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THE
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THE PREPARATION OF PAPERS

'The more words there are, the more words there are about which doubts may be entertained.'
JEREMY BENTHAM (1748–1832)

'What can be said at all can be said clearly; and whereof one cannot speak thereof one must be silent.'
LUDWIG WITTGENSTEIN: *Tractatus Logico-philosophicus* (tr. C. K. Ogden)

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(1) Papersshould be written with the utmost conciseness consistent with clarity. The best English for the purpose of the *Journal* is that which gives the sense in the fewest short words, spelt according to the *Shorter Oxford English Dictionary*.

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The following books may be found useful:

Bergey's Manual of Determinative Bacteriology, 7th edn (1957), edited by R. S. Breed, E. G. D. Murray and A. P. Hitchens. London: Ballière, Tindall and Cox.

V. B. D. Skerman, *A Guide to the Identification of the Genera of Bacteria with Methods and Digest of Genetic Characteristics* (1959). Baltimore, Maryland, U.S.A.: The Williams and Wilkins Company.

S. T. Cowan, *A Dictionary of Microbial Taxonomic Usage* (1968). Edinburgh: Oliver and Boyd.

Ainsworth and Bisby's Dictionary of the Fungi, 5th edn (1961). Kew: Commonwealth Mycological Institute.

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Influence of the Level of the Carbon Source on the Autolysis of *Aspergillus niger*

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(*Accepted for publication 21 April 1970*)

SUMMARY

The degree of autolysis of *Aspergillus niger* cultures grown and autolysed on 2·5, 5, 10, 20 and 40 g./l. initial glucose was 64, 60, 43, 43 and 43 %, respectively. The greatest loss (73 %) of mycelial nitrogen took place with the lowest initial concentration of glucose. The greatest disappearance of lipids occurred in the period up to the 8th day of autolysis irrespective of the initial sugar concentration. The percentage loss of lipids was practically constant for the first four concentrations of initial glucose assayed. The amount of total free reducing substances contained in the mycelium at the beginning of the autolytic phase increased rapidly to a high level if the initial concentration of glucose was 40 g./l., but not if it was 20 g./l. or less. The total mycelial carbohydrates determined by the anthrone reaction declined continuously irrespective of the initial sugar concentration. The percentage of mycelial constituents released during autolysis increased with initial concentration of glucose.

INTRODUCTION

The influence of certain imposed conditions on the autolytic phase of growth in cultures of filamentous fungi has been studied by Behr (1930), who was the first to describe the influence of the two types of media, physiologically acid and alkaline, upon different aspects of the autolytic phase of growth in cultures of *Aspergillus niger*. The effect of the addition of certain growth factors upon the autolytic processes of several filamentous fungi was investigated by Ritter (1955). Tandon & Chandra (1962) studied the nature of the changes induced in autolysing cultures of *Colletotrichum gloeosporioides* by the addition of antibiotics. Changes in the general pattern of autolysis brought about by a variety of different cultural conditions in *A. flavus* and in *A. terreus* were described by Lahoz (1967), Lahoz, Reyes & Beltrá (1966), Lahoz, Reyes, Beltrá & Garcia-Tapia (1967) and Lahoz & Gonzalez Ibeas (1968). In our search through the literature on the filamentous fungi we were unable to find any report of the effect of alteration of concentration of carbon source upon autolysis. The present work analyses the effect on autolysis of *A. niger* cultures of changes in the initial amounts of glucose.

METHODS

Organism. We used *Aspergillus niger* van Tieghem no. 120.49 from the Centraalbureau voor Schimmelcultures, Baarn, Holland.

Chemicals. Mineral salts used in the preparation of culture media were of analytical

grade from Probus S.A., Barcelona, Spain. Organic reagents were of analytical purity either from the British Drug Houses Ltd, Poole, Dorset, England, or from Sigma Chemical Co., St Louis, Mo., U.S.A.

Conditions of culture. Jena conical flasks (300 ml.), each containing 80 ml. of Czapek–Dox medium (g./l. distilled water: $NaNO_3$, 2; KCl, 0·5; KH_2PO_4, 1; $FeSO_4.7H_2O$, 0·01; $MgSO_4.7H_2O$, 0·5) with glucose (anhydrous) at 2·5, 5, 10, 20 and 40 g./l., were prepared and sterilized as previously described (Lahoz *et al.* 1966, 1967). At convenient intervals sets of 80, 100 or 200 flasks were in turn inoculated with 1 or 2 ml. of a spore suspension of *Aspergillus niger* prepared as previously described (Lahoz *et al.* 1966, 1967). Each batch was incubated on a reciprocating shaker at 90 oscillations/min. with an amplitude of 10 cm. at 24 to 26° in the dark. At the time of the maximum yield of mycelium at least five flasks were withdrawn from the shaker; the number of flasks taken at any particular time depended on the amount of mycelium present when harvesting. In the set containing the lowest glucose concentration samples of 10, 15 or 20 flasks had to be taken and their contents combined since growth was very scanty. During the later periods 45 flasks were needed to give sufficient mycelium for detailed analysis. In all cases the mycelium was filtered free of culture fluid, washed with water and dried at 70° to constant weight. The culture fluid was discarded after the residual glucose and the pH had been estimated.

Analytical methods. Total nitrogen, carbohydrates and lipids in mycelium were determined as in previous work (Lahoz *et al.* 1966, 1967, 1968). Methods of extraction of total free reducing substances from defatted mycelium and their estimation were as previously described (Lahoz *et al.* 1966, 1967; Lahoz & Gonzalez Ibeas, 1968; Lahoz, Beltrá & Ballesteros, 1970). The alkali soluble protein from the residue was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) using hot (80°) N-NaOH (100 mg. mycelium/25 ml. NaOH) extracts.

RESULTS

The highest yield of mycelium was attained at the 3rd, 3rd, 4th, 7th and 11th day of incubation for media containing 2·5, 5, 10, 20 and 40 g./l. initial glucose, respectively. As in previous work, the loss in mycelial dry weight was adopted as a criterion of autolysis. Therefore the day that the maximum amount of mycelium formed in the different concentrations tested was taken as the 'zero day of autolysis' and samples to be analysed were withdrawn from the shaker 8, 23, 38 and 53 days later.

The loss of weight in autolysing mycelium of *Aspergillus niger* grown at different initial glucose concentrations can be seen in Table 1. The percentage autolysis for the 2·5, 5, 10, 20 and 40 g./l. concentrations were 64, 60, 43, 43 and 43 %, respectively. Above 10 g./l. the degree of autolysis remained uniformly low as the concentration of the carbon source increased.

The total nitrogen in the autolysing mycelium of *Aspergillus niger* is shown in Table 2. With each initial concentration of glucose the nitrogen content of the mycelium (expresssed as nitrogen/unit dry weight and as nitrogen/culture) at first diminished as the culture aged, but became practically constant later. The greatest loss (73 %) of mycelial nitrogen took place at the lowest initial glucose concentration (Table 3).

The greatest disappearance of lipids from autolysing mycelium of *Aspergillus niger*

took place before the 8th day of autolysis at all the initial glucose concentrations except 40 g. glucose/l. at which the amount of fat in the mycelium remained constant throughout the whole period of autolysis (Table 4). The total fat lost during 38 days

Table 1. *Influence of initial glucose concentration on mycelial weight during autolysis of Aspergillus niger*

Initial glucose concentration (g./l.)	Time of autolysis (days)				
	0	8	23	38	53
	Weight of mycelium/flask (mg.)				
2·5	76	43	33	30	27
5	150	86	72	61	59
10	205	140	123	116	116
20	409	276	252	238	232
40	730	579	496	460	410

Table 2. *The effect of initial glucose concentration on the total nitrogen in autolysing mycelium of Aspergillus niger*

Initial glucose concentration (g./l.)	Time of autolysis (days)									
	0		8		23		38		53	
	A*	B†	A	B	A	B	A	B	A	B
2·5	4·5	3·4	3·6	1·5	3·3	1·1	3·1	0·9	—	—
5	4·9	7·3	4·0	3·4	3·7	2·6	3·7	2·3	—	—
10	4·0	8·2	3·5	4·9	3·4	4·2	3·2	3·7	3·3	3·8
20	3·8	15·4	3·3	9·1	3·1	7·7	3·0	7·3	3·0	7·0
40	2·8	20·1	2·9	16·5	2·2	11·0	2·4	11·2	2·5	10·3

* Mg. N/100 mg. dry mycelium. † Mg. mycelial N/flask.

Table 3. *Percentage loss of mycelial nitrogen during the first 38 days of autolysis of Aspergillus niger at different initial glucose concentrations*

Initial glucose (g./l.)	Zero day (mg./flask)	38th day (mg./flask)	Difference	% loss
2·5	3·4	0·9	2·5	73
5	7·3	2·3	5·0	68
10	8·2	3·7	4·5	54
20	15·4	7·3	8·1	52
40	20·1	11·2	8·9	44

Table 4. *Influence of initial glucose concentration on the amount of fat in autolysing mycelium of Aspergillus niger*

Initial glucose concentration (g./l.)	Time of autolysis (days)				
	0	8	23	38	53
	Mg. fat per flask				
2·5	3·4	0·8	1·2	1·3	—
5	6·1	3·2	2·6	2·3	—
10	8·4	4·2	5·0	3·3	2·6
20	27·9	10·0	10·1	11·0	10·1
40	18·9	17·4	19·3	18·3	17·6

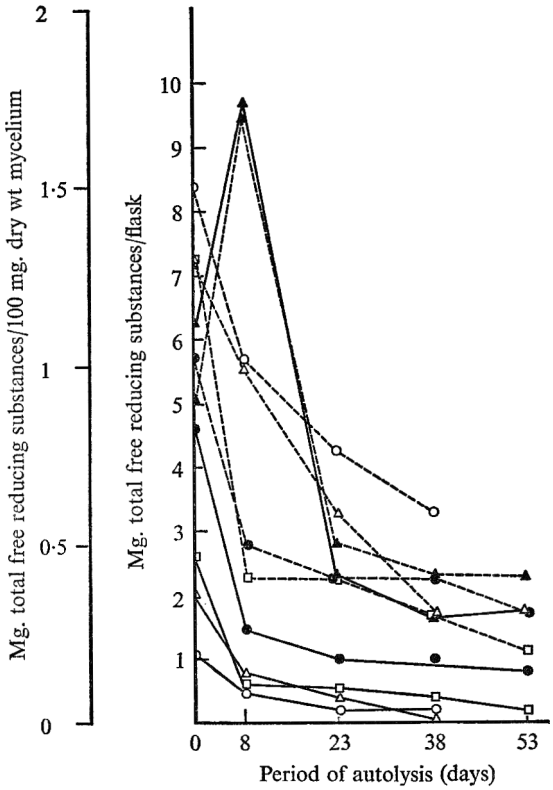

Fig. 1. Influence of initial glucose concentration on free reducing substances in the mycelium during autolysis of *Aspergillus niger* cultures. Mg./100 mg. dry weight mycelium, – – – – –; mg./flask, ————; 2·5 g./l. glucose, ○; 5 g./l. glucose, △; 10 g./l. glucose, □; 20 g./l. glucose, ●; 40 g./l. glucose, ▲.

Table 5. *Relation of total carbohydrates in autolysing mycelium of Aspergillus niger to initial glucose concentration*

Initial glucose concentration (g./l.)	Time of autolysis (days)				
	0	8	23	38	53
	Mg. anthrone postive carbohydrate/flask				
2·5	23	20	17	15	—
5	47	37	35	29	—
10	73	58	56	57	55
20	165	129	121	116	113
40	310	263	207	187	143

Table 6. *Final balance sheet of the loss of partial cellular constituents from autolysing mycelium of Aspergillus niger in cultures with different initial glucose concentrations*

mg./100 mg.	Initial concentration of glucose (g./l.)				
	2·5	5	10	20	40
Total nitrogen	5·2	5·5	5·0	4·7	3·1
Crude fat	4·2	4·2	6·5	1·0	—
Total carbohydrates	17·0	19·7	20·1	29·2	52·1
Total loss (%)	26·4	29·4	31·6	34·9	55·2

of autolysis of *A. niger*, grown and autolysed at 2·5, 5, 10 and 20 g. glucose/l. was practically uniform at 61, 62, 60 and 60% respectively. The change in total carbohydrates during autolysis can be seen in Table 5: they declined continuously at all of the initial glucose concentrations. The alkali soluble protein in the mycelium remained constant during autolysis for 53 days.

The percentage of the constituents released by autolysing cultures grown at different initial glucose levels increases with initial glucose concentration (Table 6).

DISCUSSION

Behr (1930), in his work on autolysis of *Aspergillus niger*, showed that the percentage of mycelial nitrogen continuously increases or remains constant with the ageing of the culture in physiologically alkaline media. It is shown here that the nitrogen in the mycelium decreased when expressed as N/100 mg. dry weight and N/flask (Table 2).

The increase in the content of the free reducing substances in mycelium during autolysis, which in previous work (Lahoz, 1967; Lahoz *et al.* 1970) has been attributed to an enzymic hydrolysis due to autolytic enzymes, is confirmed by the present work for the 40 g./l. level of initial glucose. The experiments showed that the initial concentration of the carbon source exerted a marked influence upon the behaviour of free reducing substances in the mycelium. A fall in free reducing substances with time was characteristic of all initial glucose levels except for the 40 g/l. (Fig. 1).

Similarly, the quantitative changes in lipids (Table 4) in autolysing mycelium seemed to be related to the initial level of the carbon source. Lipids in autolysing mycelium of *Aspergillus niger* grown in the highest initial glucose concentration (40 g./l.) remained at a constant level. A similar constancy was observed while studying autolysis in still cultures of *A. flavus*, in which a concentration of 50 glucose/l. was used. On the other hand the change in the amount of the carbon source did not exert a noticeable influence on the behaviour of total carbohydrates contained in mycelium of *A. niger* during autolysis. The pattern of continuous diminution, as autolysis proceeds, has been found previously (Lahoz & Gonzalez Ibeas, 1968; Lahoz *et al.* 1970).

The authors are indebted to Miss Teresa Raposo, who gave skilled technical assistance during the course of this work. This study is based on a portion of a thesis submitted by one of us (M. M.) in partial fulfilment of the requirements for the Ph.D. degree in the Faculty of Pharmacy, University of Madrid.

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The Relationship between Different Transmissible Plasmids Introduced by F into the Same Strain of *Escherichia coli* K12

By H. WILLIAMS SMITH AND C. L. GYLES
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(*Accepted for publication 4 May 1970*)

SUMMARY

Plasmids containing the genetic determinants for resistance to tetracyclines (Tc), streptomycin (Sm) and sulphonamide (Su), colicine production (Col) and α-haemolysin production (Hly) were transferred from four wild strains of *Escherichia coli* to *E. coli* K12 by conjugation brought about by F factor. Linkage was demonstrated between F and Tc and between Sm and Su. No linkage was noted between Col, Hly, F and Tc, and Sm and Su, which were transmitted as four independent units in no predetermined order. F and Tc were eliminated together during acridine orange and sodium lauryl sulphate treatment; the other characters were not eliminated. After mating for prolonged periods, F and Tc were most frequently found in the recipients. Recipients of other characters in the absence of F and Tc did not transmit them.

INTRODUCTION

The genetic determinants for several characters of *Escherichia coli* are now known to be located in plasmids which can be transferred from one bacterium to another by conjugation (Watanabe, 1963); it is not unusual for wild strains to contain more than one of these plasmids. The genetic elements responsible for their transfer from cell to cell, referred to hereafter as transfer factors, are probably numerous and it is not always possible to differentiate one from another. Consequently, although one may be able to detect the presence of a transfer factor in a cell by the fact that it can donate genetic material, only in certain cases can it be shown that more than one is present (Romero & Meynell, 1969). This complicates any study of the relationships between different plasmid-carried genes which may be found together in a wild strain of *E. coli*. Anderson (1965) and Anderson & Lewis (1965) reported the frequent occurrence of determinants for antibiotic resistance in the absence of transfer factors. These could be mobilized by introduction into the host bacterium of transfer factors from other sources. Plasmid-determined characters such as colicinogeny or antibiotic resistance can be readily transmitted during conjugation brought about by ColI and other transfer factors (Fredericq, 1954; Smith, Ozeki & Stocker, 1963; Anderson, 1965) and it thus seemed worth while to use the F factor to introduce a number of apparently plasmid-determined characters from different wild strains of *Escherichia coli* into the same bacterium and then to observe the relationships of one to the other in a common environment. Thus the genetic determinants for tetracycline (Tc), streptomycin (Sm) and sulphonamide (Su) resistance and colicine (Col) and α-haemolysin (Hly) pro-

duction were transferred to a single strain of *E. coli* K 12 and their relationships were assessed by their relative tendencies to associate with one another during subsequent transfer from their common host.

METHODS

Bacterial strains. Two strains of *Escherichia coli* K 12 were used in the transmission studies. One of these, strain PROTO, was prototrophic and lactose-fermenting (*lac*$^+$) and the other, strain AUXO, was auxotrophic and nonlactose-fermenting (*lac*$^-$). When one was used as donor, a mutant of the other strain resistant to nalidixic acid or ampicillin was used as recipient. The mutants were prepared by inoculating 200 ml. of nutrient broth containing 50 μg. of nalidixic acid or ampicillin/ml. with all the *E. coli* K 12 organisms that had grown on a 'medicine flat' bottle of nutrient agar that had been incubated at 37° for 24 h. The broth culture was then incubated at 37°; like all the other broth cultures in this work, it was not aerated during incubation. At daily intervals, one loopful of culture was removed, spread over a small area of a Petri plate containing MacConkey medium and a disc containing 50 μg. of the same drug that had been added to the broth was applied to the surface. The plates were incubated at 37° for 24 h. and examined for the presence of colonies growing in the immediate proximity of the disc. When such a colony was noted on any of the plates, it was removed and purified by the usual plating technique. Its resistance to the relevant drug was finally confirmed.

