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# *THE MECHANISM OF CHEMICAL MUTAGENESIS. I. KINETIC STUDIES ON THE ACTION OF TRIETHYLENE MELAMINE (TEM) AND AZASERINE**

BY V. N. IYER† AND WACLAW SZYBALSKI

INSTITUTE OF MICROBIOLOGY, RUTGERS, THE STATE UNIVERSITY, NEW BRUNSWICK, NEW JERSEY

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Quantitative studies on the kinetics of chemical mutagenesis in bacteria have not paralleled in number investigations on radiation-induced mutations.[1] The work of Demerec and Hanson[2] on chemically induced mutagenesis in *Escherichia coli* illustrates a fruitful approach to this problem and had its origin in two developments: the discovery of a mutagen of relatively low toxicity—manganous chloride—and the introduction of a simple and efficient assay system for measuring the mutation rate from streptomycin dependence to independence. The main emphasis in the work of the Demerec group,[3–4] however, subsequently shifted from quantitative studies of a particular mutational system to the evaluation of the specificity of several mutagens on a variety of genetic loci.

The present work represents an examination of two other potent mutagens—triethylene melamine (TEM) and azaserine— which are active over wide ranges of concentration and temperature without excessive killing. This study was prompted by an awareness of the advantages of a mutagenic system which would lend itself to quantitative kinetic studies of chemically induced mutational processes and thus provide a tool for deeper insight into the mechanism of events leading to a mutation.

## MATERIALS AND METHODS

The streptomycin-dependent (*sd-4*), cysteine-requiring (*cys-2*) strain Sd4-73 of *E. coli* was obtained from Dr. M. Demerec. The stock culture was periodically purified by selection from among a number of single-colony isolates on the basis of a low proportion of spontaneous back-mutants.[5] Nutrient broth (Difco) containing 20 $\mu$g/ml of streptomycin was employed for preparing liquid cultures. Three solid media served for enumeration of the total number of colonies and the proportion of respective mutants: Difco nutrient agar with and without 100 $\mu$g/ml of streptomycin, and minimal agar (7 gm. $K_2HPO_4$; 2 gm. $KH_2PO_4$; 0.5 gm. $Na_3$ citrate$\cdot 5H_2O$; 0.1 gm. $MgSO_4\cdot 7H_2O$; 1 gm. $(NH_4)_2SO_4$; 2.5 gm. glucose; 20 gm. agar) with 100 $\mu$g/ml streptomycin.

In the general procedure, an overnight culture of Sd4-73 was prepared by incubation with forced aeration at 32° C. The cells were centrifuged, washed twice, and finally suspended in distilled water, to which the desired amount of mutagen had been added prior to incubation at a particular temperature for a chosen period of time. After mutagen treatment, the cells were washed free of the mutagen,

whenever necessary, and assayed for survivors and mutants on appropriate media. The number of induced mutants was calculated in relation to the number of surviving cells and corrected by subtracting the corresponding value obtained in a parallel control experiment with the mutagen-free cell suspension. Extensions of this general procedure will be described in the discussion of individual experiments. This procedure was adapted from the method described by Demerec and Hanson, with the elimination of prewashing the cells in 0.3 *M* NaCl. The pronounced stimulation of the mutagenicity of $Mn^{++}$ ions by hypertonic treatment[2] could not be demonstrated for azaserine and TEM (Table 1).

## EXPERIMENTAL

*Effect of Mutagen Concentration (Fig. 1).*—In the initial experiments the mutagen concentration was varied, with the expectation of finding a "plateau" region in the concentration-mutagenicity curve where changes in concentration might have little or no effect on the number of mutants induced—a region which would be best suited to the study of other variables. Such a plateau has been observed for man-

