Biochemical Pharmacology, 1960, Vol. 5, pp. 206–218. Pergamon Press Ltd., Printed in Great Britain.

MUTAGENIC EFFECTS OF NITROGEN MUSTARD DERIVATIVES OF AZO-BENZENE COMPOUNDS IN *DROSOPHILA MELANOGASTER*

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(*Received* 8 *July* 1960)

Abstract—The azo-mustard 4-di-2″-chloroethylaminoazobenzene-2′-carboxylic acid was found to be mutagenic in *Drosophila melanogaster* males. The mutagenic effect was found only in pre-meiotic germ cell stages and, of these, late spermatids were the most sensitive. Sterility or egg unhatchability was found to be due to aspermia. Within the germ cell stage sterility was found to be correlated to the mutagenic effect suggesting that the differential response of germ cell stages to the mutagenic action of the mustard was not a genetic phenomenon. The induction of sex-linked recessive lethal mutation was found to be linearly related to dose.

The rate of excretion of the azo-mustard was determined by a spectrophotometric method. At least 90 per cent of the injected material appeared to be excreted in an unreduced form, in contrast to the expected mode of action of the compound which required prior reduction of the azo-bond.

A further member of the azo-mustard series, its non-mustard homologue and a possible reduction product, were also tested for mutagenic action. Only the former was shown to be mutagenic.

These results are discussed in relation to the hypothesis that reduction of the azo-linkage is a necessary prerequisite for the mutagenic activity of the azo-mustards.

INTRODUCTION

ATTEMPTS to discover chemical mutagens extend back to shortly after the re-discovery of Mendel's Laws. These early studies were of little value[1] and no conclusively positive results were obtained until the discovery, in *Drosophila*,[2] of the mutagenic effects of mustard gas ($S(CH_2CH_2Cl)_2$). Further discoveries of other chemical mutagens arose independently.[3, 4] The field rapidly expanded and a wide range of chemicals, from inorganic salts to complex organic molecules like the alkaloids and purine derivatives, is now known to be mutagenic in a wide diversity of organisms.

The discovery of the ability of certain chemicals to produce mutations led, initially, to the hope that information on the structure of the gene might be obtained by an analysis, akin to formal chemistry, of the reaction of mutagens with possible gene structures.[5] But while the chemistry of these agents has been widely studied, particularly with respect to their pharmacological properties,[6] there has as yet been no link-up

* This work represents part of the studies presented as a Thesis for the degree of Ph.D. in the University of London and was done under tenure of a Gordon Jacob Fellowship of the Royal Marsden Hospital.

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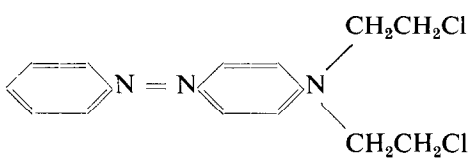
with the recent intensive work on deoxyribosenucleic acid, now widely regarded as the genic material. Furthermore, the ever increasing diversity of chemicals possessing mutagenic properties has raised the problem of whether or not their action can be interpreted as a specific and direct chemical reaction with the chromosomal material.

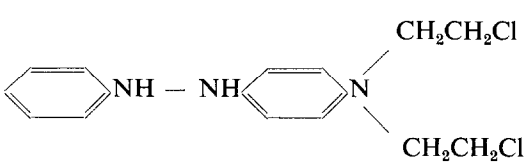
The most common method of approach for the resolution of the above problem has been to compare the effects of different agents. While many differences of effect have been demonstrated, in no case has it been established that the cause of the difference lies in the primary mutagenic action of the various agents. In a recent review, Auerbach[7] has described some secondary effects which might give rise to the observed differences between such mutagenic agents.

Since the early days of radiation mutagenesis, it has been known that different cell types vary in their sensitivity to the mutagenic action of radiation. This principle was also found to hold in chemical mutagenesis and, in addition, it was found that the pattern of genetic sensitivity depended upon whatever chemical agent was used and, in some cases, how it was used. Such variations were principally detected in the various germ cell stages present in the testis of *Drosophila*. The advantage of the *Drosophila* testis was that it contained all stages of spermatogenesis and that the sperm used in successive matings came from successively earlier stages relative to the time of treatment. A sequence of broods could be obtained by mating treated males to fresh samples of virgin females at given intervals and the mutation frequencies observed in these broods would then be an indication of the sensitivity of certain germ cell stages.

