ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1973, p. 332-336 Vol. 4, No. 3
Copyright © 1973 American Society for Microbiology
Printed in U.S.A.

Effects of Energy Metabolism on the Release of Bound Proflavine from Sensitive and Resistant *Escherichia coli*

BIPIN M. MEHTA,[1] M. JOAN GRAVELLE, AND DONN J. KUSHNER
Department of Biology, University of Ottawa, Ottawa, KIN 6N5, Ontario, Canada

Received for publication 26 March 1973

Proflavine inhibited the aerobic and anaerobic utilization of glucose by sensitive but not by resistant *Escherichia coli*. In resistant cells that had bound proflavine, glucose utilization was accompanied by release of the dye. After glucose was used up, the cells could again take up proflavine. If the amount of proflavine bound to sensitive cells was too low to inhibit glucose utilization, adding glucose to these cells caused them to release the dye. With higher proflavine concentrations, inhibitory to glucose utilization, the dye remained cell bound. Thus, metabolic energy causes the release of proflavine by both sensitive and resistant cells. In the former, energy production is inhibited by proflavine, and thus the dye prevents its own release. Chloramphenicol did not interfere with metabolically induced release of proflavine from resistant cells. Cyanide inhibited the glycerol-induced loss of proflavine, but not the glucose-induced loss. Azide and carbonyl cyanide *m*-chlorophenylhydrazone could prevent proflavine release without inhibiting glucose utilization.

Earlier studies from our laboratory showed that proflavine binding by cells of *Escherichia coli* suspended in buffer was a passive process, approximately equal in both proflavine-sensitive and -resistant strains. However, addition of any one of a number of different metabolites led to release of bound proflavine from the resistant, but not the sensitive cells (5). As a result, in growth media the former contained less proflavine than did the latter.

Sensitive and resistant cells of *Bacillus subtilis* suspended in buffer also bound the same amounts of proflavine, but adding metabolites caused no release of proflavine from these cells. When resistant cells grew in the presence of proflavine, however, their dye content decreased (1).

This work has raised questions on the nature of the cellular binding sites for proflavine, on the site(s) at which proflavine acts to inhibit cell growth, on the mechanism whereby bound proflavine is released, and on the relation between ability to release bound proflavine and resistance to this dye. Previous experiments with *E. coli* showed that spheroplasts or heated cells depleted of practically all their nucleic acids could still bind about one-half as much proflavine as intact cells (2). Thus, nucleic acids, which have been considered the main site of action of proflavine, are by no means the dye's only binding sites. Experiments on the effects of proflavine on the metabolism of *E. coli* and on the relation between metabolism and proflavine release are reported in this paper.

[1] Present address: Sloan-Kettering Institute for Cancer Research, Division of Drug Resistance, New York, N.Y. 10021.

MATERIALS AND METHODS

Cultures and culture conditions. The proflavine-sensitive and -resistant strains *E. coli* B and *E. coli* B/Pr were those previously described (5). Cultures were grown at 37 C with aeration in Erlenmeyer or Fernbach flasks (on a reciprocating shaker, 100 strokes per min) containing 0.2 volume of Trypticase soy broth (Baltimore Biological Laboratories, Baltimore, Md.). Both proflavine-sensitive and -resistant cells of *E. coli* were inoculated with 0.01 volume of an overnight culture. Growth was followed by optical density (OD) readings of the culture at 660 nm by using 18-mm cuvettes in a Coleman Junior spectrophotometer. After 4 to 6 h of incubation (OD approximately 0.5 to 0.65 at 660 nm), cells were harvested by centrifugation (8,000 $\times$ g for 10 min) and washed twice with and resuspended in the experimental buffer used to an OD of 1.0 at 660 nm (equivalent to about 2 mg/ml, dry weight).

Metabolite-induced release of proflavine. For most of the experiments, 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.5, and 0.001 M $MgCl_2$ were used. Experiments with 2,4-dinitrophenol were carried out in 0.01 M potassium phosphate buffer, pH 7.5. Reaction mixtures contained buffer with different concentrations of proflavine and 0.1 volume of cell suspension (final cell concentration about 0.2 mg/ml). Cells were incubated with proflavine for 30 min prior to the addition of the metabolite, and were further incubated for a designated length of time. Incubations were carried out at 37 C, either aerobically (shaking in air) or anaerobically (standing under N_2). Samples were withdrawn at intervals and centrifuged (10,000 $\times$ *g* for 5 min). Bound proflavine was determined, as before (5), by dissolving the pellet in 2% sodium lauryl sulfate or, in some cases, by measuring the amount remaining in the supernatant fluid. Glucose in the supernatant fluid was determined by the method of Park and Johnson (7) in Tris buffer and by the anthrone method (6) in phosphate buffer.

