Theoretical and Applied Genetics 39, 330–334 (1969)

Formaldehyde-Induced Mutations in *Drosophila melanogaster* in Dependence of the Presence of Acids

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Summary. The mutagenic activity of various combinations of formaldehyde, formic acid, acetic acid and hydrochloric acid was investigated by a sex-linked lethal test. All combinations were mutagenic and showed a mutation pattern from which it is concluded that in feeding experiments spermatocytes I are especially sensitive to the pairs of chemicals tested. In vapour experiments all germ cell stages were found to be susceptible.

The presence of volatile acids was found to be necessary for the mutagenic activity of formaldehyde in the vapour state. Mutagenic effects were also observed in larvel feeding experiments, in which only these acids were added to the medium. Experiments with stabilized pH at 7.5 did not show a significant mutagenic effect of formaldehyde.

It is postulated that the tested agents are catalase inhibitors, which promote the formation of peroxides or free radicals which interfere with DNA replication, thus producing mutations.

Introduction

The sensitivity of *Drosophila* germ cells to the mutagenic action of formaldehyde was described by RAPOPORT (1946) and confirmed by AUERBACH (1949, 1951, 1953, 1967), AUERBACH and MOSER (1953), KAPLAN (1948) and several other investigators (ALDERSON, 1960a, 1960b, 1961, 1964; KHISHIN, 1964; NAFEI and AUERBACH, 1964; SOBELS, 1956, 1958, 1962, 1963).

As briefly published earlier (STUMM-TEGETHOFF, 1964) we found that any mutagenic activity of formaldehyde depends on pH values below 7. Detailed experiments which include also the effect of impurities of commercial formaline are presented which support the earlier findings, and give information on the possible mechanisms of mutation induction.

Material and Methods

A standard strain of *Drosophila melanogaster*, Oregon-K (inbred for about 10 years) was used. This strain has a spontaneous mutation rate of 0.15% sex-linked lethals in males.

For *treatments with the vapour phase* of an agent, males were exposed for 24 hrs in specially designed bottles. The flies themselves were kept in small glass tubes (⌀: 2.5 cm), which were closed with gauze on the lower end and with cotton wool on the upper end (see figure 1). Samples of each of the agents to be tested (5 ml) were applied to the cotton wool in the bottom of the bottle (volume: 125 ml).

During *larval feeding experiments*, we used a nutrient medium consisting of 12 g of agar, 35 g of killed brewers yeast, 150 g of corn meal and 100 g of sucrose per 1000 ml distilled water. This medium was autoclaved, cooled, and supplemented with the chemicals to be tested. These were formaldehyde, formic acid, acetic acid and hydrochloric acid. The pH of the media varied from 4.2 to 5.6 depending on the added reagent. A 37% solution of formaldehyde of pH 7 was obtained by neutralization with calcium carbonate. To distinguish this formaldehyde from the commercial formaline (which is known to contain formic acid), it will be referred to as neutral "n"-formaldehyde, and the commercial formaldehyde as acid "a"-formaldehyde. Females were allowed to lay eggs for 24 to 48 hrs on the prepared medium, and were then discarded. Males, which spent their whole larval life on the medium, were collected 24 hrs after emergence (BIRD, 1952) and mated individually to two M-5 virgins.

To *test for sex-linked lethals* we employed the MULLER-5 technique (DEMEREC, 1948; SPENCER and STERN, 1948). About 50 treated males were individually crossed with one or two virgins of the M-5 strain (also inbred for about 10 years). Every third day the males were transferred to two fresh virgins in order to produce three successive broods.

All experiments were performed at a temperature of 24 °C (± 1 °C). 60–80% of the flies survive treatment with the tested agents.

In order to test the results statistically, we used the χ^2-test, the rank correlation method of KENDALL (1955), or a special test for mutation frequency of STEVENS (1942).

1. *Vapour Experiments*

The data obtained from vapour experiments are summarized in table 1. When males were treated 24 hrs after emergence with "a"-formaldehyde, formic acid, acetic acid, hydrochloric acid, or the combination of "n"-formaldehyde and the acids, all experiments produced mutations. The data were found to be significantly different ($p < 0.001$) from the spontaneous mutation rate of 0.15%.

