THE MUTAGENIC ACTION OF FORMALDEHYDE ON BACTERIA[1]

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Rapoport (1946), Kaplan (1948), and Auerbach (1949) found formaldehyde highly mutagenic for *Drosophila* when administered with its food. Auerbach (1949), however, observed negative results when *Drosophila* was exposed to formaldehyde vapor and concluded that formaldehyde acts indirectly through chemical changes brought about in food constituents. Dickey, Cleland, and Lotz (1949) found formaldehyde and a mixture of formaldehyde and hydrogen peroxide mutagenic for *Neurospora crassa*.

This paper will report investigations on the mutagenic effect of formaldehyde on bacteria.

MATERIALS AND METHODS

The mutation principally studied was that of *Pseudomonas fluorescens*, strain A3.7, wild type, I−, to a mutant, I+, which can use, as the sole source of carbon for growth, itaconic acid (methylene succinic acid), a substance not used by the parent strain.

Experiments were also conducted on the mutation of *Escherichia coli*, strain B, to resistance to phage T1. Cultures of *E. coli* and phage T1 were obtained from Dr. S. E. Luria. A phage suspension containing 2×10^{11} phage per ml was prepared by Dr. Dean Fraser.

The media used in experiments with *P. fluorescens* have been described (Englesberg and Stanier, 1949). All cultures were incubated at 30 C.

In the experiments with *E. coli*, Difco nutrient agar, with or without the addition of 1.4×10^{-4} per cent of gentian violet, was used as a plating medium. Cultures were incubated at 37 C.

Two different preparations of formaldehyde were used: a commercial product (C. P. Baker) and one made by refluxing a solution of trioxymethylene by the method of Walker (1944). The exact amount of formaldehyde present was determined by the sodium sulfite method (Walker, 1944). The mutagenic effects of both preparations were similar.

Experiments with P. fluorescens. Aliquots (0.5 or 1.0 ml) of a washed suspension of *P. fluorescens* in M/13, pH 7 phosphate buffer, were distributed into 2 test tubes, which were then placed in a water bath at 30 C. Two-tenths per cent formaldehyde at 30 C was pipetted into 1 tube to yield a final concentration of

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0.1 per cent. To the other tube the same amount of distilled water was added. Ten ml of buffer were added to both tubes 15 minutes after the formaldehyde had been added. The tubes were then centrifuged, the supernatant was discarded, and the cells were resuspended in 2 ml of buffer. (In several experiments this washing procedure was repeated without any change in results.) The total number of viable cells in each tube was determined by spreading diluted cell suspensions on the surface of yeast extract agar plates, while the number of I+ mutants was determined by spreading undiluted suspensions on 5 itaconate agar plates.

Results are presented in terms of: (1) the absolute increase in the number of mutants per 10^8 cells (the average number of mutants per 10^8 exposed cells minus the average number of "spontaneous mutants per 10^8 viable untreated cells) and (2) the relative increase in the number of mutants per 10^8 cells (the average number of mutants per 10^8 viable formaldehyde-treated cells minus the average number of "spontaneous" mutants per 10^8 viable untreated cells).

As a result of the limited growth of phenotypically I− cells on itaconate plates, this method of assay can be used only to determine "end point" mutations.

Experiments with E. coli. The general procedures of treating *E. coli*, strain B, with formaldehyde and representing results were similar to those described for *P. fluorescens*. "Zero point" mutations were determined by the method of Demerec (1946) and "end point" mutations by Beale's modification (1948) of Demerec's aerosol technique. A sixfold excess of phage particles was employed (Beale, 1948).

RESULTS

The Mutagenic Effect of Formaldehyde on P. fluorescens

I+ mutations induced by 15-minute exposure to 0.1 per cent formaldehyde. The results of a typical experiment in which I− was exposed to 0.1 per cent formaldehyde for 15 minutes are presented in table 1. There was an absolute increase of 164 I+ mutants per 10^8 cells exposed, and a relative increase of 9.0×10^5 I+ mutants per 10^8 surviving viable cells, with 0.02 per cent survivors.

