

Acridine Binding by *Escherichia coli*: pH Dependency and Strain Differences

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Received for publication 26 July 1967

Acridine dye binding by cells of *Escherichia coli* has been characterized in terms of a number of parameters. There is a temperature-dependent, readily reversible binding of acriflavine which occurs to a greater extent with acridine-sensitive mutants of *E. coli* K-12 than with wild-type *E. coli* B or K-12. There is an essentially irreversible internal binding of acriflavine which occurs when the cellular permeability barriers are destroyed or altered by heat-treatment, elevated pH, treatment with toluene or phenethyl alcohol, or infection with bacteriophage T2 or T4. Both the reversible and the irreversible binding of acridines occurs more effectively with the acridine dye acriflavine than with the related dye proflavine, and still less effectively with 9-aminoacridine and quinacrine. These properties of acridine binding can be correlated with various inhibitory effects of the dyes on the cells.

Acridine dyes have been used in a wide range of studies with microorganisms. Acridines are growth-inhibitory or lethal for bacteria (8, 17, 27) and growth inhibitory and mutagenic for bacteriophage-infected cells (6, 21). Acridines also "cure" cells of a variety of cytoplasmically inherited genetic factors (7, 14, 28).

While studying permeability changes caused by bacteriophage infection (23, 24) and phenethyl alcohol (25), we measured acridine uptake by bacterial cells. The genetic and physiological parameters which influence the uptake of acridines by normal and treated cells were varied in the course of these experiments. (i) The concentration of dye required for a biological effect depends upon the acridine used: acriflavine is more potent than proflavine, which, in turn, is more potent than 9-aminoacridine or quinacrine. These differences in effective concentration are not owing to differences in permeability but appear to be related to the binding efficiencies of the dyes to the cellular binding substrates. (ii) There are genetically controlled differences in sensitivity to acridines, which are correlated with differences in dye uptake. (iii) The curing of episomes by acridines shows a sharp pH dependency (7) which is unrelated to the pK 's of the acridines and of their presumed receptor molecules. We present here evidence that the cell envelope changes its permeability to acridines over the critical range of pH. (iv) We can distinguish between reversible (external?) binding of acridines and essentially irreversible (internal?) binding.

MATERIALS AND METHODS

The acridine dyes and the general experimental procedures have been described previously (23-25).

Strains. A variety of derivatives of *Escherichia coli* B, the usual host for bacteriophages T2 and T4, was used, including strains BBr, B/1, 5, R2/1, 5, B/4, BT⁻, and HB33/1. No essential differences in results were detected with different substrains. A variety of derivatives of *E. coli* K-12, the usual strain for studies of conjugation and curing, was used, including 58-161, K-10, Hfr Cav, W3747, JC411 rec⁺, and JC1153 rec⁻, without any essential differences in results, except for the acriflavine-sensitive mutant 201 which was furnished by R. C. Clowes (4), 18/1042 which was furnished by H. Nakamura (17), and JE2 which was furnished by Y. Sugino (27). Colicin-resistant, acridine-sensitive mutants of strain K-12, which were isolated in this laboratory by Eleanor Whitney and which are similar to strain 201, were also used.

Media. Minimal medium M9 contains (in grams): Na₂HPO₄, 6; KH₂PO₄, 3; NaCl, 0.5; NH₄Cl, 1; MgSO₄·7H₂O, 0.25; CaCl₂, 0.01; and glucose, 4 g/liter of water. Tryptone broth contains 8 g of tryptone (Difco) and 5 g of NaCl/liter of water, and has a pH of 7.0 at 25 C.

Acridine uptake. Acridine uptake was measured as previously described (23-25). Proflavine, quinacrine, and 9-aminoacridine (5-aminoacridine) were commercially available products. Purified acriflavine (10-methyl-3,6-diaminoacridine), containing only traces of proflavine, was the gift of the Pharmaceuticals Division of Imperial Chemical Industries, Ltd., Macclesfield, England. Acridines were added to bacterial cultures usually at 1 μg/ml and from time to time samples were removed and centrifuged at ca. 5,000 ×

g in a table-top centrifuge at either 25 or 4 C (which-ever temperature was closer to the incubation temperature). The residual fluorescence of the supernatant fluids was compared with that of the originally added dye in an Aminco fluoromicrophotometer (American Instrument Co., Silver Spring, Md.) equipped with a primary filter (Kodak-Wratten 47B) and a secondary filter (Kodak-Wratten 2A-12). The amount of dye taken up by the cells was usually considered to be one minus the fractional fluorescence of the supernatant fluids after centrifugation. Quantitative measurement of the amount of dye actually in the cells is complicated by fluorescence quenching and enhancement with bound dye (24). We have never observed metabolic breakdown of acridine dyes (24).

