Genet. Res. Camb. (1963), **4**, *pp.* 305–317
With 5 text-figures
Printed in Great Britain

Kinetics of *F*-curing by acridine orange in relation to the number of *F*-particles in *Escherichia coli*

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(*Received* 8 *February* 1963)

1. INTRODUCTION

In *Escherichia coli* the mating type is determined by the presence or absence of the sex factor. In F^+ strains the *F* factor is present in the autonomous, extrachromosomal state (Lederberg, Cavalli & Lederberg, 1953; Hayes, 1953a) and in Hfr strains it is attached to the chromosome (Cavalli, Lederberg & Lederberg, 1953; Hayes, 1953*b*). In F^- strains the *F* factor is absent.

Jacob & Wollman (1958) have proposed the name *episome* for genetic elements like the *F*-factor.

In 1957 Hirota and Iijima reported that F^+ cells could be converted into F^- cells by treatment with acridine dyes. However, Hirota (1960) found that acridines were without action on the *F*-particles in Hfr cells. It is thus evident that these dyes interfere with the reproduction of autonomous episomes. In the present paper the results will be reported of a study on the kinetics of the conversion of F^+ to F^- cells by acridine orange. These results indicate that acridine orange acts by inhibiting the multiplication of the *F*-particles. Our previous experiments (de Haan & Stouthamer, 1963) on the kinetics of *F*-transfer and the subsequent multiplication of sexduced cells, have shown that the number of *F*-particles per cell is small and that they are distributed in a non-random way over the daughter cells at cell division. The present results with ^{32}P-labelled episomes support this view.

2. MATERIALS AND METHODS

(i) *Bacterial strains*

The F^+ strains employed were:

5832 M^- str^s (F-gal^+) (Hirota & Sneath, 1961).

P678 (F-gal^+) obtained by infecting P678 with the F-gal^+ factor from 5832.

The F^- strains employed were:

P678 $T^-L^-B_1^-$ str^r gal^- lac^- (Jacob & Wollman, 1956).

A $T^+L^+B_1^-$ str^r gal^- lac^- derivative was obtained by crossing P678 with Hfr R_4.

58–161 M^- str^s (Tatum, 1945).

The symbols of the markers represent:

M	methionine }
T	threonine } synthesis
L	leucine } synthesis
B_1	thiamine } synthesis
gal	galactose fermentation
str^s or str^r	sensitive or resistant against streptomycin.

Stock cultures were maintained at 4° C. on Dorsett egg slopes.

(ii) *Media*

Minimal medium was as described by Winkler & de Haan (1948). Triphenyltetrazoliumchloride medium (TTZ) was as described by Lederberg (1948) with 1% galactose. The nutrient broth in which the action of acridine orange on F^+ cells was studied, was always adjusted to pH 7·6.

For the experiments with ^{32}P-labelled donor cells, the medium of Stent & Fuerst (1955) was used. The specific radioactivity in this medium was adjusted to the desired value by varying the inorganic phosphate concentration.

A small part of the phosphate in the medium was derived from the peptone and the casaminoacids. The total phosphate content of the medium was determined by the method of Fiske and Subbarow (1925). The ^{32}P was obtained from N. V. Philips-Duphar as a carrier-free isotonic solution of Na_2HPO_4. The medium for the preparation of ^{32}P-labelled 5832 cells contained 5 mC ^{32}P in 4·75 ml. H medium (specific radioactivity after inoculation 60 mC/mg.P). After mixing of the medium with the carrier-free ^{32}P solution, the medium was heated at 110°C. for 10 min. to decompose peroxides present in the ^{32}P solution. After cooling to 37°C., catalase was added (1 μg./ml.) to prevent the formation of peroxides during incubation. The protective medium for freezing ^{32}P-labelled cells was as described by Rörsch (1962).

(iii) *Methods of crossing*

(*a*) *With non-radioactive donor cells*

Overnight cultures of 5832 and P678 were prepared by inoculating 5 ml. of nutrient broth and incubating at 37° C. without aeration. Fresh cultures were prepared by diluting 1 ml. of the overnight culture into 9 ml. of prewarmed broth in screw-capped bottles. After incubating for 90 min. on an inclined turntable, the cultures were centrifuged and resuspended in 2·5 ml. prewarmed broth.

Mating mixtures were obtained by mixing equal volumes of concentrated F^+ and F^- strains to give a final concentration of about 1×10^9 cells per ml. Eighteen minutes after mixing, the non-rotated suspension was diluted by a factor 3×10^5 in prewarmed broth. Transfer was interrupted by violently shaking for 30 sec. on a microid flask shaker. The number of cells giving gal^+, gal^- and gal_v^+ colonies, was determined at intervals by plating 0·1 ml. of a suitable dilution on TTZ–gal–str

plates. All counts are recorded as the mean of five platings. The experiments were carried out in a constant temperature room maintained at 37° C.

