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Mutants of *Escherichia coli* sensitive to methylene blue and acridines

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1. INTRODUCTION

Enteric bacteria, and *Escherichia coli* in particular, can grow in the presence of comparatively high concentrations of methylene blue. For example, EMB sugar medium which is commonly used for test of sugar fermentation contains 65 μg. of methylene blue per millilitre.

Mutants of *Escherichia coli* K12 have been isolated which are sensitive to this dye and inhibited by low concentrations. These mutants were found to be sensitive also to acridine dyes, such as acridine orange.

Genetic studies of this mutation were carried out using crossing experiments, F-duction and transduction with phage P1kc.

Part of this work was reported previously (Sugino, 1963).

2. MATERIALS AND METHODS

(i) *Bacteria*

The bacterial strains used were all derivatives of *Escherichia coli* K12. They are listed in Table 1. Only relevant markers are shown under *genotype*. The meanings of symbols for genetic markers are as follows. Nutritional markers are: *thr*, threonine; *leu*, leucine; B_1, thiamine; *pur*, purine plus thiamine; *met*, methionine. + means independence, − dependence. Fermentation markers are: *lac*, lactose;

Table 1. *Strains of* E. coli *K12 used*

Strain No.	Genotype	Origin	Method of derivation or reference
W678	F^- *thr*− *leu*− B_1− *lac*− *gal*− *Mb-r str-r*		(Obtained from Dr Jacob)
JE2	F^- *thr*− *leu*− B_1− *lac*− *gal*− *Mb-s str-r*	W678	Spontaneous mutation
JE4	F^+ *pur*− *lac*− *Mb-s str-r*	JE2	Repeated crosses with males
JE16	F^- *pur*− *lac*− *Mb-s str-r*	JE4	F-elimination with acridine
JE513	F13-4$^+$/*pur*− *lac*−	W4861	Transduction with P1KC
JE519	F13-4$^+$/*pur*− *lac*− *Mb-s str-r*	JE16	Transfer of F13-4 from JE513
JE520	F^- *pur*− *lac*− *Mb-s str-r pho-*	JE16	UV irradiation
JE1031	HfrH *met*− B_1−		(Obtained from Dr Uchida)
JE1183	F13-4$^+$/*pur*− *lac*− *Mb-r str-r*	JE519	Selection with methylene blue
W3747	F13$^+$/*met*− T_6-*r*		(Hirota & Sneath, 1961)

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gal. galactose. + means fermentation, – non-fermentation. Resistance markers are: *Mb*, methylene blue; *str*, streptomycin; T_6, phage T_6. *r* means resistance, *s* sensitivity. *pho*– means alkaline phosphatase negative. HfrH is high fertility male that transfers the chromosome in the order *thr leu lac pur gal* (Hayes, 1953). F^+ and F^- are male and female respectively. F13 and F13-4 are F primes. F13 carries the chromosomal region including the *lac* and *pur* genes (Hirota & Sneath, 1961).

(ii) *Media*

Nutrient broth containing 10 g. of polypeptone, 10 g. of meat extract, and 2 g. of NaCl in 1 l. of H_2O, was adjusted to pH 7·0 with NaOH. Nutrient agar was prepared by adding 1·5% agar to nutrient broth. Lactose BTB medium contained 5 g. of polypeptone, 5 g. of NaCl, 20 g. of lactose, 15 g. of agar, and 12 ml. of 0·2% bromthymol blue in 1 l. of H_2O, adjusted to pH 7·2. Simmons glucose medium contained 3 g. of sodium glutamate, 5 g. of NaCl, 0·1 g. of $MgSO_4$, 1·5 g. of KH_2PO_4, 2·5 g. of $(NH_4)_2HPO_4$, and 3 g. of glucose per litre of H_2O. Davis minimal medium contained 7 g. of K_2HOP_4, 2 g. of KH_2PO_4, 0·1 g. of $MgSO_4$, 1 g. of $(NH_4)_2SO_4$, 0·5 g. of sodium citrate and 1 g. of glucose in 1 l. of H_2O. Lactose minimal medium was prepared by omitting glucose and glutamate from Simmons glucose medium or by omitting glucose from Davis minimal medium and adding 0·3% lactose. For solid medium 1·5 g. agar was added. EMB sugar agar and EM sugar agar are as described in Lederberg (1950).