The present study was undertaken to examine the brood sensitivity pattern produced by nitrogen mustard derivatives of azo-benzene. These compounds, derived from 4-bis-β-chlorocthylaminoazobenzene (I), were synthesized in the expectation that they would be chemically inert,[8] the chlorine atoms of the mustard group having a low reactivity as a result of the powerful electrophilic properties of the azo group. It was thought probable, however, that enzymic reduction of the azo-linkage, if it occurred, would produce the highly reactive hydrazo (II) and amino compounds (III).



(I)



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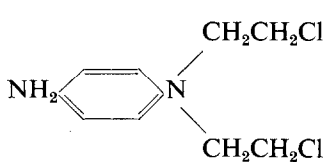


(III)

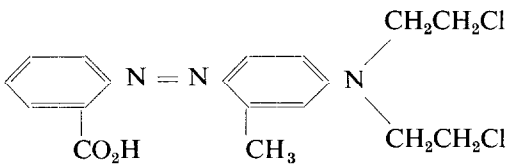
This supposition was supported by the correlation between the cytostatic effects towards the Walker rat carcinoma and the reducibility *in vitro* of compounds of this series.[9]

If, for mutagenesis, these azo-mustards require enzymic reduction, then this might have some influence on their effectiveness within the germ cell stages found in the *Drosophila* testis.

The experiments described below were designed to determine the dose effect in addition to the germ cell stage sensitivity pattern. Data on dose effects in chemical mutagenesis studies are very scanty, due mainly to the difficulty of administering a given dose of mutagen. With the technique reported below this difficulty has been partly overcome.

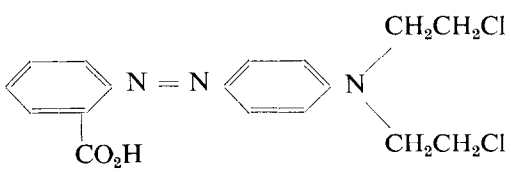
MATERIALS AND METHODS

The compound examined most extensively, and also used in the study of the dose effect, was 4-di-2″-chloroethylamino-2-methylazobenzene-2′-carboxylic acid (IV).



(IV)

Other compounds tested were 4-di-2″-chloroethylaminoazobenzene-2′-carboxylic acid (V), its non-mustard homologue 4-diethylaminoazobenzene-2′-carboxylic acid (VI) and a possible reduction product of IV, 2-methyl-4-N : N-di-2′-chloroethyl-*p*-phenylenediamine (VII).



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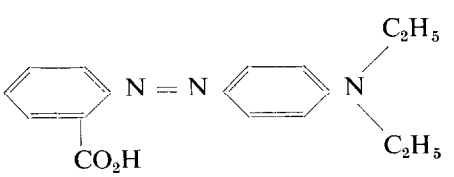
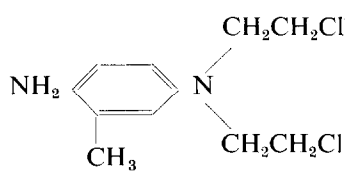


(VI)



(VII)

Compounds were injected into the abdomens of adult male *Drosophila melanogaster* of the Oregon-K strain 48±4 hr after emergence. Compounds (IV), (V) and (VI) were injected as solutions of their sodium salts in 0·4% saline. Compound (VII), which is highly unstable in aqueous solution, was dissolved and injected in arachis oil.

A micrometer syringe ("Agla" Burroughs Wellcome & Co.), equipped with glass needles drawn to a short fine point, was used for the injections. These were done under a stereoscopic microscope at a magnification of about × 20. The syringe was clamped to a stand such that the point of the needle was about ½ mm from the microscope stage. The etherized males, with wing-tips removed, were held with fine forceps and the needle was inserted mid-dorsally between the last two sclerites. The syringe mounting was sufficiently elastic to enable the required movement to be made with

ease. As a check, groups of males were weighed before and after injection. Using the above method, a group of about twenty males could be treated during a single etherization.

