_2Cl$ (R = hydrocarbon). A similar technique has been employed in the present work to study the mutagenic activity of mustard compounds.

In preliminary experiments[4] the mutagenic activity of the monofunctional sulfur mustards and nitrogen mustards ($R_2NCH_2CH_2Cl$) was tested. Despite persistent suggestions in the literature that polyfunctional molecules are necessary for mutagenic and anticarcinogenic activity[5,6,7,8,9], the monofunctional compounds were found to be quite active not only in our experiments, but also in those of AUERBACH AND MOSER[10], JENSEN, KIRK, AND WESTERGAARD[11] and DICKEY**.

The finding that these simpler molecules are active mutagens renders inadequate the suggested mechanism[7] based on a two-fold reaction of the mustard compounds. In order to obtain further information which might reveal a clue as to the true mechanism of action, a study was made of the mutagenic activity of a number of compounds chemically related to mustard gas.

EXPERIMENTAL

The test organisms were mutant strains of *Neurospora crassa* obtained through the courtesy of Dr G. W. BEADLE and staff, Division of Biology, California Institute of Technology. The three strains employed were an adenineless, colonial double mutant (derived from 70007-38701), an inositolless, colonial double mutant (derived from 70007-37401) and an adenineless, inositolless, colonial triple mutant derived from crosses of the preceding strains. Essentially the experiments consisted of observing the effects of the various compounds on the rate of reversion of the strains to adenine-independent or inositol-independent forms, by the technique described by DICKEY *et al.*[12] The conidiospores were obtained from 2- to 4-day old cultures. Unless otherwise noted a suspension of $2.5 \cdot 10^6$ to $5 \cdot 10^6$ spores per milliliter was treated with the mustard-type compound in a solution 10% in acetone, 0.1 *M* in borate buffer (pH 8), and 0.25 *M* in sodium chloride. After 30 minutes

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exposure at room temperature the spores were collected by centrifugation, washed once with 10% aqueous acetone solution and once with water. With each experiment, one aliquot of spores served as a control; this batch was treated identically except that the mustard compound was omitted from the suspension medium. The addition of acetone enabled the testing of the water-insoluble compounds in homogeneous solution. Borate was selected as buffer because it was unlikely that it would itself react appreciably with the mustard compounds, and sodium chloride was added so that the chloride ion concentration would be essentially constant throughout the experiments.

The composition of the solution was found to affect the observed mutagenic activity of a compound. It has not been possible to interpret these variations. Perhaps indirect effects, such as altered mortality of the spores, are largely responsible. It was decided in general to test all compounds under the standard conditions specified above so that comparisons could be made. Even under standard conditions of treatment, however, the results with a given compound and a given mutant strain varied somewhat from time to time, due probably to variations in the organism itself. For this reason, comparisons of activities of compounds tested at different times are only approximate and the data presented should be considered only roughly quantitative.

Certain compounds tested were obtained from the following sources: ethyl 2-chloroethyl sulfide, *tert.*-butyl 2-chloroethyl sulfide, ethyl *bis* (2-chloroethyl) amine hydrochloride, and *tris* (2-chloroethyl) amine hydrochloride from Army Chemical Center, Maryland; N, N-diethyl 2-chloroethyl amine hydrochloride and N, N-dimethyl 2-chloroethyl amine hydrochloride from Michigan Chemical Co. St. Louis, Mich.; benzyl ethyl 2-chloroethyl amine hydrochloride and dibenzyl 2-chloroethyl amine hydrochloride from Eli Lilly and Co. The samples of monofunctional nitrogen mustards were found to contain toxic impurities which were removed only by repeated recrystallization from an alcohol ether mixture.

Benzyl 2-chloroethyl sulfide, phenyl 2-chloroethyl sulfide, and *n*-butyl 2-chloroethyl sulfide were prepared by the general procedure of PATTERSON AND DU VIGNEAUD[13]. The yields of *n*-butyl 2-chloroethyl sulfide and phenyl 2-chloroethyl sulfide were comparable to the yield of the benzyl analogue previously obtained[13]. *n*-Butyl 2-chloroethyl sulfide was repeatedly recrystallized at low temperature without significant change in toxicity or mutagenic activity. The following additional compounds were prepared and recrystallized according to the procedures described in the literature: mustard gas sulfone and sulfoxide[14], phenyl 2-chloroethyl sulfone and phenyl vinyl sulfone[15], phenyl *bis* (2-chloroethyl)amine[16], mustard gas[17,18], and the sulfonium compound, (thiodiethylene) *bis* (*bis* (2-hydroxyethyl) sulfonium chloride)[19].