Identification of nontransmissible character in wild strains of Escherichia coli. Nutrient broth (Oxoid no. 2) in 10 ml. amounts was seeded with 0·02 ml. of a 24 h. broth culture of a wild strain of *Escherichia coli* resistant to one or more antibiotics or producing haemolysin or colicine, and an equal volume of one of the resistant mutants of *E. coli* K 12 strain AUXO. The mixed culture was incubated at 37° for 24 h. and then plated on media containing 30 μg. nalidixic acid or ampicillin/ml., depending on which recipient mutant was used. This prevented growth of the wild *E. coli* strain so that only colonies of the strain AUXO could develop. Haemolysin production (Hly) was detected by using 'washed blood' agar (Smith & Halls, 1967). The plates were incubated at 37° for 24 h. and examined for haemolytic colonies. To detect colicine production (Col), nutrient agar plates inoculated by streaking were incubated at 37° for 24 h. A thin layer of melted nutrient agar at 50° containing the same antibiotic as the basal layer and a few drops of broth culture of the strain AUXO was poured carefully over the colonies on the surface of the plate and incubation continued for a further 24 h. The plates were then examined to determine whether any of the colonies were surrounded by zones of inhibition of growth of the overlaid strain AUXO. To detect transmission of antibiotic resistance, the mixed culture was centrifuged and the deposit inoculated heavily in MacConkey agar + nalidixic acid or ampicillin + 15 μg./ml. of one of the antibiotics to which the wild strain had been shown to be resistant. The use of MacConkey agar, which contains lactose and a pH indicator, allowed colonies of the *lac*$^-$ AUXO strain to be distinguished from any mutant colonies of the wild strain which might arise from the large inoculum. Transfer of sulphonamide resistance was not directly tested. Strains from which Hly, Col or antibiotic resistance was not transmitted at first examination were retested, often with slight variation of technique, e.g. by varying the relative amount of the two strains used to inoculate the mixed culture

and by varying the composition of the culture medium and the time for which it was incubated. No strain was classified as unable to transfer these characters until it had been tested at least 30 times.

Mobilization by F factor. Nutrient broth, in 10 ml. amounts, inoculated with 0·02 ml. of a 24 h. broth culture of a wild strain of *Escherichia coli* containing a nontransmissible determinant and an equal volume of a broth culture of an F^+ strain of *E. coli* K12 was incubated at 37° for 24 h. Fresh broth was then inoculated with 0·2 ml. of this mixed culture and an equal volume of a broth culture of one of the resistant mutants of strain AUX0, and incubated at 37° for 24 h. Finally, this culture was examined by the methods given in the previous paragraph.

Transfer between Escherichia coli K12 mutants. Essentially the same methods were used as in testing for transfer from the wild strains of *Escherichia coli*. Mixtures of the two *lac*$^+$ and *lac*$^-$ strains were chosen to act as donor and recipient such that the donor could be eliminated by one of the two antibiotics, nalidixic acid or ampicillin, to which the recipient was resistant. After incubation, the mixed cultures were streaked on MacConkey agar containing nalidixic acid or ampicillin and isolated colonies were examined for the various characters present in the donor strain. In some experiments in which Col transmission was being studied, a mutant resistant to the colicine was used as recipient. In some other cases, a colicine-sensitive strain was used and Col$^+$ recipients were directly selected by incorporating colicine in the agar to prevent the growth of Col$^-$ bacteria. The surface of a MacConkey agar plate without antibiotic was spread with a culture of the donor strain and incubated at 37° for 24 h., after which the bacteria were killed with chloroform vapour and covered with a thin layer of MacConkey agar containing nalidixic acid or ampicillin for inoculation of the mating mixture.

Generally, the mixture of donor and recipient was incubated at 37° for 18 h. before plating. To test the effects of shorter periods of mating, 18 h. broth cultures of the donor and recipient strains were diluted 1/10 in fresh broth and, after further incubation for 150 min., a mixture of equal parts of each was incubated for 5 min. and then diluted 1/100 in broth at 37° to prevent formation of additional cell to cell contacts. Samples were removed at intervals, mating was interrupted by agitation for 2 min. in an M.S.E. macerator and suitable volumes were plated on selective media, which contained the antibiotic to which the recipient was resistant and either the colicine or the antibiotic for which the donor possessed a resistance determinant.

Transfer of antibiotic resistance was examined by selecting recipients on antibiotic-containing media or by replica-plating to MacConkey agar containing antibiotic. For sulphonamide, broth cultures were streaked up to strips of filter paper containing a high concentration of sulphadimidine on plates of D.S.T. agar (Oxoid no. CM 261). For Hly, colonies were inoculated as spots on 'washed blood' agar and examined for haemolysis after 18 h. incubation. To test for Col, colonies were similarly subcultured onto nutrient agar previously spread with a colicine-sensitive strain of *Escherichia coli* K12 F^-; colicine production was shown by the presence of a clear zone around the colony after incubation.

The presence and expression of F factor was recognized by susceptibility of cultures on nutrient agar to lysis by the F-specific phage MS2 (Davis, Strauss & Sinsheimer, 1961).

Acridine orange treatment. Escherichia coli K12 cultures were grown from small

inocula in broth containing 0·01 % (w/v) acridine orange at 37° for 24 h., a concentration that had a definite inhibitory effect.

Sodium lauryl sulphate treatment. This was done in a manner similar to that devised by Tomoeda, Inuzuka, Kubo & Nakamura (1968). Cultures were grown from small inocula in nutrient broth containing 10 % sodium lauryl sulphate at 37° for 24 h.

When the effect of passage in acridine orange and sodium lauryl sulphate was being studied, the cultures after each passage were inoculated onto Petri plates of MacConkey agar in a manner likely to yield widely separated colonies after incubation.

RESULTS

Nontransmissible antibiotic resistance and haemolysin- or colicine-production in wild strains of Escherichia coli: their transfer by means of F to a single strain of E. coli K12

Four wild strains of *Escherichia coli* were isolated that were resistant to tetracycline (Tc) or streptomycin (Sm) and sulphonamide (Su), or that produced colicine (Col) or α-haemolysin (Hly), and in which these characters were not transmissible until the strain had been infected by F factor. Using F to bring about conjugation, the five characters were transferred to a nalidixic acid-resistant mutant of *E. coli* K12 strain AUXO by two different procedures. In the first, each character was transferred to a different cuture giving four F^+ strains that were Tc^+, Sm^+Su^+, Col^+ or Hly^+ respectively. Col was then transferred to the Tc^+ strain to produce a Tc^+Col^+ strain. The latter was mated with the strain Sm^+Su^+ to produce a $Tc^+Col^+Sm^+Su^+$ strain which, in turn, was mated with the Hly^+ strain to produce a strain $Tc^+Sm^+Su^+Col^+Hly^+$. The determinants for all five characters were then transferred *en bloc* to an ampicillin-resistant mutant of *E. coli* strain PROTO; this and the *E. coli* AUXO donor are referred to subsequently as the M strains of K12 $F^+Tc^+Col^+Hly^+Sm^+Su^+$. The second procedure was to transfer each character in turn to the nalidixic acid-resistant mutant of *E. coli* strain AUXO from the four wild strains of *E. coli*. First, Tc was transferred, then Col, to produce a $F^+Tc^+Col^+$ K12 strain. A K12 F^+ strain $Tc^+Col^+Sm^+Su^+$ could not be produced by direct mating of an $F^+Tc^+Col^+$ strain with the wild Sm^+Su^+ strain but was obtained by growing a mixed culture of the two strains with a further inoculum of the nalidixic acid-resistant mutant of *E. coli* strain AUXO. Employing the usual techniques, it was not possible to introduce Hly directly from the wild strain of *E. coli*; however, F, Tc, Col, Sm and Su could be transferred together to the wild strain and subsequent mating with the nalidixic acid-resistant mutant of strain AUXO resulted in the isolation of the required K12 $F^+Tc^+Col^+Hly^+Sm^+Su^+$ strain. All the characters were then simultaneously transferred to the ampicillin-resistant strain PROTO; these two are referred to as the G strains of *E. coli* K12 $F^+Tc^+Col^+Hly^+Sm^+Su^+$. Both M and G strains were fully susceptible to lysis by phage MS 2, showing that F was present and that its function continued to be expressed.

Long-term mating of Escherichia coli K12 $F^+Tc^+Col^+Hly^+Sm^+Su^+$ with E. coli K12 F

Table 1 shows the properties of colonies of *Escherichia coli* K12 F^-, and of colicine-resistant mutants, obtained after mating for 18 h. with M and G strains. F was always found with Tc, and Sm with Su in the recipients; no other consistent associations were noted. Of the recipients that were F^-, none transmitted their characters to another F^-

strain; these could however, as before, be transferred by conjugation when F was introduced by mating with *E. coli* K12 F^+. By contrast, F^+ recipients transmitted the characters which they had received at a high rate.

Despite their different methods of preparation, there was no obvious difference between the M and G strains in the fashion in which their determinants were transmitted, except possibly in the degree of Sm and Su transfer. In all the experiments in which the colicine-resistant mutant of K12 F^- was used as recipient, the most common class was that carrying simply F and Tc. Although Table 1 shows more recipients of Hly after mating with the G strain than with the M strain, this difference was not consistently found. In the experiments in which the recipient was colicine-sensitive, all

Table 1. *The transfer of characters from the M* and G* strains of Escherichia coli K12 $F^+Tc^+Col^+Hly^+Sm^+Su^+$ during 18 h. mating with E. coli K12 F^-*

						No. of 100 colonies of the prospective recipient strain that acquired the character during mating					
						PROTO K12 F^- *col-r* and donor AUXO		PROTO K12 F^- and donor AUXO		AUXO K12 F^- and donor PROTO	
Character						M	G	M	G	M	G
F						54	62	93	81	59	55
Tc						54	62	93	81	59	55
Col						26	25	100	100	100	100
Hly						6	17	38	51	69	55
Sm						8	4	45	18	36	9
Su						8	4	45	18	36	9
None						44	37	0	0	0	0
Character pattern											
F	Tc	Col	Hly	Sm	Su						
+	+	+	+	+	+	0	2	16	12	20	3
+	+	+	+	−	−	3	9	18	29	26	27
+	+	+	−	+	+	6	1	26	4	1	2
+	+	−	−	+	+	1	1	0	0	0	0
+	+	+	−	−	−	15	13	33	36	12	23
+	+	−	+	−	−	1	5	0	0	0	0
+	+	−	−	−	−	28	31	0	0	0	0
−	−	+	+	+	+	1	0	2	2	9	0
−	−	+	−	+	+	0	0	1	0	6	4
−	−	+	+	−	−	1	0	2	8	14	25
−	−	+	−	−	−	0	0	2	9	12	16
−	−	−	+	−	−	0	1	0	0	0	0
−	−	−	−	−	−	44	37	0	0	0	0

AUXO = auxotrophic; PROTO = prototrophic; *col-r* = mutant resistant to the colicine produced by the donor.
* M and G are K12 F^+ Tc^+ Col^+ Hly^+ Sm^+ Su^+ donor strains in which the characters were introduced by different procedures (see text). The presence or absence of F was determined by MS2 phage sensitivity.

colonies were Col^+, a probable consequence of killing of Col^- bacteria by colicine present in the mixed-mating culture. The distribution of the other characters in these Col^+ colonies resembled mainly their distribution in the experiments in which the colicine-resistant mutant was the recipient. The types of colony obtained with the colicine-resistant mutant selected for Tc^+, Col^+, Hly^+ or Sm^+ after mating with the M and G strains of K12 F^+ are shown in Table 2 in which the results for the M and G

strains are pooled because they were similar. Again, complete correlation was found between the presence of F and of Tc and between Sm and Su, but between no other pairs of characters. Of 160 colonies examined, 152 had F and Tc, while the others were transmitted much less commonly.

Table 2. *The characters possessed by recipient colonies of a colicine-resistant mutant of Escherichia coli* K12 F^- *mated with E. coli* K12 $F^+Tc^+Col^+Hly^+Sm^+Su^+$ *for 18 h. and selected for different characters*

Character selected	Characters possessed by 40 selected recipient colonies					
	F	Tc	Col	Hly	Sm	Su
Tc	40	40	19	14	6	6
Col	39	39	40	16	22	22
Hly	39	39	30	40	9	9
Sm	34	34	33	19	40	40

Additional evidence that an F^+ strain donated Tc much more frequently than Sm and Su was obtained when two K12 F^+ strains, one with Tc and the other with Sm and Su, were mated with one another. The two strains could be differentiated by resistance to nalidixic acid or ampicillin. After mixed culture for 18 h., there were many more organisms of the K12 $F^+Sm^+Su^+$ strain with Tc than of the K12 F^+Tc^+ strain which had acquired Sm and Su. Both of these $F^+Tc^+Sm^+Su^+$ strains, although they had received Tc and Sm and Su in a different order, transmitted Tc with F very much more frequently than Sm and Su to an F^- strain. The same relative frequencies of transmission were observed after the donor had received Tc and Sm and Su directly from the wild strains of *Escherichia coli* irrespective of the order in which the characters had been received.

Short-term mating of Escherichia coli K12 $F^+Tc^+Col^+Hly^+Sm^+Su^+$ *with E. coli* K12 F^-

The results of mating the G strain of *Escherichia coli* K12 $F^+Tc^+Col^+Hly^+Sm^+Su^+$ for short periods with K12 F^- are shown in Tables 3 and 4. There was no great difference between the numbers of Tc^+, Sm^+ and Col^+ recipients at 5 min. (Table 3). As with mating for 18 h. all those that were Tc^+ were also F^+ and all which were Sm^+ were also Su^+; otherwise, only the selected character was usually present. After a further 20 min. of mating, additional characters appeared (Table 4) and it was noteworthy that the proportion of colonies containing Hly was approximately the same whether selection was

Table 3. *The numbers of viable organisms of Escherichia coli* K12 F^- *that acquired Sm Tc and Col during short-term mating with the G strains of E. coli* K12 $F^+Tc^+Col^+Hly^+Sm^+Su^+$

Character	Approximate no. of recipient organisms per ml. of culture that acquired the character during mating	
	5 min.	5 + 20 min.*
Tc	1,200	60,000
Sm	750	20,000
Col	1,000	80,000

* Five min. mating followed by dilution and further incubation for 20 min. before plating.

for tetracycline resistance, streptomycin resistance or colicine production. The proportions of Tc^+ and of Sm^+ colonies which produced colicine were also approximately the same, as were the proportions of Tc^+ and of Col^+ colonies which were Sm^+. None of the recipients that were F^- could transmit their characters to another F^- strain.

Table 4. *The characters acquired by colonies of Escherichia coli K12 F⁻ after short-term matings with the G strain of E. coli K12 F⁺Tc⁺Col⁺Hly⁺Sm⁺Su⁺*

						No. of 100 colonies that acquired the character during mating					
						5 min. characters selected			5+20 min.* characters selected		
Character						Tc^+	Sm^+	Col^+	Tc^+	Sm^+	Col^+
F						100	0	0	100	11	13
Tc						100	0	0	100	11	13
Sm						0	100	3	7	100	11
Su						0	100	3	7	100	11
Col						1	6	100	33	46	100
Hly						0	3	3	38	39	33
Character pattern											
F	Tc	Col	Hly	Sm	Su						
+	+	−	−	−	−	99	0	0	44	0	0
−	−	−	−	+	+	0	91	0	0	37	0
−	−	+	−	−	−	0	0	94	0	0	57
+	+	+	−	−	−	1	0	0	16	0	3
−	−	−	+	+	+	0	3	0	0	13	0
−	−	+	−	+	+	0	6	3	0	18	4
−	−	+	+	−	−	0	0	3	0	0	23
+	+	−	+	−	−	0	0	0	20	0	0
+	+	−	−	+	+	0	0	0	1	4	0
+	+	+	+	−	−	0	0	0	13	0	6
−	−	+	+	+	+	0	0	0	0	21	3
+	+	−	+	+	+	0	0	0	2	0	0
+	+	+	−	+	+	0	0	0	1	2	3
+	+	+	+	+	+	0	0	0	3	5	1

* Five min. mating followed by dilution and further incubation for 20 min. before plating.

Elimination by acridine orange and sodium lauryl sulphate

Table 5 shows the results of passaging the G strain of *Escherichia coli* K12 F^+Tc^+-$Col^+Hly^+Sm^+Su^+$ daily in nutrient broth containing acridine orange or sodium lauryl sulphate and examining 50 colonies obtained from each passage. Both agents eliminated Tc. No colonies were found that had lost Col, Hly, Sm or Su. Most of the 50 colonies examined after the first passage had lost sensitivity to lysis by phage MS2. Those still Tc^+ were able to transmit Tc and also Col, Hly, Sm and Su, but at a very much lower rate than the untreated cultures. Twenty-five MS2-insensitive cultures were passaged several times in broth, when they regained susceptibility to lysis as well as high rate of donor ability. Another culture of Tc^+ MS2-insensitive organisms which had been treated with lauryl sulphate remained MS2-insensitive and continued to transmit at low frequency after 20 passages in broth. However, cultures of other strains that had received Tc from the Tc^+ MS2-insensitive strain showed the normal

high degree of MS2 susceptibility and donor activity. No colonies were found that had lost Tc while retaining sensitivity to lysis by phage MS2. Twenty-five Tc-sensitive MS2-insensitive colonies were also passaged 20 times in broth but did not become susceptible to lysis nor regain the ability to transfer Col, Hly, Sm or Su.

Table 5. *Elimination of characters from the* G *strain of Escherichia coli* K12 F^+ Tc^+ Col^+ Hly^+ Sm^+ Su^+

	No. of colonies containing			
	Tc		Col Hly Sm Su	
*Passage no.	Expt. 1	Expt. 2	Expt. 1	Expt. 2
0	50	50	50	50
1	45	2	50	50
2	36	0	50	50
3	16	N.T.	50	N.T.
4	6	N.T.	50	N.T.
5	1	N.T.	50	N.T.
6	0	N.T.	50	N.T.
16	0	0	50	50

* Passage was continued daily in broth containing 10 % sodium lauryl sulphate (expt. 1) or 0·01 % acridine orange (expt. 2).

N.T.: not tested.

DISCUSSION

Although the M and G strains of *Escherichia coli* K12 $F^+Tc^+Col^+Hly^+Sm^+Su^+$ were prepared in different ways, the behaviour of the plasmids during subsequent mating was not influenced greatly by the order in which they had been introduced into the donor strain. The colicine had been identified as colicine E1 by Professor P. Fredericq, and ColE has been shown to require an independent transfer factor with which it is not necessarily associated in transfer (Fredericq, 1954; Smith *et al.* 1963; Watanabe, 1963). The only evidence of linkage between the characters was between F and Tc and between Sm and Su, which contrasts with the various associations of plasmid-determined characters observed to occur with another transfer factor, *A*, observed by Anderson (1968). The present results, together with those of Anderson (1965, 1968) and Anderson & Lewis (1965), indicate that plasmid-determined antibiotic resistance unlinked to a transfer factor is more common and of greater significance than was at first envisaged (see Watanabe, 1963).

Approximately equal numbers of F^+Tc^+ only, Col^+ only, and Sm^+Su^+ only, recipients were found after mating with the M and G donor strains for 5 min., indicating the absence of any predetermined sequence of transmission. This strongly suggests that, given the presence of a transfer factor to provide the necessary connecting link between the bacteria, transfer is either completely automatic, or, if not, it is the individual plasmid itself that determines how it shall be transferred.