In a series of twenty-seven sets of injections, using the above technique, the average weight of aqueous solution injected per fly per unit on the micrometer scale was 0·19 mg; one unit on the scale representing 0·2 μl. Over the twenty-seven observations the correlation between weight of solution injected and the volume as measured on the micrometer scale was 0·98; volumes from 0·1 to 0·6 μl were injected.

The Muller-5 technique[10] was used for the detection of sex-linked mutations and the brood sensitivity pattern was obtained by mating each treated male to two fresh virgin females at 3-day intervals. F_2 cultures were scored initially by observation through the glass wall of the vial using a magnification of $\times$ 20. Cultures suspected of containing a mutation were examined carefully after etherization. This examination was repeated until no further eclosion occurred. The mutations were classed as lethal if no non-Muller-5-males eclosed, or, as visible in the presence of phenotypically distinguishable flies. Mutations were confirmed in an F_3.

Unhatchability tests were made on eggs laid by Oregon-K females inseminated with sperm from treated males, the egg-laying periods of 24 hr being mid-way through the usual brood interval. Unhatched eggs were examined for the presence of dominant lethals after staining by a modified Feulgen technique.[11]

RESULTS

The azo-mustard (IV), like other chemical mutagens and X-rays, produced partial sterility in the treated males, the degree of sterility varying in different broods. Table 1 summarizes the results on sterility brood patterns.

TABLE 1. FERTILITY OF MALES INJECTED WITH THE AZO-MUSTARD (IV) EXPRESSED AS THE AVERAGE NUMBER OF OFFSPRING PER F_0 MALE IN EACH BROOD

Expt.	Brood							Dose mg $\times$ 10^{-5}/fly
	I	II	III	IV	V	VI	VII	
P_ϵ	4·6	3·1	3·0	1·4	9·2	6·4	6·8	32·2
	12·8	11·2	25·0	9·8	40·8	31·8	35·4	16·1
	61·6	53·8	51·0	31·8	29·0	47·4		0·0
P_ζ	38·9	4·1	32·0	50·7				7·8
	26·6	2·9	46·7	36·8				18·7
	24·4	1·8	16·9	21·0				22·6
P_η	53·6	97·0	102·1	105·1	93·6			0·0
P_θ	39·0	27·4	21·4	68·1	33·1	62·7		16·5
	5·4	3·2	0·8	16·7	15·3	69·4		31·4

The sterilizing action of the azo-mustard (IV) was most marked in the first three broods and, of these, brood II was frequently the most sterile. Broods IV and V were almost always highly fertile and in later broods fertility was so high that the majority of F_1 cultures were discarded in order to score the remainder thoroughly.

Table 2 summarizes the data on egg unhatchability. A cytological examination of control unhatched eggs from a different series of experiments showed that 63 per cent of these were fertile when the unhatched frequency was 23 per cent. If it can be assumed

that 63 per cent of the control unhatched eggs were fertile in the present experiment, then the calculated spontaneous contribution to the fertile and non-fertile eggs in the experimental series was twenty-four and fourteen, respectively. Subtracting these estimates from the observed numbers of fertile and non-fertile eggs indicates that in 371 induced unhatched eggs, only twenty-one (5·7 per cent) were fertile and, therefore,

TABLE 2. EGG UNHATCHABILITY INDUCED BY THE AZO-MUSTARD (IV)
(Data from broods I–III. Dose $31{\cdot}6 \times 10^{-5}$ mg/fly.)

	Experimental			Control
		Cytological examination		
		Fertile	Non-Fertile	
No. eggs laid	7087	—	—	4569
No. unhatched	6197	45	364	377
% unhatched	87·4			8·3
Ca. induced		21	350	

TABLE 3. BROOD MUTATION FREQUENCIES FOLLOWING INJECTION OF THE AZO-MUSTARD (IV)