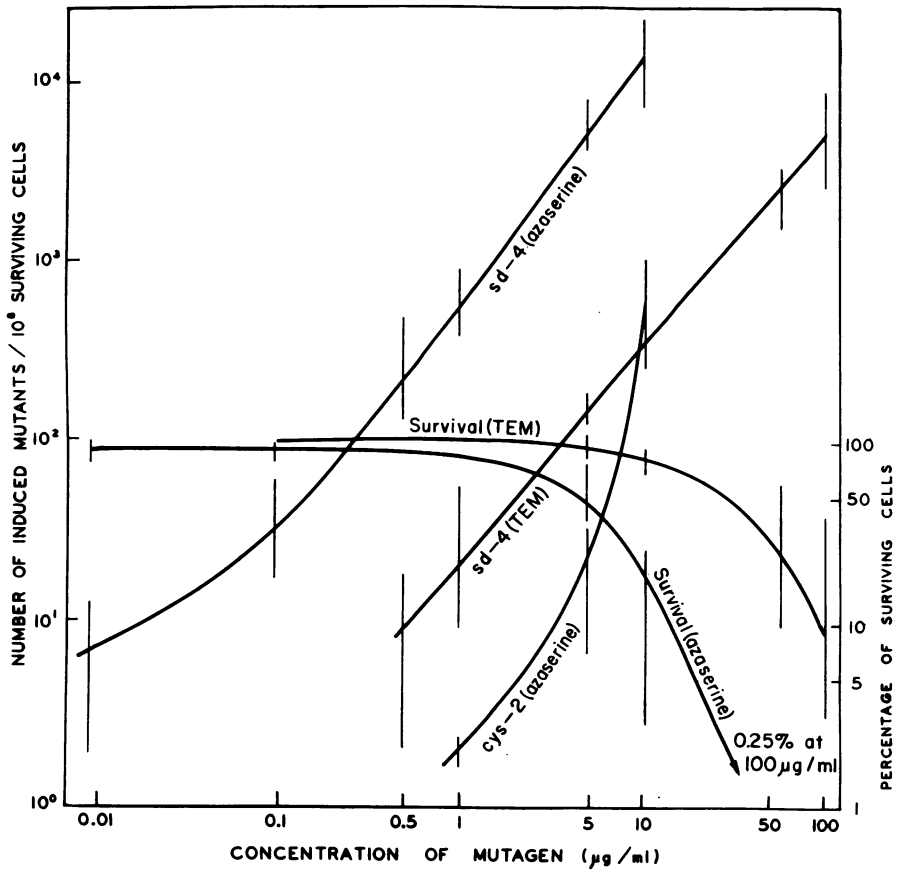


FIG. 1.—Effect of the concentration of TEM and azaserine on the number of induced mutants (*sd-4* and *cys-2* reversions) and on the percentage of survivors (2 hours exposure; 32° C.). The vertical lines denote the spread of pooled experimental results from several experiments. They were omitted in Figs. 2 and 3 to preserve the clarity of the presentation.

TABLE 1

EFFECT OF PRETREATMENT WITH 0.3 *M* NaCl ON NUMBER OF MUTANTS INDUCED BY 2-HOUR TREATMENT WITH TRIETHYLENE MELAMINE (TEM), AZASERINE, AND MANGANOUS CHLORIDE

| Mutagen (Conc.) | Cells Pre-washed with 0.3 *M* NaCl | Per Cent Survivors | Induced Mutants per $10^8$ Survivors *sd-4* | *cys-2* |
|---|---|---|---|---|
| TEM..... | − | 80.5 | 330 | 1.6 |
| (5 μg/ml) | + | 72.4 | 230 | 0.4 |
| Azaserine... | − | 68.6 | 2,200 | 66 |
| (5 μg/ml) | + | 44.7 | 3,000 | 36 |
| $MnCl_2$... | − | 100 | 14 | 0 |
| (400 μg/ml) | + | 89.5 | 1,100 | 180 |

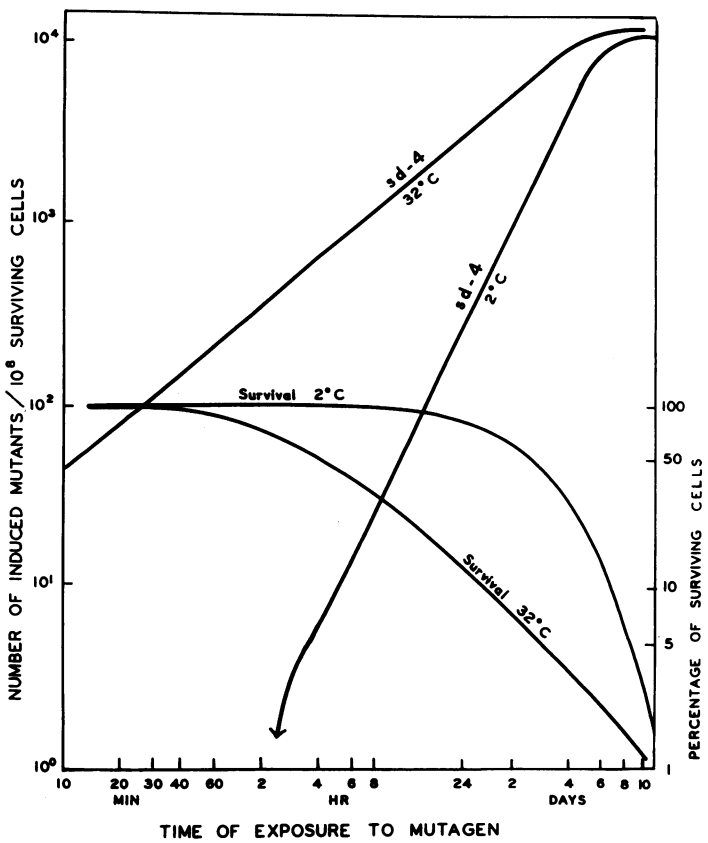


FIG. 2.—Effect of time of exposure to TEM (10 μg/ml) on the number of induced mutants (*sd-4* reversions) and on the percentage of survivors, at 32° and 2° C.

