# THE EFFECTS OF NITROGEN MUSTARD ON ESCHERICHIA COLI[1]

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A review of the genetic literature on chemically induced mutations would include many references extending back for over thirty years to the pioneer studies of T. H. Morgan (1914). The earlier studies were either negative or inconclusive. Unequivocal demonstration of a powerful chemical mutagen was not made until a group of geneticists working in England during the recent war began investigating the effects of mustard gas (Auerbach *et al.*, 1947). By means of the CLB test Auerbach and her colleagues were able to show that sex-linked lethals in *Drosophila* were induced with a frequency comparable to that previously attained only with short-wave high energy radiation. Subsequently nitrogen and sulfur mustards were successfully employed to induce genetic changes, these compounds having the general formulae: $O(CH_2CH_2 \cdot S \cdot CH_2CH_2Cl)_2$; $CH_3N(CH_2 \cdot CH_2Cl)_2$; and $N(CH_2 \cdot CH_2Cl)_3$. Their biochemical properties are thought to depend on intramolecular cyclization to form onium cations of high reactivity and possessing the ability to alkylate many cellular constituents. Possibly the alkylation of nucleoprotein through carboxyl or amino linkages is attained. The reactivity of enzymes, particularly phosphokinases, with mustard compounds and the inhibition of oxidative and glycolytic mechanisms by ethylenimonium and sulfonium cations emerge as well-established biochemical properties. A single site of action or specific substrate in all probability does not exist for these highly reactive substances, and geneticists are compelled in the present state of limited knowledge to use the mustards empirically as chemicals capable of producing a large number of diverse biochemical changes in the cell. Among these changes we may include mutations in microorganisms (Horowitz *et al.*, 1946; Tatum, 1947).

Historical retrospection in the field of induced mutation reveals that a suitable method for the demonstration of mutants is at least as important as choice of an appropriate mutagenic agent. Muller's contribution of the CLB technique in *Drosophila* and more recent analogous techniques form the keystone to all studies of mutation frequency in this organism. Controlled studies of mutation frequency in bacteria by measuring the rate of change to bacteriophage resistance represent use of but one of a variety of criteria available to the bacteriologist interested in genetic modifications (Luria, 1947). Phage resistance affords a method at least as simple as the use of altered enzyme specificities or biochemical growth requirements to measure mutation frequency.

In addition, the work of Demerec and Latarjet (1946) with X-rays and ultraviolet light as mutagenic modifiers of mutation rate to phage resistance provides

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an interesting parallel to related studies with chemicals. Therefore we have chosen the effect of nitrogen mustard on mutation to phage resistance in *Escherichia coli* as the subject for investigation.

## MATERIALS AND METHODS

The strains of bacteriophage employed were T1 and T2, in the nomenclature of Demerec and Fano (1945). Strain T2 served in testing for contaminants by the method that these authors have described. Fourteen stocks of radiation-resistant *E. coli* (B/r) were obtained from Dr. E. Witkin. Of independent origin, the B/r strains were known to be identical in their degree of resistance to X-rays and to ultraviolet light (Witkin, 1947*a*). A normal nonmotile strain of *E. coli*, designated as B, was the stock from which all cultures were originally derived.

The method employed in testing for induced mutation by chemical agents consists of two essential components: (1) detection of any change in the relative proportion of phage-sensitive to phage-resistant cells after exposure to a chemical, and (2) test to determine whether any shift in the relative proportions of phage-sensitive cells is due to differential susceptibility of resistant and sensitive individuals to the chemical agent under analysis as a potential mutagen. The actual procedure utilized in detecting alterations of relative proportion in sensitive cells (B) to cells resistant to the phage T1 involves initially the preparation of a culture containing low numbers of the resistant mutants. Since resistance is to phage T1, resistant cells are conventionally described as B/1. For simplification, mutants described in this investigation as B/1 include two classifications: cells resistant to T1 only (B/1), and cells becoming simultaneously resistant to T1 and T5 (B/1, 5). The preparation of stocks proceeds as follows: One-tenth ml of a $10^{-6}$ dilution obtained from a 24-hour, aerated, low background nutrient broth culture is used to inoculate 8 to 10 flasks containing 150 ml of medium. The inoculum will contain approximately $2 \times 10^2$ sensitive organisms, with a low probability that any resistant cell is carried in one inoculation. After forced aeration at 37 C for 24 hours the series of inoculated flasks will be found by dilution assays to contain approximately $2 \times 10^9$ bacteria per ml. By the plating of undiluted 0.1-ml aliquots from each culture on nutrient agar previously spread with 0.1 ml of undiluted phage T1 all sensitive cells are lysed. Thus only B/1 cells remain to form colonies on the agar surface. The proportion of B/1 to B is obtained by comparing for each individual culture the number of B/1 colonies obtained with the titer as determined by dilution assay on unphaged plates. This proportion is defined as the background. For example, if a culture contains $3.5 \times 10^9$ bacteria per ml and 35 colonies appear from a 0.1-ml sample plated in the presence of T1, we may say that the background is 10 B/1 per $10^8$.

