CYTOGENETIC ANALYSIS OF THE ACTION OF CARCINOGENS AND
TUMOR INHIBITORS IN DROSOPHILA MELANOGASTER.
VII. DIFFERENTIAL INDUCTION OF VISIBLE TO
LETHAL MUTATIONS BY RELATED
NITROGEN MUSTARDS

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THE analysis of the mutagenic properties of various alkylating compounds on the testis of adult *Drosophila*, has revealed some differences in their mode of action (FAHMY and FAHMY 1956). The over-all mutagenic potency was shown to depend not only on the chemical reactivity of the alkylating groups, but also on the molecular configuration of the nonalkylating (or prosthetic) moiety of the molecule. The significance of the prosthetic moiety was also manifest in the relative mutagenic response of the various cell stages of the germ line. Thus among closely related 'nitrogen mustards' the amino acid derivatives were found to be mutagenic on all stages of the male germ line, including the spermatocytes and spermatogonia, while the corresponding carboxylic acid derivatives were practically ineffective on the younger cell stages, especially the gonidia (FAHMY and FAHMY 1958b, 1959b). This difference was attributed to an intracellular 'transport' mechanism connected with protein synthesis, and favoring the conveyance of mutagens with an amino acid moiety to sites of nucleoprotein construction.

The above differences were based on the analysis of one class of mutations (the recessive lethals) and may therefore be looked upon as mainly quantitative—due to various degrees of attack on the same genic sites. However, it has now been demonstrated (FAHMY and FAHMY 1956, 1959a) that mutagens belonging to different chemical series do exert differential action on various genes, especially as regards the yield of visibles. It was, therefore, of interest to determine whether differential mutagenicity could also occur between compounds within the same chemical series, i.e., among the mustard derivatives of closely related compounds. An observation favoring this possibility is the higher ratio of visibles to lethals induced by the mustard derivatives of amino acids compared to those of related carboxylic acids (FAHMY and FAHMY 1956). Most outstanding in this connection is that the mustard derivative of phenylalanine [p-NN-di-(2-chloroethyl) aminophenylalanine] was found to induce a ratio of 3 visibles:10 lethals, which

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is significantly higher than the corresponding ratio for any other alkylating agent as well as radiation. In view of the great theoretical and practical implications of this discovery, the phenomenon was subjected to further investigation, and the data are presented in this communication. There was ample evidence that a qualitative difference occurs in the physical nature of visibles as compared to lethals, and that the ratio between them for the same mutagen was constant and independent of dose and cell stage. Variation in the visible to lethal ratio between agents, therefore, must be a genuine manifestation of differential mutability.

MATERIAL AND TECHNIQUE

All experiments were undertaken with the Oregon-K stock of *Drosophila melanogaster*. This stock has been maintained in our laboratory for many years, and gives a spontaneous sex-linked recessive mutation rate of 0.18 ± 0.05 percent lethals and 0.009 ± 0.001 percent visibles. In control experiments where the males were injected with isotonic saline (0.4 percent NaCl), the spontaneous mutation rate was unchanged.

The compounds to be considered in this communication are the mustard derivatives of amino acids, carboxylic acids, and amines, whose mutagenicity as regards the sex-linked recessive lethals has already been given (FAHMY and FAHMY 1959b). For ease of reference, the formulae and the CHESTER BEATTY (CB) code numbers for these compounds will be restated here in an abbreviated form.

Phenyl amino acid mustards:

The derivatives of phenylalanine ($n = 1$): the L-isomer CB.3025, the D-isomer CB.3026, and the DL-mixture CB.3007 and phenoxyphenylalanine, CB.3051, ($n = 1$, and the central part of the molecule = $\text{C}_6\text{H}_4\text{-O-C}_6\text{H}_4$).

Phenylcarboxylic acid mustards:

The derivatives of acetic acid CB.1331 ($n = 1$), propionic acid CB.1332 ($n = 2$), butyric acid CB.1348 ($n = 3$) and valeric acid CB.1356 ($n = 4$).

Phenylethylamine mustard:

All these compounds have been administered in solution (as sodium salts, except for CB.3034 which was given as the hydrochloride) by our microinjection technique around the testes of adult males. The progeny of the treated males was fractionated (usually at three day intervals) and the mutation rate for the individual broods was determined separately by the Muller-5 technique. The mutations considered in this communication are the sex-linked recessive visibles, scored according to our previously described technique (FAHMY and FAHMY

1955b). The F_2 mutant cultures were scrutinized throughout the full period of adult emergence. Where males of the Muller-5 genotype ($sc^{s1}B In-S w^a sc^s/Y$), and none of the potential wild type class occurred, this was taken to indicate the induction of an X chromosome lethal. Where the potential wild type class was represented by males with anomalous phenotypes, this was taken as an indication of the induction of a sex-linked recessive visible. Only mutations which were confirmed in the F_3 and all subsequent generations were considered in this analysis. Allelism tests were undertaken with the induced visibles against all the known mutants of relevant phenotype (both the α and β loci—see FAHMY and FAHMY 1959a).

A sample of the unidentified visibles, and the recessive lethals, were genetically placed by crossover experiments against a chromosome marked by the genes scute (*sc*), cut (*ct*), vermilion (*v*), forked (*f*) and carnation (*car*). A sample of the localized lethals was analysed cytologically for the detection of aberrations in the salivary gland chromosomes. All preparations were permanent aceto-orcein squashes, sometimes restrained in Feulgen.

OBSERVATIONS

Visible to lethal ratio: One of the most informative techniques in the study of comparative mutagenesis is the analysis of the relative frequency of different mutant types induced in the same treated cells, or better still in the same treated chromosomes. The Muller-5 method for detecting sex-linked recessive mutations is advantageous in this respect, since it enables the determination of both lethal and visible mutations induced in the same sample of treated X chromosomes. A serious difficulty in the classification of these two types of mutation, however, arises from the fact that some 'visible' mutants are also accompanied by reduced viability. It was, therefore, essential to draw a sharp and clear-cut distinction between visibles and lethals, if any use of their relative induction by different mutagens is to be utilized. In our laboratory, therefore, we confine the lethal class to mutations that exert complete killing in the hemizygous state, leaving the visible class to include morphological mutants irrespective of viability.