For each chemical tested, no significant deviation in mutation frequency for sex-linked lethals could be found between the results obtained with each of the three broods (STEVENS, 1942). This result allows us to consider the mutation frequencies for the total of the three broods. If we compare these data, we find significant differences ($p \leqq 0.05$) when comparing "a"-formaldehyde with acetic acid; "a"-formaldehyde with hydrochloric acid; "a"-formaldehyde with "n"-formaldehyde + formic acid; and "a"-formaldehyde with "n"-formaldehyde + hydrochlo-

Table 1. *Vapour experiments*

treatment		brood							
substance	concentration	1st		2nd		3rd		total	
	%	n[1]	%[2]	n	%	n	%	n	%
nF	7.0	—	—	—	—	—	—	3803	0.16
aF	7.0	436	0.69	712	1.97	423	1.18	1571	1.40
FA	0.1	1068	1.12	1130	1.42	850	1.41	3048	1.31
AA	0.1	1703	0.82	1359	0.66	1270	1.10	4332	0.85
HA	0.01	1047	0.67	977	0.61	396	0.50	2420	0.62
nF + FA	7.0 0.1	1491	0.80	2198	0.82	1809	0.99	5498	0.87
nF + AA	7.0 0.1	786	1.02	1004	1.29	1020	1.18	2810	1.17
nF + HA	7.0 0.01	1263	1.03	1466	0.68	852	0.70	3680	0.79
mutation control	—	—	—	—	—	—	—	2584	0.15

[1] number of chromosomes tested. — [2] percentage of sex-linked lethals found.

abbreviations:

aF = acid formaldehyde
nF = neutral formaldehyde
FA = formic acid
AA = acetic acid
HA = hydrochloric acid.

All mutation frequencies are significant at the $p < 0.001$ level when compared with the non-treated control.

ric acid. However, no significant differences were found in the comparison of "a"-formaldehyde with "n"-formaldehyde + acetic acid; "a"-formaldehyde with formic acid; and acetic acid with hydrochloric acid. It may be concluded that all germ cell stages can be influenced by "a"-formaldehyde and all the tested acids. It has been shown already in earlier investigations (STUMM-TEGETHOFF, 1964) that "n"-formaldehyde causes no mutation in vapour experiments.

2. *Larval Feeding Experiments*

It could be argued that the acid vapour treatment may interfere with the normal ventilation of the tracheal system. Therefore experiments were carried out with the mutagenic substances in the feeding substrate. As seen from table 2 all tested substances have a significant increased mutagenic effect compared with the control.

In contrast to the results of the vapour experiments the highest mutation rate of sex-linked lethals in most of the larval feeding experiments were found in the second brood. The significance of these results was determined by the rank correlation method. With 0.05% "n"-formaldehyde and 0.05% "n"-formaldehyde + formic acid, however, the highest frequency of mutation was found in the first brood. Within individual experiments there were no significant differences between the broods, as shown by the test of STEVENS (1942), except in the cases of 0.05% "n"-formaldehyde and 0.05% "n"-formaldehyde + formic acid. Therefore, totals were compared as in the vapour experiments, revealing no differences between the tested agents except for acetic acid and hydrochloric acid, which were less effective ($p < 0.05$).

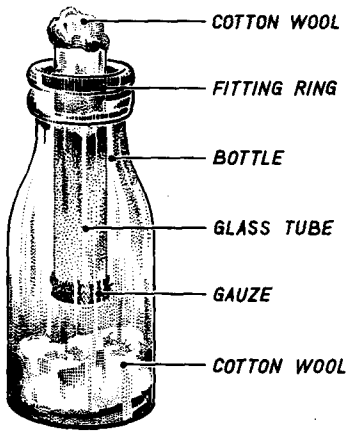

Fig. 1. Bottle for vapour experiments with *Drosophila*

The finding that "n"-formaldehyde is as mutagenic as "a"-formaldehyde for larvae, contrasts with the results of the vapour experiments. The pH of "n"-formaldehyde is 7. An explanation could be given by the following experiments.

