THE MUTAGENICITY OF SOME CARCINOGENIC COMPOUNDS FOR ESCHERICHIA COLI[1]

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OF the many substances that have been found to be mutagenic several are also carcinogenic and considerable evidence has been accumulating to indicate a relationship between these two properties.

Nitrogen and sulfur mustards were shown by AUERBACH and ROBSON (1944) to be mutagenic for Drosophila, a finding which has been confirmed by DEMEREC (1947a,b), BURDETTE (1952) and others. Nitrogen mustard was found to be mutagenic for Neurospora by BONNER (1946) and for *Escherichia coli* by TATUM (1946) and BRYSON (1948). Sulfur mustard is also mutagenic for Neurospora as demonstrated by HOROWITZ, HOULAHAN, HUNGATE and WRIGHT (1946). That the mustards are also carcinogenic has been shown by BOYLAND and HORNING (1949), HESTON (1949, 1950), and GRIFFIN, BRANDT and TATUM (1951).

Other carcinogens that have been found to be mutagenic include: 20-methylcholanthrene-endosuccinic acid, shown to be mutagenic for Neurospora by TATUM (1947); and chrysene, shown to be mutagenic for yeast by MITRA and SUBRAMANIAM (1950).

LATARJET (1948) and LATARJET, ELIAS and BUU-HOI (1949) reported the mutagenicity of the water-soluble carcinogen 1,2,5,6-dibenzanthracene-α,β-endosuccinate for *E. coli*. The results of the latter study suggested a correlation between carcinogenicity and mutagenicity in that ethyl carbamate was highly carcinogenic and highly mutagenic, while propyl carbamate and isopropyl carbamate were less strongly carcinogenic and less strongly mutagenic, and butyl carbamate was neither carcinogenic nor mutagenic. BARRATT and TATUM (1951), using Tween 80 as a solvent, reported methylcholanthrene, 9,10-dimethyl-1,2-benzanthracene, and 1,2,5,6-dibenzanthracene to increase the mutation frequency in Neurospora 4-fold, m′-methyl-*p*-dimethylaminoazobenzene, and 4′-amino-2,3-azotoluene to increase it 3-fold, and 4-dimethylaminostilbene and acetylaminofluorene to be weaker mutagens or to be inactive. They found all these substances to be much less active than nitrogen mustard and radiations. LATARJET (1948) had been unable to demonstrate mutagenicity of methylcholanthrene for *E. coli*. MAISIN (1951) and MAISIN, LAMBERT and

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VAN DUYSE (1951) have summarized the work of MAISIN and his coworkers over a period of several years in which the action of styryl 430 on yeast has been studied. They have reported that the first effect is the production of modulations and that this is followed by the production of mutations, a two-step process of mutation which parallels the two-step process of carcinogenicity.

BIRD and FAHMY (1953), using the Muller-5 technique for detecting lethal mutations in Drosophila, studied the carcinogenicity, tumor-inhibiting power and mutagenicity of eleven compounds. They found these three characteristics to be essentially parallel.

In this article, additional evidence for the mutagenicity for *E. coli* of two carcinogens, methylcholanthrene and 1,2,5,6-dibenzanthracene-9,10-α,β-endosuccinate (DBAS), and evidence for the mutagenicity of three others, 3,4-benzpyrene, 9,10-bishydroxymethyl-1,2-benzanthracene (BB) and *o*-aminoazotoluene is presented.

EXPERIMENTAL

The methods used in this work parallel those of WITKIN (1947), which were in turn based on those of DEMEREC (1946) and DEMEREC and LATARJET (1946). Suspensions or cultures of *E. coli* B/r were assayed for mutants that were resistant to T1 bacteriophage both before and after being subjected to the chemical agents. Both the *E. coli* culture and the bacteriophage strain were obtained from DR. WITKIN.

Concentrated cell suspensions were prepared by collecting cells by centrifugation from four 25-ml cultures in M9 medium (for composition see WITKIN 1947) that had been incubated for 48 hours at 37°C with shaking. The cells were washed once and resuspended in 10 ml of buffered distilled water (Standard Methods For the Examination of Dairy Products, 1948).