RESULTS

Differences between acridine dyes. The varying potencies of different acridines in episome curing and other inhibitory effects did not appear to be due to permeability effects but to differences in binding efficiency (intrinsic binding constants) between the acridines and the cellular binding substrates. This was seen in the experiment shown in Fig. 1, where *E. coli* B cells were made permeable with phenethyl alcohol (25). Such cells took up far less proflavine than acriflavine and still less 9-aminoacridine or quinacrine. The order of relative dye uptake is the same as the order of relative potency in inhibitory phenomena. Results similar to those shown in Fig. 1 have been obtained with toluene-treated and phage-infected cells of *E. coli* B (24) and with heat-killed cells of *E. coli* K-12 (*unpublished data*). Thus, the discrimination among the acridines depends on the cellular binding substrates and not on the agent used to destroy the permeability barriers. The same discrimination among acridines shown by cells treated with membrane-disrupting agents was found when the temperature-sensitive binding of acridine was studied with untreated cells (*see below*). Data on acridine orange binding are not included in Fig. 1, because the acridine orange data have been less reproducible than those with other acridines.

Strain differences in acriflavine binding. Not all *E. coli* strains showed equal sensitivities to acridines. Most *E. coli* B substrains grew only in acriflavine concentrations of 10 $\mu\text{g}/\text{ml}$ or less, whereas most *E. coli* K-12 derivatives grew in 50 to 100 μg of acriflavine per ml (Silver, *unpublished data*). We have found that the difference in sensitivity between *E. coli* B and K-12 is associated with a difference in cellular uptake of acriflavine. This strain difference persists when the uptake is increased by the addition of a variety of agents including: (i) phage, (ii) low concentrations of lipophilic reagents such as toluene and phenethyl alcohol, and (iii) alkaline pH (Table 1). Heat-

TABLE 1. *Relative uptake of acriflavine by cells of Escherichia coli B and K-12 strains: effects of various treatments*

Treatment	Added acriflavine absorbed ^a	
	Strain B	Strain K-12
	%	%
Controls (untreated cells)	26 \pm 7 (30)	13 \pm 6 (14)
Infection with bacteriophage T4	72 \pm 7 (5)	49 \pm 7 (3)
Toluene (0.25-0.33%)	74 \pm 6 (6)	55 \pm 5 (3)
Phenethyl alcohol (0.25-0.33%)	75 \pm 7 (18)	34 \pm 12 (8)
Alkaline pH (pH > 8.5)	87 \pm 11 (6)	34 \pm 12 (3)
Heating at 100 C	84 \pm 6 (5)	78 \pm 3 (4)

^a The numbers represent the average \pm standard deviation of the fraction of the acriflavine taken up by the cells at equilibrium (one minus fractional fluorescence of the supernatant fluids, as described in Materials and Methods) in different experiments on different days (number of experiments in parentheses). The bacterial concentrations ranged from 5×10^8 to $1 \times 10^9/\text{ml}$ in different experiments; several substrains of *E. coli* B and *E. coli* K-12 were used without significantly altering the differences in results between the two strains.

killed cells of *E. coli* B and K-12 absorb equal amounts of acriflavine (Table 1), and concentrations of toluene greater than those in Table 1 are required to make K-12 cells absorb maximal amounts of acriflavine (*unpublished data*). The scatter of data in Table 1 is chiefly owing to day-to-day differences in the experimental parameters, especially the cell concentration. Although several substrains of *E. coli* B were used, no reproducible differences were found. Similarly, there were no reproducible differences in acriflavine binding among several acridine-resistant derivatives of *E. coli* K-12. However, the acridine-sensitive mutants of *E. coli* K-12 absorb more acridine dye per cell than the acridine-resistant strains (16, 18; *see also* Table 2 and Fig. 5).