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Transfer Factors in *Escherichia coli* with Particular Regard to Their Incidence in Enteropathogenic Strains

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SUMMARY

Strains of *Escherichia coli* were isolated whose resistance to ampicillin (Ap), tetracyclines (Tc) or streptomycin and sulphonamides (SmSu) or whose production of colicine (Col) or α-haemolysin (Hly) could not be transmitted to *E. coli* K12 F⁻. Determinants controlling these characters (except Ap), could be mobilized in some of them after infection with transfer factors. Of 60 strains of *E. coli* isolated from the faeces of healthy pigs, cattle and human beings and selected because they did not possess R factors and did not produce colicine, α-haemolysin, porcine enterotoxin (Ent) or K 88 antigen (transmissible characteristics), 20 (33 %) contained transfer factors that could mobilize determinants from one or more of nine determinant donor strains; 8 contained at least two transfer factors, one *fi*⁻ and the other *fi*⁺. The nine determinant donor strains contained Tc, SmSu, Col or Hly determinants; some were wild strains and others were obtained from interrupted mating experiments in which *E. coli* K12 F⁻ was the recipient strain. The ability of a transfer factor to mobilize a determinant was not simply a function of the transfer factor and the determinant but was strain-dependent. Positive results were obtained most frequently when transfer was between strains of K12. Transfer factors that could mobilize determinants in determinant donor strains were found in all except one of 78 porcine enterotoxigenic strains of *E. coli* selected because they did not possess R factors or transmissible colicine. Nearly all of them produced α-haemolysin and over half of them K88 antigen. On the evidence available, the high incidence of transfer factors in these strains suggests that enterotoxin production in all of them was controlled by transmissible plasmids and that the genetic determinants controlling α-haemolysin production in some of them might be chromosomal. The linkage between Ent and the factor responsible for its transfer was not always close. Despite a previous statement to the contrary (Smith & Halls, 1968), this transfer factor was *fi*⁻; both *fi*⁻ and *fi*⁺ factors could transfer Hly. No close association between the plasmids controlling the five characteristics Neo (neomycin resistance), Ent, Col, Hly and K88 was apparent in mating experiments using as donor a porcine strain of *E. coli* carrying all five transmissible plasmids. Transfer factors were found in 15 of 21 human enteropathogenic strains and in one of five sheep enteropathogenic strains; none of these 26 strains possessed characteristics known to be transmissible.

INTRODUCTION

Most strains of *Escherichia coli* that cause diarrhoea and oedema disease in pigs produce α-haemolysin, a property also possessed by a small proportion of the non-pathogenic strains that inhabit the alimentary tract of man and domestic animals (Smith, 1963). Smith & Halls (1967*a*) found that in some strains of *E. coli* the pro-

duction of this haemolysin was controlled by a transmissible plasmid (Hly). A transmissible plasmid (Ent) was also shown to control enterotoxin production in some porcine enteropathogenic strains (Smith & Halls, 1968). Owing to the absence of sensitive selection procedures, Hly and, particularly, Ent transmission can only be demonstrated when it occurs at a high rate, very much higher than that required to demonstrate R factor transfer. This may account for the failure of Smith & Halls to detect transmission of Hly and Ent from the majority of strains they examined. Alternatively, Hly and Ent existed in these strains in a nontransmissible state.

To elucidate the situation the incidence of the genetic elements responsible for plasmid transmission, i.e. the sex factors, conjugation factors or transfer factors, in porcine enteropathogenic strains and in haemolytic nonpathogenic strains of *Escherichia coli* was examined. To do this, it was first necessary to find strains possessing mobilizable but initially nontransmissible determinants. Determinant mobilization tests (Anderson, 1965*a*, *b*; Anderson & Lewis, 1965) were employed for detecting strains containing transfer factors. The mobilizable determinants employed included those controlling antibiotic resistance since the transfer of such determinants can easily be detected. In addition, strains of *E. coli* enteropathogenic for human babies, calves and lambs were also examined for transfer factors.

Throughout this paper, a genetic element described as mobilizable is one that can only be transferred after its host has been infected with a transfer factor. A transmissible genetic element is one whose host possesses such a transfer factor.

METHODS

The identification of nontransmissible characters in strains of Escherichia coli. This was exactly as described by Smith & Gyles (1970).

The identification of strains of Escherichia coli containing mobilizable determinants. This was performed by a modification of the method of Anderson (1965*a*,*b*) and Anderson & Lewis (1965). Strains whose Hly, Col or antibiotic resistance could not be transferred directly to *E. coli* K12 F^- were inoculated into nutrient broth with *E. coli* K12 F^+ and incubated at 37° for 24 h. This mixed culture was then subcultured together with the nalidixic acid-resistant mutant of the *lac*$^-$ *E. coli* K12 F^- and incubated for another 24 h. The final culture was then examined for determinant transfer to the K12 F^- strain by the methods previously described. If transfer did not occur, the experiment was repeated using strains of *E. coli* containing transfer factors other than F in place of the K12 F^+ strain. Determinants that could be transferred by this technique but not by the usual mixed-culture technique are referred to subsequently as mobilizable and the strains containing them as determinant donor strains.

The identification of strains of Escherichia coli containing transfer factors. This was performed in the same way as the experiments designed to identify strains containing mobilizable determinants except that strains known to contain mobilizable Hly, Col or antibiotic resistance determinants were now employed as determinant donor strains, and the strain under test for carriage of a transfer factor lacked the respective property, i.e. was Hly^-, Col^- or R^-. Transfer of the character concerned to the K12 F^- recipient strain indicated that the strain under the test contained a transfer factor.

Fertility-inhibiting (fi) character of transfer factors. This was assessed by transmitting a determinant by means of the transfer factor under the test into an F^+ strain

of *Escherichia coli* K12 and then examining for susceptibility to lysis by the F-specific phage MS2 (Davis, Strauss & Sinsheimer, 1961).

The detection of enterotoxin production by porcine strains of Escherichia coli. This was achieved by injecting broth cultures into ligated segments of pig small intestine according to the methods of Smith & Halls (1967*b*).

RESULTS

The incidence of strains of Escherichia coli possessing mobilizable determinants

The incidence of strains of *Escherichia coli* whose haemolysin production and antibiotic resistance could not be transferred to *E. coli* K12 F^- in mixed culture is shown in Table 1. Most of the strains whose tetracycline, streptomycin and sulphonamide or ampicillin resistance was nontransmissible were resistant to these antibiotics only. Table 1 also shows the number of strains in which the genetic determinants for these characters could be mobilized by means of infection with F or other transfer factors. The Col as well as SmSu was mobilized in one strain, B165; the colicine was identified by Professor Fredericq of the University of Liége as E 1. Of the four strains with mobilizable Hly, two actually contained transfer factors that could mobilize SmSu but not, of course, Hly; neither strain was used in subsequent studies. None of the other strains with mobilizable determinants was shown to possess transfer factors that could mobilize other determinants.

Table 1. *The incidence of wild strains of Escherichia coli possessing nontransmissible but mobilizable determinants*

Determinant	No. of strains possessing this determinant	No. in which it was non-transmissible	No. in which it was mobilizable
Tc	60	10	5
SmSu	71	20	6
Ap	25	14	0
Cm	21	0	0
Neo	8	0	0
Hly	99	66	4

Tc, SmSu, Ap, Cm and Neo: resistance to tetracycline, streptomycin and sulphonamide, ampicillin, chloramphenicol and neomycin respectively. Hly: α-haemolysin production.

Some of the wild strains with mobilizable determinants were infected with F and their determinants transferred to K12 F^-. Matings between the resulting K12 F^+ strains and K12 F^- were then performed and some K12 strains were obtained that had received determinants but not F (Smith & Gyles, 1970). This was an additional source of strains with mobilizable determinants.

On the basis of preliminary tests with strains of *Escherichia coli* known to contain transfer factors and the strains with mobilizable determinants, eight were chosen as determinant donor strains. Five were wild strains, B165 $SmSu^+Col^+$, H121 $SmSu^+Tc^+$, B150 $SmSu^+$, H120 Tc^+ and P102 Hly^+. The remaining three, K12 F^- $SmSu^+$ (B165) Tc^+(H120), K12 F^- $SmSu^+$(H121) and K12 F^-Hly^+(P102), were K12 F^- strains into which determinants from some of the five wild strains had been introduced. The

number in brackets indicates the strain from which the determinants originated. Unlike the other two K12 F^- strains, the loss of F from K12 F^- $SmSu^+$(B165)Tc^+ (H120) occurred spontaneously during laboratory cultivation. This set of eight strains was completed by the addition of an $SmSu^+$ strain of *Salmonella typhimurium*, RT727, kindly provided by Dr E. S. Anderson of the Enteric Reference Laboratory, London, N.W.9.

The determinants possessed by seven of the nine determinant donor strains were transferred to *Escherichia coli* K12 F^- by means of F and the susceptibility to visible lysis by MS2 phage of 20 determinant-containing K12 F^+ colonies obtained from each mating was assessed; an *fi*$^-$ transfer factor had to be used to transfer the determinants from the remaining two strains, B150 $SmSu^+$ and RT727 $SmSu^+$, the recipient strain in their case being K12 F^+. All the colonies except those that had received determinants from H120 Tc^+ and K12 F^- $SmSu^+$(B165) Tc^+(H120), a strain containing the Tc determinant of H120 Tc^+, were lysed by MS2. Only one of the 20 K12 colonies that had received Tc but not SmSu from K12 F^- $SmSu^+$(B165)Tc^+(H120) and only three of the 20 colonies that had received SmSu but not Tc from this strain were lysed by MS2. When the experiments with K12F^- $SmSu^+$(B165)Tc^+(H120) were repeated and antibiotic-sensitive instead of antibiotic-resistant colonies were tested, all but one of 40 of them were lysed by MS2. Of the 20 K12F^+ colonies that had received Tc determinants from H120 Tc^+, 18 were lysed by MS2 and two were not. When cultures obtained from colonies of each type were mated with K12 F^-, no obvious difference was noted in the rate at which Tc was transmitted. All of 20 colonies obtained from the mating in which the donor was susceptible to lysis by MS2 were also susceptible to this phage; all 20 colonies obtained from the mating in which the donor was insusceptible, however, were also insusceptible. In view of the results obtained with H120 Tc^+ and K12 F^+ $SmSu^+$(B165)Tc^+ (H120) it was assumed that they possessed a transfer factor which, although unable to transfer or mobilize Tc, SmSu, Col or Hly, was able to inhibit the expression of F. For this reason they were not used subsequently to assess the *fi* character of transfer factors.

The incidence of transfer factors amongst strains of Escherichia coli isolated from the faeces of healthy pigs, cattle and human beings

The incidence of transfer factors in 60 strains of *Escherichia coli*, isolated from the faeces of healthy pigs, cattle and human beings, that could mobilize SmSu, Tc, Col or Hly from the nine determinant donor strains is illustrated in Table 2. All the mobilization tests necessary to complete this and subsequent tables were first performed once and, where considered necessary, those yielding negative results were then repeated several times before they were finally recorded as negative. During this repeat testing it was not unusual for some tests to yield positive results. The 60 strains tested were specially selected in that none of them were antibiotic-resistant or produced colicine, haemolysin, K 88 antigen, or in the case of pig strains, enterotoxin, characters that can be controlled by transmissible plasmids. In addition, none of them were latently infected with phages active against K12 F^-. The pigs, cattle and human beings from which the strains had been isolated were all from different herds or households.

Approximately the same proportion of the pig, cattle and human strains were found to contain transfer factors, 30 to 40%. There was great variation in the extent to which the different determinants in the donor strains were mobilized. For example, all

20 strains found to contain transfer factors mobilizedSmSu from K 12 F⁻ SmSu⁺(H 121) whereas only nine of them mobilized SmSu from B 150. Again, the Tc determinant in H121 SmSu⁺ Tc⁺ was mobilized by only one of the 20 strains whereas its SmSu determinant was mobilized by 13. Determinants were mobilized less frequently from wild strains than from K 12 F⁻ strains into which their determinants had been introduced. For example, Hly was mobilized in K 12 F⁻ Hly⁺(P 102) by ten strains but only four of them mobilized it from P 102 Hly⁺ itself. In the K 12 F⁻ determinant donor strains, Hly mobilization was detected less frequently than R factor mobilization, probably a reflexion of the comparatively poor methods available for selecting Hly⁺ organisms.

Table 2. *The incidence of transfer factors in strains of Escherichia coli isolated from the faeces of healthy unrelated pigs, cattle and human beings*

		No. whose transfer factors mobilized												
		SmSu from *E. coli*							Tc from *E. coli*				Hly from *E. coli*	
Source and no. of strains	No. with transfer factors	K 12 F⁻ SmSu⁺ (H121)†	B165 SmSu⁺ Col⁻	K 12 F⁻ SmSu⁺ (B165) Tc⁺ (H120)	H121 SmSu⁺ Tc⁺	RT727 SmSu⁺*	B150 SmSu⁺	Col from *E. coli* B165 SmSu⁺ Col⁺	K 12 F⁻ SmSu⁺ (B165) Tc⁺ (H120)	H120 Tc⁺	H121 SmSu⁺ Tc⁺	K 12 F⁻ Hly⁺ (P102)	P102 Hly⁺
Pigs (20)	8	8	6	5	6	5	4	5	5	1	0	2	0
Cattle (20)	6	6	5	4	3	1	1	5	6	5	1	6	2
Human beings (20)	6	6	5	5	4	4	4	5	5	3	0	3	2
Total (60)	20	20	16	14	13	10	9	15	16	9	1	11	4

Sm, Su, Tc, Col and Hly: determinants for streptomycin, sulphonamide and tetracycline resistance, and colicine and haemolysin production. P, B and H: porcine, bovine and human respectively.

* *Salmonella typhimurium* not *Escherichia coli*.

† The symbols in parentheses indicate the strains from which the determinants originated.

The 20 strains possessing transfer factors are grouped together in Table 3 according to determinant mobilizing ability; it illustrates the great difference between some of the strains in this respect. *Escherichia coli* K 12 F⁺ and K 12 F⁻ (P275), a K 12 F⁻ strain into which had been introduced the transfer factor of one of these 20 strains, P275, are included for comparison. K 12 F⁻ (P275) was a better mobilizer than was P275; its mobilizing ability, was, however, inferior to that of K 12 F⁺ and of several of the other strains. On the basis of the *fi* tests, at least eight of the 20 strains contained two transfer factors, one *fi*⁺ and the other *fi*⁻; these eight included five of the six bovine strains. There was no obvious indication that some determinants were always mobilized by *fi*⁺ transfer factors and others by *fi*⁻ ones. In the case of strains containing an *fi*⁺ and a *fi*⁻ transfer factor, it was sometimes difficult or even impossible to determine which one had been responsible for transferring a particular determinant because, in *fi* tests, transfer of the genetic elements controlling the *fi*⁺ transfer factor alters the susceptibility of the K 12 F⁺ recipient strain to the MS 2 phage, irrespective of whether the *fi*⁺ or the *fi*⁻ transfer factor is actually responsible for transferring this determinant. The position was sometimes clarified by examining a number of the K 12 F⁺ colonies that had received the determinant. If some were susceptible to lysis by the MS 2 phage and some were not, it was inferred that an *fi*⁻ transfer factor was responsible for mobili-

zing the determinant. If none of them were lysed, it was assumed that an *fi*$^+$ factor was responsible although the presence of an *fi*$^-$ factor was not thereby excluded.

To obtain further information on the specificity of the relationship existing between transfer factors and determinants, 17 nonpathogenic strains of *Escherichia coli* isolated from the faeces of 17 pigs in different herds were examined. Tc was the only known transmissible character they possessed. The transfer factors in ten of them were *fi*$^-$ and in the remaining seven were *fi*$^+$. All 17 mobilized SmSu from K12 F$^-$ SmSu$^+$ (H121) and 12, 7 *fi*$^-$ and 5 *fi*$^+$, mobilized SmSu and Col from B165 SmSu$^+$Col$^+$. Ten, five *fi*$^-$ and five *fi*$^+$, mobilized Hly from K12 F$^-$ Hly$^+$(P102) and four, all *fi*$^+$, mobilized it from P102.

Table 3. *Classification of the transfer factor-bearing strains of Escherichia coli isolated from pigs, cattle and human beings according to the ability of their transfer factors to mobilize determinants*

	Ability to mobilize												
	SmSu from:							Tc from:			Hly from:		
Identification of strains	K12 F$^-$ SmSu$^+$ (H121)	B165 SmSu$^+$ Col$^+$	K12 F$^-$ SmSu$^+$ (B165) Tc$^+$ (H120)	H121 SmSu$^+$ Tc$^+$	RT727 SmSu$^+$	B150 SmSu$^+$	Col from: B165 SmSu$^+$ Col$^+$	K12 F$^-$ SmSu$^+$ (B165) Tc$^+$ (H120)	H120 Tc$^+$	H121 SmSu$^+$ Tc$^+$	K12 F$^-$ Hly$^+$ (P102)	P102 Hly$^+$	*fi* character of transfer factors
H89	+	−	−	−	−	−	−	−	−	−	−	−	+
P275	+	−	−	−	−	−	−	−	−	−	+	−	+
P280	+	−	−	−	−	−	−	−	−	−	+	−	+ and −
P303	+	+	−	−	−	−	−	−	−	−	−	−	−
B107	+	−	+	−	−	−	−	+	−	−	+	−	−
B89, B148	+	+	−	−	−	−	+	+	+	−	+	−	Both + and −
H92	+	+	+	−	−	−	+	+	+	−	+	−	+
P284, P288, P289 P291, H97, H98	+	+	+	+	+	+	+	+	−	−	−	−	−
P276	+	+	+	+	+	−	+	+	+	−	−	−	−
B105, B149	+	+	+	+	−	−	+	+	+	−	+	+	Both + and −
B167	+	+	+	+	+	+	+	+	+	+	+	−	+ and −
H100, H110	+	+	+	+	+	+	+	+	+	−	+	+	Both + and −
K12 F$^+$*	+	+	+	+	−	−	+	+	+	+	+	+	.
K12 F$^-$ (P275)*	+	−	−	+	−	−	−	−	−	−	+	+	.

fi: Fertility inhibition. For other abbreviations see Table 2.
* Included for comparison with the 20 wild transfer factor-containing strains; K12 F$^-$ (P275) contains the transfer factor obtained from the wild strain, P275.

Transfer factors in nonpathogenic α-haemolytic strains of Escherichia coli of porcine origin

The properties of transfer factors in α-haemolytic strains of *Escherichia coli* isolated from the faeces of 33 healthy animals in different herds are shown in Table 4; they did not belong to serotypes enteropathogenic for pigs. None of them possessed R factors, Ent were colicinogenic or carried phages or produced K 88 antigen. Transfer factors that mobilized resistance determinants were found in all ten strains that were shown to possess transmissible Hly but in only five of the 23 strains that were not. It is noteworthy that the transfer factors in three of these five strains mobilized Hly from K12 F$^-$ Hly$^+$(P102) yet haemolysin production in the three strains themselves was not shown to be transmissible.