Streptomycin or methylene blue, when needed, were added at concentrations of 100 μg. per millilitre or 50 μg. per millilitre respectively.

(iii) *Isolation of mutants sensitive to methylene blue*

Such mutants were first obtained accidentally during an experiment using EMS agar plates, on which they failed to grow. Similar mutants could easily be obtained by exposure of *E. coli* cells to ultra-violet light. Upon plating on nutrient agar, sensitive mutants were located by replica-plating on nutrient agar supplemented with methylene blue.

(iv) *F-duction and F-primes*

Transfer of a chromosome fragment by incorporation in the sex factor F, is called F-duction, and such an F factor incorporating a small fragment of the bacterial chromosome, is called an F′ (F-prime) (Jacob & Adelberg, 1959; Hirota & Sneath, 1961).

The F′ used in this study, F13, carries the chromosomal region including the *pur* and *lac* genes (Hirota & Sneath, 1961). F13-4 is a derivative of F13 obtained by transduction with P1 from a strain containing F13 and selection for the *lac*+ marker. The *lac*+ gene can further be transmitted together with F to another recipient, but the other markers, i.e. *pur*, *Mb*, or T_6 no longer go together with *lac* or F. Thus F13-4 contains *lac*, but no other known markers of F13 (Nishimura, personal communication).

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(v) *Kinetics of transfer of F13*

The interrupted mating technique (Wollman, Jacob & Hayes, 1956) was used to study the kinetics of transfer of F13. 1 ml. of donor (5×10^8/ml.) and 9 ml. of recipient (2×10^8/ml.) cultures in exponential growth phase were mixed in a 200 ml. Erlenmeyer flask and kept at 37° in a water bath. 0·1 ml. samples were withdrawn at 5 min. intervals into 10 ml. of ice-cold water. The samples were immediately agitated at 0° in a homogenizer and plated on selective media.

(vi) *Crosses between Hfr and F^-*

Cultures of donor and recipient bacteria in nutrient broth in the exponential growth phase were mixed and, after 2 hours at 37°C., were plated on selective agar where only recombinants could form colonies. Recombinant colonies were picked at random and purified, and unselected markers were scored by replica plating.

(vii) *Transduction with phage P1*

The method described by Lennox (1955) was followed. Multiplicity of infection was about 0·15 phage per bacterium.

(viii) *Acridine treatment*

The method described by Hirota (1960) was followed.

3. RESULTS

(i) *Sensitivity of* Mb-s *mutant to methylene blue and related dyes*

One of the methylene blue sensitive mutants, JE2, and its derivatives, were mainly used for the following studies. Such mutants retain other characters of the

Table 2. *Sensitivity of* Mb-r *and* Mb-s *strains to methylene blue and other reagents*

		Mb-r	*Mb-s*
In broth			
Methylene blue	pH 8	100–200	< 10
	pH 7·2	100–200	< 10
	pH 6	> 200	20–30
Acriflavine	pH 7·0	10–20	2–4
Acridine orange	pH 7·0	35–40	< 5
On nutrient agar			
Methylene blue	pH 7·2	> 100	< 1
	pH 6	> 100	< 1

The bacteria used in this experiment were W678 (*Mb-r*) and JE2 (*Mb-s*).

The test of sensitivity of bacteria in liquid culture was made by inoculating one loopful of the bacteria in broth containing the indicated concentration of chemicals and incubating at 37°C. overnight.

The test on agar plates was made by plating and streaking of bacteria.

In the table are given the minimum concentrations (μg./ml.) at which no bacterial growth could be observed.

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original bacterial strain W678, and are Gram negative. They show 'cross sensitivity' to such dyes as toluidine blue, acridine orange and acriflavine, but are as resistant to eosin Y, streptomycin, and actinomycin S, as the original bacteria. Methylene blue is less active at pH 6 than at pH 7·2 or 8. These results are summarized in Table 2.

