Studies of the Origin of Bacterial Viruses

VII. *The effect of various mutagens (urethane, ethyl urethane, hydrogen peroxide, desoxycholate, maleic hydrazide, butadiene dioxide, triethylene melamine, versene, and acriflavine) on the proportion of virus-producing and streptomycin-resistant cells in cultures of B. megatherium 20 Δ*

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ABSTRACT The mutagens, urethane, ethyl urethane, hydrogen peroxide, desoxycholate, versene, maleic hydrazide, butadiene dioxide, and triethylene melamine, all increase the proportion of virus-producing cells and streptomycin-resistant cells in *B. megatherium* 20Δ cultures to about the same extent. Acriflavine has no effect on the proportion of either type of cell. Triethylene melamine appears to cause mutations to occur without cell division.

Viral nucleic acids are now known to have the same chemical and biological properties as transforming principle nucleic acids and it is probable, therefore, that they originate in the same way; *i.e.*, as a result of a mutation of the "host" cells.

From this point of view, a normal culture of bacteria consists principally of antibiotic-sensitive, virus-free, virus-resistant cells. Occasionally a cell mutates and becomes antibiotic-resistant. This cell contains transforming principle nucleic acid, which may be isolated and used to infect other cells which then become resistant also. The transforming principle nucleic acid itself may be caused to mutate by treatment with mutagens (*cf.*, for instance, Freese and Strack, 1962). These mutant cells, if cultured, sooner or later back-mutate to the original antibiotic-sensitive cell. Since these grow more rapidly than the mutant ones, the original culture will be reproduced. The

antibiotic-resistant cell occurs at random, in accordance with Poisson's ratio, and may be isolated by Lederberg's replica technique (Northrop, 1957). It is, therefore, a mutant by definition.

In a similar way, an occasional cell mutates and produces viral nucleic acid which causes the formation of phage particles, and this acid may be extracted and used to infect other cells which then produce more of the acid exactly as the antibiotic-resistant cells do. This virus DNA, like transforming principle DNA, may also be caused to mutate by treatment with mutagens (*cf.*, for instance, Silvestri, 1949; Tessman, 1959; Freese, 1961). The ability to mutate, which has been considered as a unique property of cells, turns out to be a general property of DNA. The fact that viruses may be caused to mutate, instead of distinguishing them from the transforming principle, serves to show the close relationship between them.

The virus-producing cells occur at random, in accordance with Poisson's ratio, but cannot be cultured, since they are killed in the process of producing viral nucleic acid. Some of the infected cells may back-mutate to give the original wild virus-free, virus-resistant cells (lysogenic cultures), as do the antibiotic-resistant cells.

The mutation to virus production, therefore, is harmful to the culture, as are most mutations, while the mutation to antibiotic resistance is strikingly beneficial and is, therefore, of a very rare type.

If viral DNA, like transforming principle DNA, is formed by a mutational event, then mutagenic agents, in general, should cause approximately the same increase in the proportion of virus-producing and antibiotic-resistant cells. If, on the other hand, the reactions which cause a cell suddenly to produce viral nucleic acid differ in kind from those which result in the production of transforming principle nucleic acid, then the effect of mutagens on the two processes should be different.

Previous work has shown that mutagens which were known to increase the proportion of virus-producing cells in *B. megatherium* 899 cultures also increase the proportion of antibiotic-resistant mutants (Northrop, 1958, 1960, 1961).

The present experiments show that compounds which are known to be mutagenic in other systems also increase the proportion of virus-producing cells and antibiotic-resistant cells to about the same extent in cultures of *B. megatherium* 20Δ.

The results of these experiments are shown in Table I.

All the mutagens, with the exception of acriflavine, about double the number of virus-producing cells and of streptomycin-resistant cells. Acriflavine has no effect on either.

It is surprising that a variety of mutagens of entirely different chemical

TABLE I

EFFECT OF VARIOUS MUTAGENS ON THE PROPORTION OF STREPTOMYCIN-RESISTANT AND VIRUS-PRODUCING CELLS IN B. MEGATHERIUM 20 Δ

Mutagenic agents added to logarithmic growth cultures in yeast extract peptone, grown up at 37° to 1 × 10^8 cells/ml, diluted one to ten and repeated, 6 hours. Average 6 to 12 experiments. Probable error of the means about ±20 per cent.

