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Interaction of Metronidazole with DNA Repair Mutants of *Escherichia coli*

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It has been proposed that one of metronidazole's partially reduced intermediates interacts either with DNA to exert a bactericidal effect or with water to form acetamide. To test this hypothesis we have examined the effect of metronidazole on several mutants of *Escherichia coli* that are defective in DNA repair. UV-susceptible RecA$^-$ and UvrB$^-$ point mutants have an increased susceptibility to metronidazole as manifested by both a decreased minimal inhibitory concentration and a greater bactericidal response to metronidazole in resting cultures. By these criteria, however, we find that UvrB$^-$ deletion mutants, which lack the ability to reduce nitrate and chlorate, are no more susceptible to metronidazole than is the wild type. We find, however, that these deletion mutants also lack the ability to reduce metronidazole and thus possibly to form its reactive species. When metronidazole's bactericidal effect is expressed in terms of the concurrent accumulation of acetamide derived from metronidazole, then all RecA$^-$ and UvrB$^-$ mutants are killed more efficiently than their wild types. The data are consistent, therefore, with metronidazole's lethal effect being mediated by a partially reduced intermediate on the metabolic pathway between metronidazole and acetamide. Defects in other aspects of the DNA repair system do not confer this increased susceptibility to the proposed intermediate. A Tag$^-$ mutant, for example, which is defective in 3-methyl-adenine-DNA glycosylase, does not have this increased susceptibility to the presumed precursor of acetamide. Thus, these results provide further support for the hypothesis that the bactericidal effect of metronidazole is mediated by a partially reduced intermediate in the metabolic conversion of metronidazole to acetamide and suggest that this intermediate interacts with DNA to produce a lesion similar to that caused by UV light.

Anaerobic conditions are required both for the bactericidal effect of metronidazole and for its metabolic transformation to acetamide. These phenomena appear to be related in that the log of the surviving fraction of bacteria susceptible to metronidazole varies linearly with the amount of metronidazole converted to acetamide (1, 9). The most parsimonious explanation for this kinetic relationship is that anaerobic bacteria catalyze the conversion of metronidazole to a labile, partially reduced intermediate which then has one of several possible fates. It may interact, for example, with water to form acetamide or, alternatively, with a bacterial macromolecule to initiate the bactericidal effect (1). This hypothesis suggests that the concentration of the proposed intermediate, M*, determines both the rate of formation of acetamide and the damage to a bacterial macromolecule which, if not repaired, becomes lethal (Fig. 1).

Although the chemical nature of M* is not known, its relative concentration over time, and hence the cell's exposure to it, can be inferred from the relative amounts of acetamide that accumulate. The model also enables one to distinguish between two mechanisms of resistance, one that is due to a decreased formation of the reactive form of metronidazole and another that is due to an increased resistance of the bacterial target.

The first mechanism, a decreased formation of the partially reduced, reactive form of metronidazole, appears to be responsible for the increased resistance of a clinical isolate of *Bacteroides fragilis* (4, 9). The relation between bacterial survival and acetamide accumulation for this strain is the same as that for a normally susceptible strain of *B. fragilis* (9). This suggests that the two strains are equally susceptible to metronidazole's reactive metabolite and that the reactive metabolite merely forms more slowly in the resistant strain. This explanation is supported by the finding that nitroreductase activity, which is presumably responsible for the formation of metronidazole's active form, is decreased in the more resistant strain (9, 15).

A kinetic approach based on the model in Fig. 1 has now been used to characterize metronidazole's target within susceptible bacteria. That DNA is a primary target for metronidazole is suggested by chemical (7, 8) and mutagenicity (13, 16) studies as well as by the finding (3, 5, 12) that nitroheterocyclic compounds including metronidazole are more bactericidal for *Escherichia coli* mutants that are deficient in DNA repair. Mutants defective in DNA repair should have a greater susceptibility to the reactive intermediate and thus, as proposed in Fig. 1, more susceptibility in terms of the accumulation of acetamide, derived from metronidazole. In this paper we confirm this prediction with the finding that UV-susceptible mutants with various genotypes have a steeper decline in the log of bacterial survival for a given accumulation of acetamide than the wild types from which they are derived.