When the classification of the sex-linked mutations was undertaken on the above basis, it was possible to show (FAHMY and FAHMY 1955b) that the ratio of visibles to lethals induced in the X chromosome by TEM (i.e., triethylenemelamine, or more accurately, 2:4:6-tri(ethyleneimino)-1:3:5-triazine) is reasonably constant and independent of dose and cell stage. This result has now been repeatedly confirmed with several alkylating compounds and is well illustrated by the present extensive data with the mustard derivative of phenylalanine. The effect of the cell stage on the ratio of visibles to lethals cannot be reliably analysed statistically for individual broods. The number of visibles per brood in a manageable experiment is rather small. This could magnify the inevitable error due to the missing of a few mutants because of the generally recognized difficulty in scoring small morphological changes. The problem, however, can be approached indirectly—through the analysis of the over-all ratio in large mutant

samples derived from experiments involving different germ cell stages. If the relative frequency of the two classes of mutation is independent of the cell stage, the over-all ratio between them should be reasonably homogeneous, irrespective of the fraction of the germ line from which the mutations were recovered. Table 1 summarizes the over-all ratio of visibles to lethals in 28 separate experiments undertaken with the phenylalanine mustard. It is to be noted that the experiments were with different doses of the two isomers and their mixture, and they gave widely different lethal rates. Moreover, the mutants were detected in offspring recovered at various times after treatment (0–21 days) and must have been induced in different stages of spermatogenesis. In the greatest majority of the experiments, however, the progeny tested was recovered within the first 12 days, and evidence was available that during this period sperm derived mainly from the postmeiotic stages of the germ line are utilized. Within the last fraction of this period (10–12 days) a total of 33 visibles was detected, but none were in clusters. In contrast, within the following fraction (13–15 days), there were only eight visibles consisting of two small clusters (a pair each) and four single mutants. This indicates that the premeiotic stages are not appreciably utilized earlier than 13 days after treatment. In experiments extending to only 12 days, therefore, no complication due to germinal selection against the lethals occurs, since this process is ineffective among the postmeiotic sperm. Some experiments (extending beyond 12 days) contributed mutations induced in premeiotic stages, where germinal selection could operate. The cytotoxic (killing) effect of the phenylalanine mustard on the premeiotic stages (FAHMY and FAHMY 1959b), however, meant that the contribution from these cells to the over-all mutation pool was extremely small (2.5 percent of the total mutations). In this series of experiments, therefore, the effect of germinal selection can be safely ignored.

The relative frequency of visibles to lethals in the different experiments given in Table 1, has been subjected to extensive statistical analysis by the contingency table technique. The ratio of the two types of mutation was homogeneous for the L-isomer ($\chi^2 = 12.39$, degrees of freedom = 13, $P = 0.5$), as well as for the D-isomer ($\chi^2 = 2.26$, degrees of freedom = 5, $P = 0.8$), but a slight heterogeneity occurred with the DL-mixture, ($\chi^2 = 14.39$, degrees of freedom = 7, $P \approx 0.05$). The heterogeneity was traced to three anomalous experiments (21, 22 and 27, Table 1) which were undertaken with a faulty sample of the compound. This sample was the first attempt at the conversion of the amino acid mustard to its sodium salt and contained an excess alkali (NaOH) as indicated by its excessively high pH. Biologically, also, this sample gave high toxicity, and abnormally low lethal rates. It is highly probable that this faulty sample was inactivated, in part at least, by the hydrolysis of some of its chloroethyl groups. The excess free alkali could also have affected the biological mode of action. It is of interest to note, however, that the few anomalous experiments did not conceal the over-all homogeneity of the ratio of visibles to lethals on the basis of all experiments ($\chi^2 = 33.14$, degrees of freedom = 27, $P \approx 0.2$). A comparison has also been undertaken of the relative yield of visibles to lethals in

TABLE 1

Ratio of sex-linked recessive visibles to lethals induced by the mustard derivatives of phenylalanine at different concentrations and in various stages of spermatogenesis

Experiment No.	Dose ($\times 10^{-2}M$)	Days after treatment	Lethal* rate percent	Chrom. tested	Visibles v	Lethals l	Ratio v/l
L-isomer, CB.3025							
1	0.9	0-3	5.0	515	10	26	0.38
2	1.07	0-9	5.4	167	2	9	0.22
3	0.9	0-9	5.5	692	9	38	0.24
4	0.9	0-9	5.8	928	12	54	0.22
5	0.8	0-12	6.0	1,763	36	105	0.34
6	0.9	0-9	6.3	679	16	43	0.37
7	1.07	0-15	6.5	1,177	34	77	0.44
8	1.2	0-18	7.0	1,591	46	112	0.41
9	0.9	0-9	7.7	754	25	58	0.43
10	1.5	0-6	8.4	345	14	29	0.48
11	0.9	0-9	8.5	707	24	60	0.40
12	0.9	0-9	8.9	1,438	29	128	0.23
13	1.07	0-12	9.9	992	35	98	0.36
14	1.07	0-3	14.0	235	12	33	0.36
Total				11,983	304	870	0.35
D-isomer, CB.3026							
15	0.8	0-12	4.6	1,599	21	73	0.29
16	1.07	0-9	7.6	1,023	20	78	0.26
17	0.9	0-6	8.0	399	9	32	0.28
18	1.07	0-9	9.0	927	24	83	0.29
19	1.5	0-6	10.6	198	9	21	0.43
20	1.2	0-9	13.4	179	4	24	0.17
Total				4,325	87	311	0.28
DL-mixture, CB.3007							
21	0.8	0-9	1.7	1,147	4	20	0.20
22	0.51	0-12	2.0	1,793	3	35	0.09
23	0.45	0-21	3.0	2,111	19	64	0.30
24	0.9	0-18	6.1	1,916	43	116	0.37
25	1.2	0-21	6.1	1,851	38	112	0.34
26	1.2	0-12	7.0	660	18	46	0.39
27	1.5	0-6	8.4	885	10	74	0.14
28	1.5	0-9	12.4	322	10	40	0.25
Total				10,685	145	507	0.29
Grand total				26,993	536	1,688	0.32

