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CROSS-RESISTANCE RELATIONSHIPS IN *ESCHERICHIA COLI* BETWEEN ULTRAVIOLET RADIATION AND NITROUS ACID

ANTONIO ZAMPIERI AND JOSEPH GREENBERG

Palo Alto Medical Research Foundation, Palo Alto, California

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ABSTRACT

ZAMPIERI, ANTONIO (Palo Alto Medical Research Foundation, Palo Alto, Calif.), AND JOSEPH GREENBERG. Cross-resistance relationships in *Escherichia coli* between ultraviolet radiation and nitrous acid. J. Bacteriol. 87:1094-1099. 1964.—A number of radiosensitive and radioresistant strains of *Escherichia coli* were tested for sensitivity to injury by nitrous acid. All the radioresistant strains, including 13 radioresistant mutants of strain S, B/r, Bpr5, and K-12, were found to be significantly more resistant to nitrous acid than were the radiosensitive strains S and B. The radioresistant mutants of strain S, Bpr5, and K-12 displayed similar responses to nitrous acid and were less resistant than was strain B/r. Strains B and S were indistinguishable on the basis of nitrous acid sensitivity. The survival curves of all strains examined were similar in shape to corresponding survival curves after ultraviolet radiation. The sensitivity to nitrous acid of the radiosensitive strains S and B, but not that of the radioresistant strains, was found to be greater on Tryptone medium than on Penassay medium, and greater on Penassay medium than on glucose-salts medium. Between 2 and 3% of the strain S survivors of nitrous acid treatment were radioresistant; 46 such radioresistant mutants were isolated and found to be identical in cross-resistance pattern with radioresistant types (R_3, R_4, or R_6) previously described. The proportions in which these radioresistant types were found to occur were similar to those observed after selection by other radiomimetic agents.

Most of the mutants of *Escherichia coli* S resistant to mitomycin C (Greenberg, Mandell, and Woody, 1961*a*), nitrogen mustard and nitromin (Woody, Mandell, and Greenberg, 1961), nitrosoguanidines and azaserine (Greenberg et al., 1961*b*; Mandell, Woody, and Greenberg, 1961), and nitrofurazone (Woody-Karrer and Greenberg, 1963) are resistant to the other chemicals of this group and to ultraviolet and X radiation (Greenberg and Woody-Karrer, 1963). These diverse agents also have in common the property of being mutagens. The relationship implied is that all agents which exhibit a cross-resistance relationship with ultraviolet radiation are mutagens, and vice versa.

Nitrous acid is a mutagen in bacteria (Kaudewitz, 1959, 1963; Zamenhof, 1961), bacteriophage (Tessman, 1959; Vielmetter and Wieder, 1959), tobacco mosaic virus ribonucleic acid (Gierer and Mundry, 1958), *Pneumococcus* deoxyribonucleic acid (DNA; Litman and Ephrussi-Taylor, 1959), and *Bacillus subtilis* DNA (Anagnostopoulos and Crawford, 1961; Luzzati, 1962). If nitrous acid is, in fact, a radiomimetic agent, radioresistant mutants of *E. coli* should be cross-resistant with nitrous acid. Conversely, mutants selected with nitrous acid should exhibit concomitant resistance to radiation and radiomimetic chemicals. Rörsch et al. (1962) found that one radioresistant mutant of *E. coli* B was resistant to nitrous acid.

Earlier studies showed that there are 18 different radiation-resistant mutants of *E. coli* S (Woody-Karrer and Greenberg, 1963), identified as types R_1 through R_{18}; each is distinguishable by its distinctive cross-resistance pattern, i.e., its degree of resistance to ultraviolet radiation and to radiomimetic agents. Types R_3 and R_4 have been isolated as majority populations after treatment with nitrogen mustard, mitomycin C, azaserine, alkylnitrosoguanidines, nitrofurazone, and proflavine; the other R-types occur less frequently.

E. coli B (Roberts and Aldous, 1949) and *E. coli* S (Woody et al., 1961) are much more resistant to ultraviolet radiation when plated on glucose-salts medium than on complete (Tryptone) medium. Most of the radioresistant mutants of *E. coli* B and S, on the other hand, exhibit an identical degree of resistance to ultraviolet radiation, and the degree of resistance is not affected by whether they are plated on glucose-salts or complete medium.