ganous chloride by Demerec and Hanson.[2] In our experiments, cells were exposed to varying concentrations of mutagen at an arbitrarily chosen temperature of 32° C. for 2 hours. The results for TEM and azaserine were as follows: (1) Increasing the concentration of either TEM or azaserine did not produce a mutagenic

response of the type characteristic of manganous chloride;[2] for both mutagens, the relationship between the logarithms of the two variables was nearly linear, with a slope of approximately 1. (2) Over wide concentration ranges in which the mutagens were active (TEM, 1–50 $\mu$g/ml; azaserine, 0.01–5 $\mu$g/ml), survival was close to 100 per cent, falling off rapidly beyond these limits. (3) Azaserine-induced mutations of *cys-2* to prototrophy paralleled those of *sd-4* to streptomycin independence, although the number of mutants was smaller by a factor of 25–40. The effect of TEM on *cys-2*, under the experimental conditions, was too small for evaluation.

The absence of a "plateau" region necessitated rigid control of the concentration level in subsequent experiments, a condition rather difficult to fulfill with unstable substances. For the same culture, the results were reproducible with a variation of less than $\pm 25$ per cent. In experiments performed on different cultures, the mutagenic response varied within much wider limits, which are indicated by the vertical bars corresponding to each point on the curves (Fig. 1).

*Effects of Time and Temperature (Figs. 2 and 3).*—Experiments in this series were conducted at two arbitrarily chosen temperatures (32° and 2° C.). The selected concentrations of 10 $\mu$g/ml of TEM and 5 $\mu$g/ml of azaserine were known from previous experiments to produce low bactericidal effects at sufficiently high mutagenicity (Fig. 1).

With TEM, an almost linear response (log-log scale) was obtained for exposure times ranging from 10 minutes to 3 or 4 days at 32° C., the number of mutants per $10^8$ survivors increasing by approximately 150 every hour. After this period, the curve leveled off, and the fraction of mutants per $10^8$ survivors remained constant (at $1\text{–}2 \times 10^4$). The number of survivors steadily decreased during the entire period of observation, even after the "plateau" for induced mutants was reached.

In the assay of mutagenicity at low temperatures, the cells were never exposed to a temperature higher than 2° C. during the entire operation, including prewashing, treatment with mutagen, centrifuging, removal of the mutagen, and plating. During the first few hours, no induced mutants were perceptible. In experiments extended over a period of days, it soon became apparent not only that TEM was capable of exerting mutagenicity at low temperatures but also that the maximum number of induced mutants was identical at 2° and 32° C. This indifference to temperature renders invalid the otherwise legitimate objection that, during prolonged treatment, slow multiplication of non-dependent mutants at the expense of a dying parental population may have been involved, by analogy with the interpretation of the origin of streptomycin resistance in quasi-stationary populations.[6]

The shape of the survival curve at 2° C. was indicative of a lower toxicity of the mutagen at this temperature and closely approximated the curve obtained when the cells were stored in distilled water at 2° or 32° C. in the absence of mutagen. In these control experiments an increase in the number of spontaneous mutants was noted, with doubling of the proportion of mutants at 32° C. over an 8-day period and an almost tenfold increase in the same time span at 2° C. The indicated mutagenic effect of cold was also observed by Ryan[7] and Wainright,[8] and can perhaps be better understood through a consideration of the paradoxical results presented in Figure 5 and discussed in the corresponding section of this paper.

The effect of time and temperature on the action of azaserine is depicted in Figure 3. The maximum effect of this mutagen at 32° C. was reached in a period of 10–20 hours, i.e., much earlier than for TEM. At 2° C., the final number of mutants produced by azaserine at a concentration of 5 μg/ml was somewhat lower than at 32° C.

These data extend the results of Demerec and Hanson,[2] who reported a marked decrease in the short-term mutagenicity of $MnCl_2$ at low temperatures but did not study the effect of the mutagen over a prolonged period of time.

