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Induction of Delayed Mutations by Benzene and Ethylene Dibromide in Drosophila

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Two carcinogens, ethylene dibromide and benzene, were used to induce delayed (germinal mosaic) sex-linked recessive lethal mutations in spermotozoa and spermatids of adult Drosophila males. Significant numbers of delayed mutations (in F_3) were scored in absence of conventional (in F_2) mutations. A large proportion of nonlethal F_2 cultures carried delayed mutations, so much so thot, in some cultures, all F_2 females were carriers of mutotions. The mechanism through which single strand damage to treated X chromosomes can result in such deloyed lethals is discussed. These observations indicate thot the delayed mutation test should be used for testing the mutagenicity of environmental compounds, especially carcinogens, which tested negative in the conventional sex-linked recessive lethal mutotion test. The data will support the relotionship between mutagenesis and carcinogenesis and, also will further enhance the sensitivity of the Drosophila mutation assay.

Key words: Drosophila, mosaic, delayed, mutations, carcinogens

INTRODUCTION

Genetic tests using Drosophila have long been recognized as indicators of the potential of chemicals to cause heritable gene mutations and chromosome aberrations in animal germ cells. Among the several tests available in Drosophila, the sex-linked recessive lethal test (SLRL) is the most sensitive. If a chemical is negative in this test, the probability of its being a carcinogen is low. Nevertheless some very potent carcinogens have indeed been shown to be negative in this test and this reflects negatively on the utility of Drosophila in carcinogen prediction. Thus, after screening hundreds of chemicals, Foureman et al. [1994] conclude that the SLRL test is specific but not as sensitive. The test has been removed from the revised list by the EPA Office of Toxic Substances test scheme [Bentley et al., 1994]. However, the Drosophila literature documents examples where chemicals negative in the SLRL test performed in a conventional manner may still prove to be mutagens when tested for their delayed action [Aurbach, 1946, 1976; Lee et al., 1970; Shukla and Auerbach, 1979]. It is, therefore, possible that other compounds that tested negative may induce delayed mutations. Similarly, very low doses of mutagens or carcinogens found negative in the conventional SLRL test may produce delayed mutations which appear in the F_3 generation. We tested this hypothesis using ethylene dibromide (EDB) and benzene and report that this indeed is true.

MATERIALS AND METHODS

Canton-S strain was used for treatment and *Basc* was used for mutation detection. One-day-old adult males were kept in a 1,000 ml glass jar with a small aperture in the lid. For ethylene dibromide (EDB) treatment, 10 ml of saturated vapor was injected in the treatment jar. After 30 min the jar was ventilated in a hood and the males were transferred to fresh food for 1 day before first matings. The exposure was approximately 125 ppm as calculated for our previous EDB experiments [Kale and Baum, 1979]. No dose-frequency response was required and therefore precise dose was not a factor in these treatments.

The procedure was similar for benzene treatment except instead of saturated vapor, 0.1 ml liquid benzene was dropped on a blotting paper in the jar. Benzene is volatile and forms vapor immediately. After 1 hr the jar was ventilated. The males were transferred to fresh food and the survivors were stored for 1 day. We had used a similar exposure previously [Kale and Baum, 1983] and found that this amount of benzene generates 27,000 ppm by volume.

The treated males were individually mated to six *Basc* virgins for 2 days to obtain brood 1. Three such broods were cultured in a period of 6 days. The F_1 progeny was pair-mated separately for each male in order to identify clusters. The F_2 cultures were scored as usual for complete lethals. These data are given in Table I. In EDB experiments, F_2 non-lethal cultures were chosen randomly and from each of these, three to ten pair-matings were set up for scoring the delayed mutations in F_3 generation (Table II). In the case of benzene (Table III), a different experimental method was used. In order to test if any genetic (heritable) instability can be induced in some of the treated males, the mutation induction was followed in each serially numbered individual male for the three broods. Unlike EDB experiments where between three and ten pair matings were made from one random F_2 vial. in the case of benzene only one F_2 vial was chosen from each treated male. From this F_2 vial, three to twenty F_3 vials were cultured. As can be seen in Table III for spermatozoa, male No. 1 produced 65 F_2 pair-matings. None of these was a lethal. One of these was used to generate twenty pair-matings for F_3. For spermatids this male produced 21 F_2 vials and one of these was used to generate twenty F_3 vials. The purpose was to see if the same male produces delayed lethals in each successive brood. Indeed, this appears to be the case in males Nos. 2 and 7. The number of F_2 cultures

