Proflavine Uptake and Release in Sensitive and Resistant *Escherichia coli*¹

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Received for publication 29 April 1968

Both *Escherichia coli* B and a proflavine-resistant mutant, *E. coli* B/Pr, took up the same amounts of proflavine when suspended in buffer containing the dye. In growth media, however, sensitive cells took up more proflavine than did resistant cells. Adding growth media or any one of several constituents of these media, including amino acids, glycerol, pyruvic acid, and metabolizable sugars, to resistant cells that had taken up proflavine in buffer caused them to lose the dye, but had less or no effect on sensitive cells. Certain salts caused an equal release of proflavine from resistant and sensitive cells. Proflavine released from resistant cells by glucose was not changed chemically. The effects of temperature and metabolic inhibitors suggest that proflavine uptake is a passive process but that its release may be an active one, dependent on metabolism. Glucose had more effect on the proflavine binding of *E. coli* B grown in a minimal medium than on that of cells grown in a complex medium. *E. coli* B was less susceptible to proflavine when growing in a minimal medium. The effects of other synthetic media on proflavine susceptibility of *E. coli* B were also studied. Deoxyribonucleic acid and envelopes from sensitive and resistant cells bound the same amounts of proflavine, and no difference was seen in the site of dye binding when sensitive and resistant cells that had taken up proflavine in buffer were sonically broken and fractionated. The results suggest that sensitive and resistant cells are equally permeable to proflavine but differ in the ease with which metabolites cause them to release bound proflavine. So far, however, these differences do not account completely for the ability of resistant cells to grow in high proflavine concentrations.

The mutagenic action of certain acridine dyes may be explained by their combination with deoxyribonucleic acid (DNA), a reaction that has been studied in detail by elegant physico-chemical methods (2, 8). DNA binding may be the cause of growth inhibition by higher concentrations of acridines, although these dyes also bind to ribonucleic acids (RNA) and to other macromolecules and cell structures and presumably affect the cell in doing so (10, 17). Study of the entry of acridine dyes into the intact cell is more complex and difficult than study of their binding to isolated cell components, but it is necessary for understanding the effects of the dyes on the living cell.

Work has appeared recently on the effects of bacteriophage infection and of treatment with agents that affect cell membranes on acridine dye binding (14, 18, 19). Some information on the control of dye entry may also be gained by

studying acridine-resistant cells. Trypanosomes resistant to "acriflavine" take up less dye than do sensitive trypanosomes (6, 7). [Commercial acriflavine is a mixture of 3,6-diamino acridine (proflavine) and 3,6-diamino-10-methyl acridinium chloride. The latter compound is sometimes called acriflavine.] On the other hand, Peacocke and Hinshelwood (15) observed that proflavine-resistant *Aerobacter aerogenes* took up slightly more proflavine than did sensitive cells. We had begun an investigation of proflavine resistance in *Escherichia coli* and had observed that sensitive and resistant cells bound the same amount of the dye, when a paper appeared by Nakamura (12), stating that acriflavine-resistant *E. coli* bound substantially less acriflavine than did sensitive cells. We thought it unlikely that different mechanisms would exist for resistance to such closely related compounds. In the course of resolving this apparent contradiction, we found that proflavine *binding* is a passive process, but that its *release* from cells to which it is bound

¹ Part of this work is included in a thesis presented for the MSc degree by S. R. Khan.

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may be an active one, dependent on metabolism. Sensitive and resistant cells differ in the latter process.

A study was made of the effects of different metabolites on the proflavine-binding ability of cells, effects that must be considered in interpreting any treatment in terms of altered "permeability" to proflavine and (presumably) other acridine dyes.

This work also compares proflavine binding by subcellular fractions of sensitive and resistant *E. coli*. A preliminary report has appeared (D. J. Kushner and S. R. Khan, *Bacteriol. Proc.*, p. 32, 1968).

MATERIALS AND METHODS

Sources of strains. *E. coli* B was obtained from D. Mount of the Defense Research Board, Ottawa, and was maintained with biweekly transfers on Trypticase-soy-agar. The compositions of this and other growth media are given below. The resistant strain, *E. coli* B/Pr, obtained as described below, was maintained on this agar containing 10^{-3} M proflavine (302 μ g/ml).

Growth media. Trypticase soy broth (TSB, from Baltimore Biological Laboratories, Baltimore, Md.) contains 17 g of Trypticase (USP pancreatic digest of casein), 3 g of Phytone (USP soy peptone), 5.0 g of NaCl, 2.5 g of glucose per liter; final pH, 7.4. Trypticase-soy-agar (TSA) was prepared by adding 2% agar (Difco) to TSB. In certain experiments, Trypticase broth was used. This contained Trypticase and NaCl, as above, and 2.5 g per liter of glucose or lactose, as specified; Phytone was omitted. The minimal medium used contained (per liter): 1 g of KH_2PO_4 , 1 g of K_2HPO_4 , 1 g of NaCl, 4 g of $(\text{NH}_4)_2\text{SO}_4$, 5 g of NH_4Cl , 0.7 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, final pH adjusted to 7.0. Glucose, lactose, or certain amino acids were autoclaved separately and added to these salts to a final concentration of 0.5%.