MATERIALS AND METHODS

Materials. Crystalline metronidazole (mp, 158 to 160°C) was a gift from G. D. Searle and Co. (Chicago, Ill.). [2-^{14}C]metronidazole (18.6 mCi/mmol) was a gift from May and Baker Ltd. (Dagenham, England). [1-^{14}C]acetamide (1.0 mCi/mmol) was purchased from California Bionuclear Corp. (Sun Valley, Calif.).

Cultures. The strains of *E. coli* used are described in Table 1. Strain AB1885, containing a single point mutation (*uvrB5*), and strain SR58, containing a double point mutation (*uvrB5 recA56*), are otherwise isogenic to the wild-type AB1157 from which they were derived. Strain EE128 contains a point

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TABLE 1. Descriptions of *E. coli* strains used

Strain	Relevant genotype	Comments	Source (reference)
AB1157	*uvr*$^+$ *rec*$^+$		American Type Culture Collection
P90C	*uvr*$^+$ *rec*$^+$		E. Eisenstadt
AB1885	*uvrB recA*$^+$	AB1157 *uvrB5*	E. Eisenstadt
SR58	*uvrB recA*	AB1157 *uvrB5 recA56*	E. Eisenstadt
EE128	*uvrB*$^+$ *recA*	P90C *recA56*	E. Eisenstadt
EE348	Δ*uvrB*	Deletion through *uvrB* locus derived from P90C; decreased nitrate and chlorate reductase activities	E. Eisenstadt
EE349	Δ*uvrB*	Deletion through *uvrB* locus derived from P90C; decreased nitrate and chlorate reductase activities	E. Eisenstadt
SM172	Δ*uvrB*	*thy*; otherwise isogenic to EE349	E. Eisenstadt
NH5016	*tag*$^+$	AB1157 *xth metE*	T. Lindahl (6)
PK432 · 1	*tag-1* (Ts)	NH5016 *tag-1 argE*$^+$; deficient in 3-methyladenine-DNA glycosylase at 43°C but not at 30°C	T. Lindahl (6)

mutation (*recA56*) but is otherwise isogenic with the wild-type P90C from which it was derived. EE348, EE349, and SM172 are deletion mutants derived from P90C that manifest a decreased ability to reduce nitrate to nitrite and chlorate to chlorite. SM172, which is *thy*, is otherwise isogenic with EE349. Strain PK432 · 1 [*tag-1*(T_s)] is a single point mutant, derived from strain NH5016, which has a temperature-sensitive defect in 3-methyladenine-DNA glycosylase (6), an enzyme necessary for repairing DNA damage due to alkylating agents. Unless otherwise specified, bacteria were propagated in prereduced brucella medium (Difco Laboratories, Detroit, Mich.) supplemented with 5 μg of hemin (Nutritional Biochemicals Corp., Cleveland, Ohio) per ml.

Other bacterial methods. To determine metronidazole's bactericidal effect and its metabolism to acetamide, bacterial incubations were conducted in an oxygen-free atmosphere provided by a Virginia Polytechnic Institute anaerobic culture system as previously described (1). Bacteria were propagated overnight at 37°C in prereduced, anaerobically sterilized medium which also contained 1 μg of resazurin (Matheson, Coleman and Bell, East Rutherford, N.J.) per ml, to yield a culture in stationary phase. Metronidazole was added at a final concentration of 100 μg/ml; cultures, incubated at 37°C, were sampled at intervals to assay for acetamide and viable bacteria (1).

UV susceptibility was determined on bacteria that had been freshly streaked from a 24-h colony and then irradiated for various periods with a 30-W UV germicidal lamp. Observation at 24 h confirmed that all strains mentioned except the wild types (AB1157, P90C, and NH5061) and the Pk432-1 mutant had an increased susceptibility to UV light.

