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ROLE OF SULFHYDRYL COMPOUNDS IN THE BACTERICIDAL EFFECT OF METRONIDAZOLE*

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Abstract—The bactericidal effect of metronidazole on *Escherichia coli* and *Bacteroides fragilis* can be partially reversed by cysteamine under conditions that lead to the formation of an adduct, the thioether, 4-(2-aminoethyl)thio-2-methylimidazole-1-ethanol (4-ATME). This adduct, which is not mutagenic for the Ames histidine auxotrophs of *Salmonella typhimurium*, forms at a rate that is independent of live bacterial cells and, therefore, can not be shown to relate to the biological effect of cysteamine. When treated with Raney nickel, this adduct yields 2-methylimidazole-1-ethanol. To determine whether a structurally related adduct forms with bacterial protein, a culture of *B. fragilis* was incubated with radiolabelled metronidazole and then treated with 5% trichloroacetic acid. That the radiolabel in the precipitate did not yield 2-methylimidazole-1-ethanol when treated with Raney nickel suggests that binding of metronidazole to cellular macromolecules does not involve thioether formation.

The biological actions of metronidazole, such as its lethal effect on anaerobic microorganisms [1–3] and its cytotoxicity for hypoxic mammalian cells [4, 5], appear to depend on reduction of the nitro group. As nitro group reduction proceeds through a radical anion [6], and possibly other transient intermediates, it has been suggested that one of these active intermediates is the actual mediator of the biological effects of metronidazole and the other nitromidazoles.

Some of the biological effects of the nitromidazoles are inhibited by compounds containing a sulfhydryl group. Thus, the toxicity of metronidazole and other nitromidazoles for cultured hypoxic mammalian cells is diminished by the addition of cysteamine or glutathione [7–9]. The importance of sulfhydryl groups is also demonstrated by the observation that lower concentrations of the 2-nitroimidazole misonidazole are required by anoxic glutathione-deficient mutants of *Escherichia coli* than by the wild type to obtain equivalent radiosensitization [10].

The mechanism by which sulfhydryl compounds protect the cell is unclear. They may act as free radical scavengers or they may interact with another reactive form of the nitroimidazole to form a covalently bonded adduct. A model of the second possibility is the recent demonstration of a reaction between cysteamine and various nitroimidazoles under anaerobic conditions in which the nitro group is displaced and a thioether formed [11].

The present studies were undertaken to investigate the possibility that metronidazole might interact with thiol groups of biologically important molecules to form such an adduct *in vivo*. Bacteria were used in the investigation because it was felt that their growth to high density and rapid metabolic rate would facilitate the formation of such adducts in quantities sufficient to isolate.

MATERIALS AND METHODS

Chemicals. Crystalline metronidazole was a gift from G. D. Searle & Co. (Chicago, IL). [2-^{14}C]Metronidazole (18.6 mCi/mmole) and 2-methylimidazole-1-ethanol were donated by May & Baker, Co. (Dagenham, England). [2-^{14}C]Metronidazole was purified on an AG 50W-X4 (H^+ form) column [12] before use. *N*-Acetylcysteamine was purchased from the Cyclo Chemical Co. (Los Angeles, CA) and Raney nickel from the Aldrich Chemical Co. (Milwaukee, WI). 4-(2-Aminoethyl)thio-2-methylimidazole-1-ethanol (4-ATME) was a gift from Dr. J. D. Wuest at the University of Montreal. Other reagents were obtained from either the Sigma Chemical Co. (St. Louis, MO) or the Fisher Scientific Co. (Medford MA).

Bacterial Cultures. Escherichia coli, isolated from rat cecum, and *Bacteroides fragilis*, isolated from human feces, were strains used previously [13–15]. Both were cultivated anaerobically in pre-reduced [16] brucella broth (Difco Laboratories, Detroit, MI) and enumerated on plates of the same medium [14]. Incubation at 37° overnight yielded a culture in stationary phase.