In the tests, 2 ml of the concentrated cell suspension were added to 2 ml of the stock carcinogen suspension and 16 ml of suspending medium. This mixture, together with a control consisting of 2 ml of the cell suspension and 18 ml of suspending medium, was incubated at 37°C with shaking. Total cell counts and counts of B/r/1 mutants were made before incubation and after various intervals of incubation. Mutagenicity was judged by the ratio of mutants in the experimental and control cultures after incubation. Control experiments were run with a noncarcinogen, anthracene.

Except for the water-soluble carcinogen, DBAS, the stock carcinogen suspensions contained 2.04 mg/ml of distilled water. These suspensions were stored in the refrigerator in the dark. Because of low solubility of these carcinogens the bacteria were exposed to saturated solutions containing extra carcinogen in suspension. The stock solution of the water-soluble carcinogen contained 1.0 mg/ml. Total cell counts were made by plating on nutrient agar.

For determining the concentration of B/r/1 cells, 0.1 ml portions of the suspension or culture were deposited on the surfaces of ten nutrient agar plates that had been prepared 24 hours previously and stored at room temperature with porous clay tops. To each plate was added 0.1 ml of a freshly prepared

bacteriophage suspension with a titer of 5×10^9 to 1×10^{10}. This was thoroughly mixed with the culture with a glass rod and the mixture was spread over the surface of the agar. The plates were allowed to dry for 15 to 20 minutes, and were then inverted and incubated at 37°C for 48 hours.

Experiments were considered valid only if the number of mutants in the control, "background number," was small. The mutagenic effect was measured by dividing the percent B/r/1 cells in the experimental culture by the percent B/r/1 cells in the control. It was assumed that if no mutagenic action occurred the ratio would be 1.00. All ratios that have been considered to indicate mutagenicity have been distinctly greater than this figure.

Experiments with 9,10-bishydroxymethyl-1,2-benzanthracene (BB)

The first compound to be tested was BB. It was kindly supplied by DR. ALEXANDER HADDOW of the Royal Cancer Hospital, London. It had been synthesized by KENNAWAY and had been shown to display marked carcinogenic activity for mice (BADGER and COOK 1940). This compound was selected because it has greater solubility than other compounds with the benzanthracene nucleus. The results of a series of experiments with this compound are shown in table 1.

In the first test the suspending solution used was buffered distilled water. After an incubation period of three hours the total count on the control suspension had dropped to 28.8 percent of that before incubation and the total count on the suspension with the carcinogen had dropped to 9.7 percent of the original, indicating toxicity of the carcinogen. The ratio of 6.95 obtained indicates definite mutagenicity, apparently in the form of "zero point" mutations since growth can hardly be expected to have taken place within a three-hour period in buffered distilled water. In the second test M9 medium was used as a suspending medium. After 2½ hours the total count had fallen to 8.2 percent of the original although the count on the control was 92 percent of the original, and the mutant ratio was 12.9. There was not only an increase in the percentage of mutants in the suspensions but also a significant increase in the actual number of mutants ($P < .02$). This is considered to be important since, as critics of experiments on mutagenesis have so frequently pointed out, an increase in percentage of mutants might so easily be due to selective toxicity.

In the next test the same experimental setup was used, with total counts and counts of B/r/1 mutants being made after 2, 4, 12 and 24 hours of incubation. From the table it can be seen that toxic effects of the carcinogen were evident for the first 4 hours, with the total count dropping to 2.2 percent of the original after this period of time. Growth evidently began at about this time since the count after 12 hours was considerably increased. The maximum mutant ratio occurred at the same time at which the maximum toxicity was evident and decreased after growth began. This result was unexpected since it was anticipated that growth would give an opportunity for "end point" mutations to become evident. In an experiment performed in an attempt to partially explain these results it was found that cells that had been

TABLE 1

Mutagenicity as measured by production of E. coli B/r/1 mutants.