(b) *With radioactive donor cells*

Overnight cultures of 5832 and $T^+L^+B_1^-$ *lac*$^-$*gal*$^-$*str*^{r} were prepared by inoculating 5 ml. of H medium and incubating at 37° C. on an inclined turntable. Then 0·25 ml. of the overnight culture of 5832 was added to the prewarmed ^{32}P-containing medium and 0·25 ml. of the overnight culture of $T^+L^+B_1^-$ *lac*$^-$*gal*$^-$*str*^{r} was added to 4·75 ml. prewarmed non-radioactive H medium. Both cultures were incubated for 3 hr. on an inclined turntable and then centrifuged at room temperature. The sediment of $T^+L^+B_1^-$ *lac*$^-$*gal*$^-$*str*^{r} was taken up in 1·25 ml. of prewarmed H medium containing 100 μg. streptomycin/ml. and incubated in a 37°C. waterbath. The sediment of 5832 was washed with 5 ml. H medium of 4°C., supplemented with 100 μg. streptomycin/ml. to remove the ^{32}P. The streptomycin was added to prevent the 5832 cells from synthesizing nucleic acids during the washing procedure. After centrifugation the sediment was taken up in 1ml. of prewarmed streptomycin supplemented H medium. Equal volumes of the 5832 and $T^+L^+B_1^-$ *gal*$^-$*lac*$^-$*str*^{r} cultures were mixed and incubated for 18 min. at 37°C.

Then the mating mixture was diluted by a factor 100 in streptomycin supplemented H medium, blended and afterwards diluted 1 in 10 in precooled (0°C.) protective medium. One-millilitre samples of this suspension in screw-capped bottles were then rapidly frozen by immersion in a dry ice–acetone mixture. The bottles were kept under dry ice in Dewar vessels. At intervals the content of a bottle was thawed for the determination of the total number and the number of sexduced cells. The total number was determined by plating on TTZ–gal–str in a suitable dilution and the number of sexduced cells by plating without dilution on minimal agar supplemented with thiamine (2·5 μg./ml.), streptomycin (100 μg./ml.) and galactose (0·2%).

(iv) *Test for mating type*

A fresh culture of 58–161 F$^-$ was centrifuged, washed twice with saline and the pellet resuspended in saline (half of the original volume). Colonies from TTZ plates were suspended in 0·1 ml. of saline and one drop (0·03 ml.) of these suspensions were spotted on minimal plates inoculated with 0·1 ml. of the concentrated 58–161 F$^-$ suspension.

3. RESULTS

(i) *Action of acridine orange on cells with a limited number of* F-gal$^+$ *particles*

(a) *Experiments with non-dividing cells*

Immediately after the transfer of the *F-gal*$^+$ factor from a donor strain to a recipient strain, the number of *F*-particles in the recipient cells is small. A large fraction of the *gal*$^+$ cells form variegated colonies on the TTZ–gal indicator plates (de Haan & Stouthamer, 1963). It was found that the fraction of variegated colonies

decreased in the presence of chloramphenicol, indicating that episome multiplication took place in the absence of growth.

In such an experiment episome multiplication can be studied without the interference of growth. This kind of experiment was thought to be very suitable for the study of the mechanism in which acridine orange interferes with the multiplication of the *F*-factor.

A mating mixture of log-phase cells of 5832 and P678 was diluted 18 min. after mixing and blended immediately thereafter. The mixture was then further diluted