Analysis by high pressure liquid chromatography. High pressure liquid chromatography (HPLC) was performed with a Waters Associates (Milford, MA) chromatograph model ALC/GPC 204, using either a C-18 reverse phase μ-Bondapak column (Waters

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Associates, Milford, MA) or a C-18 reverse phase Partisil ODS-2 column (Whatman Inc., Clifton, NJ). Compounds were detected by their absorbance at 254 nm with a Waters Associates UV-detector, model 440, or at other wavelengths with a Kratos (Westwood, NJ) model 770 variable wavelength detector, and the results were recorded by means of a Hewlett–Packard (Lexington, MA) model 3380A integrator. Radioactivity in the eluate was assayed by collecting fractions at 15-sec intervals and assaying them for radioactivity in 10 ml of Aquasol (New England Nuclear Corp., Boston, MA).

Metronidazole was quantified by its u.v. absorption at 254 nm after its seperation by means of a C-18 μ-Bondapak column that was eluted with 10% methanol in 5 mM potassium phosphate, pH 4.0, at a flow rate of 2 ml/min. In this system, metronidazole had a retention time of 7.8 min.

To quantify 4-ATME, it was added to a C-18 μ-Bondapak column which was first eluted (at a rate of 2 ml/min) for 10 min with 20% methanol in water and then by 20% methanol in 5 mM potassium phosphate at pH 6.8. 4-ATME, with a retention time of 7.7 min from the start of the second solvent system, was quantified on the basis of its radioactivity.

2-Methylimidazole-1-ethanol was assayed on a Whatman Partisil ODS-2 column which was eluted at 1.5 ml/min with 20% methanol in 5 mM potassium phosphate at pH 5.5. The compound, with a retention time of 5.3 min, was detected either by its u.v. absorption at 210 nm or, if synthesized from radiolabeled metronidazole, by its radioactivity.

Thin-layer chromatography (TLC). Thin-layer chromatograms on an Eastman 13254 cellulose TLC sheet that contained a fluorescent indicator were developed with ethanol–water–NH_3 (80:15:5); compounds were detected by their fluorescence, by the color developed with ninhydrin (1% in methanol), or by their radioactivity. Radioactivity was detected by dividing the sheet into 1 cm strips which were assayed by scintillation spectrometry in 10 ml of Aquasol.

Gas–liquid chromatography (GLC). Compounds that had been acetylated by trifluoroacetic anhydride (Pierce Chemical Co., Rockford, IL) were chromatographed on a Hewlett–Packard model 7620A gas–liquid chromatograph with a 6 ft 10% SP-100/1% H_3PO_4 on 100/200 Chromasorb W AW column (Supelco, Bellefonte, PA). The column was maintained at 100° for 4 min and then taken by a linear temperature gradient of 10°/min to 200°, at which temperature it was maintained for an additional 10 min. N_2 (60 ml/min) was the carrier gas.

Formation and isolation of 4-ATME from E. coli *cultures.* [2-^{14}C]Metronidazole (400 μg/ml, 10,000 cpm/ml) and cysteamine (15 mg/ml) were incubated anaerobically in brucella broth with a stationary phase culture of *E. coli.* After 24 hr at 37°, the medium was centrifuged (7700 *g* for 20 min at 4°), filtered (0.45 μm, Millex Filter, Millipore, Bedford, MA), reduced in volume by rotary evaporation at 37°, and then chromatographed on an AG 50W-X4 column (20 cm × 1 cm). The column was then eluted initially with 30 ml of water and then with 100 ml of 1 N NH_4OH. Fractions (2 ml) were collected and 0.1 ml from each fraction was added to 4 ml of Aquasol and assayed for radioactivity. Metronidazole was eluted between 56 and 62 ml. The eluate between 10 and 15 ml contained the solvent front and a number of acidic and neutral compounds that are not bound to the column. The fractions eluting between 100 and 130 ml, which contained a peak of radioactivity, were pooled, and the water was removed by rotary evaporation. The residual material was resuspended in methanol, the suspension filtered with a Millipore 0.5 μm FH filter at room temperature, and the filtrate further purified by HPLC using a C-18 μ-Bondapak column that was eluted at 2 ml/min with a solvent system that consisted initially of 20% methanol in water. After 10 min this was changed to 20% methanol in 20 mM ammonium acetate buffer at pH 5.5. 4-ATME, detected by its u.v. absorption at 254 nm, had a retention time of 4.7 min from the start of the second solvent. The eluate in this peak was collected, the solvent removed by rotary evaporation, and the residue redissolved in methanol.

