Genet. Res., Camb. (1967), **10**, *pp.* 241–249
With 3 text-figures
Printed in Great Britain

Effects of acriflavine on the transfer of episomes and bacterial chromosome in *Escherichia coli* K-12

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(*Received* 23 *June* 1967)

1. INTRODUCTION

Acridine dyes are known to inhibit the synthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (Hurwitz *et al.*, 1962) and to act as mutagenic agents (Orgel & Brenner, 1961). Jacob *et al.* (1963) and Cuzin & Jacob (1966) reported that acridine orange causes specific inhibition of the transfer of F factor and bacterial chromosome. This finding was claimed to support their replicon hypothesis. The transfer of R factor (see Watanabe, 1963) is also inhibited by acridine dyes (Akiba *et al.*, 1964; Hayashi *et al.*, 1965). We have previously proposed that the inhibition of transfer of R factor by acriflavine may be caused by the specific inhibition of the DNA synthesis required for episome transfer (Takano *et al.*, 1966). It has recently been reported, however, that acridine dyes may not act on the transfer itself, but may inactivate the transferred DNA immediately after its transfer to recipient bacteria, with colicinogenic factors (Lorkiewicz *et al.*, 1964, 1965), and with bacterial chromosome (Brinton *et al.*, 1966).

We have studied the effects of acriflavine on the transfer of various episomes and of the bacterial chromosome using acriflavine-sensitive and -resistant strains of *Escherichia coli* K-12 as donors and recipients. The episomes studied were F′, R and colicinogenic factors I and B. The transfer of bacterial chromosome was studied in an Hfr strain of *E. coli* K-12.

2. MATERIALS AND METHODS

(i) *Media and drugs*

Liquid cultures were prepared in M9 (glucose-salts) medium (Adams, 1959), supplemented with 1% casamino acids (Difco) and adjusted to pH 7·7 with a phosphate buffer (=enriched M9 medium). Plating media were nutrient agar and bromothymol blue (BTB)-lactose or -galactose agar (containing 1% sugar). Dihydrostreptomycin sulphate (Sankyo Co.), chloromycetin powder (Parke, Davis, and Co.), and kanamycin sulphate (Tanabe Co.) were added to the media in the concentrations described previously (Watanabe *et al.*, 1964*b*). Acriflavine hydro-

chloride (Tokyo Kasei Kogyo Co.) was used at concentrations to be described below. These drugs will be abbreviated as Sm, Cm, Km and Af, respectively.

(ii) *Method of isolation of acriflavine-resistant mutants of bacteria*

Five millilitres of enriched M9 broth containing Af in two-fold dilutions was inoculated with a loopful of bacterial culture. Selection of Af-resistant (Af^r) mutants was made as follows: The bacteria which grew in the broth containing the highest concentrations of Af were transferred to the broth containing higher concentrations of Af. In this way, five to six stepwise selections gave Af^r mutants. No Af^r mutants could be obtained by one-step selection. Minimal inhibitory concentrations of Af were 10–20 μg./ml. in sensitive, wild-type bacteria and 50–60 μg./ml. in resistant mutants. Af resistance levels of these Af^r mutants were stable.

(iii) *Bacterial strains*

E. coli K-12; W3350 (*lac*$^-$, *gal*$^-$, F$^-$), W2252 (*met*$^-$, Hfr: transfers its chromosome in the order *lac-pro-leu-thr*...) and W3102 (*gal*$^-$, *lac*$^-$, Sm-resistant, phage λ-resistant, F$^-$), and Af-resistant mutants of these strains (Af^s, Af^r, respectively). Colicin I- and B-resistant (/IB) strains were prepared from these strains. F'_8-galactose factor (F'-*gal*) (Hirota & Sneath, 1961) and R factor 222 carrying the markers of resistance to Sm, Cm, tetracycline and sulfonamide (Watanabe *et al.*, 1964*a*) were introduced into Af^s and Af^r strains of W3350. Colicinogenic factor I or B (*col* I, *col* B, respectively) (Ozeki *et al.*, 1962) was introduced into Af^s and *Af* strains of W3350/IB. As an indicator strain for colicins, *E. coli* ϕ/Sm^r was used. This strain was obtained from Dr H. Ozeki, National Institute of Health, Japan.