RESULTS AND DISCUSSION

In Table I are listed the compounds tested for mutagenic activity toward the adenineless, colonial double mutant. In the case of mustard gas and the monofunctional compound *n*-butyl 2-chloroethyl sulfide, results are reported for experiments at several concentration levels to indicate the change in mutagenic activity with concentration. In other cases, only the average and range of results of all experiments on each compound are given. The "induced mutation rate" as defined by DICKEY *et al.*[12] is the number of mutants observed divided by the number of spores plated, corrected for the spontaneous mutation rate of a sample of untreated spores from the same batch. The genetic nature of the reversions has not been studied in this paper. However, KOLMARK AND WESTERGAARD[20] have shown genetically that the reversion of the adenineless, colonial mutant to the adenine-independent colonial mutant induced by methyl *bis* (2-chloroethyl) amine is in all probability a true back mutation.

As shown in Table I, all of the 2-chloroethyl sulfides are highly active, the monofunctional compounds being at least as active as mustard gas itself, with the possible exception of phenyl 2-chloroethyl sulfide.

In confirmation of previous reports, the bifunctional and trifunctional 2-chloroethyl amines show distinct though moderate mutation rates. Phenyl *bis* (2-chloroethyl) amine, on the other hand exhibits questionable activity. In the case of the monofunctional compounds, there are wide variations in activity. Diethyl 2-chloroethyl amine and dimethyl 2-chloroethyl amine are tolerated by the spores at quite high concentrations

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TABLE I

EFFECTS OF MUSTARD-TYPE COMPOUNDS ON ADENINELESS COLONIAL MUTANT OF *Neurospora crassa*

Compound	No. of Expts	Concentration*	Mortality**	Induced Mutation** Rate · 10^7
		M	per cent	
n-$C_4H_9SCH_2CH_2Cl$	3	0.0008	75 (60–85)	159 (71–227)
n-$C_4H_9SCH_2CH_2Cl$	5	0.0006	35 (0–50)	154 (81–246)
n-$C_4H_9SCH_2CH_2Cl$	18	0.0004	25 (0–60)	98 (26–162)
n-$C_4H_9SCH_2CH_2Cl$	5	0.0002	10 (0–20)	11 (3–18)
n-$C_4H_9SCH_2CH_2Cl$	2	0.00005–0.0001	0	0.5 (0.5–0.5)
$ClCH_2CH_2SCH_2CH_2Cl$	2	0.0004	93 (90–95)	18 (13–22)
$ClCH_2CH_2SCH_2CH_2Cl$	1	0.0003	65	38
$ClCH_2CH_2SCH_2CH_2Cl$	5	0.0002	35 (0–75)	69 (44–100)
$C_2H_5SCH_2CH_2Cl$	4	0.0002–0.0004	60 (35–70)	171 (22–345)
$C_6H_5SCH_2CH_2Cl$	5	0.0004–0.0006	40 (20–60)	27 (4–49)
$C_6H_5CH_2SCH_2CH_2Cl$	5	0.0004–0.0008	60 (30–90)	59 (9–113)
tert.-$C_4H_9SCH_2CH_2Cl$	2	0.0004–0.0008	35 (20–50)	162 (131–193)
$N(CH_2CH_2Cl)_3$	4	0.00005–0.0002	55 (0–90)	1.9 (0–3.5)
$C_2H_5N(CH_2CH_2Cl)_2$	4	0.0004–0.001	50 (30–80)	3.2 (1.4–6.7)
$CH_3N(CH_2CH_2Cl)_2$	4	0.0002–0.001	40 (25–75)	1.9 (1.1–3.6)
$(CH_3CH_2)_2NCH_2CH_2Cl$	6	0.004–0.02	40 (0–100)	12.4 (6–18)
$(CH_3CH_2)_2NCH_2CH_2Cl$	2	0.02–0.04	55 (25–85)	22.5 (13–32)***
$(CH_3)_2NCH_2CH_2Cl$	2	0.03–0.05	30 (10–50)	35.5 (23–48)
$(CH_3)_2NCH_2CH_2Cl$	4	0.015–0.08	15 (0–35)	54 (27–100)***
$C_6H_5N(CH_2CH_2Cl)_2$	7	0.00005–0.0002	60 (0–100)	0.4 (0–1.0)
$(C_6H_5CH_2)_2NCH_2CH_2Cl$	7	0.0001–0.005	70 (20–94)	0.2 (0–0.8)
$C_6H_5CH_2N(CH_2CH_3)CH_2CH_2Cl$	12	0.0005–0.002	54 (0–95)	1.6 (0–5.4)
none	80	—	—	0.2 (0–1.5) §

* When the results of experiments at different levels are averaged, the range of concentrations is indicated.