Synthesis of radiolabeled 4-ATME. Radiolabeled 4-ATME was synthesized by incubating [2-^{14}C]metronidazole with cysteamine in anaerobic 0.2 M potassium phosphate buffer, pH 6.0, at 37° overnight. The product was then purified as described above.

Assay of metronidazole bound to macromolecules of B. fragilis. A 30-ml culture of *B. fragilis* grown to 10^9 cells/ml was concentrated by centrifuging it at 7700 *g* for 10 min and resuspending the pellet in 3 ml of pre-reduced brucella broth. Radiolabeled metronidazole (23 nmoles, 754,000 cpm) was added, and the mixture was incubated anaerobically at 37° for 48 hr. The reaction mixture was then centrifuged at 7700 *g* for 20 min at 4°. The pellet was washed twice with 0.9% NaCl and then precipitated with 5 ml of 5% trichloroacetic acid (TCA). The precipitate was washed with additional aliquots of 5% TCA (eight times) until the supernatant solution contained no radioactivity. The precipitate was then freed of TCA by washing it twice with 5-ml aliquots of distilled water and solubilized by incubating it overnight at room temperature in 1 ml of 1 N NaOH. The solution was neutralized with glacial acetic acid and assayed for radioactivity by scintillation spectrometry. Alternatively, the TCA precipitated pellet was suspended in 2 ml of water which was added to 0.5 ml of Raney nickel suspension (50% slurry, pH 10). This mixture was shaken vigorously for 45 min at room temperature. The supernatant solution obtained after centrifugation at 20,000 *g* for 30 min was then assayed for radioactivity and examined for the presence of 2-methylimidazole-1-ethanol by the HPLC method described previously.

Formation of 2-methylimidazole-1-ethanol from 4-ATME. Raney nickel cleaves the 4-(2-aminoethyl)thiol side chain from 4-ATME to yield 2-methylimidazole-1-ethanol [11]. For this reaction 20 mg of radiolabeled 4-ATME (105,000 cpm) in 0.9 ml H_2O was shaken vigorously with 0.1 ml of 50% Raney nickel in water for 45 min at room temperature. Raney nickel was then removed by centrifugation and filtration, and the clarified solution was analyzed by HPLC as described previously. The yield of this reaction was greater than 95%.

Table 1. Effect of cysteamine on the bacterial activity of metronidazole for *E. coli**

Cysteamine concn (mg/ml	Log (viable bacteria/ml) Without metronidazole	With metronidazole
0	9.38	0
1	9.30	0
5	9.40	6.40
10	8.95	6.40
15	9.70	8.66
25	8.90	6.90

* Cysteamine at the indicated concentrations was added to a culture of *E. coli* in stationary phase that contained either no metronidazole or metronidazole at a concentration of 200 μg/ml. After a 24-hr incubation at 37° under anaerobic conditions, the viable cells were enumerated.

Ames mutagenicity test. Mutagenicity was assayed with a frame shift mutant (TA98) and a base pair mutant (TA100) of *Salmonella typhimurium* either with or without activation by the rat liver S9 fraction [17].

Other techniques. Anaerobic buffer was prepared as previously described [18]. Radioactivity was assayed in a Packard (Downers Grove, IL) model 3003 Tri-Carb scintillation spectrometer. Mass spectra, recorded on a Hitachi spectrophotometer, model RMU 6E, were provided by Mr. E. Hanson of Children's Hospital Medical Center.