* This estimate involves the assumption that mitotic proliferation in spermatogonia does not alter the ratio of normal to mutant chromosomes.

the over-all pool of mutants under the L-isomer, the D-isomer and the DL-mixture and no significant difference was detected ($\chi^2 = 4.44$, degrees of freedom = 2, $P > 0.1$). There is enough justification, therefore, to utilize the data from all isomers for an accurate determination of the ratio of visibles to lethals for the phenylalanine mustard. The mean value for this ratio is 0.32, with a

standard deviation as estimated from the data of 0.02, which confirms, our earlier estimate (FAHMY and FAHMY 1956).

According to our scheme of classification, morphological mutants, irrespective of viability, are considered as "visibles." A small proportion of these were of low viability and would perhaps be classified by other workers as semilethal. It was thought appropriate, therefore, to test how far this difference in classification could account for the high ratio of visibles to lethals observed under the effect of the amino acid mustard. An analysis of the relative frequency of mutant to Muller-5 males in all the F_2 cultures from which the visibles were obtained is given in Table 2. It can be seen that only nine percent of the observed visibles had a viability less than ten percent that of the Muller-5 males in the same cultures, and might be classified as semilethal. Transferring this proportion from the visible to the lethal class, gives an over-all relative frequency of 486:1738, a ratio of 0.28, which is still not significantly different from the over-all ratio ascertained by our more accurate method of scoring ($\chi^2 = 3.18$, for one degree of freedom, $P = 0.08$).

The relative yield of visibles to lethals under the various other nitrogen mustards investigated are given in Table 3. This yield for another amino acid mustard, *viz.* the phenoxypyphenylalanine compound (CB.3051), is not significantly different from the over-all ratio for the nonsubstituted phenylalanine derivative ($\chi^2 = 0.20$, for one degree of freedom, $P > 0.5$). Under the four carboxylic acid mustards the visible to lethal ratio is extremely consistent ($\chi^2 = 1.55$, degrees of freedom = 3, $P > 0.5$) and is of the order of one visible to ten lethals. The amine mustard gives an over-all ratio of 0.17 which is significantly higher than that for the carboxylic acid mustards ($\chi^2 = 8.02$ for one degree of freedom, $P < 0.01$) but is grossly lower than that for the amino acid mustards (compared with the phenylalanine ratio, $\chi^2 = 18.09$ for one degree of freedom, $P < 0.001$).

Among the nitrogen mustards, therefore, the phenylalanine derivative is clearly the most effective agent in the induction of visibles relative to lethals. It is also more efficient in this respect than X-radiation. In experiments with the physical agent comparable to those undertaken by the mustard, as regards experimental conditions and method of scoring, there were two visibles to ten lethals, a ratio which was shown to be significantly lower than that for the amino acid mustard (FAHMY and FAHMY 1957a). More irradiation experiments both with X-rays and γ -rays (from a cobalt⁶⁰ source) have now been undertaken,

TABLE 2

Viability of the hemizygous "visible" mutants relative to the Muller-5 males in the same F_2 cultures

Percent viability	100	99-50	49-10	< 10	Total
No. of visibles	194	134	158	50	536
Percent of total	36.2	25.0	29.5	9.3	100.0

TABLE 3

Ratio of sex-linked recessive visibles to lethals induced by the mustard derivatives of an amino acid CB.3051, and amine CB.3034, and various carboxylic acids, CB.1348, 1331, 1356 and 1332

Experiment No.	Dose ($\times 10^{-2}M$)	Days after treatment	Lethal rate percent	Chrom. tested	Visibles v	Lethals l	Ratio v/l
CB.3051							
1	1.2	0-12	2.6	2,059	15	54	0.28
CB.3034							
1	0.19	0-21	3.2	3,268	20	105	0.19
2	0.29	0-21	4.0	3,271	19	130	0.15
3	0.38	0-21	5.4	1,660	16	89	0.18
4	0.77	0-12	11.4	211	4	24	0.17
5	1.15	0-12	19.1	68	3	13	0.23
Total				8,478	62	361	0.17
CB.1348							
1	0.2	0-12	3.0	1,665	4	50	0.08
2	0.6	0-12	7.4	653	5	48	0.10
3	0.6	0-12	5.6	1,128	4	63	0.06
4	1.0	0-12	13.0	453	4	59	0.07
Total				3,899	17	220	0.08
CB.1331							
1	0.3	0-6	3.2	620	3	20	0.15
2	0.7	0-3	3.6	358	2	14	0.14
3	0.7	0-3	4.6	261	..	12	...
4	1.0	0-3	6.5	309	2	20	0.10
Total				1,548	7	66	0.11
CB.1356							
1	0.5	0-12	1.9	1,504	4	29	0.14
2	0.7	0-12	4.3	1,220	5	52	0.10
3	1.0	0-18	3.0	1,706	7	52	0.13
Total				4,430	16	133	0.12
CB.1332							
1	0.3	0-12	1.5	1,105	2	17	0.12
2	1.2	0-3	2.0	294	..	6	...
3	1.6	0-3	3.9	258	1	10	0.10
Total				1,657	3	33	0.09

and it was possible to confirm the homogeneity of the above ratio in different experiments and its independence of dose and cell stage. In nine radiation experiments there was an over-all of 200 visibles to 978 lethals which when compared to the over-all for the phenylalanine mustard (last row, Table 1) proved to be very significantly lower ($\chi^2 = 22.98$ for one degree of freedom, $P < 0.001$).