Growth experiments. These were carried out in Erlenmeyer flasks, containing one-fifth volume of growth medium and fitted with an 18-mm diameter side arm for turbidimetric measurements, or in 18-mm diameter tubes containing (usually) 10 ml of medium and placed on a shaker at a 45° angle. Tubes or flasks were shaken reciprocally 120 times per min at 37 C. Growth was measured as turbidity at 660 nm in a Coleman Junior spectrophotometer. An optical density of 1.0 corresponded to 2.0 mg (dry weight) of cells per ml, and an optical density of 0.5 corresponded to 0.66 mg of cells per ml. Experiments on proflavine binding were carried out, unless otherwise specified, with overnight cultures (16 to 20 hr) in TSB.

Buffers. For most experiments, 0.01 M potassium phosphate buffer (pH 7.0) was used. This is usually designated as "buffer." In some specified experiments, 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.2), Michaelis veronal-acetate buffer (pH 7.0) (11), or a mixture of 0.01 M H_2PO_4 and 0.01 M Tris (final pH 6.9) was used.

Proflavine and measurement of its binding. Proflavine

sulfate (Allied Chemicals) was recrystallized from alcohol-water before use. Solutions were stored in red glass flasks to prevent photochemical decomposition; in all experiments, light was excluded as much as possible. No loss of color attended exposure to normal laboratory illumination for 60 min, the longest time necessary to measure the absorbance of a large number of proflavine solutions. Proflavine was measured by its absorbance at 445 nm. The spectra of proflavine solutions were measured on a Beckman DB recording spectrophotometer, or manually on a Beckman DU spectrophotometer.

Bound proflavine was extracted quantitatively from cells or subcellular particles by treatment with 2% sodium lauryl sulfate (SLS) in water or buffer for 10 min at 60 C, a procedure that lysed the cells and dissolved almost all subcellular fragments. Corrections were made for material absorbing light at 445 nm extracted from control cells that had not been exposed to proflavine. Recovery of absorbed proflavine was complete, provided that the cell concentration was not higher than 1 mg (dry weight) per ml. We also attempted to extract bound proflavine by ethyl alcohol, acid ethyl alcohol, acetone, and 0.1 M HCl, or NaOH; none of these removed all the dye. In preliminary experiments, cells that had absorbed proflavine were held at 100 C for 10 min to break down their permeability barriers. After this treatment, all the dye could not be removed, even by SLS.

Most experiments on the ability of different substances to release cell-bound proflavine were carried out as follows. Cells (about 0.2 mg, dry weight, per ml) were incubated 2 hr at 37 C in buffer containing 2×10^{-5} M proflavine. The suspension was dispersed in centrifuge tubes, and one-tenth volume of the material to be tested was added. After incubation at 37 C for another 2 hr (unless specified otherwise), cells were centrifuged down (10 min at $10,000 \times g$) and proflavine remaining in the supernatant fluid was measured to determine the amount bound. The amount bound to cells treated only with distilled water is taken as 100%.

Preparation of subcellular fractions. Cells were broken sonically, with a Branson sonic oscillator model S-125, by exposing the suspension to four successive 15-sec bursts of 20 kc/sec at maximal power and cooling in ice between bursts. Phase-contrast examination showed that this procedure broke at least 99% of the cells.

Cell envelopes were also prepared by mechanical disintegration. Cell suspensions (20 ml) in distilled water, containing about 30 mg of cells per ml, were broken in a Bronwill apparatus with 16-g Ballotini glass beads (grade 12) and a drop of octanol to reduce foaming (4). Shaking was carried out for 4 min and the suspensions were cooled with CO_2 during shaking. Beads and intact cells were removed by centrifuging at $4,500 \times g$ for 10 min and envelopes were sedimented at $15,000 \times g$ for 30 min. Alternate slow and fast centrifugations were repeated several times to remove all intact cells; the envelopes were finally washed three times with distilled water.

DNA was isolated by the method of Marmur (9) and its phosphorus content was measured by Allen's

(3) method. The specific absorbance, Ep at 260 nm, that is, absorbance at 260 nm $\times$ light path in cm gram-atoms of phosphorus per liter (5) was 6,102 for DNA from sensitive cells and 6,320 for DNA from resistant cells; the difference is not significant.

RESULTS

Selection of proflavine-resistant cells. *E. coli* B did not grow in the presence of 10^{-3} M proflavine (302 μ g/ml) even after a long incubation in broth (TSB) or agar (TSA). However, some cells did grow after a long lag in the presence of 10^{-4} M proflavine. These were then grown successively in the presence of 2×10^{-4} and 5×10^{-4} M proflavine in TSB, then streaked on TSA containing 10^{-3} M proflavine. After 24 hr at 37 C, several colonies appeared. One of these was selected and the cells were maintained by biweekly transfers on Trypticase-soy-agar containing 10^{-3} M proflavine. These cells (designated *E. coli* B/Pr) retained their resistance to proflavine, as measured by the ability to grow on agar containing 10^{-3} M proflavine, after several passages through TSB in the absence of proflavine. Growth of resistant cells in the absence of proflavine was slower than that of sensitive cells (see Fig. 2) and the cells were smaller. They gave typical *E. coli* colonies on eosine-methylene blue plates and fermented lactose. *E. coli* B/Pr was also resistant to "acriflavine" (300 μ g/ml).