In order to account for the lag in growth produced by formaldehyde action (table 1), yeast extract agar plates containing formaldehyde-treated cells were incubated for 72 hours before colony counts were made. This growth lag was also evident on itaconate plates, extending the time of appearance of colonies on these plates from the usual 48 hours (required for the growth of "spontaneous" I+ mutants) to approximately 72 hours. Since I+ mutant colonies continue to appear on further incubation, the number of colonies on the 48-hour control plates was compared with the number on the 72-hour experimental plates.

Consideration of the factor of growth. The absolute increase in the number of I+ mutants may be considered as proof of the mutagenicity of formaldehyde if it can be shown that the slight growth on itaconate agar plates of formaldehyde-treated I− is no greater than that of untreated I−. To test this, the growth of formaldehyde-treated and untreated cells was followed in itaconate

liquid medium. This experiment also provided another estimate of the increase in number of itaconate mutants as a result of formaldehyde treatment. The procedure used in the preliminary portion of the experiment was similar to that in the previous experiment. The experimental and control suspensions, after being assayed in the usual manner, subsequent to formaldehyde treatment of the experimental suspension, were diluted in itaconate mineral liquid medium and distributed in 5-ml amounts into a series of 25 test tubes. Growth of the wild type was followed daily in these tubes, and the time of appearance of tur-

TABLE 1

I+ mutations induced by 15 minute exposure to 0.1 per cent formaldehyde

	CONTROL TUBE			EXPERIMENTAL TUBE		
	Days after inoculation			Days after inoculation		
	1	2	3	1	2	3
Total Viable Count						
Colonies on yeast agar/0.2 ml of 2 ml cell suspension	65 88	65 88	65 (10^{-6})* 88 (10^{-6})*	0 0	135 164	140 (10^{-2})* 168 (10^{-2})*
Average cells/2 ml of cell suspension	7.7×10^8			1.5×10^6		
Per cent survivors	100			0.02		
Mutant Count						
Colonies on itaconate agar/0.2 ml of 2 ml cell suspension	0 0 0 0 0	9 4 7 15 10	15 7 11 27 20	0 0 0 0 0	0 0 0 0 0	124 120 138 141 151
Average I+/2 ml of cell suspension	90			1350		
Absolute number of I+ induced by formaldehyde/10^8 exposed cells: 164						
Number of induced I+/10^8 viable cells: 9.0×10^6						

0.5 ml of 0.2 per cent formaldehyde was added to 0.5 ml of cell suspension. After washing, cells were resuspended in 2 ml of buffer.

* This indicates the dilutions plated.

bidity in the tubes was recorded. I+ requires approximately 48 hours to produce visible turbidity in itaconate liquid medium (Englesberg and Stanier, 1949). Subsequent experiments have shown that in the presence of 10^6 to 10^7 wild-type cells, I+ produces visible turbidity in 36 to 48 hours. Therefore, tubes appearing turbid by the second day were recorded as containing mutants at zero time. Since formaldehyde causes an approximate 24-hour lag in the growth of treated cells, tubes containing formaldehyde-treated cells appearing turbid on the third day were also considered to contain mutants at zero time.

The distribution of the number of mutants in the 25 tubes, being a chance phenomenon, should follow the Poisson distribution. Thus, the most probable number of mutants may be calculated from the equation: $\frac{q}{n} = e^{-x}$ (q = number of tubes containing no mutants, n = total number of tubes used, x = most probable number of mutants per tube). The results of this experiment (table 2) offer direct experimental evidence for an absolute increase in the number of mutants, under conditions of equal growth of the wild type in both control and formaldehyde-treated suspensions.

Susceptibility of I− and I+ to formaldehyde treatment. Mutagenicity can be reliably expressed in relative terms only if I− and I+ cells are equally susceptible to formaldehyde treatment and if viable counts on yeast extract agar accurately indicate the number of viable cells plated on the mutant assay plates (itaconate agar plates). With regard to susceptibility, two I+ mutants were isolated from itaconate plates, inoculated with formaldehyde-treated cells, and exposed in parallel with I− to 0.1 per cent formaldehyde for 15 minutes. I+ mutants produced by formaldehyde action were just as susceptible to the killing action of formaldehyde as I−.