In some instances, the effect of methylene blue on *Mb-s* bacteria on EMB sugar agar differs depending on the kind of sugar used. Thus JE520 can grow in EMB galactose agar to some extent, but is completely inhibited on EMB lactose agar.

In buffer, methylene blue and acridine orange inactivate even *Mb-r* cells, but *Mb-s* cells are more sensitive than *Mb-r* cells, as shown in Table 3.

Table 3. *Survival of* Mb-r *and* Mb-s *after 30 min. in acridine orange or methylene blue at indicated concentrations*

Acridine orange			Methylene blue		
	Fraction surviving			Fraction surviving	
μg./ml.	*Mb-r*	*Mb-s*	μg./ml.	*Mb-r*	*Mb-s*
0	1·0	1·0	0	1·0	1·0
5	$3·6 \times 10^{-1}$	$2·9 \times 10^{-2}$	1	$6·6 \times 10^{-1}$	$4·8 \times 10^{-1}$
10	$3·9 \times 10^{-2}$	$2·4 \times 10^{-4}$	5	$6·8 \times 10^{-3}$	$1·5 \times 10^{-4}$
30	10^{-4}	10^{-6}	10	$3·8 \times 10^{-4}$	$8·0 \times 10^{-6}$
60	10^{-5}	10^{-7}	30	$1·5 \times 10^{-5}$	—

Strains JE1183 (*Mb-r*) and JE519 (*Mb-s*) were suspended in buffer (Davis minimal medium minus glucose) containing the indicated concentrations of reagent, and incubated 30 min. at 37°C. Viable cell counts were made by diluting and plating. Initial cell counts: *Mb-r* $6·6 \times 10^6$, *Mb-s* $6·2 \times 10^6$ per millilitre.

(ii) *Reverse mutation*

Reverse mutants to methylene blue resistance were obtained at the rate of about 10^{-5} by plating a culture of *Mb-s* bacteria on nutrient agar containing methylene blue. Such mutants had simultaneously regained resistance to acridine dyes. Conversely, reverse mutants obtained by selection with acridine orange were also found resistant to methylene blue.

This result shows that resistance to acridines and methylene blue are determined by the same genetic locus, and consequently there is something common in the mechanism of resistance to these dyes.

(iii) *Joint F-duction of the* Mb-r *gene with the* lac+ *gene*

An F-duction experiment was carried out with W3747 as donor and JE2 as recipient. W3747 carries F13 which contains the *lac*+, *pho*+, T_6-*r*, and *pur*+ genes. JE2 is a mutant sensitive to methylene blue. 0·1 ml. of overnight culture of JE2 was added to 3 ml. of overnight culture of W3747. After further incubation for 24 hours at 37°C., the mixture was diluted and plated on lactose BTB medium containing streptomycin which eliminated W3747 cells. Of the JE2 cells that

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formed colonies on this medium, about 50% had become *lac*+; that is, they had received F13 from W3747. The plates were replica-plated on to nutrient agar containing methylene blue to check the *Mb* marker. Most of the *lac*+ colonies were *Mb-r*, whereas most of *lac*+ colonies remained *Mb-s*. This result shows that the *Mb-r* and *lac*+ genes are jointly F-duced with F13; i.e., the chromosomal fragment of F13 contains the genetic locus determining resistance to methylene blue.

(iv) *Segregation of* Mb-s *from F-duced cells; dominance of* Mb-r *over* Mb-s

lac+ *Mb-r* colonies obtained by F-duction as described in the previous section were examined for segregation of *Mb-s* cells. Out of eight isolates tested, one gave no *Mb-s* in 358 colonies, six gave 0·2 to 0·7% *Mb-s*, and one gave 3·8% *Mb-s*. This result indicates that methylene-blue resistant cells obtained by F-duction segregate sensitive cells. All such *Mb-s* segregants were also *lac*−. This means that in the original resistant cell, the sensitive allele *Mb-s* of the *Mb* gene must have been present in the chromosome, whereas the resistant allele was carried by F13, and the resistant allele *Mb-r* is dominant over the sensitive allele *Mb-s*.