However, there is a group of R types (e.g., R_6, R_{11}, R_{12}, and R_{13}) which exhibit a degree of resistance to ultraviolet radiation intermediate

between that of *E. coli* S and that of the other R-types. These strains of intermediate ultraviolet resistance, like *E. coli* S, appear to be more resistant to ultraviolet light when plated on glucose-salts medium than when plated on complete medium.

There are also mutants which are significantly resistant only to the selecting agent. These have been termed chemoresistant.

This report will show that the 13 radioresistant R types of *E. coli* S tested, as well as *E. coli* B/r (Witkin, 1947), Bpr5 (Alper and Gillies, 1960), and K-12 (naturally radioresistant), were all more resistant to nitrous acid than was *E. coli* B or S. The R types of S, as well as strains Bpr5 and K-12, displayed similar responses to nitrous acid and were less resistant than was B/r. *E. coli* B and S could not be distinguished on the basis of sensitivity to nitrous acid. Furthermore, between 2 and 3% of the strain S survivors of nitrous acid treatment were radioresistant; 46 such radioresistant mutants were found to be identical in cross-resistance patterns with one of three radioresistant types already described. The proportions in which these radioresistant types were found to occur were similar to those observed after selection by other radiomimetic agents. Finally, the sensitivity to nitrous acid of *E. coli* S and B, but not that of radioresistant mutants of S, was found to be greater when the treated bacteria were plated on Tryptone medium rather than on glucose-salts medium. Survival on Penassay medium was intermediate between that on Tryptone medium and that on glucose-salts medium.

MATERIALS AND METHODS

Bacterial strains. Most of the strains of bacteria used, their source, derivation, and properties, have been described in detail (Woody-Karrer and Greenberg, 1963). Strain W1895 is a derivative of K-12 obtained from Joshua Lederberg.

Compounds. Sodium nitrite was purchased from Mallinckrodt Chemical Works, New York, N.Y. The sources of the other compounds were described by Woody-Karrer and Greenberg (1963). To produce nitrous acid, 0.005 M sodium nitrite was dissolved in 0.1 M acetate buffer (pH 4.2).

Media. The media used contained the following (per liter of distilled water). Tryptone agar: Tryptone, 10.0 g; glucose, 1.0 g; sodium citrate, 2.0 g; sodium chloride, 8.0 g; and agar (BBL), 12.0 g adjusted to pH 7.0 (T7) with sodium hydroxide or pH 5.5 (T5.5) with hydrochloric acid. M9 agar: dibasic sodium phosphate, 5.8 g; monobasic potassium phosphate, 3.0 g; ammonium chloride, 1.0 g; sodium chloride, 0.5 g; glucose, 2.0 g; magnesium sulfate ($7H_2O$), 250 mg; calcium chloride, 14 mg; 1% gelatin solution, 10 ml; and agar, 8.0 g (Ionagar, Oxo Ltd., London). Peptone broth: peptone, 10.0 g; beef extract, 3.0 g; glucose, 1.0 g; and sodium chloride, 5.0 g.

Phosphate-buffered saline was 1% sodium chloride in 0.02 M phosphate buffer (pH 6.8). Tryptone Glucose Extract Agar was a commercial (Difco) preparation on which cultures were preserved. Penassay Agar was a commercial (Difco) preparation.

Measurement of resistance to chemical agents. The method used to measure the degree of resistance to chemical agents has been described (Mandell et al., 1961). Isolated clones were grown overnight in peptone broth at 37 C, adjusted with a model 9 Nepho-colorimeter to 3.5×10^8 cells per ml, and streaked on gradient plates according to the method of Szybalski and Bryson (1952). Gradient plates for testing the radiomimetic agents 1-methyl-3-nitro-1-nitrosoguanidine, mitomycin C, and nitrogen mustard were made with Tryptone agar (pH 5.5). The minimal inhibitory concentration was determined as follows: (length of solid growth)/(total length of streak) $\times$ maximal concentration of test compound (μg/ml).

It was not possible to demonstrate any difference between radioresistant and radiosensitive strains of *E. coli* on gradient plates at pH 5.5, the lowest pH compatible with growth. Presumably, at this pH so little nitrous acid was formed that death of the cells was attributable to other factors.