** Each value represents the average of all experiments. The figures in parentheses indicate the range of values obtained.

*** In these experiments the acetone was omitted from the standard test solution.

§ Spontaneous mutation rate. Two experiments showed a high spontaneous rate; both were discarded.

TABLE II

EFFECTS OF MUSTARD-TYPE COMPOUNDS ON INOSITOLLESS, COLONIAL MUTANT OF *Neurospora crassa*

Compound	No. of Expts	Concentration	Mortality	Induced Mutation Rate · 10^7
		M	per cent	
n-$C_4H_9SCH_2CH_2Cl$	12	0.0001–0.001*	65** (5–85)*	0.5** (0–1.7)*
$ClCH_2CH_2SCH_2CH_2Cl$	4	0.0001–0.0002	65 (45–100)	0.7 (0–1.0)
$C_2H_5SCH_2CH_2Cl$	4	0.0001–0.0004	45 (0–80)	0.5 (0–1.0)
$C_2H_5N(CH_2CH_2Cl)_2$	9	0.0002–0.002	30 (0–50)	2.2 (0–4.0)
$CH_3N(CH_2CH_2Cl)_2$	2	0.0002–0.001	40 (30–50)	1.8 (1.4–2.1)
$(CH_3CH_2)_2NCH_2CH_2Cl$	5	0.0075–0.036	50 (0–80)	0.3 (0–0.4)
$(CH_3)_2NCH_2CH_2Cl$	3	0.003–0.06	40 (25–50)	0.8 (0.5–1.3)
$C_6H_5N(CH_2CH_2Cl)_2$	3	0.0001–0.0002	65 (40–100)	0.3 (0–1.3)
None	21	—	—	0.08 (0–0.3)***

* Range of values. ** Average. *** Spontaneous mutation rate.

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and at these levels are highly active. Dibenzyl 2-chloroethyl amine could be tested only at low concentrations and showed little, if any, mutagenic activity, while N-benzyl, N-ethyl 2-chloroethyl amine was active. The latter compounds were tested because of interest in them as adrenergic blocking agents[21].

In Table II are listed the compounds tested for activity toward the inositolless, colonial double mutant. The induced mutation rates are in general much lower with this mutant than with the previous one. However, the spontaneous mutation rate is also very low. It was zero in 26 of 28 experiments and $0.3 \cdot 10^{-7}$ in the remaining two experiments. It therefore seems safe to conclude that both the monofunctional and bifunctional 2-chloroethyl amines and sulfides are active for the inositolless mutant.

A more elegant comparison of the effects of a given compound on the two mutant genes was provided in tests on the adenineless, inositolless, colonial triple mutant. This type of test, involving two mutant genes in the same organism, was used previously by GILES AND LEDERBERG[22]. Aliquots of the same batch of treated spores can be tested for reversions of the two types. The results obtained in this way are shown in Table III.

TABLE III

EFFECTS OF MUSTARD-TYPE COMPOUNDS ON ADENINELESS, INOSITOLLESS COLONIAL MUTANT OF *Neurospora crassa*

Compound	No. of Expts	Concentration	Mortality	Induced Mutation Rate $\cdot 10^7$	
				Inositolless	Adenineless
		M	per cent		
n-$C_4H_9SCH_2CH_2Cl$	6	0.0002–0.0005*	50** (35–70)*	0.7** (0–1.6)*	71** (30–124)*
$ClCH_2CH_2SCH_2CH_2Cl$	10	0.00005–0.0003	70 (20–100)	0.9 (0–3.1)	42 (9–118)
$CH_3N(CHCH_2Cl)_2$	5	0.0002–0.001	45 (40–50)	1.2 (0.8–1.5)	2.5 (0–4.6)
$(CH_3)_2NCH_2CH_2Cl$	2	0.02–0.06	30 (20–40)	0.6 (0–1.1)	29 (19–39)
None	10	—	—	0.04 (0–0.4)***	0.4 (0–1.1)***

* Range of values.
** Average.
*** Spontaneous mutation rate. Two experiments showing a high spontaneous mutation rate were discarded.