Mutagen	0	Urethane	Hydrogen peroxide	Desoxy-cholate	Versene	Maleic hydrazide	Butadiene dioxide	Ethyl urethane	Triethylene melamine TEM	Acriflavine
Concentration	0	20 to 40 mg/ml	3×10^{-5} M	20 to 40 γ/ml	100γ/ml	2 to 3 mg/ml	2 to 4 mg/ml	10 to 20 mg/ml	0.5 to 1.0 mg/ml	0.01 to 10 mg/ml
Relative increase in proportion of mutants in presence of mutagens										
Streptomycin-resistant cells	1	3.5	2.3	2.3	2.2	2.2	1.5	1.5	3	1.1
Virus-producing cells	1	1.8	3.1	2.7	5.4	2.0	10.0	1.2	5	1.0
Mutagen reference		Oelkers, 1943	Demerec, Bertani, and Flint, 1951	Witkin, 1947	Eversole and Tatum, 1956	McLeish, 1953		Oelkers, 1943	Lorkiewicz and Szybalski, 1961	Witkin, 1947

properties should have the same effect on the proportion of various mutants. This result indicates that the increase in mutation rate is caused, not by any specific reaction between the mutagen and the nucleic acid,[1] but by some nonspecific reaction, similar possibly to denaturation.

Higher concentrations of hydrogen peroxide, butadiene dioxide, or triethylene melamine (TEM) cause nearly complete lysis of the culture; *i.e.*, nearly all the cells produce virus. The increase in the streptomycin-resistant cells cannot be determined under these conditions.

Higher concentrations of the other mutagens result in slower growth rates with no further increase in the proportion of either streptomycin-resistant mutants or of virus.

In order to determine the mutation frequency rate constant, or time rate constant, from the proportion of mutants given in Table I, it is necessary to know the effect of the mutagen on the growth rate of the mutants and of the wild cells, since the mutation frequency rate constant $\lambda = (M/W)_e[(A - B)/2A]$ and the time rate constant $C = (M/W)_e(A - B)$ (Northrop and Kunitz, 1957). A is the growth rate of the wild, B, the growth rate of the mutant, and $(M/W)_e$, the equilibrium ratio of M/W.

The simplest test for changes in $(A - B)$ is to maintain a mixed culture of mutants and wild cells in continuous logarithmic growth (by repeated dilution) and to determine the change in the ratio of M/W. Under these conditions, $(A - B) = [\ln(M_0W_t/M_tW_0)]/t$, where M_0 is the number of mutants at the start and M_t, the number of mutants after t hours, while W_0 and W_t are the number of wild cells (Northrop and Kunitz, 1957).

When this test was carried out with the various mutagens, it was found that maleic hydrazide and desoxycholate have a much greater effect on the growth rate of the wild cells than on the streptomycin-resistant cells. Urethane has a slightly greater effect. These mutagens, therefore, select streptomycin-resistant cells and part of the effect noted in Table I is probably due to selection. This complicates the results with these mutagens.

Hydrogen peroxide, versene, and triethylene melamine, on the other hand, have no selective effect and the results reported are due to an increase in the mutation rate. This conclusion has been confirmed for hydrogen peroxide and triethylene melamine (*cf.* below) by the null fraction method of determining the mutation rate. This method is the only one which is independent of either selective killing or selective growth (Lederberg, 1951).

[1] Nitrous acid does react specifically with nucleic acid (Mundry and Gierer, 1958), but the effect is the same as that of all other mutagens tested (Northrop and Cavallero, 1961).

Denatured transforming principle nucleic acid, treated with nitrous acid, also increases the proportion of several mutants (Horn and Herriott, 1962).

The Effect of Triethylene Melamine[2]

This mutagen is unusual in that it causes marked mutation with little or no effect on the viability or even on the growth rate of the culture. The effect is greater with slowly growing cultures than with rapidly growing cultures and there is some evidence that the mutations occur without cell division (*cf.* Szybalski, 1960). This is undoubtedly true as regards virus production and the present results indicate it is also true, in this case, in regard to the production of streptomycin-resistant cells.

Determination of the mutation rate by the null fraction method confirms the conclusion that the increase in the proportion of mutants is due to an increase in the mutation rate and not to selection. The mutation time rate constant of the streptomycin-resistant cells in 5 per cent yeast extract peptone is 20×10^{-7} hr.$^{-1}$ and in the presence of 1 mg triethylene melamine/ml, 50×10^{-7} hr.$^{-1}$. The mutation rate of the virus-producing cells increases from 1×10^{-3} to 10×10^{-3} hr.$^{-1}$.