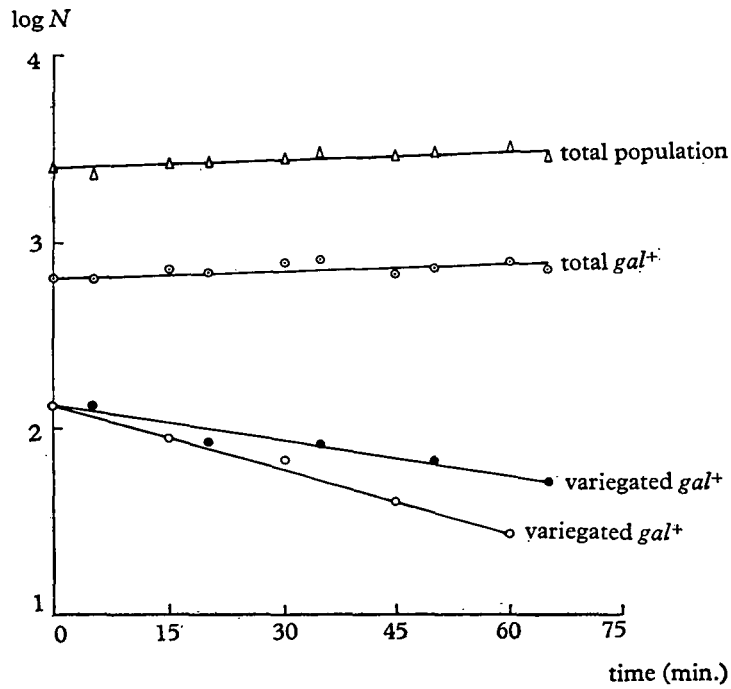

Fig. 1. The effect of acridine orange and chloramphenicol on the number of cells forming variegated colonies on TTZ–gal–str plates. A mating mixture of 5832 and P678 was blended and diluted by a factor 3×10^5 into broth supplemented with chloramphenicol (25 μg./ml.) and acridine orange (20 μg./ml.). The control contained only chloramphenicol.

△ = total population with or without acridine orange
⊙ = total *gal*+ with or without acridine orange
● variegated *gal*+ in the presence of acridine orange
○ = variegated *gal*+ in the absence of acridine orange

into nutrient broth (pH 7·6) supplemented with chloramphenicol (25 μg./ml.) and acridine orange (20 μg./ml.). The control contained no acridine orange. Samples were withdrawn at intervals and plated on TTZ–gal–str plates. It may be seen from Fig. 1 that acridine orange has no influence on the total number of *gal*+ cells. This result is in accordance with the findings of Hirota (1960), who showed that no curing of F+ occurred in the absence of growth. A mechanism which is based on inactivation of *F*-particles to explain the curing effect of acridine orange on F+ cells is thus

unlikely. In the absence of acridine orange a logarithmic decrease in the number of *gal*+ cells which form variegated colonies on the indicator plates is observed. In the presence of acridine orange and chloramphenicol this decrease is also observed showing that multiplication of episomes in the presence of acridine orange is possible. The difference in slope of the two curves shows the inhibition of the multiplication of the *F-gal*+ factor.

(b) *Action of acridine orange on growing cells*

Preliminary experiments have shown that the presence of acridine orange in a mating mixture has only a small influence on contact formation and the subsequent

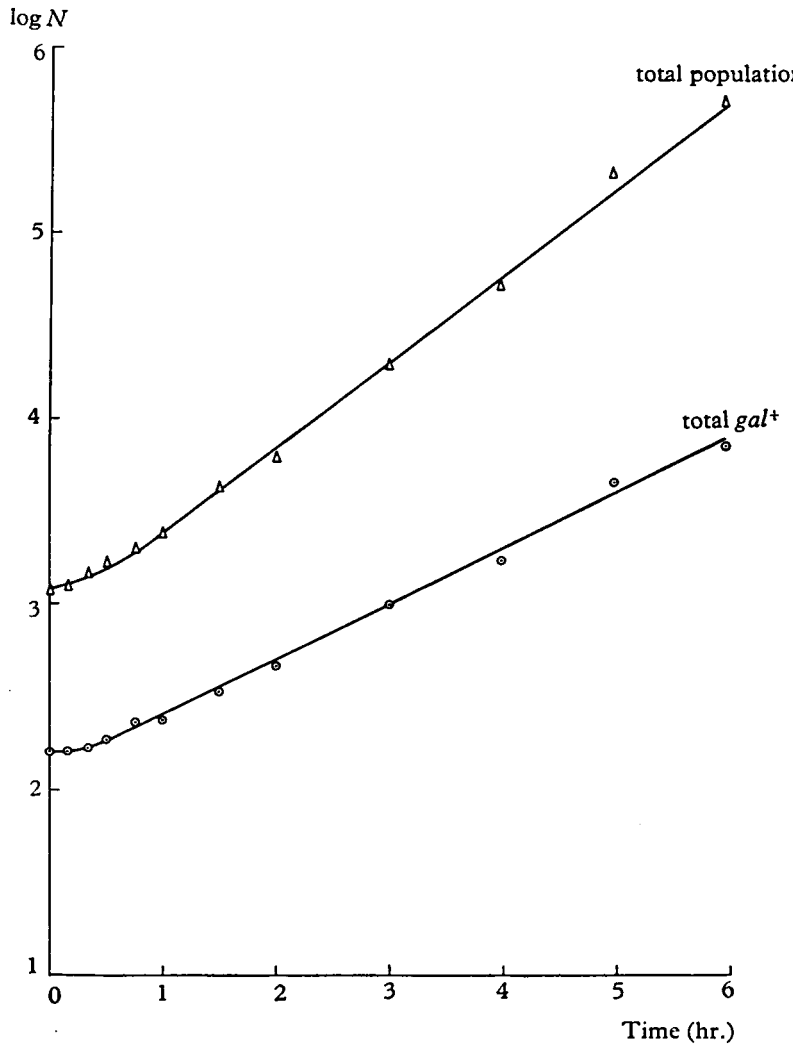

Fig. 2. Multiplication of sexduced cells. A mating mixture of 5832 and P678 in acridine orange supplemented broth was diluted by a factor 3×10^5 in the same medium, 18 min. after mixing.