TABLE IV

EFFECTS OF SULFONES ON ADENINELESS COLONIAL MUTANT OF *Neurospora crassa*

Compound	No. of Expts	Concentration	Medium	Mortality	Induced Mutation Rate $\cdot 10^7$
		M		per cent	
$C_6H_5SO_2CH_2CH_2Cl$	6	0.005–0.02*	0.1 *M* borate buffer	10–95*	0.1** (0–0.5)*
$C_6H_5SO_2CH=CH_2$	4	0.01–0.02	0.1 *M* borate buffer	55–75	0.1 (0–0.4)
$ClCH_2CH_2SO_2CH_2CH_2Cl$	7	0.0002–0.002	0.1 *M* borate buffer	0–80	0
$C_6H_5SO_2CH=CH_2$	3	0.01–0.025	Water	75–100	0
$ClCH_2CH_2SO_2CH_2CH_2Cl$	7	0.001–0.008	Water***	0–85	0

* Range of values.
** Average
*** The solution was kept at neutrality by the addition of 0.05 *N* NaOH

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It is clear that the pronounced difference in mutation rate between the two mutants which was observed in the earlier experiments with mustard gas and *n*-butyl 2-chloroethyl sulfide is fully substantiated by the results obtained with the triple mutant.

In view of the mutagenic activity of a variety of 2-chloroethyl sulfides, all of which are vesicants, it seemed of interest to test the activity of some vesicant sulfones. In Table IV are listed the compounds tested and conditions employed. No mutagenic activity was detected with any of the sulfones.

In the course of our study, we had occasion to test the mutagenic activity of solutions of the mustard-type compounds which had been allowed to stand either buffered or unbuffered for long periods of time. WYSS *et al.*[23] have reported that nutrient broth treated with *tris* (2-chloroethyl) amine is mutagenic, and have inferred that a reaction with some component of the broth is responsible. In the present experiments, the mustard compounds were allowed to react with water for a given length of time; after removal of the unreacted compound the aqueous solution was tested for mutagenic activity. The results, shown in Table V, demonstrate that mutagenic substances remain in the "aged" solutions of mustard gas or *n*-butyl 2-chloroethyl sulfide. The hydrolysis product of mustard gas, thiodiglycol, is inactive. The other probable reaction products are sulfonium compounds. The most readily available sulfonium salt, $[(HOCH_2CH_2)_2SCH_2CH_2SCH_2CH_2S(CH_2CH_2OH)_2]\,Cl_2$, was therefore prepared and tested. As shown in Table VI, the compound is highly mutagenic. Since the molecule reacts rather slowly[19], it is not surprising that it has to be added at relatively high concentration levels. The finding of mutagenic activity of sulfonium compounds provides a reasonable explanation of the activity of "aged" solutions of the sulfur mustards.

The ready availability of a water soluble, crystalline sulfur mustard derivative of considerable mutagenic activity may be of use in future studies.

TABLE V

EFFECT OF AGED SOLUTIONS ON ADENINELESS, COLONIAL MUTANT OF *Neurospora crassa*

The mustard compound, in the concentrations indicated below, was added to distilled water. The resulting mixture was shaken frequently and kept neutral by the addition of 0.1 *N* NaOH. After the specified length of time the solution was extracted repeatedly with petroleum ether to remove any traces of the unreacted compound, and aliquots of the aqueous solution tested.

Compound	No. of Expts	Initial Concentration	Period of Ageing	Mortality	Induced Mutation Rate · 10^7
		M	hours	per cent	
n-$C_4H_9SCH_2CH_2Cl$	2	0.015	3½	80*	23* (13–33)**
n-$C_4H_9SCH_2CH_2Cl$	2	0.01	3½	30	2.1 (2–2.2)
n-$C_4H_9SCH_2CH_2Cl$	2	0.015	0	0	0
$ClCH_2CH_2SCH_2CH_2Cl$	1	0.03	3½	0	17
$ClCH_2CH_2SCH_2CH_2Cl$	2	0.02	3½	30	5 (2.8–7.2)
$ClCH_2CH_2SCH_2CH_2Cl$	1	0.01	3½	10	0.3
$ClCH_2CH_2SCH_2CH_2Cl$	2	0.03	0	0	0

* Average of values.
** Range.

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TABLE VI

EFFECTS OF SULFONIUM COMPOUND ON MUTANTS OF *Neurospora crassa*

Group No.	Mutant	No. of Expts	Concentration	Mortality	Induced Mutation Rate · 10^7
			M	per cent	
1***	Adenineless	3	0.03–0.06*	20** (20–20)*	48** (40–64)*
2	Adenineless	3	0.03–0.06	25 (10–30)	1.0 (0–2.1)
3***	Inositolless	3	0.03–0.08	30 (0–70)	2.9 (1.3–3.1)
4	Inositolless	2	0.03–0.06	5 (0–10)	0.4 (0.3–0.6)

* Range of Values
** Average
*** In these experiments, sodium chloride was omitted from the standard medium.