RESULTS

Protection of E. coli *from the bactericidal effect of metronidazole by cysteamine.* Table 1 shows that cysteamine at concentrations above 5 mg/ml protected *E. coli* from the bactericidal effect of metronidazole (200 μg/ml), while having little or no effect on the viability of *E. coli.* Under these conditions cysteamine was repeatedly observed to exert its maximal protective effect at a concentration of 15 mg/ml. Cysteamine at this concentration also partially protected *B. fragilis* from the bactericidal activity of metronidazole as shown in the typical experiment, one of three, portrayed in Fig. 1.

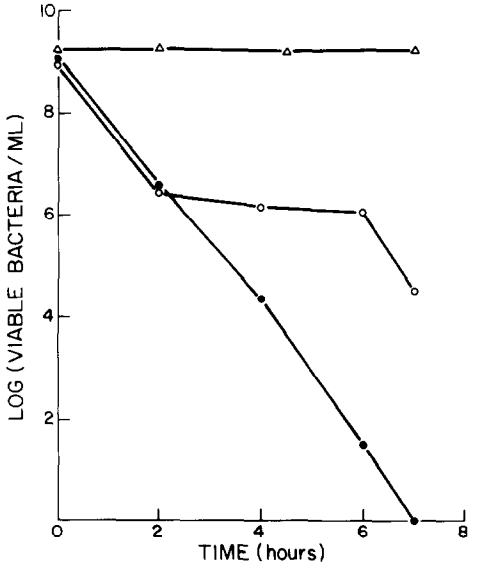

Fig. 1. Effect of cysteamine on the bactericidal activity of metronidazole for a stationary phase culture of *B. fragilis.* additions to the cultures were as follows: none (△); metronidazole (100 μg/ml) (●); and metronidazole (100 μg/ml) and cysteamine (15 mg/ml) (○).

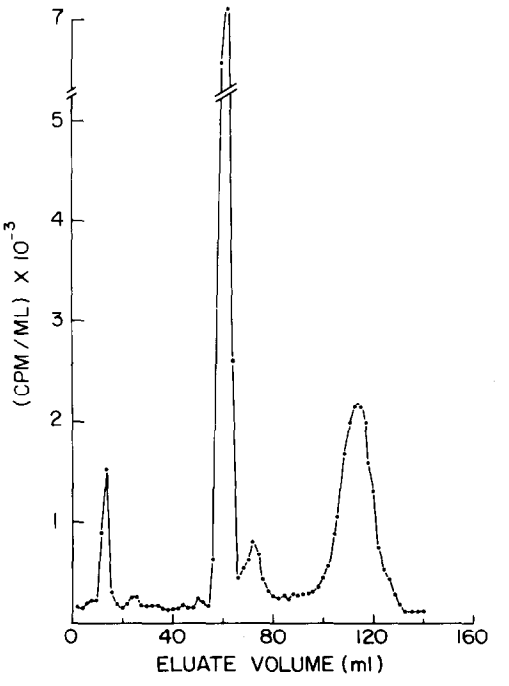

Fig. 2. Fractionation on an AG 50W-X4 column of the products of anaerobic metabolism of metronidazole (400 μg/ml) by *E. coli* in the presence of cysteamine (15 mg/ml). The column was eluted with 30 ml of water followed by 100 ml of 1 N NH_4OH.