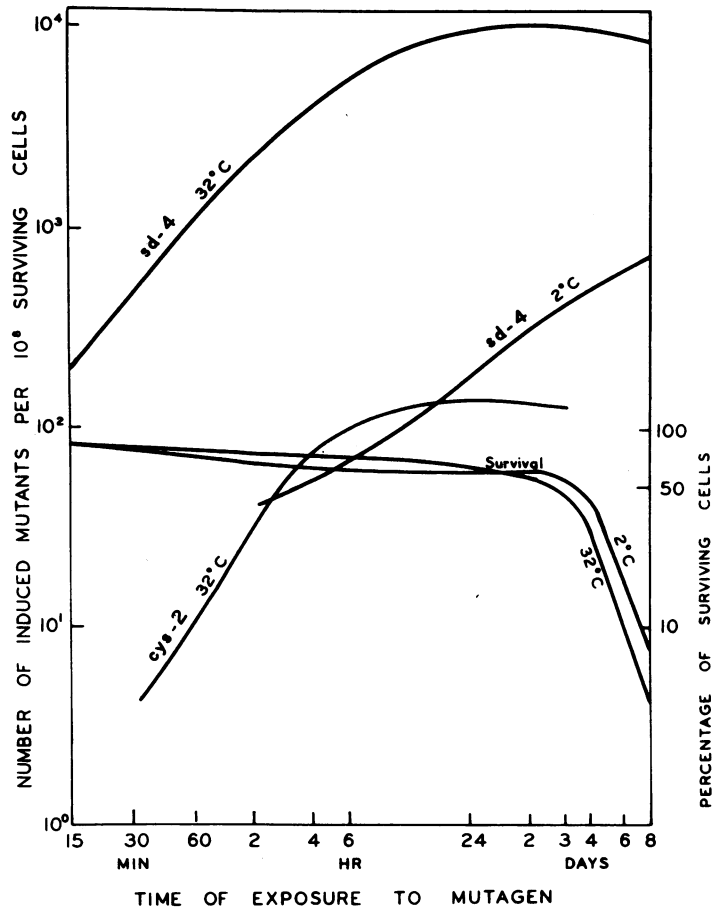


FIG. 3.—Effect of the time of exposure to azaserine (5 μg/ml) on the number of induced mutants (*sd-4* and *cys-2* reversions) and on the percentage of survivors at 32° and 2°.

Further experiments were aimed at determination of the fate of the mutagen and the stability of the new mutation before its expression.

*Effect of Retreatment with Freshly Prepared Mutagen (Fig. 4).*—The purpose of these experiments was to examine the possibility that mutagen might either be used up or decay during the treatment and that its renewal might increase the mutagenic response.

The production of mutants was first followed for 3–4 hours at 32° C., after which period the suspension was divided in half. The cells in one portion were centrifuged and washed and immediately resuspended in the same volume of freshly prepared mutagen solution at 32° C., after which periodic assays on both suspensions were resumed. As may be seen in Figure 4, this procedure had no effect on the action of TEM, while it increased the mutagenicity of azaserine by a factor of 3–4, a result which points to the instability of azaserine in contact with the bacterial suspension under these conditions.