△ = total population ⊙ = total *gal*+

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transfer of the episome. The effect of acridine orange may thus be studied with cells which have just received the *F-gal*$^+$ factor by conjugation.

The result of such an experiment in which the mating was performed in the presence of acridine orange is given in Fig. 2. To prevent further contact formation,

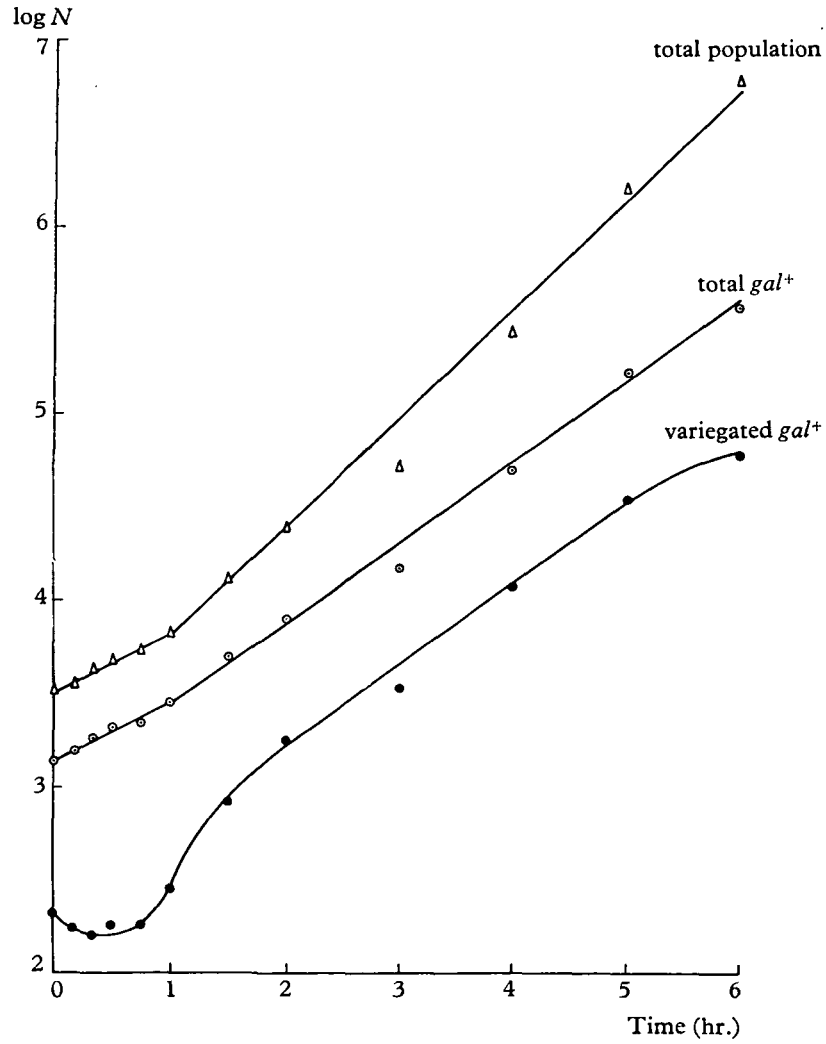

Fig. 3. Multiplication of sexduced cells. A mating mixture of 5832 and P678 in broth was diluted by a factor 3×10^5 into acridine orange supplemented broth, 18 min. after mixing.

△ = total population ⊙ = total *gal*$^+$ ● = variegated *gal*$^+$

the mating mixture was diluted into acridine orange supplemented broth and blended 18 min. after mixing. At regular intervals thereafter samples of the suspension were plated on TTZ–gal–str. It may be seen from Fig. 2 that the growth of the recipient strain is slightly inhibited for about one hour. After this period the cells multiply with the normal growth rate.

