

THE EFFECT OF ACRIDINE DYES ON MATING TYPE FACTORS IN
*ESCHERICHIA COLI**†

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In *Escherichia coli*, female strains are designated as F^- ; male strains are of two kinds, designated F^+ and Hfr, respectively. The male determinant in Hfr strains behaves as a chromosomal factor allelic to F^- .¹⁻³ In F^+ strains, however, maleness is determined by a factor "F," with remarkable properties, notably its easy, contagious transmission to F^- cells.⁴⁻⁶ Cells carrying F can be disinfected by treatments with cobalt ion and with acridine dyes.^{7, 8} These properties support the conclusion that F is a plasmid, an extrachromosomal particle, which is readily transferred during mating contacts. It has been suggested that Hfr strains represent the incorporation of F as an element of the chromosome.^{1, 2, 4, 5, 9} Jacob and Wollman introduced the term "episome" for a plasmid that has a facultative association with the chromosome.⁹ The present paper reports further evidence for this conception, namely on the mechanism by which F is eliminated by the acridine dyes.

Materials and Methods.—Two acridine dyes, proflavine (PF, 2:8-diaminoacridine), and acridine orange (AO, 2:8-bisDimethylaminoacridine) were used. Stock solutions containing 100 μg per ml of PF or 500 μg per ml of AO in water were autoclaved and stored in the dark for periods up to a week.

EM-sugar agar¹¹ was used as a selective medium. To grow on this medium, a recombinant must be prototrophic and also be able to ferment the sugar, e.g., lactose. Nutrient medium used for acridine treatment consisted of Difco peptone, 10 and Difco meat extract, 10 gm per liter. The pH of the medium was adjusted with sodium hydroxide solution using the Beckman pH meter. Difco penassay broth (Antibiotic assay medium number 3) was used routinely for bacteriological work.

Strains of *E. coli* used in these experiments are mutants derived from strain K-12. The production, origin, and characteristics of these mutants are summarized in Table I. The strain used for acridine treatment was mainly W6.

Recombination technique: Overnight cultures of tester strains are streaked on EM-sugar medium and they are cross-brushed against one loopful of the culture being tested. Recombinants arise at the junction of the two cultures only in compatible combinations.⁴

Acridine method: An overnight F^+ culture is diluted to 10^4 cells per ml in a

TABLE 1

A SUMMARY OF THE STRAINS USED			
Strain Number	Genotype	Original Strain	Reference
W6	F ⁺ M ⁻	K-12	⁴
W1895	Hfr ₁ M ⁻	W6	Type Hfr ⁵
W2979	F ⁻ Mal ₁ ⁻ Xyl ₂ ⁻ Gal ₂ ⁻ Ara ₂ ⁻		J-11 (supplied by Dr. Cavalli) ¹
W3133	F ⁻ Lac ⁻		Ann Cook ¹⁰
W4164	F ⁺ Lac ⁻	W3133	Obtained by F infection
W4171	F ⁺ M ⁻	W1895	Spontaneous reversion (given by Dr. A. Novick)
W-3399	F ⁺ M ⁻	W1895	F-refractory ¹⁵ (selected in soft agar); ¹⁶ Low fertility X F ⁻ and does not infect F ⁻ to produce F ⁺

nutrient broth (pH 7.6) containing 20 micrograms per ml of acridine orange (abbreviated AO-20) and incubated overnight at 37°C.