	Period of exposure	Total count per ml	% of original count	B/r/1 mutants per ml	B/r/1 mutants per 10^6 cells	Experimental/control ratio
A. Tests with BB* in buffered distilled water						
Control	0	5.95×10^7		21	35.30	
Control	3 hr.	1.71×10^7	28.8	6	35.10	
Experimental	3 hr.	5.75×10^6	9.7	14	244.00	6.95
B. Tests with BB in M9 medium						
Control	0	5.7×10^8		650	114.00	
Control	2½ hr.	5.3×10^8	92	635	120.00	
Experimental	2½ hr.	4.7×10^7	8.2	727	550.00	12.90
C. Tests with BB in M9 medium						
Control	0	4.9×10^9		187	3.82	
Control	24 hr.	7.4×10^{10}	1510	2970	4.01	
Experimental	2 hr.	4.6×10^8	9.4	130	28.20	7.39
Experimental	4 hr.	1.1×10^8	2.2	52	47.30	12.38
Experimental	12 hr.	1.6×10^9	32.6	245	15.40	3.84
Experimental	24 hr.	3.3×10^9	67.4	415	12.60	3.14
D. Tests with methylcholanthrene in M9 medium						
Control	0	5.5×10^8		1802	328.00	
Control	3 hr.	4.6×10^8	83.6	1625	353.00	
Experimental	3 hr.	4.3×10^8	78.2	1607	373.00	1.06
E. Tests with methylcholanthrene in M9 medium + 20% sheep plasma						
Control	0	3.2×10^8		429	134.00	
Control	4 hr.	12.5×10^6	3.9	18	144.00	
Experimental	4 hr.	3.7×10^6	1.1	20	541.00	3.76
F. Tests with anthracene (noncarcinogenic control) in M9 medium						
Control	0	6.7×10^8		702	105.00	
Control	3 hr.	14.1×10^8	210	1418	101.00	
Experimental	3 hr.	12.4×10^8	185	1322	107.00	1.06

*9,10-bishydroxymethyl-1,2-benzanthracene.

incubated for 36 hours in the presence of the carcinogen were resistant to its toxic effects. If it be assumed that surviving cells are likewise resistant to the mutagenic effects of the carcinogen then it may be postulated that the rate of production of mutants decreases as soon as growth begins to occur in the culture. If this decrease in rate of production of mutants is accompanied by the development of selective conditions which favor the wild type over the mutants then results similar to those obtained might be expected.

A series of control experiments was next run to eliminate as far as possible the possibility that the increased mutant ratios were a result of something other than mutagenesis. Comparative toxicity tests with one of the spontaneous B/r/1 mutants and with the parent B/r strain indicated that the mutant was

not more resistant to the toxic effects of the carcinogen. Before the toxicity tests were made the mutant culture was subjected to extensive tests to prove that it was a pure B/r/1 strain. A suspension of the mutant in buffered distilled water that was incubated with shaking for three hours in the presence of the carcinogen yielded 23.8 percent survivors as compared to 46.2 percent in the control without carcinogen. In another test in which a suspension containing equal numbers of B/r and B/r/1 cells was incubated for three hours in the presence and in the absence of the carcinogen, 48.0 percent B/r/1 cells were found in the suspension with carcinogen and 46.5 percent in the control without carcinogen. The possibility always remains after experiments of this type that the comparative toxicities might be different in a suspension in which a very small percentage of B/r/1 cells was present, or that other B/r/1 strains might be more resistant to the carcinogen.

Growth curves of the B/r and B/r/1 cultures in M9 medium were apparently identical. This indicates that the results obtained were not due to differences in growth rates, although it is possible that the growth rates might be different in a mixed culture or in the presence of the carcinogen.

Lack of toxicity of the carcinogen for the bacteriophage was indicated by the fact that when equal amounts of carcinogen suspension, 24-hr. broth culture of *E. coli* B/r, and bacteriophage suspension were mixed and spread on the surfaces of agar plates no reduction in the number of plaques was found as compared with control plates made by substituting buffered distilled water for the carcinogen suspension.

As can be seen in table 1 the non-carcinogenic control, anthracene, yielded a mutant ratio of 1.06 after an exposure period of three hours in M9 medium and cannot, therefore, be judged to be mutagenic.

