THE MUTAGENICITY OF FORMALDEHYDE MEDIUM TO *DROSOPHILA MELANOGASTER* LARVAE

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Summary

There is a linear decrease in the mutagenicity of formaldehyde medium with aging, whether it is preworked or not.

There is little or no decrease in the concentration of formaldehyde in the medium from preparation to 7 days later.

It is suggested that formaldehyde itself is the mutagen, but that it forms a reaction product with some food constituent, though presumably not with protein. This compound probably breaks down in the larvae, releasing mutagenically active formaldehyde.

I. Introduction

Auerbach (1953), Auerbach and Moser (1953), and Herskowitz (1954) have based their ideas on the mutagenic action of formaldehyde medium on the expectation that the mutagenicity would be zero after 24 hr working by larvae. However, Barker and Davern (1956) showed that 0·1 per cent. formaldehyde medium (dead yeast fortified) remains mutagenic for at least 10 days, and that there is a linear decrease in the mutagenic response with increased preworking period. Further, they suggested that this decreased response with time may be merely an effect of aging of the food. This possibility has been tested and results are presented in this paper.

Additional evidence has been obtained by chemical estimation of the formaldehyde concentration in the medium at intervals after its preparation.

II. Materials and Methods

The experimental material was the Oregon-R-C stock of wild-type *Drosophila melanogaster*. All cultures were maintained at 25±1°C, except when removed from the constant-temperature room for short periods for mating and inspection. The flies were kept on dead yeast fortified medium and all cultures were seeded with live yeast. The frequency of sex-linked recessive lethal mutations was used as an index of mutation rate. These were detected by use of the Muller-5 stock; F_2 vial cultures were scored as lethals when no wild-type males were present. Vials with less than 20 flies or 10 males were discarded from the score.

To prepare the formaldehyde medium, the measured amount of C.P. formaldehyde (36 per cent. w/v HCHO) was thoroughly stirred in when the boiled medium

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cooled to about 60°C. The amount added was calculated to give 0·1 per cent. formaldehyde as volume percentage of the liquid medium. The mutagenicity of this medium was determined after it had remained at 25°C for 0, 2, 4, 6, and 8 days. The procedure used was that of experiment 4 of Barker and Davern (1956).

For the chemical estimation, 0·1 per cent. formaldehyde medium was prepared. The first analysis was done as soon as the medium solidified. The remaining medium was maintained in $\frac{1}{4}$-pint culture bottles at 25°C, and the analysis was repeated daily for 7 days. Each day's estimation was duplicated (taking two bottles at random), using the following procedure. A sample of 1 g of medium was homogenized in 80 ml of distilled water. This suspension was divided into two 40-ml portions, of which one was made to 50 ml with distilled water and the other acidified to pH 1 with 2N sulphuric acid, then brought to 50 ml. Both solutions were then filtered. The non-acidified medium will give free formaldehyde and the acidified free formaldehyde plus formaldehyde bound to protein. Two 0·5-ml aliquots of each solution were taken and 5·0 ml chromotropic acid reagent (MacFadyen 1945) added to each. These were heated for 30 min on a boiling bath and the optical densities then read at wavelengths of 520, 575, and 615 mμ. These wavelengths were determined by deriving the absorption curve, where maximum absorption was observed at 575 mμ, while at 520 and 615 mμ absorption was one-half the maximum. A colour correction for irrelevant absorption was carried out on the optical densities at these wavelengths (Morton and Stubbs 1946). The corrected optical density (O.D.) was taken as $2\times$(O.D. at 575 mμ)$-$(O.D. at 520 mμ+O.D. at 615 mμ). The corrected optical densities were converted to μg formaldehyde per ml of medium solution by comparison against readings from a stock formaldehyde solution. Preliminary tests of the estimation method showed that the recovery of formaldehyde is satisfactory. This was done by adding a known amount of formaldehyde solution to the media solution immediately before heating in the boiling bath.

III. Results

The effects of aging fortified formaldehyde medium on the mutagenic response are shown in Table 1, where the results of Barker and Davern (1956) for preworked fortified formaldehyde medium are included for comparison. The values for angle response in the two missing plots were fitted by regression analysis.

Analysis of variance of the results (see Table 1) showed no significant differences between days or between treatments and the linear interaction term was not significant. The linear mean square between days is significant when tested against the theoretical variance, but is not when tested against the non-linear interaction as error. The regression coefficient (b) of mutagenic response on days is $-0{\cdot}702$, where b is the change in angle response per day. Since this is similar in magnitude to that found by Barker and Davern (1956), it is reasonable to infer that there is a negative regression of mutagenic response on days, but that it is the same whether the medium is worked or not.