(iv) *Conjugation conditions and selection of clones which received episomes and bacterial chromosome*

Bacteria were grown in enriched M9 medium at 37°C. with gentle shaking to 2 to 5×10^8 cells/ml. 0·5 ml. of a donor culture was mixed with 4·5 ml. of a recipient culture in a 100 ml. Erlenmeyer flask and kept at 37°C. for 30 min. without aeration. To study the effect of Af on mating, Af in a final concentration of 20 μg./ml. was added to a mating mixture at the time of mixing donor and recipient. For the detection of the transfer of F'-*gal* and of the bacterial chromosome, mating mixtures were plated on BTB-galactose and -lactose agar containing 1000 μg./ml. of Sm. F-ductants and recombinants were scored as Sm^r *gal*$^+$ and Sm^r *lac*$^+$ colonies, respectively. The transfer of R was detected as Sm^r Cm^r colonies on BTB-galactose agar containing 1000 μg./ml. of Sm plus 25 μg./ml. of Cm. The donor bacteria without the chromosomal Sm^r gene and with R factor carrying Sm^r marker cannot grow on this medium, because the level of Sm resistance conferred by the R factor is rather low. Mating mixtures for *col* I or *col* B transfer were plated on nutrient agar containing 1000 μg./ml. of Sm. The production of colicin by colonies was detected with the method of Frédéricq (1948) using *E. coli* ϕ/Sm^r as an indicator. The effect

Table 1. *The effects of acriflavine on the transfer of episomes and bacterial chromosome in* Escherichia coli *K-12*

Af sensitivity		Af in	Episomes and Bacterial chromosome									
			R (222)		F′-*gal*		Hfr		*col* I		*col* B	
Donor	Recipient	mating mixture	*Cm*^{r}*Sm*^{r}/ Donor	Af +/−	*gal*$^+$*Sm*^{r}/ Donor	Af +/−	*lac*$^+$*Sm*^{r}/ Donor	Af +/−	*Col I*$^+$*Sm*^{r}/ Donor	Af +/−	*Col* B$^+$*Sm*^{r}/ Donor	Af +/−
Af^s	*Af*^{s}	−	$1{\cdot}4\times10^{-2}$	—	$1{\cdot}1\times10^{0}$	—	$1{\cdot}6\times10^{-1}$	—	$8{\cdot}3\times10^{-4}$	—	$6{\cdot}3\times10^{-2}$	—
		+	$2{\cdot}6\times10^{-5}$	$1{\cdot}9\times10^{-3}$	$1{\cdot}3\times10^{-3}$	$1{\cdot}2\times10^{-3}$	$5{\cdot}0\times10^{-4}$	$3{\cdot}1\times10^{-3}$	$4{\cdot}2\times10^{-5}$	$5{\cdot}1\times10^{-2}$	$1{\cdot}3\times10^{-3}$	$2{\cdot}1\times10^{-2}$
Af^s	*Af*^{r}	−	$9{\cdot}3\times10^{-3}$	—	$1{\cdot}2\times10^{0}$	—	$1{\cdot}4\times10^{-1}$	—	$3{\cdot}1\times10^{-3}$	—	$2{\cdot}8\times10^{-2}$	—
		+	$7{\cdot}5\times10^{-5}$	$8{\cdot}1\times10^{-3}$	$2{\cdot}5\times10^{-3}$	$2{\cdot}1\times10^{-3}$	$4{\cdot}3\times10^{-4}$	$3{\cdot}1\times10^{-3}$	$5{\cdot}4\times10^{-4}$	$1{\cdot}7\times10^{-1}$	$1{\cdot}1\times10^{-3}$	$3{\cdot}9\times10^{-2}$
Af^r	*Af*^{s}	−	$7{\cdot}7\times10^{-3}$	—	$8{\cdot}9\times10^{-1}$	—	$6{\cdot}6\times10^{-1}$	—	$5{\cdot}3\times10^{-3}$	—	$2{\cdot}9\times10^{-2}$	—
		+	$1{\cdot}9\times10^{-3}$	$2{\cdot}5\times10^{-1}$	$1{\cdot}6\times10^{-1}$	$1{\cdot}9\times10^{-1}$	$1{\cdot}6\times10^{-1}$	$2{\cdot}4\times10^{-1}$	$5{\cdot}9\times10^{-4}$	$1{\cdot}1\times10^{-1}$	$3{\cdot}7\times10^{-3}$	$1{\cdot}3\times10^{-1}$
Af^r	*Af*^{r}	−	$6{\cdot}2\times10^{-3}$	—	$1{\cdot}4\times10^{0}$	—	$4{\cdot}6\times10^{-1}$	—	$4{\cdot}7\times10^{-3}$	—	$1{\cdot}3\times10^{-2}$	—
		+	$3{\cdot}3\times10^{-3}$	$5{\cdot}4\times10^{-1}$	$3{\cdot}7\times10^{-1}$	$2{\cdot}6\times10^{-1}$	$2{\cdot}4\times10^{-1}$	$5{\cdot}2\times10^{-1}$	$3{\cdot}6\times10^{-3}$	$7{\cdot}7\times10^{-1}$	$3{\cdot}4\times10^{-3}$	$2{\cdot}6\times10^{-1}$