The physical nature of the lethals: It is now firmly established that recessive lethals induced by the alkylating mutagens are associated with a lower frequency

of major chromosome aberrations than those induced at the same over-all rate by X-radiation (SLIZYNSKA and SLIZYNSKI 1947; BIRD and FAHMY 1953; FAHMY and BIRD 1953; FAHMY and FAHMY 1956). Even under the polyethyleneimine (TEM), which was shown to be more efficient than X-rays in the induction of chromosome breaks (FAHMY and FAHMY 1954, 1957b), a high proportion of these breaks was eliminated as dominant lethals, leaving a lower rate among viable sperm (FAHMY and FAHMY 1955a) and recessive lethals. On the other hand, the rate of induction of small aberrations (deficiencies and small deletions) under the alkylating mutagens was nearly double that occurring among X-ray lethals induced at the same over-all rate (mainly, FAHMY and FAHMY 1956, 1957b). Formaldehyde-food was also shown (SLIZYNSKA 1957) to induce a high frequency of moderate size deletions relative to other major rearrangements in the salivary chromosomes of the F_1 viable larvae. It is not known, however, whether this efficiency in the induction of deletions could be extended to the smaller deficiencies (five bands or less), nor to what extent these aberrations are recovered among the formaldehyde recessive lethals.

Differences occurred among the alkylating mutagens investigated as regards the proportion of lethals with major chromosome aberrations produced at mutagenically equivalent doses (FAHMY and FAHMY 1956). The most pronounced difference in this connection occurred between the polyethyleneimine (TEM) and the phenylalanine mustard. In lethal samples produced at practically the same over-all rate (8–10 percent), there were 20 percent gross rearrangements under TEM, as compared to two percent under the amino acid mustard. It was, therefore, of interest to determine whether comparable differences occur between mutagens of closer molecular structure. The cytological analysis was accordingly extended to a sample of lethals induced by the most active of the carboxylic acid mustards (p-NN-di-2-(chloroethyl)aminophenylbutyric acid), and the results were compared with those for the amino acid derivative (Table 4). The proportion of major structural rearrangements is significantly higher under the carboxylic mustard (χ^2 with continuity correction = 3.85 for one degree of freedom, $P = 0.05$). The frequency of the deficiencies, on the other hand, is approximately the same for the two mustards.

TABLE 4

Chromosome aberrations among sex-linked recessive lethals induced at roughly the same rate (8–10 percent) by the "mustard" derivatives of phenylalanine and phenylbutyric acid

Mutagen	Stocks examined	Gross rearrangements						Total stocks with chromosome aberrations		
		Trans.	Inv.	Del.	Total		Deficiencies		No.	%
					No.	%	No.	%		
Phenylalanine mustard	134			3	3	2.2	50	37.3	53	39.6
Phenylbutyric acid mustard	48	1	1	3	5	10.4	17	35.4	22	45.8

This is further evidence in favor of the hypothesis (FAHMY and FAHMY 1957b) that the induction of deficiencies by the alkylating agents is independent of the process of chromosome breakage, and is most probably due to gene inactivation *in situ*.

The loci of induction of recessive lethals along the X chromosome do not seem to differ under the mustard derivatives of phenylalanine as compared to the phenylbutyric acid. The distribution of the genetically placed lethals under these two agents and between the five localization markers are given in Table 5, and it is clear that they are almost identical ($\chi^2 = 0.40$, degrees of freedom = 4, $P > 0.98$). The distributions within shorter segments (three crossover units groupings) are given in Table 6. The small numbers in the individual cells necessitated the application of the ψ test (SMITH 1952) for the analysis of the correspondence of the two distributions and again revealed no significant difference ($P = 0.74$).

The cytologically detectable loci of chromosome damage (deficiencies and breaks) among the sex-linked recessive lethals induced by the phenylbutyric acid mustard were localized within the lettered subunits of the salivary X chromosome, and their distribution was compared with those of the phenylalanine mustard. The small lethal sample analysed for the phenylbutyric acid derivative, naturally meant a small number of detectable loci of damage and necessitated gross pooling for the statistical test. Table 7 shows the relative frequency of these loci for the two agents, pooled between the markers used in the genetical placings. The application of ψ test to the above data revealed no significant difference in distribution ($P = 0.54$).

In previous studies (FAHMY and FAHMY 1956) we have also demonstrated that no major differences occur in the distribution of the lethals, as well as the larger loci of chromosome damage, under the effect of the phenylalanine mustard, diepoxybutane and a polyethyleneimine (TEM). It would seem, there-

TABLE 5

The distribution of the sex-linked recessive lethals induced by the mustard derivatives of phenylalanine and phenylbutyric acid between the markers of the genetical localization

Segment	Phenylalanine mustard	Phenylbutyric acid mustard	Total	χ^2
<i>sc</i>				
0 -19.9	67	14	81	0.026
<i>ct</i>				
20 -32.9	42	8	50	0.133
<i>v</i>				
33 -56.6	73	18	91	0.201
<i>f</i>				
56.7-62.4	12	3	15	0.004
<i>car</i>				
62.5+	25	5	30	0.035
Total	219	48	267	0.399

TABLE 6
The distribution of the sex-linked recessive lethals induced by the mustard derivatives of phenylalanine and phenylbutyric acid among 24 segments of three crossover units each

