Genet. Res., Camb. (1960), **1**, *pp.* 173–188
With 3 *text-figures*
Printed in Great Britain

Cytogenetic analysis of the action of carcinogens and tumour inhibitors in *Drosophila melanogaster*

VI. THE MUTAGENIC CELL STAGE RESPONSE OF THE MALE GERM LINE TO THE 'NITROGEN-MUSTARD' DERIVATIVES OF AMINO-ACIDS, CARBOXYLIC ACIDS AND AMINES

BY O. G. FAHMY AND MYRTLE J. FAHMY
Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London, S.W.3

(*Received* 3 *July* 1959)

I. INTRODUCTION

The biochemical mechanism of genetic change is perhaps the most important aspect in the field of mutagenesis, and unfortunately it is the least understood. Chemical mutagens offer an advantage over radiation in the solution of this problem. Mutations induced chemically must be initiated by a reaction: either within the hereditary nucleoproteins, or with a vital molecule in their environment which is involved in gene synthesis or replication. It would seem feasible, therefore, that a comparative analysis of the mutagenic and biochemical properties of closely related compounds would ultimately lead to the solution of the molecular mechanism of mutation.

The quantitative interpretation of the chemical mutagenesis results, however, is charged with difficulties and pitfalls. Apart from the experimental artifacts of administration, there are the biological factors: mainly toxicity and cellular penetration, and also the biochemical complications: due to reactions in the cytoplasm before the active mutagen reaches the genetic material inside the nucleus. In *Drosophila*, the feeding technique is obviously of very limited value in the analysis of the biochemistry of mutagenesis. Reactions are bound to occur between the mutagen and the food constitutents, as well as with the contents of the alimentary canal and haemocoel, before any active compound reaches the gonad, which is the organ that is subjected to the genetic study. In order to exclude some of these unanalysable reactions, we developed our micro-injection technique, whereby the active mutagen is introduced in measured amounts directly round the male germ-cells. The genetic experiments are then designed for the analysis of the influence of the intracellular environment, during sperm differentiation, on the mutagenic yield. This information, coupled with a knowledge of the chemical properties and metabolic fate of the compounds tested, does enable the assessment of the major biological and biochemical factors affecting mutagenicity. The value of this approach has already been demonstrated in our study of the genetic effects of the alkyl methanesulphonates (Fahmy & Fahmy, 1956, 1957 *a*). In this communication it is intended to analyse the mutagenic properties of a series of closely related

https://doi.org/10.1017/S0016672300000173 Published online by Cambridge University Press

nitrogen-mustards, with a view to the elucidation of how far slight changes in the chemical structure of a mutagenic molecule could affect the relative mutation yield of the cell-stages of the male germ line.

II. MATERIAL AND TECHNIQUE

The Oregon-K wild type strain of *Drosophila melanogaster* was used for the mutagenic tests. Males were subjected to treatment at an age of 30 ± 5 hours after eclosion, and were of roughly the same size and weight. About 200–500 males were selected for each experiment, and they were then partially dewinged and weighed on an analytical chemical balance. In the present set of experiments the average weight per male was $0{\cdot}85 \pm 0{\cdot}05$ mg. An aqueous solution of the compound under test was prepared, and injected by a calibrated micrometer-syringe around and within the testes of the weighed males, so that each fly received the same volume of solution. The injected males were then reweighed. The difference in the average weight per male before and after injection gave the weight injected, which for dilute solutions is roughly equal to the volume. In the present experiments the average volume of solution received per male was $0{\cdot}3\,\mu l$.

The compounds analysed for mutagenicity in the present study are the 'N-mustard' derivatives of amino-acids and carboxylic acids as well as an aromatic amine. All compounds were synthesized in the Chemistry Department of the Chester Beatty Research Institute; their code numbers, formulae, and full chemical names are given below.

Phenylamino-acid mustards:

CB. 3007, CB. 3025, CB. 3026: $(ClCH_2CH_2)_2N.C_6H_4.CH_2CH(NH_2)COOH$;
p-NN-di-(2-chloroethyl)aminophenylalanine, the DL-mixture (Merphalan), the L-isomer (Melphalan), and the D-isomer (Medphalan).

CB. 3051: $(ClCH_2CH_2)_2N.C_6H_4\text{-}O\text{-}C_6H_4.CH_2CH(NH_2)COOH$;
p-/*p'*-NN-di-(2-chloroethyl)aminophenoxy/phenylalanine.

CB. 1385: $(ClCH_2CH_2)_2N.C_6H_4.CH_2CH_2CH(NH_2)COOH$;
α-amino-γ-*p*-(NN-di-2-chloroethyl amino) phenylbutyric acid.

Phenylcarboxylic acid mustards:

$(ClCH_2CH_2)_2N.C_6H_4.(CH_2)_nCOOH$

CB. 1331: $n = 1$; *p*-NN-di-(2-chloroethyl)aminophenylacetic acid.

CB. 1332: $n = 2$; *p*-NN-di-(2-chloroethyl)aminophenylpropionic acid.

CB. 1348: $n = 3$; *p*-NN-di-(2-chloroethyl)aminophenylbutyric acid, or 'Chlorambucil'.

CB. 1356: $n = 4$; *p*-NN-di-(2-chloroethyl)aminophenylvaleric acid.

Phenylethylamine mustard:

CB. 3034: $(ClCH_2CH_2)_2N.C_6H_4.CH_2CH_2(NH_2)$;
p-NN-di-(2-chloroethyl)aminophenylethylamine.

https://doi.org/10.1017/S0016672300000173 Published online by Cambridge University Press

These compounds are insoluble in water and could, therefore, be administered by injection only as a suspension. Previous experience, however, has shown the gross inadequacy of such a procedure, since it is impossible to ensure the homogeneity of dose per male in the injected volume. The ethylamine-mustard (CB. 3034) was injected in solution as the hydrochloride. All the other compounds were converted to the sodium salts, and were in complete solution at the concentrations utilized in the present experiments. It should be noted, however, that the sodium salts are very unstable, thus necessitating the preparation of the sample for each set of experiments separately, just before administration. Some differences did occur in the activity of the various samples, resulting in some variation in replicate experiments both as regards toxicity and mutagenic response. These variations, however, did not obscure the major mutagenic characteristics of any of the compounds examined.