Donors: W3350 (R or F′-*gal*), W3350/IB (*col* I or *col* B) and W2252 (Hfr).

Recipients: W3102 or W3102/IB.

0·5 ml. of a donor culture was mixed with 4·5 ml. of a recipient culture in the presence (+) or absence (−) of Af (20 μg./ml.) and incubated for 30 min. at 37°C. in a 100 ml. Erlenmeyer flask. The transfer of each episome or bacterial chromosome was detected as described in Materials and Methods. The frequencies of transfer are indicated as those per input donor cell, and the effect of Af on the transfer is shown as the ratio of the frequencies of transfer in the presence (+) and absence (−) of Af.

of Af on episomes and the bacterial chromosome immediately after transmission was examined in the following way: At various intervals, samples of mating mixtures were taken and transferred to enriched M9 medium containing 1000 μg./ml. of Sm and 1000 μg./ml. of Sm plus 20 μg./ml. of Af, immediately agitated in a blender, and then incubated at 37°C. for 30 min. Selection of clones which received episomes and bacterial chromosome was then made by the above methods. Af sensitivity of *col*$^+$ bacteria immediately after its transfer was examined in the following way: After 30 min. of mixed incubation, the mating mixture was blended and diluted in enriched M9 medium containing 1000 μg./ml. of Sm to kill donor bacteria and to prevent further *col* transfer. This culture was incubated at 37°C. At various intervals, samples were taken and treated with Af in the same way as above.

(v) *Af sensitivities of bacteria carrying various episomes*

Bacteria were grown in enriched M9 medium to 5×10^8 cells/ml. Cells were washed by centrifugation and resuspended in fresh enriched M9 medium containing 20 μg./ml. of Af to a concentration of 10^8 cells/ml. At various intervals, colony-forming cells were scored on nutrient agar. To study the Af sensitivities of bacteria in buffer, washed cells were resuspended in M9 buffer, that is the M9 medium without glucose, casamino acids and gelatin, and starved for 30 min. at 37°C. before 20 μg./ml. of Af was added.