Mutagen	Genetical map of the X chromosome																								Total
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
Phenylalanine mustard	37	8	1	5	9	4	8	11	5	13	8	8	10	4	9	3	7	19	19	1	11	4	10	5	219
Phenylbutyric acid mustard	10	1	1	1	1	1	1	2	3	3	2	2	2	1	1	1	2	8	2	2	1	2	2	1	48
Total	47	8	2	5	10	5	9	13	5	16	11	10	12	4	10	4	9	27	21	3	12	6	12	6	267

TABLE 7
The distribution of the loci of chromosome damage (breaks and deficiencies) among the sex-linked recessive lethals induced by the mustard derivatives of phenylalanine and phenylbutyric acid among the chromosome segments between the markers of the genetical localization

Genetical map	sc	ct	v	f	car	Total
Genetical map	0-19.9	20-32.9	33.0-56.6	56.7-62.4	62.5 +	
Cytological map	1A-7B _{1,2}	7B ₃ -9F ₁₃	10A _{1,2} -15E ₇	15F _{1,2} -19A _{3,6}	19B _{1,2} -centromere	
No. of bands	301	178	343	157	49	1,028
Loci of damage						
phenylalanine mustard	39	25	45	6	23	138
phenylbutyric acid mustard	6	4	7	1	8	26

fore, that the loci of lethal induction along the X chromosome are essentially the same under the effect of various alkylating mutagens.

The sites of induction of visibles and lethals: On the basis of the view that visibles and lethals are different phenotypic expressions of the same mutations, no differences in their sites of induction would be expected to occur. It has now been ascertained that the distribution of the lethals along the X chromosome is essentially the same under different alkylating mutagens. If the assumption of physical identity of the two mutant types is to be satisfied, the distribution of visibles should also be comparable among various alkylating mutagens and similar to that of the lethals, at least for the same agent. However, it has already been shown (FAHMY and FAHMY 1959a) that differences do occur in the relative distribution of visible alleles under different alkylating mutagens. There now remains to determine the relationship of the distributions of the visibles relative to the lethals under the same compound.

The data for the phenylalanine mustard are sufficiently extensive for the examination of the above problem. But, before any reliance can be placed on the relative distribution of the two mutant types, it is essential to determine how consistent is the distribution of each separately, particularly under different doses. In order to guard against the possible artifacts of placing in the linkage experiments, we confined the analysis to the distribution of the mutants among the five segments between the localization markers (*sc-ct-v-f-car-centromere*). The placed lethals (Table 8) were recovered from three concentrations: 1.5, 0.9

TABLE 8

*The distribution of the sex-linked recessive lethals induced by the mustard derivative of phenylalanine between the localization markers, under different concentrations.
(The expected numbers are given in brackets)*

Segment	Concentration ($\times 10^{-2}M$)			Total
	1.5	0.9	0.8	
<i>sc</i>				
0 -19.9	22 (23.2511)	18 (18.9680)	27 (24.7808)	67
<i>ct</i>				
20 -32.9	19 (14.5753)	11 (11.8904)	12 (15.5342)	42
<i>v</i>				
33 -56.6	26 (25.3333)	20 (20.6667)	27 (27.0000)	73
<i>f</i>				
56.7-62.4	1 (4.1644)	5 (3.3973)	6 (4.4384)	12
<i>car</i>				
62.5+	8 (8.6758)	8 (7.0776)	9 (9.2466)	25
Total	76	62	81	219

$$\chi^2_{[8]} = 6.46; P > 0.5.$$

and $0.8 (\times 10^{-2}M)$, and the numbers occurring in the various segments were reasonably adequate for the statistical analysis. The number of placed visibles from the above experiments, on the other hand, was much too small for the statistical tests, but a great many visibles were placed from other experiments. In order to make full use of all the data, and thus make the analysis more reliable, pooling of visibles from experiments at close doses was necessary. This was undertaken to correspond most closely to the concentrations inducing the lethals and in such a way as not to prejudice the possibility that lethal induction may be favored by stronger treatment. The experiments were, therefore, classified into three groups with the concentration ranges: 1.5–1.2; 1.1–0.9 and 0.8–0.5 ($\times 10^{-2}M$), and these were considered to be equivalent to the three doses inducing the lethals. The over-all visibles falling in the five marked segments for the three dose ranges are given in Table 9. The lethal data in Table 8 were subjected to a 3×5 contingency test and revealed complete homogeneity ($\chi^2 = 6.46$, degrees of freedom = 8, $P > 0.5$). The same analysis was applied to the visible data (Table 9) and also revealed no significant heterogeneity ($\chi^2 = 13.28$, degrees of freedom = 8, $P = 0.1$). It is, therefore, certain that the distribution of the lethals by themselves, and the visibles by themselves, among the five marked segments of the X chromosome are homogeneous and independent of the strength of the treatment. This distribution for each category of mutations, therefore, must be a function of the compound.

TABLE 9

The distribution of the sex-linked recessive visibles induced by the mustard derivative of phenylalanine, between the localization markers, under different concentrations.
(The expected numbers are given in brackets)

Segment	Concentration ($\times 10^{-2}M$)			Total
	1.5–1.2	1.1–0.9	0.8–0.5	
<i>sc</i>				
0 –19.9	36 (48.3852)	71 (66.2667)	35 (27.3481)	142
<i>ct</i>				
20 –32.9	25 (22.1481)	27 (30.3333)	13 (12.5185)	65
<i>v</i>				
33 –56.6	52 (46.3407)	66 (63.4667)	18 (26.1926)	136
<i>f</i>				
56.7–62.4	19 (13.9704)	14 (19.1333)	8 (7.8963)	41
<i>car</i>				
62.5+	6 (7.1556)	11 (9.8000)	4 (4.0444)	21
Total	138	189	78	405

$$\chi^2_{[8]} = 13.28; P = 0.1.$$

The lethal and visible data (Tables 8 and 9) were then statistically analysed together, to determine whether the distributions of the two mutant types differ relative to one another. This analysis was undertaken by YATES' (1955) recent technique for the combination of probabilities from a set of 2×2 tables. The frequency of mutants within each segment to those in the rest of the chromosome (for the lethals as well as the visibles at each dose) was compared in a 2×2 table, and a χ^2 for the deviation was determined. In this study, the direction, as well as the magnitude of the deviation, is important. χ , (i.e., $\sqrt{\chi^2}$), was therefore calculated with sign: +ve when the proportion of lethals was higher, and -ve when the excess was in favor of the visibles. In the combination test from all doses, the values of χ were algebraically summed, before the assessment of the significance of the overall trend. The summary of the statistical analysis is given in Table 10. A decisive heterogeneity in the relative distribution of the two classes of mutation occurs in the last two segments of the chromosome. A disproportionate excess of visibles is induced between *f-car*, whereas relatively more lethals occur beyond *car*.