Expt.		Brood								Dose mg × 10^{-5} fly
		I	II	III	IV	V	VI	VII	VIII	
P_λ	No. tests	327	507	520	345	336				0·0
	No. lethals	1	1	—	—	1				
	%	0·31	0·19	0·0	0·0	0·3				
P_μ	No. tests	5042	3376	5336						3·5
	No. lethals	92	111	87						
	%	1·82	3·26	1·63						
P_ζ	No. tests	930	97	749	449					7·8
	No. lethals	26	2	17	8					
	%	2·8	2·1	2·3	1·8					
P_ϵ	No. tests	501	134	85	62	159	145	102	30	16·1
	No. lethals	27	9	2	—	—	—	—	—	
	%	5·4	6·7	2·4	0·0	0·0	0·0	0·0	0·0	
P_θ	No. tests	656	415	246	589	466	502			16·5
	No. lethals	29	18	10	5	1	—			
	%	4·4	4·3	4·1	0·8	0·2	0·0			
P_λ	No. tests	969	879	661	816	344	361			18·5
	No. lethals	37	38	21	13	—	—			
	%	3·8	4·3	3·2	1·6	0·0	0·0			
P_ζ	No. tests	761	75	845	342					18·7
	No. lethals	30	4	21	5					
	%	4·0	5·3	2·5	1·4					

true dominant lethals. It is obvious that dominant lethality contributed little to the total induced egg unhatchability.

Sex-linked recessive mutation

The data on the induction of sex-linked recessive lethals by IV are summarized in Tables 3 and 4. Irregularities in the number of tests per brood in each experiment

were due to lack of F_1 offspring, conditioned by sterility in the earlier broods, and F_0 male mortality in the later broods. In the majority of experiments the highest mutation frequency was found in brood II, broods I and III were also highly sensitive to the treatment, brood IV was very much less so and in later broods there was no evidence of induced mutation. In all but the experiment with the lowest dose, the differences between mutation frequencies in broods I and II were not statistically significant. The high degree of sterility in brood II made it impossible to put up sufficient tests to obtain an efficient estimate of the mutation frequency. In two experiments using doses of 1·65 and 1·85 mg $\times$ 10^{-4}/fly (Table 3) where large numbers of tests were put up in brood II, mutation frequencies in the first three broods showed little variation. It seems plausible that in these experiments some overlapping occurred so that high sterility and mutagenic sensitivity were shared among the broods.

TABLE 4. BROOD MUTATION FREQUENCIES FOLLOWING INJECTION OF THE AZO-MUSTARD (IV)—(*contd.*)

Expt.		Brood I	II	III	IV	V	VI	VII	VIII	Dose mg $\times$ 10^{-5}/fly
$P\zeta$	No. tests	923	51	340	342					22·6
	No. lethals	45	4	15	2					
	%	4·9	7·9	4·4	0·6					
$P\varsigma$	No. tests	169	104	410	388					31·2
	No. lethals	16	7	17	4					
	%	9·5	6·7	4·1	1·1					
$P\theta$	No. tests	111	3	—	190	231	277			31·4
	No. lethals	12	—	—	—	2	—			
	%	10·8	—	—	0·0	0·9	0·0			
$P\eta$	No. tests	88	40	—	320	106	43			31·6
	No. lethals	4	2	—	4	1	—			
	%	4·6	5·0	—	1·3	0·9	0·0			
$P\epsilon$	No. tests	174	81	19	82	146	231	48	5	
	No. lethals	11	8	—	—	1	—	—	—	32·2
	%	6·3	9·9	—	0·0	0·7	0·0	0·0	—	
$P\zeta$	No. tests	20	—	35	255					46·2
	No. lethals	—	—	—	3					
	%	—	—	—	1·2					

The experiment at the lowest dose (expt. $P\mu$, Table 3) was designed to clarify the situation with respect to mutagenic sensitivity in the first three broods. The dose of 0·35 mg $\times$ 10^{-4}/fly was chosen in the hope that there would be little sterility in these particular broods. The choice proved successful and there was no lack of offspring in any of the broods. It was decided to make a lower number of tests in brood II on the grounds that if the mutation frequency was highest here, then a greater number of tests in broods I and III would improve the efficiency of the experiment. The total number of tests set up was the maximum that could be handled. In brood II the mutation frequency was almost double that in broods I and III. Comparing the three broods for homogeneity gave a χ^2 of 30 for 2 d.f. ($P < 0{\cdot}0001$). This highly significant result demonstrates conclusively that the germ cells contributing predominantly to brood II were the most sensitive to the mutagenic action of the azo-mustard IV.