3. RESULTS

The effects of Af on the transfer of various episomes and bacterial chromosome by conjugation are summarized in Table 1. The inhibition of transfer of these elements by Af was, in all cases, remarkable in the matings of Af^s donors, regardless of whether the recipients were Af^s or Af^r. In the case of *col* transfer, however, the inhibitory effect of Af was reduced to some extent by using Af^r recipients.

Next, the effects of Af on these episomes in recipient bacteria immediately after their transfer were examined. Samples of mating mixtures in Af-free medium were taken at various intervals and treated with 20 μg./ml. of Af at 37°C. for 30 min. In the case of R, samples treated with Af were divided into two portions, and one was plated as above and the other was washed and reincubated in Af-free medium for 20 min. at 37°C. before plating in order to allow the phenotypic expression of drug resistances, because phenotypic expression was not possible on the selective media. Phenotypic expression of other episomes occurred on selective plates. The kinetics

Fig. 1. The kinetics of transfer of episomes and the effects of acriflavine on episomes after transfer in *Escherichia coli* K-12.

●—● Normal kinetics. ○—○ Treated with 20 μg./ml. of acriflavine for 30 min. at 37°C. and no time allowed for phenotypic expression. × — × Treated with acriflavine and phenotypic expression allowed in Af-free broth at 37°C. for 20 min. after Af treatment.

The coordinates are the frequency of transfer per input donor cell. a: R(222); b: F′-*gal*; c: *col* I; d: *col* B.

https://doi.org/10.1017/S0016672300011009 Published online by Cambridge University Press