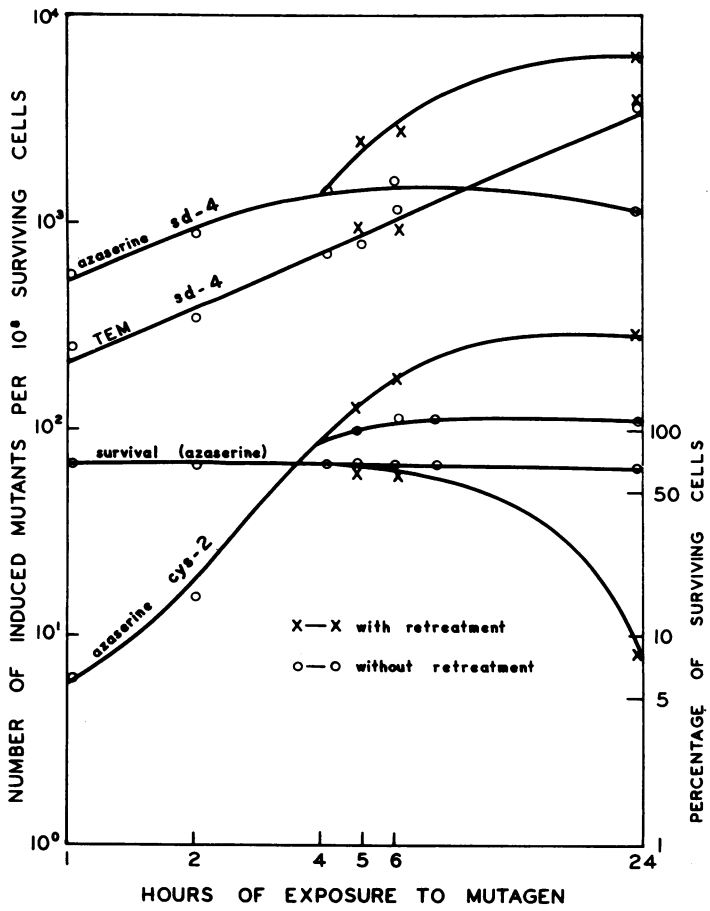


FIG. 4.—Effect of retreatment with freshly prepared mutagen (TEM 10 μg/ml; azaserine 3 μg/ml) on the number of induced mutants (*sd-4* and *cys-2* reversions); 32° C.

*Effect of Various Posttreatments* (*Tables 2 and 3*).—Following the demonstration that TEM- and azaserine-induced mutations could be conveniently studied in *E. coli*, attempts were made to analyze individual steps in mutagenesis with the aid of procedures capable of retarding, blocking, or reversing this process. The mutagen was removed from the cell environment and replaced with water or different salt solutions.

TABLE 2

EFFECT OF REMOVAL OF MUTAGEN AND POSTTREATMENT WITH DISTILLED WATER ON NUMBER OF MUTANTS INDUCED BY TEM (10 μG/ML), AZASERINE (5 μG/ML), AND $MnCl_2$ (400 μG/ML), AT 32° C.

| MUTAGEN | TIME OF PREVIOUS EXPOSURE TO MUTAGEN | | MUTANT | INTERVAL (HOURS) OF POSTTREATMENT IN WATER AFTER PREVIOUS EXPOSURE TO MUTAGEN: 0 | 0.5 | 1 | 2 | 4 | 6 | 8 | 24 hrs |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TEM | 2 hours | Percentage of survivors | | 100 | 77 | 76 | 74 | 74 | ... | ... | 60 |
| Azaserine | | | | 62 | 63 | 42 | 19 | 18 | ... | ... | 13 |
| $MnCl_2$ | | | | 100 | 88 | 73 | 43 | 23 | ... | ... | 6.7 |
| TEM | | No. of induced mutants per $10^8$ survivors | *sd-4* | 400 | 780 | 720 | 670 | 680 | ... | ... | 880 |
| Azaserine | | | *sd-4* | 2,000 | 2,200 | 2,300 | 3,500 | 1,300 | ... | ... | 1,600 |
| Azaserine | | | *cys-2* | 5 | 17 | 11 | 58 | 52 | ... | ... | 57 |
| $MnCl_2$ | | | *sd-4* | 500 | 550 | 500 | 940 | 670 | ... | ... | 300 |
| $MnCl_2$ | | | *cys-2* | 83 | 55 | 70 | 110 | 250 | ... | ... | 70 |
| TEM | 18 hours | Percentage of survivors | | 2.2 | 3.1 | 2.5 | 2.0 | 1.3 | 1.5 | ... | 2.3 |
| Azaserine | | | | 47 | 43 | 40 | 29 | 27 | 26 | 33 | 28 |
| $MnCl_2$ | | | | 32 | 9.7 | 9.3 | 9.3 | 4.6 | 4.6 | 4.4 | 1.65 |
| TEM | | No. of induced mutants per $10^8$ survivors | *sd-4* | 5,500 | 3,600 | 3,400 | 4,300 | 6,100 | 5,700 | ... | 3,700 |
| Azaserine | | | *sd-4* | 1,600 | 2,600 | 2,500 | 3,500 | 2,900 | 1,700 | 1,500 | 1,900 |
| Azaserine | | | *cys-2* | 47 | 65 | 62 | 120 | 130 | 130 | 80 | 80 |
| $MnCl_2$ | | | *sd-2* | 250 | 1,100 | 800 | 800 | 240 | 180 | 190 | 310 |
| $MnCl_2$ | | | *cys-2* | 23 | 105 | 50 | 21 | 23 | 18 | 12 | <15 |

TABLE 3

EFFECT OF REMOVAL OF MUTAGEN AND POSTTREATMENT WITH SALTS OF MONO- AND BI-VALENT METALS ON NUMBER OF MUTANTS (*sd*-4) INDUCED BY 2 HOURS OF PREVIOUS TREATMENT WITH TEM OR AZASERINE AT 32° C.