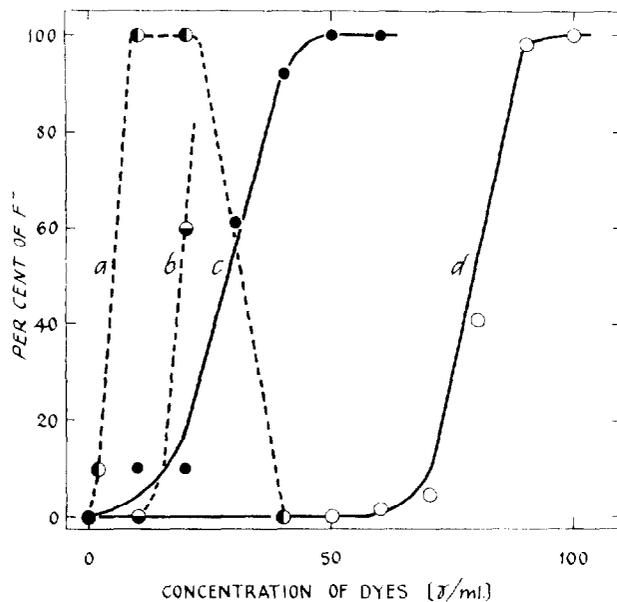


FIG. 1.—Effect of pH on the effective concentration of acridines. (a) Proflavine, pH 7.65. (b) Proflavine, pH 7.20. (c) Acridine orange, pH 7.60. Acridine orange, pH 7.20. An overnight culture of W6 was inoculated into broth (10^7 cells/ml, pH 7.20 or pH 7.6) containing the indicated dye and incubated at 37°C for 20 hours before plating.

sex-compatibility after growth in acridine broth was observed by a standard test of sex-compatibility on isolated clones. The results are generally expressed in terms of the conversion fraction, i.e., the proportion of clones, after treatment which have become stably F⁻. The pH influences the effect of the dyes; for example, AO-50 gives 100 per cent F⁻ at pH 7.6 but none at pH 7.2. Generally speaking, the minimum effective concentration of acridine is low at a high pH and vice versa (Fig. 1). All the colonies formed in the untreated control remained F⁺.

Proflavine exerts its action at lower concentrations than that of AO at the same pH. However, the rate of conversion is decreased at higher (bacteriostatic) concentrations of PF. These pH effects indicate that either the drug cation or an

The use of acriflavine for the removal of F has been briefly reported in an earlier publication.⁸ AO has proved to be more useful than acriflavine, proflavine, or acridine yellow by virtue of its low toxicity.

The F⁻ clones obtained in these experiments have been repeatedly studied. The F⁻ strains so obtained are genetically stable for this mating type, like the other F⁻ reported. If any F⁺ cells had reappeared in the F⁻ culture by spontaneous mutation, they would be amplified by cross-infection during repeated passages.

Experimental Results.—

1. Environmental effects on the action of acridines:

The conversion of sex-

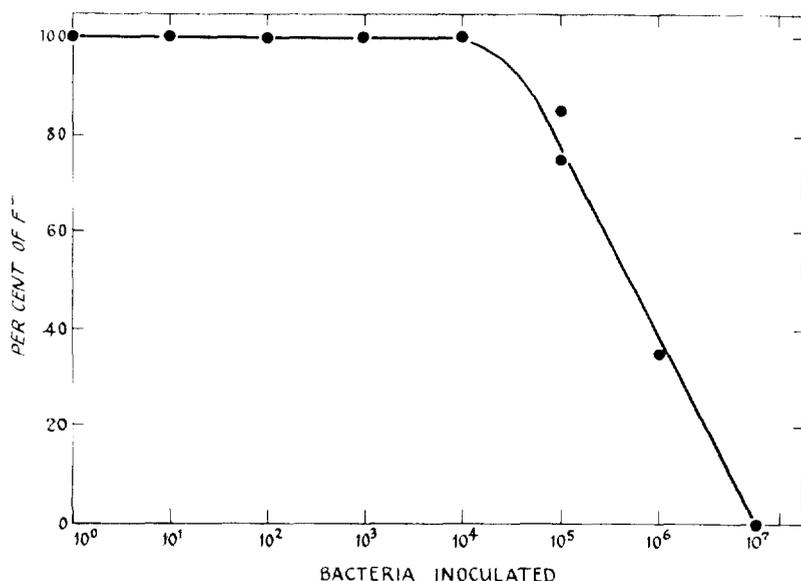


FIG. 2.—Effect of inoculum size on conversion of sex-compatibility. Various dilutions of an overnight culture of W6 were inoculated into broth containing AO (pH 7.6, 20 μ g./ml) and incubated overnight at 37°C.