The effect of dose

The data on dose effect for sex-linked recessive lethals can be seen in Tables 3 and 4 where the experiments have been listed in order of increasing dose of mutagen. In Fig. 1 the results are represented graphically, the weighted regressions being fitted by maximum likelihood such that $y = a + bx$, where y is the frequency of lethals per cent, and x is the dose of mutagen administered, in mg $\times 10^{-5}$. The parameters, their

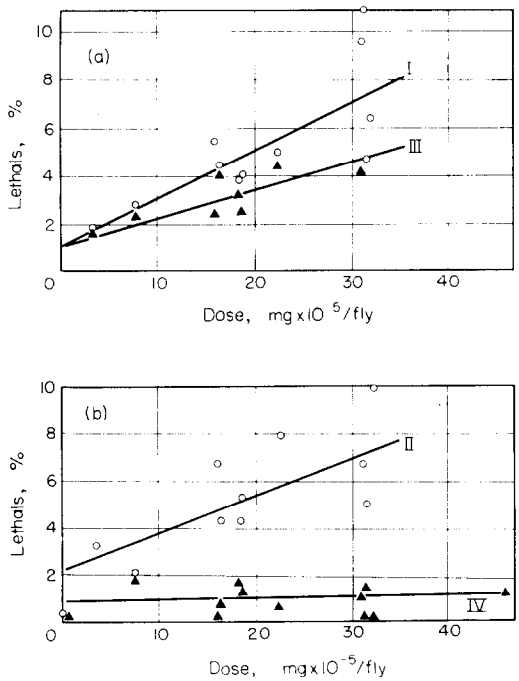


FIG. 1. Dose effect for sex-linked recessive lethals induced by the azo-mustard (IV). Straight lines fitted according to the parameters listed in Table 5. A. Brood I ——— ○. B. Brood II ——— ○. Brood III ——— ▲. Brood IV ——— ▲.

standard errors and a χ^2 for goodness of fit, are included in Table 5. The regression coefficients were highly significant except in brood IV. In this brood the mutation rate was low and the results were not sufficient to supply a precise estimate. The χ^2 for the sum total of broods I–IV showed a highly significant deviation from linearity. This heterogeneity is to be expected because of the variations between broods, with respect to induced mutation rates and sterility. Similarly, in brood II there was a high χ^2 which was just significant at the 5 per cent level. This could be a chance effect but is more probably due to the fact that selective toxicity towards the germ cells was most evident in this brood. In the remaining broods the χ^2 values showed no deviation from the hypothesis of a linear effect. In no instance could a plausible case for a curved regression be put forward. The data are consistent, therefore, with the hypothesis that the mutation frequency increases linearly with dose.

During the above experiments it was noted that the orange-coloured azo-benzene compound could be observed, within 30 min of injection, in the faecal pellets of

treated males. The amount of the azo-compound excreted was, therefore, determined spectrophotometrically. The optical density of a solution of the excreted azo-compound was measured in a Unicam S.P. 500 spectrophotometer, $\lambda = 530$ mμ, using as a blank a similar solution from saline-injected controls. The amount excreted was determined by interpolation, using a graph relating optical density to concentration

TABLE 5. STATISTICAL ANALYSIS OF THE DATA ON DOSE EFFECT WHERE $y = a + bx$; y = FREQUENCY OF LETHALS IN %, x = DOSE IN MG $\times 10^{-5}$/FLY

Brood	a	s.e.	b	s.e.	χ^2	d.f.
I	1·065	0·212	0·198	0·021	12·98	10
II	2·233	0·460	0·157	0·047	14·76*	7
III	1·078	0·190	0·116	0·021	10·10	7
IV	0·906	0·302	0·004	0·014	5·24	5
I–IV	1·338	0·236	0·164	0·025	34·76***	11

as measured with the solution originally made up for the injections. The results of three such experiments (Table 6) showed not only that excretion was rapid, but also that between 90 and 100 per cent of the injected azo-mustard was excreted as an azo-compound. These results were surprising in that the expected mode of action of the azo-mustards involved reduction.