(v) *Kinetics of transfer of F13*

W3747 was used as donor of F13, and JE16, an F^- *pur*− *Mb-s lac*− strain, was used as a recipient in an interrupted mating. The number of colonies that appeared on plates seeded with samples of a mixture of these two strains taken at various times is shown in Fig. 1. Samples of colonies that appeared on the selective medium (eighty for each time point, except where less than eighty appeared, when all colonies were examined) were restreaked on the same medium as used for selection, and *lac*, *Mb*, and *pur* characters were determined by replica plating. Figure 2 shows the percentage transfer of unselected markers among colonies selected as *pur*+ *str-r*.

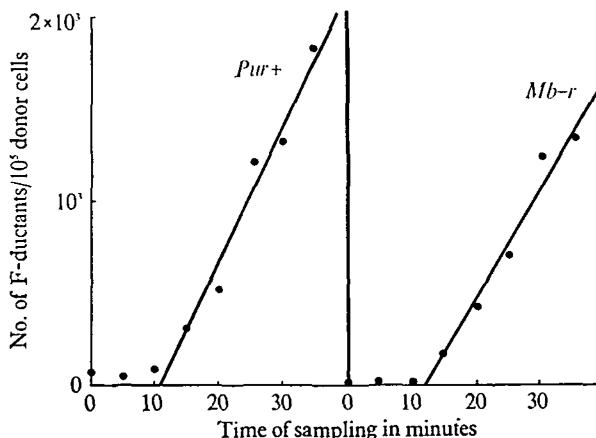

Fig. 1. Kinetics of transfer of the F13 markers *pur*+ and *Mb-r*. The left curve represents *pur*+ F-ductants, and the right curve, *Mb-r* F-ductants. For experimental procedure, see Methods Section.

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Figure 3 shows the percentage transfer of unselected markers among *Mb-r str-r* colonies. Thus the *pur*+ gene seems to be transferred at about 10 min. after the time of mixing. Extrapolations of the curves of Fig. 1 and Fig. 3 give almost identical values for time of beginning of appearance of *pur*+ colonies. *Mb-r str-r* colonies begin to be recovered at about the same time or slightly later. Figure 2

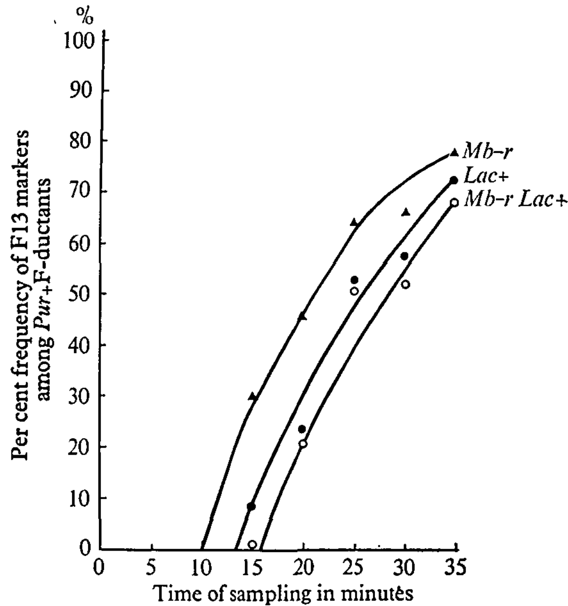

Fig. 2. Unselected markers of *pur*+ *str-r* F-ductants. For explanation, see text.

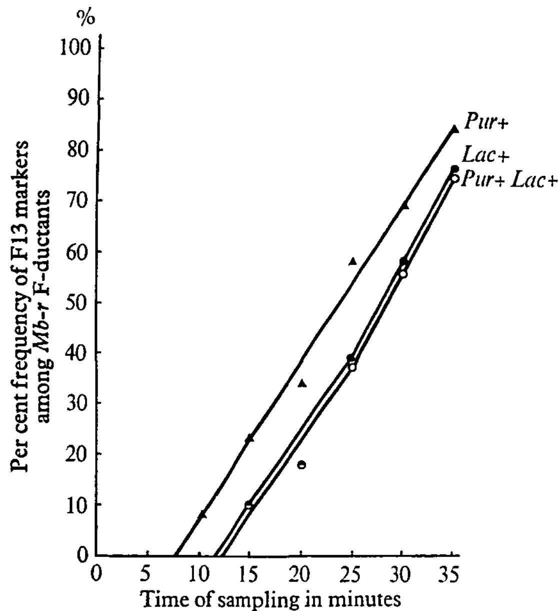

Fig. 3. Unselected markers of *Mb-r str-r* F-ductants. For explanation, see text.