The incidence of transfer factors in enteropathogenic strains of Escherichia coli of porcine origin

The results of examining 97 porcine enterpathogenic strains of *Escherichia coli* for transfer factors are summarized in Table 5. The determinant donor strains were chosen from those studied previously as probably being the most useful for differentiating the transfer factors possessed by those 97 strains. The latter had been isolated from

Table 4. *Transfer factors in nonpathogenic α-haemolytic strains of Escherichia coli of porcine origin*

			No. with transfer factors that mobilized							
			SmSu from		Tc from:	Hly from			*fi* character of transfer factors	
Transmissibility of Hly	No. of strains	No. with transfer factors	K 12 F⁻ SmSu⁺ (H 121)	B165 SmSu⁺ Col⁺	H120 Tc⁺	K12 F⁻ Hly⁺ (P102)	P102 Hly⁺		*fi*⁺	*fi*⁻
+	10	10	10	9	1	N.T.	N.T.		2	8
−	23	5	5	4	1	3	2		0	2*

* Only 2 strains tested. N.T. = Not tested

Table 5. *The incidence and character of transfer factors in enteropathogenic strains of Escherichia coli of porcine origin*

Property of strains						No. with transfer factors that mobilized						
Ent		Hly		No. with these properties	No. with transfer factors*	SmSu from B165 SmSu⁺ Col⁺	B150 SmSu⁺	Tc from H120 Tc⁺	Hly from P102 Hly⁺	*fi* character† *fi*⁺	*fi*⁻	*fi*⁺ and *fi*⁻
Produced	Transmissible	Produced	Transmissible									
+	+	+	+	6	6	6	3	3	N.T.	0	1	3
+	+	+	−	3	3	3	2	1	0	0	2	1
+	+	−	−	2	2	2	2	2	2	0	0	2
+	−	+	+	24	24	24	15	4	N.T.	2	2	0
+	−	+	−	43	42	42	27	5	11	0	18	3
−	−	+	−	15	1	1	1	0	0	0	1	0
−	−	−	−	4	1	1	1	0	0	.	.	.

Forty-one of the enterotoxigenic strains possessed K88 antigen; 56 did not.
* Identified by their ability to mobilize determinants.
† Not all the strains were tested. N.T. = Not tested.

different outbreaks of diarrhoea or oedema disease under conditions strongly indicating that they were playing an aetiological role. None of them possessed R factors, or transmissible Col. They all belonged to antigenic types generally accepted as being enteropathogenic for pigs, 41 to types that possessed the K 88 antigen, types O8:K87, 88ab (4 strains), O8:K87, 88ac (11 strains), O45:K?, 88ac (4 strains), O141:K85ab, 88ab (4 strains), O147:K89, 88ac (5 strains) and O149:K91, 88ac (13 strains) and 56 to types that did not possess this antigen, types O9:K9 (2 strains), O45:K? (4 strains), O138:K81 (17 strains), O139:K82 (19 strains), O141:K85ab↔ac (6 strains) and O141:K85ac (8 strains). The K 88 antigen was known to be transmissible in two of the 41 strains that possessed it; transmissibility was not examined in the remaining 39.

All except one (P 102) of the 76 strains that produced enterotoxin and haemolysin, and the two strains that produced enterotoxin only, possessed transfer factors that could mobilize SmSu in B 165 SmSu$^+$Col$^+$; most also mobilized this determinant in B 150 SmSu$^+$ but only a few mobilized Tc in H 120 Tc$^+$. In 46 of these strains, haemolysin production was not found to be transmissible, yet 11 of the 46 mobilized Hly from P 102 Hly$^+$. No difference was detected between the transfer factors in the 41 enterotoxigenic strains that possessed the K 88 antigen and those in the 37 that did not, either in their mobilizing ability or in their *fi* character. In contrast to the enterotoxigenic strains, no transfer factors were detected in 14 of the 15 α-haemolytic strains that did not produce enterotoxin or in three of the four strains that produced neither enterotoxin or haemolysin; they had been tested with the good determinant donor strain K 12 F$^-$ SmSu$^+$(H 121), as well as with the four strains referred to in Table 5. All these 19 nonenterotoxigenic strains belonged to antigenic types O 139:K 82 (15 strains) and O 45:K ? (4 strains), types that usually cause oedema disease, a disease in which enterotoxin is not aetiologically involved. Of a total of 19 O 139:K 82 strains examined, only four produced enterotoxin; in all four it was transmissible.

Because the transfer factors in most of the enterotoxigenic strains illustrated in Table 5 were *fi*$^-$ although Smith & Halls (1968) had reported the transfer factors concerned with Ent transmission to be *fi*$^+$, the strains possessing a transmissible Ent were submitted to closer examination. Six of nine such strains (Table 5) were found to contain an *fi*$^+$ as well as an *fi*$^-$ transfer factor. During these examinations, a nontransfer factor-containing strain of *Salmonella typhimurium* was grown in mixed culture with *Escherichia coli* P 19, a nonhaemolytic O 9:K 9 strain possessing a transmissible Ent. Attempts to mobilize the determinants in three determinant donor strains with the transfer factors in 19 clones of *S. typhimurium* obtained from this mixed culture (Table 6), suggest that the *S. typhimurium* organisms had either received no transfer factors, one transfer factor or two transfer factors from P 19, one mobilizing Tc from

Table 6. *The determinant mobilizing ability of clones of a strain of Salmonella typhimurium that had been grown in mixed culture with Escherichia coli P 19 Ent*$^+$

No. of clones	Ent	Ability of their transfer factors to mobilize: SmSu from B 150 SmSu$^+$	Tc from H 120 Tc$^+$	Hly from P 102 Hly$^+$
5	+	+	+	+
5	+	+	−	−
1	−	+	+	+
1	−	−	+	+
7	−	−	−	−

H 120 and Hly from P 102 and the other mobilizing SmSu from B 150, this one being responsible for Ent transmission. This was confirmed by showing that the transfer factor that mobilized SmSu from B 150 was *fi*$^-$ and would not mobilize Tc from H 120 or Hly from P 102; while the transfer factor that had mobilized Tc from H 120 would not mobilize SmSu from B 150 but it would mobilize Hly from P 102.

Likewise the transfer factor that mobilized Hly from P 102 would not mobilize SmSu from B 150 but it would mobilize Tc from H 120; this transfer factor was *fi*$^+$. Although the *fi*$^-$ transfer factor appeared to be responsible for Ent transmissions, its relationship

with Ent was not close because it was present in two of the *S. typhimurium* clones that were Ent$^-$.

Similar results to those described above were obtained in studies on another four Ent$^+$ strains. In three of them, Hly as well as Ent was transmissible; in these three, the *fi*$^+$ transfer factor was responsible for Hly transfer. In contrast to these five strains, a sixth was found to possess only an *fi*$^-$ transfer factor which mobilized SmSu in B 150 but not Tc and Hly in H 120 and P 102 respectively.

The relationship between the transmission of different determinants from enterotoxigenic strains of porcine Escherichia coli

Because in the studies reported above the transfer factors responsible for Ent transmission were able to mobilize SmSu, the SmSu resistance in 12 wild enterotoxigenic strains of *Escherichia coli* were transmitted to *E. coli* F 11, a strain containing no transfer factors but able to receive Ent. *E. coli* K 12 F$^-$, although a good recipient of Ent, was not used for this purpose because it is not easy to identify Ent$^+$ organisms of this strain (Smith & Halls, 1968). Single and pooled SmSu$^+$ colonies of F 11 obtained from each of the 12 mixed cultures were examined for Ent; none was positive. Fifty-three cultures prepared from single or pooled colonies of F 11 to which SmSu, Tc or Hly had been transferred from B 150 SmSu$^+$, H 120 Tc$^+$ or P 102 Hly$^+$ respectively by

Table 7. *The distribution of determinants among 200 colonies of Escherichia coli F 11 that had been grown in mixed culture with E. coli P233 Ent$^+$ Hly$^+$ Neo$^+$ Col$^+$ K 88$^+$*

To obtain a high transfer rate, the mixed culture of P 233 and the recipient strain F 11 was passaged twice in broth; each passaged broth culture was incubated for 24 h.

Determinant	No. of colonies
Col	90
Neo	89
Ent	51
Hly	51
K 88	1

Determinant pattern					
Col	Neo	Ent	Hly	K 88	
+	+	+	+	−	34
+	+	+	−	−	17
+	+	−	+	−	16
+	+	−	−	−	18
+	−	−	−	−	4
−	+	−	−	−	3
+	+	−	+	+	1

the transfer factors possessed by a variety of enterotoxigenic pig strains of *E. coli* also yielded negative results when tested for Ent. In another experiment, R factors, single and multiple, coding for resistance to antibiotics such as tetracyclines, neomycin, chloramphenicol, streptomycin and sulphonamides were transmitted to *E. coli* H 1 from 30 enterotoxigenic α-haemolytic pig strains, 14 of which produced K 88 antigen; H 1 was a human enteropathogen able to receive Ent. None of the 30 resulting antibiotic-resistant strains of H 1 was either Ent$^+$, Hly$^+$ or K 88$^+$. Hly, also, was found in

only one of 12 strains of K12 F⁻ or F11 to which Tc or SmSu had been transmitted from H120 Tc⁺ or B150 SmSu⁺ by transfer factors possessed by haemolytic enterotoxigenic strains.

Absence of close linkage between Ent and Hly and between them and K 88, Col and neomycin resistance in a wild strain of *Escherichia coli*, P233, antigenic formula O8:K87, 88ac, is illustrated in Table 7. At least two transfer factors were contained in P233, one *fi*⁺ and the other *fi*⁻.

Transfer factors in strains of Escherichia coli enteropathogenic for human babies, lambs or calves

The results of examining strains of *Escherichia coli* isolated from different outbreaks of diarrhoea in human babies, lambs and calves for transfer factors are summarized in Table 8. The human strains belonged to serotypes generally accepted as being enteropathogenic for babies; severe diarrhoea has been produced in calves and lambs by oral inoculation of the calf and lamb strains. None of the 28 strains was antibiotic-resistant or carried phages active against *E. coli* K12 F⁻. A few were colicinogenic and one, from a lamb, was haemolytic; none of these characters was found to be transmissible. The transfer factors in the human strains differed, as a group, from those found in the porcine enterotoxigenic strains in that only five of the 15 strains possessing them mobilized SmSu in B165 SmSu⁺Col⁺ and only three mobilized it from B150 SmSu⁺; nearly half of them, too, were *fi*⁺. The distribution of transfer factor-containing strains amongst the different antigenic types was O26 (1 of 2 strains examined), O55 (2 of 5), O11 (1 of 1), O119 (5 of 5), O125 (1 of 1), O127 (0 of 1) and O128 (5 of 6).

Table 8. *Transfer factors in strains of Escherichia coli enteropathogenic for human babies, lambs and calves*

Source of strains	No.	No. with transfer factors*	No. with transfer factors that mobilized: SmSu from K12 F⁻ SmSu⁺ (H121)	SmSu from B165 SmSu⁺ Col⁺	Tc from H120 Tc⁺	Hly from P102 Hly⁺	*fi*† character of their transfer factors: *fi*⁺	*fi*⁻
Human Babies	21	15	15	5	2	0	7	8
Lambs	5	1	1	1	0	1	1	0
Calves	2	2	2	2	1	0	1	0

* Demonstrated by ability to mobilize determinants.

† The *fi* character of the transfer factors of only one lamb and one calf strain was examined.

The incidence of strains of Escherichia coli in which colicine production was transmissible

Of 137 strains of *Escherichia coli* isolated from the faeces of unrelated healthy human beings, calves and pigs, 31 produced colicine active against *E. coli* K12 F⁻. Its production in 10 of 20 tested strains was shown to be transmissible. Among 36 strains from healthy pigs, 11 produced colicine: its production in 5 of 9 tested strains was transmissible. Of 221 strains of *E. coli* enteropathogenic for pigs, 83 produced colicine; production in 7 of 36 tested strains was transmissible.

DISCUSSION

The incidence of transfer factors, 33%, amongst the 60 strains of *Escherichia coli* isolated from the faeces of healthy cattle, pigs and human beings, and selected because they were neither antibiotic-resistant nor produced colicine, haemolysin, K 88 antigen or porcine enterotoxin (characteristics that may be transmissible), was considerably higher than the 7% found by Lewis (1968) in 135 antibiotic-sensitive, noncolicine-producing strains of *E. coli* isolated from the general human population; it approached that in salmonellae (Anderson, 1968). One reason for this difference might be that Lewis used only one determinant donor strain whereas we used nine, one of which K12 F^- $SmSu^+$(H121), was particularly good. Results of the determinant mobilization tests were not always reproducible. The ability to detect the presence of a transfer factor within a cell was not simply a function either of the transfer factor itself or of the mobilizable determinant. It varied with the bacterial strain containing the transfer factor, the strain containing the determinant and that used as the final recipient. Mobilization was achieved most easily when all three strains were K12.

It seems likely, then, that the actual incidence of transfer factors in the 60 strains we examined was higher than 33%. Apart from the technical difficulties in demonstrating transfer factors, it remains possible that some of the strains possessed transfer factors which would not mobilize any of the determinants in the nine determinant donor strains. One of the donor strains, H120 Tc^+, possessed a transfer factor, also present in another donor strain derived from it, K12 F^- $SmSu^+$ (B165) Tc^+(H120), that would neither transmit the determinants in these two strains nor mobilize those in the other donor strains. This transfer factor was only detected by the fact that it inhibited the expression of F in K12 F^+. Since it was not linked to the determinants in the two strains, it is more logical to regard it simply as unable to facilitate the transfer of these determinants rather than to suppose that it had lost the ability to do so, because it was defective (Cuzin & Jacob, 1965).

At least two transfer factors were identified in eight of the 20 strains tested on the basis of the fertility-inhibition (*fi*) test. The *fi* test only permits classification of transfer factors into two groups, fi^+ and fi^- (Watanabe, 1963), and the presence of more than one fi^+ or more than one fi^- transfer factor in the same bacterial cell cannot be readily detected.

Transfer factors that mobilized determinants for antibiotic resistance were found in only five of the 23 nonpathogenic strains of *Escherichia coli* whose α-haemolysin production could not be shown to be be transmissible, an incidence no higher than that found in the 60 nonpathogenic strains that did not possess characters known to be transmissible. Since the transfer factors in all of the 10 nonpathogenic strains whose haemolysin production was transmissible were able to mobilize these antibiotic resistance determinants, this is evidence that the haemolysin production in most of the 23 strains was really nontransmissible and not that its rate of transfer was too low to be detected by our methods. The transfer factors in three of the five strains which possessed them were able to mobilize Hly from the Hly^+ donor strains as did 11 of the 44 porcine enteropathogenic strains whose haemolysin production was not shown to be transmissible. It is possible, then, that the genetic elements coding for haemolysin production in at least these 14 strains may not be located in plasmids at all but in the chromosome. In regard to the actual transfer factors involved in Hly transmission,

those examined by Smith & Halls (1967*a*) were *fi*$^+$. Examination of those in the ten nonpathogenic strains referred to above, however, revealed that some were *fi*$^+$ and some were *fi*$^-$.

All except one of the 78 porcine enterotoxigenic strains of *Escherichia coli* that did not possess R factors or transmissible Col possessed transfer factors that mobilized determinants from our donor strains. These transfer factors were known to be responsible in some of the strains for the transmission of K 88, haemolysin production and enterotoxin production. It would be extremely difficult by the methods presently available to determine the exact extent to which these three properties were controlled by transmissible plasmids because they can be detected only if the rate of transfer is high. For the reasons given in the previous paragraph, it seems unlikely that the transfer factors in the 77 enterotoxigenic strains were principally concerned with Hly transmission, or with K 88 transmission, because 37 of them did not possess this antigen. It is possible, then, that the transfer factors were principally concerned with Ent transmission, thus implying that enterotoxin production in all the strains was controlled by transmissible plasmids. The observations on the 19 α-haemolytic strains of serotype O139:K82, a serotype mainly incriminated in oedema disease rather than in diarrhoea, supported this view in that transfer factors could be demonstrated only in the four that produced enterotoxin.

As for the 20 nonpathogenic strains that possessed transfer factors, it was not unusual to find both *fi*$^+$ and *fi*$^-$ transfer factors in the same enterotoxigenic strain. In the present work, both *fi*$^+$ and *fi*$^-$ transfer factors were found in the strains that had led Smith & Halls (1968) to the erroneous conclusion that the factors mediating Ent transfer were *fi*$^+$. It is probable that in their investigations the presence of the *fi*$^+$ factor had masked that of the *fi*$^-$ factor transmitting Ent.

Even in the porcine strains shown to contain a transmissible Ent plasmid, the linkage between plasmid and transfer factor was not close. In clones of *Escherichia coli* F11 recovered after the mating with strain P233 the Ent plasmid was not linked to any of the plasmids controlling the production of haemolysin, colicine and K 88 antigen or neomycin resistance. These facts provide possible explanations for our failure to isolate Ent$^+$ recipient organisms from mating mixtures by selecting those that had received antibiotic resistance or Hly from donor strains that produced enterotoxin but whose enterotoxin production could not be shown to be transmissible by the usual procedure.

Amongst the strains enteropathogenic for animals other than pigs, the fact that only one of the five sheep strains contained transfer factors is taken as evidence that transmissible plasmids do not control any characteristic common to this kind of strain. The incidence of transfer factors in the 21 human enteropathogenic strains was considerably higher than that in the comparable group of human nonpathogenic strains, 71% compared with 30%. It may be that the human enteropathogens, as a group, possess characteristics that are controlled by transmissible plasmids.

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A Study of the Antigenic Relationships of Isolates of *Trypanosoma brucei* Collected from a Herd of Cattle Kept in One Locality for Five Years

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SUMMARY

During surveys of the prevalence of trypanosomiasis, 37 isolates of trypanosomes of the subgenus *Trypanozoon* were collected over a period of five years from a herd of cattle kept on a farm in an area in Nigeria where human and animal trypanosomiasis is endemic. Thirty-three were typical isolates of *Trypanosoma brucei* but four were unusual in several respects and their identity was uncertain.

The antigenic relationships of the trypanosomes were determined by agglutination tests using antisera to the predominant variant antigens of each isolate. Comparisons of groups of isolates obtained in two of the surveys showed that some of the animals in the herd were infected with antigenically related trypanosomes. Four isolates obtained in one survey had antigens in common and probably belonged to one trypanosome strain, while 26 isolates obtained in another survey seemed to have been derived from three different strains. At least ten animals examined in the latter survey were infected with two strains of *Trypanosoma brucei* at the same time. Comparisons of trypanosomes obtained in successive surveys showed that one isolate was very closely related to four isolates obtained from different animals two years later. Agglutination and neutralization tests, and a study of variant antigen production by clones prepared from the first isolate and one of the later group of four isolates, established the antigenic similarity of these trypanosomes and provided direct evidence of the long-term antigenic stability of a trypanosome strain under field conditions. Apart from this notable exception, there was little antigenic relationship among the trypanosomes isolated from year to year and at least seven antigenically distinct strains of *T. brucei* were isolated from the herd in the five-year observation period.