TABLE 6. THREE SEPARATE MEASUREMENTS OF THE AMOUNTS OF INJECTED MUSTARD (IV) EXCRETED BY *Drosophila* MALES

No. males injected	44	58	79
Total weight mustard injected $\times 10^{-4}$ mg	224·6	139·2	157·0
Weight excreted: 0–24 hr	141·5	106·0	158·0
24–48 hr	11·5	6·0	8·0
residue in fly	40·5	30·5	0·0
Total weight excreted + residue	203·5	142·5	164·0
Recovered (%)	90·0	101·3	104·4

The compounds (V), (VI) and (VII) were tested in an attempt to distinguish between the possibilities that the mutagenic action of (IV) was a property of:

(i) The mustard group *per se*.
(ii) The azobenzene group *per se*.
(iii) The mustard group when activated through reduction of the azo-linkage; the reduction occurring: (a) in the germ cells affected, or (b) at other sites, followed by diffusion of the products into the germ cells.

The results of these mutation experiments are presented in Table 7; the average dose per fly for each compound is included in the last column.

The non-mustard azo-compound (VI) showed no mutagenic activity, while its mustard derivative (V) produced a highly significant increase in mutation frequency at an equivalent molar dose.

The reduction product (VII), injected in arachis oil, likewise showed no mutagenic activity. A similarly non-toxic dose of (IV) had given a mutation frequency of 2·1 per cent (Table 3, $P\mu$). If one assumes: (a) a linear relationship between dose of mutagen and mutation frequency; (b) that 10 per cent of the injected azo-compound was

TABLE 7. BROOD MUTATION FREQUENCIES FOLLOWING INJECTION INTO ADULT *Drosophila melanogaster* MALES OF *A*, *B* AND *C*

Brood	I	II	III	IV	Totals I–IV	Dose mg × 10^{-5}/fly
A. 4-*diethylaminoazobenzene*-2′-*carboxylic acid* (VI)						
Chromosomes tested	739	738	735	712	2924	
Lethals	3	2	—	2	7	21·0
% lethals	0·41	0·27	0·00	0·28	0·24	
B. 4-*di*-2″-*chloroethylaminoazobenzene*-2′-*carboxylic acid* (V)						
Chromosomes tested	930	874	892	906	3602	
Lethals	33	23	20	18	94	19·4
% lethals	3·55	2·63	2·24	1·99	2·61	
C. 2-*methyl*-4-*N* : *N*-*di*-2′-*chloroethyl*-p-*phenylenediamine* (VII)						
Chromosomes tested	722	715	749	560	2746	
Lethals	2	2	—	1	5	2·6
% lethals	0·28	0·28	0·00	0·18	0·18	

reduced to the amine VII; and (c) that the amine was the active agent, the molar dose of the amine would be equivalent to a molar dose of (IV) that produced 15·6 per cent sex-linked recessive lethals. This expectation must be modified, however, since the amine was administered in arachis oil, and under such conditions mutagens are generally only about one-tenth as efficient as when they are administered in aqueous solution.[12] Thus a mutation frequency of about 1·6 per cent would be expected. The observed frequency was only 0·18 per cent ± 0·17, this is significantly lower than expectation and does not differ significantly from the spontaneous mutation frequency which, in the stock used, was about 0·3 per cent.

DISCUSSION

In the testes of newly hatched male *Drosophila* all germ cell stages up to non-motile spermatozoa are present. The immature spermatozoa mature rapidly and, at the time of treatment (48 ± 4 hr after emergence) cell stages from spermatogonia to fully-mature spermatozoa are represented.

Information on the identity of germ cell stages sampled in successive broods of offspring from treated males is somewhat contradictory. The significant part of the controversy concerns the position of that brood in which the sperm is derived from cells undergoing meiosis at the time of treatment. Some observations on the induction of dominant lethals by X-rays have suggested that meiotic cells are sampled in brood IV[13] and others, of a similar nature, suggested brood III.[14, 15] Histological studies on the testes of males of the wild type stock used in the present work suggested, however, that meiosis is sampled in brood V.[16] This conclusion being supported by a study of germinal selection in the brood sequence[17] and by an analysis of autosomal lethal mutation clusters.[18] Clusters were frequent in broods VI onwards, but absent from the earlier broods. Such clusters arise when mutations are induced in dividing spermatogonia.