The data in Table 2 show a difference in dye uptake between Clowes' acridine-resistant and -sensitive strains. Furthermore, they show additivity between the uptake controlled by the bacterial gene and the acriflavine uptake controlled by the bacteriophage T4 *ac* gene (23, 24). Inhibition of viral growth occurs at a lower concentration of acriflavine with T4-infected acridine-sensitive cells than with T4-infected acridine-resistant cells (8; Silver, *unpublished data*).

pH dependency of cellular permeability to acri-

TABLE 2. Uptake of acriflavine by acriflavine-sensitive and acriflavine-resistant strains of *Escherichia coli* K-12: effect of bacteriophage infection

Bacteria with or without bacteriophage	Added acriflavine absorbed ^a
	%
Uninfected <i>ac^r</i>	22 ± 4
<i>ac^r</i> + T4.....	41 ± 2
Uninfected <i>ac^s</i>	56 ± 2
<i>ac^s</i> + T4.....	79 ± 2

^a Percentage of cell-bound acriflavine at equilibrium as measured by the fractional fluorescence of the supernatant fluids after centrifugation. For each culture, two samples each were centrifuged 41, 56, and 71 min after the addition of 0.75 μ g of purified acriflavine per ml (average \pm standard deviation for the six samples). The acriflavine-resistant strain (*ac^r*, 58-161) and its acriflavine-sensitive derivative (*ac^s*, 201) were described by Clowes (4). The bacterial cultures at 5×10^8 cells/ml were infected with wild-type T4 bacteriophage at a multiplicity of five phage per bacterium.

flavine. Although acridine orange has been the dye of choice in most curing experiments because of its low toxicity (7), we used acriflavine in the uptake studies because it binds more efficiently to cellular constituents (24; Fig. 1). Cells of *E. coli* B showed a striking pH dependency for uptake of acriflavine (Fig. 2). When the bacterial cells were suspended in a medium of acid pH, very little dye was absorbed; when the pH was alkaline, more dye was absorbed. The "pK" of this absorption appeared to be about pH 7.8 in broth. The pH-dependent uptake of dye is more readily seen with cells of *E. coli* B than with *E. coli* K-12 cells (compare Table 1 and Fig. 2).

The pH effect in Fig. 2 could be owing to either a pH-dependent binding of acriflavine to the cellular binding substrate or to a pH-dependent permeability change in the cell membrane. These alternatives were tested in three experiments. (i) Heating for 10 min at 100 C makes cells fully permeable but does not reduce the cellular binding capacity. When heat-killed cells were resuspended in a series of buffers at different pH, there was no pH-dependent difference in dye absorption above pH 5.6 (Table 3). (ii) When heat-killed cells were allowed to absorb acriflavine while suspended at pH 7.0 and then resuspended in different buffers, there was no pH-dependent difference in the amount of dye released during a subsequent 1 hr of incubation, at least none above pH 5.6 (Table 3). It has also been found (Spielman, unpublished data) that there is no pH-dependent change in binding of acriflavine to purified deoxyribonucleic acid (DNA) over the range pH 5.5

to 8.5. (iii) To eliminate the possibility that the acriflavine-binding components of heat-killed cells might differ from those of viable cells, increased acriflavine uptake was induced by phenethyl alcohol in one case and high pH in another, and the pH dependence of the release of absorbed dye was then measured. Again, there was no pH dependence of dye release (unpublished data). We conclude that the pH effect on dye uptake seen in Fig. 2 was the result of increased permeability to the dye at high pH, thus making available more cellular binding sites, rather than a direct pH effect on the efficiency of binding by these sites.

