The Elusive Permeability Barriers and Binding Sites for Proflavine in Escherichia coli

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Cells of proflavine-sensitive and -resistant Escherichia coli strains were altered in different ways, and the proflavine binding of the changed material was studied. Spheroplasts prepared from sensitive and resistant cells bound similar amounts of proflavine at saturation, whether or not they were osmotically protected by 10% sucrose. Intact cells bound approximately the same amounts of proflavine as spheroplasts. On addition of glucose, osmotically protected resistant but not sensitive spheroplasts released proflavine; unprotected spheroplasts did not release bound proflavine. Thus, osmotically protected membranes are not required for proflavine binding (a passive process) but are required for proflavine release (an active process). The presence of sucrose reduced proflavine binding by resistant cells. Adding glucose to cells in 20% sucrose did not cause a release of residual proflavine, though glucose caused a release of proflavine from cells suspended in 0 or 10% sucrose. On treatment of heated cells or ruptured spheroplasts with nucleases and Pronase, practically all nucleic acids were removed. Proflavine-binding ability of such preparations fell by only 30 to 50%. Washing heated cells with ethanol did not reduce their proflavine-binding ability. There appear to be important binding sites in cells aside from nucleic acids.

It has been widely assumed that the acridine dyes such as proflavine and acriflavine act primarily on nucleic acids (reviewed in 2, 11, 13, 20). There is certainly no doubt that such dyes can bind to nucleic acids, but they can also bind to other polyatomic cellular compounds (16-18). At present, the relative importance of these different sites in the binding of acridine dyes is unknown. This question was studied by means of experiments, reported here, in which the proflavine binding of intact cells of Escherichia coli and of heat-treated cells and spheroplasts freed from nucleic acids was compared.

A second purpose of this work was to study the relationship between permeability and proflavine binding. This seems especially appropriate since uptake of acridines and other basic dyes is used as one index of envelope permeability (15-18). Previous work from this laboratory has shown that resting cells of proflavine-sensitive and -resistant E. coli strains bind proflavine rapidly by a passive process and that resistant cells can release bound proflavine by an energy-dependent process (10); these results have cast a different light on what "permeability" may mean. The present experiments with osmotically protected and lysed spheroplasts have permitted us to look further at the relation between permeability and proflavine binding and release.

MATERIALS AND METHODS

Cultures and culture conditions. The proflavine-sensitive and -resistant strains E. coli B and E. coli B/Pr were previously described (10). Cultures were grown with shaking in conical flasks containing one-fifth volume of growth medium at 37 °C in Trypticase Soy Broth (BBL). Growth was measured as optical density (OD) of cultures at 660 nm in a Coleman Junior spectrophotometer, with the use of 18-mm diameter tubes and a medium blank.

Preparation and testing of spheroplasts. Cells were transformed to spheroplasts with ethylenediaminetetraacetic acid (EDTA) and lysozyme, essentially as described by Murray et al. (14). For binding experiments, whole cells were suspended at about 2 mg (dry weight)/ml in solution A [0.01 M tris(hydroxymethyl)aminomethane buffer, pH 7.4, plus 0.001 M MgCl₂]. Spheroplasts, which were protected by 20% (w/v) sucrose from osmotic lysis during preparation, were centrifuged (12,000 × g for 10 min) and resuspended in solution B (solution A containing 20% sucrose) to the same volume as the cell suspension from which they were derived. Subsequent calculations are based on the weight of cells from which spheroplasts were derived.

Microscopically, spheroplast formation seemed
complete. This was also checked by measurements of osmotic fragility. We reasoned that spheroplasts exposed to solution A (of low osmotic pressure) should release as much inorganic phosphate as could be released by a complete disruption of the cell with acid (see 9 for a discussion of this method of testing completeness of lysis). Equal volumes of spheroplasts in solution B were centrifuged. One pellet was resuspended in 10 ml of water plus 1.0 ml of 50% trichloroacetic acid and centrifuged to yield supernatant 1. The second pellet was resuspended in 10 ml of solution A and centrifuged. To 9 ml of its supernatant fluid was added 0.9 ml of 50% trichloroacetic acid, and the precipitated material was centrifuged, to yield supernatant 2. The inorganic phosphate (Pi) contents of supernatants 1 and 2 were compared, by use of Bartlett’s (5) modification of Allen’s (3) method.