The mutations analysed in this communication are the sex-linked recessive lethals as detected by the Muller-5 technique. The scoring method used in our laboratory has already been described in detail (Fahmy and Fahmy, 1955). It should be re-emphasized, however, that mutants scored as lethals are those that exert *complete* killing in the hemizygous state. F_2 lethal cultures, therefore, contain males of the Muller-5 genotype ($sc^{S1} B (InS) w^a sc^8/Y$) and none of the potential wild type class. Sex-linked recessive visibles were observed in all experiments and will be considered in a later publication.

The progeny of the treated males was fractionated into broods, to enable the assessment of the effect of cell stage on mutagenicity. This has been undertaken by our standard method which has already been described (Fahmy & Fahmy, 1954, 1955). Treated males are mated to a succession of virgin females at 3-day intervals, and the mutation rates in the successive broods are determined separately. Under our experimental conditions, the post-meiotic stages (sperm and spermatids) are predominantly utilized within the first four broods (0–12 days after treatment), whereas the meiotic and premeiotic stages (spermatocytes and spermatogonia) are mainly utilized in later broods (see section IV). In some instances, experimental convenience necessitated slight deviations from the standard progeny fractionation procedure. Such deviations are indicated in the relevant tables in the experimental section.

III. OBSERVATIONS

1. *The amino-acid mustards*

Of the amino-acid mustards studied, the phenylalanine derivatives (the L-isomer, CB. 3025, the D-isomer, CB. 3026, and the DL-mixture, CB. 3007) proved to be the most suitable for the genetic analysis and were used extensively (Tables 1–3 and Fig. 1). The naturally occurring L-isomer gave the most consistent brood-mutation curves (CB. 3025, Fig. 1A). Here it is abundantly clear that the mutagenic response is higher in the later broods. The cytotoxic effect of the compound (as manifested by sterility through lack of sperm) also increases the later is the brood, and only small progenies were recovered from the fourth brood onwards. Sterility through

https://doi.org/10.1017/S0016672300000173 Published online by Cambridge University Press

cytotoxicity is most pronounced with the D-isomer, so that males treated even with low doses become gradually more infertile with time after treatment, and completely failed to produce offspring after the fourth brood. The brood-mutation analysis for the D-isomer, therefore, could only be undertaken for the first 12 days after treatment (Fig. 1B). Though the picture here is not as complete as with the L-isomer, the overall trend seems to be the same: there is a tendency for a higher mutagenic yield

Table 1. *Sex-linked recessive lethals induced by the mustard derivative of L-phenylalanine*

Conc. ($\times 10^{-2}$M)	Brood No.*	Chromosomes	Lethals No.	Lethals %
0·8	I	588	17	2·9
	II	426	24	5·6
	III	539	44	8·2
	IV	210	20	9·5
	Total	1763	105	6·0
0·9	(*a*) I	564	42	7·4
	II	568	53	9·3
	III	306	33	10·8
	Total	1438	128	8·9
	(*b*) I	355	14	3·9
	II	323	16	5·0
	III	250	24	9·6
	Total	928	54	5·8
	(*c*) I	328	22	6·7
	II	185	16	8·6
	III	241	20	8·3
	Total	754	58	7·7
	(*d*) I	360	19	5·3
	II + III	347	41	11·8
	Total	707	60	8·5
	(*e*) I	342	19	5·6
	II + III	350	19	5·4
	Total	692	38	5·5
	(*f*) I	345	20	5·8
	II + III	334	23	6·9
	Total	679	43	6·3
	(*g*) I	515	26	5·0
1·07	(*a*) I	451	15	3·3
	II	491	29	5·9
	III	161	21	13·0
	IV	71	12	16·9
	V	3	—	—
	Total	1177	77	6·5
	(*b*) I	396	33	8·3
	II	372	37	9·9
	III	179	23	12·8
	IV	45	5	(11·1)
	Total	992	98	9·9
	(*c*) I	74	6	8·1
	II	80	2	2·5
	III	13	1	(7·7)
	Total	167	9	5·4
	(*d*) I	235	33	14·0
1·2	I	531	29	5·5
	II	504	37	7·3
	III	313	24	7·7
	IV	147	11	7·5
	V	85	10	11·8
	VI	11	1	(9·1)
	Total	1591	112	7·0
1·5	I	255	15	5·9
	II	90	14	15·6
	Total	345	29	8·4

*Broods I–VI start, respectively, on days 0, 4, 7, 10, 13, 16.

the later is the brood. With the DL-mixture, the sterilizing effect on the treated males (particularly at low doses) was not very marked, and some offspring was obtained as late as the sixth and seventh broods (16–21 days after treatment). It is also to be noted that the brood-mutation curves for the DL-mixture (Fig. 1C) are more confused than for either of the pure isomers. On the whole, however, the general trend is not incompatible with the picture conveyed by the pure isomers. There is an appreciable mutation rate in all fractions of the progeny, including the late broods. In some experiments with the lower doses, however, the mutation

https://doi.org/10.1017/S0016672300000173 Published online by Cambridge University Press

yield in the later progeny fractions sometimes falls below that occurring in the first. This is partly a sampling error, due to the low rates, and the few viable flies available for the genetic tests in the later broods. In part also, this is the outcome of germinal selection that operates against the recovery of mutants induced in the young gonia. Obviously the role of this selection becomes more apparent at the lower doses, where only a few mutations occur, than at high doses where the high mutation yield due to cell sensitivity, supersedes and conceals the reduction in mutation rate due to germinal selection. One of the experiments with the DL-mixture (expt. at $0{\cdot}9 \times 10^{-2}$ M, Table 3) was markedly anomalous, yielding the highest mutation rate in the first brood. This is obviously an artifact, probably due to exceptional physico-chemical circumstances leading to excessive penetration of the drug into the sperm. It is of interest to note, however, that even in this anomalous experiment, the mutation yield in the late progeny (recovered in the fifth and sixth broods) was reasonably high, confirming the positive response of the young germ-cells.