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The multiplication of the *gal*$^+$ cells shows a lag of 20–30 min. During this period all the *gal*$^+$ cells segregate a F$^-$ cell at cell division. It is thus very likely that during this period the *gal*$^+$ cells contain only one copy of the episome. After this period the number of *gal*$^+$ cells increases logarithmically. The difference in slope between the two logarithmic curves shows that the *gal*$^+$ cells segregate F$^-$ cells with a constant probability of about 50%. When the experiment was performed in the absence of acridine orange, the number of *gal*$^+$ cells increased immediately and with the same slope as the total population, indicating that the *gal*$^+$ cells at the end of the transfer period contained at least two copies of the *F-gal*$^+$ factor (de Haan & Stouthamer, 1963). Thus the observed lag in the multiplication of the *gal*$^+$ cells when the mating was performed in the presence of acridine orange, shows that acridine orange inhibits the multiplication of the episome instantaneously.

The influence of acridine orange on *gal*$^+$ cells, which had received the *F-gal*$^+$ factor in the absence of acridine orange, was also studied. The result of such an experiment is shown in Fig. 3. After (18 min.) conjugation and transfer in the absence of acridine orange, the mating mixture was diluted into acridine orange supplemented broth and blended. The growth of the recipient strain is again slightly inhibited during the first 60 min.

Thereafter normal growth is observed. The most important fact is that the number of *gal*$^+$ cells increases immediately and with the same slope as the initial slope of the total population. The absence of a lag in the multiplication of the episome confirms the idea that the episome multiplies immediately after transfer. About 1 hr. after blending the increase in the number of *gal*$^+$ cells becomes slower. Thereafter the *F-gal*$^+$ cells have a constant probability of about 30% to segregate a F$^-$ cell.

(ii) *Action of acridine orange on cells with several copies of the* F-gal$^+$ *factor*

A stock culture of strain P678 (*F-gal*$^+$) was used for this experiment. A fresh culture of this strain was diluted into acridine orange supplemented broth and at intervals samples were withdrawn and plated on TTZ–gal plates (Fig. 4). It may be seen from this figure that again the acridine orange is slightly inhibitory during the first hour but normal growth of the population is obtained after this period. Very few *gal*$^-$ or *gal*$^+$ colonies are found during the first 60 min. After this period a rapid increase in the number of *gal*$^-$ and *gal*$^+$ colonies is observed. On the other hand the curve for the *gal*$^+$ cells shows a bend, indicating that the probability that only one of the two daughter cells obtains an episome increases with time. After 5–6 generations (about 3½ hr.) the curves for the F-*gal*$^+$ and for *gal*$^-$ become logarithmic. This result shows that from this time on the *gal*$^+$ cells segregate *gal*$^-$ cells with a constant probability. This probability (0·3) can be calculated from the slopes of the curves for the total number and the number of F-*gal*$^+$ cells.

A number of *gal*$^+$ and *gal*$^-$ colonies obtained at the end of the experiment were tested for mating type. All *gal*$^+$ cells were found to be F$^+$ and all *gal*$^-$ cells were F$^-$. This indicates that the method of plating on TTZ–gal–str for the detection of the

presence of the episome is correct even at the end of the experiments. Cells with intermediate fertility, as found in acridine orange treated F+ cultures by Hirota & Iijima (1957), were not detected in our case. When the *gal*+ cells obtained at the