SUMMARY

A study was made of the mutagenic activity of a number of compounds chemically related to mustard gas. Biochemical mutants of *Neurospora crassa* were tested for reversion to nutritionally independent forms. Under the conditions of the tests, monofuctional compounds of the type $RSCH_2CH_2Cl$ showed activity comparable to that of mustard gas. The aliphatic monofunctional nitrogen mustards were found to be more active toward the adenineless mutant and less active toward the inositolless mutant than the corresponding bifunctional compounds. Nitrogen mustards with aromatic substituents exhibited low to insignificant activity.

With the few compounds tested, similar results were obtained with the inositolless, adenineless mutant.

The vesicant 2-chloroethyl sulfones and vinyl sulfones were found to be inactive under the conditions of the experiments.

The sulfonium compound (thiodiethylene)*bis*(*bis*(2-hydroxyethyl) sulfonium chloride) proved to be an effective mutagen. The mutagenic activity of sulfonium compounds may provide an explanation for the mutagenic activity of "aged" solutions of mustard gas and related compounds.

RÉSUMÉ

Nous avons étudié l'activité mutagène d'un certain nombre de composés chimiques reliés au gas moutarde. Des mutants biochimiques de *Neurospora crassa* ont été examinés en vue de leur retour à des formes indépendantes de la nutrition. Dans les conditions de ces expériences, des composés monofonctionels du type $RSCH_2CH_2Cl$ ont montré une activité comparable à celle du gas moutarde. Nous avons trouvé que les composés monofonctionels aliphatiques ressemblant au gas moutarde, mais contenant de l'azote au l'eu du soufre, ($R_2NCH_2CH_2Cl$) étaient plus actifs vis à vis du mutant qui dépend de la présence d'adénine et moins actifs vis à vis du mutant qui dépend de la présence d'inosite que les composés bifonctionels correspondants. Les composés semblables à substituants aromatiques montrèrent une activité faible ou insignifiante.

Avec le peu de composés étudiés nous avons obtenu des résultats semblables pour le mutant qui dépend de la présence d'inosite et d'adénine.

Nous avons trouvé que les 2-chloroéthylsulfones et les vinylsulfones vésicantes sont inactives dans les conditions de nos expériences.

Le composé sulfonium $[(HOCH_2CH_2)_2SCH_2CH_2SCH_2CH_2S(CH_2CH_2OH)_2]Cl_2$ est un mutagène actif. L'activité mutagène des composés sulfonium pourrait servir d'explication à l'activité mutagène des solutions "vieillies" de gas moutarde et de composés semblables.

ZUSAMMENFASSUNG

Die mutagene Aktivität einer Anzahl von Verbindungen, welche chemisch dem Senfgas nahe stehen, wurde untersucht. Biochemische Mutanten von *Neurospora crassa* wurden auf Rückkehr zu nahrungs-unabhängigen Formen untersucht. Unter den Versuchsbedingungen zeigten monofunktionelle Verbindungen vom Typus $RSCH_2CH_2Cl$ eine, dem Senfgas vergleichbare Aktivität. Die

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senfgas-ähnlichen aliphatischen monofunktionellen Verbindungen, welche statt Schwefel Stickstoff enthalten ($R_2NCH_2CH_2Cl$), zeigten sich dem adenin-abhängigen Mutanten gegenüber aktiver, und dem inosit-abhängigen Mutanten gegenüber weniger aktiv als die entsprechenden bifunktionellen Verbindungen. Ähnliche Verbindungen, welche aromatische Substituenten enthalten, wiesen eine geringe oder unbedeutende Aktivität auf.

Die wenigen Verbindungen, welche wir auf den adenin- und inositabhängigen Mutant einwirken liessen, ergaben ähnliche Resultate.

Die blasenziehenden 2-Chloräthylsulfone und Vinylsulfone erwiesen sich, unter den verwendeten Versuchsbedingungen, als inaktiv.

Die Sulfoniumverbindung $[(HOCH_2CH_2)_2SCH_2CH_2SCH_2CH_2S(CH_2CH_2OH)_2]Cl_2$ war ein aktives Mutagen. Diese Aktivität der Sulfoniumverbindungen könnte die Aktivität der "gealterten" Lösungen von Senfgas und verwandten Verbindungen erklären.

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