Binding of proflavine to sensitive and resistant cells in buffer. Cells (about 0.4 mg/ml) were suspended in 0.01 M potassium phosphate buffer, pH 7.0, containing different proflavine concentrations for 2 hr at 37 C and centrifuged down for 10 min at $10,000 \times g$. Measurement of proflavine remaining in the supernatant fluid showed that there was no significant difference in the amount bound by sensitive and resistant cells over most of the concentration range studied (Fig. 1). In the experiment shown, sensitive cells took up more proflavine from a 10^{-6} M solution than did resistant cells, but, in other experiments, both took up the same amount.

Binding of proflavine to sensitive and resistant cells in growth medium. A quite different result was obtained when the proflavine-binding power of sensitive and resistant cells was compared in a complex growth medium (TSB). In this experiment, the effect of different proflavine concentrations on growth was observed. Then cells were centrifuged down and proflavine binding was determined by dissolving the pellet in SLS. Sensitive cells bound four to six times as much proflavine as resistant cells, whether or not the dye inhibited growth (Fig. 2).

Another experiment measured the proflavine-binding capacity of resistant cells growing in

FIG. 1. Proflavine uptake by cells and envelopes of sensitive and resistant *E. coli*. (■) Sensitive; (○), resistant. Solid lines, cells; broken lines, envelopes. Sensitive cells (0.40 mg/ml) or resistant cells (0.43 mg/ml) were incubated in buffer containing the proflavine concentration shown for 2 hr at 37 C. Envelopes (0.35 mg/ml) were incubated for 1 hr at 37 C and removed by centrifuging 30 min at $10,000 \times g$.

yet higher concentrations of the dye. A culture in TSB with a cell density of 0.1 mg/ml was treated with 0 , 1×10^{-4} , 2×10^{-4} , and 3×10^{-4} M proflavine. After 4.5 hr of growth, to culture densities of 0.60 mg/ml (0.56 in the highest proflavine concentration), bacteria growing in the above concentrations of proflavine had bound 1.40×10^{-2} , 2.79×10^{-2} , and 7.03×10^{-2} μ moles of cells per mg, respectively. This experiment showed that resistant cells were able to grow while binding amounts of proflavine that would inhibit the growth of sensitive cells.

Experiments were then performed to determine the effect of growth medium on cells that had already bound proflavine while suspended in buffer. Cells were incubated for 2 hr at 37 C with different proflavine concentrations, a sample was removed to determine the amount bound, and to the rest of the suspension was added an equal volume of double-strength TSB containing a proflavine concentration equal to that to which the cells had originally been exposed. The suspension was shaken, growth was measured turbidimetrically, and samples were removed at intervals and centrifuged to measure cell-bound proflavine in the sediment. Adding TSB caused a rapid decrease in the proflavine bound to both sensitive and resistant cells (Fig. 3), but the fall was faster and greater in resistant cells. The amount bound per milligram of cells leveled off after 1 hr to a value dependent on the cell type and the external proflavine concentration.

FIG. 2. Effect of proflavine on growth of sensitive and resistant *E. coli* in TSB. At times shown by the arrows, the concentrations of proflavine shown were added. Figures in parentheses show proflavine binding in 10^{-2} μ mole per mg of cells.

Growth began, if at all, after such a level value was reached. Although not shown in Fig. 3, *E. coli* B/Pr in 10^{-4} M proflavine began to grow after 4 hr of incubation.

Effect of different constituents of growth media on proflavine binding. These results showed that the growth medium had a dramatic effect on proflavine binding, an effect that was greater with resistant cells. Experiments were next carried out to find what constituents of TSB, or of other media that would support growth, were responsible for the release of bound proflavine. A general survey (Table 1) showed that several salts released proflavine from sensitive and resistant cells, that a mixture of amino acids (Casamino Acids) released more proflavine from resistant than from sensitive cells, and that glucose released much more proflavine from resistant than from sensitive cells. Magnesium ions were also slightly more effective on resistant than sensitive cells. In an experiment comparing the effects of a wide range of Mg^{++} concentrations, resistant cells bound more proflavine than sensitive cells in the absence of Mg^{++} , and Mg^{++} concentrations above 10^{-4} M caused a slightly greater loss of proflavine from resistant cells (Fig. 4).

A more detailed study of the effects of different carbon compounds (Table 2) showed that glucose, galactose, ribose, lactose, maltose, and glycerol lowered the binding of proflavine to resistant cells to varying extents, but these compounds had no effect, or much smaller effects, on the binding to sensitive cells. Pyruvate was especially active on resistant cells but not on sensitive cells. Sodium acetate and sodium formate affected

FIG. 3. Proflavine release and cell growth after addition of TSB. Upper curves, proflavine binding; lower curves, growth. The proflavine concentrations are shown at each curve.

both resistant and sensitive cells, whereas sodium succinate and sodium citrate (cf. Table 1) hardly affected proflavine binding.