The excess lethals in the proximal chromosome segment (*car*-centromere) is indeed most impressive and consistent. The experiments at the various dose levels show the deviation in the same direction (in favor of the lethals) and to roughly the same degree. This strongly suggests that the relative induction of lethals to visibles is independent of the strength of the treatment, at least within the dose range utilized (roughly threefold). In view of the narrowness of this range, however, it was thought appropriate to subject the above conclusion to a more stringent test, in an indirect way. If lethals are simply the more severe counterparts of visibles, then by comparing the proportion of lethals at the low doses with that of the visibles at the high doses, the difference should be concealed, or, at least, its magnitude reduced. The same applies when comparing the lethal proportion at the high doses with that of the visibles at the low doses. We, therefore, applied this double switch for the proportion of the lethals and visibles in the proximal

TABLE 10

The distribution of χ for the relative proportions of lethals and visibles induced by the mustard derivative of phenylalanine, between the localization markers, under different concentrations

Segment	Concentration ($\times 10^{-2}M$)			Total χ	No. of estimations	P
	1.5-1.2	1.1-0.9	0.8-0.5			
<i>sc</i>						
0 -19.9	0.4510	-1.2207	-1.4900	-2.2597	3	0.10
<i>ct</i>						
20 -32.9	1.1916	0.6589	-0.3206	1.5299	3	0.19
<i>v</i>						
33 -56.6	-0.5049	-0.3833	1.4353	0.5471	3	0.37
<i>f</i>						
56.7-62.4	-2.9950	0.1697	-0.6338	-3.4591	3	0.02
<i>car</i>						
62.5+	1.7493	1.8303	1.3748	4.9544	3	0.002

segment for the two extreme doses, leaving the data for the medium doses unchanged. With these new combinations, the sum of χ for the three estimates increased from 4.95 to 4.99, thus indicating an even greater significance for the relative excess in lethals.

A consideration of the over-all data (from all doses) brings out the phenomenon of differential response within the last two chromosome segments: *f-car* and *car-centromere*, most clearly (Table 11). The proportion of mutants proximal and distal to *f* (i.e., *sc-f* and *f-centromere*) is amazingly comparable for both the visibles and the lethals, giving a rough percentage ratio of 85:15. When the actual data for the distribution of the two classes of mutation between the above segments were compared in a 2×2 table, no significant difference was detectable ($\chi^2 = 0.27$, for one degree of freedom, $P \approx 0.6$). However, on examining the distribution of lethals and visibles between the two marked subsections proximal to *f* (i.e., *f-car* and *car-centromere*), a very major difference becomes immediately discernible. The lethal proportion is twice as high beyond, as before, *car* (5.5:11.4 percent), while the visibles show the reverse proportions (10.1:5.2 percent) within the same segments. When only the mutants that occurred beyond *f* were considered, and the relative frequencies of the visibles and lethals on either side of *car* were compared in a 2×2 table, a very significant difference was detected ($\chi^2 = 10.58$, for one degree of freedom, $P \approx 0.001$). There can hardly be any doubt, therefore, that a differential induction of visibles to lethals occurs proximal to *f*, with a relative excess of visibles in the chromosome segment *f-car*, and excess lethals in *car-centromere*.

DISCUSSION

The fascinating discovery of differential gene response to mutagens has already been discussed in detail (FAHMY and FAHMY 1959a). The phenomenon is

TABLE 11

The over-all distribution of the sex-linked recessive visibles and lethals induced by the mustard derivative of phenylalanine, showing the differential yield proximal to forked

Segment	Visibles v	Lethals l	Total	Proportion (percent)		P [χ^2]
				v	l	
<i>sc</i>						
0 -56.6	343	182	525	84.7	83.1	0.001 } 0.6
<i>f</i>						
56.7-62.4	41	12	53	10.1	5.5	
<i>car</i>						
62.5+	21	25	46	5.2	11.4	0.001 } 0.6
<i>f-centromere</i>						
56.7+	62	37	99	15.3	16.9	
Total	405	219	624	100.0	100.0	

amply manifested in the present data, by the variation of the ratio of visibles to lethals induced in the X chromosome even under chemically related mutagens. Foremost in this connection is the observation that the mustard derivative of phenylalanine induces three visibles to ten lethals, which was found to be significantly higher than the ratio of 1:10 induced by the majority of the other difunctional alkylating mutagens (FAHMY and FAHMY 1956, 1958a) and also higher than the ratio of 2:10 occurring with radiation. It cannot be overemphasized, however, that these ratios are mean values, based on large scale experiments. Particularly in the case of the phenylalanine mustard, consideration was given to variation due to optical isomerism, dose and cell stage, as well as to the uncontrollable experimental errors. As was demonstrated (Table 1), the mean value of this ratio is 0.32, with a standard deviation as estimated from the experimental data of ± 0.02 .