In the experiments reported, all (100% ± 5%) Pi was released when spheroplasts were suspended in solution A. Intact cells suspended in solution A lost 5% or less of their Pi. To assess the osmotic protection given by different sucrose concentrations, leakage of cell constituents was measured as loss of ultraviolet (UV)-absorbing substances (OD at 260 nm). This index of leakage is more sensitive and better suited to small quantities of spheroplasts, as well as more convenient, than measuring Pi loss. The loss of UV-absorbing substances (approximately an OD of 2.0 per mg of spheroplasts) in solution A after 30 min of incubation at room temperature was taken as 100%. In a typical experiment with 10% sucrose added, sensitive spheroplasts lost 16% of their UV-absorbing substances, and resistant spheroplasts lost 41%; with 20% sucrose, the losses from sensitive and resistant spheroplasts were, respectively, 13 and 26%. Later experiments (see Table 1) showed that 20% sucrose prevented the glucose-induced release of proflavine from resistant cells, and 10% sucrose did not. Therefore, 10% sucrose was used to stabilize spheroplasts in proflavine-binding experiments even though this concentration gave resistant spheroplasts only moderate stability.

Treatment for lowering nucleic acid and protein content of spheroplasts and heated cells. Suspensions of E. coli B spheroplasts in solution B were divided into three 25-ml lots, sedimented, and lysed by resuspension in 20 ml of solution A. One lot was treated with DNAase [Deoxyribonuclease I, 2 × crystalline (Beef Pancreas), Nutritional Biochemicals Corp., Cleveland, Ohio] and RNAase [Ribonuclease (Beef Pancreas), Nutritional Biochemicals Corp.], 125 μg of each per ml, for 30 min at 37 °C, centrifuged, and then washed three times with solution A. The second lot of spheroplasts was treated with DNAase and RNAase as above, followed by a further digestion with Pronase (125 μg/ml, Pronase B grade, Calbiochem, Los Angeles, Calif.) for 90 min at 37 °C, and then was washed three or four times with solution A. The third lot of spheroplasts was treated with Pronase alone and washed. Cells heated to 100 °C for 10 min were also treated in a similar manner to reduce nucleic acids and proteins.

Determination of macromolecules. The shortened trichloroacetic acid extraction procedure of Beppu and Arima (6) was followed. Ribonucleic acid (RNA) was determined as ribose by the orcinol reaction (1), and deoxyribonucleic acid (DNA), as deoxyribose by the method of Webb and Levy (21) or by the diphenylamine reaction (7). The material remaining after hot trichloroacetic acid extraction was dissolved in 0.5 N NaOH, and the protein content was determined by the method of Lowry et al. (12), with bovine serum albumin as a standard. The arbitrary nature of this means of determining protein may account for the fact that the sum of cellular protein, DNA, and RNA was slightly greater than the dry weight of the cells analyzed (see Table 2).

Proflavine binding. For measuring proflavine binding under conditions of dye saturation, 0.1 ml of a suspension of cells (about 2 mg (dry weight)/ml) treated, or spheroplasts was pipetted into 10 ml of solution A containing different proflavine concentrations (usually 2, 5, 8, 10, 20, 30, and 40 × 10⁻⁵ m) and sucrose as indicated. After incubation for 1 hr at 37 °C, cells or spheroplasts were centrifuged, the pellet was drained, the tube was wiped dry, and bound proflavine was determined colorimetrically after dissolving the pellet in 2% sodium lauryl sulfate (10). As before (10), the degree of concentration of proflavine in the sedimented material was so high that corrections for the very small amounts in any adhering supernatant fluid were not necessary. Free proflavine was calculated by subtracting the amount bound from that originally present. Binding experiments were carried out in triplicate, and the individual measurements did not differ by more than 2 to 3% from the mean values, which are given in the results.