anionic bacterial receptor is effective in the elimination of F. The same relationship between bacteriocidal action of acridine ion and pH effect has been extensively studied by Albert.¹² The inoculum size of F⁺ cells also influences the rate of conversion, which decreases with larger inocula (Fig. 2). With an inoculum of about 1 cell per ml, 100 per cent F⁻ can be obtained in nutrient broth at pH 7.6 with AO 10 to AO-20. The lowest concentration at which any effect was noted was AO-1 to AO-5.

The rate of elimination is reduced at lower temperatures and was essentially zero at 5°C (Table 2). The viability of the cells obtained from the treated culture and the untreated culture at low temperature is the same.

TABLE 2
EFFECT OF TEMPERATURE ON CONVERSION OF SEX-COMPATIBILITY FROM F⁺ TO F⁻

Temperature (°C):	—Treated with AO—		—Untreated Control—	
	37	5	37	5
Total colonies tested:	110	110	110	110
F ⁻ obtained:	91	4	0	3
Per cent of F ⁻ :	82.7	3.6	0.0	2.7

10⁷ cells per ml of an overnight culture of W6 were inoculated into nutrient broth pH 7.6 and treated for about 20 hours with 50 micrograms of acridine orange, or without it as control.

Figure 3 shows the conversion fraction in treated cultures plated on EMB lactose agar at various times. F⁻ clones first appear after a lag of 1 to 2 hours, and their incidence then increases rapidly. The concentration of AO, pH of the medium, and cultural age of the treated cells influence the rate of conversion and the time for removal of F.

Acridines are ineffective on cells growing in a synthetic medium.¹³ However, peptone 0.02 per cent, restored the effect (Table 3). A systematic analysis of

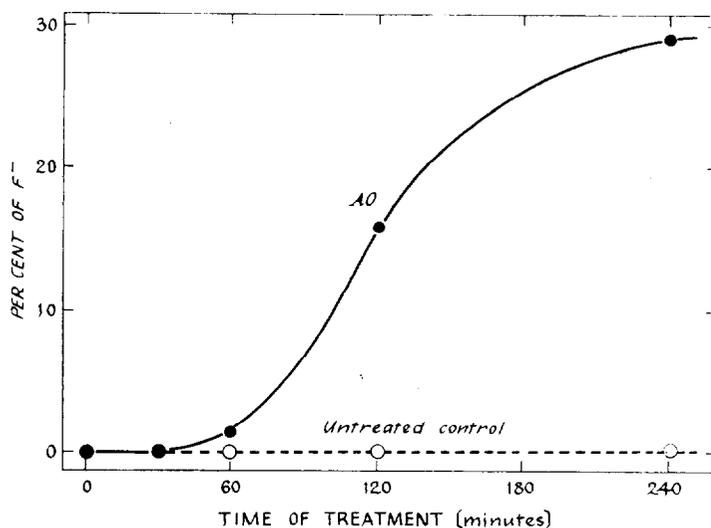


FIG. 3.—Time analysis of acridine treatment. An exponentially growing, W6 culture was inoculated into penassay broth (pH 7.2) with or without AO (36 $\mu\text{g.}/\text{ml}$). The F^+ cultures (10^7 cells/ml), were shaken gently at 37°C . Samples were diluted and plated on EMB agar. The following morning single colonies were tested for their compatibility. The figure summarizes five experiments. About 2,000 colonies from untreated controls were all F^+ .

likely constituents of complex media showed the necessity for the simultaneous presence of several co-factors: amino acids plus a nuclein base. AO eliminated F from F^+ bacteria in a defined medium containing the following supplements: serine, aspartic acid, isoleucine, valine, and cytosine. The significance of these co-factors for the cure of maleness remains for future studies. The specific supplements required for growth of auxotrophic mutants were also essential for F elimination.