Minimal inhibitory concentrations (MICs) were determined by inoculating 1.0 ml of an overnight culture, which had been diluted to a concentration of 10^6 CFU/ml, into tubes that contained 1.0 ml of broth with various concentrations of metronidazole. The loosely capped tubes were then incubated anaerobically in GasPak jars (BBL Microbiology Systems, Cockeysville, Md.).

Acetamide analysis. Radiolabeled acetamide derived from [2-^{14}C]metronidazole was assayed by means of a high-pressure liquid chromatograph (model ALC/GPC 204; Waters Associates, Milford, Mass.) equipped with a model 660 solvent programmer and containing a 5-μm Supelcosil LC-18 column (250 by 4.6-mm inside diameter; Supelco Inc., Bellefonte, Pa.). The column was eluted at a flow rate of 1.4 ml/min with 10 mM potassium phosphate buffer (pH 7.5), and the eluate was collected in scintillation vials during successive 15-s intervals. Radioactivity from acetamide, which eluted at 3.0 min, was assayed by liquid scintillation spectrometry after adding 10 ml of Aquasol (New England Nuclear Corp., Boston, Mass.). With this method the recovery of authentic [1-^{14}C]acetamide was 100 ± 4% (mean ± standard deviation) when added to brucella broth at concentrations of 0.25 to 10 mg/ml. Other experiments indicated that acetamide measurements obtained by this high-pressure liquid chromatographic method did not differ significantly from those obtained previously by means of cation-exchange column chromatography (1).

Metronidazole assay. Metronidazole in the culture medium was also assayed by high-pressure liquid chromatography. A 0.5-ml aliquot of culture medium containing metronidazole was extracted with 5 ml of ethyl acetate, and the mixture was clarified by centrifugation. An aliquot (50 μl) of the ethyl acetate phase was analyzed in an HP1084B liquid chromatograph equipped with a model 79842A autosampler and model 79841A variable volume injector (Hewlett-Packard, Lexington, Mass.). The 5-μm Supelcosil LC-18 column was eluted with 25% methanol in water at a flow rate of 1.5 ml/min. Metronidazole, monitored at 314 nm with an HP1040A diode array spectrophotometric detector, was eluted at 3.5 min and was quantified from the integrated area under the peak in comparison with known standards.

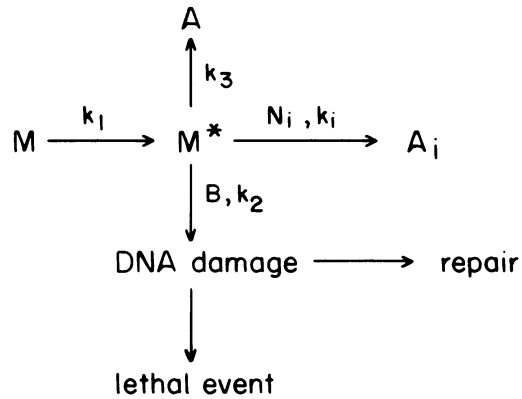

FIG. 1. Model relating metronidazole's metabolism and its lethal effect on bacteria. Metronidazole (M) is metabolized with rate constant k_1 to a labile intermediate (M*) which can react with a bacterium (B) with rate constant k_2 to cause DNA damage. Only when this damage exceeds the capacity for DNA repair will it result in a lethal effect. M* may also react with water to form acetamide (A) or with other compounds (N_i) in the medium to yield other metabolites (A_i).