Reactivation phenomenon. The possibility that the number of viable formaldehyde-treated cells determined on yeast extract agar might not accurately reflect the number of viable cells plated on itaconate agar was suggested since a variety of media yielded different viable counts with the same suspension of formaldehyde-treated cells while yielding the same viable counts with the untreated suspension. (The per cent survivors determined on yeast autolysate, 0.5 per cent yeast extract, 3 per cent yeast extract, and mineral asparagine agar were 3.2×10^{-4}, 3.2×10^{-1}, 2.3, and 6.2, respectively.)

Since I− does not form visible colonies on itaconate agar, direct evidence of the variation in viable counts on itaconate and yeast extract agar was not available. This matter was further investigated by using the I+ mutant. Viable counts of I+ on yeast extract and on itaconate agar prior to formaldehyde treatment were equal, as expected, but after exposure to 0.1 per cent formaldehyde for 15 minutes, there were 0.02 per cent survivors on yeast extract agar and 9.0 per cent on itaconate agar. The same would probably be true of the formaldehyde-treated I−.

Thus, the validity of expressing this mutagenic effect of formaldehyde in terms of relative increase of mutants remains uncertain. The differences in viable counts of formaldehyde-treated cells on different media indicate that certain media contain substances that can reactivate formaldehyde-inactivated cells. This phenomenon cannot be explained by the neutralization of extraneous formaldehyde since counts were based on 10^{-5} or 10^{-6} dilutions of formaldehyde-treated cells washed at least once prior to dilution.

Possible mechanisms of the reactivation phenomenon were investigated by Fraenkel-Conrat (1951), who showed that in neutral solutions, formaldehyde reacts immediately and reversibly with the free amino groups of proteins and becomes more firmly bound only with longer periods of contact. In alkaline

TABLE 2

Comparison of the growth of formaldehyde-treated and untreated Pseudomonas fluorescens I— and the demonstration of the mutagenic effect of formaldehyde treatment by the tube assay method

	DILUTION	ORGANISMS PER TUBE AT ZERO TIME, 5 ML OF DILUTION	AVERAGE VIABLE COUNT PER TUBE			TUBES WITH MUTANTS		MOST PROBABLE NO. OF MUTANTS PER TUBE	ABSOLUTE NO. MUTANTS PER 0.1 ML OF ORIGINAL SUSPENSION	MUTANTS PER 1×10^8 VIABLE CELLS
			Days after incubation			Zero time	1st day			
			1	2	3					
Control	10^{-3}	1.25×10^7	—	—	—	2	5	0.083	1.6	0.6
	0.5×10^{-4}	6.25×10^5	3.1×10^6	3.0×10^6	2.8×10^6	0	0	—	—	—
Experimental	0.5×10^{-3}	1.35×10^4	1.1×10^6	$6.8 \times 10^{6*}$	(All tubes I+)	25	—	—	—	—
	0.5×10^{-4}	1.35×10^3	9.0×10^4	3.0×10^6	2.8×10^6	6	2	0.274	110†	2.0×10^4

The results of a corresponding plate assay indicated an absolute increase of 152 mutants per 10^8 cells exposed and a relative increase of 7.3×10^4 mutants per 10^8 viable treated cells.

* High viable counts were due to presence of large number of I+ mutants.

† Absolute number of mutants induced per 10^8 cells exposed to formaldehyde is 44.

solutions, not only the amino, but also the indole, amide, and guanidyl groups add formaldehyde rapidly. The amino compound is reversibly dissociable under these conditions, while the other compounds are more stable. Formaldehyde forms methylene linkages between amino groups, amides, and other reactive groups of the protein molecule. These reactions can occur when neither the amino nor the other polar groups by themselves give stable formaldehyde addition products, or when they do so only slowly. This chemical evidence not only lends support to the reversibility of the formaldehyde effect, demonstrated with the *Pseudomonas*, but also indicates the specificity of its action. The reversibility of the "killing" action of formaldehyde has been shown by Ross and Stanley