TABLE 3
CO-FACTOR FOR REMOVAL OF F BY AO

Minimal plus Indicated Supplement	Per Cent of F^- Obtained	
	W6 (M^-)	W4164 (Prototroph)
No supplement	0	0
Peptone* plus AO	100	...
Mixture†	71	100
“ minus AO	0	0
“ minus serine	4.1	10.1
“ “ cytosine	3.7	0
“ “ methionine	0	33.4

* 200 micrograms per ml.

† Concentration of each supplement: cytosine 100, serine 20, aspartic acid 20, isoleucine 20, valine 20, methionine 20, AO 7.5 (micrograms/ml).

Overnight cultures of W6 and W4164 were washed with sterilized water, treated in several media, purified on EMB agar, and their compatibility tested by the cross-brushing method. The experimental conditions were as follows: inoculum size about 10^4 cells per ml, time of treatment 20 to 24 hours at 37°C .

Methylene blue and thionine which are structurally analogous (Fig. 4) to AO antagonize the elimination of F but not the inhibition of growth by AO. These dyes alone have no dramatic activity on F^- disinfection; F^- clones have appeared infrequently in treatments with methylene blue. Albert¹² has summarized analogous examples of “therapeutic interference” in the typanocidal action of these dyes.

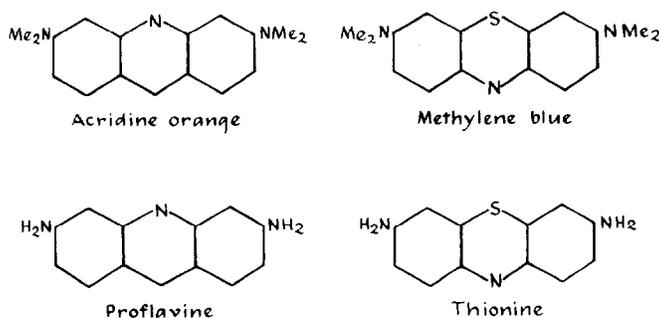


FIG. 4.—Comparison of the chemical structure of several dyes.

Propamide isethionate (Baker and May Ltd., 4:4'-diamidino-diphenoxypropane di-(β -hydroxyethane sulfonate)) is another chemotherapeutic agent which supposedly reacts with nucleic acids. At 50 micrograms per ml in penassay broth it was found to produce about 15 per cent F^- after overnight treatment, and is therefore a weak agent affecting F^- .

It is concluded that acridine mainly produces its effect on actively multiplying cells: in the absence of cell multiplication, i.e., with large inocula, low temperature, nutritional limitation, or bacteriostasis by excess dye, there is little or no loss of F^- .

2. *Mode of action of acridine:* The increase in the proportion of F^- after acridine treatment could be attributed in principle either to mutagenic effects of acridine dyes or selective enrichment of F^- cells already present among the F^+ population. No differential in growth of F^+ vs. F^- cells could be found even remotely approaching the dramatic differences needed to account for complete conversions (see Table 4). However, the most decisive experiments concerned the fate of small numbers of cells inoculated in AO medium.

TABLE 4
RECONSTRUCTION EXPERIMENT

	Input Mixtures				Mixed Cultures after Incubation			
	Lac ⁺ F ⁻	Lac ⁺ F ⁺	Lac ⁻ F ⁻	Lac ⁻ F ⁺	Lac ⁺ F ⁻	Lac ⁺ F ⁺	Lac ⁻ F ⁻	Lac ⁻ F ⁺
A Treatment	0.329 (W2979)	0	0	0.671 (W4164)	0.471 (W2979)	0	0.529 F-elimination	0
Untreated Control	0.363 (W2979)	0	0	0.637 (W4164)	0.246 (W2979)	0.152 F-infection	0	0.602 (W4164)

Overnight cultures of W4164 (F^+) and W2979 (F^-) were mixed and about 10^4 cells/ml inoculated into broth, pH 7.6, with or without AO, and incubated overnight at 37°C. The proportion of Lac⁺ and Lac⁻ bacteria in the mixed cultures was diagnosed on EMB lactose agar before and after the treatments; the F^- status of samples of isolated colonies was tested by crosses against a standard F^- strain (see Materials and Methods). The figures in this table show the fraction of colonies in each class.