For the interpretation of the brood pattern produced by the azo-mustard (IV), it is immaterial whether meiosis is related to brood IV or V. In either case the mutagen was highly effective towards spermiogenic stages and, except for a very low mutagenic effect in brood IV, ineffective in the earlier stages of spermatogenesis. The stage most sensitive to the mutagenic activity of the azo-mustard was that sampled in brood II, probably late spermatids. Both mature spermatozoa and early spermatids were somewhat less sensitive.

The genetic sensitivity pattern was closely paralleled by the toxic effect of the treatment as measured by egg unhatchability or sterility. The induced egg unhatchability was due chiefly to the absence of sperm and since quite drastic chromosomal aberrations do not render sperm inviable, it would appear that the sterility effect was a general cellular toxicity and not a genetic phenomenon. In this case, the close parallel of genetic and toxic effects would suggest that they are both determined by some general aspect of the treatment such as penetration of the mutagen into the cell. The different degrees of genetic sensitivity of the germ cell stages, the brood pattern, were merely the response of the chromosomes in the respective cell types to different concentrations of the mutagen. A similar parallel was found between the mutation and the sterility brood patterns for another class of chemical mutagens (amino acid mustard[19]) in which the actual pattern differed from that above.

Dose effect

Analysis of the results with respect to individual broods showed that, for the azo-mustard (IV), the mutation rate was constant over the dose range 0·0 to $32·3 \times 10^{-5}$ mg/fly (Table 5).

A linear dose–mutation frequency relationship has been described for triethylene-iminotriazine.[16, 20] This was based on total mutation frequencies over a series of broods. The interpretation of this relationship is, however, complicated by the fact that the broods sampled were markedly heterogeneous with respect to the induced mutation frequency and sterility and that the number of broods included in the sample varied at different doses. In particular the higher doses were tested only over the first four broods, the lower doses[16] were tested in up to seven broods.

Muller[21] reviewed the problems associated with the linear dose-relationship in radiation genetics with particular respect to the "target" theory. One major objection to this theory was that radiations may produce some of their effect through the formation of a chemical intermediate, since certain irradiated materials proved mutagenic

to bacteria.[22] The results of the present study lend some support to this objection, since the formation of a chemical intermediate need have no marked effect on the rate of increase of mutation frequency with respect to dose, provided that the production of the chemical intermediate is itself linearly related to the dose of radiation given. This latter point is difficult to demonstrate in living cells. Peroxides, however, have been closely linked with the genetical effects of radiation and it is significant that, at least at low doses, the production of H_2O_2 in irradiated water is linearly related to dose.[23] A linear relationship between dose and effect for nitrogen mustards, however, cannot be extrapolated to include other mutagens like peroxides. A conclusive proof of the above point would require an exhaustive knowledge of the chemical changes induced in the cell by radiation, and of the quantitative mutagenic effects of the possible secondary mutagens.

A further criticism of the "target" theory concerns the dose effect on small deficiences. These apparent two-break effects followed a single hit rule:[21] in other words, the mutagenic effect of a single event was spatially separated in the chromosome. One explanation of this anomaly was that the linear dose relationship for the two-break small aberrations arose through the breaking of the chromosome in two places by separate ionizations within the ion cluster of the final track of an ionizing particle,[24] the frequency of these ion clusters being proportioned to dose. It has been shown, however, that the frequency of small deficiencies produced by mustard gas is similar to that produced by X-rays at mutagenically equivalent doses.[25] If the azo-mustards are comparable to mustard gas in their production of small deficiencies, as seems *a priori* probable, then for chemicals also, the frequency of small deficiencies is proportional to dose. Deficiencies form a large part of the sex-linked lethal count (20 per cent) and any increase proportional to the square of the dose should produce a measurable effect on the total lethal frequencies: this was not observed. The hypothesis that small deficiencies induced by radiations are the result of two breaks produced by separate ions in an ion cluster is, therefore, unnecessary since it cannot be argued that the primary mutagenic action for chemicals is similarly non-randomly distributed.