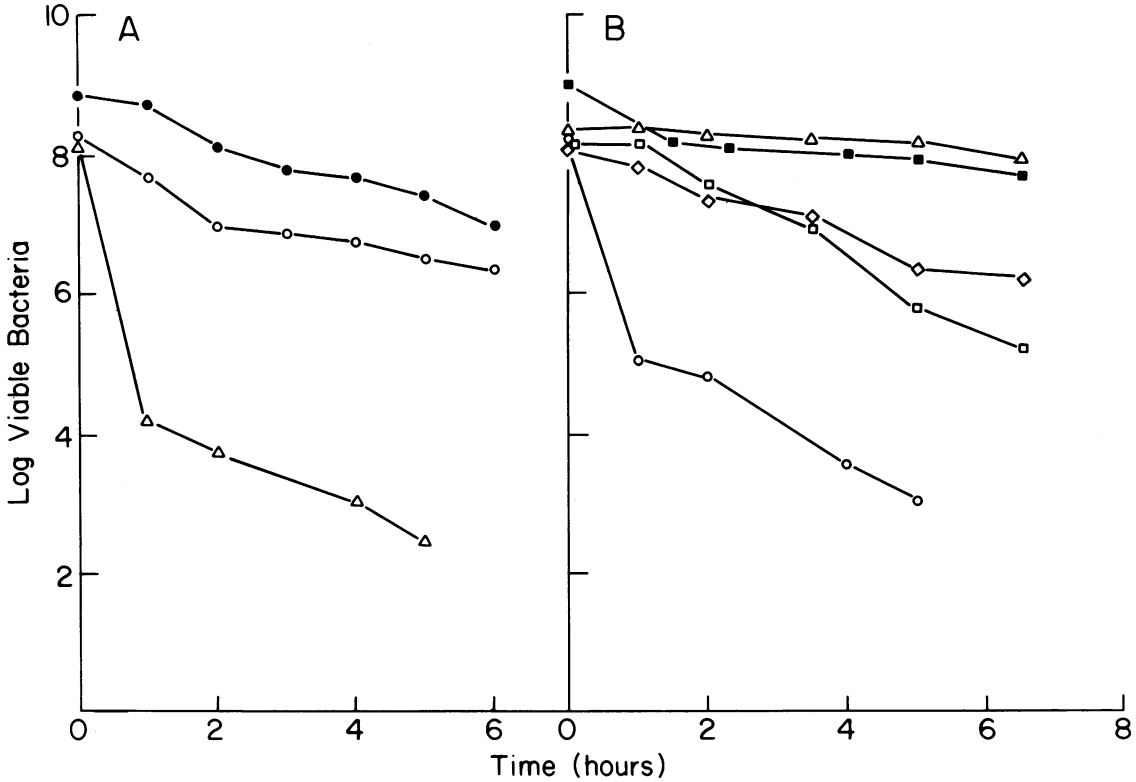

FIG. 2. Bacterial viability in the presence of metronidazole. Shown are the time courses of the survival of wild types (closed symbols) and DNA repair-deficient mutants (open symbols) of *E. coli* in the presence of 100 μg of metronidazole per ml. Symbols: (A) ●, AB1157; ○, AB1885; △, SR58; (B) ■, P90C; ○, EE128; △, EE348; □, EE349; ◇, SM172.

RESULTS

The susceptibility to metronidazole of the various *E. coli* mutants was determined by MIC (Table 2) and by survival curves in the presence of metronidazole at a concentration of 100 μg/ml (Fig. 2). Both methods indicated that *recA* point mutants, EE128 and SR58, have an increased susceptibility to metronidazole. There is no consistent pattern, however, in the susceptibilities of UvrB$^-$ mutants. The *uvrB* point mutant AB1885 appears to be slightly more susceptible to metronidazole than its parent strain in terms of MIC, but there is little difference in its survival in stationary phase in the presence of metronidazole. On the other hand, the *uvrB* deletion mutants, EE348, EE349, and SM172, are no more susceptible than the parent strain. Thus, conventional measures of antibiotic susceptibility fail to reveal a correlation between susceptibility to metronidazole and that to UV light.

A possible reason for the discrepancy between the susceptibility to metronidazole and that to UV light may be found in some of the other characteristics of the mutants. EE348, EE349, and SM172, for example, are deletion mutants that reduce nitrate and chlorate at diminished rates. It is therefore of particular interest that these deletion mutants also reduce metronidazole more slowly than the other strains (Fig. 3) and thus according to our model should form metronidazole's active intermediate more slowly. In other words, the greater vulnerability of mutants EE348, EE349, and SM172 may be obscured by their subnormal rate of formation of metronidazole's reactive species.