The mutagenic effect of AO was tested more directly by cultivating one or two cells in a microdroplet of penassay broth, with or without acridine dye, in an oil-chamber as described by Lederberg.¹⁴ The microcultures were then allowed to grow for several generations. Each microclone was transferred to EMB lactose agar by a capillary pipette and plated out to give single colonies arising from individual cells. The colonies were then tested for sex-compatibility. The entire clone was plated; thus, differential growth of spontaneous F^- mutants could alter the proportion of F^- progeny within a clone but should not affect the number of

TABLE 5

INDUCTION OF F ⁻ FROM SINGLY ISOLATED F ⁺ CELL AND F ⁺ CELLS		
Number of Cells in Drop Initially	Number of Drops with Induced F ⁻	Number of Drops Tested
Acridine-treated cell(s)		
1	3*	25
2	5†	20
Untreated Control		
1	0	25
2	0	20

An exponential culture of strain W6 was grown at 37°C in penassay broth with gentle shaking. This culture contained 10⁹ cells per ml. Single cells were then isolated in microdroplets by a simple modification of De Fonbrune's oil chamber method.¹⁴ Droplets were dispensed by free hand manipulation from a suspension containing 10⁶ cells per ml. with or without AO-40. The droplets were then examined microscopically, and those which were verified to contain precisely one cell or two cells then recorded. They were incubated at 37°C for 4 hours. At the end of this time, the cells that had grown in each drop were counted, then collected by a capillary pipette and spread on EMB agar. All the colonies formed on the agar were tested for sex-compatibility. If one or more F⁻ colonies were observed, this was recorded as "containing induced F⁻".

By Fisher's exact test, P = 0.0056.
Total numbers of F⁻ cells in "induced drops" were:

* 1,1,1.

† 20,2,4,1,2.

and the total cell numbers of those induced droplets were:
33,20,10; 72,10,34,11,71, respectively.

clones in which F⁻ progeny are found. As shown in Table 5, AO increases the proportion of F⁻-containing clones from 0/45 in controls to 8/45 in the treated series. This supports the conclusions that AO induces the loss of F from growing F⁺ cells.

3. *The action of AO on various male strains:* A wide variety of F⁺ strains mutants of *E. coli* K-12 have been tested. The markers such as nutritional requirements, drug resistance, lysogenicity, phage resistance, sugar fermentation seem to have no bearing on the effect of AO nor are they altered by the treatment. However, Hfr males differ markedly from F⁺. For example, Table 6 shows a result of

TABLE 6

ACCESSIBILITY OF TWO STATES OF F IN VARIOUS STRAINS TO DISINFECTING ACTION OF ACRIDINE TREATMENT

	W6 F ⁺	W1895 Hfr	W4171 F ⁺ Reversion from W1895	W4399 F ⁻ -Refractory
Can infect standard F ⁻	+	-	+	-
Mode of inheritance of maleness	Nonsegregational	Segregational	Nonsegregational	Segregational
Compatibility with standard F ⁻	+	+++	+	±
Compatibility status affected by acridine treatment	yes	no	yes	no