The results of the chemical analyses of formaldehyde media are shown in Table 2. Each day the acidified and non-acidified media were compared using

samples derived from the one bottle. Since these samples, therefore, are not independent, it would be expected that the error associated with such comparisons is smaller than that associated with comparison of bottles. The analysis of variance therefore follows the split-plot form (Cochran and Cox 1957), the days differences

TABLE 1

LETHAL MUTATION FREQUENCIES INDUCED BY 0·1 PER CENT. FORMALDEHYDE WHERE THE MEDIUM AGED WITH AND WITHOUT LARVAL WORKING

Treatment	Data	Period of Aging (days)					
		0	2	4	6	8	10
Medium worked by larvae*	No. of lethals/total tested	27/228	7/274	—	16/503	24/817	23/781
	Percentage lethals	11·842	2·555	—	3·181	2·938	2·945
	Angle response	20·16	9·155	(12·600)	10·272	9·882	9·896
Medium not worked by larvae	No. of lethals/total tested	15/279	12/211	7/201	4/153	3/122	—
	Percentage lethals	5·38	5·69	3·48	2·61	2·46	—
	Angle response	13·39	13·80	10·72	9·26	8·97	(7·214)

Analysis of Variance

Source of Variation	Degrees of Freedom	Mean Square
Between days	(5)	
Linear	1	69·016
Remainder	4	4·513
Between treatments	1	6·179
Interaction	(3)	
Linear	1	0·154
Remainder	2	16·832
Theoretical variance†	∞	2·30

*Results of this treatment are from Barker and Davern (1956).

†Theoretical variance = $820{\cdot}7/n$, where n = average group size = 356·9.

being tested against one source of error and the media differences against another. Analysis of variance (see Table 2) shows that there are significant differences between bottles within days and between media, but that there is no difference between days. This suggests there is little or no loss of formaldehyde from the medium for

at least 8 days. However, there is considerable loss during preparation. The medium was prepared to contain 1083 μg formaldehyde per ml of media. This is equivalent to the 0·1 per cent. concentration used in the mutation tests. With a 1/100 dilution, the estimation solution will be expected to contain 10·83 μg per ml. The results in Table 2 show that an average of 40 per cent. of the added formaldehyde is lost during medium preparation.

TABLE 2

CHEMICAL ANALYSIS OF SAMPLES OF MEDIA TAKEN DAILY FOR 8 DAYS

Results given as μg formaldehyde/ml 1/100 media solution

Medium	Bottle No. (two replications)	Days							
		0	1	2	3	4	5	6	7
Acidified	1	5·66	—	7·22	7·16	6·36	6·74	6·62	5·82
		5·72	—	6·64	6·86	6·42	6·60	6·34	6·06
	2	7·10	—	7·46	7·08	6·64	6·24	6·88	6·76
		7·00	—	7·38	6·90	6·58	6·42	7·38	7·24
Non-acidified	1	5·36	—	6·58	6·70	5·74	6·20	5·60	5·52
		5·84	—	6·82	6·94	6·18	6·60	6·80	5·88
	2	6·74	—	7·16	6·80	6·00	6·40	6·20	6·64
		6·72	—	7·42	6·82	5·88	6·18	6·60	6·68

Analysis of Variance

Source of Variation	Degrees of Freedom	Mean Square	F
Between days	6	0·8876	1·001
Between bottles within days (error A)	7	0·8868†	
Between media	1	1·2244	18·439***
Media × bottles within days	7	0·0276	
Media × days	6	0·0540	
Between replicates (error B)	28	0·0664†	

*** $P<0·001$. † $F = 13·355$***

The acidified solution contains significantly more formaldehyde than the non-acidified. However, the average difference (i.e. the protein-bound formaldehyde) amounts to only about 5 per cent. of the total. Thus there is very little loss of formaldehyde from the media with storage, even though only a small fraction is protein-bound. It is possible that some formaldehyde may be bound to food constituents other than proteins.

IV. Discussion

The results show two contrasting features. First, there is little or no decrease in the concentration of formaldehyde in the medium from preparation to 7 days later. Secondly, there is a linear decrease in the mutagenic effectiveness of the medium with aging, whether it is preworked or not. These results need to be related to ideas on the mode of action of formaldehyde.