Experiments with 20-methylcholanthrene

A mutant ratio of 1.06 was obtained for methylcholanthrene (Eastman Kodak Co.) after an exposure period of three hours in M9 medium (table 1). In this test the methylcholanthrene showed little or no toxicity. Total counts showed that 78.2 percent of the cells survived the exposure period as compared to 83.6 percent of the cells in the control without methylcholanthrene. At this point it was postulated that the lack of activity of the methylcholanthrene might be due to its insolubility. Therefore the experiment was repeated with enough of the M9 medium being replaced by sheep plasma to give 20 percent plasma in the final suspension. It was found that after 4 hours incubation the methylcholanthrene was definitely toxic and a mutant ratio of 3.76 was obtained. It is assumed, although not proven, that the effect of the plasma was to increase the solubility of the methylcholanthrene. The lack of solubility of methylcholanthrene may explain LATARJET's (1948) failure to obtain an increase in number of bacteriophage-resistant mutants of *E. coli* B. BARRATT and TATUM (1951) used Tween 80 in the medium as a solvent and were able to demonstrate mutagenicity in Neurospora.

TABLE 2

Mutagenicity as measured by production of E. coli B/r/1 mutants.

	Exposure medium	Period of exposure	Total count per ml	B/r/1 mutants per ml	B/r/1 per 10^6 cells	Experimental/ control ratio
		A. Tests with 3:4-benzpyrene and DBAS*				
Control	Buffered distilled water	4 hr.	4.5×10^8	80	17.80	
Control	Buffered distilled water	16 hr.	5×10^6	16	320.00	
Control	Nutrient broth	4 hr.	4.3×10^{10}	662	1.54	
Benzpyrene	Buffered distilled water	4 hr.	4.5×10^8	312	69.30	3.90
Benzpyrene	Buffered distilled water	16 hr.	3×10^6	29	967.00	3.02
Benzpyrene	Nutrient broth	4 hr.	19.5×10^9	1485	7.60	4.95
DBAS*	Buffered distilled water	4 hr.	4.3×10^8	404	94.00	5.30
DBAS	Buffered distilled water	16 hr.	3.3×10^6	43	1300.00	4.06
DBAS	Nutrient broth	4 hr.	14.3×10^9	2101	14.90	9.66
		B. Tests with o-aminoazotoluene and anthracene (noncarcinogenic control)				
Control	Buffered distilled water	6 hr.	4×10^8	17	4.25	
Control	Buffered distilled water	16 hr.	9×10^7	14	15.60	
Control	Nutrient broth	4 hr.	1.1×10^8	39	35.50	
o-aminoazotoluene	Buffered distilled water	6 hr.	6×10^8	45	7.50	1.76
o-aminoazotoluene	Buffered distilled water	16 hr.	9.8×10^7	26	26.50	1.63
o-aminoazotoluene	Nutrient broth	4 hr.	9×10^7	61	67.80	1.91
Anthracene	Buffered distilled water	6 hr.	5×10^8	17	3.40	0.80
Anthracene	Buffered distilled water	16 hr.	1×10^8	13	13.00	0.83
Anthracene	Nutrient broth	4 hr.	1.2×10^8	35	29.20	0.83

*1,2,5,6-dibenzanthracene-α,β-endosuccinate.

Experiments with 3,4-benzpyrene, 1,2,5,6-dibenzanthracene-α,β-endosuccinate (DBAS) and o-aminoazotoluene

The 3,4-benzpyrene was obtained from A. D. MACKEY, Pure Chemicals-Metals and Minerals, 198 Broadway, N. Y., and the *o*-aminoazotoluene (2-amino-5-azotoluene) from the Eastman Kodak Co. The 1,2,5,6-dibenzanthracene-α,β-endosuccinate was synthesized according to the method of Bachmann and Kloetzel (1938).

Mutant ratios were determined for 3,4-benzpyrene and DBAS after four and sixteen hours of incubation in buffered distilled water and for *o*-aminazotoluene after six and sixteen hours of incubation. Mutant ratios were also determined for each substance after 4 hours incubation in nutrient broth.