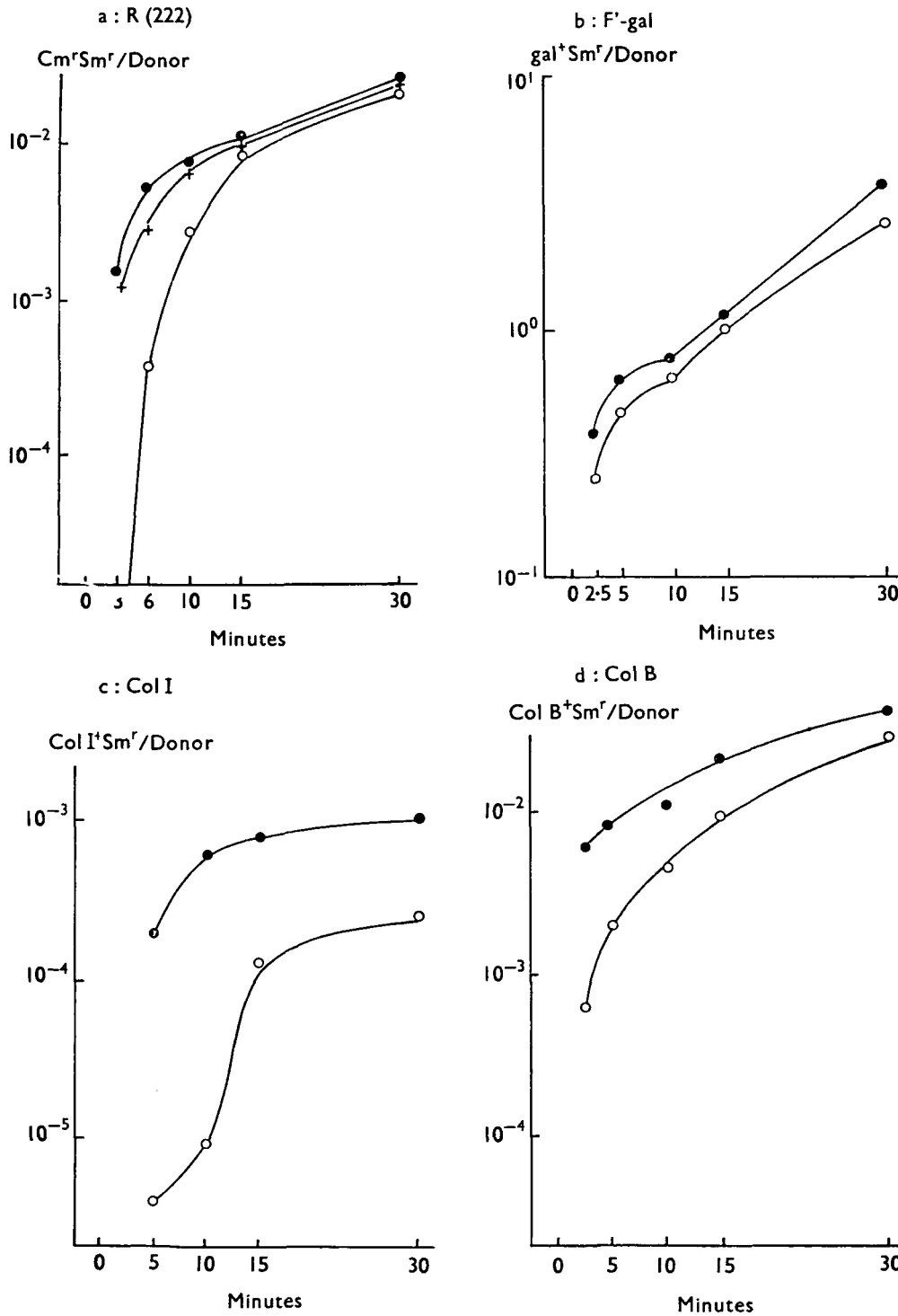

Fig. 1.

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of transfer of episomes and the effects of Af on the biological activity of episomes immediately after their transfer are shown in Fig. 1. As seen here, Af treatment exerted almost no effect on R and F after their transfer to recipient bacteria. In the case of R, it was found that the phenotypic expression of drug resistances was inhibited by Af. In contrast, bacteria with *col*, especially *col* I, were considerably reduced by Af treatment.

In order to study the mechanism of reduction of *col*+ bacteria by Af, mating for *col* transfer was interrupted by blending after 30 min. of mixed incubation, and 0·1 ml. of the blended mating mixture was transferred to 10 ml. of enriched M9 medium containing 1000 μg./ml. of Sm to kill the donor bacteria and to prevent further *col*

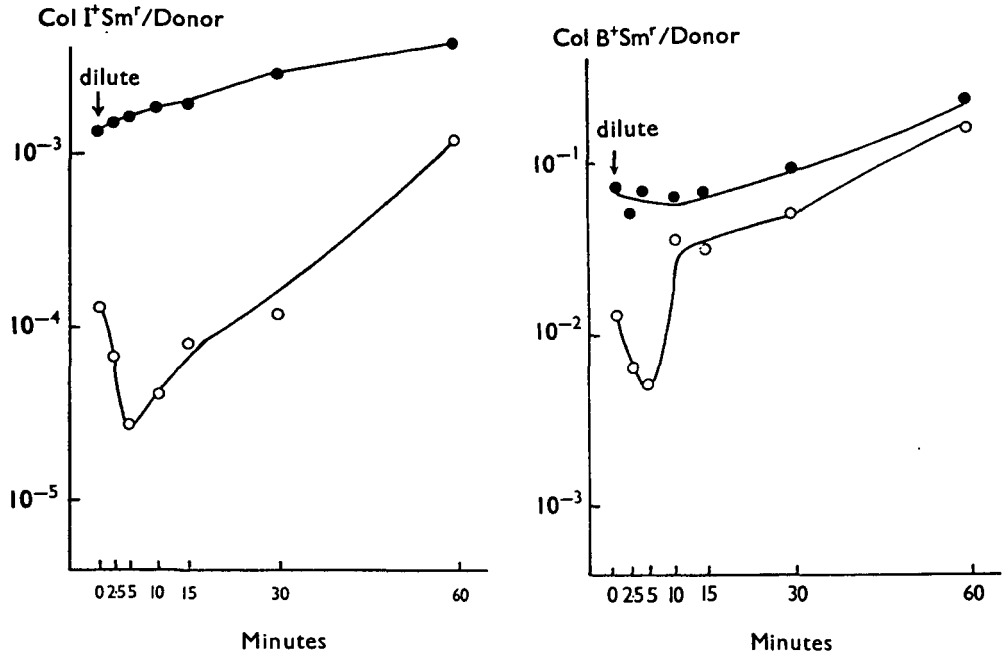

Fig. 2. Acriflavine sensitivity of *col*+ *Escherichia coli* K-12 immediately after *col* transmission.