An obvious aid in studies of mutation is a low number of mutants spontaneously present in the culture to be tested. A low background number of less than 20 B/1 per $10^8$ is obtained only when mutation of B to B/1 occurs rarely in the growth of a culture and late enough in the growth phase so that descend-

ants of the mutated cells do not eventually constitute a large proportion of the saturated population. In an actively growing broth culture spontaneous mutation of B to B/1 occurs with a frequency in the order of 1 per $10^8$. The occasional appearance of spontaneous mutants in the early growth of cultures inevitably provides a proportion of high background preparations that are discarded as unsuited for the desired experimental purpose.

Low background cultures are centrifuged, and the sedimented cells from several independent broth preparations are pooled to provide at least 3 ml of cells. Five-tenths ml of the sedimented cells are added to an equal volume of saline and 1 ml of broth, giving a total volume of 2 ml with a titer of 2 to 5 $\times$ $10^{10}$ per ml. This sample is assayed to determine exact titer and background. The addition of saline and broth assumes a meaning when we compare the assay titer and background with the remaining aliquot of cells obtained by the original centrifugation and set aside for experimentation. This remaining aliquot undergoes a dilution corresponding to the sample employed for determination of titer and background. The first dilution occurs when an equal volume of buffered nitrogen mustard is added to the cell suspension. The material is then incubated at 37 C for 1 hour. A later dilution takes place at the termination of chemical treatment by the addition of a quantity of chilled nutrient broth equal to the combined volume of cells and saline. In each aliquot the cellular suspension thus constitutes 25 per cent of the total liquid volume. Originally, addition of broth to the experimental preparation was intended to retard the reaction between cells and chemical by dilution and by reduction of the temperature from the experimental level of 37 C. Later it was found that, when cells have been incubated at 37 C with 0.1 per cent nitrogen mustard for 1 hour, the reaction between chemical and substrate has proceeded at a decelerated rate to a point where further exposure produces insignificant change in survival.

*Determination of zero-point mutations.* Following treatment it is necessary to determine the extent of killing. Routine assay tubes are set up at dilutions of $10^{-2}$, $10^{-4}$, and $10^{-6}$, providing an index of survival by subsequent plating. One-tenth ml of the chemically treated cells is then placed on each of 20 nutrient agar plates that have been previously prepared by the surface spreading of 0.1 ml of undiluted T1 phage. After incubation of the plates intended for assay it is possible to determine the number of viable cells per ml following chemical treatment. The quantity of viable cells plated on 20 phaged nutrient agar plates must correspond to twice the assay number (0.1 ml $\times$ 20). The number of B/1 cells is determined directly by counting the colonies on phaged plates following their incubation, permitting the calculation of B/1 per $10^8$ survivors. A comparison may then be made of the proportion of B/1 per $10^8$ cells before and after chemical treatment. Simple subtraction of the background number from the final relative number of resistant cells provides a direct index of mutation induced in the nondividing cells during chemical exposure, providing selection is ruled out by the necessary experiments to be conducted concurrently. For example, if a stock containing 3 B/1 cells per $10^8$ as background is treated by a chemical known to be nonselective, with a final proportion of 250 B/1 per $10^8$

chemically treated viable organisms, the number of induced mutants is 250 minus 3, or 247 B/1 per $10^8$ cells. In experiments with radiation Demerec and Fano (1945) described the mutations that occur in nondividing cells as zero-point mutations. Refrigeration of all cells at 12 C for at least 12 hours prior to experimental use ensures the absence of cell division during 1-hour exposure to the chemical and places mutations attained by this technique in the category of zero-point mutants.