Sensitivity to ultraviolet radiation. The ultraviolet radiation source was a single 15-w General Electric germicidal lamp with a maximal output at 2,537 A. Calibrated with bacteriophage T_2 according to the method of Latarjet, Morenne, and Berger (1953), this lamp delivered 15.4 ergs mm^2 per sec. Cultures grown overnight in peptone broth were washed twice with buffered saline (pH 6.8), and were exposed with gentle agitation in 50-mm petri dishes containing 1 ml of the bacterial suspension. Exposures were made at a distance of 51.5 cm from the ultraviolet radiation source. Appropriate dilutions in cold phosphate-

buffered saline were plated in duplicate on both Tryptone agar (pH 7.0) and M9 agar, incubated at 37 C for 24 and 48 hr, respectively, and counted. All manipulations subsequent to irradiation were carried out in subdued light to minimize photoreactivation.

To determine rapidly whether survivors of nitrous acid were radioresistant, isolated clones from assay plates were grown overnight in peptone broth, streaked on Tryptone agar (pH 7.0), exposed to 77 ergs mm^2 of ultraviolet radiation, incubated 1 to 2 hr at 37 C, re-exposed to 230 ergs mm^2, and then incubated overnight at 37 C. Streaks with sparsely isolated colonies were considered as sensitive as parent strain S; with heavy, confluent growth, as fully radioresistant; with granular growth, as intermediate in radioresistance (type R_6).

Sensitivity to nitrous acid. To test for sensitivity to nitrous acid, bacteria were grown overnight in peptone broth, washed in buffered saline, and diluted to 10^7 bacteria per ml of acetate buffer (0.1 M; pH 4.2) containing 0.005 M sodium nitrite; 1 ml of the bacterial suspension in nitrite was shaken for varying times in a 50-ml flask, in a water bath at 37 C. Samples were diluted in buffered saline, plated on appropriate medium, and incubated 24 to 48 hr at 37 C; the number of colonies were then counted.

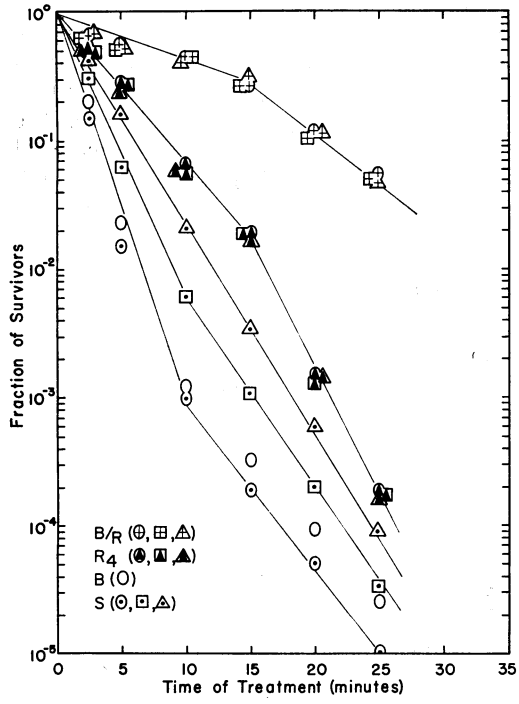

FIG. 1. *Survival of Escherichia coli S and B and radioresistant strains R_4 and B/r exposed to nitrous acid. Symbols: circles, plated on Tryptone (pH 7.0); squares, plated on Penassay; triangles, plated on M9 agar.*

RESULTS

Resistance to nitrous acid of previously isolated radioresistant mutants of E. coli. The survival of *E. coli* S and B and their radioresistant mutants, R_4 and B/r, as a function of duration of exposure to nitrous acid (0.005 M; pH 4.2) is shown in Fig. 1. All bacteria were plated on Tryptone agar.

The response of *E. coli* S and B was identical. Relative to *E. coli* S and B, B/r and R_4 were resistant to nitrous acid. However, B/r was significantly more resistant than was R_4 over the exposure period examined. All the survival curves had two components. With the radiosensitive nitrous acid-sensitive strains, *E. coli* S and B, the initial component had the more acute slope, indicating a more rapid rate of kill. With the radioresistant strains, the second component had the more acute slope. The general shape of the survival curves is strongly reminescent of those resulting from ultraviolet irradiation (Greenberg and Woody-Karrer, 1963).

Additional (12) radioresistant mutants of *E. coli* S, as well as strain K-12 and Bpr5 (Alper and Gillies, 1960), were tested for survival after exposure for 10 sec to nitrous acid (0.005 M; pH 4.2 at 37 C). All the strains were resistant to nitrous acid to approximately the same degree as was radioresistant type R_4 of *E. coli* S (Table 1).