RESULTS

Effects of proflavine on glucose utilization. Previous work showed (5) that adding glucose and other metabolites to resistant cells that had bound proflavine caused the dye to be released, but that no such release occurred with sensitive cells. Because the effects of glucose on resistant cells were inhibited by low temperatures and metabolic inhibitors (5), it seemed likely that glucose utilization was needed for proflavine release, and experiments were carried out to investigate this directly.

In resistant cells, adding glucose led to the immediate release of proflavine from the cells, as well as the progressive disappearance of glucose itself from the external medium. After most of the glucose had been used, proflavine was again taken up by the cells (Fig. 1A and B). The rate of glucose utilization was the same in the presence and absence of proflavine (up to 10 $\times$ 10^{-5} M, the highest concentration tested); glucose disappeared more quickly anaerobically, but less proflavine was released anaerobically than aerobically. After the glucose was used up, less rebinding of proflavine by the cells occurred anaerobically than aerobically.

In contrast, the glucose utilization of sensitive cells was inhibited by proflavine in concentrations of 2 $\times$ 10^{-5} M and higher (Fig. 2). The degree of inhibition was greater under anaerobic than under aerobic conditions. Aerobically, even the highest proflavine concentration did not completely inhibit glucose utilization. These experiments also showed that if the amount of proflavine bound to the sensitive cells was too low to inhibit glucose utilization

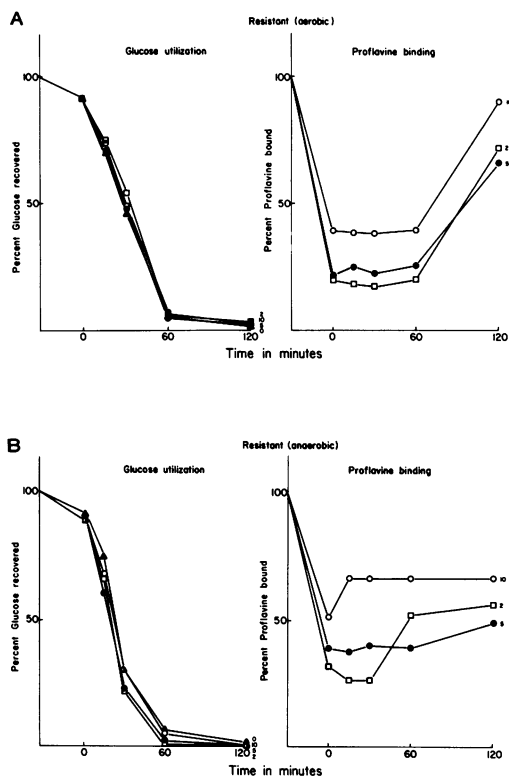

FIG. 1. *Glucose utilization and release of bound proflavine by resistant cells. A, Aerobic incubation; B, anaerobic incubation. Cells were incubated in the presence of different proflavine concentrations (the concentrations, $\times 10^{-5}$ M, are indicated by the curves). After measuring bound proflavine (100% binding), glucose (0.01% final concentration) was added. A sample was centrifuged immediately (0 time) and at intervals for measuring proflavine bound to the cells and glucose remaining in the supernatant fluid. The small decrease in glucose and large decrease in bound proflavine in the 0 time samples show the changes occurring while cells were being centrifuged. 100% Proflavine contents of cells ($\times 10^{-2}$ µmol per mg, dry weight) for added concentrations of 2, 5, and 10 $\times$ 10^{-5} M, respectively, were (A) 2.2, 4.9, and 14.1; and (B) 2.5, 6.2, and 14.3.*

strongly, then adding glucose caused this bound proflavine to be released. This suggests that both sensitive and resistant cells are able to expel bound proflavine, but that in the sensitive cells proflavine itself inhibits the metabolic events responsible for its release.

Effects of metabolic inhibitors and uncoupling agents on resistant cells. It was previously shown that fluoride and iodoacetate prevented the release of proflavine from resistant cells by glucose (5). The effect of certain other inhibitors has been studied. Chloramphenicol