For experiments showing the effect of glucose on release of bound proflavine, cells or spheroplasts were first exposed to proflavine (2 × 10⁻⁴ m), with or without sucrose, for 30 min at 37 °C. Then glucose (0.5 %, w/v, final concentration) or water (for a control) was added. Samples were removed at intervals and centrifuged, and bound proflavine was measured in the pellet.

RESULTS

Relation between osmotic stability and proflavine binding. Proflavine binding was measured in the presence and absence of 10% sucrose at a range of external dye concentrations, up to those which saturated the cells or spheroplasts (Fig. 1). In the absence of sucrose, saturated sensitive cells bound only 5% more proflavine than osmotically lysed spheroplasts. In the presence of 10% sucrose, saturated cells bound about 17% less proflavine than in the absence of sucrose. Osmotically protected spheroplasts could bind about 16% more proflavine than the cells they were derived from, but in subsaturating proflavine concentrations cells could bind more than spheroplasts (Fig. 1a).

A somewhat different result was observed in resistant cells and spheroplasts. Without sucrose,
Proflavine release from cells and spheroplasts.

It was previously found that the addition of glucose and other metabolites caused resistant, but not sensitive, cells to release bound proflavine (10). The ability of cells and spheroplasts suspended in different sucrose concentrations to release bound proflavine was next examined (Table 1). Sucrose reduced proflavine binding by intact cells. Glucose caused a profound decline in the amount of proflavine bound to resistant cells suspended in 0 or 10% sucrose, but caused little or no release of proflavine from cells suspended in 20% sucrose.

Addition of glucose to spheroplasts of resistant cells suspended in 10% sucrose led to a small but definite decrease in the amount of bound proflavine. When glucose was added to lysed resistant spheroplasts that had bound proflavine in the absence of sucrose, no more dye was released than when water was added. Similar experiments showed that, as before (10), glucose caused no release of proflavine from sensitive cells. Glucose also caused no proflavine release from sensitive spheroplasts, whether or not sucrose was present.

These experiments suggest that, though the possession of an intact (that is, an osmotically supported) membrane is not essential for proflavine binding, it is essential for metabolically induced proflavine-release.

Relation between proflavine binding and content of macromolecules in heated cells and spheroplasts.

A comparison of DNA, RNA, and protein contents and proflavine-binding abilities of intact cells and of cells and spheroplasts subjected to various treatments is shown in Table 2.

The most clear-cut results were obtained with cells heated at 100 C for 10 min. When these were suspended in buffer, they lost 25% of their RNA, only 6% of their protein, and none of their DNA. The proflavine-binding capacity of these cells increased by 49%. Such increase of proflavine binding on heating has been previously demonstrated for a number of acridines in E. coli (17) and for sensitive (but not resistant) Bacillus subtilis cells (4). Treatment of heated cells with nucleases or with nucleases plus Pronase reduced DNA by about 90% and RNA by about 99%, and lowered proflavine binding to approximately half that of the intact cells (or to one-third that of the heated cells not treated with enzymes). Heated cells treated with Pronase alone lost more protein (88%) than nucleic acids; their proflavine-binding capacity was reduced by about one-third. Finally, washing heated cells prewashed with 95% ethanol, a procedure that would be expected to remove membrane lipids, reduced RNA by 72% and had

**FIG. 1.** Saturation of cells and spheroplasts with proflavine in the presence and absence of 10% sucrose. Cells and spheroplasts (18.5 μg [dry weight]/ml for sensitive and 20.0 μg [dry weight]/ml for resistant) were incubated in solution A, containing different concentrations of proflavine, as shown. After 1 hr of incubation, 10-ml samples were centrifuged, and proflavine was determined in the supernatant fluid and in the pellet. (a) Sensitive (E. coli B); (b) resistant (E. coli B/Pr). Curves A and B represent cells and spheroplasts, respectively, in the absence of sucrose. Curves C and D represent cells and spheroplasts, respectively, in the presence of 10% sucrose.

Saturated cells and spheroplasts bound the same amounts of proflavine. Under nonsaturating conditions, spheroplasts usually bound more than cells. In the presence of sucrose, cells bound about 25% more proflavine at saturation than spheroplasts. At lower proflavine concentrations, spheroplasts could bind more proflavine than cells (Fig. 1b).