The protective effect of cysteamine might be related to the non-enzymatic reaction between cysteamine and metronidazole that has been shown to result in the formation of either 4- or 5-(2-aminoethyl)thio-2-methylimidazole-1-ethanol (4- or 5-ATME) [11]. To determine whether one of these adducts is formed in the bacterial culture media, [2-^{14}C]metronidazole (400 μg/ml, 10,000 cpm/ml) was added along with cysteamine (15 mg/ml) to 10 ml of an *E. coli* culture in stationary phase. *E. coli,* rather than *B. fragilis,* was used because its relative resistance to metronidazole made it possible to use high concentrations of metronidazole as a means of facilitating the formation of any adduct. After an incubation of 24 hr at 37° under anaerobic conditions, the reaction mixture was clarified by centrifugation, filtered, and an aliquot (2 ml) chromatographed on an AG 50W-X4 column (20 cm × 1 cm) (Fig. 2). The eluate revealed two radiolabeled peaks (a minor one in the fractions eluated between 66 and 80 ml and a major one in the fractions between 100 and 130 ml) that were not present in incubation mixtures that lacked cysteamine. The relative small amount of

Table 2. Mutagenicity of 5-ATME and metronidazole for histidine auxotrophs of *S. typhimurium*

	No. of colonies/plate			
	TA98		TA100	
	+S9	−S9	+S9	−S9
Control	51	27	182	225
Metronidazole (0.58 μmole)*	ND†	ND	ND	1109
5-ATME (0.58 μmole)	ND	ND	ND	238
Metronidazole (1.46 μmoles)	42	27	2171	2157
5-ATME (1.46 μmoles)	79	65	207	220
Metronidazole (2.92 μmoles)	ND	ND	ND	4390
5-ATME (2.92 μmoles)	ND	ND	ND	217

* The amount of drug added per plate is indicated in parentheses.
† Not done.

radiolabeled material that eluted in the earlier peak was not characterized.

Identification of 4-*ATME.* The fractions of eluate collected between 100 and 130 ml were pooled, purified as described in Materials and Methods, and found to have properties identical to authentic 4-ATME by TLC, HPLC, GLC and mass spectroscopy. TLC on cellulose plates revealed a single spot ($R_f = 0.79$) which fluoresced under short-range ultraviolet light and reacted distinctly with ninhydrin spray. HPLC analysis with a C-18 μ-Bondapak column that was eluted at a flow rate of 2 ml/min with 20% methanol in 5 mM potassium phosphate (pH 6.8), and monitored at an absorbance of 254 nm, showed a single peak with a retention time of 6.8 min. After acetylation, both authentic 4-ATME and the material purified from the column eluate had retention times of 15.9 min on GLC (for conditions, Materials and Methods). The material purified from the *E. coli* incubation also gave the same mass spectrum as authentic 4-ATME, the parent peak having an *M/e* ratio of 201 (the molecular weight of 4-ATME) and additional major peaks with *M/e* ratios of 185, 172, 171, 158, 139, and 60.

Ames mutagenicity test. As shown in Table 2, 4-ATME was not mutagenic for either a frame shift (TA98) or a base pair (TA100) mutant of the Ames histidine auxotrophs of *Salmonella typhimurium* when tested either with or without the rat liver activation systems (S9 fraction). At the same concentrations, metronidazole was mutagenic for strain TA100 in either the presence or the absence of the S9 fraction.

Formation of 4-*ATME under other conditions.* To determine whether the formation of 4-ATME was accelerated by either the presence of *E. coli* or some constituent of its medium, metronidazole (400 μg/ml) and cysteamine (15 mg/ml) were incubated anaerobically under the following conditions: (1) a culture of *E. coli* in brucella broth, (2) a culture of *E. coli* as used previously but autoclaved at 121° for 20 min, (3) brucella broth without the addition of bacteria, and (4) anaerobic distilled water. At the end of a 24 hr incubation period, 4-ATME was detected by HPLC in all of these systems. Furthermore, the kinetics of the formation of 4-ATME and the disappearance of metronidazole were the same under all conditions (Fig. 3). Thus, the reaction between metronidazole and cysteamine appeared to be independent of bacteria and required neither bacterial enzymes nor the reducing environment provided by a viable culture. In addition, the reaction occurred in water even in the presence of air. Reaction kinetics were not studied under these conditions, however, because of the possible complication that cysteamine's free sulfhydryl group would be oxidized in air.