| POSTTREATMENT MEDIUM | | INTERVAL (HOURS) OF POSTTREATMENT AFTER EXPOSURE TO: TEM (10 μG/ML) 0 | 1 | 4 | 24 hrs | AZASERINE (5 μG/ML) 0 | 1 | 4 | 24 hrs |
|---|---|---|---|---|---|---|---|---|---|
| Distilled water | Percentage of survivors | 70 | 62 | 52 | 19 | 74 | 44 | 41 | 26 |
| 0.1 *M* NaCl | | ... | 66 | 89 | 29 | ... | 54 | 33 | 8 |
| 0.1 *M* KCl | | ... | 62 | 56 | 30 | ... | 50 | 44 | 12 |
| 0.1 *M* $MgCl_2$ | | ... | 55 | 57 | 26 | ... | 16 | 7 | 5 |
| 0.1 *M* $CaCl_2$ | | ... | 71 | 54 | 20 | ... | 50 | 1.3 | 1.1 |
| 0.1 *M* $ZnCl_2$ | | ... | 17 | 1.6 | <0.01 | ... | <0.01 | <0.01 | <0.01 |
| 0.1 *M* $MnCl_2$ | | ... | 53 | 46 | 19 | ... | 1.2 | 0.6 | 0.1 |
| Distilled water | No. of induced mutants per $10^8$ survivors | 1,100 | 1,500 | 1,300 | 3,100 | 2,400 | 3,800 | 6,900 | 2,600 |
| 0.1 *M* NaCl | | ... | 1,500 | 850 | 550 | ... | 4,000 | 6,300 | 2,600 |
| 0.1 *M* KCl | | ... | 1,600 | 850 | 840 | ... | 4,400 | 1,700 | 320 |
| 0.1 *M* $MgCl_2$ | | ... | 1,700 | 1,100 | 900 | ... | 9,300 | 14,000 | 2,500 |
| 0.1 *M* $CaCl_2$ | | ... | 1,600 | 4,800 | 1,200 | ... | 4,200 | 950 | |
| 0.1 *M* $ZnCl_2$ | | ... | 800 | 5,100 | 0* | ... | 0* | 0* | 0* |
| 0.1 *M* $MnCl_2$ | | ... | 1,650 | 1,200 | 1,200 | ... | 11,500 | 11,000 | 15,000 |

* Excessive killing prevented the determination of mutagenicity.

In the experiments summarized in Tables 2 and 3, cells were treated at 32° C. for either 2 hours or 18 hours. After treatment, they were centrifuged, washed free of extraneous mutagen, and suspended in distilled water or various salt solutions; assays were made periodically over a period of 24 hours. As can be seen from Table 2, the number of mutants could not be reduced by this procedure, designed to allow any unbound intracellular mutagen to leach out of the cell. Indeed, the number of mutants seemed to increase approximately twofold during the first few hours of this presumed leaching process. The 24-hour readings, however, on the average were not significantly changed in most experiments.

The experiments in which salt solutions provided the posttreatment followed a generally similar course during the first hours of "leaching." Some salts even slightly or moderately increased the mutagenic action of TEM or azaserine during