Of the amino acids tested, glutamate was especially active, releasing bound proflavine from both sensitive and resistant cells; its activity was followed by that of serine and lysine, which

TABLE 1. Substances that affect the release of bound proflavine from *E. coli*^a

Additions	Proflavine bound (10^{-2} μ mole)	
	Sensitive	Resistant
Expt 1		
Water	4.0	3.5
Glucose (0.5%)	3.7 (0.3)	1.4 (2.1)
Casamino Acids (0.2%)	2.2 (1.8)	0.4 (3.1)
Salts ^b	0.0 (4.0)	0.0 (3.5)
Glucose + salts + Casamino Acids	0.0 (4.0)	0.0 (3.5)
Expt 2		
Water	4.9	6.3
Salts ^b	0.4 (4.5)	1.9 (4.4)
NaCl (0.5%)	1.7 (3.2)	3.0 (3.3)
MgSO ₄ (0.034%)	2.7 (2.2)	3.2 (3.1)
NH ₄ Cl (0.5%)	1.3 (3.6)	2.5 (3.8)
Sodium citrate (0.05%)	5.0 (-0.1)	6.8 (-0.5)

^a Suspensions (7 ml) in buffer containing 2×10^{-5} M proflavine were incubated at 37 C for 150 min (experiment 1) or 120 min (experiment 2). Additions were made, in 3.0-ml volume, to the final concentrations shown and tubes were incubated an additional 120 min before centrifuging down cells and measuring proflavine binding. Dry weight of cells used: experiment 1, 0.56 mg; experiment 2, 1.4 mg. Figures in parentheses show fall in proflavine binding caused by each addition.

^b The composition of salts used is shown in experiment 2.

also affected binding in sensitive cells slightly. Alanine, proline, leucine, and glycine, in decreasing order of effectiveness, reduced proflavine binding in resistant cells only.

Effect of growth medium composition on the response of proflavine binding to sugars. Table 3 shows the effect of certain monosaccharides and disaccharides on proflavine binding of sensitive and resistant cells grown in Trypticase broth containing glucose or lactose, and also on those grown in a synthetic medium with glucose or lactose as the sole carbon source. If resistant cells were grown in Trypticase plus glucose, proflavine binding in the washed cells was lowered by glucose and galactose but not by lactose; if these cells were grown in Trypticase plus lactose, however, binding was also lowered by lactose. The same pattern appeared in resistant cells grown in minimal media containing glucose or lactose, except that galactose had little effect on proflavine binding of glucose-grown cells. Sucrose did not affect the binding in such cells, and maltose affected it slightly.

Binding to sensitive cells grown in Trypticase medium containing either glucose or lactose was not affected by sugars. However, sensitive

FIG. 4. Effect of MgSO₄ on proflavine release from sensitive and resistant cells. Cells (0.19 mg/ml) were incubated 160 min at 37 C with 2×10^{-5} M proflavine; one-tenth volume of water or MgSO₄ was then added to give the final concentrations shown, and incubation was continued for 70 min.

cells grown in synthetic media did respond to certain sugars. This response varied from experiment to experiment but was generally less than that of resistant cells. The pattern of response to individual sugars depended on the sugar present in the synthetic medium, as it did with resistant cells. Galactose did not affect glucose-grown sensitive cells but did affect those grown in lactose minimal medium. In one experiment, growth in the presence of lactose made binding of sensitive cells especially responsive to sugars, but an increased response of lactose-grown over glucose-grown cells was not found in all experiments. Sensitive cells grown in glucose or lactose minimal media responded to alanine and pyruvate, though their response was less than that of similarly grown resistant cells.

These results suggest that a given sugar will affect proflavine binding only in those cells that can metabolize the sugar. Galactose and lactose affect binding of resistant cells grown in TSB in which glucose is the only added carbohydrate, and maltose has a greater effect on such cells than on those grown in synthetic media (Tables 2 and 3). Possibly, the soy peptone in TSB contributed carbohydrates that can induce β -galactosidase and α -glucosidase activity.

TABLE 2. *Effect of different compounds on proflavine binding^a*

Addition	Proflavine bound (%)	
	Sensitive	Resistant
Water	100	100
Glucose (0.1%)	95	51
Glucose (0.5%)	100	51
Galactose (0.5%)	95	78
Ribose (0.5%)	100	65
Lactose (0.5%)	128	69
Maltose (0.5%)	110	64
Sucrose (0.5%)	95	109
Glycerol (0.5%)	97	61
Sodium pyruvate (0.1%)	94	42
Sodium succinate (0.1%)	100	88
Sodium formate (0.5%)	33	38
Sodium acetate (0.1%)	67	73
Sodium acetate (0.5%)	33	33
Glycine (0.45%)	100	86
DL-Alanine (0.45%)	97	53
DL-Serine (0.45%)	88	36
Sodium-L-glutamate (0.45%)	59	22
L-Leucine (0.18%)	106	75
L-Lysine HCl (0.45%)	75	53
L-Proline (0.45%)	100	56

^a All cells were grown on Trypticase soy broth. Figures represent several typical experiments in which 100% binding was 0.033 to 0.045 μ mole of proflavine per milligram of cells.