Reaction of metronidazole with other sulfhydryl

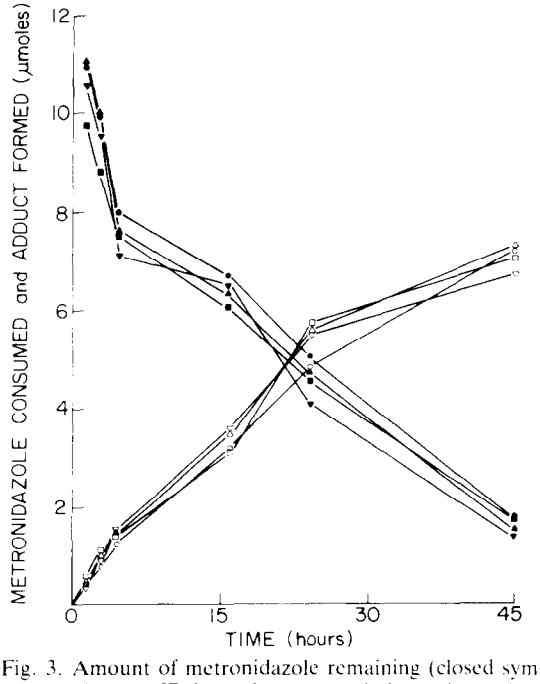

Fig. 3. Amount of metronidazole remaining (closed symbols) and 5-ATME formed (open symbols) under various conditions. Key: stationary phase culture of *E. coli* in brucella broth (□); stationary phase cultures of *E. coli* in brucella broth but autoclaved at 121° for 20 min, (△); pre-reduced brucella broth (△); and anaerobic distilled water (○).

Table 3. Reactivity of various sulfhydryls with metronidazole*

Sulfhydryl	% Metronidazole remaining†
Cysteine	56, 59
Homocysteine	86, 88
Cysteamine	64, 76
Glutathione	82, 81
2-Mercaptoethanol	74, 77
N-Acetylcysteine	101, 93
N-Acetylcysteamine	97, 94

* The sulfhydryl compounds indicated (1.5 mM) were incubated with metronidazole (0.3 mM) anaerobically at 37° in 0.2 M sodium phosphate buffer, pH 6.0. After 20 hr unreacted metronidazole was measured by HPLC as described in Materials and Methods.
† Results from two experiments are shown.

compounds. In order to further clarify its reactivity, metronidazole (0.3 mM) was incubated in anaerobic 0.2 M sodium phosphate buffer at pH 6.0 with various sulfhydryl compounds that had been adjusted to a thiol concentration of 1.5 mM as determined by titration with Ellman's reagent. After 20 hr of anaerobic incubation, a time at which the reaction with cysteamine was almost half complete (Fig. 3), the remaining metronidazole was quantified by HPLC. The results, summarized in Table 3, indicate that the various compounds reacted with metronidazole to different extents. Cysteine was the most active, while *N*-acetylcysteamine and *N*-acetylcysteine were the least reactive.

The observation that metronidazole interacts with a variety of sulfhydryl compounds suggests that sulfhydryl groups on bacterial molecules might be a target for metronidazole. This possibility was examined by incubating [2-^{14}C]metronidazole with a culture of *B. fragilis* that had been concentrated to a density of 10^{10} bacteria/ml in order to facilitate the proposed interaction. After 48 hr at 37° under anaerobic conditions the cells were precipitated by 5% TCA, and the pellet was washed to remove non-bound radioactive material. The pellet, dissolved in 1 N NaOH, contained 19,000 cpm or 2.5% of the added radiolabeled metronidazole. To determine whether this radiolabeled material contained a thioether analogous to that in 4-ATME, it was treated with Raney nickel under conditions (described in Materials and Methods) that release 2-methylimidazole-1-ethanol from 4-ATME. No radioactive compound with the chromatographic properties of 2-methylimidazole-1-ethanol was found. It was also established that 4-ATME is stable in 5% TCA and 1 N NaOH so that the presumed adduct should have survived the treatment it received prior to its treatment with Raney nickel.