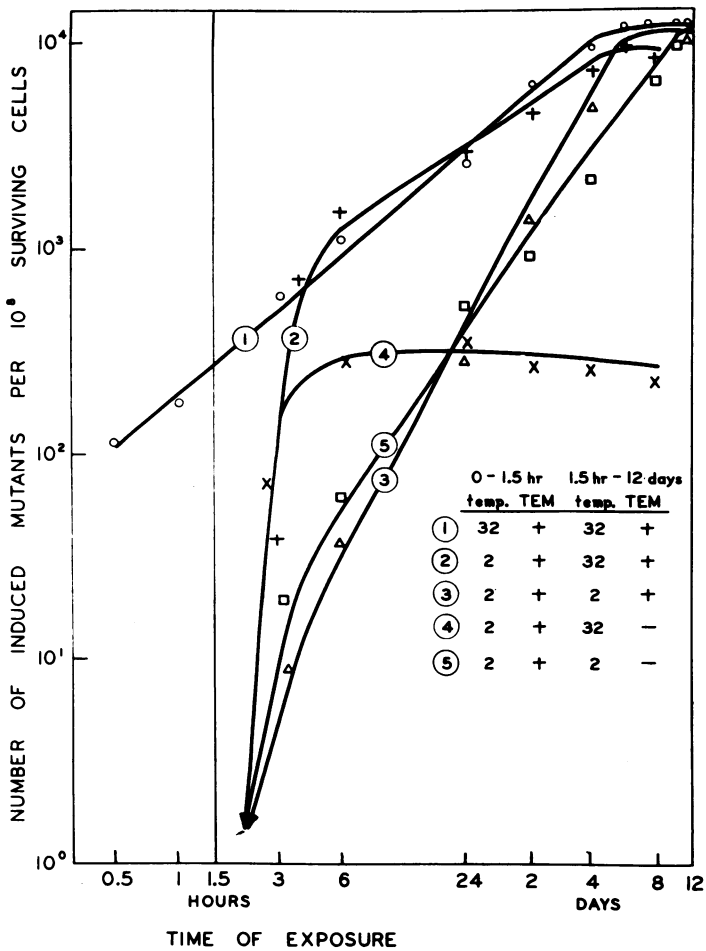


FIG. 5.—The effects of withdrawal of mutagen (TEM; 10 μg/ml) and change in temperature (2°, 32° C.) on the number of induced mutants (*sd-4* reversions). TEM was present throughout experiments 1–3 but only in the first hour and a half in experiments 4 and 5. Points represent one experiment, but comparable results were obtained in two other identical experiments.

1–4 hours of posttreatment. After 24 hours, however, the mutagenic effect in most cases was less than that obtained with distilled-water treatment. This was especially true for TEM, in which case most of the salts reduced the final number of mutants; only 0.1 KCl showed a considerable antagonistic effect against azaserine.

The most pronounced and permanent synergistic effect on the mutagenic action of azaserine was produced by $MnCl_2$, which, however, did not influence the mutagenicity of TEM. Magnesium ions caused a similar, although less permanent, effect. More detailed analysis of these interactions has been described elsewhere.[9]

*Effect of Early Withdrawal of Mutagen (Fig. 5).*—The results of earlier experiments had indicated that an appreciable increase in mutants could be expected when mutagen-treated cells were washed and held in water or salt solution. This increase was noted both at 32° and at 2° C. (Table 4). Experiments were therefore designed to study the effects of short-term exposure to mutagen at low temperature, followed by prolonged treatment in the same suspending medium (distilled water). TEM was selected as the mutagen because of the instability of azaserine evident from Figure 4 and Tables 2–4.

TABLE 4

EFFECT OF WITHDRAWAL OF MUTAGEN AND CHANGE IN TEMPERATURE ON NUMBER OF MUTANTS INDUCED (*sd-4* TO STREPTOMYCIN INDEPENDENCE) IN 18 HOURS, AT 2° C. BY EXPOSURE TO TEM (10μG/ML) OR AZASERINE (5 μG/ML)

| MUTAGEN | | INTERVAL (HOURS) AND TEMPERATURE OF POSTTREATMENT IN WATER AFTER PREVIOUS EXPOSURE TO MUTAGEN | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Temp. | 0 | 0.5 | 1 | 2 | 4 | 6 | 24 hrs. |
| TEM | Percentage of survivors | 2°C. | 100 | 84 | 90 | 86 | 80 | 65 | 67 |
| TEM | | 32 | 100 | 98 | 86 | 82 | 80 | 67 | 55 |
| Azaserine | | 2 | 90 | 63 | 64 | 71 | 63 | 62 | 60 |
| Azaserine | | 32 | 90 | 68 | 73 | 86 | 70 | 67 | 59 |
| TEM | No. of induced mutants per $10^8$ survivors | 2°C. | 80 | 80 | 72 | 75 | 105 | 110 | 170 |
| TEM | | 32 | ... | 139 | 194 | 184 | 166 | 177 | 264 |
| Azaserine | | 2 | 11 | 24 | 27 | 28 | 38 | 45 | 44 |
| Azaserine | | 32 | ... | 36 | 59 | 48 | 40 | 61 | 35 |

Figure 5 represents the results of an experiment in which the cell suspension was treated for $1^1/_2$ hours with 10 μg. of TEM at 2° C., after which period it was divided into four equal portions. Two were incubated further in the presence of TEM, one at 2° C. (curve *3*) and the other at 32° C. (curve *2*). Both curves reached plateaus at about $10^4$ mutants per $10^8$ surviving cells, but curve *2* climbed much faster within the first few hours after transfer of the cells to 32° C. and then followed control curve *1* representing cells treated from the beginning at 32° C. These results are similar to those shown in Figure 2.

Somewhat paradoxical results were obtained with the remaining two portions of the cell suspension. Mutagen was removed from both by centrifugation and washing of the cells at 2° C., before reincubation in distilled water at 2° C. (curve *5*) and at 32° C. (curve *4*). Curve *5* followed the same general course as curve *3*. Curve *4* first resembled curve *2*, but the number of mutants soon became constant at a level approximately 30–40 times lower than that recorded for any of the other three portions. Thus cell suspensions exposed to mutagen at low temperature for only a limited period seemed to yield a higher number of mutants at low temperature than at 32° C.