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shows that the time of transfer of *Mb-r* is not very much earlier than the time at which *Mb-r str-r* colonies are recovered on direct selection on plates containing methylene blue. The *lac*+ gene seems to be transferred at about 2 or 3 min. after the *Mb-r* or *pur*+ genes. Most of the *pur*+ *lac*+ colonies are also *Mb-r*, and most of *Mb-r lac*+ colonies are *pur*+ even at early time points. But the reverse is not the case; i.e., only low percentages of *pur*+ or *Mb-r* colonies are *lac*+ at early time points.

(vi) *Cross of an* Mb-r *Hfr and an* Mb-s F^-

The results of F-duction of the *Mb-r* gene with F13 showed that the *Mb* locus was situated near the *pur* and *lac* loci. The results of a cross involving an *Mb-r* Hfr strain and an *Mb-s* F^- strain confirm this and show that the *Mb* locus lies between *pur* and *pho* (Table 4).

Table 4. *Segregation patterns of unselected markers among recombinants from cross JE1031 × JE520, after selection of various markers*

Selected markers											
(1) *lac*+ *met*+ *str-r*				(2) *Mb-r str-r*				(3) *pur*+ *str-r met*+			
pho	*Mb*	*pur*	%	*lac*	*pho*	*pur*	%	*lac*	*pho*	*Mb*	%
1	1	1	44	1	1	1	45	1	1	1	53
1	1	0	11	1	1	0	28	0	0	1	16
1	0	0	24	0	0	0	17	0	0	0	24
0	0	0	16	0	0	1	6	0	1	1	3
Others			5	Others			4	Others			4
Total			100				100				100
Recombinants scored			95				89				87

The alleles of markers derived from the Hfr parent are represented by 1, and those derived from the F^- parent, by 0.

The results are compatible only with the order of the markers *lac pho Mb pur*. For in Table 4, the genotypes that require only the minimum numbers of crossing-overs from the postulated order of markers occur at high percentages, whereas those requiring multiple crossing-overs, here grouped as 'others', occur at low percentages. Other assumptions on the position of the *Mb* locus would require that a triple crossing-over occurs more frequently than single crossing-over.

(vii) *Transduction with phage P1kc*

A transduction experiment was performed with W3747 (*Mb-r pur*+ *lac*+ T_6-*r*) as donor and JE4 (*Mb-s pur*− *lac*− T_6-*s*) as recipient. Selections for (1) *pur*+, (2) *lac*+, and (3) *pur*+ *lac*+ transductants were made by plating on appropriate selective media. Yield of *pur*+ or *lac*+ transductants was of the order of 10^{-6} per phage. Of 189 *pur*+ transductants obtained, three had also received the *Mb-r* gene from the donor; this confirms the linkage of *pur* and *Mb*. The *lac* or T_6 markers were not jointly transduced with *pur*. None of 361 *lac*+ transductants obtained

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had received the *Mb*, *pur* or T_6 marker. No colonies appeared on selection for *pur* + *lac* +.

Lack of linkage of the *pur* and T_6 genes in the transduction suggests that, of the two loci *Mb* and T_6, the former is nearer to the *pur* locus than the latter is. This, together with the results of crossing experiments which indicated that the *Mb* locus lies between *pur* and *pho*, allows us to place the *Mb* locus on the chromosome map as in Fig. 4.