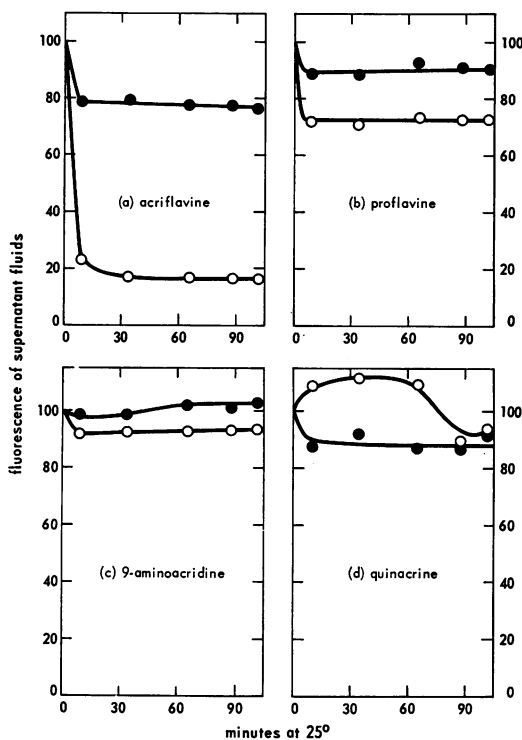


FIG. 1. Effect of phenethyl alcohol on the uptake of different acridine dyes. M9-grown *Escherichia coli* B was centrifuged to remove fluorescent materials excreted during growth and was resuspended in fresh medium. The resuspended cells were divided in half and 0.33% (v/v) phenethyl alcohol was added to one portion. The treated and untreated cultures were divided into four fractions and 1.0 μ g/ml of (a) purified acriflavine, (b) proflavine, (c) 9-aminoacridine, or (d) quinacrine was added. Samples were removed and centrifuged as indicated and the fractional fluorescence (fluorescence of the supernatant fluids divided by the fluorescence of the originally added 1.0 μ g/ml of acridine) was measured. (●) Untreated control cells; (○) phenethyl alcohol-treated cells.

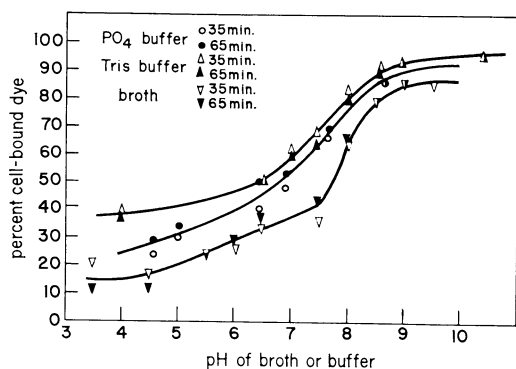


FIG. 2. Effect of pH on cellular uptake of acriflavine. Tryptone broth-grown *Escherichia coli* B was centrifuged and resuspended at 5×10^8 /ml in a series of broths, phosphate buffers, or Tris buffers. The pH of tryptone broth was adjusted by the addition of concentrated HCl or NaOH; 0.067 M phosphate buffers were made by mixing varying proportions of 0.067 M KH_2PO_4 and Na_2HPO_4 ; the pH of 0.12 M Tris buffer was adjusted by adding concentrated HCl. Purified acriflavine was added at 1.0 $\mu\text{g}/\text{ml}$ and samples were removed and centrifuged after 35 and 65 min at 25 C. The fluorescence of the supernatant fluids was measured. The standards with 1 $\mu\text{g}/\text{ml}$ of acriflavine in broth showed about 65% of the fluorescence of the Tris and phosphate standards due to the absorption of light by the broth. The series of standards with 1 μg of acriflavine per ml in buffers with varying pH showed no significant changes (less than 5%) in fluorescence of the free dye over the pH range in question.

If cells of *E. coli* B are first exposed to alkaline pH for 45 min and are then resuspended in buffers of varying pH in the presence of acriflavine, results similar to those in Fig. 2 are obtained (*unpublished data*). Therefore, the pH-dependent change in permeability is reversible, although the binding within permeable cells is not readily reversible.

Permeability versus external binding. The results in the previous sections of this paper were interpreted in terms of alterations in cellular permeability followed by intracellular binding of acriflavine. We also measured external binding of acridines to the cell surface and operationally distinguished between external binding (readily reversible) and internal binding (not readily reversible). External binding was studied by Nakamura (18), who found a readily reversible, temperature-dependent binding of acriflavine which differed between acriflavine-sensitive and acriflavine-resistant strains. *E. coli* cells absorbed more acriflavine at low temperatures (ca. 0 C) than at intermediate temperatures (25 or 37 C) (Fig. 3-5). If this were owing to a temperature-dependent effect on cellular permeability, the dye

absorbed at low temperatures would not have been released when the temperature was raised. But the acriflavine which was absorbed at low temperatures was released when the temperature was raised (Fig. 3; ref. 18). The ready reversibility of this low temperature-induced binding distinguished it from the pH, phenethyl alcohol, or heat-induced dye uptake described above, which was not readily reversible. We were able to repeat Nakamura's (18) results not only with his strain but also with Sugino's (27) acridine-sensitive strain (Fig. 3) and with the colicin-resistant, acridine-sensitive strains (data similar to that in Fig. 3). Similar results, although less dramatic because less dye was absorbed, were obtained with *E. coli* B and acridine-resistant K-12.