In contrast to our data on the phenylalanine mustard, there is the ALTENBURGS' (1959) single experiment yielding 27 lethals (with three presumed 'clusters') and no visibles, whereas on the basis of our mean ratio (3:10) a minimum of six visibles would be expected. Statistically such a deviation is significant. It is to be noted, however, that the above expectation has been estimated on the basis of the binomial distribution and completely ignores the experimental variation, which could be quite substantial due to difficulty in scoring visibles. Obviously the ALTENBURGS' single experiment cannot supply the range of their experimental variation, thus making their statistical test of little biological value. In our experiments, it is certain that the experimental error did not play a major role, as indicated by the small standard deviation of the mean as estimated from the whole data. Nevertheless, one of our atypical experiments (experiment 22, Table 1), which is most comparable in size and lethal rate to the ALTENBURGS', gave an anomalous ratio of 0.09, on the basis of which the absence of visibles in an experiment giving 27 lethals would be insignificant. This illustrates the danger of basing any conclusions as regards the induction of visibles on a single experiment with a low mutation rate. It is perhaps suggestive that the anomalous visible to lethal ratios in our experiments occurred with a faulty sample of the racemic mixture, which was partly inactivated by hydrolysis. This may indicate that the quality and purity of the ALTENBURGS' compound may have been substandard. Whatever the reason for the discrepancy of the visibles in these authors' experiment, it is highly unlikely to be the different method of scoring developed in our laboratory (FAHMY and FAHMY 1955b, see also MATERIAL AND TECHNIQUE). As already stressed (see OBSERVATIONS), even when visibles of low viability were classified as full lethals, the ratio of visibles to lethals under the phenylalanine mustard was not substantially decreased below the postulated 3:10 mean.

The significance of the variation in the ratio of visibles to lethals under different mutagens, has recently been discussed, on purely theoretical grounds, by both AUERBACH (1958) and the ALTENBURGS (1959). To these authors the classification of mutations into visibles and lethals is merely a matter of con-

venience, which is "neither strict nor biologically very meaningful" (AUERBACH 1958). They maintain that a "lethal" is a "visible" of very low viability. As a phenotypic description of the two types of mutation, this is perhaps tenable, since there is a continuous spectrum of viability from visibles through semilethals (including some visibles) to full lethals. This is, indeed, the reason why in our laboratory we confine the lethal class to mutations that exert *complete* killing in the hemizygous state, leaving the visible class to include morphological mutants irrespective of viability. In spite of the "merging" in the viability expression of the two mutant classes, however, it has long been known that differences do occur in their physical nature (as manifested in the salivary chromosomes). Lethals are often associated with chromosome aberrations, while visibles are mostly point mutations unaccompanied by such changes. This has been amply illustrated in the radiation mutagenesis work. In a sample of recessive lethals and visibles induced at specific X chromosome loci in the same series of X-ray experiments (DEMEREK 1937), nearly 40 percent of the lethals were shown to occur at one of the breakage points of major chromosome rearrangements, while none of the visibles were caused by this mechanism. Furthermore, viable "visible" changes in the white-Notch region (DEMEREK 1939) were not only devoid of major chromosome rearrangements, but also unassociated with small deficiencies which are known to occur most frequently in this region. The radiation mutability results at specific loci on the autosomes (third chromosome, WARD and ALEXANDER 1957) are closely parallel to the earlier results with the X chromosome. The fully viable visibles gave a percentage ratio of cytologically detectable aberrations to apparently normal of 20:80, while this ratio for the lethals was 60:40. Extensive mutability tests at various X chromosome loci under the effect of the phenylalanine mustard have now been undertaken (FAHMY and FAHMY 1959a), and the cytological analysis of the mutants produced is still in progress. Nevertheless, all the fully viable visibles so far observed proved to be point mutations, whereas the lethals were associated with deficiencies or major aberrations.

The above considerations show that the viability scale of the visible-lethal mutants induced in a given chromosome sample does bear some relationship to the physical nature of the genetic damage induced. The scale devised in our laboratory is to refer the frequency of the morphological mutants to those which produce extreme depression of viability so as to result in complete elimination of the hemizygous adults. If, on the basis of this classification, two mutagens induce different relative frequencies of the two classes of mutation, it would seem reasonable to infer that their mode of action on the hereditary material must be somehow different. The question then arises as to the exact mechanism responsible for this difference. The apparent association between chromosome breakage and recessive lethals led to the conclusion (mainly CATCHESIDE 1948) that these mutations are indeed breaks which either reconstitute or undergo exchange. Visibles, on the other hand, are assumed to be intragenic changes. On this basis, the visible to lethal ratio will be highest for mutagens with the lowest potency in chromo-

some breakage. The amino acid mustards are, indeed, the weakest breakers and they do give the highest visible to lethal ratio (3:10). On the other hand, the greatest majority of the other alkylating mutagens (imines, epoxides and carboxylic acid mustards) give a ratio of one visible to ten lethals, which was found to be lower than that given by radiation (2:10). This is so in spite of the fact that the majority of the above compounds were shown to induce significantly less major rearrangements among viable sperm as well as among recessive lethals than mutagenically equivalent doses of radiation. It would seem, therefore, that the ratio of visibles to lethals is not entirely controlled by the agent's efficiency in the induction of intra- to intergenic mutations though obviously this must be playing a partial role.

Another mechanism which affects the yield of lethals is the mutagen's potency in the induction of small deficiencies (less than one percent of the length of a salivary chromosome arm). Among sex-linked recessive lethals induced at the same over-all rate, the alkylating agents gave roughly the same proportion of small deficiencies which was double that for X-radiation. Yet, differences do occur in the ratio of visibles to lethals among the alkylating mutagens. Therefore, the efficiency in the induction of deficiencies is not the controlling factor in the variation of the visible to lethal ratio.