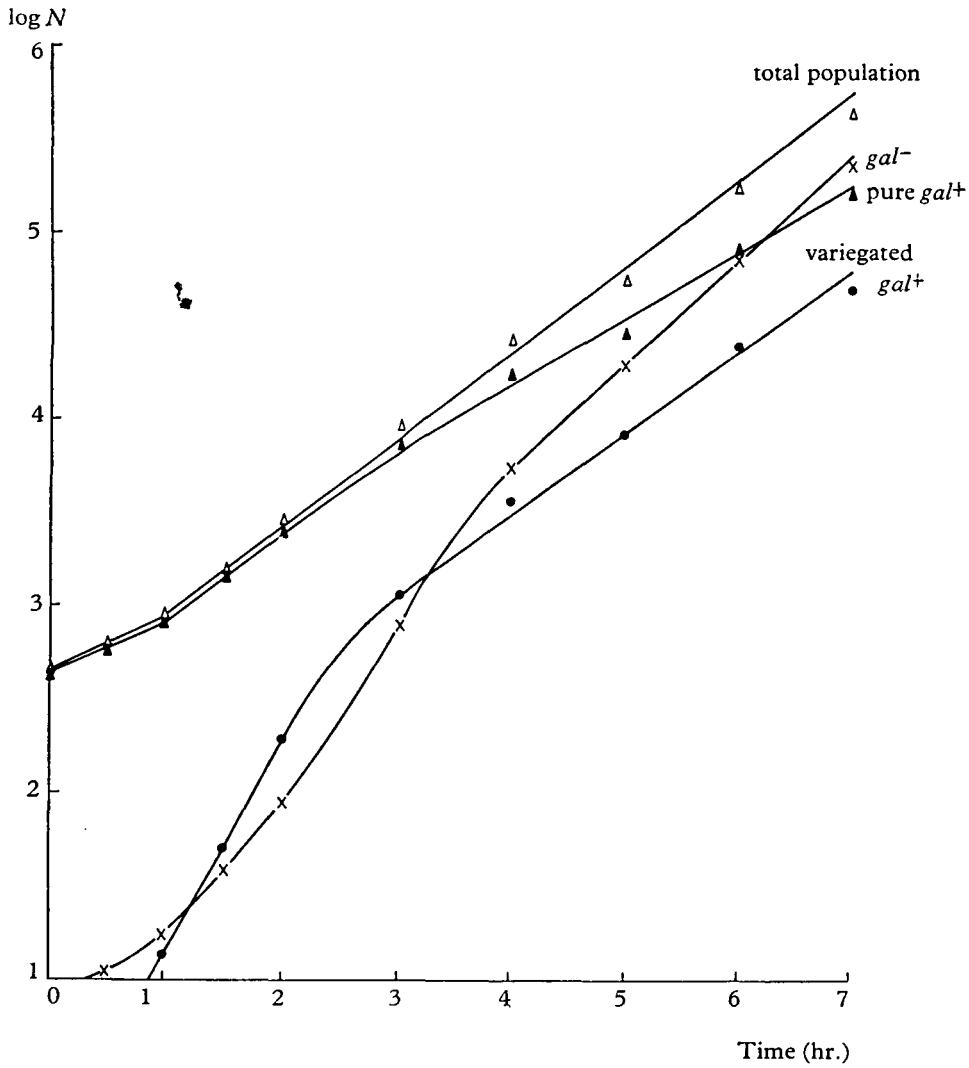

Fig. 4. The curing effect of acridine orange on strain P678 *F-gal*+. A fresh culture of this strain was diluted by a factor 10^5 into acridine orange supplemented broth.

△ = total population
▲ = pure *gal*+
× = pure *gal*−
● = variegated *gal*+

end of the experiments were treated again with acridine orange, the same result was obtained as in Fig. 4. This indicates that at the end of the experiment we have not selected a fraction of cells, which are more resistant to curing by acridine orange.

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(iii) *Decay of* ^{32}P *in the* F-gal$^+$ *factor*

It is known that bacteria grown in a medium containing large quantities of ^{32}P are inactivated exponentially as a function of the fraction disintegrated ^{32}P atoms and proportional to the specific radioactivity of the medium (Fuerst & Stent, 1957). It has been shown that this inactivation is due to the disintegration of ^{32}P atoms in DNA. This method has been used to estimate the size of several DNA containing particles.

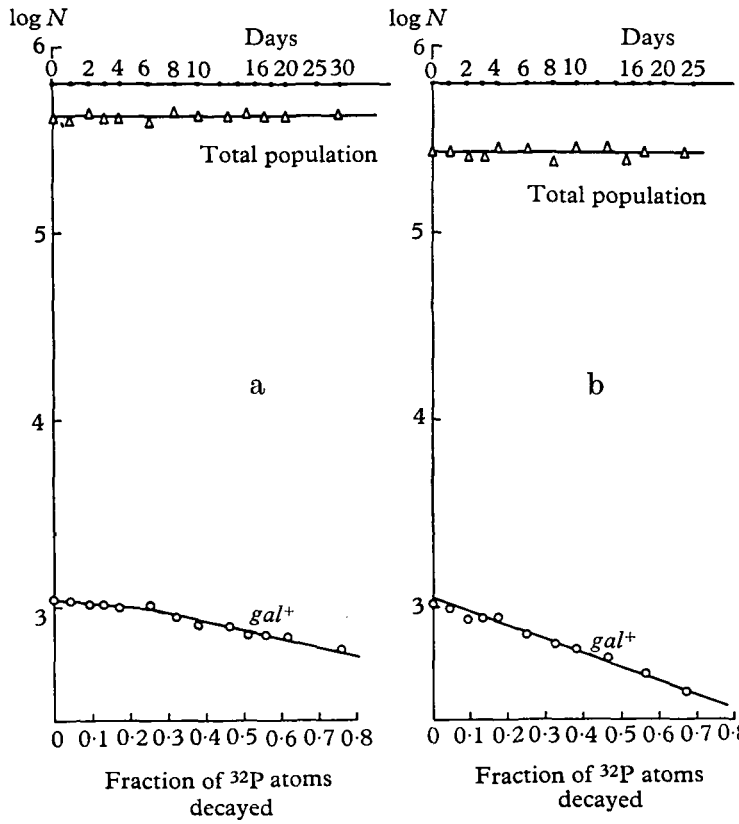

Fig. 5. The effect of ^{32}P decay on the number of sexduced cells. For experimental details see methods. The mating was performed in the absence (5*a*) or in the presence (5*b*) of acridine orange.