The model proposed in Fig. 1 indicates that compensation for this lesion in the deletion mutants can be achieved by focussing on M* rather than the metronidazole from which it is derived. Thus, the model predicts that bacteria unable to repair the damage induced by the reactive intermediate, M*, should be more susceptible to M* than those with an intact repair system. Unfortunately, this prediction cannot be tested directly because the concentration of M* cannot be measured. However, the integral of the concentration of M* over time and hence the exposure of the culture to M* are reflected in the amount of acetamide accumulated. Thus, mutants defective in their ability to repair DNA damage caused by M* should be killed more efficiently in terms of acetamide accumulation than the wild type from which they are derived. This prediction has been examined (Fig. 4).

Figure 4A indicates that UvrB$^-$ strain AB1885 is more susceptible to metronidazole in terms of acetamide accumulation than is its parent strain. Susceptibility measured in these terms seems to be even greater in the double mutant SR58 (*uvrB recA*). Figure 4B confirms the increased susceptibility of another UV-susceptible strain by showing that EE128 (RecA$^-$) is more susceptible in terms of acetamide accumulation than is its parent strain. This form of data presentation is of particular interest for the UvrB$^-$ deletion mutants EE348, EE349, and SM172 (Fig. 4B) because it reveals how they differ from the wild type in a way that is obscured by the more traditional methods of susceptibility assessment that are portrayed in Fig. 2 and Table 2. Thus, portraying the lethal effect of metronidazole in terms of acetamide accumulation, and therefore in terms of presumed exposure to metronidazole's reactive form, provides a more selective probe of metronidazole's action than application of the usual methods of assessing antibiotic susceptibility.

Deficiencies in DNA repair do not necessarily confer an

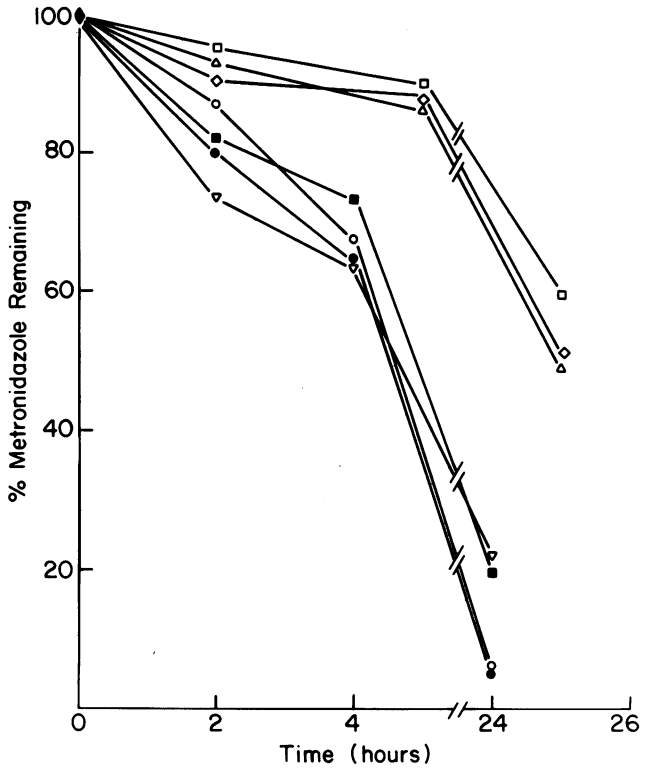

FIG. 3. Rate of disappearance of metronidazole in stationary-phase cultures of various strains of *E. coli*. Cultures initially contained 10^8 bacteria per ml and metronidazole at a concentration of 100 μg/ml. Symbols: ●, AB1157; ■, P90C; ○, AB1885; ▽, EE128; △, EE348; □, EE349; ◇, SM172. (In the absence of bacteria, metronidazole disappeared from the culture medium at a rate of approximately 10% per 24 h.)

enhanced susceptibility to metronidazole in terms of acetamide accumulation. Thus, the Tag$^-$ mutant PK432 · 1 exhibits the same relationship between bacterial survival and acetamide accumulation as its parent strain (Fig. 5). Indeed, this mutant does not manifest increased susceptibility to metronidazole in terms of either MIC (Table 2) or survival in the presence of metronidazole.