When hatched larvae were transferred every day to fresh nutrient medium which was adjusted to pH 7.5 with glycine-NaOH buffer (table 3), no mutation was found in the second brood for 0.025% formaldehyde, and one mutation (0.27%) was found for 0.05% formaldehyde.

In another experiment with 0.1% formic acid in the food, but stabilized on pH 7.5 a mutation rate

Table 2. *Larval feeding experiments A*

treatment		brood							
substance	concentration	1st		2nd		3rd		total	
	%	n[1]	%[2]	n	%	n	%	n	%
nF	0.025	910	0.77	717	1.40	737	0.68	2364	0.93
nF	0.05	1266	1.26	1503	0.33	1601	0.31	4370	0.59
aF	0.025	1287	0.70	1213	0.91	1089	0.73	3589	0.78
aF	0.05	754	1.06	802	1.75	901	0.78	2457	1.18
FA	0.1	786	1.15	522	1.34	571	0.88	1879	1.11
AA	0.1	981	0.61	1007	0.60	1157	0.43	3145	0.54
HA	0.01	757	0.40	668	0.75	800	0.50	2225	0.55
nF + FA	0.025 0.1	839	0.95	857	1.17	587	1.19	2283	1.09
nF + FA	0.05 0.1	981	1.33	1352	0.96	1574	0.76	3907	0.97
nF + AA	0.025 0.1	716	0.98	782	1.28	652	0.77	2150	1.02
nF + AA	0.05 0.1	770	0.91	840	1.79	1092	1.10	2702	1.25
nF + HA	0.025 0.01	478	1.05	594	1.18	548	0.91	1620	1.05
nF + HA	0.05 0.01	446	2.02	514	1.95	447	1.12	1407	1.71
Mutation control	—	—	—	—	—	—	—	2584	0.15

[1] number of chromosomes tested. — [2] percentage of sex-linked lethals found.

[1] abbreviations:

aF = acid formaldehyde
nF = neutral formaldehyde
FA = formic acid
AA = acetic acid
HA = hydrochloric acid.

All mutation frequencies are significant at the $p < 0.001$ level when compared with the non-treated control.

Table 3. *Larval feeding experiments B*

substance	concentration	pH of the medium	tested chromosomes	mutations found	mutation frequency
nF	0.025%	7.5	398	0	0.00%
nF	0.05%	7.5	366	1	0.27%
FA	0.1%	7.5	544	2	0.38%
Mutation control	—	7.0	2584	4	0.15%

For these experiments a special prepared medium of pH > 7 was used (see "Materials and Methods"). Only the 2nd brood was analyzed. Concentration of the glycine-NaOH buffer: 0.1 M. The mutation frequency shows no significant deviation from the (untreated) control (0.15%) in contrast to the results obtained by testing the same substances in an acid medium.

of 0.38% was observed in the second brood. These data are not significantly different from the spontaneous mutation frequency.

Comparing the results of the feeding experiments one can conclude that there is a positive correlation between the low pH of the nutrient medium and the mutation frequency over all broods.

Discussion

AUERBACH (1951), KAUFMANN and GAY (1963) and KHISHIN (1964) proved that during larval development spermatogenesis stops with spermatocytes I. Therefore, only this type of germ cells (plus spermatogonia) is capable of reacting with mutagenic agents present in the larval food. These findings are in agreement with our results from larval feeding experiments. No clusters of identical lethals from the same male have been found. Therefore, with great probability, only spermatocytes I are influenced (AUERBACH, 1953; SOBELS, 1954). In comparison with the controls, all broods showed significant higher mutation frequency.

The highest mutation rate occurred in the larval feeding experiments (table 2) in the second brood, which is not in accordance with the data from AUERBACH and MOSER (1953), who found the highest

mutation frequency in the first brood. This is perhaps due to a difference in sensitivity of our standard strain and to the fact that our larvae remained their whole larval lifetime on the treated medium (older larvae incorporate more of the substances than younger ones). All these explanations assume that the males mate only once with the M-5 females within one brood, as suggested by MANNING (1962).