TABLE 3

Relationship between the exposure of Pseudomonas fluorescens I− to different concentrations of formaldehyde and the I+ mutant count based on 10^0 and 10^{-1} dilutions

PER CENT FORMALDEHYDE	VIABLE CH_2O-TREATED CELLS/0.1 ML	I+ COUNT/0.1 ML, DILUTIONS PLATED ON ITACONATE		$\frac{\text{I+ COUNT } (10^{-1})}{\text{I+ COUNT } (10^0)} \times 100$
		10^0	10^{-1}	
0.1	1.3×10^5	437	122	28
0.082	1.6×10^6	538	500*	93
0.067	6.8×10^6	396	252	64
0.05	3.2×10^7	343	208	61
0.02	9.1×10^7	171	232	136
0.01	4.6×10^8	102	158	155
Control	5.7×10^8	59	108 (10^{-2})500† (10^{-3})4000‡	183

5.7×10^8 cells were exposed to the per cent formaldehyde indicated for 15 minutes. The procedure was that previously described. All counts are based on the average of 5 plates. Both experimental and control plates were incubated for 72 hours before colonies were counted.

* The inoculum on the itaconate plates, on the basis of which this value was determined, had not been distributed evenly over the agar surface.

† Count based on 0.1 ml of a 10^{-2} dilution (5.7×10^6 viable cells).

‡ Count based on 0.1 ml of a 10^{-3} dilution (5.7×10^5 viable cells).

(1938) with tobacco-mosaic virus. A simple dialysis at pH 3 reactivated formaldehyde-inactivated virus. This reactivation was correlated with the freeing of formaldehyde-bound amino groups.

The significance of the reactivation phenomenon with regard to the mutagenic effect of formaldehyde is yet to be explored. It is hoped that the relative chemical specificity of formaldehyde may be of value in elucidating the formaldehyde effect.

The dilution effect. In assaying for induced I+ mutants it was sometimes necessary to count colonies on itaconate plates containing a 10^{-1} dilution of the original suspension. Counts based on 10^{-1} dilutions were considerably less than those based on 10^0 dilutions (table 3). This differs from the 10^{-1} dilution count

of I+ mutants in a suspension of untreated cells (controls), in which the count based upon 10^{-1} dilution plates is higher than the count based upon plates inoculated with the same undiluted suspension.

In one experiment (table 3) the same numbers of cells were exposed to different concentrations of formaldehyde for the same time. With one exception, the I+ count based upon the 10^{-1} dilution is low in comparison to the I+ count based upon the 10^{0} dilution. As the number of viable cells per plate increases, the count based upon the 10^{-1} dilution progressively approaches and then passes the count based on the undiluted sample. The exception occurred on the 10^{-1} plate in which the inoculum was not spread out over the entire surface of the plate. With the cells so concentrated the 10^{-1} plate count became almost equal to that of the 10^{0} plate.

Ryan and Schneider (1949), in counting the "spontaneous" histidine-independent mutants in clones of histidine-dependent *E. coli*, observed a phenomenon similar to the one described with counts of "spontaneous" I+ mutants in clones of the wild type. They explain the disproportionately greater counts based on higher as compared to lower dilutions to result from plate mutations subsequent to slight growth of the parent organism on the mutant assay medium; plate mutations have a better chance of forming visible mutant colonies when the total number of wild-type microcolonies per plate is small.

Although this appears to explain the dilution effect with the control suspension, it does not explain the dilution effect with formaldehyde-treated cells. The latter phenomenon is not the result of dilution of some extraneous material carried over with the cells, e.g., formaldehyde. This has been amply demonstrated in experiments in which double washings were used after treatment with formaldehyde. In addition, counts based on the 10^{-1} dilution were greater in suspensions on which the concentrations of formaldehyde were low (table 3). Apparently the factor or factors responsible for this phenomenon are directly related to the number of living cells plated.