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TABLE I. Induced Sex-Linked Recessive Lethal Mutations in Three Successive 2-Day Broods From *D. melanogaster* Males Treated With EDB and Benzene

Germ cell stage	No. of— Males brooded	Males sterile	Chs. tested in F_2	Mutations in F_2	Percent mutations in F_2	No. of— Chs. tested in F_3	Mutations in F_3	Percent mutations in F_3
EDB								
Spermatozoa	24	2	816	***0***	***0***	2,738	29	1.06
Late spermatids	21	***0***	**1,251**	4	0.32	603	9	**1.49**
Early spermatids	20	3	637	**21**	3.29	1,659	101	6.09
Benzene								
Spermatozoa	40	14	1,967	3	**0.15**	483	**4**	0.83
Late spermatids	39	14	**1,046**	1	***0.09***	455	5	**1.09**
Early spermatids	38	20	**420**	***0***	0.00	162	2	1.23
Control								
Spermatozoa	24	***0***	591	1	0.16	482	***0***	0
Late spermatids	24	***0***	***580***	***0***	***0***	528	***0***	0
Early spermatids	23	***0***	707	**1**	0.14	616	1	0.16

TABLE II. Delayed Mutations (recovered in F_3) Induced by Ethylene Dibromide in Drosophila Spermatozoa and Spermatids* (Mutations; N = total number of chromosomes tested)

Spermatozoa			Late spermatids			Early Spermatids					
Brood 1 F_2 vial	No. of F_3 vials cultured N	No. of F_3 vials L	Brood 2 F_2 vial	No. of F_3 vials cultured N	No. of F_3 vials lethal L	Brood 3 F_2 vial	No. of F_3 vials cultured N	No. of F_3 vials lethal L	Brood 3 F_2 vial	No. of F_3 vials cultured N	No. of F_3 vials lethal L
1	7	7	1	6	***5***	1	10	10	19	3	1
2	6	6	2	7	**1**	2	9	9	20	10	1
3	**4**	4	3	10	1	3	**10**	8	21	7	1
4	10	**1**	4	9	1	4	7	7	22	10	1
5	10	1	***5***	8	1	5	10	6	23	10	1
6	**10**	1	6 to 76	563	***0***	6	10	6	24	10	1
7	7	1				7	***5***	***5***	25	**10**	1
8	10	1				8	10	***5***	26	10	1
9	10	**1**				9	5	4	27	10	1
10	**10**	1				10	10	4	28	10	1
11	**10**	1				11	10	4	29	10	1
12	**10**	**1**				***12***	10	3	30	10	1
13	7	1				13	10	3	31	10	1
14	**10**	1				14	**10**	3	32	10	1
15	8	1				15	**10**	2	33	10	1
16 to 313	2,609	***0***				16	10	2	34	10	1
						17	10	2	35 to 178	1,303	0
						18	10	2			
Total	2,738	29	76	603	9					1,659	101
Percent delayed mutations	1.6			**1.49**						6.09	

*L. lethal mutations; N, total number of chromosomes tested.

used (only one for each treated male) is small. However, the proportion of these cultures carrying a delayed lethal is quite high. In Table IV, we have summarized the data from **both** the chemicals and also provide the frequencies of F_2 cultures which carry delayed lethals.

We perform one control experiment every month. One of them coincided with the EDB and one with the benzene experiment. These data were used as concurrent controls. The frequency in these controls were not significantly different from each other or from our historical control (0.067%). The control data are given in Table I.