It is virtually certain, therefore, that no one physical mechanism for the production of lethals can be singled out, as the sole, or even the major, cause in the variation of the relative induction of visibles to lethals by different mutagens. This variation must be a function of the mutagen's ability to induce visibles as a class, relative to lethals as an integral group, irrespective of the latter's detailed constitution as regards chromosome breaks, deficiencies and point mutations. The only mechanism that can embrace the three physical causes for lethal production is that of genetic loss of microscopic or submicroscopic (i.e., molecular) dimensions. The alternatives for the visibles, would be either a lower degree of genetic loss, or an intramolecular reorientation within the genes without any loss of material. The fact that the ratio of visibles to lethals for any one compound is independent of dose (FAHMY and FAHMY 1955b; Present paper, Tables 1 and 3) argues against the possibility that the difference is entirely a matter of degree and favors a genuine qualitative difference between the two mutant classes.

The difference in the nature and loci of induction of visibles and lethals became abundantly clear through the study of their distribution along the X chromosome under the phenylalanine mustard. The distribution of mutants belonging to each class was consistent and independent of dose, but the relative distribution of the two mutant classes was decisively different. The differential response occurred mainly in the proximal part of the X chromosome (*f*-centromere). Within this segment the relative proportions of the two mutant types showed an excess of visibles distal to *car* (*f-car*) and an excess of lethals nearer the centromere (beyond *car*). It was further possible to show that the relative excess of the lethals beyond *car* was again independent of the strength of the

treatment. There is no doubt, therefore, that some gene sites do show a varied susceptibility to the induction of the two classes of mutation.

The excess in the relative yield of lethals compared to visibles close to the centromere (beyond *car*) may perhaps be attributed to its large heterochromatic content. Heterochromatin is relatively inert, and it would, therefore, be conceivable that the proximal segment contains few genes that can affect morphological characters (i.e., visibles). While this possibility cannot be entirely excluded, it does not seem to be supported by the data on the visible loci so far mutated. By radiation and a diversity of alkylating mutagens, an over-all of 73 sex-linked visible loci have so far been mutated beyond *f*. Of these, there were 40 between *f-car*, and 33 beyond *car*—a relative frequency which is not significantly different from equality ($\chi^2 = 0.67$, for one degree of freedom, $P > 0.3$).

Another feature that may be attributed to heterochromatin, is that it may consist of a less rigid variety of nucleoprotein, which is more vulnerable to damage by loss, thus favoring the induction of lethals. This phenomenon could certainly be ascertained by a consideration of the cytological nature of the lethals induced by the phenylalanine mustard (see Observations). The frequency of the loci of chromosome damage (breaks and deficiencies) can be taken as a function of the efficiency of gene elimination, while the number of salivary chromosome bands is perhaps the best estimate of genic content. Within the same sample of lethals analysed (Table 7) there were six loci of damage between *f-car*, compared to 23 beyond *car*, indicating a significant excess in the latter segment ($\chi^2 = 9.97$, for one degree of freedom, $P < 0.01$). This is so in spite of the fact that the physical length of the *f-car* segment includes 157 bands while the segment beyond *car* contains only 49 bands. Furthermore, the number of loci of damage detected beyond *car* is probably an underestimate since a great many rearrangements induced in segment 20 of the salivary X chromosome are apt to be missed because of the confused chromocentre. It is, therefore, certain that the frequency of the loci of damage relative to the gene content is very grossly higher beyond *car* than between *f-car*. The relative excess of lethals to visibles beyond *car*, therefore, could reasonably be attributed to the ease of gene elimination within the heterochromatin.

The excess in the proportion of visibles relative to lethals occurring in the *f-car* segment is indeed of great biological significance. The structure of this segment is exclusively euchromatic, or, at any rate, it is the freest from heterochromatin. This clearly shows that the differential induction of visibles to lethals is by no means restricted to the heterochromatic chromosome segments, but could also occur in the euchromatin. This differential gene response may well be brought about by a mechanism analogous to that inferred for the segment beyond *car*, only in the opposite direction. The genes within the *f-car* segment are probably more susceptible to molecular reorientation, rather than mutation by material loss.

The establishment that lethal and visible mutations are qualitatively different in nature and distribution along the chromosome introduces a problem as to

whether they are to be considered as occurring within the same or different genes. The recent discovery of the composite nature of the gene (BENZER 1956), both as regards structure and function, reduces any distinction between the hereditary units to the level of convenient terminology. Applying BENZER's nomenclature, lethals and visibles must now be looked upon as the manifestations of different mutons, since they could obviously involve different mutational sites. It is not known, however, whether these sites occur within the same or different functional subunits: cistrons, or even whether they belong to the same or different larger units: genes. Theoretically, it is conceivable that the genome of an individual could be differentiated into genes for the vegetative (metabolic) functions controlling viability: mutating to lethals, and others governing morphogenesis: mutating to visibles. In practice, however, it is difficult to be certain that any one gene does either of the above functions to the exclusion of the other. It would, therefore, seem convenient to think of visibles and lethals as different changes within the same genes. The differential yield of visibles to lethals, therefore, could best be visualized as resulting from reactions with various molecular receptors of the genetic material. An attack of a certain type results only in molecular reorientation sufficient to alter the transmission of the genetic code, thus resulting in a visible. Another type of attack is sufficiently different to result in the partial, or complete, elimination (or inactivation) of the gene, thus resulting in a lethal.

SUMMARY

The ratio of visibles to lethals induced in the same sample of treated X chromosomes shows significant variation even among closely related mutagenic compounds. For the same compound, however, this ratio is constant and independent of dose and cell stage. The distribution of each of the mutant classes under the same compound (the phenylalanine mustard) along the X chromosome is also constant and independent of dose. The relative distribution of the two mutant classes, however, is significantly different. Between *f-car*, the proportion of visibles induced is higher than that of the lethals, while beyond *car*, the excess is in the proportion of the lethals. The whole data indicate a qualitative difference in the physical nature of visibles and lethals. A hypothesis is formulated that visibles are the result of intramolecular reorientation within genes while lethals involve material loss on the genic or subgenic level.

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