One explanation of this dilution effect is the possibility of a phenomic lag in the expression of the formaldehyde-induced itaconate mutations. This investigation demonstrated a lag in the phenotypic expression of formaldehyde-induced phage-resistant mutants of *E. coli* (to be shown later). Demerec (1946), Demerec and Latarjet (1946), and Newcombe and Scott (1950) found such a phenomenon in ultraviolet-induced phage-resistant mutants of this organism. Davis (1950) observed a phenomic lag in the expression of ultraviolet-induced back mutations of *E. coli* to tryptophan independence. Applied to the itaconate mutant, this would mean that the I+ mutant would have to divide one or more times before it could use itaconate for growth. For this primary growth the mutant would depend on the utilizable impurities in the medium and any substance produced in the medium by the slight growth of the wild type or of other itaconate mutants. Such growth might change the agar medium to facilitate the growth of itaconate mutants not at that time equipped to utilize itaconate. The more numerous these active cells and the more substance utilizable for growth of these cells, the greater the chance for the nonutilizing itaconate mutants to

grow and become itaconate utilizers. (This would mean that the counts on 10^{-1} itaconate plates in the range of high killing may indicate the number of "zero point" mutants.)

The count of itaconate-induced mutants in liquid media, however, presents a different picture. Tube counts of the number of itaconate mutants induced, although lower than corresponding counts based on 10^0 plates, were considerably higher than one would expect if the initial number of viable cells was the only factor involved in the growth of I+ mutants. Because individual cells and their metabolic products are not isolated in liquid media, growth of a few active cells may serve the same purpose as growth of a great many more such cells on an agar medium in converting phenotypically I− to I+.

The possibility that reactivation may be partly responsible for the 10^{-1} effect should be considered. The initial growth of some formaldehyde-treated cells and the subsequent change in the medium may reactivate formaldehyde-inactivated cells, both I− and I+. Thus, as the number of viable cells per itaconate plate increases, reactivation increases and, therefore, the number of I+ mutants per plate is also greater.

The relationship between the killing effect of formaldehyde, the increase in absolute number of mutations, and the time of exposure to 0.01 per cent formaldehyde. The killing effect of 0.01 per cent formaldehyde, determined by viable counts on yeast extract agar, appears to be an exponential function of time of exposure (figure 1).[3] The absolute number of mutations increases sharply on initial contact with formaldehyde, remains fairly constant for over an hour, and then falls with increasing time of exposure.

The Mutagenic Effect of Formaldehyde on E. coli, Strain B

In 5 experiments in which the number of "zero point" mutations was determined there was no apparent absolute increase in the number of B/1 mutants. In 1 of the 5 experiments the relative number of mutants increased slightly.

To permit comparison with the studies on *Pseudomonas fluorescens*, several generations of growth of the formaldehyde-treated cells were permitted before the number of B/1 mutants was assayed. This allowed for the possible lag in the phenotypic expression of formaldehyde-induced mutations. In 1 such experiment about 6 divisions of the formaldehyde-treated cells were required for the phenotypic expression of all formaldehyde-induced mutations (table 4). Under such conditions, the relative increase was 5.35×10^3 B/1 mutants per 10^8 viable cells. Both B and formaldehyde-induced B/1 (isolated after exposure of a culture of B to formaldehyde) were equally susceptible to formaldehyde killing.

A comparison of these results with those of Bryson (1948) indicates that formaldehyde is about ten times as effective as nitrogen mustard in inducing "end point" mutations in *E. coli* to phage resistance under conditions of approximately equal killing.

[3] A plot of the log of the per cent survivors against time from data in table 3 would yield a curve slightly concave downward. This, however, is probably the result of additional killing during washing (a proportionately greater number of organisms are killed during washing of cells originally exposed to higher concentrations of formaldehyde).