The fact that AUERBACH (1949) did not succeed in mutation induction with formaldehyde vapour is possibly due to a higher concentration employed, which did not allow a longer time of treatment than 2 hrs.

During the larval feeding experiments, it was observed that addition of neutral formaldehyde to the nutrient medium caused a pH decrease, thus producing a mutagenic effect similar to that obtained with "a"-formaldehyde. We suggested earlier (STUMM-TEGETHOFF, 1964) that a part of the formaldehyde is oxidized to formic acid, which is also mutagenic.

In the larval feeding experiments, and in some of the vapour experiments, the most sensitive germ cell stages are the spermatocytes I. Probably, DNA replication in these cells is disturbed by the agents (NAFEI and AUERBACH, 1964).

SOBELS (1954, 1958, 1963) suggested that the formation of peroxides was the cause of mutations, a result which is confirmed by other investigators for lower organisms (DICKEY, CLELAND and LOTZ, 1949; JENSEN, KIRK, KOLMARK and WESTERGAARD, 1951). Normally, *Drosophila* larvae contain catalase of high activity. Inhibition of catalase by acids or acidic formaldehyde solutions must lead to an accumulation of mutagenic peroxides. If acids and combinations of formaldehyde with these acids are used in vitro (STUMM-TEGETHOFF and VAN DER LOO, 1969), the resulting catalase inhibition shows a good correlation to the mean mutation frequency obtained in vivo. These findings suggest that, as a consequence of catalase inhibition, peroxides and free radicals are responsible for the mutation. In this regard, BUTLER and CONWAY (1953) proved that OH-radicals are able to break the connection between sugar and phosphate or sugar and base in DNA, and that they are able to oxidize the sugar or the base of nucleosides. In addition, FREESE (1961) found in T-phages that the formation of transversions or transitions by depurinating agents is dependent on a low pH, and can be imitated by a buffer of the same low pH. There may be some relationship between these reports and our results. The absence of a mutagenic effect in the experiments with a buffered medium of pH 7.5 can also be due to the formation of a glycine-formaldehyde complex.

With regard to the larval feeding experiments and the results obtained from the 3rd brood of the vapour experiments, formaldehyde and/or acids may inhibit catalase, as a consequence peroxides and free radicals accumulate; by this process DNA synthesis is disturbed, which leads to mutation. Mutations occurring in the 1st and 2nd broods of the vapour experiments may occur as a consequence of a disturbance of chromosome segregation during meiosis, thus producing repeats and deletions. Other theories of the mutagenic activity of formaldehyde are, however, also compatible with our findings (ALDERSON, 1960a, 1960b, 1961, 1964, 1967).

Acknowledgements

I am indebted to Miss L. VAN SCHELTINGA for her skilled assistance during the course of the experiments. Thanks are also due to Prof. Dr. S. J. GEERTS for critically reading the manuscript and to Prof. Dr. A. SCHWARTZ for checking the English text.

Zusammenfassung

Mit Hilfe des Muller-5-Tests wird bei *Drosophila melanogaster* die mutagene Aktivität von Formaldehyd, Ameisensäure, Essigsäure und Salzsäure allein und in verschiedenen Kombinationen von Formaldehyd mit den Säuren geprüft. Alle Kombinationen sind mutagen. Da keine Mutagenität mehr nachweisbar ist, wenn die Fliegen mit Formaldehyd mit einem pH $\geqq 7$ behandelt werden, ist anzunehmen, daß die mutagene Wirkung von Formaldehyd von einem sauren Milieu abhängig ist. Die untersuchten Substanzen werden den Tieren entweder in der Gasphase oder durch Beimischung zum Nährboden verabreicht. Im ersten Fall werden alle Keimzellstadien beeinflußt, während die Applikation im Nährboden nur die Spermatocyten I angreift. Der Wirkungsmechanismus der untersuchten Stoffe wird als eine Katalase-Hemmung gedeutet, die zur Bildung von u. a. freien Radikalen und damit — über Störung der DNS-Synthese — zum Entstehen von Mutationen führt.

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Received August 1969

Communicated by H. F. LINSKENS

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