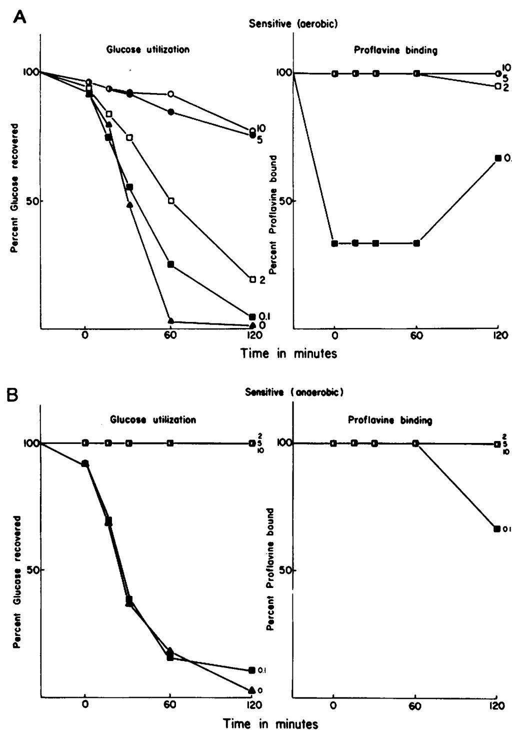

FIG. 2. *Glucose utilization and release of bound proflavine by sensitive cells. A, Aerobic incubation; B, anaerobic incubation. Conditions were as in Fig. 1. 100% Proflavine contents of cells ($\times 10^{-2}$ µmol per mg, dry weight) for added concentrations of 0.1, 2, 5, and 10 $\times 10^{-5}$ M, respectively, were (A) 0.3, 2.8, 6.2, and 14.6; and (B) 0.3, 2.4, 7.0, and 13.3*

(100 µg/ml) did not inhibit proflavine release from resistant cells after the addition of glucose, ribose, or glycerol; hence, proflavine release does not depend on protein synthesis. Cyanide prevented the glycerol-induced release of proflavine, but not that induced by glucose (Fig. 3). This may reflect the fact that glucose can yield energy by both aerobic and anaerobic metabolic pathways, but glycerol only by aerobic pathways. Sodium azide can inhibit cytochrome oxidase and adenosine triphosphatase and act as a proton conductor (3). This compound could prevent proflavine release while permitting glucose utilization. Dissociation of the two processes was seen most clearly under anaerobic conditions (Table 1).

The uncoupling agent (3) carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 10^{-4} M) partly inhibited anaerobic utilization of glucose and completely inhibited proflavine release. Aerobically, this concentration of CCCP stimulated glucose utilization and strongly inhibited proflavine release (Table 1). A lower CCCP concentration (10^{-6} M) affected neither glucose utilization nor proflavine release. It was also found that CCCP increased the amount of proflavine bound before glucose was added (Table 1). Another uncoupling reagent (3), 2,4 dinitrophenol (5 $\times 10^{-3}$ M), strongly inhibited both glucose utilization and proflavine release under aerobic and anaerobic conditions and could not be used to separate the two phenomena.

DISCUSSION

One of the main points to emerge from the present work is that proflavine acts as a metabolic inhibitor, affecting glucose metabolism more strongly in sensitive than resistant cells. Preliminary experiments have shown that ribose and glycerol oxidation are also inhibited to a greater extent by proflavine in sensitive than in resistant cells. Proflavine was previously shown to inhibit glucose metabolism in yeast (8).

Both sensitive and resistant *E. coli* are able to release bound proflavine, but the former can do so only if the amount bound is too low to inhibit glucose utilization. This explains our earlier finding (5) that glucose causes proflavine release when a given amount of the dye is added to a large amount of sensitive cells, but not when it is added to a smaller amount of such cells. If the cell concentration is high, less proflavine will be bound per unit weight of cells. Thus, calculations from Table 4 of reference 5 show that a proflavine content of 1 $\times 10^{-2}$ µmol per mg of cells permitted glucose-induced release; a concentration of 5.4 $\times 10^{-2}$ µmol per mg of cells prevented this release. These results emphasize the importance of expressing the

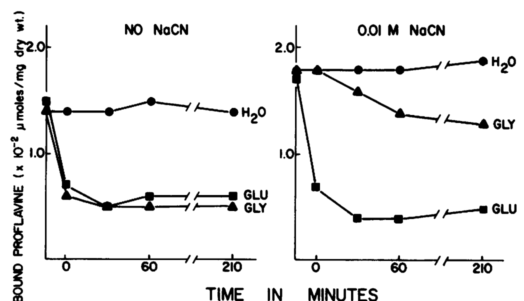

FIG. 3. *Effect of NaCN on glucose- and glycerol-induced release of proflavine from resistant cells. Cells were first incubated in 2 $\times 10^{-5}$ M proflavine for 30 min. At 0 time, a 0.1 volume of H_2O, 5% glucose, or 5% glycerol was added. Incubation was continued aerobically and bound proflavine was determined at intervals.*

TABLE 1. *Effect of inhibitors on proflavine release and glucose utilization by resistant cells of Escherichia coli*[a]