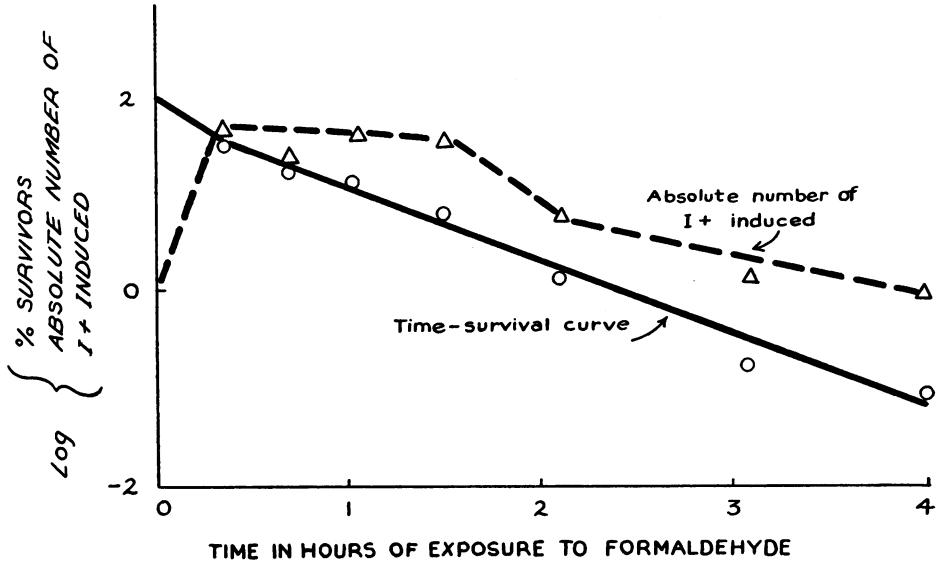

Figure 1. Absolute number of I+ mutants induced in *Pseudomonas fluorescens* by exposure to 0.01 per cent formaldehyde and time-survival curve.

TABLE 4

Number of zero-point and end-point mutations produced by exposure of Escherichia coli, strain B, to 0.1 per cent formaldehyde for 20 minutes

HOURS AFTER INCUBATION WHEN VIABLE COUNTS WERE MADE	VIABLE COUNTS PER PLATE	HOURS AFTER INCUBATION WHEN PHAGE WAS SPRAYED	AVERAGE MUTANTS PER PLATE	MUTANTS INDUCED PER 10^8 VIABLE CELLS
0	6.10×10^5	0	2*	—
6	1.75×10^6	6	7*	—
9	2.15×10^7	9	34 (−0.2)†	5.35×10^3
10½	8.90×10^7	10½	27 (−0.8)†	4.11×10^3
12	3.50×10^8	12	35 (−3.1)†	5.04×10^3

The cell suspension used contained, prior to formaldehyde treatment, 6.15×10^7 viable cells and 119 B/1 mutants per 0.1 ml. Mutant counts are based on an average of 4 plates.

* Figures not considered significant.

† Figure in parentheses is the correction for the number of spontaneous mutations on the assay plates during growth. It was calculated from the spontaneous mutation rate of 8.9×10^{-9}, as determined in this experiment.

DISCUSSION

These investigations indicate advantages of formaldehyde as a mutagenic agent in bacteria over other chemical mutagens: greater mutagenicity, ease of handling, and possible specificity of action. Furthermore, evidence that formaldehyde is an intermediary compound in the metabolism of glycine (Paretsky and Werkman, 1950) and sarcosine (Handler, Bernheim, and Klein, 1941; Mackenzie and du Vigneaud, 1949) opens the question of the role of this metabolite in "spontaneous" mutation.

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SUMMARY

The mutagenicity of formaldehyde in the bacteria is demonstrated by: (1) the large absolute increase in "end point" itaconate-positive mutants of *Pseudomonas fluorescens* and (2) the relative increase in "end point" mutants of *Escherichia coli*, strain B, to phage resistance (T1), under conditions in which the factors of growth and selection were excluded.

Large differences in viable counts of formaldehyde-treated cells on different media indicate that certain media reactivated formaldehyde-inactivated cells.

Mutant counts of formaldehyde-treated cells based on 10^{-1} dilution plates were considerably lower than counts based on 10^{0} plates in the range of low survival. As the concentration of organisms per plate increased, this discrepancy disappeared. This may be the result of the phenomic lag in the expression of formaldehyde-induced mutations or a manifestation of the reactivation phenomenon.

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