*Determination of delayed mutations.* By a supplementary method the class of B/1 known as delayed mutations may be obtained. Such mutations appear in the descendants of treated cells with a frequency greatly exceeding the spontaneous rate of occurrence. They are thought to result from labile modifications of genetic structure requiring the intervention of one or more cell divisions before expression. Delayed mutations are obtained by allowing treated cells to multiply on *unphaged* plates for sufficient time to extend beyond the stationary phase (4 to 6 hours). Assay for total cell number is then made by washing cells carefully from the agar surfaces, with dilution at varied levels to provide an estimate of total cell number attained by incubation from the original treated 0.1 ml spread on the plate. Another series of plates is phaged by exposure to T1, aerosolized from a commercial nebulizer (DeVilbiss no. 40). Aerosol avoids disturbance of bacterial colonies which must remain *in situ* for accurate determination of delayed B/1 mutants. Each colony that appears after delayed phaging represents either a zero-point mutation or a mutant that appears during the reproduction of sensitive cells through division. If the number of zero-point mutants has been determined in another series of plates, the delayed mutants may be derived by comparing the number of additional mutants with new cells arising during the incubation period and assayed by unphaged controls (Demerec and Latarjet).

One advantage of the technique for determining delayed mutations is that selection cannot play a role in the experimental result. All delayed mutants must have arisen *de novo* since they represent a number larger than the total number of phage-resistant cells (B/1) available for selection to act upon, as revealed by the total number of colonies present on plates phaged without prior incubation.

*Activity of methyl-bis (beta-chloroethyl)amine hydrochloride as related to pH.* It is known that aqueous solutions of the salts of nitrogen mustards are acid and relatively stable. At physiological hydrion concentrations the solution becomes highly reactive with rapid union of the ethylenimonium cation and available anionic groups. It has been a routine procedure to buffer the chemical prior to the addition of bacteria. Reaction between nitrogen mustard and buffer will rapidly utilize available ethylenimonium cations, as will reaction with the solvent. Consequently, the buffering of an aqueous solution of methyl-bis(beta-chloroethyl)amine hydrochloride, or HN-2, by $Na_2HPO_4$ results in a very rapid alteration in the toxicity of the solution. Since any test for mutagenesis requires some measure of control over toxicity of the mutagenic agent, an investigation of the effect of $Na_2HPO_4$ on HN-2 assumes significance. Addition of 8 ml of

M/4 $Na_2HPO_4$ to 2 ml of 1 per cent HN-2 results in a rise of pH from 4.5 to 6.8. Addition of equal volumes of this freshly prepared 0.2 per cent buffered HN-2 to a cell suspension with subsequent incubation at 37 C for 1 hour gives a survival rate of 1.3 per $10^8$. If the buffered HN-2 is not added to another sample of cells until 5 minutes after the addition of buffer, the survival value becomes $1 \times 10^5$ per $10^8$. The reduced physiological activity of buffered HN-2 dependent upon elapsed time before the addition of cells is represented graphically in figure 1.

It is apparent that the toxic effects of HN-2 in buffered solution decrease at a decelerating rate with time and that after 15 minutes' contact with buffer only a minor residual toxicity remains. The earlier experiments conducted in this study were made without knowledge of this fact, accounting in part for variations in the degree of killing obtained by identical concentrations of buffered HN-2.