DISCUSSION

We have demonstrated that cysteamine protected anaerobically cultured *E. coli* and *B. fragilis* from the bactericidal activity of metronidazole. A cysteamine–metronidazole adduct, 4-ATME, was formed under these conditions. However, the biological importance of this reaction in the detoxification of metronidazole remains uncertain since the formation of the adduct depended neither on the presence of live bacteria nor on a reduced environment.

Furthermore, we found no evidence that metronidazole formed thioethers with protein sulfhydryl groups analogous to those that it formed *in vitro* with cysteamine. However, another limitation of these experiments must be recognized. Although the product indicative of this adduct was not isolated after treating the macromolecules with Raney nickel, only limited experience indicates that Raney nickel interacts with macromolecules as it does with analogous small molecules [19]. It is possible, therefore, that Raney nickel is not a dependable reagent to cleave a thioether on a protein. Furthermore, the adduct may have been formed in quantities too small to detect.

REFERENCES

1. D. I. Edwards, M. Dye and H. Carne, *J. gen. Microbiol.* **76**, 135 (1973).
2. R. M. J. Ings, J. A. McFadzean and W. E. Ormerod, *Biochem. Pharmac.* **23**, 1421 (1974).
3. M. Muller and D. G. Lindmark, *Antimicrob. Agents Chemother.* **9**, 696 (1976).
4. J. K. Mohindra and A. M. Rauth, *Cancer Res.* **36**, 930 (1976).
5. G. E. Adams, E. D. Clarke, R. S. Jacobs, I. J. Stratford, R. G. Wallace, P. Wardman and M. E. Watts, *Biochem. biophys. Res. Commun.* **72**, 824 (1976).
6. E. Perez-Reyes, B. Kalyanaraman and R. P. Mason, *Molec. Pharmac.* **17**, 239 (1980).
7. J. D. Chapman, A. P. Reuvers, J. Borsa and C. L. Greenstock, *Radiat. Res.* **56**, 291 (1973).
8. E. J. Hall, M. Astor, C. Geard and J. Biaglow, *Br.J. Cancer* **35**, 809 (1977).
9. Y. C. Taylor and A. M. Rauth, *Br. J. Cancer* **41**, 892 (1980).
10. B. D. Michael and H. A. Harrop, in *Radiation Sensitizers: Their Use in Clinical Management of Cancer* (Ed. L. W. Brady), p. 14. Masson, New York (1980).
11. P. Goldman and J. D. Wuest, *J. Am. chem. Soc.* **103**, 6224 (1981).
12. E. J. T. Chrystal, R. L. Koch and P. Goldman, *Molec. Pharmac.* **18**, 105 (1980).
13. L. A. Wheeler, F. B. Soderberg and P. Goldman, *J. Pharmac. exp.Ther.* **194**, 135 (1975).
14. E. J. T. Chrystal, R. L. Koch, M. A. McLafferty and P. Goldman, *Antimicrob. Agents Chemother.* **18**, 566 (1980).
15. M. A. McLafferty, R. L. Koch and P. Goldman, *Antimicrob. Agents Chemother.* **21**, 131 (1982).
16. L. V. Holdeman, E. P. Cato and W. E. C. Moore (Eds.), *Anaerobe Laboratory Manual*, 4th Edn. p. 141. Virginia Polytechnic Institute and State University, Blacksburg, VA. (1977).
17. B. N. Ames, W. E. Durston, E. Yamasaki and F. D. Lee, *Proc. natn. Acad. Sci. U.S.A.* **70**, 2281 (1973).
18. R. L. Koch and P. Goldman, *J. Pharmac. exp. Ther.* **208**, 406 (1979).
19. F. Chapeville, F. Lipmann, G. von Ehrenstein, B. Weisblum, W. J. Ray, Jr. and S. Benzer, *Proc. natn. Acad. Sci. U.S.A.* **48**, 1086 (1962).