It was previously shown that, in buffer, both sensitive and resistant E. coli cells bound the same amounts of proflavine (10). These earlier experiments were carried out under conditions in which cells were not saturated with the dye. Under conditions of saturation, sensitive cells bound no more proflavine than resistant cells (curves A, Fig. 1a and 1b). Indeed, in these experiments they bound somewhat, though probably not significantly, less.
PROFLAVINE BINDING IN E. COLI

Table 1. Effect of glucose on proflavine binding by resistant cells and spheroplasts

<table>
<thead>
<tr>
<th>Material</th>
<th>Sucrose concn (%)</th>
<th>Addition</th>
<th>Proflavine bound (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Before addition</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Before</td>
</tr>
<tr>
<td>Cells</td>
<td>0</td>
<td>Water</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Glucose</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Water</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>Glucose</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>Water</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>Glucose</td>
<td>14</td>
</tr>
<tr>
<td>Spheroplasts</td>
<td>0</td>
<td>Water</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Glucose</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Water</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Glucose</td>
<td>24</td>
</tr>
</tbody>
</table>

* Cells or spheroplasts (0.2 mg/ml) were allowed to bind proflavine (2 × 10⁻⁵ M) during 30 min at 37°C in solution A with or without sucrose. Samples were removed, and proflavine binding was measured. Each remaining suspension was divided into two parts. One-tenth volume of water was added to one part, and one-tenth volume of glucose was added to the other (final glucose concentration, 0.3%). Incubation was continued at 37°C. At the intervals shown, samples were removed for measurement of proflavine binding.

Table 2. Macromolecular contents and proflavine binding in treated cells and spheroplasts of E. coli B

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Heated cells (100°C for 10 min)</th>
<th>Spheroplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNA</td>
<td>RNA</td>
</tr>
<tr>
<td>Untreated cells (control)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Heated cells or spheroplasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>In solution A</td>
<td>111</td>
<td>75</td>
</tr>
<tr>
<td>In solution A + 10% sucrose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated with nuclease alone</td>
<td>13</td>
<td>1.4</td>
</tr>
<tr>
<td>Treated with nuclease and Pronase alone</td>
<td>13</td>
<td>1.4</td>
</tr>
<tr>
<td>Washed 10 times with solution A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Washed 3 times with 95% ethanol</td>
<td>94</td>
<td>28</td>
</tr>
</tbody>
</table>

* DNA, RNA, and protein contents for intact cells were (in µg/mg, dry weight): 35.7, 498, and 455, respectively, for the experiment with heated cells, and 37.4, 520, and 474, respectively, for the experiment with spheroplasts. Binding experiments were carried out with 10 × 10⁻⁵ µmole of proflavine per ml (10⁻⁴ M) and 0.02 mg of cells or material derived from this weight of cells per ml.

much less effect on DNA and protein, but did not reduce proflavine binding at all.

These experiments show that nucleic acids, though important, are by no means the only binding sites for proflavine.

Similar conclusions as to the importance of nucleic acids were reached by using spheroplasts. Transforming cells to osmotically protected spheroplasts (in 10% sucrose) did not significantly lower their DNA, RNA, or protein, but...
did lower their proflavine-binding power. In the absence of sucrose, spheroplasts lost 30 to 40% of their macromolecules, but proflavine binding was at least as great as that of intact cells. Treatment with nuclease reduced the nucleic acid content of spheroplasts by 90% or more and their protein content by about 70%. Treatment with Pronase alone, however, caused a substantial reduction in nucleic acid as well as protein content, and these macromolecules could also be reduced by washing spheroplasts several times in solution A without any enzyme treatment. This suggests that lysis of spheroplasts caused much of the loss of macromolecules. Such lysis complicates interpretation of these experiments, but the results do show that the fall in proflavine binding did not parallel the fall of either nucleic acid or protein. Spheroplasts with only 10% nucleic acid remaining bound almost half as much proflavine as intact cells.

DISCUSSION

The main points made by the present work are (i) that proflavine binding by E. coli is changed little by transforming cells to spheroplasts or by increasing spheroplast permeability and (ii) that cells or spheroplasts can lose almost all of their nucleic acids without losing much of their proflavine-binding ability.