INTRODUCTION

The development of improved methods for determining the antigenic relationships of isolates of *Trypanosoma brucei* and *T. rhodesiense* (Cunningham & Vickerman, 1962; Gray, 1966) and for the long-term preservation of trypanosomes at low temperatures (Walker, 1966) has facilitated immunological studies on several aspects of the epidemiology of African trypanosomiasis. Although there are major antigenic differences between strains of *T. brucei* isolated in widely separated areas, close relationships frequently occur among organisms isolated in restricted geographical localities (Broom & Brown, 1940; Cunningham & Vickerman, 1962; Gray, 1966). Comparisons of the variant antigens of strains of *T. brucei* and *T. rhodesiense* have indicated that it

may sometimes be possible to differentiate between these morphologically identical species by serological tests (Weitz, 1962; Taylor, 1968).

Such findings suggest that it might eventually be possible to immunize animals against trypanosomiasis in certain areas and to use serological methods to determine whether pleomorphic trypanosomes of the subgenus *Trypanozoon* (Hoare, 1964) isolated from animals in the course of epidemiological studies are *Trypanosoma brucei*, *T. rhodesiense* or, possibly, *T. gambiense*. At present, *T. brucei* can be distinguished from the other two species with certainty only by its inability to infect man, while *T. rhodesiense* and *T. gambiense* are identified on the basis of their geographical origin, sensitivity to certain trypanocidal drugs and pathogenicity in man and experimental animals (Ashcroft, 1959). It is generally acknowledged that much more information on the numbers of antigenically different strains of these three species occurring in selected areas and on the antigenic stability of trypanosome strains in the field is needed before such suggestions can be properly evaluated (Lumsden, 1965).

In 1961 an exceptionally high prevalence of trypanosomiasis was discovered in a herd of cattle kept on a government farm in Benue Province, Nigeria (Godfrey, Leach & Killick-Kendrick, 1964). Variations in the prevalence of trypanosome infections in this herd were studied on four further occasions during the next five years and numerous trypanosome isolates, including 37 classified in the subgenus *Trypanozoon*, were collected for study in the laboratory. This paper describes an investigation to find the number of antigenically different strains of this subgenus which infected the cattle in the five-year observation period and a search for evidence of antigenic stability in trypanosome strains in their natural environment.

METHODS

Origin and ecology of the trypanosomes. The trypanosomes were isolated from a herd of cattle kept on a government farm at Raav, in Benue Province, Nigeria. They were pleomorphic organisms belonging to the subgenus *Trypanozoon* and were identified on the basis of their morphology and bovine origin as *Trypanosoma brucei*.

The farm consisted of 6000 acres of land south of the River Benue in the Guinea Savannah Vegetation Zone (Keay, 1953). Low numbers of the tsetse flies *Glossina palpalis* and *G. tachinoides* occurred in the area and human and animal trypanosomiasis was endemic. No attempt was made to prevent trypanosome infections in the cattle, but when necessary, severely affected animals were treated with therapeutic trypanocidal drugs such as homidium chloride, B.Vet.C., and diminazene aceturate, B.Vet.C.

The trypanosomes were collected during surveys of the prevalence of trypanosomiasis in the animals, carried out between November 1961 and April 1966 by methods described by Godfrey & Killick-Kendrick (1961). Five surveys were made and many animals were found to be infected with *Trypanosoma vivax*, *T. congolense* and *T. brucei*. A full account of variations in the prevalence of trypanosome infections found in the first three surveys has been given elsewhere (Godfrey *et al.* 1964). A summary of the findings in all five surveys in relation to *T. brucei* is shown in Table 1.

Isolation and adaptation of the trypanosomes. The trypanosomes were isolated by inoculating blood from infected cattle into white rats. Each isolate was identified by the number of the survey in which it was collected and by the number of the rat inoculated with blood from the bovine host. Thus, isolate 5/28 was collected in survey 5 in the 28th rat inoculated with ox blood. Only two of the 20 isolates of *Trypanosoma*

brucei found in the herd in survey 5 and none of the three isolates infecting the animals in survey 9 were available for study, but most of the organisms found in the later surveys 17, 22 and 30 were examined serologically (Table 1).

Thirty-two of the isolates were collected from animals with pure *Trypanosoma brucei* infections, but three were obtained from cattle infected with *T. brucei* and *T. vivax* and two from animals infected with *T. brucei* and *T. congolense*. Pure lines of *T. brucei* were obtained from mixed isolates of *T. brucei* and *T. vivax* by passaging the trypanosomes repeatedly in rats, which are not readily infected with *T. vivax*, and from mixtures of *T. brucei* and *T. congolense* by preparing clones of the former species by the method of Inoki (1960).

Table 1. *Trypanosoma brucei infections found in five surveys of trypanosomiasis in a herd of cattle and the number of isolates of the trypanosome examined serologically*

			Types of infections					
No. of survey	Date of survey	No. of animals examined	*T. brucei* alone	*T. brucei* and *T. vivax*	*T. brucei* and *T. congolense*	*T. brucei*, *T. vivax* and *T. congolense*	No. of animals infected with *T. brucei*	No. of isolates examined serologically
5	23. xi. 61	99	0	4	13	3	20	2
9	8. xi. 62	77	1	1	1	0	3	0
17	28. xi. 63	95	4	0	2	0	6	6
22	27. i. 65	100	1	0	0	0	1	1
30	26. iv. 66	107	29	1	1	0	31	28

A few of the trypanosomes were very pathogenic when first isolated from the cattle and gave rise to acute infections and massive parasitaemias in rats and mice within 3 to 4 days, but in most cases the primary rodent infections were mild. As heavy suspensions of trypanosomes were required for serological tests, the less virulent isolates were passaged repeatedly at short intervals in rats and mice until they adapted to these hosts. They were then frozen and stored at $-80°$ as antigens for agglutination tests (Cunningham & Vickerman, 1962). In three instances, the degree of adaptation induced in isolates by prolonged passage was very low and the trypanosomes were frozen and stored in an incompletely adapted state. Fuller details of the number of times particular isolates were passaged in rats and mice are given below where relevant.

Determination of the antigenic relationships of the trypanosomes

The antigenic relationships of the trypanosomes were determined mainly by agglutination tests, but serum neutralization tests were used in part of the work.

Agglutination tests. Antigens for agglutination tests were prepared from syringe-passaged lines of the isolates by the method of Cunningham & Vickerman (1962), and stored at $-80°$. In a few instances, antigens were also prepared from cyclically transmitted trypanosomes with basic strain antigens (Gray, 1965).

Agglutinating antisera were prepared by infecting rabbits with syringe-passaged or cyclically transmitted trypanosomes. Antisera obtained from animals which had been infected for 3 to 4 weeks and which had high titres of agglutinins to the predominant variant antigens of the isolates (see Gray, 1966) were used in most of the experiments, but pools of sera collected from animals in the second, third and fourth weeks of infection were used occasionally. All antisera were stored at $-20°$.

Agglutination tests were carried out by the method of Cunningham & Vickerman (1962) with minor modifications (Gray, 1962). In the description of the results of the tests the term 'strain' has been used to include all trypanosome isolates and derived clones which produced similar agglutinogenic variant antigens in the first 3 to 4 weeks of infections in rabbits.

Neutralization tests. Antigens for neutralization tests were prepared by diluting antigens for agglutination tests, containing approximately 1 to 3×10^8 live organisms/ml., 1/100 with a balanced solution of inorganic salts buffered at pH 8·0 by a borate buffer system (Lumsden *et al.* 1965).

Neutralizing antisera were obtained from rabbits which had been infected with trypanosomes transmitted by tsetse flies. The rabbits were bled for normal serum before infection and for antiserum 14 days afterwards. Neutralizing antisera were also prepared in rats. Groups of twenty 100 to 150 g. rats were infected with approximately 2×10^6 trypanosomes from frozen stocks of appropriate isolates. The rats were treated 3 days later, when the parasitaemias were heavy, with diminazene aceturate (0·5%, w/v) in distilled water at a dose rate of 2·0 mg./100 g. rat, administered by intraperitoneal injection. A group of uninfected rats was treated with the drug at the same dose rate to provide control serum. The rats were bled for serum 9 days after drug treatment.

Neutralization tests were performed by the method of Cunningham & van Hoeve (1963). Normal sera and antisera were diluted 1/10 with the borate-buffered balanced salts solution described by Lumsden *et al.* (1965), chilled to 0 to 2° and dispensed in 1·8 ml. volumes in rows of eight tubes. Volumes of 0·2 ml. of the suspension of trypanosomes used as the antigen were added to the first tubes in each row and left for 30 min. at 0 to 2°. Seven tenfold serial dilutions of the suspensions of trypanosomes after exposure to the sera and antisera were then prepared and 0·1 ml. volumes of each dilution were inoculated into groups of six mice which were examined for 8 days for trypanosome infections. The infectivity of each test suspension, expressed as the number of 63% infective doses (ID_{63}) per ml., was then estimated from the proportion of mice infected of those inoculated at each dilution by reference to tables published by Lumsden *et al.* (1963).

RESULTS

The relationships of trypanosomes isolated from different animals in individual surveys

When two or more isolates of *Trypanosoma brucei* were obtained from different cattle in a survey they were compared by agglutination tests to find if the animals were infected with related or unrelated organisms.

Survey 5. Isolates 5/28 and 5/65 were virulent when they were first isolated and they adapted to maintenance in rats and mice after 8 and 10 passages, respectively. They were transmitted in the laboratory by *Glossina morsitans* to obtain trypanosomes with basic strain antigens for use in agglutination tests. Tests with these antigens and antisera from rabbits which had been infected with the trypanosomes transmitted by both syringe passage and by tsetse flies for periods up to 4 weeks did not reveal any antigenic relationship between the isolates.

Survey 17. Six isolates were obtained in survey 17. Isolates 17/14, 17/30, 17/70 and 17/96 adapted readily to maintenance in rats and mice after 8 to 10 passages, but isolates 17/8 and 17/58 were much less virulent and caused only light, relapsing parasitaemias after prolonged passage.

Clones were prepared from the six isolates. Those derived from isolates 17/14, 17/30, 17/70 and 17/96 were cyclically transmitted to provide trypanosomes with basic strain antigens. Agglutination tests with antisera from rabbits infected by tsetse flies with these four clones, and antisera from rabbits infected by syringe passage with all six clones, showed that clones 17/14, 17/30, 17/70 and 17/96 produced variant antigens in common and thus probably originated from the same strain, but clones 17/8 and 17/58 were antigenically distinct. A fuller account of the antigenic relationships of this group of isolates has been given elsewhere (Gray, 1966).

Survey 30. Twenty-eight isolates were obtained in survey 30, and 27 were adapted to rats and mice by repeated passage. The number of passages required for the adaptation of different isolates varied from 7 to 28 (average 15) and the time required for adaptation varied from 37 to 153 (average 88) days. The exceptional isolate, 30/53, had not adapted completely to rats and mice after 48 passages extending over 203 days. Antisera were prepared by infecting rabbits with syringe-passaged trypanosomes of each isolate, harvested from the blood of infected rats after 3 to 6 passages, when the trypanosomes were still incompletely adapted. Antigens for agglutination tests were prepared after further passage when the isolates were fully adapted to rats and mice.

Antisera obtained from rabbits after 3 weeks of infection were diluted 1/40 and tested for agglutinins to antigens of 27 of the 28 isolates. The antisera could not be tested for agglutinins to isolate 30/53 because the isolate had not adapted to rats. All of the antisera agglutinated three or more of the antigens, indicating that many of the trypanosomes were antigenically related (see Table 2). However, the isolates had probably been derived from only three trypanosome strains each of which produced a number of characteristic variant antigens. These strains have been called Strains 1, 2 and 3 for ease of reference in the following account.

Sixteen isolates were collected from cattle infected with trypanosomes of apparently just one of the three different strains. Three isolates were classified as Strain 1, two as Strain 2 and 11 as Strain 3. Antisera to isolates of Strain 1 did not agglutinate pure isolates of Strains 2 and 3; antisera to isolates of Strain 2 did not agglutinate pure isolates of Strains 1 and 3; and antisera to isolates of Strain 3 did not agglutinate pure isolates of Strains 1 and 2.

Ten isolates were collected from cattle infected with trypanosomes of apparently at least two strains. In nine instances the evidence for the mixed composition of the isolate was clear; either the antiserum to the isolate agglutinated pure isolates of two different strains or the antigen of the isolate was agglutinated by antisera to pure isolates of two strains. Antiserum to isolate 30/94, however, agglutinated isolates of Strain 2 whereas the antigen of the isolate was agglutinated only by antisera to isolates of Strain 3. Also the antisera to three mixed isolates, 30/69, 30/94 and 30/97, failed to agglutinate the homologous trypanosomes. In all, two isolates seemed to consist of organisms of Strains 1 and 2, three isolates seemed to be a mixture of Strains 1 and 3, and five a mixture of Strains 2 and 3. There was no strong evidence that any of the isolates were a mixture of trypanosomes of all three strains, but the reaction of the antiserum to isolate 30/14 (probably a mixture of Strains 1 and 2) with one antigen of Strain 3, and the reaction of the antiserum to isolate 30/42 (probably a mixture of Strains 1 and 3) with one antigen of Strain 2, indicated that this was a possibility.

The trypanosomes of the remaining antigen 30/20 were lysed in heat-inactivated normal rabbit serum and in antisera to 26 of the isolates, and the isolate could not be

Table 2. *Agglutination of isolates of Trypanosoma brucei collected in survey 30 by antisera prepared in rabbits.*

The antisera were collected from the rabbits when they had been infected for 3 weeks and were tested at a dilution of 1/40. The reactions of the antisera with clones derived from four of the isolates are also shown.

Trypanosomes used as antigens	Trypanosome strain nos.	Reactions of antisera to the isolates: Isolates consisting of trypanosomes of one strain																Isolates consisting of trypanosomes of two strains										Unclassified isolates	
		73	84	103	31	105	11	17	58	63	74	77	78	80	81	99	107	14	66	42	51	97	33	37	69	83	94	20	53
Isolate 30/73	1	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−
Isolate 30/84		+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−
Isolate 30/103		+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	+	−
Clone 30/73		+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+	−	−	−	−	−	−	−	−
Isolate 30/31	2	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	+	−	−	+	+	−
Isolate 30/105		−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−
Clone 30/31		−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	+	−	−	+	+	−
Isolate 30/11	3	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	+	+	−	−	+
Isolate 30/17		−	−	−	−	−	+	+	+	+	+	−	+	−	−	+	+	−	−	−	+	+	+	−	+	−	−	−	−
Isolate 30/58		−	−	−	−	−	+	+	+	−	−	+	+	+	+	+	+	−	−	−	+	+	−	−	−	−	−	−	−
Isolate 30/63		−	−	−	−	−	+	+	+	+	−	−	−	−	−	+	+	−	−	−	−	+	−	−	+	−	−	−	−
Isolate 30/74		−	−	−	−	−	+	+	+	−	+	+	+	+	+	+	+	−	−	+	+	+	+	−	+	−	−	−	−
Isolate 30/77		−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	−	+	+	−	−	−
Isolate 30/78		−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	−	+	+	−	−	+
Isolate 30/80		−	−	−	−	−	+	+	+	+	−	+	−	+	+	+	+	−	−	−	+	+	+	−	+	+	−	−	−
Isolate 30/81		−	−	−	−	−	−	+	+	−	−	+	+	+	+	+	+	−	−	+	+	+	+	−	+	+	−	−	−
Isolate 30/99		−	−	−	−	−	−	−	+	−	−	+	+	+	−	+	−	−	−	−	−	+	−	−	+	−	−	−	−
Isolate 30/107		−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	−	−	−	+	+	−	−	+
Clone 30/77		−	−	−	−	−	+	+	+	−	+	+	+	+	+	+	+	−	−	−	+	+	+	−	+	+	−	−	−
Isolate 30/14	1, 2	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	+	−
Isolate 30/66		+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+	−	−	−	−	−	−	−	−
Isolate 30/42	1, 3	−	−	−	−	−	+	+	+	−	+	+	+	+	+	+	+	−	−	+	+	+	+	−	−	+	−	−	−
Isolate 30/51		−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	−	+	+	−	−	+
Isolate 30/97		+	+	+	−	−	−	+	+	−	−	−	−	−	−	+	+	+	+	−	+	−	−	−	+	−	−	−	−
Clone 30/97		−	−	−	−	−	+	−	+	−	−	−	+	−	−	+	+	−	−	−	+	−	−	−	+	−	−	−	−
Isolate 30/33	2, 3	−	−	−	+	+	+	+	−	−	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+	+	+	−	−
Isolate 30/37		−	−	−	+	+	+	+	−	−	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+	+	+	−	−
Isolate 30/69		−	−	−	+	+	−	−	−	−	+	−	−	−	−	+	−	+	−	−	−	−	−	+	−	−	+	−	−
Isolate 30/83		−	+	−	+	+	+	−	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+	+	+	−	−
Isolate 30/94		−	−	−	−	−	+	−	−	−	+	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−

+ = Agglutination. − = No agglutination.

properly classified. However, the antiserum to isolate 30/20 reacted with a few of the antigens of the other isolates and it may have been related to isolates of Strains 1 and 2. Similarly, reactions of the antiserum to isolate 30/53 with heterologous antigens indicated that isolate 30/53 was related to isolates of Strain 3.

To extend these observations, clones were prepared from isolates 30/73, 30/31 and 30/77, which were representative of Strains 1, 2 and 3, respectively, and from isolate 30/97 suspected to be a mixture of trypanosomes of Strains 1 and 3. When the antisera to the isolates were tested for agglutinins to these trypanosomes, clones 30/73, 30/31 and 30/77 reacted in almost exactly the same way as the original isolates (Table 2). Further tests with antisera from rabbits infected with the three clones confirmed that they consisted of trypanosomes of different strains (Table 3). Tests with the antisera to the original isolates (Table 2) and antiserum from a rabbit infected with clone 30/97 (Table 3) indicated that clone 30/97 was related only to the isolates of Strain 3 and clone 30/77, unlike the original isolate 30/97 which was agglutinated by antisera to isolates of Strains 1 and 3.