RESULTS AND DISCUSSION

In our previous work on chemical mutagenesis [Kale and Baum, 1979, 1983], we used both EDB and benzene. In the present investigation our intent was to use very low exposures in order that significant numbers of conventional SLRL mutations are not induced. This was

TABLE III. Complete (F_2) and Delayed (F_3) Mutations Induced by Benzene in Drosophila Spermatozoa and Spermatids*

	Spermatozoa				Late spermatids				Early spermatids			
	F_2		F_3*		F_2		F_3		F_2		F_3	
Male No.	N	L	N	L	N	L	N	L	N	L	N	L
1	65*	0	20	0	21	0	20	0	—	—	—	—
2	67	0	18	1	43	0	20	0	26	0	19	0
3	54	0	20	0	35	0	16	0	20	0	18	0
4	133	0	18	0	62	0	20	0	2	0	—	—
5	77	0	19	0	45	0	19	0	53	0	10	0
6	91	0	20	0	47	0	18	0	32	0	10	0
7	83	0	19	1	21	0	19	1	—	—	—	—
8	79	0	20	0	43	1	19	1	34	0	18	0
9	80	0	20	0	104	0	20	0	9	0	—	—
10	43	0	19	0	24	0	18	0	13	0	—	—
11	64	0	18	1	—	—	—	—	—	—	—	—
12	117	1	18	0	61	0	20	0	4	0	—	—
13	—	—	—	—	4	0	—	—	—	—	—	—
14	84	0	20	0	56	0	20	1	28	0	10	0
15	103	0	20	0	23	0	19	1	36	0	10	0
16	105	1	20	0	44	0	17	1	19	0	10	0
17	69	0	17	1	23	0	19	0	22	0	10	0
18	84	0	20	0	22	0	19	0	—	—	—	—
19	35	0	20	0	—	—	—	—	—	—	—	—
20	64	0	20	0	21	0	20	0	—	—	—	—
21	76	0	18	0	93	0	20	0	17	0	9	1
22	120	1	20	0	—	—	—	—	—	—	—	—
23	—	—	—	—	46	0	18	0	—	—	—	—
24	65	0	19	0	45	0	19	0	40	0	10	0
25	101	0	20	0	76	0	18	0	41	0	19	0
26	41	0	—	—	29	0	20	0	—	—	—	—
27	28	0	20	0	—	—	—	—	—	—	—	—
28	79	0	20	0	58	0	19	0	24	0	9	0
	1,967	3	483	4	1,046	1	455	5	420	0	162	2
Percent Mutations	0.15		0.82		0.09		1.09		0.0		1.23	

*L, lethal mutations; N, total number of chromosomes tested.
*Only one F_2 culture vial from these 65 was used from each treated male to generate a maximum of 20, F_3 cultures.

TABLE IV. Comparison of Conventional (F_2) and Delayed (F_3) Mutations Induced by EDB and Benzene in Drosophila Spermatozoa and Spermatids

	EDB			Benzene		
	Spermatozoa	Late spermatids	Early spermatids	Spermatozoa	Late spermatids	Early spermatids
No. of chromosomes tested in F_2	816	1,251	657	1,967	1,046	420
No. of complete (F_2) mutations	0	4	21	3	1	0
% complete (F_2) mutations	0.0	0.32	3.29	0.15	0.09	0.0
No. of chromosomes tested in F_3	2,738	603	1,659	483	455	162
Number of delayed (F_3) mutations	29	9	101	4	5	2
Percent delayed (F_3) mutations	1.06	1.49	6.09	0.83	1.10	1.23
No. of F_2 non-lethal cultures used to test delayed mutations	313	76	170	25	23	13
Number of F_2 non-lethal cultures carrying one or more delayed mutations	15	5	34	4	5	2
Percent F_2 cultures carrying one or more delayed lethal mutations	4.79	6.57	20.00	16.00	21.73	15.38

achieved in the first two broods of EDB and for benzene treated flies (Table I).