△ = total population
⊙ = total *gal*$^+$

In the experiments of Lavallé & Jacob (1961) an immediate decrease in the number of sexduced cells was found, when a suspension of cells, which had recently received ^{32}P-labelled episomes, was kept frozen. This result indicates that in their experiment only one copy of the episome was present in the cells at the moment of freezing. Because they used a transfer period of 20 min. (in our experiments 18 min.) it is evident that their result is in contradiction with our previous results (de Haan & Stouthamer, 1963) and the results of the experiments with acridine orange.

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Therefore the experiments of Lavallé & Jacob (1961) were repeated with our strains under our growth and mating conditions. The result of such an experiment is shown in Fig. 5*a*.

After freezing 90–95% of the cells survived. The curve for the total number shows that the survival was about the same in all bottles. The number of *gal*$^+$ cells remained nearly constant until about 20–25% of the ^{32}P atoms had decayed, then a slow decline was found. The slope of this logarithmic decline was $-0{\cdot}38$ (decrease in log *gal*$^+$ after all the ^{32}P atoms have decayed). The curve in this experiment and in a number of similar experiments was a typical double-hit curve, indicating that two-labelled *F*-particles were present. This might be due to one division of the transferred episome. In the preceding section we described that the sexduced cells most probably contained only one episome, when the mating was performed in the presence of acridine orange. The experiment was therefore repeated with cells which had received the episome in the presence of acridine orange (Fig. 5*b*). In this experiment an immediate decline in the number of *gal*$^+$ cells is found. The slope of this logarithmic decline was $-0{\cdot}67$, nearly twice the slope of the curve for the number of *gal*$^+$ cells in Fig. 5*a*. This result confirms that only one episome is transferred and that, when the transfer is performed in the absence of acridine orange, most cells contain two copies of the episome at the end of the transfer period.

4. DISCUSSION

In a previous paper we have shown that the number of *F*-particles per cell is small and that the distribution of the *F*-particles among the daughter cells is non-random (De Haan & Stouthamer, 1963). There are thus two possible explanations for the curing effect of acridine orange on *F*$^+$ cells;

(i) the dye inhibits the multiplication of the *F*-factor, lowering the number of copies to less than two per cell, or
(ii) the dye does not inhibit episome multiplication, but interferes with the mechanism which provides each daughter cell with at least one copy of the episome.

All results presented in this paper suggest that acridine orange acts by inhibiting the multiplication of the *F*-factor rather than by interfering with the non-random distribution of the episomes at cell division. The inhibition of multiplication was clearly shown in the absence of growth (Fig. 1). The growth inhibition by chloramphenicol excluded a possible interference of acridine orange with the distribution of the episomes among the daughter cells.

The experiments with growing cells (Fig. 2, 3 and 4) show that it is very unlikely that the distribution of the *F*-particles at cell division is disturbed in the presence of the drug. With F$^+$ cells, which contained the normal number of *F*-copies (Fig. 4), it was found that the probability for segregation of a F$^-$ cell at cell division increases with time. This increase in probability shows that the number of episomes per cell

decreases during the experiment. After one to two generations there is already a rapid increase in the number of *gal*$^-$ cells and cells giving rise to gal_v^+ colonies.

This indicates that the population is inhomogeneous with respect to the number of *F*-particles per cell and that a fraction of the cells contain already fewer episomes than the mean number at the start of the experiment. After five to six generations the probability for segregation of a daughter cell without a *F*-particle becomes constant (0·3). In all experiments with growing cells (Figs. 2, 3 and 4) the probability for the segregation of a F$^-$ daughter cell became constant after various times (0·5, 0·3 and 0·3 respectively); the interval during which the probability increases being dependent on the number of episomes originally present in the cells at the beginning of the experiment. When the mating was performed in the absence of acridine orange, a constant segregation probability was obtained after about two generations. The absence of a lag in the multiplication of the *gal*$^+$ cells shows that at the end of the transfer period, the cells contained at least two copies of the *F-gal*$^+$ episome. On the other hand the number of episomes per cell is lower than the number found in normal F$^+$ cells, as is indicated by the short interval during which the segregation probability increases. A lag (20 to 30 min.) in the multiplication of the *gal*$^+$ cells was found, when the mating was performed in the presence of acridine orange (Fig. 2), indicating that the cells contain less than two copies of the *F*-factor at the beginning of the experiment. The presence of acridine orange during the mating procedure thus immediately inhibited the multiplication of the transferred episome.