acridine treatment on four male types all derived from W6. Mutants which show chromosomal segregation and non-infectivity of the male character are not sensitive to the disinfecting action of acridine dyes. This inaccessibility is observed both in stable Hfr and the F^r mutants (summarized by Lederberg and Lederberg).¹⁵ Hirota and Iijima⁸ reported that AO gave F⁻ types not only from F⁺ but also from Hfr₂.⁶ However, this culture has proven to be extremely mutable and spontaneously produces many F⁺ reversions. The F⁻ obtained from cultures labelled Hfr₂ can be accounted for by their origin from such F⁺ reversions. The F⁺ state of the mutable Hfr₃² is also accessible to this treatment. Stable Hfr strains, e.g., Hfr₁ have not been influenced by exposure to AO. On the other hand, the other

mutants which maintain a contagious F are readily disinfectable by this treatment. F elimination by AO thus differs from the correlated selection of highly motile F⁻ bacteria in soft agar, which selects F⁻ not only from F⁺ but also from stable Hfr strains.¹⁶

Discussion.—In this phenomenon, many hypotheses may be considered. From the above experiment, however, it seems difficult to escape the conclusion that induction of F⁻ from F⁺ is the result of the loss of F particles endowed with genetic continuity. Whether the relative multiplication of F is decreased, or the F particles are agglutinated by combination with the acridinium cation, growing cells exposed to AO eventually produce bacteria without F.

Stability of F⁻ means a permanent loss of F, and conversely the stable maintenance of the F⁺ trait and the remarkable infectivity of F in usual media signify that F multiplies faster than the host cell. The transfer of F unlinked to the host chromosome, and the non-segregational uniform inheritance of F in the cross F⁺ × F⁻,⁴⁻⁶ would most logically correspond to cytoplasmic transfer of the male determinant.^{17, 18} That is, the factor in an F⁺ cell is a plasmid.^{5, 17, 18} Other plasmids, such as the respiratory factors in yeast, kinetoplasts in trypanosome are also known to be accessible to acridine treatment.¹⁹⁻²²

On the other hand, maleness in Hfr cells is inherited chromosomally as a gene linked to certain markers in genetic recombination tests.¹⁻³ Correlated with this is the fact that Hfr males are resistant while infective F⁺ males are accessible to the acridine treatment (Table 6). These facts suggest that in Hfr strains this male determinant is bound to the particular chromosome site, and thus accounts for the non-infectivity and segregational inheritance of maleness, and its inaccessibility to the disinfecting action of acridine dyes.

This situation parallels the lysogenic system of bacteria in which there are two states, vegetative phage, free in the cytoplasm and prophage, bound to the chromosome. The term episome has been used for this type of particle.⁹

Acridine dyes are reported to cure virus infection, produce defective virus, and induce virus mutants respectively.²³⁻²⁷ However, they do not affect the chromosomally bound prophage of temperate phages.²⁸ The detailed mechanism of action is, however, not known. These dyes do possess the unique property of straining certain nucleic acid-containing particles, nuclei, and the other particles of living cells, in several organisms.²⁹ Nucleotides, nucleic acid, and polyadenylic acid form complex salts with acridine dyes.^{30, 31} The effect of acridine orange on the function of DNA as a primer for its enzymatic replication has not been studied.

The co-factors required for F-disinfection may act in part by supporting the growth of the host cell in its particulate components in the presence of AO. The F particle itself may have a negative charge, so that acridine exerts its effect only as a cation.

Summary.—Acridine orange converts F⁺ (male) clones of *E. coli* into stable F⁻ (female) forms. The main factors which affect the rate of conversion of F⁺ to F⁻ are the concentration of acridinium ions in the treating medium and the growth of the treated cells in the presence of the dye. Furthermore, the accessibility of F to acridine treatment depends upon the state of F in the host cell.

Acridine orange increases the frequency of conversion from F⁺ to F⁻ directly without appreciable selective growth. The conversion from F⁺ to F⁻ is irreversible,

as expected for the loss of a genetic particle. Hfr males are resistant to the disinfecting action of acridine dyes. These results are well accounted for by the dual nature of F, chromosomal F, and plasmid F.

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