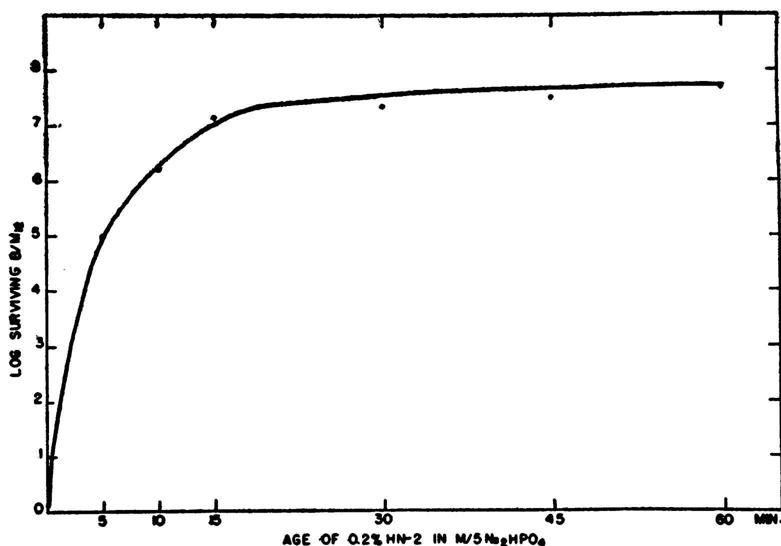


*Figure 1.* Effect of aging HN-2 in buffer before addition to *E. coli.*

INDUCTION OF HN-2 RESISTANCE IN E. COLI STRAIN B

Preliminary experiments established the fact that no unequivocal mutagenic effect of HN-2 on strain B is demonstrable at concentrations of 0.005 or 0.04 per cent with 1-hour exposure at 37 C. At the higher concentration survival was reduced to 0.04 per cent, yielding only two colonies in 20 plates (vol. 2 ml of treated cell suspension). The obvious method of attempting to increase the proportion of delayed mutants (eliminating selection) by increased concentration or time of exposure was made impractical by the extreme toxicity of the compound. Since strains of bacteria resistant to toxic agents may be obtained by the selection of mutant cells, the possibility arises that the production of a strain of *E. coli* resistant to HN-2 would allow higher concentration of the chemical to be employed in tests for mutagenesis. Several strains of mustard-resistant *E. coli* were produced by seeding ca. $2 \times 10^9$ cells in petri plates, which were then

filled with 10 ml of proteose no. 3 agar at HN-2 concentrations ranging from 0.1 to 0.4 per cent. One colony from the plate having the lowest number of survivors was picked to provide a new strain of cells. Strains have been designated as B/M1, B/M2, . . . B/M12, the numerical notation indicating the number of exposures to HN-2 preceding the isolation of a particular mustard-resistant stock. Thus strain B/M12 was derived from B/M11. Increase in resistance to HN-2 was found to occur progressively and is not an all-or-none phenomenon. A comparison of different strains of *E. coli* exposed to HN-2 is presented in table 1.

TABLE 1

*Resistance of E. coli strains to HN-2*

| STRAIN | SEEDING | COLONIES AT VARIED CONCENTRATIONS OF HN-2 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0.35 | 0.3 | 0.25 | 0.12 | 0.15 | 0.1 | 0.05 | 0.025% |
| | *Bact. per 0.5 ml* | | | | | | | | |
| B | $2 \times 10^9$ | — | — | 2 | 41 | 557 | $>10^4$ | $>10^4$ | $>10^4$ |
| B/M1 | $3 \times 10^9$ | — | 1 | 10 | 4,100 | $>10^4$ | $>10^4$ | — | — |
| B/M2 | $4 \times 10^9$ | — | 30 | 215 | $>10^4$ | $>10^4$ | $>10^4$ | — | — |
| B/M7 | $1 \times 10^9$ | 202 | 4,500 | $>10^4$ | $>10^4$ | — | — | — | — |
| B/r* | $1 \times 10^9$ | 22 | 1,500 | $>10^4$ | $>10^4$ | — | — | — | — |

* Radiation resistant.

TABLE 2

*Induction of phage-resistant mutants of B/M12 by 0.1 per cent HN-2, aged three, two, and one minute in* M/5 $Na_2HPO_4$ *before addition to cells*

| PER CENT SURVIVAL | AGE OF BUFFERED HN-2 | SEEDING | ZERO-POINT MUTANTS |
|---|---|---|---|
| | *min* | | |
| 0.9 | 3 | $9.2 \times 10^7$ | $1.1 \times 10^1$ per $10^8$ |
| 0.007 | 2 | $4.1 \times 10^5$ | $2.4 \times 10^2$ per $10^8$ |
| 0.0004 | 1 | $1.5 \times 10^4$ | $2.3 \times 10^3$ per $10^8$ |

It is apparent that strains exposed to HN-2 should provide through selective mechanisms a form suitable for further studies of mutagenesis, permitting higher concentrations of HN-2 to be used. Although a high degree of resistance was attained in strain B/M7, transfers and selection of surviving colonies were continued to derive strain B/M12, which was used for further study. HN-2 was at the same time found to be relatively nontoxic to a radiation-resistant strain (B/r), an observation extended by additional experimentation (Bryson, 1947).