Table 3. *Agglutination of clones prepared from four isolates of Trypanosoma brucei collected in survey 30 by antisera prepared in rabbits (the antisera were tested at a dilution of 1/40 only)*

Trypanosomes used as antigens	Strain represented (see Table 2)	Reactions of antisera to clones			
		30/73	30/31	30/77	30/97
		Antisera collected after 2 weeks of infection			
Clone 30/73	Strain 1	+	−	−	−
Clone 30/31	Strain 2	−	+	−	−
Clone 30/77	Strain 3	−	−	+	−
Clone 30/97	Strain 3	−	−	−	+
		Antisera collected after 4 weeks of infection			
Clone 30/73	Strain 1	+	−	−	−
Clone 30/31	Strain 2	−	+	−	−
Clone 30/77	Strain 3	−	−	+	+
Clone 30/97	Strain 3	−	−	−	+

+ = Agglutination. − = No agglutination.

The relationships of trypanosomes isolated in different surveys

At intervals during this work, newly isolated trypanosomes were compared serologically with those collected in earlier surveys.

The relationship between isolates 5/65 and 17/14. A close relationship was found between isolate 5/65, collected from an infected ox in November 1961, and the clone prepared from isolate 17/14, collected from a different animal in November 1963. Agglutinating antisera from rabbits infected with either the isolate or the clone had high titres of antibodies to both antigens (Table 4), which indicated that the trypanosomes were derived from the same strain. The antigenic similarity of these trypanosomes, isolated from the herd at an interval of two years, was of considerable epidemiological significance and the relationship was investigated in greater detail. A clone was prepared from a line of isolate 5/65 which had been maintained first at −80° for 15 months and subsequently in an ox for 9 months. Clones 5/65 and 17/14 were then cyclically transmitted by *Glossina morsitans* to rats from which trypanosomes with basic strain antigens were obtained and stored at −80° as antigens for agglutination

tests. Rabbits were also infected with the clones by the tsetse flies to provide agglutinating and neutralizing antisera.

Agglutination tests with antisera taken from the rabbits after 10 and 14 days of infection confirmed earlier indications of the close relationship between the isolates and indicated that the cyclically transmitted trypanosomes of the derived clones had antigens in common.

Table 4. *Agglutination of isolates of Trypanosoma brucei collected from a herd of cattle over a period of five years by antisera prepared in rabbits*

Trypanosomes used as antigens	Titres of agglutinins in antisera to isolates and clones (as reciprocals of serum dilutions)								
	Isolate 5/28	Isolate 5/65	Clone 17/14	Clone 17/8	Clone 17/58	Isolate 22/24	Isolate 30/73	Isolate 30/31	Isolate 30/77
Isolate 5/28	1280	.	.	.	.	.	.	.	.
Isolate 5/65	.	1280	2560	.	.	.	.	20	.
Clone 17/14	.	640	2560	.	.	.	.	20	.
Isolate 22/24	.	.	.	.	.	2560	.	.	.
Isolate 30/73	.	.	.	.	40	.	320	.	.
Isolate 30/31	.	.	.	.	.	.	.	2560	.
Isolate 30/77	.	.	.	.	.	.	.	.	1280

. = No agglutination.

Table 5. *The effect of neutralizing antisera from rats and rabbits on the infectivity of suspensions of trypanosomes of clone 5/65 and clone 17/14*

Origin of sera	Infectivity of trypanosomes after incubation in normal sera or antisera*	
	Clone 5/65	Clone 17/14
Normal rabbits	4·8 ± 0·3	5·1 ± 0·5
Rabbit infected with clone 5/65	None	1·8 ± 0·3
Rabbit infected with clone 17/14	2·0 ± 0·3	None
Normal rats	4·9 ± 0·5	5·3 ± 0·5
Rats immunized against clone 5/65	0·8 ± 0·5	1·7 ± 0·3
Rats immunized against clone 17/14	2·1 ± 0·3	None

* Expressed as the $\log_{10}$ of the number ID_{63} per ml. of suspension and the standard errors according to Lumsden *et al.* (1963).

In neutralization tests, antiserum obtained from a rabbit which had been infected with clone 5/65 for 14 days neutralized the infectivity of a suspension of cyclically transmitted trypanosomes of clone 5/65 and considerably reduced the number of infective organisms in a suspension of clone 17/14. A comparable result was obtained when antiserum from the rabbit infected with clone 17/14 was tested for neutralizing activity against trypanosomes of both clones (Table 5). Further tests with neutralizing antisera prepared by infecting rats with cyclically transmitted trypanosomes of the clones and treating them with therapeutic doses of diminazene aceturate confirmed these findings. Both the rat and the rabbit antisera reduced the infectivity of suspensions of the heterologous clone to approximately the same value which indicated that the cyclically transmitted population of each clone probably consisted of trypanosomes with at least two variant antigens, only one of which occurred in both clones.

Production of variant antigens by the clones was also compared in chronic infections in rabbits. The rabbits were infected with 2×10^6 cyclically transmitted trypanosomes from the frozen clone stocks and three variant populations of each clone were isolated by injecting 0·5 ml. of infected rabbit blood into mice at the ends of the first, second and third weeks of infection. The variants isolated from the rabbit infected with clone 5/65 were designated 5/65a, 5/65b and 5/65c and those from the animals infected with clone 17/14, 17/14x, 17/14y and 17/14z. Sera were collected from the rabbits before infection, at 2-day intervals during the first week of infection and at 7-day intervals for a further three weeks. The sera were tested for agglutinins to both clones and the six derived variants.

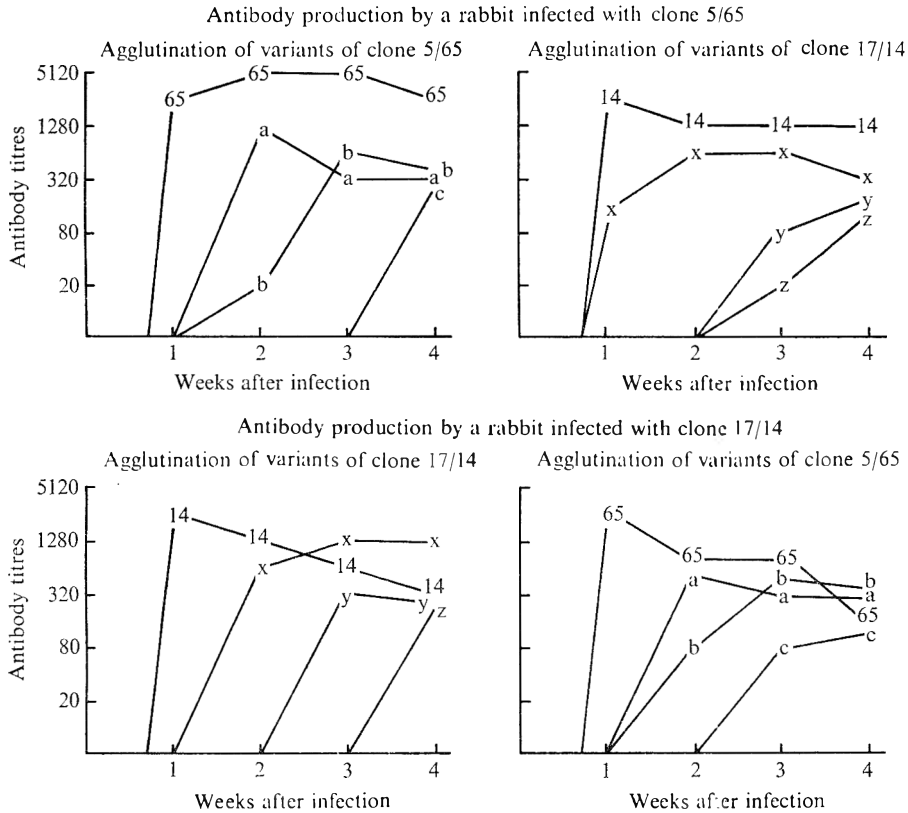

Fig. 1. The antigenic similarity of two clones of *Trypanosoma brucei* illustrated by comparing the production of agglutinating antibodies by rabbits infected with clone 5/65 and clone 17/14 to antigens prepared from the clones and derived variants. 65—65, a—a, b—b and c—c, production of agglutinins to clone 5/65 and variants 5/65a, 5/65b and 5/65c. 14—14, x—x, y—y and z—z, production of agglutinins to clone 17/14 and variants 17/14x, 17/14y and 17/14z.

The clones produced similar variant antigens during the infections because the antisera from the rabbit infected with clone 5/65 agglutinated the trypanosomes isolated from the rabbit infected with clone 17/14 and vice versa. The patterns of antibody production in the infected rabbits also indicated that, in general, variant antigens were produced in a similar sequence by both clones (Fig. 1).

Other relationships among the isolates. The results of agglutination tests to determine the general relationships of the trypanosomes isolated in four of the five surveys are shown in Table 4. The main characteristics of eight of the nine isolates used in these tests have already been described. The remaining isolate, 22/24, was the only isolate of *Trypanosoma brucei* obtained from the herd in survey 22. Clone 17/14 was used to represent the antigenically related isolates 17/14, 17/30, 17/70 and 17/96 obtained in survey 17, and isolates 30/73, 30/31 and 30/77 were used to represent the three strains of trypanosomes obtained in survey 30. The antisera used were pools of equal volumes of sera obtained from rabbits infected with the isolates at the ends of the second, third and fourth weeks of infection. Each pool was tested for agglutinins to the homologous and heterologous isolates with the exceptions of the antisera to clones 17/8 and 17/58 which were tested only against heterologous trypanosomes, as the homologous antigens were not available.

There was little evidence of antigenic similarity among the isolates with the exception of the close relationship between isolate 5/65 and clone 17/14, which has been described above. Low titres of antibodies in the antisera to clone 17/58 and isolate 30/31 suggested that the former was possibly related to isolate 30/73 and the latter to isolate 5/65 and clone 17/14. No evidence to support these possible relationships was obtained when further samples of antisera to clone 17/58 were tested for antibodies to isolate 30/73 and the related isolates 30/84 and 30/103 (see Table 2), or when a new batch of antiserum to clone 17/14 was tested for agglutinins to antigens of isolate 30/31 and the related isolate 30/105.

DISCUSSION

This study has shown that a majority of isolates of *Trypanosoma brucei* can be classified by agglutination tests using antisera to the predominant variant antigens of the trypanosomes to yield results of considerable practical value. In earlier work, such tests were used to demonstrate antigenic differences between isolates of *T. brucei* from widely separated geographical localities and similarities between trypanosomes isolated from different animals kept in one area (Gray, 1966). In the present work, similar methods have been used to show that 26 of 28 isolates of *T. brucei* obtained from a herd of cattle on one day consisted of trypanosomes of only three antigenically distinct strains. The techniques used were also sufficiently sensitive to suggest that ten isolates had been obtained from cattle infected with two different strains of *T. brucei* at the same time, a finding which was confirmed in one instance when a clone was prepared from a suspected mixed isolate.

Four of the isolates studied differed from the majority of the trypanosomes in some respects. Three did not adapt to maintenance in rats and mice, even after prolonged syringe passage, and the fourth was so sensitive to lysis by inactivated serum from both normal and immune rabbits that it could not be used as an antigen in agglutination tests. Furthermore, antisera to the predominant antigens of the four isolates reacted with few of the antigens of the other trypanosomes. Human trypanosomiasis is endemic in the area in which the trypanosomes were isolated (Duggan, 1962) and it is possible that these unusual isolates were the human pathogen *Trypanosoma gambiense* rather than *T. brucei*, particularly since the tsetse flies *Glossina palpalis* and *G. tachinoides*, which are normally associated with the transmission of *T. gambiense* in Nigeria, were

probably also the vectors of bovine trypanosomiasis in the experimental area. The problems involved in accurate determination of the species of pleomorphic trypanosomes of the subgenus *Trypanozoon* isolated from animals are well known (see Ormerod, 1967) and it would be difficult to substantiate the above suggestion without testing the infectivity to man of the four unusual isolates. Typical isolates of *T. brucei* and *T. gambiense* usually differ significantly in a number of ways, notably in their pathogenicity for experimental animals, the production of posteronuclear forms, and their sensitivity to tryparsamide and to the lytic effect of human serum, but tests based on such properties are of limited taxonomic value because they may change during the maintenance of isolates in the laboratory and because trypanosomes with intermediate characteristics occur in the field (Lester, 1933).

The advisability of conducting infectivity tests with poorly characterized trypanosomes in human volunteers is questionable, and no attempt was made to test the infectivity to man of the four unusual isolates. It was simply assumed for the purposes of the experiments that all the trypanosomes were *Trypanosoma brucei* on the grounds that they were isolated from cattle and that *T. gambiense* has not yet been isolated with certainty from any naturally infected vertebrate host in Nigeria other than man. In view of the recent isolation of the closely related human pathogen *T. rhodesiense* from cattle in East Africa (Onyango, van Hoeve & de Raadt, 1966), this assumption may have been wrong and it is to be hoped that current research on the antigenic relationships of organisms of the subgenus *Trypanozoon* will lead to unequivocal methods of differentiating between *T. brucei* and *T. gambiense* in the future.

One of the main objectives of the present work was to seek evidence of the antigenic stability of strains of *Trypanosoma brucei* in their natural environment from year to year. Earlier experiments on antigenic variation have indicated that the characteristic basic and predominant variant antigens of each strain of *T. brucei* always develop at an early stage of infection when a strain is transferred to a new host by blood passage or by a tsetse fly (Gray, 1965). Strains might therefore be expected to persist in a recognizable antigenic form in the field despite the extensive antigenic variation which occurs in trypanosomes during infections. The antigenic similarity of isolates 5/65 and 17/14 which were obtained from different animals at an interval of 2 years has afforded direct evidence in support of this suggestion. Although the populations of trypanosomes used in the comparative tests were not immunologically identical, the results of the investigation left little doubt that the isolates belonged to the same strain. Despite a careful search, no other examples of such long-term antigenic continuity were detected, but the single positive finding has emphasized the value of serological methods for comparing trypanosomes in epidemiological studies.

The significance of the antigenic diversity of the strains isolated during the 5-year observation period remains to be considered. There were at least seven different strains of trypanosomes among the 37 isolates examined and even more strains might have been found if a more representative sample of the trypanosomes infecting the herd in the first two years of the experiment had been available for study. Only two isolates were available from 20 infected animals in 1961, and none from three animals infected when the herd was examined in 1962. The policy of treating severely affected animals with therapeutic drugs from time to time also undoubtedly influenced the results by eliminating trypanosomes from the cattle between surveys. The findings that cattle

kept in a relatively small area under conditions of light tsetse challenge were exposed to infection with at least seven antigenically different strains of one trypanosome species and that animals were occasionally infected with two strains of the same species simultaneously, seem to render the possibility of immunization against trypanosomiasis remote.

I wish to thank Mr C. J. Roberts for much useful discussion and for his continued interest and help during this work.

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Pathogenicity of *Pseudomonas morsprunorum* in Relation to Host Specificity

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SUMMARY

Differences in phage type between plum and cherry strains of *Pseudomonas morsprunorum* were stable during passage through the homologous and heterologous hosts. In experiments controlled by phage typing reisolates from lesions, both strains were specific for the homologous plant when inoculated into leaves at low concentrations ($< 10^5$/ml.) but not at high concentrations ($> 10^6$/ml.). No specificity was observed in wound inoculations of branches made during the winter.

Autumn inoculation of cherry leaf scars with cherry strains resulted in severe disease, with a dose response type of relationship between inoculum concentration and infection. Plum strains were ineffective through cherry leaf scars and inhibited infection by cherry strains when present in the same inoculum. The inhibitory effect was not observed with plum strains killed by heat or streptomycin, or with plum strains inoculated live in the presence of bacteriostatic levels of streptomycin.

INTRODUCTION

Pseudomonas morsprunorum Wormald causes bacterial canker disease of cultivated Prunus species and is an important factor limiting the successful cultivation of the cherry (*Prunus avium*) and the plum (*P. domestica*) in Britain. It invades the stems and branches in the autumn causing extensive necrosis in the cortex and phloem. The resultant cankers are active until early the following summer, when the bacteria die in the affected tissues. Most cankers on cherry originate through leaf scars and hence are located on the branches. Cankers on plum trees usually affect the main stem, where the bacteria apparently gain access through small wounds. The pathogen survives the summer as an epiphyte on the foliage, but it may also infect the foliage, causing small necrotic leaf spots. The epidemiology of this disease is described in detail by Crosse (1966).

Plum and cherry isolates of *Pseudomonas morsprunorum* are indistinguishable biochemically (Garrett, Panagopoulos & Crosse, 1966) and under certain experimental conditions they will cross-infect (Wormald, 1938). Phage typing, however, has shown them to be distinct forms with high specificity in the field for their homologous hosts (Crosse & Garrett, 1963; Garrett & Crosse, 1963). The experiments described below compared the pathological behaviour of plum and cherry strains through different avenues of infection and at different levels of inoculum concentration. Their object was to investigate the role of the pathological characters in determining the host distribution of these organisms.

METHODS

Organisms. Cultures of *Pseudomonas morsprunorum* were isolated from necrotic leaf spots and from the leaf surfaces (Crosse, 1959) of plum and cherry trees at East Malling Research Station between 1955 and 1961, and maintained on nutrient agar containing 2% glycerol (NGA) and stored at 4°.

Isolations from plant material. Necrotic tissues from the margins of advancing lesions were comminuted in sterile distilled water and the resulting suspensions plated on nutrient agar containing 5% sucrose. On this medium *Pseudomonas morsprunorum* produces characteristic colonies after 3 to 4 days at 25° (Crosse, 1959).

Phage typing. The media and procedures were those described by Crosse & Garrett (1963). Typing was done with the virulent phages A3, A7, A32 and A15, originally from soils (Crosse & Garrett, 1963) and with the temperate phages N5/A5 and C6/C22 isolated from lysogenic strains of *Pseudomonas morsprunorum* (Garrett & Crosse, 1963).

Preparation of inoculum. Growth from a 24 h. slope culture on NGA was quickly suspended in 5 ml. sterile distilled water, decanted immediately into an empty sterile tube and diluted to the required concentration by reference to a calibration standard relating optical density to viable cell numbers.

Plant material. This consisted of 6- to 10-year-old field trees of the cherry cultivars Napoleon and Roundel and of the plum cultivar Victoria. A few experiments were done in the greenhouse on 3-year-old potted trees of these varieties.

Methods of inoculation. Branches were inoculated in autumn and winter through scalpel wounds in the bark and, in cherry, also through leaf scars on the fruiting spurs (120 to 180 spurs/treatment), by methods described by Crosse & Garrett (1966). Leaves (20/treatment) were inoculated by spraying bacterial suspensions on the under surfaces from a hand atomizer. Immediately before inoculation leaves were sprayed with water under 15 lb./in.^{2} pressure until the intercellular spaces of the leaves were visibly water-congested, to aid penetration of bacteria through the stomata. In all experiments a fully replicated control treatment, with sterile water substituted for bacterial suspension, was included to estimate the level of 'background' infection from 'wild' strains of *Pseudomonas morsprunorum* present on the tree surfaces.