There are four ways of mutagenic action of formaldehyde when taken into the body via the digestive tract of larvae:

(i) Free formaldehyde is the mutagen that is taken into the body and reaches the gonads as such. This is unlikely in view of the high reactivity of formaldehyde.

(ii) Formaldehyde is itself the mutagen but it forms a labile compound with some food constituent and is released either in the gonads or even in the gonadal cells themselves. Auerbach (1951) suggested this mode of action and further suggested that protein was the food constituent involved. The results of Alderson (1957) certainly indicate that formaldehyde is itself the effective mutagen.

(iii) Formaldehyde may react with a food constituent when it is added to the medium at about 60°C to produce the mutagen.

(iv) The mutagen may be formed by some reaction in the body either during digestion or in the germ cells themselves.

The second of the above hypotheses is the simplest and the most attractive. Auerbach (1952) showed that 0·3 per cent. formaldehyde solution injected into adult males gave rise to 3–4 per cent. sex-linked lethals, while Herskowitz (1955) obtained 2·2 per cent. sex-linked lethals following a sperm bath with 2·0 per cent. formaldehyde solution. By immersing pupae for 3 hr in a 10 per cent. aqueous solution of formaldehyde, Khishin (1956) obtained 1·33 per cent. sex-linked lethals. In these cases, formaldehyde itself is most likely to be the mutagen. On the other hand, a 0·2 per cent. concentration of formaldehyde in the medium gives an average of 6–7 per cent. sex-linked lethals. Further, the direct methods of formaldehyde application have a different sensitivity pattern to that obtained from formaldehyde in the larval food. Therefore, even though formaldehyde itself is probably the mutagen in both cases, differences in the mutagenic mechanisms are indicated.

Auerbach (1951) has suggested that the formaldehyde in the feeding method is reversibly bound to protein, being released either into the food, or the digestive tract, or even the germ cells themselves. However, chemical analysis of the medium has shown that only about 5 per cent. of the formaldehyde in the medium is protein-bound. This does not mean that this is not the effective mutagenic fraction but, if it is, the difference between the feeding method and direct application methods in their mutagenic effectiveness becomes even more striking. On the other hand, 40 per cent. of the formaldehyde added to the medium is not accounted for as free or protein-bound formaldehyde immediately after medium preparation. Some of this loss is due to vaporization but it is probable that most reacts with a food constituent to form a new compound. This may break down later to reform formaldehyde.

The mutation tests have shown that the rate of decrease of mutagenicity of formaldehyde media is the same whether it is preworked or not. Thus there is a decrease in mutagenic effectiveness with aging even though the chemical analysis shows there is little or no decrease in the formaldehyde concentration in the same period of time. If formaldehyde is the mutagen, one would expect the decrease in mutagenicity to be due to its loss from the medium. There is no simple reason why the formaldehyde should decrease in efficiency with no concurrent decrease in its concentration. This may mean that formaldehyde forms a reaction product in the food with something other than protein. This compound may break down with aging, releasing free formaldehyde into the food at about the same rate as it is lost by vaporization. If this is the case, then one cannot determine the real concentration of mutagenically active formaldehyde in the medium.

The results here support the conclusion of Barker and Davern (1956) that formaldehyde food does not lose its mutagenicity in 24 hr. However, Auerbach (1956) has shown that the larvae develop some sort of tolerance to the mutagenic activity of the medium in which they develop and that this tolerance develops within about 24 hr. However, this medium remains mutagenic to previously untreated larvae, while if treated larvae are transferred to fresh food, their mutation rate is further increased. It would be interesting to see if this latter occurs when treated larvae are transferred to a different vial of worked medium.

The development of this larval tolerance may help to explain the results of Herskowitz (1954). In this case, individuals 0–12 hr old (counting from time of egg laying) showed higher mutation rates than individuals 12–24 hr old. Both these groups are probably subject to the same mutagenic action for the same period of time. That is, 12–24-hr individuals will be freshly emerged larvae that will be subject to mutagenic action for 24 hr or so. Similarly, the 0–12-hr individuals will be subject to mutagen for this period when they emerge. Because of decline in the mutagenicity of the medium with time, one would expect the latter to show a lower mutation rate. The difference between these expected results and the actual ones is probably due to the method of treatment—adding an aqueous solution of formaldehyde to the top of the food in each bottle. This formaldehyde may not diffuse equally through the food in all bottles so that the results obtained could be quite fortuitous.

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