*Mutagenic effects of HN-2 on B/M12.* A 0.1 per cent solution of HN-2 in M/5 $Na_2HPO_4$ was selected as the optimum concentration for tests of induced phage resistance by mutation of B/M12 to B/M12/1. The toxicity of HN-2 was varied by allowing different intervals of time to elapse before cells were added to the buffered chemical. A summary of three experiments is given in table 2.

Experiments were conducted by seeding 20 phaged plates, each with 0.1 ml

of HN-2-treated cells. Chemical treatment was for 1 hour at 37 C. It is observed that the proportion of zero-point mutants increases with decreased survival. To what extent this is a real and not an apparent induction of mutation depends on the relative sensitivity of B/M12 and B/M12/1 to HN-2. Supplementary experiments show that the phage-sensitive stock is more resistant to HN-2, eliminating the possibility of selection in the interpretation of data presented in table 2. Delayed mutation where selection is not a factor gave 370 delayed mutants per $10^8$ at 0.007 per cent survival with 8.3 cell divisions, and 440 per $10^8$ at 0.9 per cent survival with 2.2 cell divisions. The rate of induced and spontaneous mutation falls off in cell divisions following a stationary phase, being highest in the first division.

*Resistance of B/M12 to ultraviolet light.* The effect of nitrogen mustard on cells and tissues has often been compared to that of short-wave radiation. An interesting question therefore arises: Is strain B/M12 more resistant to radiation than the normal B cells? The question was investigated by exposing cells spread over the surface of nutrient agar plates to the emanations of a G. E. mercury vapor arc lamp. There can be no doubt that HN-2-resistant strains are also resistant to the lethal effects of ultraviolet light. Five of the numerous ultraviolet-resistant strains provided through the courtesy of Dr. E. Witkin were tested simultaneously. There appears to be no significant difference between HN-2 and radiation-resistant stocks (B/r nos. 34, 14, 23, 32, and 37). Simultaneous protection against the toxic properties of both ultraviolet and nitrogen mustard through mutation suggests a common biochemical effect of these two agents. The similarity in resistance is shown graphically in figure 2. Survivors per $10^8$ cells are indicated by the ordinate. The common pattern of resistance has been discovered independently by the Italian geneticist, L. Cavalli (1948).

*Reciprocal relationship of resistance to HN-2 and radiation.* The demonstration that strains of *E. coli* resistant to HN-2 also resist the toxic effects of ultraviolet light suggests that the protective mechanism may be identical. Theoretically four alternative situations might exist: HN-2 resistance and radiation resistance; HN-2 resistance and radiation sensitivity; HN-2 sensitivity and radiation resistance; and sensitivity to both agents. If HN-2 resistance and radiation resistance are identical or coupled, only two states may prevail, either resistance or sensitivity *in toto*. As a test for the four alternative states, nutrient agar plates were spread with approximately $10^6$ cells of eight representative bacterial strains. One-half-inch disks of filter paper were placed on each side of the petri plates after saturation with 0.03 ml of 0.25 per cent HN-2. One side of each plate, including a disk, was then shielded while the remaining surface was exposed to 50 ergs/mm$^2$/second of ultraviolet light. After 5 hours of incubation at 37 C the exposed half-plate was again irradiated at 1,800 ergs (double irradiation technique of Witkin). The diagram shown in figure 3 gives the four possible consequences of experimental treatment. Crosshatched areas indicate the portion of plate shielded from radiation. As shown in figure 3, only two categories of plates were found. Cells resistant to HN-2 as revealed by the growth

of cells in contiguity with the filter paper disks were also resistant to radiation, allowing survival on the unshielded portion of the plate. Thus all tested strains were included in two categories: (1) sensitive to both HN-2 and ultraviolet, or