Table 2. *Sex-linked recessive lethals induced by the mustard derivative of* D-*phenylalanine*

Concentration ($\times 10^{-2}$ M)		Broods		Chromosomes	Lethals	
		No.	Days		No.	%
0·8		I	0–3	559	19	3·4
		II	4–6	432	18	4·2
		III	7–9	493	25	5·1
		IV	10–12	115	11	9·6
		Total		1599	73	4·6
0·9		I	0–3	202	14	6·9
		II	4–6	197	18	9·1
		Total		399	32	8·0
1·07	(*a*)	I	0–3	482	31	6·4
		II	4–6	490	44	9·0
		III	7–9	51	3	5·9
		Total		1023	78	7·6
	(*b*)	I	0–3	461	34	7·4
		II	4–6	427	45	10·5
		III	7–9	39	4	(10·3)
		Total		927	83	9·0
1·2		I	0–3	110	16	14·5
		II	4–6	40	3	(7·5)
		III	7–9	29	5	(17·9)
		Total		179	24	13·4
1·5		I	0–3	78	7	9·0
		II	4–6	120	14	11·7
		Total		198	21	10·6

The statistical analysis of the fluctuation in mutation yield in successive broods is presented in Table 4. The relative frequency of mutant to normal chromosomes for

https://doi.org/10.1017/S0016672300000173 Published online by Cambridge University Press

Table 3. *Sex-linked recessive lethals induced by the mustard derivative of* DL-*phenylalanine*

Conc. ($\times 10^{-2}$M)	Brood No.*	Chromosomes	Lethal No.	Lethal %
0·45	I	406	13	3·2
	II	441	11	2·5
	III	433	20	4·6
	IV	456	15	3·3
	V	204	4	2·0
	VI	137	1	0·7
	VII	34	—	—
	Total	2111	64	3·0
0·51	I	458	7	1·5
	II	457	12	2·6
	III	434	6	1·4
	IV	444	10	2·3
	Total	1793	35	2·0
0·8	I	466	11	2·4
	II	465	7	1·5
	III	216	2	0·9
	Total	1147	20	1·7
0·9	I	377	43	11·4
	II	448	18	4·0
	III	459	18	3·9
	IV	361	24	6·6
	V	217	9	4·1
	VI	54	4	7·4
	Total	1916	116	6·1

Conc. ($\times 10^{-2}$M)	Brood No.*	Chromosomes	Lethals No.	Lethals %
1·2	(*a*) I	555	12	2·2
	II	563	35	6·2
	III	282	29	10·3
	IV	244	20	8·2
	V	167	13	7·8
	VI	34	1	(2·9)
	VII	6	2	—
	Total	1851	112	6·1
	(*b*) I	342	18	5·3
	II	245	19	7·8
	III	69	9	13·0
	IV	4	—	—
	Total	660	46	7·0
1·5	(*a*) I	176	22	12·5
	II	125	15	12·0
	III	21	3	(14·3)
	Total	322	40	12·4
	(*b*) I	434	41	9·4
	II	451	33	7·3
	Total	885	74	8·4

*Broods I–VII start, respectively, on days 0, 4, 7, 10, 13, 16, 19.

Table 4. *Distribution of χ for the increase in mutation rate between consecutive broods under the mustard derivative of phenylalanine*

	Broods I–II	II–III	III–IV	IV–V	V–VI
L-isomer					
Total χ	9·9437	9·3133	0·9885	1·0954	−0·0265
No. of estimations	9	8	4	1	1
P	0·0005	0·0005	0·31	0·14	0·48
D-isomer					
Total χ	4·0410	1·0985	1·8385		
No. of estimations	6	4	1		
P	0·05	0·29	0·03		
DL-mixture					
Total χ	−1·1131	3·4498	0·8800	−2·3472	−0·9323
No. of estimations	8	7	4	3	3
P	0·35	0·10	0·33	0·09	0·29

https://doi.org/10.1017/S0016672300000173 Published online by Cambridge University Press