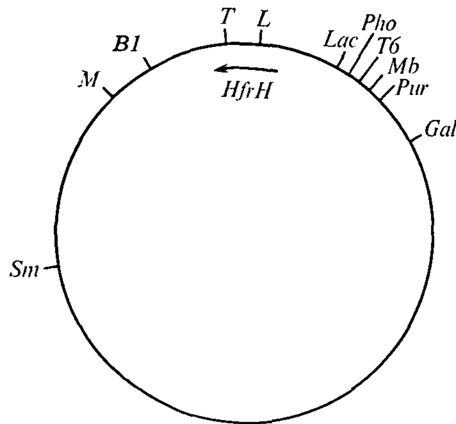

Fig. 4. Chromosome map of *Escherichia coli* K12.

(viii) *Sensitivity of the* Mb-s *mutant to F-elimination with acridine orange*

Not only is an *Mb-s* mutant more sensitive to the growth inhibitory action of acridine orange, but it is disinfected of the F factor at lower concentration of the

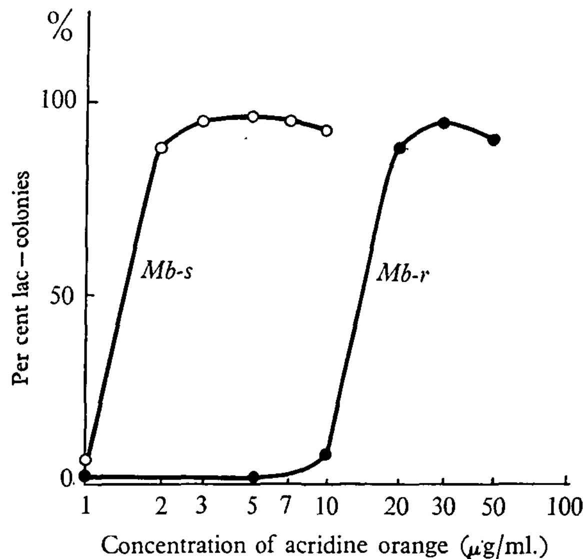

Fig. 5. F-elimination of *Mb-r* and *Mb-s* cells with acridine orange. The abscissa is in log scale; the ordinate gives the percentage of *lac* – colonies among the total colonies. For experimental procedure, see text.

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dye than *Mb-r* bacteria. This is shown in Fig. 5. One of the strains used for this experiment, JE519, is *Mb-s*, and a heterogenote for the *lac* locus, carrying the *lac* − allele on the chromosome, and *lac* + allele on F13-4: the other strain, JE1183, is an *Mb-r* revertant of JE519.

The cultures were treated with acridine orange, and diluted and plated on lactose BTB medium plus streptomycin, which was added to avoid contamination. On this medium cells that had lost F13-4 form white colonies, because they do not ferment lactose, whereas those retaining F13-4 form yellow colonies, because they

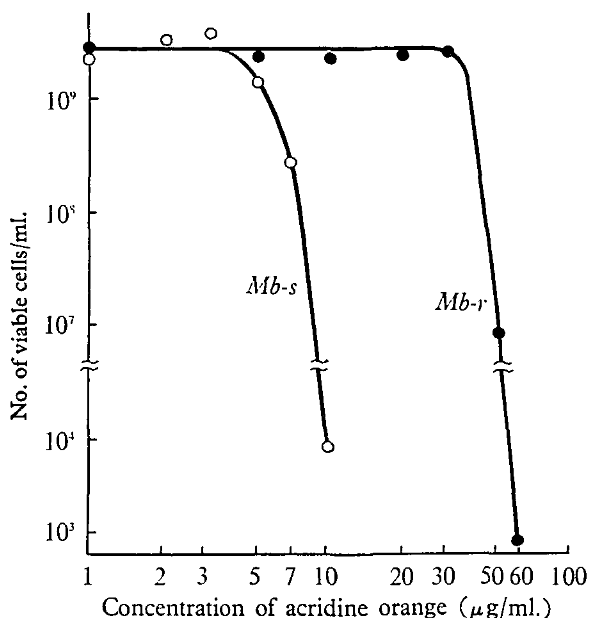

Fig. 6. Survival of *Mb-r* and *Mb-s* cells after treatment with acridine orange. The cell concentrations of the same cultures as in Fig. 5 are represented in this figure. Both the abscissa and ordinate are in log scale.

ferment lactose. Thus the percentage of *lac* − colonies corresponds to the percentage of cells disinfected of F13-4. Figure 6 gives the viable cell numbers of the same overnight cultures as Fig. 5. Not only the F′ factor but also the ordinary F factor can be eliminated from *Mb-s* bacteria with lower concentrations of acridine orange than from *Mb-r* bacteria.