Disease assessment. The number and size of cankers from branch inoculations were recorded after 22 weeks, in May of the year following inoculation. Cankers from wound inoculations were sized by length and those from leaf scar inoculations by a rating scale ranging from 1 for a micro-canker localized in the region of a leaf scar, to 10 for large cankers which killed the fruiting spur and spread more than 4 cm. along the axis of the branch. Since many fruiting spurs were completely necrotic at the time of recording, it was impossible to determine how many of the 7 to 9 scars on each spur were initially infected. We therefore estimated this from the product

$$\text{percentage infected spurs} \times \text{mean rating for canker size.}$$

This procedure was justified by the fact that canker size is largely dependent on the number of scars infected (Crosse, 1957). The resultant values were termed 'the rate of leaf scar infection' and were a measure of the infectivity of the strains through the leaf scar route.

Leaf infections were recorded 10 to 24 days after inoculation as number of necrotic spots/cm.^{2} leaf.

RESULTS

Phage relations of the bacteria

Phage typing. The phage types and lysogenicity of the experimental cultures are shown in Table 1. Plum strains were generally distinguished from cherry strains by their insensitivity to A3 and to the related phage A7 (Crosse & Garrett, 1963). An exception in this instance was D17, one of two anomalous plum isolates amongst approximately 200 examined in the course of this and previous investigations, which behaved like a cherry strain in phage typing. Cherry strains were divisible into subtypes according to their reactions to phages A32, A15, N5/A5 and C6/C22, but the plum strains behaved uniformly with these phages and only the lysogenic strain D2 could be distinguished from the others on the basis of its phage relations.

Table 1. *Phage types of plum and cherry strains of Pseudomonas morsprunorum*

Strains		Phages (at R.T.D.)					
Host	Code no.	A3	A7	A32	A15	N5/A5	C6/C22
Cherry	NA3*, N5*, C12, C41*	+	+	+	+	−	+
	C5*, C9*	+	+	−	+	+	−
	N7	+	+	+	−	−	+
	C17, C22	+	+	+	+	+	+
	C46	+	+	+	−	+	+
Plum	D17*	+	+	+	+	−	+
	D1, D2,* D4, D5, D7, D10, D12, D18	−	−	−	+	−	−

* Lysogenic strains. +, Confluent lysis. −, No reaction.

Stability of phage types during host passage. After eight successive passages through the fruitlets of each host, followed in some instances by three further passages through leaves, the phage type of each of the strains except D12 was unchanged. This strain lost sensitivity to phage A15 after the first passage through plum leaves and retained this character in subsequent passages. Strain D12 has been observed to have an abnormally high rate of mutation to resistance to A15 *in vitro*.

After a single passage of 22 weeks duration in plum and in cherry branch tissues, no change in sensitivity to A3 and A7 was detected in 14 of the 19 strains, but some re-isolates of N7 were now sensitive to phage N5/A5 (Table 2). Inoculations with the remaining four strains, N5, C5, D7 and D12, yielded some re-isolates of the heterologous strain type. Since these were mostly observed in the heterologous combination, i.e. where a plum strain was inoculated to cherry and a cherry strain to plum, these re-isolates were probably homologous 'wild' strain contaminants from the plant surfaces. None of these strains was highly virulent, while three of them, N5, C5 and D12, were amongst the least virulent tested (Fig. 1). The results of this experiment confirmed earlier work indicating that phage type was a stable and reliable marker for *Pseudomonas morsprunorum* in host passage experiments (Crosse & Garrett, 1966). No changes in their lysogenicity were observed in passage experiments.

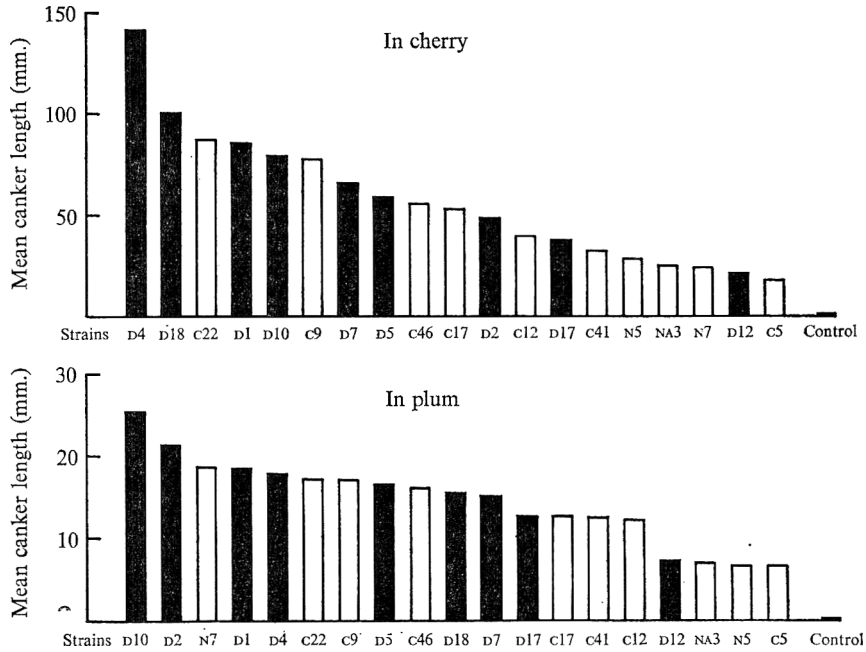

Fig. 1. Length of cankers in branches 22 weeks after inoculation through wounds during winter with individual plum strains (black columns) and cherry strains (white columns) of *Pseudomonas morsprunorum* (*c.* 10^7 cells/ml.).

Table 2. *Stability of plum and cherry phage types of Pseudomonas morsprunorum during host passage*

Ten replicated inoculations of each strain were made into each host and bacteria recovered from necrotic tissues at the margins of cankers after 22 weeks. Three of the ten re-isolates in each strain + host combination were selected at random and phage typed.

Strain	No. of isolates (max. 3) of the heterologous type (differing from parent strain in sensitivity to phages A3 and A7)	
	From cherry	From plum
NA3, N7*, C9, C12, C17, C22, C41, C46, D17, D1, D2, D4, D5, D10, D18	0	0
N5	0	2
C5*	0	1
D7	1	0
D12	2	3

* The majority of isolates were identical to the parent strain in all their phage reactions, but one isolate each of C5 and N7 from cherry and two of N7 from plum differed from the parent in sensitivity to the temperate phage N5/A5.

Infection experiments

Leaf infections. The first fully expanded leaf (no. 1) and the three leaves below (nos. 2 to 4 inclusive) on young growing shoots of field plum and cherry trees were inoculated through the stomata with the cherry strain C46 or the plum strain D 10 separately and in combination. All inocula were at a concentration of 10^4 cells/ml., giving a concentration for each strain in the combined inocula of 5×10^3 cells/ml.

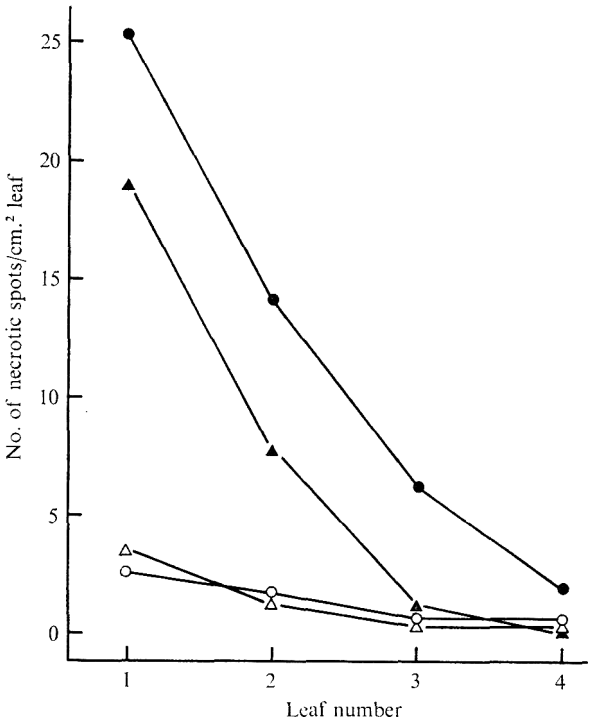

Fig. 2. Rate of leaf-spot infection by *Pseudomonas morsprunorum* in the first fully expanded leaf on cherry shoots (no. 1) and the three successive leaves below (nos. 2 to 4 incl.). ●, Cherry strain C46; ○, plum strain D 10; ▲, C46 + D 10; △, control (water). Total inoculum concentration in all treatments *c.* 10^4 cells/ml.

The results for cherry leaves are summarized in Fig. 2. As is common in plant diseases, the rate of infection decreased progressively down the shoot, i.e. with increasing leaf maturity. The cherry strain (C46) caused severe leaf spotting, especially on the younger leaves. The rate of infection with the heterologous plum strain (D 10) was approximately equal to that in the controls, suggesting that it was probably due to 'wild' cherry strains present on the leaf surfaces at the time of inoculation. This was subsequently confirmed by phage typing isolates from the D 10 inoculations (Table 3).

Because penetration of inoculum into plum leaves was not uniform, lesions appeared as irregularly distributed patches of necrosis rather than as discrete spots. For this reason, no detailed data are shown for plum experiments, but the results were generally in line with those from cherry. Thus the homologous strain (D 10) was outstandingly the more infective, causing necrosis of more than 50% of the total lamina area in most

inoculated leaves. The heterologous strain (C46) affected fewer leaves and never more than 25% of the lamina area. The level of infection with heterologous strain, was, however, higher than in the controls; this evidence of infection of plum by the heterologous strain was confirmed by the recovery of re-isolates exclusively of the C46 type from leaves inoculated with this strain (Table 3).

Table 3. *Incidence (%) of plum and cherry strains of Pseudomonas morsprunorum in leaf lesions*

Leaves were inoculated with C46 (cherry strain) and D 10 (plum strain), separately and in combination. Bacteria were recovered from lesions after 10 days and from 28 to 57 isolates per treatment tested with the full range of typing phages.

Inoculation treatment (10^4 cells/ml.)	Cherry leaves		Plum leaves	
	Cherry strains	Plum strains	Cherry strains	Plum strains
C46	.	.	100*	0
D 10	98†	2	.	.
C46+D 10	100*	0	23*	77
Controls (water)	100†	0	12†	88

* Identical to C46 in all phage reactions except one isolate from the C46+D 10 inoculations of plum.

† A mixture of phage types indicating infection by 'wild' cherry strains.

The higher infectivity of C46 in the heterologous host showed in the results from the mixed inoculations, this strain accounting for 23% of the re-isolates from plum compared with 0% of the re-isolates of D 10 from cherry. In the controls, where there was ample opportunity for cross-infection because of the very close proximity of the trees, 'wild' cherry strains were recovered from 12% of the lesions on plum leaves but no plum strains were recovered from cherry leaves. This suggests that higher infectivity in the heterologous host may be a general character of cherry strains.

The host specificity of the two strains was further examined in experiments with mixed inocula at extreme concentrations (10^2 and 10^7 to 10^8 cells/ml.). The results showed that specificity was influenced by inoculum concentration and was only pronounced at the lower concentration (Table 4). At the higher concentration the cherry strain was predominant in both hosts and in the plum it apparently interfered with the development of homologous plum strains. In leaf infections bacteria pass through the stomata and grow initially within the confines of the substomatal vesicle. At lower inoculum concentrations there would tend to be few mixed infections of the substomatal vesicles and hence less direct interference between the strains. This probably accounts for the higher incidence of the plum strain in both hosts at the lower concentration. The unusually high incidence of the plum strain in cherry leaves inoculated with the lower inoculum concentration (Table 4) was associated with the absence of any disease symptoms. The leaves used in this experiment were fully mature, and in such leaves it seems that host specificity factors may not operate effectively, or growth of bacteria in the tissues after infection is insufficient to reveal these factors fully.

Inoculation of branches through wounds. These were made in December when plum and cherry branches tend to be most susceptible to wound infection. Each of the experimental cultures was inoculated (10^7 cells/ml.) once on each of ten trees of each

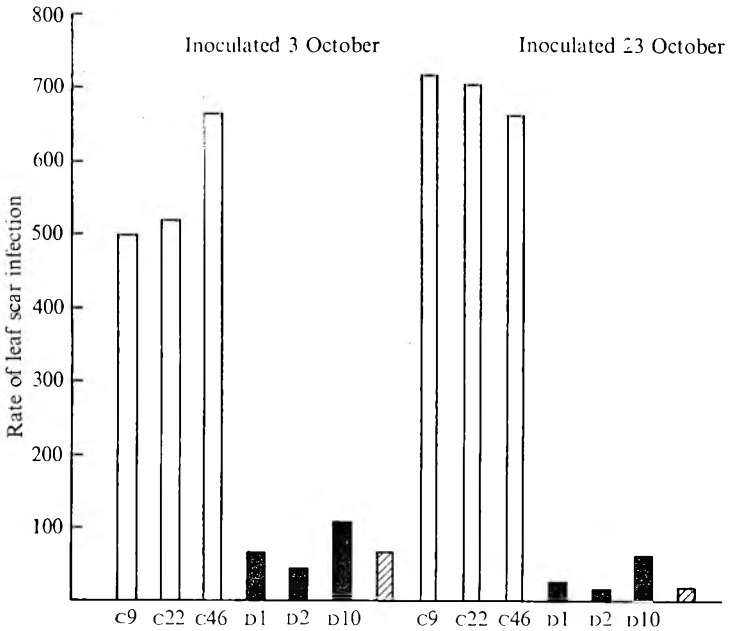

Fig. 3. Rate of cherry leaf-scar infection by individual cherry strains (white columns) and plum strains (black columns) of *Pseudomonas morsprunorum* on two occasions in the autumn (c. 10^7 cells/ml.). Hatched columns denote water controls.

Table 4. *Host selection of homologous strain from mixed strain inocula of Pseudomonas morsprunorum*

At each inoculum concentration there were approximately equal cell numbers of C46 (cherry strain) and D 10 (plum strain). Bacteria were recovered from lesions 11 days after inoculation and approximately 100 single colony isolates from each treatment tested with phage A 7 (C46 sensitive) and phage A 15 (D 10 sensitive) to identify the strain.

Expt. no.	Total inoculum concentration (cells/ml.)	% homologous bacteria recovered from: Cherry leaves	Plum leaves
1	10^8	100	30
	10^2	95	73
2	10^7	98	5
	10^2	63*	100

* No symptoms occurred in this treatment; bacteria were recovered from macerates of undiseased leaf tissue.

host at randomly selected sites on 2- to 3-year-old branches. The mean lengths of the cankers in the following May are shown in Fig. 1. The plum and cherry strains readily cross-infected and without sign of host specificity. Among the eight strains producing the longest cankers in cherry, five were originally from plum; in plum, three of the more virulent strains were from cherry. All inoculations were successful except with the less virulent strains D 12, C 5 and N 7 in cherry, where 60, 50 and 10 % respectively of the inoculations failed. It was concluded that factors determining host specificity in plum and cherry strains did not operate effectively under these conditions of inoculation.

Leaf scar infections of cherry fruiting spurs. Figure 3 shows the rate of leaf-scar infection with the cherry strains C9, C22 and C46 and with the plum strains D1, D2 and D10, on two separate occasions in the autumn (3 and 23 October). At the high concentration of inoculum employed (10^7 cells/ml.) each of the cherry strains induced severe disease, but the plum strains were largely ineffective (Fig. 3). Indeed, comparison with natural infection in the uninoculated controls suggested that many of the cankers recorded for the plum strains were probably due to 'wild' cherry strains. This was confirmed by phage typing bacteria from the cankers. All the isolates from the D1 cankers, and 50% and 20% from the D2 and D10 cankers respectively, phage-typed as 'wild' cherry strains.

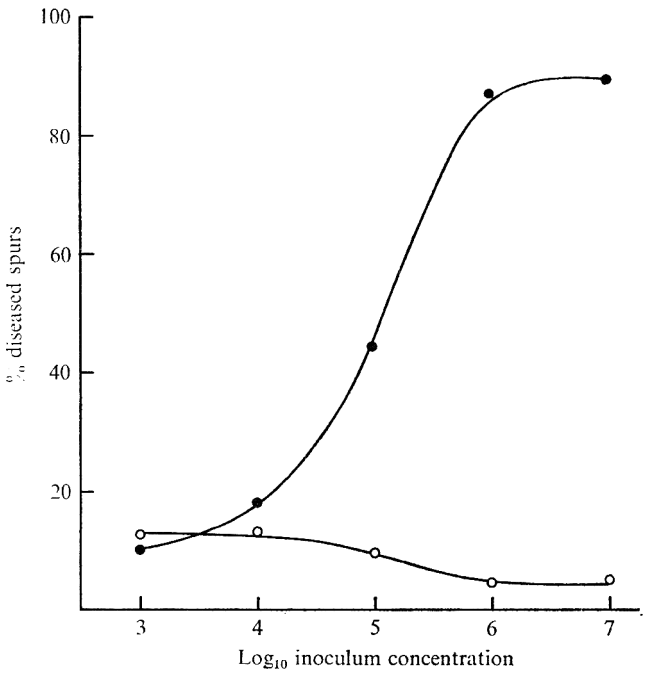

Fig. 4. Percentage diseased spurs resulting from leaf-scar infection with a mixture of five cherry strains of *Pseudomonas morsprunorum* (●) and with a mixture of five plum strains (○) over a range of inoculum concentrations.

The next experiment compared a mixed inoculum of five cherry strains (C9, C12, C17, C22 and C46) with a mixed inoculum of five plum strains (D1, D2, D4, D5 and D10) over a logarithmic series of concentrations from 10^3 to 10^7 cells/ml. inclusive. The cherry strains again resulted in severe disease with a dose-response type of relationship between the log inoculum concentration and the percentage diseased spurs (Fig. 4). A few small cankers were recorded for the plum strains and these were subsequently shown by phage typing to be due to 'wild' cherry strains. There were indications that these 'wild' strain infections were inhibited at the higher inoculum concentrations of the plum strains (Fig. 4). This was supported by the data on canker size (Fig. 5). Thus with cherry strains canker size increased with the inoculum concentration, but with the plum strains it decreased. The latter anomalous result can be explained by assuming that the plum strains inhibited most readily those 'wild' strain

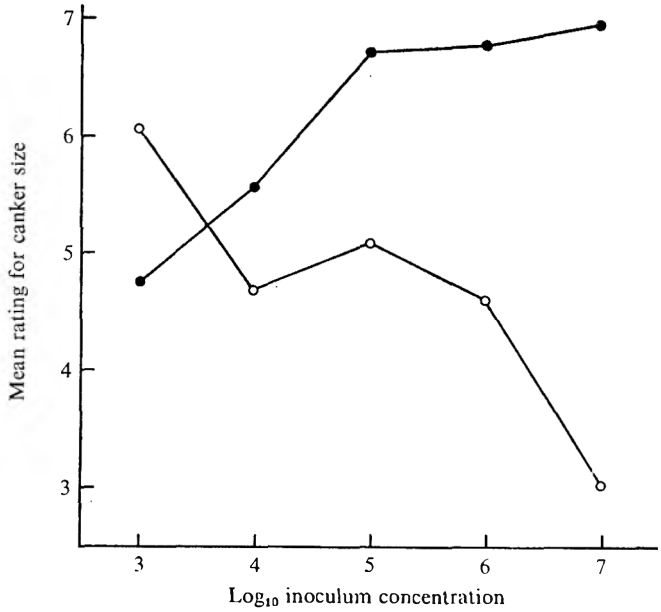

Fig. 5. Mean rating for canker size. Details otherwise as in Fig. 4.