The constant probability for the segregation of a F$^-$ daughter cell, which is reached after various intervals of growth in the presence of acridine orange, show that the episome multiplies at a constant rate. In all experiments different probabilities (0·5, 0·3 and 0·3) were found however. These differences are probably due to different experimental conditions. The mean probability for the segregation of a F$^-$ cell at cell division in the three experiments is about 0·4, indicating that 60% of the cells contain two copies and 40% only one copy of the *F*-particle.

The average increase in the number of episomes during one generation is therefore 60%. The mean number of episomes in normal cells (Fig. 4) may then be calculated from the generation time of the episome and the interval during which the probability for F$^-$ segregation increases. Under the assumption that episomes are more or less equally distributed over the daughter cells an average number of 3–4 episomes is calculated for normal F$^+$ cells just after cell division. This number is very close to the number calculated by Jacob, Schaeffer & Wollman (1960) from the amount of β-galactosidase present in *F-lac*$^+$ cells.

The experiments with ^{32}P-labelled episomes confirm these results. In the experiment in which the decay of ^{32}P-labelled episomes after transfer in the presence of acridine orange was studied, an immediate decrease in the number of *gal*$^+$ cells was found (Fig. 5*b*). This result shows that the cells contain only one copy of the *F*-particle at the end of the transfer period. When the transfer was performed in the absence of acridine orange the number of *gal*$^+$ cells decreased very slowly until 20–25% of the ^{32}P atoms had disintegrated (Fig. 5*a*). Thereafter the number of

gal$^+$ cells decreased logarithmically with about half the slope of the curve in Fig. 5*b*. This result proves that at the end of the transfer period the majority of the *gal*$^+$ cells contain two copies of the episome, each having about half the number of ^{32}P atoms of the transferred episome. This result confirms that only one episome is transferred from donor to acceptor cell and that the transferred episome divides very rapidly after transfer. If episome multiplication should continue at this rate, the ultimate number of particles in the cell would become very large. As the number of episomes in normal F$^+$ cells is small, a control mechanism must adjust the number of *F*-particles in the cell to a fixed number.

Lavallé & Jacob (1961) found an immediate decrease by ^{32}P decay in the number of F$^+$ cells after transfer of the labelled episome to non-labelled acceptor cells. This difference with our results is most probably due to the use of other strains or to different experimental conditions. Lavallé & Jacob (1961) found however,, that colicinogenic factors labelled with ^{32}P are very resistant to ^{32}P decay. From the slope of their curves for the number of colicinogenic cells they concluded that the maximum nucleotide content of these factors is approximately 10^4 nucleotides per particle. Silver & Ozeki (1962) who used a different method for the determination of the size of a number of colicinogenic factors, found that the size of these particles is between 3 and 7×10^4 nucleotides per particle. This large difference is most probably also due to a rapid multiplication of the episomes after transfer. The size of the *F-gal*$^+$ episome may be accurately determined from the decay of the cells obtained from the acridine orange supplemented mating mixture. Under our conditions the constant for the fraction of ^{32}P disintegrations which are lethal is not known. The values found in the literature are 0·02 (Fuerst & Stent, 1956); 0·027 (Apelgot & Latarjet, 1962) and 0·015 (Rörsch, 1962). The values for the size of the episome calculated with these constants and the formula of Hershey, Kamen, Kennedy & Gest (1951) are respectively $3{\cdot}8 \times 10^5$, $2{\cdot}8 \times 10^5$ and $5{\cdot}0 \times 10^5$ nucleotides per particle. A preliminary evaluation of the constant under our conditions gave a value which was very close to the one obtained by Rörsch (1962), indicating that 5×10^5 is the most probable number of nucleotides per particle. This number is very close to the number of $4{\cdot}5$–$5{\cdot}0 \times 10^5$ nucleotides per particle as found by Lavallé & Jacob (1961).

5. SUMMARY

Acridine orange does not interfere with the distribution of the *F*-particles among the daughter cells but inhibits the multiplication of the episome.

In growing cells the generation time of the episome in the presence of acridine orange is longer than the generation time of the cell.

This lowers the number of episomes per cell to less than two copies at cell division.

F-particles rapidly multiply after transfer until a relatively small fixed number (3–4) is reached.

The authors are indebted to Prof. Dr V. V. Koningsberger for facilities for ^{32}P experiments and to Dr A. Rörsch for a gift of catalase. The excellent technical assistance of Miss A. K. Mol is gratefully acknowledged.

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