Although live cells, and particularly acriflavine-sensitive cells, showed a striking temperature-

TABLE 3. pH dependency of acriflavine uptake and release by heat-killed cells^a

pH of medium	1st expt, cell-bound acriflavine at equilibrium	2nd expt, previously bound acriflavine released
	%	%
4.5	75	33
5.6	83	26
6.5	85	20
7.0	84	22
7.9	83	23
8.7	83	22

^a A tryptone broth culture of *E. coli* B at 7×10^8 cells/ml was centrifuged and concentrated 50-fold in water. After heating for 10 min at 100 C, the cells were diluted to 7×10^8 cells/ml in a series of 0.1 M potassium phosphate buffers. *First experiment.* The cells were diluted into buffers of varying pH; 1 μg of purified acriflavine per ml was added, and two samples of each were centrifuged after 20 and 40 min at 25 C. The fluorescence of supernatant fluids was measured and the percentage of cell-bound dye was calculated. Data are the averages for the four samples at each pH. *Second experiment.* The cells were diluted into pH 7.0 phosphate buffer and 1 μg of purified acriflavine per ml was added. After 20 and 40 min at 25 C, a series of samples were centrifuged (83% of the acriflavine had been absorbed by the cells) and the cellular pellets were resuspended in a series of buffers of varying pH. After 30 additional min at 25 C for release of dye, the cells were centrifuged again and resuspended in the same buffers. After an additional 30 min of incubation at 25 C, the cells were centrifuged for a third time. Percentage released is the sum of the fluorescence of the supernatant fluids after the second and third centrifugations divided by the fluorescence of the 0.83 μg absorbed per ml of the cells.

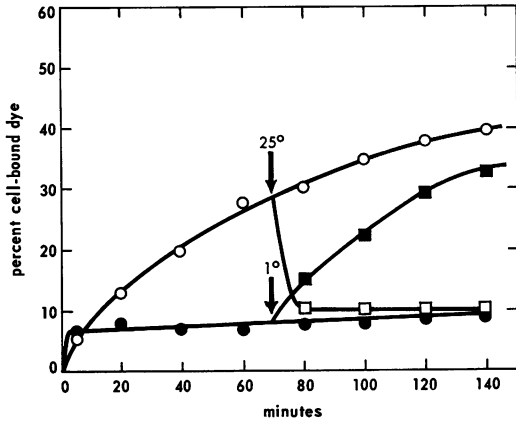


FIG. 3. Temperature-dependent reversible binding of acriflavine. Broth-grown *Escherichia coli* JE2 at 3×10^8 cells/ml was distributed in broth and cooled to 1°C (○) or 25°C (●). Purified acriflavine at 1 μg/ml was added at zero time and samples were removed and centrifuged. After 70 min, a portion of the 1°C culture was transferred to 25°C (□) and a portion of the 25°C culture was transferred to 1°C (■). Additional samples were removed and centrifuged, and the fraction of the dye in the cells was determined by measuring the residual fluorescence of the cell-free supernatant fluids.