Further characterization of the response of sensitive and resistant cells to glucose. The difference between the response to glucose of proflavine binding in sensitive and resistant cells (grown in TSB) is evident only at high external dye concentrations. This was shown by incubating different amounts of cells with a fixed concentration of proflavine and, consequently, reducing the external proflavine concentration to different extents. At the lowest cell (and highest external proflavine) concentration—conditions similar to those in most of the experiments given above—glucose affected only resistant cells (Table 4), but, as the cell concentration was increased and the external proflavine lowered, glucose also affected sensitive cells. Thus, glucose caused proflavine release from sensitive cells into a low but not into a high external proflavine concentration.

Effect of temperature. Temperature affects proflavine uptake and release in different ways. More proflavine was taken up at 0 C than at 37 C by cells suspended in buffer. However, when glucose was added, more proflavine was released at the higher temperature (Fig. 5). These results suggest that proflavine binding is passive but that glucose-stimulated release is an active

TABLE 3. *Effect of growth in different media on response of proflavine binding to carbohydrates and other substances*

Growth medium	Additions	Proflavine bound (%) ^a	
		Sensitive	Resistant
Trypti-case + glucose	Water	100	100
	Glucose (0.1%)	95	30
	Galactose (0.1%)	97	42
	Lactose (0.1%)	97	94
Trypti-case + lactose	Water	100	100
	Glucose (0.1%)	97	41
	Galactose (0.1%)	100	48
	Lactose (0.1%)	100	45
Minimal + glucose ^b	Water	100	100
	Glucose (0.5%)	85	51
	Galactose (0.5%)	103	86
	Lactose (0.5%)	110	91
	Maltose (0.5%)	98	93
	Sucrose (0.5%)	104	105
	DL-Alanine (0.45%)	85	68
	Na-pyruvate (0.1%)	73	56
Minimal + lactose	Water	100	100
	Glucose (0.5%)	53	69
	Galactose (0.5%)	62	65
	Lactose (0.5%)	76	72
	Maltose (0.5%)	90	86
	Sucrose (0.5%)	88	106
	DL-Alanine (0.45%)	85	63
	Na-pyruvate (0.1%)	60	46

^a In most experiments, 100% proflavine binding was 0.035 to 0.050 μ mole of proflavine per mg of cells.

^b Results with sugars added to cells grown in minimal media are the mean values of two experiments.

process dependent on metabolism. The dependence on metabolism had already been implied by studies of the efficiency of different sugars (*see above*) and was further indicated by studies with inhibitors.

Effect of inhibitors on proflavine uptake and release. The following substances had no effect on proflavine uptake at 37 C by sensitive and resistant cells: NaCN, NaF, and sodium iodoacetate, all in 10^{-3} M concentration. However, glucose-stimulated release of proflavine from resistant cells was affected slightly by NaF and more strongly by iodoacetate (Table 5). Iodoacetate had a relatively stronger inhibitory effect on proflavine release from cells incubated in standing tubes than from those shaken in flasks. If proflavine release requires energy, these results may indicate that energy production by glycol sis

TABLE 4. Effect of cell concentration on response of proflavine binding to glucose in sensitive and resistant *E. coli*^a

Cell concn (mg/ml)	External proflavine concn (10^{-5} M)			
	Sensitive		Resistant	
	Water added	Glucose added	Water added	Glucose added
0.00	1.82	1.82	1.82	1.82
0.083	1.37	1.37		
0.091			1.17	1.55
0.165	1.26	1.37		
0.182			1.05	1.53
0.413	0.93	1.20		
0.455			0.80	1.43
1.49	0.34	1.17		
1.64			0.33	1.12

^a Cells were incubated for 2 hr at 37 C with 2×10^{-5} M proflavine and for 2 hr after adding water or glucose. Figures given for cell concentrations and proflavine concentration in the absence of cells are adjusted for this dilution. Final glucose concentration, 1.0%.

is inhibited in standing tubes but that increased oxidative metabolism permits the cells to partly bypass the effects of iodoacetate.

p-Chloromercuribenzoate (2.5×10^{-4} M) and NaN_3 (1.1×10^{-3} M) appear to inhibit proflavine release stimulated by glucose, pyruvate, and glycerol. However, interpretation of the effect of these inhibitors is complicated by the fact that they themselves stimulate the release of bound proflavine.

Is released proflavine changed chemically? To answer this question, we incubated resistant cells for 2 hr at 37 C with 10^{-4} M proflavine, centrifuged down and resuspended the cells in buffer, and stimulated release of the dye by adding 1% glucose and incubating 2 hr more. The spectrum of the released proflavine in the range 240 to 520 nm was identical to that of pure proflavine at pH values of 4.7, 6.15, and 7.7. Chemical alterations of the acridine nucleus cause considerable changes in its spectrum (2); the unchanged spectrum of released proflavine indicates that it is released as such, neither chemically altered nor bound to a protein or nucleic acid.