After mixed incubation for 30 min. at 37°., mating was interrupted, and the culture diluted and further incubated at 37°C. At various intervals, samples were taken and treated with 20 μg./ml. of acriflavine at 37°C. for 30 min.

●—● Not treated. ○—○ Treated.

transmission. This culture was incubated at 37°C., and, at various intervals, samples were taken and treated with 20 μg./ml. of Af at 37°C. for 30 min. It is seen in Fig. 2 that the reduction of *col*+ bacteria by Af treatment reached a maximum at 5 min. after interruption and recovered gradually.

Then, in order to study whether the reduction of *col*+ bacteria was due to the inactivation of *col*+ cells or the inactivation or elimination of *col* itself, immediately after its transmission, Af sensitivity of bacteria with various episomes was examined (Fig. 3).

https://doi.org/10.1017/S0016672300011009 Published online by Cambridge University Press

In broth, *col*$^+$, especially *col* I$^+$, bacteria were killed far more rapidly than other bacteria, but in buffer, *col*$^+$ bacteria were inactivated at the same rate as *col*$^-$ bacteria.

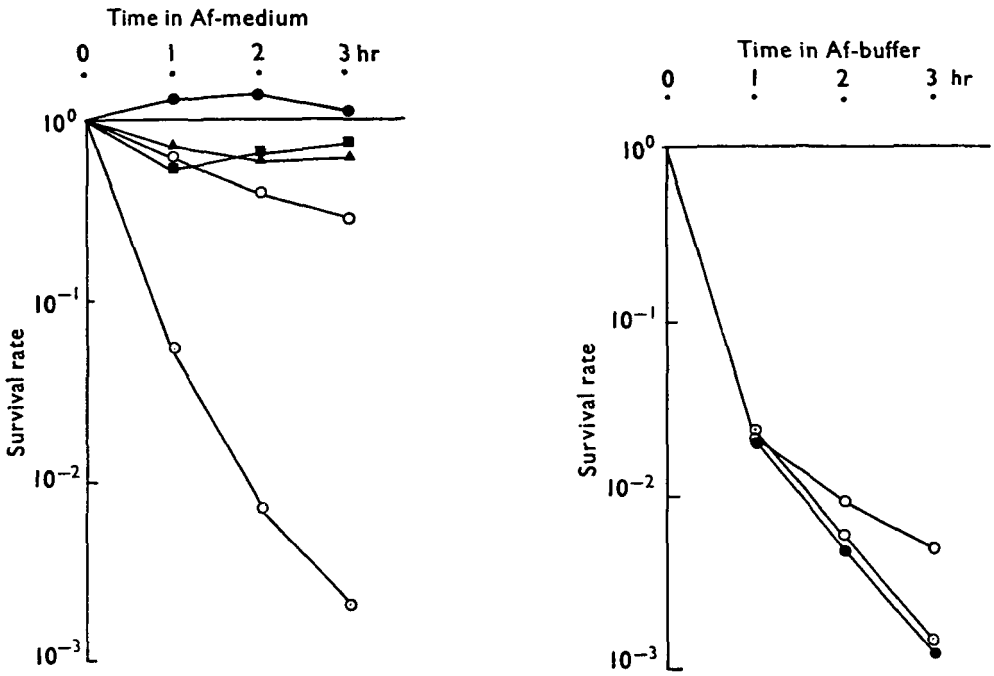

Fig. 3. The effects of colicinogenic factors and other episomes on acriflavine sensitivity of *Escherichia coli* K-12.