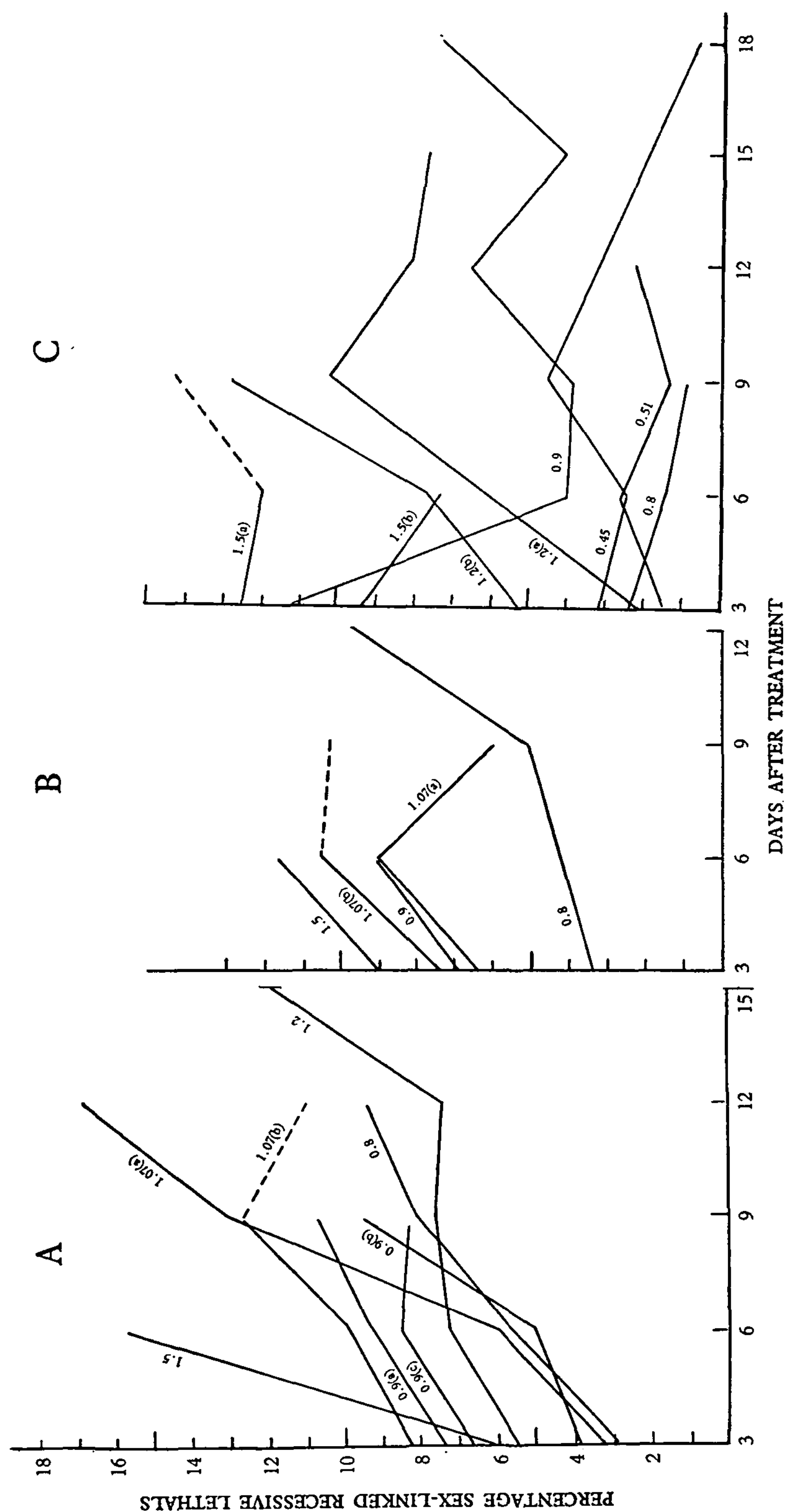

Fig. 1. The brood-mutation curves for the mustard derivatives of phenylalanine: A, the L-isomer, B, the D-isomer, and C the DL-mixture. The injected concentrations ($\times 10^{-2}$ M) are indicated for the separate experiments.

https://doi.org/10.1017/S0016672300000173 Published online by Cambridge University Press

each pair of consecutive broods in the same experiment was compared in a 2×2 contingency table and a χ^2 for the significance of the deviation was calculated. The primary interest in this analysis, however, is to determine whether the deviation in the mutation rate for any two consecutive broods is in a common direction in experiments with different doses. The significance level P for each pair of broods in each experiment, therefore, should be based on one tail only of the corresponding distribution. As was shown by Yates (1955), χ (i.e. $\sqrt{\chi^2}$) for the individual tables, without correction for continuity, gives a good approximation to a normal deviate corresponding to the probability for a single tail. Yates has further pointed out that the values of χ represent deviations with unit standard deviation, and their sum is a normal deviate with a standard deviation $= \sqrt{n}$, where n is the number of estimations. This would, therefore, provide a means for testing association from a set of 2×2 tables, as occurs in the present study. The significance levels for the pairs of consecutive broods for the L-isomer reveal a decisive brood-mutation pattern. The mutation rate rises sharply from the first to the third brood, and is maintained at a high level in the later progeny. This pattern, however, is not clearly revealed by the

Table 5. *Sex-linked recessive lethals induced by the mustard derivatives of phenoxyphenylalanine (CB. 3051) and phenylamino-butyric acid (CB. 1385)*

Mutagen and concentration ($\times 10^{-2}$M)		Broods No.	Broods Days	Chromosomes	Lethals No.	Lethals %
CB. 3051						
1·2		I	0–3	635	14	2·2
		II	4–6	621	16	2·6
		III	7–9	720	23	3·2
		IV	10–12	83	1	1·2
		Total		2059	54	2·6
CB. 1385						
0·3		I	0–3	273	4	1·5
0·5		I	0–3	258	5	1·9
0·7	(*a*)	I	0–3	189	3	1·6
	(*b*)	I	0–3	219	6	2·7
1·0	(*a*)	I	0–3	112	4	3·6
	(*b*)	I	0–3	95	4	4·2

analysis of the data for the D, and DL forms. Nevertheless, here again there is clear evidence that the mutation rate in the later broods is of roughly the same order as in the earlier ones.

An attempt at the analysis of the brood-mutation relationship was also undertaken with two other amino-acid mustards (Table 5, and Purdom, 1957), viz. the derivatives of phenoxyphenylalanine, CB. 3051, and aminobutyric acid: CB. 1385. This analysis, however, was seriously handicapped by the severe toxic and sterilizing effects these agents exert on the treated males. With the aminobutyric acid derivative, no treated males survived more than 3 days, thus only permitting the determination of the mutagenicity on the mature sperm. With the phenoxyphenylalanine

https://doi.org/10.1017/S0016672300000173 Published online by Cambridge University Press

derivative, toxicity was not so drastic, but infertility was pronounced as early as the third brood, and was very severe at the fourth and later broods. It was, therefore, often difficult to recover sufficient offspring for a firm determination of the mutation rate among sperm derived from the earlier germ-cells. Within the analysable first three broods, however, it was clear that under the phenoxy derivative, as with the phenylalanine mustard, the mutation rate tends to rise the later is the brood (Table 5). It is also of interest that in one of Purdom's experiments (*loc. cit.*, fig. 8, table 8), where it was possible to recover and test a reasonable number of sperm derived from

Table 6. *Sex-linked recessive lethals induced by the mustard derivatives of various phenylcarboxylic acids*