Proflavine released from resistant cells was still able to inhibit growth of sensitive cells, but quantitative comparison with pure proflavine was difficult, partly because much of the dye was adsorbed to the filters used to prepare sterile media and partly because glucose, used to release proflavine from resistant cells, affected the sus-

FIG. 5. Effect of temperature on proflavine binding and glucose-induced proflavine release from resistant cells. Glucose or water was added at 120 min (arrow). (●) Incubation at 0 C; ○, incubation at 37 C. Solid line, water added; broken line, glucose added. Cells (0.17 mg/ml) were incubated in 120 ml of buffer containing 2×10^{-5} M proflavine and 7-ml samples were removed at 30-min intervals. Then suspensions were divided into 40-ml lots to which 4.0 ml of water or 5.5% glucose was added. Figures are corrected for this dilution.

TABLE 5. Effect of inhibitors on proflavine release from resistant cells^a

Additions	Proflavine bound (%) ^b	
	Standing	Shaken
Water	100 (2.6)	100 (3.1)
Sodium iodoacetate (10^{-4} M)	104	100
NaF (10^{-3} M)	96	100
Glucose (0.5%)	50	32
Glucose + iodoacetate	89	58
Glucose + NaF	58	35

^a Cell concentration, 0.35 mg/ml. The second incubation of 5.5 ml was carried out in standing tubes (18-mm diameter) or in shaken 50-ml flasks.

^b Figures are given as percentage of water control. Parentheses show binding observed as 10^{-2} μmole per mg of cells.

ceptibility of sensitive cells (see below and reference 13).

Effect of medium composition on susceptibility of cell growth to proflavine. These experiments showed that both metabolites and ions can influence proflavine binding to cells, and that, in general, metabolites lowered binding more in resistant than in sensitive cells. In addition, the

response of washed sensitive cells to certain metabolites was affected by the medium in which these cells had been grown. To evaluate further the role of proflavine binding in the action of this dye on cells, we studied the effect of proflavine on growth of sensitive and resistant cells, using media or experimental treatment which might influence the dye-binding power of the cells.

A straightforward result was obtained with media containing different concentrations of Mg^{++} , an ion that had been shown to reduce proflavine binding in both sensitive and resistant cells (Table 1 and Fig. 4), presumably by competing for negatively charged groups in the cell (16). Inhibition of sensitive cell growth in glucose minimal medium by 10^{-4} M proflavine was partly reversed by 10^{-2} M $MgCl_2$, but not by 10^{-4} M. Resistant cells were not affected by this concentration of proflavine, even in the absence of added $MgCl_2$.

Of several amino acids tested, glutamate and lysine markedly lowered proflavine binding by sensitive cells, whereas serine only slightly lowered it (Table 2). The effects of proflavine on growth of *E. coli* B in media in which amino acids were the only sources of carbon are shown in Table 6. Of the amino acids tested, only alanine, serine, and glutamic acid supported growth in 22 hr, though there was slight growth after 48 hr in a proline-containing medium (not shown). Cells were more susceptible to proflavine in serine- or alanine-containing media than in those containing glutamate or glucose. The inoculum for the experiment shown was washed cells from an overnight culture in TSB; the cells, when suspended in buffer, released proflavine on addition of glutamate but not on glucose addition. Thus, there was not a clear-cut correlation between the ability of these carbon compounds to drive proflavine from cells and to support growth in the presence of proflavine. However, the chemical environment of any growing culture will change, and it is difficult to predict from the initial composition of the culture medium what effect the later composition of the medium will have on proflavine binding.

Because sensitive cells grown in a minimal medium released proflavine more readily on addition of glucose and other metabolites than those grown in TSB, we compared the effect of adding proflavine to cells growing in each medium. Those growing in glucose minimal medium were less susceptible to proflavine than those growing in TSB and, at the end of the experiment, had bound much less dye (Fig. 6). The amount of proflavine associated with glucose-grown *E. coli* B was comparable to that of TSB-grown *E. coli* B/Pr (Fig. 2). The difference in susceptibility

TABLE 6. Effect of proflavine on growth of *E. coli* B in different defined media^a

Carbon source	Growth (per cent of control) at proflavine concn (M) of				
	0	2×10^{-5}	5×10^{-5}	10^{-4}	3×10^{-4}
DL-Alanine...	100(0.265) ^b	91	1	0	0
DL-Serine...	100(0.370)	83	5	0	0
Monosodium L-glutamate	100(0.210)	131	30	7	0
Glucose.....	100(0.231)	66	100	32	1

^a Cells from an overnight culture in TSB were washed twice in sterile buffer and suspended to a density of 1.5 mg/ml (OD 0.89). One drop of this suspension was added to 18-mm tubes containing minimal medium (without $MgSO_4$ in this experiment), the carbon sources shown in 0.5% final concentration, and proflavine as shown (final volume, 10 ml). Incubation time, 22 hr. Figures shown are corrected for the initial turbidity (about 0.01) due to the inoculum.

^b Figures in parentheses show turbidity in control tubes (absence of proflavine). Presumably, traces of Mg^{++} in the other salts of the minimal medium permitted the small amount of growth observed.

between *E. coli* B and B/Pr was still apparent in glucose minimal medium: the growth of the latter was not affected by 3×10^{-4} M proflavine, a concentration that completely inhibited the growth of *E. coli* B.