The change from virus-resistant to virus-producing cells must occur without cell division, since under some conditions the rate of production of these cells is greater than the growth rate (Northrop, 1958). Cells infected with virus transform to virus-producing cells without cell division (Price, 1947; Herriott and Price, 1948; Krueger *et al.*, 1948) and cells infected with transforming principle nucleic acid also transform to antibiotic-resistant cells without growth (Fox, 1959; Voll and Goodgal, 1961). It will be assumed, therefore, in order to conserve hypotheses, that all the mutations occur at a time rate constant C, whether the culture is growing or not. Since in these experiments C (except for triethylene melamine) is small compared to the growth rate, $C = 2\lambda A$ and there is little practical difference between the mutation time rate constant and mutation frequency constant.

If this assumption is correct and the mutations do occur without cell division, it follows that the appearance of new DNA's in the cell is the result of reaction with the mutagen or of turnover, rather than interference with the replication system (*cf.* Novick and Szilard, 1950; Ryan, 1955; Ryan *et al.*, 1959).

The results of three experiments calculated in this way are shown in Figs. 1, 2, and 3.

The experiment shown in Fig. 1 was carried out by adding 1 mg triethylene melamine/ml to a slowly growing culture in 5 per cent yeast extract peptone containing 5×10^8 cells/ml and the culture was shaken at 37°. The triethylene melamine has very little effect on the growth rate.

[2] The writer is indebted to Dr. Ruegsegger of the Lederle Laboratories for samples of triethylene melamine.

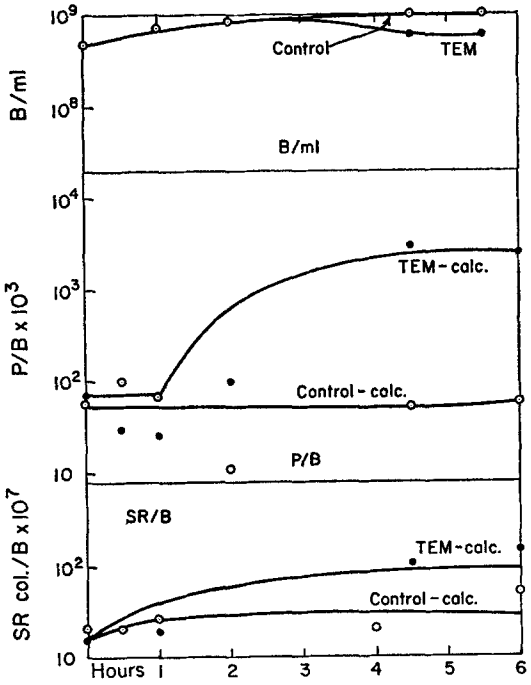

FIGURE 1. Suspension in yeast extract peptone (5×10^8 cells/ml) ± 1 mg triethylene melamine/ml, shaken at 37°.

Calculation: $P/W = lC(e^{(C-A)t} - 1)/(C - A) + (P_0/W_0)e^{(C-A)t}$; l = burst size = 200

	Control	+TEM
A	0.3	0.3
B	0	0
C	0.12×10^{-3}	5×10^{-3}

t = (elapsed time − 1 hr.)

Streptomycin-resistant $(SR)M/W = C(e^{(B+C-A)t} - 1)/(B + C - A) + (M_0/W_0)e^{(B+C-A)t}$

	Control	+TEM
A	0.3	0.3
B	0.2	0.2
C	2×10^{-7}	20×10^{-7}

The increase in streptomycin-resistant cells does not occur for several hours at which time the growth has ceased. The curves were calculated by means of the equations derived by Northrop and Kunitz for the rate of appearance of mutants at a constant fraction (C) of the cells per hour. The time used for calculation of the virus is 1 hour less than the elapsed time,