Bacteria were grown in enriched M9 medium and washed. Washed cells were resuspended in enriched M9 medium or M9 buffer to a final concentration of 10^8 cells/ml. and incubated at 37°C. after which acriflavine was added to a final concentration of 20 μg./ml. At various intervals, samples were taken and colony-forming cells were counted on nutrient agar.

● W3102. ▲ W3102 (*F'-gal*). ■ W3102 (R).
○ W3102/IB (*col* B). ⊙ W3102/IB (*col* I).

The experiments were carried out in the room light.

4. DISCUSSION

The fact that the inhibition of transfer of R, F, *col* I, *col* B and bacterial chromosome by Af occurs mainly in donor bacteria seems to support the hypothesis that the transfer of these elements requires specific DNA synthesis for their transmission and that Af inhibits this DNA synthesis for transfer (Jacob *et al.*, 1963). The replication of these episomes and bacterial chromosome for transfer seems to be equally sensitive to Af.

Viable *col*$^+$ bacteria were reduced in number by Af treatment. This reduction may be due to killing of the host cell by Af rather than to the inactivation of the colicinogenic factor itself. Lorkiewicz *et al.*, (1964, 1965) observed the killing effect of Af on

col$^+$ bacteria, not the inactivation of colicinogenic factor itself. We have further shown that the sensitivity of *col*$^+$ bacteria to Af is increased up to 5 min. after transmission and then decreased gradually. Inactivation with Af was not observed in R and F immediately after their transfer to recipient bacteria. Thus, colicinogenic factors may be considered exceptional in this respect. It has been reported that acridine orange sensitizes λ-lysogenic cells to induction by visible light (Freifelder, 1966). It may be possible to consider that the Af inactivation of colicinogenic bacteria is also due to the induction of colicin production by Af, because only the colicinogenic factors were inducible among the episomal elements we have employed. We have studied the effect of Af on the transfer of a leading marker of an Hfr strain, but no inactivation was detected, unlike the report of Brinton *et al.* (1966). The reason for this discrepancy is not known at the present moment.

In addition, the experiments on R transfer have shown that Af inhibits not only its replication but also its phenotypic expression. The inhibition of the phenotypic expression of R by Af may be explained by the inhibition of transcription (Hurwitz *et al.*, 1962).

It is known that acridine dyes eliminate F at high efficiencies (Hirota, 1960) and R at lower frequencies (Watanabe & Fukasawa, 1961). It has not been possible to eliminate *col* I with acridine dyes (Ozeki *et al.*, 1962). These facts indicate that the replication of episomes and bacterial chromosome for cell division may have different sensitivities, although their sensitivities to Af revealed by the inhibition of transfer seems to be equal.

We are now studying the cause of the differences in Af sensitivities among various episomes and bacterial chromosome in their replication for transfer and in their replication for cell division.

SUMMARY

The effects of acriflavine on the transfer of various episomes and of the bacterial chromosome were studied using acriflavine-sensitive and -resistant strains of *E. coli* K-12 as donors and recipients. It was found that the inhibition of transfer of these elements by acriflavine was only slight when donor bacteria were resistant to this acridine dye, regardless of whether the recipient bacteria were acriflavine-sensitive or -resistant. This result seems to support the view that acriflavine inhibits the replication of these elements in donor bacteria which is considered essential for their transfer. With colicinogenic factors, in contrast to F and R, the results were affected by the sensitivity or resistance of the recipient and were in support of the killing by acriflavine of the cells which received the colicinogenic factors.

We are indebted to D. Ushiba for his encouragement.

This investigation was supported by U.S. Public Health Service Research Grant AI-04740 from the National Institute of Allergy and Infectious Diseases, and by a research grant from the Ministry of Education, Japan.

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