4. CONCLUSIONS AND DISCUSSION

The following conclusions can be drawn from the above results:

1. The genetic locus determining resistance or sensitivity to methylene blue is situated between the T_6 and *pur* loci on the chromosome map of *Escherichia coli*.

2. The chromosomal fragment carried by F13 contains the *Mb-r* gene. Thus F13 now has the following five known markers, *lac pho* T_6 *Mb pur*, associated with its F factor. (Markovitz (1964) also identified two other genes on F13).

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3. The *Mb-r* allele is dominant over *Mb-s*: a diploid heterogenote with the genetic structure *Mb-s/Mb-r*, where the *Mb-r* allele is carried by F13 and the *Mb-s* allele is carried by the chromosome, is resistant to methylene blue, but segregates out bacteria sensitive to methylene blue.

4. *Mb-s* bacteria are also more sensitive to the growth inhibitory action of acridine dyes than *Mb-r* bacteria. There is something common in the mechanism of resistance to these dyes.

5. *Mb-s* bacteria are disinfected of F factor at a lower concentration of acridine orange than *Mb-r* bacteria.

That the *Mb-s* mutation affects sensitivity not only to growth inhibitory action, but also to the F-eliminating activity of acridines suggests that this mutation increases the permeability of the cell surface to these dyes, or, in other words, destroys the surface barrier to acridines and other dyes that might be present in *Mb-r* bacteria. Another possibility is that the *Mb-r* gene synthesizes some substance that antagonizes the action of these dyes, or it may be concerned with a specific detoxication or excretion system. The dominance of *Mb-r* over *Mb-s* suggests that the *Mb-s* mutation involves the loss of some function. The kinetic experiment of transfer of F13 shows that this function is expressed very soon after the transfer of the *Mb-r* gene under our experimental conditions.

The results of the experiment demonstrating the kinetics of transfer of F13 show some peculiarities that must be noted. Thus in Fig. 2, the percentage of *Mb-r* colonies among colonies selected as *pur*+ *str-r* rises steadily, while in Fig. 3, the percentage of *pur*+ colonies among colonies selected as *Mb-r str-r* rises in a similar way. If the *pur*+ gene is transferred earlier than the *Mb-r* gene, the former result is expected, but the latter result is not easy to explain. If the *Mb-r* gene is transferred earlier than the *pur*+ gene, the former result is difficult to explain. However, it must be pointed out that in our experiment no distinction was made between the chromosomal or episomal location of the *pur*+ or *Mb-r* genes. Those *pur*+ or *Mb-r* genes that have been separated from the F sex factor of F13 might be unable to multiply autonomously, and would have to be integrated in the chromosome to be perpetuated. In the event of integration, the linkage between *pur*+ and *Mb-r* might be apt to be broken in our experimental conditions. The increase with time in linkage between *pur*+ and *Mb-r* that we observe may reflect the kinetics of transfer of whole intact F13, in which the *pur*+, *Mb-r* and *lac*+ genes are replicated as a unit with the F factor.

Recently, similar mutants were isolated by Dr Nakamura of Konan University (personal communication).

SUMMARY

A mutant (*Mb-s*) of *Escherichia coli* K12 which is more sensitive than wild-type (*Mb-r*) to such dyes as methylene blue or acridines was studied. The *Mb-s* mutant can also be disinfected of F factor with lower concentrations of acridine orange than *Mb-r* bacteria. The *Mb* locus was mapped by crosses between Hfr and F^-, F-duction with F13, and transduction with phage P1. It was found to be situated between

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the *pur* and T_6 loci on the chromosome. Moreover, it was found to be contained in the chromosomal fragment carried by the F-prime, F13. In a heterogenote obtained by F-duction, the *Mb-r* gene is dominant over the *Mb-s* gene. The *Mb* gene was transduced jointly with the *pur* gene by phage P1.

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