Plating medium response. E. coli S, R_4, and B/r were plated after nitrous acid treatment on three different plating media, M9, Penassay, and T7 (Fig. 1). *E. coli* S was most resistant to nitrous acid when plated on M9, and most sensitive when plated on T7. The response, when plated on Penassay, was intermediate between that on M9 and T7. On the other hand, neither R_4 nor B/r exhibited any significant differences in sensitivity on any of the three plating media.

Selection of radioresistant mutants of strain S after nitrous acid treatment. E. coli strain S was treated with nitrous acid (0.005 M) for 25 sec (pH 4.2; 37 C), and was plated on T7 agar. Of the 10^{-4} surviving colonies, 2,300 were tested by the rapid method for detecting resistance to

ultraviolet radiation; 46 were found to be resistant: 45 exhibited resistance equivalent to *E. coli* S type R_3 or R_4, and 1 was equivalent to type R_6. All of these radioresistant mutants were tested by the gradient plate method for their resistance to 1-methyl-3-nitro-1-nitrosoguanidine, mitomycin C, and nitrogen mustard; 33 (72%) had cross-resistance patterns identical with radioresistant type R_4; 12 (26%) with type R_3; and 1 (2%) with type R_6 (Table 2). Ultraviolet survival curves were obtained for four of the R_4 strains and three of the R_3 strains, and were found to be identical with those of the R_4 and R_3 prototypes. No plating-medium response was exhibited. The R_6-like mutant had an ultraviolet survival curve identical with a prototype R_6, and exhibited plating-medium response.

No nitrous acid-resistant mutants were discovered among ten radiosensitive survivors of nitrous acid treatment examined.

DISCUSSION

It was shown that radioresistant mutants of *E. coli* were resistant to nitrous acid, and that 2 to 3% of the survivors of nitrous acid treatment were resistant to radiation and radiomimetic chemical agents. The radioresistant mutants isolated from among survivors of nitrous acid were identical in cross-resistance patterns to types previously isolated from among survivors of other radiomimetic agents. As with other selecting agents (Table 2), the majority of radioresistant mutants were type R_4; about 25% were R_3; and about 2% were R_6. Nitrous acid, therefore, belongs to a class of chemical agents, called radiomimetic, which exhibit cross-resistance with radiation and with each other.

It has been shown (Greenberg, *personal communication*) that radiation sensitivity in *E. coli* S is associated with a gene closely linked to the locus for resistance to coliphage T6 and that, when resistance to radiation is transmitted from radioresistant donors to radiosensitive recipients, resistance to radiomimetic agents is transmitted concomitantly. Resistance to radiation and radiomimetic chemicals is, therefore, the result of a shared genetic and physiological mechanism.

There is corollary evidence in this report that *E. coli* responds to injury inflicted on the cell by nitrous acid as it responds to injury caused by ultraviolet radiation. For both ultraviolet radiation (Greenberg and Woody-Karrer, 1963) and nitrous acid, the survival curves of *E. coli* S and B can be described as convex upward, or having two components, the first being steeper in slope than the second. For both ultraviolet light and nitrous acid, the survival curves for radioresistant mutants of *E. coli* B and S can be described as convex downward, or as having two components, the first being steeper in slope than the second. Furthermore, *E. coli* B and S are less sensitive to both nitrous acid and ultraviolet radiation when

TABLE 1. *Lethal effect of nitrous acid on radioresistant strains of Escherichia coli**

Strain	Radioresistant type	Log per cent of survivors
S	R_1	5×10^{-2}
	R_2	5×10^{-2}
	R_3	6×10^{-2}
	R_4	6×10^{-2}
	R_5	5×10^{-2}
	R_6	6×10^{-2}
	R_7	5×10^{-2}
	R_8	6×10^{-2}
	R_9	5×10^{-2}
	R_{10}	6×10^{-2}
	R_{11}	4×10^{-2}
	R_{12}	6×10^{-2}
	R_{13}	6×10^{-2}
K12		6×10^{-2}
Bpr5		6×10^{-2}

* Procedure: 2×10^7 cells per ml of overnight culture were treated in acetate buffer (0.1 M; pH 4.2) containing 0.005 M nitrous acid and shaken at 37 C. At 10 sec, samples were taken, diluted in buffered saline, and 0.1 ml was placed on Tryptone agar (pH 7.0). The plates were incubated at 37 C overnight.