Expt	Conditions of incubation	Inhibitor added	Glucose remaining (%) at (min):			Proflavine bound (%)			
			0	30	60	Before adding glucose	After adding glucose (min) 0	30	60
1	Aerobic	Nil	90	10	8	100 (2.9)[a]	32	36	60
		0.01 M NaN_3	90	8	6	100 (2.8)	39	55	66
		0.1 M NaN_3	90	8	6	100 (2.9)	70	74	78
2	Anaerobic	Nil	90	11	10	100 (2.6)	38	33	71
		0.01 M NaN_3	97	76	56	100 (2.1)	100	100	106
		0.1 M NaN_3	100	94	85	100 (2.2)	100	97	103
3	Aerobic	Nil	81	60	46	100 (2.9)	28	44	36
		10^{-4} M CCCP	79	25	2	100 (4.8)	95	78	79
4	Anaerobic	Nil	98	42	6	100 (2.9)	54	59	65
		10^{-4} M CCCP	99	91	68	100 (5.2)	100	100	100

[a] Cells were incubated for 30 min with proflavine (2×10^{-5} M) and inhibitors as shown. Then glucose (0.01% final concentration) was added and proflavine liberation and glucose utilization followed as in Fig. 1 and 2. Figures in parentheses indicate amount bound before glucose was added ($\times 10^{-2}$ μmol per mg of cells).

effective dye concentration as the amount bound per weight of cell rather than as the amount added to the medium.

The fact that proflavine released during glucose metabolism may be taken up again after glucose disappears suggests that a continuous supply of energy is needed to keep this dye out of the cell.

It was noted earlier that although resistant cells bind less proflavine than do sensitive cells in growth medium, they can still grow while containing enough of the dye to inhibit growth of sensitive cells (5). This indicates that ability to expel proflavine is not the only mechanism of resistance.

Comparison of the figures found here and in our earlier paper (5) indicates that proflavine levels that cause little or no inhibition of glucose utilization do not inhibit growth; concentrations inhibiting utilization by 50% also inhibit growth by 80 to 90%; when glucose utilization is 80% or more inhibited, growth is completely inhibited. It seems quite possible that proflavine affects growth in these cells primarily through its action on metabolic processes. Resistance at the metabolic level may determine whether or not growth is inhibited. Metabolically induced release of bound proflavine may be regarded as a second line of defense. Such release would presumably diminish the amount of proflavine available for binding to nucleic acids and other sites that may also be sensitive to this dye (2).

In *Streptococcus faecalis*, which depends entirely on fermentation for its energy, CCCP and other uncouplers prevent the active transport of cations into the cell (4). We have already considered the possibility that metabolic energy stimulates transport of metallic cations into the cell, displacing bound proflavine (5). Uncoupling agents, which we have found to inhibit proflavine release, would presumably block such transport. However, it was shown earlier that glucose stimulated proflavine release from resistant cells in a Tris-phosphate buffer, and we have found (unpublished data) that such release also occurs in a Tris-hydrochloride buffer (pH 7.4) and in an imidazole buffer (pH 8.0). Thus, metallic cations do not seem to be needed for proflavine displacement.

Our experiments have shown that when proflavine inhibits glucose utilization, it also inhibits its own release. Other inhibitors can prevent proflavine release while permitting glucose utilization and, presumably, energy production. The latter seems necessary, but not sufficient, for proflavine release.

ACKNOWLEDGMENTS

This work was supported by a grant to D. J. Kushner from the National Research Council of Canada.

LITERATURE CITED

1. Barabas, G., B. M. Mehta, and D. J. Kushner. 1970. Proflavine binding and release by sensitive and resistant *Bacillus subtilis*. Can. J. Microbiol. **16**:973-981.
2. Gravelle, M. J., B. M. Mehta, and D. J. Kushner. 1972. The elusive permeability barriers and binding sites for proflavine in *Escherichia coli*. Antimicrob. Ag. Chemother. **1**:470-475.
3. Harold, F. M. 1972. Conservation and transformation of energy by bacterial membranes. Bacteriol. Rev. **36**:172-230.
4. Harold, F. M., and J. R. Baarda. 1968. Inhibition of membrane transport in *Streptococcus faecalis* by uncouplers of oxidative phosphorylation and its relation to proton conduction. J. Bacteriol. **96**:2025-2034.
5. Kushner, D. J., and S. R. Khan. 1968. Proflavine uptake

and release in sensitive and resistant *Escherichia coli*. J. Bacteriol. **96**:1103–1114.

6. Morris, D. L. 1948. Quantitative determination of carbohydrates with Dreywood's anthrone reagent. Science **107**:254–255.
7. Park, J. T., and M. Johnson. 1949. A submicrodetermination of glucose. J. Biol. Chem. **185**:149–151.
8. Witt, I. B., B. Neufang, and H. Muller. 1968. The effect of proflavine on yeast cells. Biochim. Biophys. Acta **170**:216–218.