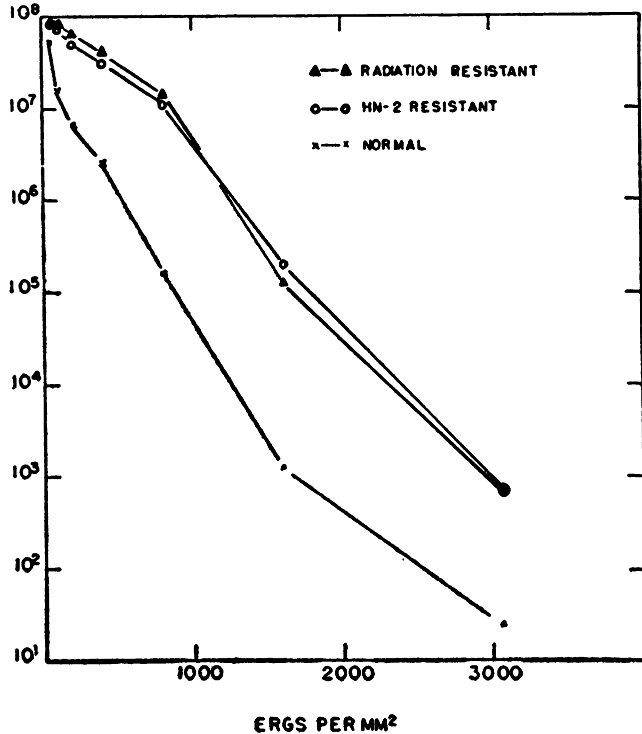


*Figure 2.* Relative resistance of **normal, radiation-resistant, and mustard-resistant** strains of *E. coli* to ultraviolet light.

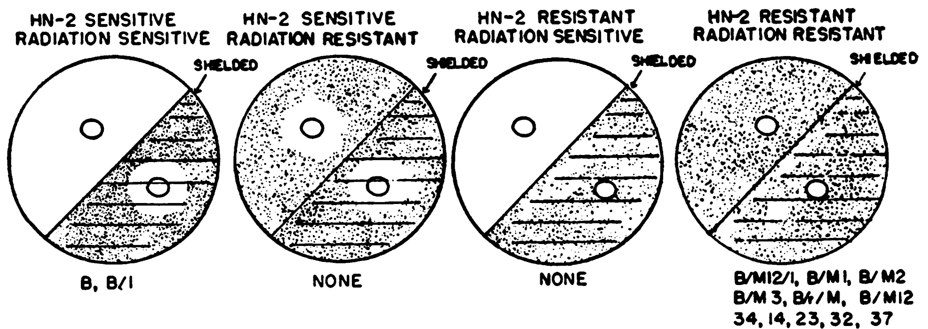


*Figure 3.* Distribution of *E. coli* strains exposed to methyl-bis(beta-chloroethyl)amine hydrochloride and ultraviolet radiation.

(2) resistant to both agents. The resistant strains consisted of three independent radiation cultures (B/r) and three cultures that had been exposed to the effects of HN-2 in previous experiments.

One of the HN-2-resistant strains was also phage-resistant—B/M12/1. Zones of inhibition surrounding the paper disks averaged 5 mm in width, measured radially from the disk border. A few resistant colonies could be found in HN-2- and ultraviolet-inhibited zones, but in insufficient quantity to affect the validity of the test.

As a further procedure, representative strains were inoculated from slants into nutrient broth and aerated at 37 C for 24 hours. Cultures were then added to petri dishes at a dilution of $10^{-6}$ in 0.5-ml quantity. Exactly 10 ml of proteose no. 3 agar containing varied concentrations of HN-2 were then mixed in

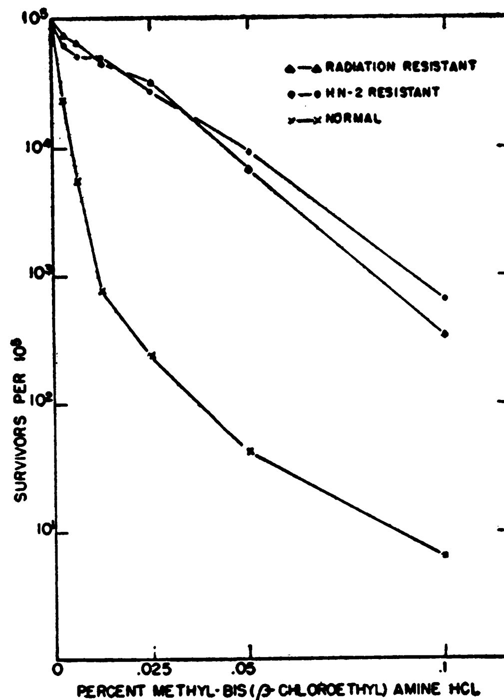


*Figure 4.* Relative resistance of normal, radiation-resistant, and HN-2-resistant strains of *E. coli* to methyl-bis(beta-chloroethyl)amine hydrochloride.