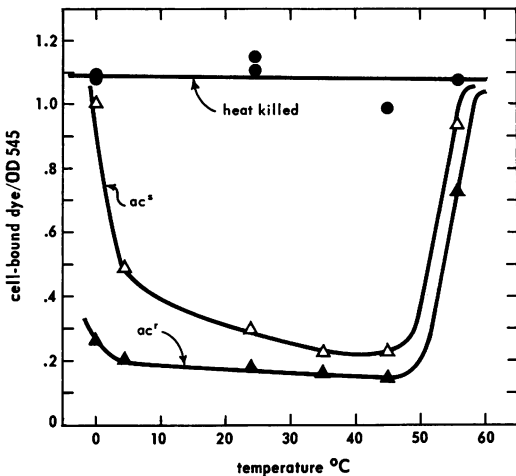


FIG. 4. Temperature dependence of acriflavine binding: the difference between live and heat-killed cells. Broth-grown *Escherichia coli* JE2 was centrifuged and concentrated 50-fold. One portion of the cells was heat-killed (100°C for 15 min) and both the live and the heat-killed cells were diluted to original concentration (2.5×10^8 cells/ml) in a series of broths (pH 7) equilibrated at different temperatures and containing 1.0 μg of purified acriflavine per ml. Samples were removed and centrifuged after 20 and 50 min and the percentage of dye in the cells was determined by measuring the fluorescence of the cell-free supernatant fluids. (●) Heat-killed cells, average for two samples each at 20 and 50 min; (△) live cells, 20-min samples; (▲) live cells, 50-min samples.

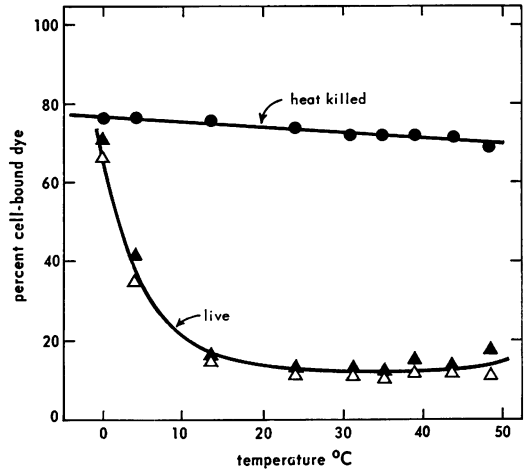


FIG. 5. Temperature dependence of acriflavine uptake: dye-sensitive versus dye-resistant cells. Broth-grown cells of *Escherichia coli* W3747 (*ac^r*) and JE2 (*ac^s*) were centrifuged and concentrated 50-fold. One portion of the *ac^r* cells was heat-killed (100°C for 15 min); the cells were diluted in broth and samples were taken as in Fig. 4. The percentage of cell-bound dye was determined by measuring the fluorescence of the cell-free supernatant fluids, and these values were normalized by dividing by the optical density at 545 mμ as measured in a Zeiss PMQ II spectrophotometer. Neither the broth nor the acriflavine contributes significantly to the OD₅₄₅. (●) Heat-killed *ac^r*; (△) live *ac^r*; (■) live *ac^s*.

dependence of acriflavine uptake, heat-killed cells did not (Fig. 4 and 5). The differences between the temperature-dependent binding by acridine-sensitive and acridine-resistant strains and between live and heat-killed cells are shown in Fig. 5. The living acridine-sensitive cells absorbed more acriflavine than did the acridine-resistant cells, and the difference between the strains was more pronounced at lower temperatures. After heat-killing, we could detect no significant difference in the dye-binding capacities of the sensitive and resistant cells.

DISCUSSION

Our goal in studying acridine binding by *E. coli* is to obtain some understanding of the molecular biology of the cell envelope. But this understanding is still at such an early stage that we cannot be sure whether the primary changes, which ultimately affect dye uptake, occur in the cell membrane or in some overlying layer of the cell wall. Nevertheless, studies of mutants such as those utilized here should help to clarify this problem. In addition, we have found explanations for some of the characteristics of acridine-inhibitory phenomena.

Acridine binds more effectively to the treated cells (Fig. 1) than does proflavine, which, in turn, binds more effectively than 9-aminoacridine or quinacrine. This order of relative binding efficiencies is the same with untreated cells as with cells treated in a variety of ways to increase dye uptake (24); this is the same order as that for relative effectiveness in most acridine-inhibitory effects. However, the acridines bind to DNA with equal efficiencies according to Drummond et al. (5). It seems likely that acridine binding to substances other than DNA is involved, but the evidence is not conclusive and this has been discussed at length by Silver (24).

The differences between strains B and K-12, which are summarized in Table 1, are specific for acriflavine uptake. *E. coli* B and K-12 do not differ in the breakdown of the permeability barriers to potassium ions by phenethyl alcohol or toluene (25); there is also no significant difference between B and K-12 with regard to bacteriophage-induced leakage of potassium (Silver, Levine, and Spielman, *in preparation*). K-12 strains must possess a barrier to acriflavine entrance to the cellular interior in addition to the barrier attacked by phage, phenethyl alcohol, and toluene. This extra barrier is not affected by the more "gentle" treatments, but can be removed by higher concentrations of toluene or by heating. The difference between strains B and K-12 may reside in the cell membrane or in the multiple layers of the cell wall (2, 15). Differences in fatty acid composition between strains B and K-12 have been reported (11); differences in cell wall composition have not been reported but seem likely.