Earlier work showed that proflavine binding in both E. coli and B. subtilis was a passive process (4, 10). The experiments with spheroplasts have now shown that an osmotically protected membrane is not needed for proflavine binding by E. coli, nor does it present a barrier to such binding. However, an osmotically protected membrane does seem necessary for proflavine extrusion by spheroplasts of resistant cells. Spheroplasts that had bound proflavine in 10% sucrose released some of the dye when glucose was added. In the absence of sucrose, they could bind proflavine but not release it. This supports our conclusion that such release is a metabolically active process. Possibly, osmotic lysis of spheroplasts causes loss or inactivation of enzymes responsible for the release of bound proflavine.

Resistant spheroplasts were not completely protected by 10% sucrose, but higher concentrations could not be used in studying proflavine release because these reduced proflavine binding in intact cells and inhibited proflavine release on glucose addition. Possibly, such effects were caused by withdrawal of intracellullar water and concentration of intracellular salts.

Degradative studies showed that almost all of the nucleic acid could be removed from heated cells or spheroplasts without a corresponding loss in proflavine-binding ability. Thus, nucleic acids are not the only cellular binding sites for proflavine. Are they the most important ones? Proflavine and other acridine dyes can be shown to bind to isolated nucleic acid molecules and to ribosomes. Biochemical and genetic evidence indicates that the nucleic acids are important sites of action of such dyes (11). However, there is increasing evidence that acridines may inhibit growth by acting on sites other than nucleic acids. Witt et al. (22) found that proflavine inhibits glycolysis in yeast. Proflavine acts as a competitive inhibitor of the action of chymotrypsin against acetyl-1-valine methyl ester (8). Quite recently, Wolfe et al. (23) showed that Nitroakridine 3582 and quinacrine exerted bactericidal action that was independent of their effects on DNA synthesis. Our own current work (unpublished data) has shown that proflavine inhibits glucose utilization by sensitive but not resistant E. coli cells.

Sliver and his associates (16–18) pointed out that proflavine can bind to several cellular polyanions in addition to nucleic acids, including proteins, mucopolysaccharides, and phospholipids. It also seems possible that removal of one binding site can open up others. Miall and Walker (13) observed that treatment with EDTA greatly increased the proflavine-binding power of E. coli ribosomes. They thought this was caused by unfolding of the RNA, but it could also have been due to exposure of fresh protein binding sites to the dye. With binding sites of so many chemical natures available, it seems very unlikely that any selective degradation process could remove all such sites and leave any cell structure.

To add to these complications, proflavine binding must not be considered only as an attraction of a positively charged dye for a negatively charged cell site. Even with isolated DNA, proflavine may be bound both ionically and by a type of stacking in which one bound dye molecule attracts another (19). Possibly, the degree of stacking could be affected by the free space available around the binding site. Such considerations explain why it does not seem possible to make a correlation between content of any one class of polyanions and quantitative ability of intact cells to bind proflavine. Speculations on the binding sites of proflavine in intact cells should still be made cautiously.

When considering the entrance of acridines, and possibly of other basic dyes, the term "permeability" must also be used with caution. Increased uptake of acridines by E. coli after bacteriophage infection or treatment with phenethyl alcohol (16–18), or of gentian violet after mutation in the envA locus of E. coli (15), have
been considered evidence of increased permeability to these dyes. However, in this work considerable amounts of dyes were taken up by cells in growth media, even without addition of agents that are thought to increase permeability. In our own work, *E. coli* cells in buffer bound more proflavine than cells in growth media. Isolated walls bound relatively little proflavine. Since alcohol extraction does not lower proflavine-binding ability of heated cells, membrane lipids may not constitute quantitatively important binding sites. Thus, proflavine binding does not appear to be confined to the outer surface of the cell. It might be very difficult to distinguish between increased membrane permeability and opening up of new cellular binding sites. For the present, we suggest as a working hypothesis that the cytoplasmic membranes of *E. coli*, and possibly other organisms, do not pose an effective barrier to the entry of acridine dyes, and that the amounts actually taken up by such cells may depend on the availability of binding sites.

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LITERATURE CITED