Mode of action of azo-mustards

The azo-mustards differ from previously tested mustard compounds in that the chlorine atoms have a very low reactivity. One hypothesis on their mode of action was that the azo-linkage might be enzymically reduced *in vivo* to produce the highly reactive amine mustards. An alternative hypothesis was that the mustard group was not implicated in the mutagenic action, but that this was a property of the azo-benzene group, the azo-compound 4-dimethylamino-azobenzene (butter yellow) being mutagenic in *Drosophila*.[26] The lack of any measurable mutagenic effect of the non-mustard azo-compound (VI) at a dose at which the mustard homologue (V) produced up to 3·5 per cent sex-linked recessive lethals was, however, conclusive evidence against this alternative hypothesis. The mutagenic properties of the azo-mustards are clearly dependent on the presence of the chloroethylamino group(s).

Two hypotheses have been put forward to explain the mutagenic properties of the mustard group in the above azo-mustards, either that the mustard group may react directly with the genetic material, or that it may require activation by reduction of the azo-bond. We will consider the second of these hypotheses.

If the reduction occurred at sites remote from the germ cells, the highly reactive reduction product would have to diffuse intact, perhaps for a considerable distance. The treatment of flies with a solution of the mustard (V) in arachis oil was an attempt to reproduce this condition. The absence of any mutagenic effect in this experiment is, therefore, evidence against the hypothesis that the mutagenic action of the azo-mustards depends on reduction of the azo-linkage outside the germ cells.

Unlike the inhibition of tumour-growth, which may be due to diverse reactions at various sites, the production of mutations appears to be a specific phenomenon. One must suppose that the mutagenic potentialities of a compound can be realized only if it can penetrate the nucleus in its active form. Thus a highly reactive chemical would appear non-mutagenic because of its inability to reach the chromosomes unchanged. In view of this possibility a simple correlation between chemical reactivity and mutagenicity should not be expected. In fact, such a correlation has not been found except in closely related members of homologous series.[27, 28] In view of this, the failure of the highly reactive reduction product (IV) to produce mutations may be plausibly attributed to an inability to reach the genetic material in sufficient concentration to produce a measurable effect. This interpretation is supported by the observation that less reactive, but otherwise similar, arylhalogenoalkylamines have been shown to be mutagenic in *Drosophila*.[27]

It is necessary, therefore, to suppose that if reduction of the azo-linkage is a prerequisite for the mutagenic action of the azo-mustards, it must take place inside the germ cells and therefore close to the chromosomes. This would require the presence of the appropriate enzymes in the germ cell. Little appears to be known about metabolism in the sperm of insects, but in mammalian sperm the middle cytoplasmic area has been shown to be rich in enzymes.[29] This hypothesis is, therefore, at least plausible. The fact that the fly was able to excrete at least 90 per cent of the azo-compound in an unreduced form, plus the fact that the mutation frequency—dose relationship was linear, suggested, that reduction of the azo-bond was not a necessary prerequisite for its mutagenic action. The brood sensitivity pattern did not seem to be correlated to cell metabolism since the more actively dividing cells proved resistant to the mutagenic action of the azo-mustards. The brood pattern would appear to be determined, in part at least, by secondary characters such as the degree of penetration of the mutagen into the cells, such that this lack of effect in actively metabolizing cells cannot be viewed as evidence against the hypothesis that the azo-mustards exert their mutagenic effect only after intracellular reduction.

A possible explanation of the mutagenic efficiency of the azo-mustard might be that their relatively low reactivity enables them to penetrate the cell easily. Thus the chromosomes might be exposed to a high actual concentration of the compound, whereas, with the more reactive mustards only a very small fraction of the given dose actually reaches the chromosomes. Such an hypothesis, although tentative, would be supported by the linear dose effect and would also explain the excretion of the azo-compound largely unreduced.

Acknowledgements—I wish to thank Dr. G. P. Warwick for his interest in this work and for his advice on the compounds tested. I am also indebted to Dr. J. F. Scott of the Unit of Biometry, Oxford for the statistical analysis of the dose relationship data.

This investigation has been supported by grants to the Chester Beatty Institute (Institute of Cancer Research, Royal Marsden Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, United States Public Health Service.

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