each inoculated plate with the bacterial suspension and incubated. Control plates were inoculated simultaneously, yielding an average number of 680 bacteria per plate. Thirteen independent radiation-resistant stocks showed 2 to 77 survivors at a concentration of 0.1 per cent HN-2. Stock B/M12 yielded 7 survivors (colonies). No cells of strain B survived a concentration of 0.025 per cent HN-2.

It is concluded that all 14 radiation-resistant strains are able to grow in concentrations of HN-2 that inhibit the growth of the normal B strain. If a larger quantity of normal cells are used, some survivors may be found in plates containing 0.025 per cent HN-2 (30 survivors in 94,000).

To perform an experiment analogous to the ultraviolet exposure data pre-

sented in figure 2 it was necessary to determine the required inoculum size to yield a countable plate after treatment of cells with high concentrations of HN-2 in proteose no. 3 agar. After considerable experimentation, killing limits were determined for varied concentrations and the resulting data are given in figure 4. Because HN-2 loses its toxicity with time after addition to proteose no. 3, the experiment was duplicated reversing the order in which HN-2-containing media were added to the different strains of cells. Points on the curves represent the average of six HN-2-resistant stocks compared with six radiation-resistant stocks. Strain B and B/1 served as a control, their values before averaging being less than the mean survival of the HN-2- and radiation-resistant strains. Inspection of figure 4 amply justifies the conclusion that radiation-resistant *E. coli* closely resembles HN-2-resistant cells in relative insensitivity to toxic effects of the chemical.

## DISCUSSION

The conclusion may be drawn that although mutation of B to B/1 is not certainly induced by HN-2, the conversion is readily accomplished in a strain resistant to the chemical; in the presence of methyl-bis(beta-chloroethyl)amine hydrochloride, B/M12 cells may be changed to B/M12/1. Previous reports have indicated that HN-2 has the capacity to induce mutation (Auerbach *et al.*, 1947). The studies of Dr. E. Witkin (1947*b*) indicate that the variety of chemicals capable of inducing mutation is greater than had been anticipated. No practical advantages attend these findings at the present time, since the mutagenic influence of chemicals may more easily be duplicated with X-rays or ultraviolet radiation. Continued research with microorganisms may eventually uncover differences in the types or relative frequencies of genetic changes depending on the mode of induction, as previously observed by geneticists comparing the effects of radiation of varied wave length. A continued search in the field of chemically induced mutation offers the best hope for directed mutation, analogous to the transmutation effect of desoxyribonuclease on pneumococci as described by Avery, MacLeod, and McCarty (1944). Compared with the random action of ionizing radiations, specific chemicals should offer a more precise mechanism for altering the chemical structure of the cell to produce directed genetic modification. Highly reactive chemicals, including HN-2, would be expected to offer the least specificity.

It is believed that the most interesting result of this study is the finding that resistance to radiation and to methyl-bis(beta-chloroethyl)amine hydrochloride may involve an identical mechanism. A number of interesting hypotheses may be advanced to account for protection against these agents by a single genetically determined biochemical change. However, there exist no biochemical facts on which a sound hypothesis might be based. Until such facts are available, it therefore appears unduly speculative to indulge in interpretations of the mechanism of resistance. Present efforts should be directed rather to the analysis of enzyme activity, metabolic processes, and polymerization characteristics of the resistant cell structure.

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SUMMARY

Methyl-bis(beta-chloroethyl)amine hydrochloride (HN-2) is capable of inducing mutations to phage resistance in a strain of *Escherichia coli* specifically resistant to the toxic effects of this chemical.

The resistance of selected strains of *E. coli* to the lethal effects of HN-2 is identical or related to ultraviolet radiation resistance. Conversely, strains of *E. coli* that have mutated to ultraviolet resistance are simultaneously relatively insensitive to killing by HN-2.

Resistance to HN-2 and ultraviolet light therefore appears to result from related genetic changes and suggests a similarity in the mode of action of these two agents on the bacterial cell.

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