TABLE 2. *Types of radioresistant mutants of Escherichia coli S selected after treatment with radiomimetic chemicals*

Chemical agent	No. examined	Radioresistant types			
		R_3	R_4	R_6	Others
Nitrous acid	46	26*	74	2	0
Proflavine	50	20	50	2	28
Nitrofurazone	64	23	47	3	27
Mitomycin C	85	40	54	1	5

* Indicates per cent of all radioresistant mutants.

plated on glucose-salts medium than when plated on complex media.

Radiation and nitrous acid, as well as other radiomimetic chemicals, probably inflict their damage on the same target; this damage is prevented, repaired, or bypassed by the same resistance mechanism. Radiation and radiomimetic agents also share the property of being mutagens. The common target would appear to be DNA. Yet, the nature of the common damage to DNA was not deduced from what is known of the chemical changes in DNA produced by the agents, which include, among others, radiation, both ultraviolet and X-ray, nitrous acid, mono- and bifunctional alkylating agents, acridine dyes, and nitrofurazone. Ultraviolet radiation, but apparently not X radiation, injures DNA by causing the formation of thymine dimers (Beukers and Berends, 1961; Setlow and Setlow, 1962). Nitrous acid is a deaminating agent, injuring DNA by deaminating aminopurines and aminopyrimidines (Vielmetter and Schuster, 1960). Alkylating agents alkylate ring nitrogens of purines, especially guanine, leading to the depurination of the polymer (Brookes and Lawley, 1960) or alternatively causing cross-linking of DNA strands (Brookes and Lawley, 1961). The acridine dye, proflavine, is supposed to intercalate itself between adjacent bases on DNA, leading, on replication, to deletion or insertion of bases (Brenner et al., 1961; Crick et al., 1961; Lerman, 1963). The action of nitrofurazone is unknown, but its chemical structure and reactivity suggest none of these mechanisms for injuring DNA. Because of the diversity of the initial chemical effects of these agents, it does not seem likely that the radioresistance mechanism is involved in the repair of the many, specific, initial chemical effects.

It is known that the rate of activity of DNA polymerase is reduced by radiation-induced injury on primer DNA (Bollum and Setlow, 1963). Although it has not been demonstrated that injury to primer by radiomimetic chemicals inhibits polymerase, it is possible that the DNA-replicating mechanism of resistant strains is less sensitive to chemically induced aberrations in DNA structure than is the replicating mechanism of sensitive strains. The hypothesis that the kind of radiation resistance with which this report is concerned results from a greater tolerance in the replicating mechanism for errors in the template might also explain the multiplicity of radioresistant strains differentiated by their cross-resistance patterns. It is conceivable that there could be a variety of mutant DNA polymerases with different sensitivities to different kinds of injuries to DNA, or to the degree of such injuries. This would account for the different cross-resistance patterns of the various radioresistant mutants. This hypothesis could be tested by examining the sensitivity to injuries induced on primer DNA of polymerases extracted from radiosensitive and radioresistant strains of *E. coli*.

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LITERATURE CITED

ALPER, T., AND N. E. GILLIES. 1960. The relationship between growth and survival after irradiation of *Escherichia coli* strain B and two resistant mutants. J. Gen. Microbiol. **22**:113–128.

ANAGNOSTOPOULOS, C., AND I. P. CRAWFORD. 1961. Transformation studies on the linkage of markers in the tryptophan pathway in *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S. **47**:378–390.

BEUKERS, R., AND W. BERENDS. 1961. The effects of UV irradiation on nucleic acids and their components. Biochim. Biophys. Acta **49**:181–189.

BOLLUM, F. J., AND R. B. SETLOW. 1963. Ultraviolet inactivation of DNA primer activity. I. Effect of different wavelengths and doses. Biochim. Biophys. Acta **68**:599–607.

BRENNER, S., L. BARNETT, F. H. C. CRICK, AND A. ORGEL. 1961. The theory of mutagenesis. J. Mol. Biol. **3**:121–124.

BROOKES, P., AND P. D. LAWLEY. 1960. The reaction of mustard gas with nucleic acids *in vitro* and *in vivo*. Biochem. J. **77**:478–484.

BROOKES, P., AND P. D. LAWLEY. 1961. The reaction of mono- and difunctional alkylating agents with nucleic acids. Biochem. J. **80**:496–503.

CRICK, F. H. C., L. BARNETT, S. BRENNER, AND R. J. WATTS-TOBIN. 1961. General nature of the genetic code for proteins. Nature **192**:1227–1232.