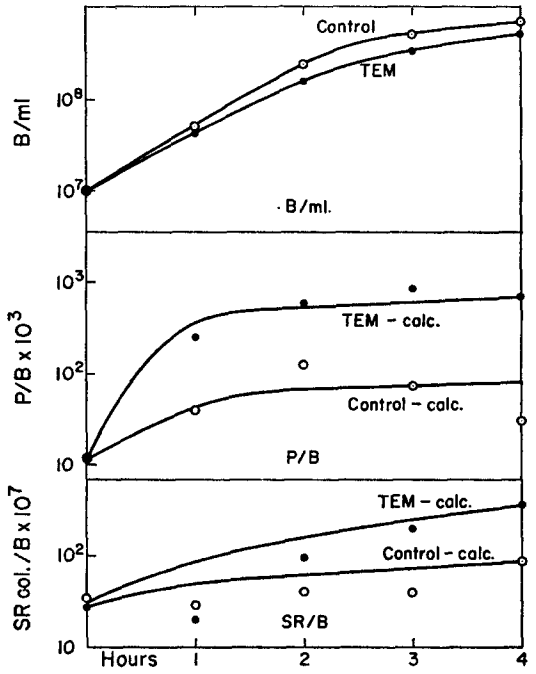

FIGURE 2. Logarithmic growth suspension 3 × 10^8 cells/ml in yeast extract peptone ±1 mg triethylene melamine/ml, 3 tubes each, 1 hr. at 25°, diluted to 1 × 10^7 cells/ml in yeast extract peptone, shaken at 37°.

P calculated

	Control	TEM
l	200	200
A	0.6	0.6
B	0	0
C	0.3×10^{-3}	2.4×10^{-3}
C by null fraction	1×10^{-3}	10×10^{-3}

Streptomycin-resistant cells calculated

	Control	TEM
A	0.6	0.6
B	0.45	0.45
C	30×10^{-7}	175×10^{-7}
C by null fraction	20×10^{-7}	50×10^{-7}

since about 1 hour elapses from the time virus nucleic acid appears in the cell and free virus appears.

In the second experiment (Fig. 2) the triethylene melamine was added to a rapidly growing culture containing 1 × 10^7 cells/ml. The mutagen has little or no effect on the growth rate, but causes a marked increase in the

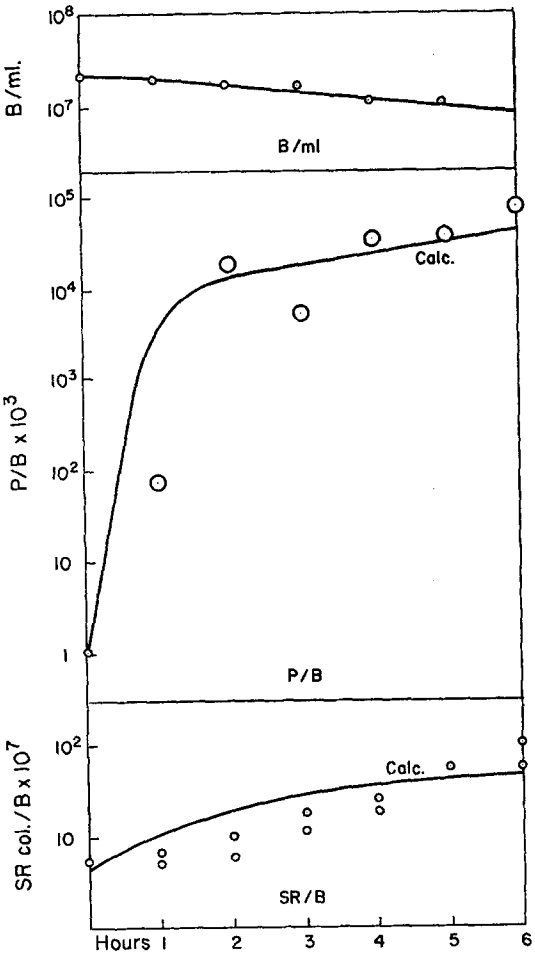

FIGURE 3. Effect of triethylene melamine on the proportion of virus-producing and streptomycin-resistant cells. Two mg triethylene melamine/ml added to suspension of logarithmic growth cells (3×10^8 cells/ml) 1 hr. at 25°. Centrifuged, washed in 0.01 per cent yeast extract peptone, diluted to 2×10^7 cells/ml in 0.01 per cent yeast extract peptone, and shaken at 37°.

	Phage calculated	Streptomycin-resistant cells calculated
A	0	0
B	0	0
C	2.5×10^{-3}	8×10^{-7}
C by null fraction	10×10^{-3}	50×10^{-7}

The values for *C* are smaller than those found by the null fraction method, but the conditions are different, since the null fraction test was carried out with very dilute suspensions in 5 per cent yeast extract peptone, while this experiment was carried out with a concentrated suspension in 0.01 per cent yeast extract peptone.

proportion of virus-producing and of streptomycin-resistant cells. If the culture is maintained in continuous logarithmic growth by repeated dilution, the mutagen has less effect on either the virus or streptomycin-resistant cells. It differs in this respect from all other mutagens tested with this system.