Cells of *E. coli* B, growing in glucose minimal medium containing 5×10^{-5} M or 10^{-4} M proflavine were, on the average, one and one-half times as long as cells growing without proflavine. Part, but not all, of the increase in turbidity in the presence of 5×10^{-5} M proflavine may have been due to cell elongation.

pH changes caused by sensitive and resistant cells. Hydrogen ions lower the binding of acridine dyes to cells and cell constituents (16, 19), and production of hydrogen ions was considered as a possible mechanism of resistance. However, resistant cells produced no more acid on growth than did sensitive cells. With both cells, in TSB, the pH fell from 7.4 to 6.0, when a cell density of 0.8 mg/ml was reached, and afterwards rose toward 7.0. In glucose minimal medium, the pH dropped to about 4.0 in 24 hr. In most experiments in which sugars or other metabolites were added to cells in buffer, the pH did not change or fell only 0.1 to 0.2 unit during incubation.

Proflavine binding sites in sensitive and resistant cells. We compared the binding sites of cells that had taken up proflavine in buffer by measuring the amount of the dye associated with

FIG. 6. Effect of proflavine on *E. coli* B growing in TSB or glucose-minimal medium. Cells from a 17-hr culture in TSB were washed twice in sterile distilled water and the cell density was adjusted to 1.6 mg/ml. A 1-ml amount of this suspension was added to 100 ml of TSB or glucose minimal medium in 500-ml Erlenmeyer flasks and shaken at 37 C until growth began. At the times indicated by arrows, 9.0-ml portions of each culture were added to 18-mm tubes containing 1.0 ml of water or sterile proflavine to the final concentrations shown by each curve, and shaking was continued. Figures in parentheses show bound proflavine, expressed as 10^{-2} μ mole per mg of cells, determined at the end of the growth period. Final pH in each culture (determined by indicator paper) was approximately 6.5 for cells grown in TSB with 0 and 10^{-5} M proflavine and 7.0 for higher concentrations, 6.0 for cells grown in glucose minimal media with 0 or 10^{-5} M proflavine, and 6.5 for higher concentrations.

different cell fractions. In suspensions of sonically broken cells, the location of bound proflavine depended on the amount taken up. In 1.5×10^{-5} M proflavine, almost all the bound dye could be recovered from the soluble portion of the broken-cell suspension (Table 7); it was not centrifuged down after 1 hr at $160,000 \times g$. As the external proflavine concentration was increased, more proflavine was bound to the cell. A greater proportion of this appeared in the material sedimenting at $160,000 \times g$ and at lower forces. There was no real difference in proflavine localization in sensitive and resistant cells.

Envelopes isolated from sensitive and resistant cells and then exposed to proflavine also bound it to the same extent (Fig. 1). On a comparative basis (micromoles of proflavine per milligram of protein or per milligram dry weight), envelopes bound only about one-sixteenth as much as intact cells at each concentration studied.

The binding of proflavine to isolated DNA was measured by the decrease in intensity and the shift in the proflavine absorption maximum from 445 nm toward 460 nm (20). No difference was found between the dye-binding capacity of DNA from sensitive and resistant cells by this method (Fig. 7).

TABLE 7. Location of bound proflavine in fractions from sonically broken cells^a

Proflavine concn in medium (M)	Proflavine bound		
	Total (10^{-2} μ mole)	Per cent of total	
		In super- natant fluid after 10 min at 6,000 $\times$ g	In super- natant fluid after 1 hr at 160,000 $\times$ g
Sensitive cells			
1.5×10^{-5}	11.6	96	95
10×10^{-5}	83	90	52
50×10^{-5}	392	68	13
Resistant cells			
1.5×10^{-5}	12.1	97	95
10×10^{-5}	76	92	57
50×10^{-5}	341	71	12

^a Cells (7.5 mg) were incubated for 2 hr at 37 C with proflavine concentrations shown in a 10-ml volume, then centrifuged down, resuspended in 10 ml of buffer, broken sonically, and fractionated. Total proflavine was determined on the sonic extract before fractionating.

DISCUSSION

These experiments explain the apparent difference between our first results and those of Nakamura (12), who worked with several acridine-resistant *E. coli* strains and studied uptake

FIG. 7. Shift in proflavine spectrum caused by adding DNA from sensitive and resistant cells. DNA ($10 \mu\text{g}$ of *P* per ml) was dissolved in "dilute saline citrate" (9). The volumes shown were added to 3.0 ml of 3×10^{-6} M proflavine.

in the presence of growth medium (nutrient broth and minimal medium containing glucose). Our proflavine-sensitive and -resistant *E. coli* strains also take up quite different amounts of proflavine in the presence of such growth media, though in buffer they usually take up the same amounts.

Indeed, our results suggest that cells in buffer are freely permeable to proflavine and are able to concentrate the dye. In the binding experiment shown in Fig. 1, for example, at most concentrations, about 90% of the added proflavine becomes cell-bound. Growth media release bound proflavine, and the presence of different metabolites can greatly influence the apparent "permeability" of cells to the dye.