The mutations altering acriflavine sensitivity studied by Nakamura and Sugino map near the gene controlling sensitivity and resistance to bacteriophage T6 (17, 27). Clowes' mutants are unlinked to the T6 gene, but map near the histidine operon (4, 16). The two classes of acridine-sensitive mutants also differ in other physiological characteristics (4, 16-20, 27), and the only common factor between the mutants may be the increased absorption of acridines. Nakamura (19) has reported that the same gene which makes strain 18/1042 sensitive to acriflavine and makes it bind higher amounts of acriflavine also results in an increased sensitivity to phenethyl alcohol, a reagent which acts by altering cellular permeability (25). This phenethyl alcohol sensitivity is also dependent upon pH in the range between pH 6 and 8. We do not know what elements of the cell surface are altered in these mutants, although we hope to determine this. Differences in acriflavine-induced clumping have been used

to distinguish between rough and smooth strains of bacteria (3), and these differences presumably occur in the outer cell wall layers.

We can operationally distinguish between what appears to be external binding of acridines and intracellular binding on the basis of the ready reversibility of the binding to the external receptors and the essentially irreversible binding of dye after the cellular permeability barriers are removed. We can make this distinction because the internal (essentially irreversible) binding sites bind the acriflavine much more effectively than do the external (reversible) sites. For example, Nakamura (18) measured the reversible binding of 0.4 to 0.5 μg of acriflavine per mg (dry weight) of cells when 1 μg of acriflavine per ml was added to cells in broth. Permeable cells bind about 7 μg of acriflavine per mg (dry weight) when exposed to 1 μg of added acriflavine per ml, or more than 10 times the external binding (Table 1). We never saturated the dye-binding capacity of permeable cells, but, when phenethyl alcohol-treated cells were exposed to 80 μg of acriflavine per ml (24), about 450 μg of acriflavine was bound per mg (dry weight) of cells.

One common use of acridine dyes in studies of bacterial genetics involves the growth of cells in acridines to "cure" them of extrachromosomal episomes (7, 14, 28). The mechanism of this curing is assumed to be a preferential inhibition of the replication of episomal DNA relative to the replication of chromosomal DNA (7, 9); indeed, acridines bind strongly to all DNA (5, 12, 13, 22, 26). One difficulty in understanding the mechanism of acridine curing comes from its sharp pH dependency. Growth of F^+ bacteria in 50 μg of acridine orange per ml results in essentially 100% curing at pH 7.6, but no curing at pH 7.2 (7). However, acridine orange has a pK of 10.1 (proflavine pK 9.3; 9-aminoacridine pK 9.6; acriflavine $pK > 12$; ref. 1); whereas, DNA does not contain groups with pK 's between pH 5 and 10 (10). The pH results in this paper do not add to the understanding of the actual mechanism of curing, but do explain one puzzling attribute of the curing reaction. It appears that the cell envelope changes its permeability to acridines over the critical pH range.

The pH dependency of acriflavine uptake is more readily measured in strain B than in strain K-12 (Table 1); but most curing experiments have been performed with K-12 derivatives. However, the material which binds the bulk of the acridines need not be responsible for curing, and is probably not DNA. It is possible that the pH-dependent change in dye uptake, which we see more readily in strain B than in strain K-12, is suffi-

cient in K-12 to allow access for the acridines to the critical substrate (episomal DNA?) but not to allow access to the bulk binding material (the cellular nucleic acids). This hypothesis introduces a notion of compartmentalization which is common with higher cells but is not generally required to explain phenomena in microorganisms.

Although the results in this paper seem to have raised more questions than they have answered, the direction for further work is clear. The combination of genetic and physiological studies with mutants with altered elements in the cell envelope will increase our understanding of the molecular biology of the various layers of the cell surface and the substructure of these layers. But our understanding will be grossly deficient until we can biochemically isolate and identify the altered structures.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant GM 12524-01 from the National Institute of General Medical Sciences to D. A. Glaser and by Natural Science Foundation Grant GB 5922.

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