DISCUSSION

This paper provides additional evidence consistent with a model of metronidazole's bactericidal activity in which metronidazole forms a reactive intermediate that may interact either with bacterial DNA to exert a lethal effect or with another molecule, probably water, to form acetamide. That the amount of acetamide accumulating when bacteria are incubated with metronidazole varies linearly with the log of the fraction of surviving bacteria is consistent kinetically with an intermediate that is common to both bactericidal activity and the formation of acetamide. That the lethal effect is greater, in terms of the accumulation of acetamide,

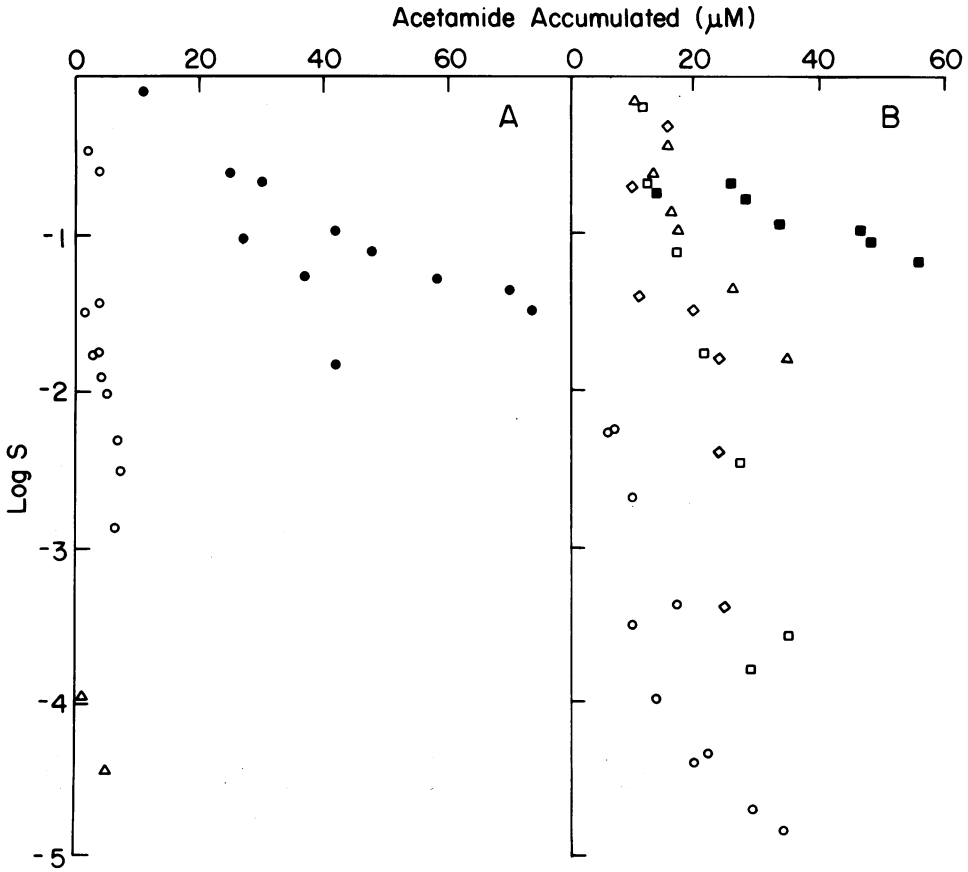

FIG. 4. Relation between acetamide formation and log of the fraction of surviving bacteria for wild types (closed symbols) and DNA repair-deficient mutants (open symbols) of *E. coli*. The concentration of metronidazole was 100 μg/ml. Symbols: (A) ●, AB1157; ○, AB1885; △, SR58; (B) ■, P90C; ○, EE128; △, EE348; □, EE349; ◇, SM172.