In these studies counts of B/r/1 mutants in the controls were made only after the same periods of incubation as in the controls since counts on the suspensions previous to incubation did not appear to give essential information. Although in the previous studies the " background number " of mutants had remained constant in spite of incubation, in these studies the " background number " increased between the 4th or 6th and 16th hours of incubation. This does not appear to have obscured the effects of the test substances.

From the results in table 2 it can be seen that the water-soluble carcinogen, DBAS, was the strongest mutagen, especially when it is recalled that it was used in a considerably lower concentration. The 3,4-benzpyrene was also a strong mutagen but the *o*-aminoazotoluene was the weakest mutagen of the carcinogens tested. The anthracene (noncarcinogenic) again proved to be nonmutagenic.

Total counts showed no toxicity for the benzpyrene and the DBAS in distilled water after the 4-hour exposure but slight toxicity after 16 hours. Counts on the suspension with *o*-aminoazotoluene in distilled water were higher than counts on the control. Inhibition of growth was evident in all the 4-hr. nutrient broth cultures containing carcinogens but not in that containing anthracene. The degree of inhibition was parallel to the degree of mutagenicity, being strongest for the DBAS and weakest for the *o*-aminoazotoluene.

As can be seen in table 2, the mutant ratios for the suspensions in buffered distilled water were slightly lower after 16 hours than after 4 or 6 hours, in every case. The mutant ratios for the nutrient broth cultures were distinctly higher than those for the water suspensions, possibly indicating " end point " mutations in the growing cultures.

Growth curves were determined for the parent B/r culture and for a B/r/1 strain (a different strain from that used in the studies with BB) in nutrient broth. The rate of growth was identical for the first six hours, after which the parent B/r culture grew more rapidly and reached a higher maximum population. The effects of each of the carcinogens on the growth of the parent B/r culture and the B/r/1 strain were next determined. Each of the carcinogens was inhibitive to the B/r/1 strain but not to the parent B/r culture. These results appear to indicate that both growth rates and the selective effects of

the carcinogens favored the parent type over the mutants in the nutrient broth cultures. This enhances the evidence for mutagenicity.

Benzpyrene was shown to have no effect on the plaque count produced by a bacteriophage suspension or on its ability to select B/r/1 mutants when the suspension was subjected to the carcinogen for 4 hours.

Fifteen of the B/r/1 mutants from the various experiments were tested for purity and then tested for resistance to T1, T2, and T5 bacteriophages. All were resistant to T1 and sensitive to T2. Six were resistant to T5. The abilities of all 15 mutants to grow in a synthetic medium without added tryptophane were tested. The results with fourteen agreed with the finding of DEMEREC and FANO (1945) that only the strains that are resistant to both T1 and T5 bacteriophages can grow without added tryptophane. One strain that was resistant to both bacteriophages failed, upon repeated tests, to grow in the absence of tryptophane, although it grew well in its presence.

SUMMARY

Evidence has been presented that the carcinogens, 20-methylcholanthrene, 1,2,5,6-dibenzanthracene-α,β-endosuccinate (DBAS), 3,4-benzpyrene, 9,10-bishydroxymethyl-1,2-benzanthracene (BB) and *o*-aminoazotoluene increased the rate of production of B/r/1 mutants from *Escherichia coli* B/r. A noncarcinogen, anthracene, had no effect. Mutagenesis was apparently demonstrated in buffered distilled water suspensions, in M9 medium, and in nutrient broth cultures, the strongest effects being in the latter. 3,4-Benzpyrene, DBAS, and BB appeared to be strong mutagens. Mutagenic effects of methylcholanthrene could only be demonstrated when 20 percent sheep plasma was added to the suspending M9 medium, the effect probably being one of increasing the solubility of the carcinogen. *o*-Aminoazotoluene was weakly mutagenic. The mutagenicity of the compounds tested appeared to parallel their reported carcinogenicity when their solubility was taken into account. Toxicity and growth rate studies with the parent B/r culture and with two of the B/r/1 mutants supported the view that the effects observed represent true mutagenesis.

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