In the third experiment the triethylene melamine was added to a logarithmically growing culture containing 3×10^8 cells/ml and allowed to stand 1 hour at 25°. The cells were then centrifuged and washed in 0.01 per cent yeast extract peptone, suspended in the 0.01 per cent yeast extract peptone, and shaken at 37°. The cell concentration decreased slowly and there was a marked increase in streptomycin-resistant cells after 1 to 2 hours. The virus concentration increased very rapidly at first and then slowly, at about the same rate as did the streptomycin-resistant cells.

The initial rapid increase is due to the fact that the virus present in the culture at equilibrium concentration at the start of the experiment is removed by centrifuging. This initial rapid increase is not due to the triethylene melamine and occurs in the control tube as well, for the same reason. The streptomycin-resistant cells, on the other hand, are not removed by the centrifuging, and hence, are present at the start in the equilibrium concentration of the control and the subsequent increase is due to the triethylene melamine. This experiment is not always reproducible. Some tubes show no increase in either virus or streptomycin-resistant cells.

Similar experiments with *megatherium* 899, however, result in significant increases in virus-producing cells and terramycin-resistant cells without any measurable growth or cell division (unpublished data).

The increase in the proportion of mutants under these conditions cannot be accounted for by either differential killing or differential growth rates.

EXPERIMENTAL PROCEDURE

Culture The organism studied (*B. megatherium* 20Δ) was isolated by Dr. James S. Murphy from a sample of mud from San Francisco Bay (personal communication). It produces a phage which lyses the KM indicator strain and also *megatherium* 899, the lysogenic strain. It is not infected by the *megatherium* 899 phages. When cultured by transfer either in liquid or solid media, the culture produces large, clear plaques. If grown continuously in the steady state apparatus, a number of different phages are produced, as in the case of *megatherium* 899 (Northrop and Murphy, 1956) and lysogenic KM (Northrop, 1961).

The proportion of streptomycin-resistant mutants present in the culture is also dependent on the temperature and conditions of growth. In continuous logarithmic growth in yeast extract peptone, the growth rate of the wild cells is 1.3 $hr.^{-1}$ and that of the streptomycin-resistant cells, 1.2 $hr.^{-1}$ and the equilibrium ratio of streptomycin-resistant colonies to cells is about $1/10^6$. If the culture is allowed to stand at 25°, however, the streptomycin-resistant cells grow more rapidly than the wild cells with

the result that an almost pure culture of streptomycin-resistant cells is obtained. This is another example of the emergence of an antibiotic-resistant culture without exposure to the antibiotic. A similar result was obtained with *B. megatherium* 899 when grown in the steady state apparatus (Northrop, 1962).

A spore culture of the organism was prepared as described by Grelet (1951). A loopful of this culture was transferred to yeast extract peptone and allowed to stand at 25° for 18 hours. The tube was then shaken at 37° to about 3×10^8 cells/ml. It was then diluted one to ten and grown up again.

Calculation of the Rate of Appearance of the Mutants (Northrop and Kunitz, 1957)

The proportion of mutants to wild cells is $M/W = 2\lambda A(e^{(B-A)t} - 1)/(B - A) + (M_0/W_0)e^{(B-A)t}$, if the mutants appear as a result of cell division, and $\lambda \ll 1$.

If the mutation occurs at the rate C, independent of cell division, the proportion is

$$M/W = C(e^{(C+B-A)t} - 1)/C + B - A + (M_0/W_0)e^{(C+B-A)t}$$

In the case of the virus-producing cells, $B = 0$ and $P/l = M$, where l is the burst size and P is the virus concentration.

If there is no growth of the culture, $A = 0$, $B = 0$, and $M/W = (e^{Ct} - 1) + (M_0/W_0)e^{Ct}$ or if $W = W_0$, $M/W_0 = (1 - e^{-Ct}) + (M_0/W_0)$ or $C = -[\ln (1 - (M - M_0)/W)]/t$ or if $[(M - M_0)/W_0] \ll 1$, $C = (1/t)[(M - M_0)/W_0]$.

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