TABLE 2. Metronidazole MIC of the *E. coli* strains[a]

E. coli strain (phenotype)	Metronidazole MIC (μg/ml)
AB1157 (Uvr$^+$ Rec$^+$)	>200
P90C (Uvr$^+$ Rec$^+$)	>200
AB1885 (UvrB$^-$ Rec$^+$)	100
SR58 (RecA$^-$ UvrB$^-$)	13
EE128 (Uvr$^+$ RecA$^-$)	25
EE348 (UvrB$^-$ Rec$^+$)	>200
EE349 (UvrB$^-$ Rec$^+$)	>200
SM172 (UvrB$^-$ Rec$^+$)	>200
NH5016 (Uvr$^+$ Rec$^+$ Tag$^+$)	>200
PK432 · 1 [Uvr$^+$ Rec$^+$ Tag$^-$ (Ts)]	>200

[a] MICs were determined under anaerobic conditions as described in the text except for strains NH5016 and PK432 · 1, which were incubated at 40°C rather than at 37°C.

for mutants of *E. coli* that are defective in DNA repair provides additional evidence that DNA is the primary target for the reactive form of metronidazole.

DNA repair mutants that are susceptible to UV light are also susceptible to the reactive form of metronidazole, which suggests that both agents produce similar lesions in DNA. Although the characteristics of this lesion are uncertain, its repair does not depend on 3-methyladenine-DNA glycosylase because a Tag$^-$ mutant, which lacks this enzyme, is more susceptible neither to metronidazole itself nor to its postulated reduced intermediate. Perhaps a systematic study of the susceptibility of DNA repair mutants in terms of acetamide accumulation might clarify the nature of the lesion produced by metronidazole on bacterial DNA.

That the susceptibility to metronidazole is best expressed in terms of the accumulation of acetamide further supports the concept that nitroreductase activity is obligatory for the activation of metronidazole (2, 10, 13). However, the chemical structure of the partially reduced form of metronidazole that is suggested by these kinetic experiments remains difficult to elucidate. One candidate is the radical anion whose existence has been shown by electron spin resonance techniques (11); another is the nitroso derivative whose existence has been suggested from kinetic evidence (11). A third candidate is the hydroxylamine derivative which has been synthesized and characterized by nuclear magnetic resonance spectroscopy (A. M. Rauth, personal communication). A fourth candidate is the amino derivative (14). However, all of these reduced forms of metronidazole are so labile at physiological pH that it will be difficult to design experiments that relate one or another of them to metronidazole's biological activity. Thus, at the moment, M* remains a kinetic concept that cannot be assigned the structure of any intermediates postulated to arise in the reduction of metronidazole.

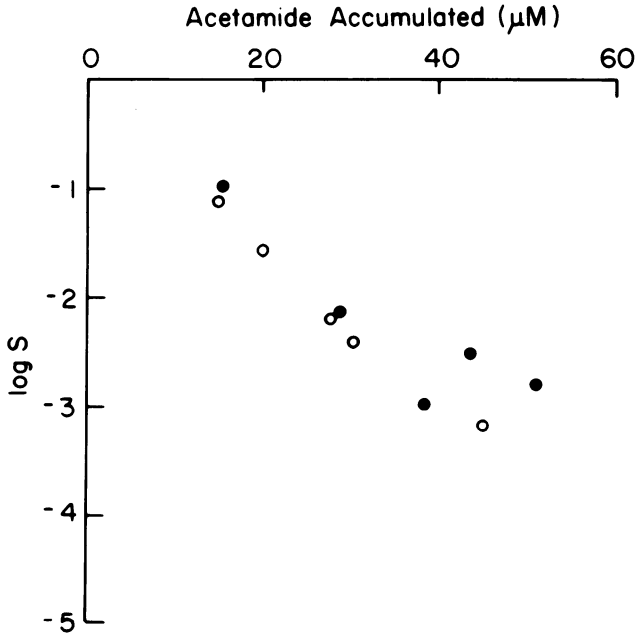

FIG. 5. Relation between acetamide formation and log of fraction of surviving bacteria for strains NH5016 (●) and PK432 · 1 (○). Conditions were as in the legend to Fig. 2 except that the incubations were at 40°C.

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