Mutagen and concentration ($\times 10^{-2}$M)		Brood No.*	Chromosomes	Lethals No.	Lethals %
CB. 1332	0·3	I	356	4	1·1
		II	378	6	1·6
		III	259	6	2·3
		IV	112	1	0·9
		Total	1105	17	1·5
	1·2	I	294	6	2·0
	1·6	I	258	10	3·9
CB. 1356	0·5	I	387	8	2·1
		II	412	6	1·5
		III	376	12	3·2
		IV	329	3	0·9
		Total	1504	29	1·9
	0·7	I	317	11	3·5
		II	324	14	4·3
		III	287	25	8·7
		IV	292	2	0·7
		Total	1220	52	4·3
	1·0	I	289	13	4·5
		II	282	7	2·5
		III	292	28	9·6
		IV	276	3	1·1
		V	266	—	—
		VI	301	1	0·3
		Total	1706	52	3·0
CB. 1331	0·3	I	458	13	2·8
		II	162	7	4·3
		Total	620	20	3·2
	0·7	(*a*) I	358	14	3·6
		(*b*) I	261	12	4·6
	1·0	I	309	20	6·5
CB. 1348	0·2	I	424	12	2·8
		II	467	16	3·4
		III	398	17	4·3
		IV	376	5	1·3
		Total	1665	50	3·0
	0·6	(*a*) I	361	19	5·3
		II	354	21	5·9
		III	215	21	9·8
		IV	198	2	0·8
		Total	1128	63	5·6
		(*b*) I	249	14	5·6
		II	215	19	8·8
		III	139	15	10·8
		IV	50	—	—
		Total	653	48	7·4
	1·0	I	267	33	12·4
		II	186	26	14·0
		Total	453	59	13·0

*Broods I–VI start, respectively, on days 0, 4, 7, 10, 13, 16.

treated spermatogonia (occurring in the seventh brood), a mutation rate of the same order as that occurring in the first three broods was obtained. This is so in spite of the fact that the mutation rate in a brood as late as the seventh could easily have been depressed by germinal selection. It would seem highly probable, therefore, that the brood-mutation relationship for the phenoxyphenylalanine mustard is of essentially the same type as that for the phenylalanine derivative.

https://doi.org/10.1017/S0016672300000173 Published online by Cambridge University Press

2. The carboxylic-acid mustards

Experiments were undertaken to test the mutagenic variation in the successive broods of males treated with the mustard derivatives of homologous phenylcarboxylic acids. The detailed data for the separate compounds are given in Table 6 and

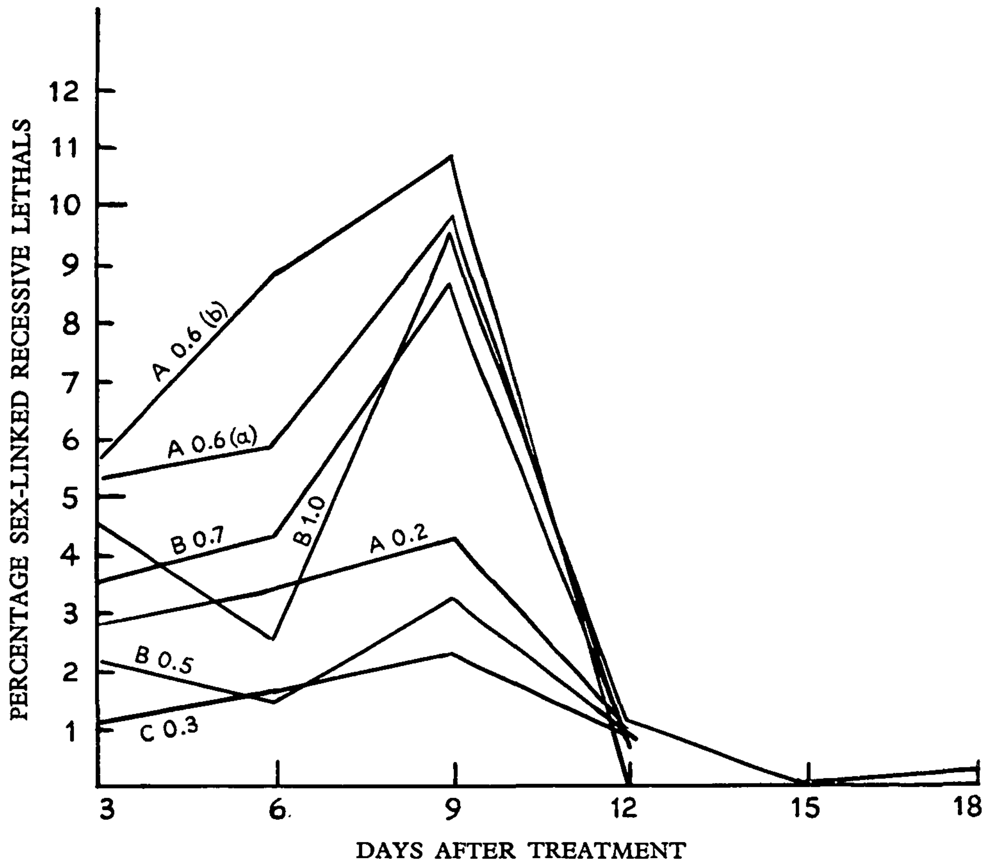

Fig 2. The brood-mutation curves for the mustard derivatives of various phenylcarboxylic acids: A, CB.1348; B, CB.1356; C, CB.1332. The injected concentrations ($\times 10^{-2}$ M) are indicated for the various experiments.

Fig. 2. In all experiments the mutation rate was high for the first three broods and then dropped to a very low level in the fourth. Furthermore, when the progeny fractionation was extended beyond this brood only the control mutation rate was recovered. It is certain, therefore, that compounds of this series are inactive on the germ-cells supplying sperm for the later progeny. The genetic analysis, therefore, was confined to the first four broods, 10–12 days after treatment. In some instances, however, and particularly at the higher doses, it was only possible to test one or two broods, as the treatment resulted in the partial sterilization and early death of the males.

The brood-mutation fluctuations for the various compounds tested have been analysed statistically by the χ method and the results are given in Table 7. A general

https://doi.org/10.1017/S0016672300000173 Published online by Cambridge University Press

fluctuation pattern is discernible, and is best illustrated in experiments with the butyric and valeric acid derivatives (CB. 1348, 1356). Roughly the same mutation rate occurs in the first two broods, and this is followed by a sharp rise to a peak in the third brood, and then a drastic drop at the fourth brood, almost reaching the control level.