We have used our earlier experiments, which were especially performed to study the differential germ cell sensitivity [Kale and Baum, 1979], to relate the broods with germ cell stages. We equate brood 1 to treated spermatozoa, brood 2 to late spermatids, and brood 3 to early spermatids. Table I gives the F_3 mutation frequencies in the three cell types for both the compounds which appear to be quite high. However, these numbers should be kept in perspective for EDB since up to ten pair-matings were made from each F_2 culture. Therefore, the underlying detailed data are given in Table 11. A total of 313 randomly chosen F_2 cultures from treated spermatozoa produced 29 F_3 (delayed) lethals in 2,738 vials. Out of the 313 analyzed, 298 were non-lethal and fifteen were lethal. Of these 15, twelve (Nos. **4-15)** produced one lethal in ten F_3 cultures, whereas in the remaining three, all F_3 cultures were lethal (see Table 11). In the next two broods, especially in early spermatids, the frequency of such high numbers (from one F_2 culture) is very high, so much so that 18 F_2 cultures (Nos. 1–18) produced two or more delayed lethals. In several of these cases all F_2 females in the vial carried a delayed lethal which was scored as a full lethal in F_3. Table IV shows the proportion of F_2 cultures carrying one or more delayed lethals. These numbers are quite high (4.79%, 6.58%. and 20%) and demonstrate that a large number of conventional F_2 cultures may appear normal (non-lethal) but can indeed carry delayed lethals.

The results clearly indicate that **1)** low doses of EDB which do not induce conventional SLRL mutations do induce a highly significant number of delayed mutations in F_3 and 2) potent carcinogens such as benzene, which has been declared negative in the conventional SLRL test, do induce a highly significant number of delayed mutations.

There is evidence in the literature [Lee et al., 1967a, 1970; Shukla and Auerbach, 1979] demonstrating that delayed lethals can be detected in F_3 in the absence of significant numbers of conventional lethals in F_2. Thus Lee et al. [1967b] observed that incorporation of P^{32} in Drosophila spermatozoa resulted in significant increase in the frequency of complete mutations. Similarly, Shukla and Auerbach [1979] documented the delayed mutagenic action of hydroxylamine which produces mainly one-strand mutations. The sex-linked recessive lethal mutations in this experiment were scored in the usual way. In order to test for delayed lethals, as in the case of our experiments, one female from each of a large number of apparently lethal-free F_2 vials was mated to a Mueller-5 male and the progeny of this cross (F_3) was scored for lethals. The frequency of lethals in F_3 was clearly more than that in the control. According to the authors, the preponderance of delayed lethals after treatment with a substance like hydroxylamine strongly supports the suggestion that, in tests for potential mutagens in Drosophila, a negative or doubtful result in F_2 should be tested for delayed lethals in F_3. After all, if lethals are considered a genetic risk, it makes no difference whether they occur first as completes.

The mechanism underlying the induction of delayed mutations has been well illustrated by Jenkins [1975]. Lethal hits in both complementary DNA strands are required to induce a conventional SLRL mutation. However, if only one strand is hit and mutates, upon replication and division, two DNA molecules will be generated, one mutant and one non-mutant. Thus in an F_1 daughter of a treated male there are two kinds of cells: 1) cells with the mutant chromosome which are mutant, and 2) the cells with the non-mutant chromosome which are normal. In the next (F_2) generation, this female will produce wild type males and the culture will be scored as non-lethal even when several females in the culture carry a mutation. If tested for an additional (F_3) generation, cultures from these females will be scored as mutations. It is likely that weak mutagens and low doses of strong mutagens, as in the case of benzene and EDB, can induce single strand damage which can be scored only in the F_3 generation.

At higher doses, the probability of hitting both strands is more than at lower doses. This should result in lower frequency of delayed lethals at higher doses. Epler [1966] testing ethyl methanesulphonate tested the dose-response of complete and mosaic mutations induced in the X chromosomes of Drosophila. Indeed, the frequency of complete mutations increased linearly with the dose but the frequency of mosaics declined sharply at higher doses.

Although the Drosophila SLRL assay is being used successfully to predict carcinogenicity of chemicals, some potent carcinogens and other important environmental chemicals have been shown to be negative in this assay. This has resulted in the recent removal of the assay from the battery of short-term tests [Bentley et al., 1994]. However, this apparent insensitivity may be due to an inappropriate end-point chosen to test some chemicals. We have provided evidence that the conventional SLRL assay (carried up to the F_2 generation) fails to identify the mutagenicity of some potent carcinogens and weak mutagens unless the test is extended for one more generation. At present, our evidence includes only the two chemicals being reported here. It will be worthwhile to test additional so-called ''negative compounds" to re-establish the sensitivity of Drosophila in genotoxicity testing and carcinogen prediction.

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