A survey of a number of compounds showed that some influenced the proflavine binding of both sensitive and resistant cells equally, but that many had a much greater effect on resistant cells. Monovalent and divalent salts and salts of certain organic acids, such as sodium acetate and sodium formate, belong to the first group. The cations of these salts probably displace the positively-charged proflavine molecule from the site to which it is bound, as has been observed for its binding to isolated nucleic acids (16). Glycerol, pyruvate, a number of amino acids, and several metabolizable sugars affect the proflavine binding of resistant cells much more than that of sensitive cells. The effect of glucose was studied in detail.

Glucose causes resistant cells to release bound proflavine. It can also affect the binding of proflavine by sensitive cells when the external dye concentration is low, but its effect is greater on resistant cells in higher external proflavine concentrations. The response to glucose and other sugars apparently depends on their metabolism. Though proflavine binding takes place more

readily at 0°C than at 37°C, release by glucose is much more effective at the higher temperature. Nakamura (12) showed that cells in broth took up more acriflavine at 0°C than at 37°C, and those that had taken up acriflavine at 0°C released it on being warmed to 37°C. Presumably, he would also have found that this release depended on the metabolites supplied by nutrient broth. Certain metabolic inhibitors block the glucose-induced release of proflavine but not its uptake by cells in buffer. Finally, galactose and lactose affect proflavine binding most strongly in cells grown under conditions which would be expected to induce enzymes capable of attacking these sugars.

In sensitive cells grown in minimal medium containing glucose or lactose, the proflavine binding was more sensitive to glucose and other sugars; this is reflected in a reduced sensitivity to proflavine. These changes may have been caused by the low pH values of cultures in minimal media. Nakamura (13) recently found that adding glucose to a complex medium, and hence lowering the final pH, decreased acriflavine binding and the susceptibility of *E. coli* grown in this medium to acriflavine.

The mechanism of metabolite-induced proflavine release is still unknown. It is not due to acid production. It was thought possible that energy was used to transport monovalent cations inside the cell and displace bound proflavine, but no evidence has been found for this. Glucose released proflavine from resistant cells suspended in a Tris-phosphate buffer lacking both K^+ and Na^+ .

The possibility was also considered that glucose caused a greater permeability alteration in resistant than in sensitive cells. Abrams (1) showed that glucose increased the permeability of spheroplasts and intact cells of *E. coli* to

sucrose, leading to a swelling of suspensions in sucrose solution and a fall in turbidity. We also found that glucose lowered the turbidity of *E. coli* suspensions in sucrose, but it had no greater effect on resistant than on sensitive cells, and no other evidence (through loss of inorganic phosphate or ultraviolet-absorbing substances) was found for a greater permeability increase in resistant than sensitive cells when glucose was added.

We believe that the greater release induced by metabolites in resistant cells is the basis of proflavine resistance. Though this is not a completely satisfactory explanation, it still seems the only possible one. Resistant cells do not destroy proflavine. The absorbed dye can be quantitatively eluted by hot sodium lauryl sulfate, and proflavine released from resistant cells by glucose is chemically unchanged.

There seems to be no difference between the proflavine-binding sites in sensitive and resistant cells. Isolated envelopes and DNA from each kind of cell had the same proflavine binding capacity. No difference was found in binding sites when sensitive and resistant cells that had taken up proflavine in buffer were broken sonically and fractionated. Admittedly, the last experiments are difficult to interpret. At lower external concentrations, the proflavine taken up was found entirely in that portion of the cell homogenate remaining soluble after 1 hr at $160,000 \times g$, a force that we would expect to sediment most or all the DNA and RNA. Does this mean that proflavine is bound, if at all, to soluble proteins or low molecular weight substances in the intact cell? Probably not. When cells in suspension are broken, their internal contents are diluted many times. The fact that proflavine does not sediment with nucleic acids, ribosomes, and cell membranes until there is a high concentration of the dye in the broken cell preparation is probably due to its binding with these cell structures being an equilibrium process, reversible on dilution.

The arguments linking proflavine resistance with ability to expel the bound dye are as follows. (i) Both sensitive and resistant cells are equally permeable to proflavine in buffer but are affected differently by growth media and individual metabolites. (ii) In growth media, at any given proflavine concentration, resistant cells bind much less proflavine than do sensitive cells, whether or not growth is inhibited. (iii) Treatments that cause sensitive cells to lose proflavine (high Mg^{++} concentrations or previous growth in glucose minimal medium) lower their proflavine susceptibility.

Despite the generally greater ability of resistant cells to expel bound proflavine, these cells can still grow while they contain enough bound proflavine to inhibit growth of sensitive cells (see Fig 3 and Fig. 2 legend). We do not know where this proflavine is located. A calculation, based on the assumption that the dry weight is one-tenth the wet weight of a cell pellet and that the pellet contains about 30% intercellular fluid, shows that only 2 to 5% of the bound proflavine can be assigned to intercellular spaces. Our experiments with isolated envelopes indicate that their binding capacity is less than 10% that of intact cells, but it is possible that these layers can bind more proflavine while the cell is intact, and that such proflavine is still excluded from the cell's interior. This seems open to experimental study.

ACKNOWLEDGMENTS

We are indebted to Yves Patenaude and Carol Rossman for technical assistance. This work was supported by the Medical Research Council of Canada Grant, no. MA-1943.

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