Table 7. *Distribution of χ for the increase in mutation rate for consecutive broods under the mustard derivatives of various phenylcarboxylic acids*

	Broods		
	I–II	II–III	III–IV
CB. 1331			
Total χ	0·9179		
No. of estimations	1		
P	0·18		
CB. 1332			
Total χ	0·5417	0·6650	
No. of estimations	1	1	
P	0·29	0·25	
CB. 1348			
Total χ	2·7214	2·9533	−6·3386
No. of estimations	4	3	2
P	0·09	0·04	0·000004
CB. 1356			
Total χ	−1·4090	7·4019	−11·1297
No. of estimations	3	3	3
P	0·21	0·00001	$< 10^{-9}$

3. *The phenylethylamine mustard*

From the previous two sections it is clear that a difference in the brood-mutation curves occurs with the carboxylic, as compared to the amino-acid, mustards. The mutation rate remains at a high level in sperm recovered later than the ninth day after treatment with the amino-acid mustards, while it drops drastically and consistently after the same period under the carboxylic acid mustards. In order to assess how far this is a function of the presence of an amino (—NH_2) group in the molecule, the mutagenicity of the mustard derivative of an aromatic amine (CB. 3034: *p*-NN-di(2-chloroethyl)aminophenylethylamine) was tested. This is the amine corresponding to the phenylalanine mustard, but devoid of the carboxyl group.

The brood-mutation relationship for this amine mustard is given in Table 8 and Fig. 3, and the statistical analysis of the significance of the fluctuations is given in Table 9. The overall data show that there is roughly the same mutation rate in the first two broods, leading to a significant rise in the third, a gradual though insignificant fall through the fourth and fifth broods, which is followed by a very drastic fall in later broods. It is clear, therefore, that the presence of an amino-group in the mustard-molecule results in an increase of the mutagenic response of some of the

https://doi.org/10.1017/S0016672300000173 Published online by Cambridge University Press

Table 8. *Sex-linked recessive lethals induced by the mustard derivative of phenylethylamine*

Conc. (× 10^{-2}M)	Brood No.*	Chromosomes	Lethals No.	Lethals %
0·19	I	417	17	4·1
	II	514	12	2·3
	III	485	31	6·4
	IV	507	31	6·1
	V	495	12	2·4
	VI	504	2	0·4
	VII	346	0	0·0
	Total	3268	105	3·2
0·29	I	388	16	4·1
	II	524	22	4·2
	III	488	28	5·7
	IV	486	29	6·0
	V	535	29	5·4
	VI	514	6	1·2
	VII	336	0	0·0
	Total	3271	130	4·0
0·38	I	419	29	6·9
	II	553	46	8·3
	III	64	3	4·7
	IV	75	7	9·3
	V	8	2	—
	VI	377	2	0·5
	VII	164	0	0·0
	Total	1660	89	5·4
0·77	I	62	8	12·9
	II	139	15	10·8
	III	9	—	—
	IV	1	1	—
	Total	211	24	11·4
1·15	I	43	7	(16·3)
	II	24	5	(20·8)
	III	—	—	—
	IV	1	1	—
	Total	68	13	19·1

*Broods I–VII start, respectively, on days 0, 4, 7, 10, 13, 16, 19.

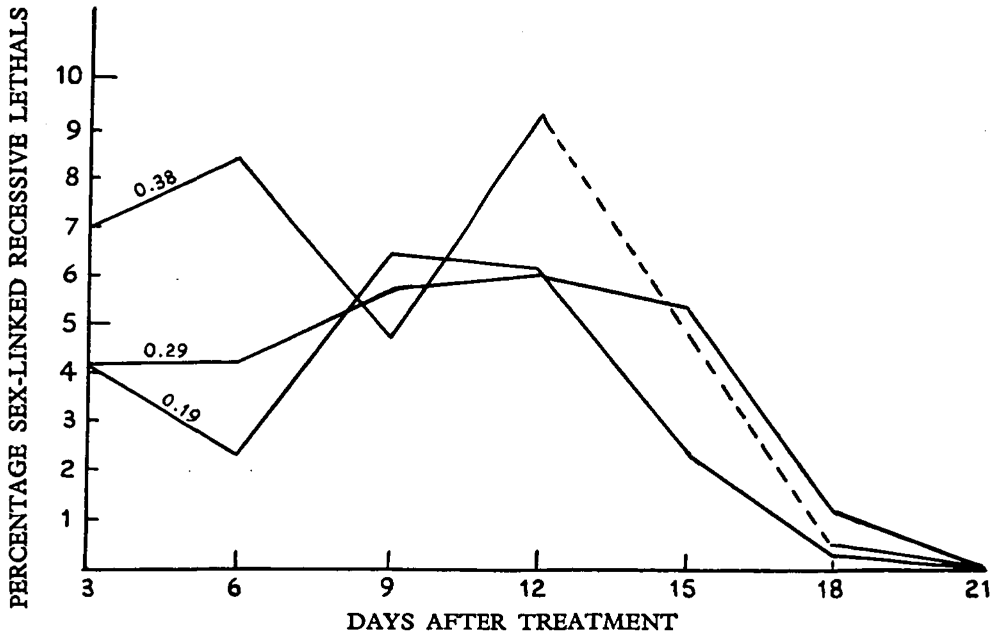

Fig. 3. The brood-mutation curves for the mustard derivative of phenylethylamine. The injected concentrations (× 10^{-2} M) are indicated for the separate experiments.

https://doi.org/10.1017/S0016672300000173 Published online by Cambridge University Press

early germ cells. The drastic and consistent fall in the mutation rate later than the fifteenth day after treatment, however, shows that the earliest stages of the male germ-line are refractory to the amine mustard. In contrast, the amino-acid mustards did possess decisive mutagenic effect on the very young germ cells: recovered later than the fifteenth day after treatment (Tables 1 and 3). It would follow, therefore, that the response of these early germ-cells to the amino-acid mustard is not entirely a function of the presence of an amino-group in the mutagen.