## COMMENTS

The experimental data here presented substantiate the previously suspected complexity of induced mutational processes. Diffusion of the mutagen into the cell is presumably the first step of the mutational event. For TEM and azaserine, diffusion proceeds unaided, while a violent osmotic decompression appears to be required to introduce $MnCl_2$ into the cell[2, 10] (Table 1). The observed maximum expression of mutagenicity after a rather short exposure to TEM at 2° C. (Fig. 5, curve *5*) is consistent with a high efficiency of diffusion at low temperatures. The same experiment also indicates the existence of a second step, a semireversible binding of the mutagen in a form in which it is not lost during subsequent incubation in distilled water at 2° C. and its effect is gradually expressed to the same degree as if the extraneous mutagen were not removed. Curve *4*, in the same experiment, however, suggests a partial reversibility of the second step at a higher temperature, as an explanation of the paradoxically lower final number of mutants at 32° C. These experiments, however, do not permit a definite distinction between true reversal with loss of intracellular mutagen and a secondary reaction that may involve the decay of some mutagenic principle.

The processes of diffusion and semireversible binding take place very rapidly at 2° and 32° C., but the next step, which is manifested as an increase in mutants after removal of the mutagen, is temperature-dependent to a high degree. Measurement of the rate of the third step is possible because of the nature of the assay system, in which the final number of mutant colonies is determined at the time the treated cells are plated on the selective agar medium. This step seems to be arrested when the cells are transferred from water to the solid growth medium lacking only one growth factor, where, as shown by earlier experiments,[2] they can undergo a few terminal divisions. The situation thus roughly resembles that encountered by Witkin[11] in her studies of ultraviolet-induced mutations, a short-term exposure to TEM at 2° C. being analogous to a mutagenic dose of irradiation; in both cases the final number of mutants is highly dependent on the events intervening between the exposure to mutagen and the end of the first cell division on the selective medium. Determination of the fate of intracellular mutagen awaits biochemical and radioactive tracer[10] studies.

We would like to acknowledge the very skilful technical assistance of Miss Clara E. Biro.

## SUMMARY

Triethylene melamine and azaserine are mutagens particularly suited to the quantitative evaluation of mutagenic processes, because within wide limits of effective concentration the survival of treated cells is close to 100 per cent. The maximum yield of over $10^4$ mutants per $10^8$ surviving cells produced by TEM does not depend on temperature, although the rate of appearance of the mutants is much lower at 2° than at 32° C. Only a short period of exposure to TEM at low temperature is required to obtain subsequent full expression of mutagenicity in the absence of the mutagen at 2° C.; transfer to 32° C. causes the effect of the mutagen to be partially lost, presumably as a result of secondary decay of a mutagen-cell complex. The kinetic data point to the intricate nature of the mutagenic response with participation of the postulated steps: diffusion and semireversible binding,

which are rapid even at low temperatures, and a temperature-dependent reaction which proceeds at a comparatively low rate in a nutrient-free, aqueous medium. The events which follow transfer of the cells to solid media were not elucidated.

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† Postdoctoral Fellow (U. S. Government, Fulbright and Smith-Mundt Act). Present address: S. B. Garda College, Navsari, Surat Dt., Bombay, India.

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## *TRIVIALITY OF VECTOR BUNDLES OVER THE AFFINE SPACE $K^2$*

By C. S. Seshadri

TATA INSTITUTE OF FUNDAMENTAL RESEARCH, BOMBAY, AND
CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE, PARIS

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In his paper "Faisceaux algébriques cohérents,"[1] J. P. Serre has proposed the problem: Is every algebraic vector bundle over the affine space $K^n$ trivial? Or, equivalently, is every finitely generated projective module $P$ over the ring of polynomials $K[x_1, \ldots, x_n]$ in $n$-variables free? For the case $n = 1$, it is well known that the answer is in the affirmative. In this note it is shown that the answer is still in the affirmative for the case $n = 2$; in fact, we prove that every finitely generated projective module over $A[x]$, the ring of polynomials in one variable over a principal ideal ring $A$, is free. The author is indebted to Professor J. P. Serre, who suggested the demonstration presented here after having seen the original "non-intrinsic" demonstration of the author for the case $A[x] = K[x_1, x_2]$, $K$ algebraically closed.

Let $\sigma$ be a commutative integrity domain with unit. We denote by $SL^*(n, \sigma)$ the group of inversible matrices of order $n$ over $\sigma$ with determinant $\pm 1$ and by $SL(n, \sigma)$ its subgroup of matrices with determinant $+1$.