GIERER, A., AND K. W. MUNDRY. 1958. Production of mutants of tobacco mosaic virus by chemical alteration of its ribonucleic acid *in vitro*. Nature **182**:1457–1458.

GREENBERG, J., J. D. MANDELL, AND P. L. WOODY.

1961*a*. Resistance and cross-resistance of *Escherichia coli* mutants to radiomimetic agents. A preliminary report. Cancer Chemotherapy Rept. **11**:51–56.

GREENBERG, J., J. D. MANDELL, AND P. L. WOODY. 1961*b*. Resistance and cross-resistance of *Escherichia coli* mutants to antitumour agent mitomycin C. J. Gen. Microbiol. **26**:509–520.

GREENBERG, J., AND P. L. WOODY-KARRER. 1963. Radioresistant mutants of *Escherichia coli* B (ORNL). Radiation Res. **20**:350–363.

KAUDEWITZ, F. 1959. Production of bacterial mutants with nitrous acid. Nature **183**:1829–1830.

KAUDEWITZ, F. 1963. Lethality and inactivation after treatment of *Escherichia coli* cells with nitrous acid. Biochem. Biophys. Res. Commun. **11**:416–421.

LATARJET, R., P. MORENNE, AND R. BERGER. 1953. Un appareil simple pour le dosage des rayonnements ultraviolets émis par les lampes germicides. Ann. Inst. Pasteur **85**:174–184.

LERMAN, L. S. 1963. The structure of the DNA-acridine complex. Proc. Natl. Acad. Sci. U.S. **49**:94–102.

LITMAN, R. M., AND H. EPHRUSSI-TAYLOR. 1959. Inactivation et mutation des facteurs génétiques de l'acide désoxyribonucléique du pneumocoque par l'ultraviolet et par l'acide nitreux. Compt. Rend. **249**:838–840.

LUZZATI, D. 1962. The action of nitrous acid on transforming deoxyribonucleic acid. Biochem. Biophys. Res. Commun. **9**:508–516.

MANDELL, J. D., P. L. WOODY, AND J. GREENBERG. 1961. Resistance and cross-resistance of *Escherichia coli* mutants to anticancer agents. 1-methyl-3-nitro-1-nitrosoguanidine. J. Bacteriol. **81**:419–424.

ROBERTS, R. B., AND E. ALDOUS. 1949. Recovery from ultraviolet irradiation in Escherichia coli. J. Bacteriol. **57**:363–375.

RÖRSCH, A., A. EDELMAN, C. VAN DER KAMP, AND J. A. COHEN. 1962. Phenotypic and genotypic characterization of radiation sensitivity in *Escherichia coli* B. Biochim. Biophys. Acta **61**:278–289.

SETLOW, R. B., AND J. K. SETLOW. 1962. Evidence that ultraviolet-induced thymine dimers in DNA cause biological damage. Proc. Natl. Acad. Sci. U.S. **48**:1250–1257.

SZYBALSKI, W., AND V. BRYSON. 1952. Genetic studies on microbial cross resistance to toxic agents. I. Cross resistance of Escherichia coli to fifteen antibiotics. J. Bacteriol. **64**:489–499.

TESSMAN, I. 1959. Mutagenesis in phages ϕX174 and T4 and properties of the genetic material. Virology **9**:375–385.

VIELMETTER, W., AND H. SCHUSTER. 1960. Die Basenspezifität bei der Induktion von Mutationen durch salpetrige Säure im Phagen T2. Z. Naturforsch. **15b**:304–311.

VIELMETTER, W., AND C. M. WIEDER. 1959. Mutagene und inaktivierende Wirkung salpetriger Säure auf freie Partikel des Phagen T2. Z. Naturforsch. **14b**:312–317.

WITKIN, E. 1947. Genetics of resistance to radiation in *Escherichia coli*. Genetics **32**:221–248.

WOODY, P. L., J. D. MANDELL, AND J. GREENBERG. 1961. Resistance and cross-resistance of *Escherichia coli* mutants to anticancer agents. Nitrogen mustard and nitromin. Radiation Res. **15**:290–297.

WOODY-KARRER, P., AND J. GREENBERG. 1963. Resistance and cross resistance of *Escherichia coli* S mutants to the radiomimetic agent nitrofurazone. J. Bacteriol. **85**:1208–1216.

ZAMENHOF, S. 1961. Gene unstabilization induced by heat and by nitrous acid. J. Bacteriol. **81**:111-117.