Table 9. *The distribution of* χ *for the increase in mutation rate between consecutive broods under the mustard derivative of phenylethylamine*

	Broods				
	I–II	II–III	III–IV	IV–V	V–VI
Total	−0·6275	3·1254	−1·0863	−1·9017	−13·3162
No. of estimations	5	3	3	3	3
P	0·40	0·04	0·26	0·14	$< 10^{-9}$

IV. DISCUSSION

The interpretation of the brood-mutation curves in terms of cell-stage response is by necessity only inferential, and is bound to be very approximate. The germ line presents an uninterrupted spectrum of differentiation, and any attempt at its fractionation into separate cell stages by repeated matings can never be absolute. As was demonstrated in our work with TEM (2:4:6-tri(ethyleneimino)-1:3:5-triazine, Fahmy & Fahmy, 1954 and 1955), even under standard experimental conditions (as regards culture conditions, age of male and brood interval), alterations in the brood-mutation curves did occur, mainly due to cell killing and variation in speed of germ line differentiation. Nevertheless, through extensive histo-genetic studies (Fahmy & Fahmy, 1954), it was possible to show that in our experiments with TEM, post-meiotic stages of the testis are mainly utilized within the first four broods (i.e. 0–12 days after treatment), and the meiotic and pre-meiotic stages are utilized in later broods. Evidence is now available that this timing is approximately applicable to the majority of the alkylating mutagens tested in our laboratory. With 2-chloroethyl methanesulphonate (Fahmy & Fahmy, 1956 and 1957 *a*) which is strongly mutagenic on the early germ-cells, 'clusters' of identical visibles were detected in the fifth brood (13–15 days after treatment), indicating that at least some of the sperm utilized in this period was derived from treated spermatogonia. In our study of the visibles induced in the present experiments with the phenylalanine mustard (Fahmy & Fahmy, 1959) a large sample of visibles was recovered in the fourth brood, but no 'clusters' were detected. In the fifth brood, however, though fewer mutations were recovered (because of sterility through cytotoxicity), visible clusters did occur. Most significant also are our results with S-chloroethyl cysteine (Fahmy & Fahmy, 1957 *b* and under publication) which exerts specific mutagenicity on the early germ-cells. Here again clusters of identical sex-linked visibles occurred in the fifth brood, indicating that the sperm producing this progeny

https://doi.org/10.1017/S0016672300000173 Published online by Cambridge University Press

came from treated spermatogonia capable of mitotic proliferation. Independent evidence pointing to the same conclusion came from the analysis of the time of onset of germinal selection. The study of the X-chromosome mutations revealed a disturbance in the visible to lethal ratio (in favour of the visibles) at the fifth brood, simultaneous with the appearance of the visible clusters. This suggested that germinal selection did operate against the recovery of a proportion of the lethals induced in proliferating spermatogonia. A comparative study of the autosomal and sex-linked lethals induced in the early germ-cells by the cysteine mustard, also showed that selection against the X-chromosome hemizygous mutations is first noticeable in the fifth brood. This confirms the timing of the onset of germinal selection deduced on the basis of the sex-linked mutations. Furthermore, it indicates that proliferating spermatogonia (supplying the hemizygous lethals against which selection operates) must also contribute to the sperm utilized in the fifth brood. There is little doubt, therefore, that under our experimental conditions, and with the most diverse alkylating agents, some of the sperm derived from treated spermatogonia starts to be utilized 13–15 days after treatment. This reasonable consistency in the separation of the spermatogonial from the post-spermatogonial stages probably indicates that under moderate doses of the alkylating mutagens the speed of the male germ-line differentiation is not drastically disturbed. It is highly improbable, however, that even this gross separation is achieved in every progeny fractionation experiment. In some instances the utilization of spermatogonia may begin as early as the fourth, or may be delayed until after the fifth brood.

In the light of the above considerations, it would be possible to give a broad interpretation of the brood-mutation curves for the compounds analysed in terms of cell-stage response. Under the carboxylic acid mustards, there is a peak of maximal mutability in the third brood followed by a sharp fall to almost the control level in the fourth and later broods. These compounds, therefore, give maximal activity on a post-meiotic stage, most probably an early spermatid. In this respect, it would appear that their mode of action is fundamentally comparable to X-radiation (Glass's 1956 review, and Fahmy & Fahmy—unpublished), as well as a diversity of chemical mutagens (e.g., mustard gas: Auerbach, 1950; diepoxybutane: Bird & Fahmy, 1953; and TEM: Fahmy & Fahmy, 1955). In contrast, under the amino-acid mustards, the overall trend is towards a rise in the mutation rate until the third brood, and the maintenance of the high rate in later progeny. This indicates that the later stages of spermatogenesis (i.e. the sperm and later spermatids) are less responsive to this series than the earlier stages (early spermatids, spermatocytes and spermatogonia). The brood-mutation curve for the amine mustard is nearer that for the amino-acid, than the carboxylic-acid mustards. The mutation rate reaches its maximum at the third brood, and is maintained at a high level through the fourth and fifth, but falls decisively to the control level in later broods. The earliest stages of the male germ line, therefore, seem to be refractory to the mutagenic action of the amine mustard. It is difficult to be certain, however, if this refractory stage represents the whole of the spermatogonial population, or only the earlier stages (i.e. the primary spermatogonia). It is perhaps of significance that no visible

https://doi.org/10.1017/S0016672300000173 Published online by Cambridge University Press

'clusters' were detected in the fifth brood with the amine mustard. This may suggest that in this series of experiments the recovery of sperm from spermatogonia may have been delayed, in part at least, until the sixth brood. On this basis, it would appear that practically all the spermatogonia are refractory to the mutagenicity of the amine mustard.

The most significant fact that emerges from the present analysis is that while the amino-acid derivatives possess decisive mutagenic activity on the spermatocytes and spermatogonia, the carboxylic-acid mustards are practically ineffective on these cells. This does not seem to be entirely due to the mere presence of an amino (NH_2) group in the mutagenic molecule, since the phenylethylamine mustard is still inactive on spermatogonia (at least the earlier stages). It would seem, therefore, that the response of the gonia is a feature of the whole amino-acid moeity of the mutagen. Of interest in this connexion are the biochemical results (Cohn, 1957) that ^{14}C-labelled phenylalanine mustard, and its hydrolysates, become bound *in vivo* with the protein cell fractions of metabolically active tissues (rat liver and kidney). This binding is probably physical (through adsorption) rather than due to chemical incorporation (through a peptide linkage), since it has now been shown (Brookes, 1959) that the labelled pure dihydroxy-derivative (*p*-di-(2-hydroxy-$^{14}C_2$-ethyl)-amino-L-phenylalanine) is not incorporated in the protein fraction of metabolizing cells. Of all the stages of spermatogenesis, it is only the spermatocytes and the spermatogonia which are endowed with any anabolic activity. The mutagenic response of these cells to the amino-acid mustards, suggests some intracellular 'transport' mechanism associated with protein synthesis. This may proceed along the same pathway as that ensuring the supply of the unsubstituted amino-acid for nucleoprotein synthesis during chromosome replication, but without leading to the successful incorporation of the 'unnatural' molecule. The outcome of this transport mechanism, as regards mutagenesis, would be the conveyance of the reactive molecule through the cytosome to the hereditary nucleoproteins during chromosome reproduction. This could lead to a higher frequency of 'encounters' between the active groups of the mutagen and the genes, and would account for the observed mutagenic response.

SUMMARY

The analysis of the variation in the mutation rate in the fractionated progeny of treated males, revealed a marked differential cell stage response to the various chemical series investigated. The mustard derivatives of amino-acids (particularly L-phenylalanine) exert their minimal mutagenicity on mature sperm, but possess an appreciable activity on other stages of spermatogenesis, including spermatogonia. The carboxylic-acid mustards produce their maximal effect on an early spermatid, but are practically ineffective on spermatocytes and spermatogonia. The amine mustard corresponding to the phenylalanine derivative is effective on the stages of spermiogenesis (including the early spermatids) as well as on the spermatocytes, but is inactive on the spermatogonia (at least the primary stages). The response of the

https://doi.org/10.1017/S0016672300000173 Published online by Cambridge University Press

gonia, therefore, is a function of the amino-acid moeity of the mutagen, and is not merely due to the presence of an amino-group in the molecule.

We are indebted to Professor A. Haddow, F.R.S., for his interest, and to our colleagues of the Chemistry Department, particularly Professor F. Bergel, F.R.S., and Dr W. C. J. Ross, and Dr J. A. Stock, for the supply of the mutagens used, and for their helpful discussion.

This investigation has been supported by grants to the Chester Beatty Research Institute 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 Institute of Health, U.S. Public Health Service.

REFERENCES

Auerbach, C. (1950). Differences between effects of chemical and physical mutagens. *Pubbl. staz. zool. Napoli* (Suppl.), **22,** 1.

Bird, M. J. & Fahmy, O. G. (1953). Cytogenetic analysis of the action of carcinogens and tumour inhibitors in *Drosophila melanogaster*. I. 1:2, 3:4-diepoxybutane. *Proc. roy. Soc.* B, **140,** 556.

Brookes, P. (1959). Studies on the incorporation of an unnatural amino-acid, *p*-di-(2-hydroxy/$^{14}C_2$/ethyl)amino-L-phenylalanine into proteins. *Brit. J. Cancer,* **13,** 313.

Cohn, P. (1957). The distribution of radioactivity in tissues of the rat following the administration of a nitrogen mustard derivative (*p*-di-(2-chloroethyl)amino-DL-phenyl/β-^{14}C/-alanine). *Brit. J. Cancer,* **11,** 258.

Fahmy, O. G. & Fahmy, M. J. (1954). Cytogenetic analysis of the action of carcinogens and tumour inhibitors in *Drosophila melanogaster*. II. The mechanism of induction of dominant lethals by 2:4:6-tri-(ethyleneimino)-1:3:5-triazine. *J. Genet.* **52,** 603.

Fahmy, O. G. & Fahmy, M. J. (1955). Cytogenetic analysis of the action of carcinogens and tumour inhibitors in *Drosophila melanogaster*. IV. The cell stage during spermatogenesis and the induction of intra- and intergenic mutations by 2:4:6-tri-(ethyleneimino)-1:3:5-triazine. *J. Genet.* **53,** 563.

Fahmy, O. G. & Fahmy, M. J. (1956). Mutagenicity of 2-chloroethyl methanesulphonate in *Drosophila melanogaster*. *Nature, Lond.,* **177,** 996.

Fahmy, O. G. & Fahmy, M. J. (1957*a*). Mutagenic response to the Alkyl-methanesulphonates during spermatogenesis in *Drosophila melanogaster*. *Nature, Lond.,* **180,** 31.

Fahmy, O. G. & Fahmy, M. J. (1957*b*). Selective cell-stage response to the mutagenicity of S-chloroethylcysteine in *Drosophila melanogaster*. *Drosophila Inform. Serv.,* **31,** 118.

Fahmy, O. G. & Fahmy, M. J. (1959). Cytogenetic analysis of the action of carcinogens and tumour inhibitors in *Drosophila melanogaster*. VII. Differential induction of visible to lethal mutations by related 'Nitrogen-mustards'. *Genetics* (in the press).

Glass, B. (1956). Differences in mutability during different stages of gametogenesis in *Drosophila*. *Brookhaven Symp.,* in *Biol.,* **8,** 148.

Purdom, C. E. (1957). Comparative study of the effect of dose and cell stage on the mutagenicity of different alkylating agents in *Drosophila melanogaster*. Ph.D. Thesis, University of London.

Yates, F. (1955). A note on the application of the combination of probabilities test to a set of 2×2 tables. *Biometrika,* **42,** 404.

https://doi.org/10.1017/S0016672300000173 Published online by Cambridge University Press