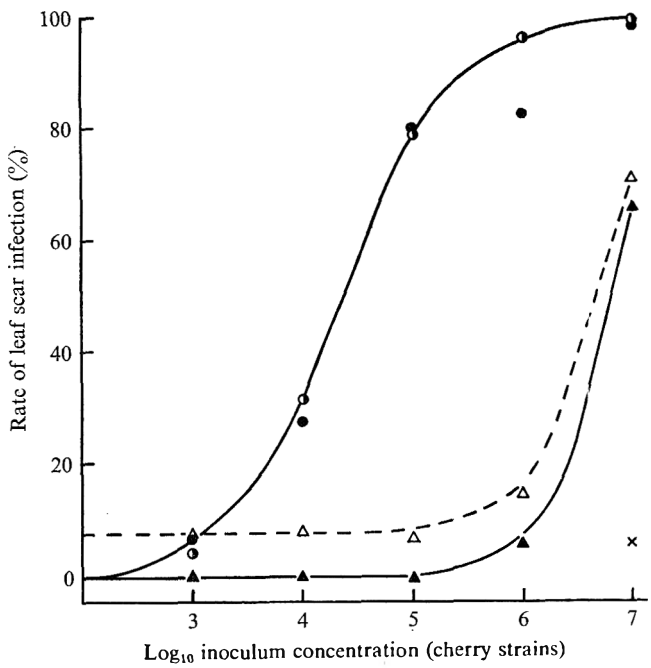

Fig. 6. Rate of cherry leaf scar infection %* with different inoculum concentrations of a mixture of five cherry strains of *Pseudomonas morsprunorum* in the presence and absence of a fixed concentration (c. 10^7 cells/ml.) of a mixture of five plum strains. (●), Cherry strains alone; (▲), plus live plum strains; (◐), plus heat-killed plum strains; (×), incidence of pinpoint necroses with plum strains alone; continuous lines, typical symptoms; broken line, all symptoms, including pinpoint necroses.

Corrected for natural infection, and expressed as percentage of the rate of infection at the highest inoculum concentration (see text).

infections which would have given rise to the smallest cankers. The result would be to increase mean canker size in the successful 'wild' strain infections.

Inhibition of cherry by plum strains in leaf-scar infections on cherry. Figure 6 compares the infectivity of the mixture of cherry strains over the inoculum concentration range 10^3 to 10^7 cells/ml. inclusive, in the presence and absence of a fixed concentration of 10^7 cells/ml. of a mixture of plum strains.

The data indicated that the rate of infection with cherry strains at 10^7 cells/ml. was the maximum possible in this experiment. All other values were therefore expressed

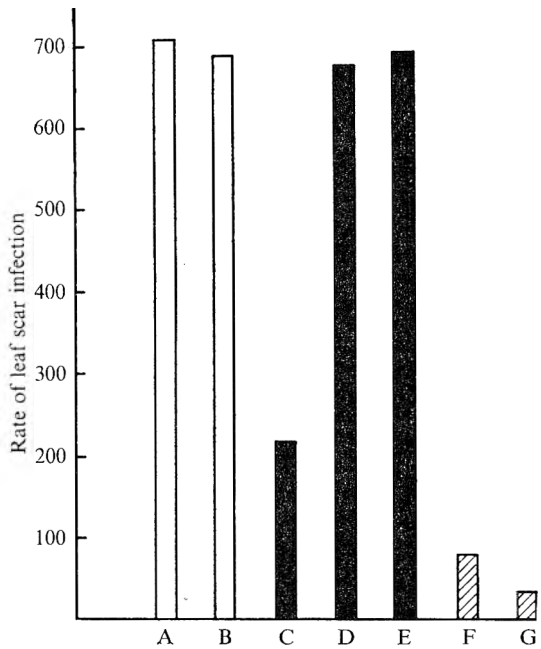

Fig. 7. Rate of leaf-scar infection with a streptomycin-resistant mutant of the cherry strain C22 of *Pseudomonas morsprunorum* (*c.* 10^6 cells/ml.) in the presence and absence of the streptomycin-sensitive plum strain, D10 (*c.* 10^7 cells/ml.). A, Cherry strain alone (white columns) in water; B, in streptomycin; C, cherry strain plus plum strain (black columns) in water; D, in streptomycin; E, with plum strain killed with streptomycin. ▨, Controls; F, water; G, streptomycin. Concentration of streptomycin in B, D and G, 0·72 i.u./ml.

as a percentage of this and then corrected for natural infection by the method described by Fisher & Yates (1953). The continuous lines refer to dark brown, water-soaked lesions typical of infection by cherry strains. In the absence of plum strains the rate of infection percentage with cherry strains followed a dose-response pattern and this was not affected by the presence in the inoculum of plum strains which had been killed by heat treatment at 60° for 10 min. The presence of live plum strains, however, reduced infection by the cherry strains at the higher concentrations of inoculum and totally inhibited it at the lower concentrations. The inhibitory effect of the plum strains was thus proportional to their relative numbers in the inoculum.

Fruiting spurs inoculated with live plum strains in this experiment produced leaf and blossom normally in the following spring, but several spurs showed a dry pinpoint necrosis, centred around the vascular traces in one or two of the leaf scars. This

feature was noted whenever live plum strains were present in inoculum and especially with cherry strains at the lower concentrations. The broken line in Fig. 6 refers to all infections, including pinpoint necroses. At concentrations of the cherry strains less than 10^6 cells/ml. the incidence of infections, mostly pinpoint necroses, remained constant at a level slightly above that observed with plum strains alone. The rate at which plum strains induced pinpoint necrosis was therefore not affected by the presence of cherry strains in the inoculum.

The inhibitory activity of the plum strain D 5 against a streptomycin-resistant mutant of the cherry strain C 22 was tested (*a*) with bacteriostatic levels of streptomycin sulphate (0·72 i.u./ml.) in the inoculum and (*b*) after the plum strain had been killed with streptomycin sulphate (18.0 i.u./ml. for 45 min. followed by three washes in sterile distilled water). The effect of these treatments on the leaf-scar infectivity of mixed inocula which contained the plum and cherry strain at concentrations of 10^7 and 10^6 cells/ml. respectively are summarized in Fig. 7. When present live in the inoculum, D 5 reduced the incidence of typical lesions caused by the cherry strain by approximately 70%, but had no effect in the presence of streptomycin or after it has been killed by streptomycin. Streptomycin had no discernible effect on the infectivity of the cherry strain but reduced natural infection by 'wild' cherry strains in the control treatment.

DISCUSSION

Although indistinguishable when cross-inoculated through wounds in branches, plum and cherry strains behaved as distinct pathotypes with marked specificity for the homologous (natural) host in stomatal and leaf-scar inoculations. These results indicate that host distribution of *Pseudomonas morsprunorum* strains in the field is primarily determined by pathological characters. They further suggest that pathogenicity in this organism is not a simple 'all-or-none' character but a complex attribute involving more than one virulence factor. As to the nature of the factors responsible for host specificity, we can at present only speculate. The fact that specificity in stomatal inoculations was evident only at lower inoculum concentrations suggests that in leaf tissues it may be determined by factors controlling infection by these organisms from low inoculum doses. Ercolani & Crosse (1966) reached similar conclusions from study of the effects of inoculum dose on the rate of growth of *P. morsprunorum* and related bacteria *in vivo*. They found the rate of growth of homologous organisms was independent of the dose. Comparable growth rates with heterologous organisms were observed only at the highest inoculum concentrations and growth was terminated early by a hypersensitivity reaction in the host.

The hypersensitivity reaction in bacterial diseases, originally described by Klement (1963), appears now to be a general response in leaves of non-host plants injected with phytopathogenic bacteria (Klement, Farkas & Lovrekovich, 1964; Klement & Goodman, 1967). It is characterized by loss of water from plant cells and the rapid appearance of atypical necrotic symptoms coincident with the abrupt cessation of bacterial growth in the tissues (Klement & Lovrekovich, 1961, 1962; Ercolani & Crosse, 1966).

There is evidence that the failure of heterologous plum strains to infect through cherry leaf scars, and their inhibitory effect on cherry strains, may have been due to a hypersensitivity reaction. The dry pinpoint necroses of the vascular leaf traces associated with the presence of plum strains in the inoculum was strongly reminiscent

of this type of reaction. Plum strains also failed to inhibit when killed by heat or inactivated by streptomycin. Klement & Goodman (1967) have shown that phytopathogenic bacteria so treated are unable to induce the hypersensitivity response in leaf tissues.

There have been several reports of bacteria, either saprophytic forms (Teliz-Oritz & Burkholder, 1960; Crosse, 1965; Goodman, 1965) or avirulent strains of pathogens (Averre & Kelman, 1964; Goodman, 1967; Lippincott & Lippincott, 1969), inhibiting plant infection by phytopathogenic bacteria. Averre & Kelman (1964) attributed the inhibition of virulent by avirulent strains of *Pseudomonas solanacearum* to the hypersensitivity re action. As in our experiments, the inhibitory strain wa most effective when present in the inoculum in high numbers relative to the infecting strain. Lippincott & Lippincott (1969) found, however, that the inhibitory effect of an avirulent on a virulent strain of *Agrobacterium tumefaciens* was a function of the absolute concentration of the strains in the inoculum and not of their relative numbers. They concluded from this and other evidence that inhibition was due to exclusion of the virulent strain from specific attachment sites within the host tissues. The fact that the inhibitor strains in their experiments were equally effective after heat treatment is also evidence that a hypersensitivity reaction was not involved.

Why plum and cherry strains of *Pseudomonas morsprunorum* should infect nonspecifically through wounds in branches is not clear. These inoculations differed from leaf-scar inoculations in three major respects. The effective inoculum dose was probably massive compared with that in leaf-scar infections (Crosse, 1966); the inoculations resulted in mechanical damage to tissues at the site of infection; and they were made at a time (December) when branch tissues were fully dormant and in their most susceptible condition (Shanmuganathan, 1962). It is possible that a hypersensitivity reaction was induced by heterologous bacteria in wound inoculations but was not effective against them at the very high inoculum doses employed. Alternatively, the effects of this reaction may have been neutralized by some artefact resulting from damage to the plant tissues, but it would then be difficult to explain how heterologous bacteria were able subsequently to invade the undamaged tissues. Finally, it is possible that in fully dormant tissues heterologous bacteria behave as homologous bacteria, i.e. they do not induce a hypersensitivity reaction or any other form of host defensive response.

These experiments confirm that there is a correlation between virulence characters and phage sensitivity in *Pseudomonas morsprunorum*, similar to that reported by Williams (1962) for staphylococci. Thus only strains sensitive to A7 and related phages are able to infect through cherry leaf scars. We have found no exceptions to this amongst English isolates and very few amongst isolates from other countries. The plum isolate D17, for example, which phage-typed as a cherry strain, has behaved exactly as a cherry strain in leaf-scar inoculations. In recent experiments cherry strain mutants resistant to phage A7 were found to have lost infectivity through leaf scars, suggesting that virulence factors and phage sensitivity may be linked as they are in *Salmonella typhi* (Craigie & Yen, 1938). There is no proof of this, however, and the differences in phage type between plum and cherry strains of *P. morsprunorum* may simply reflect a different history of phage infection in the field. Cherry strains may have failed to acquire resistance to A7 and related phages because these are absent from cherry, or their activity is in some way inhibited.

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Genetic Mapping of a Locus for Triosephosphate Isomerase on the Genome of *Escherichia coli* K12

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SUMMARY

A gene specifying triosephosphate isomerase (*tpi*) has been located between *rha* and *glpK* on the *Escherichia coli* chromosome. Very recently a gene specifying fructose-6-phosphate kinase (*pfk*) has been located in this same area and we have found that the *tpi* and *pfk* are highly cotransducible. This finding provides the first indication of any close chromosomal association between genes specifying glycolytic enzymes.

INTRODUCTION

The selection of mutants of *Escherichia coli* K12 deficient in triosephosphate isomerase activity (TPI) has been described previously (Anderson & Cooper, 1969). The pleiotropic characteristics of such mutants showed that only one species of TPI was involved in both glycolysis and gluconeogenesis. In this paper the position of the *tpi* locus on the *E. coli* genome has been determined to see how it relates to the known genes which specify other glycolytic or gluconeogenic enzymes (Böck & Neidhart, 1966; Brice & Kornberg, 1967; Fraenkel, 1967; Morrissey & Fraenkel, 1969).

METHODS

Bacterial strains. Table 1 lists the strains used. The symbol *tpi* has been chosen for the gene specifying triosephosphate isomerase.

Media. Bacteria were maintained on Oxoid nutrient agar slopes supplemented with L-methionine and thymine (40 μg./ml.). Selective plates contained minimal medium (Ashworth & Kornberg, 1966) solidified with 1·5% (w/v) Oxoid no. 1 agar and the appropriate carbon source, at a final concentration of 50 mM (D,L-sodium lactate) or 25 mM (D-glucose, sodium D-gluconate and mannitol). Where required, amino acids were added at a final concentration of 80 μg./ml. The medium used for phage propagation and transduction (Z-medium) consisted of Oxoid nutrient broth or nutrient agar supplemented with $MgCl_2$ (10 mM) and $CaCl_2$ (2·5 mM). Soft minimal agar was minimal medium containing 0·6% (w/v) agar. The eluting fluid used for harvesting the phage was 0·8% NaCl + 0·8% Oxoid bacteriological peptone in chloroform-saturated water.

Bacteria were grown in liquid minimal medium or nutrient broth as described by Ashworth & Kornberg (1966).

Bacterial crosses. Log-phase cells growing in nutrient broth were mixed at 37° to give approximately 1×10^8 Hfr cells and 5×10^8 F^- cells/ml. After 5 min. gentle

agitation the mating mixture was diluted into minimal medium containing gluconate and the appropriate amino acids (de Haan & Gross, 1962). Conjugation was interrupted by 1 min. agitation of a 2 ml. sample using a Vortex Jun. Mixer (Scientific Industries, Inc., Springfield, Mass., U.S.A.). Samples were spread on to the appropriate selective plates containing streptomycin sulphate (100 μg./ml.) to select against the Hfr cells.

Table 1. *Characteristics of Escherichia coli K12 strains*

Strain	Sex	Genotype*	Source or reference
K10	HfrC	*thi, str-s*	F. C. Neidhardt
AA200	HfrC	*tpi-1, thi, str-s*	From K10 by mutagenesis (Anderson & Cooper, 1969)
AA201	HfrC	*tpi-2, thi, str-s*	From K10 by mutagenesis (Anderson & Cooper, 1969)
AA202	HfrC	*tpi-3, thi, str-s*	From K10 by mutagenesis (Anderson & Cooper, 1969)
PA309	F$^-$	*thi, argHBCE, his, leu, thr, trp, str-r* (*gal, xyl, man, mal*)	R. H. Walmsley
AA210	F$^-$	*thi, tpi-1, argHBCE, leu, thr, trp, str-r*	AA200 × PA309 by selection for His$^+$ (this paper)
P10	HfrJ$_4$	*thr, leu, str-s* (*lac, mal*)	F. Jacob
AB2297	Hfr	*ilv, ade, str-s* (*xyl*)	E. A. Adelberg
AT2444	HfrH	*metE, thi, str-s*	A. L. Taylor
K1	HfrH	*metBF, thy, str-s*	H. L. Kornberg
K1.17	HfrH	*metBF, thy, glpK, str-s*	From K1 by mutagenesis (this paper)
K1.17.18	HfrH	*metBF, thy, glpK, rha, str-s*	From K1.17 by mutagenesis (this paper)
K1.1.5^c.16	HfrH	*metBF, thy, pfk, str-s* (*pps, ici^c*)	H. L. Kornberg
PA374	F$^-$	*thr, leu, thi, his, metE, argH, str-r*	N. Glansdorff
AB1911	F$^-$	*metBF, argHBCE, str-r*	H. L. Kornberg

Abbreviations used: *thi* = thiamin; *arg* = arginine; *his* = histidine; *leu* = leucine; *thr* = threonine; *trp* = tryptophan; *ilv* = isoleucine and valine; *met* = methionine; *thy* = thymine; *ade* = adenosine; *gal* = galactose; *xyl* = xylose; *man* = mannose; *mal* = maltose; *lac* = lactose; *rha* = rhamnose; *str-r* = resistance to streptomycin; *str-s* = sensitivity to streptomycin; *tpi* = triosephosphate isomerase; *glpK* = glycerol kinase; *pfk* = fructose 6-phosphate kinase; *pps* = phosphoenolpyruvate synthetase; *icl^c* = constitutive formation of isocitrate lyase; *fda* = fructose diphosphate aldolase.

* Non-relevant carbohydrate markers are given in parentheses.

Transduction. Phage P1*kc* lysates were prepared by confluent lysis of donor bacteria on Z-agar plates. About 2×10^9 donor bacteria in Z-broth were mixed with 2 to 4×10^8 P1*kc* particles. After 20 min. at 37° the mixture was diluted sixfold into soft minimal agar at 46° and 3 ml. samples were plated as an overlay on to Z-agar plates. This surface layer was collected after 6 h. incubation at 37°, homogenized in 2 ml. of

eluting fluid and the debris was removed by centrifugation at 12,000*g* for 5 min. at 2°. The lysates were stored at 4° over chloroform and generally contained about 10^{10} plaque-forming units/ml. when titrated with K10 as the indicator strain.

Recipient bacteria were grown on 20 ml. nutrient broth to about 1×10^9 bacteria/ml. then harvested and resuspended in 2 ml. of Z-broth. The phage were added (multiplicity = 1) and adsorption was allowed to proceed for 30 min. at 37° without aeration. The mixture was centrifuged, the bacterium pellet resuspended in 1 ml. of nutrient broth and suitable dilutions were plated on to the appropriate selection media. The colonies obtained after incubation for about 48 h. at 37° were streaked on to fresh plates and then restreaked on to the appropriate plates to score the inheritance of unselected markers. In each experiment, controls were run to check reversion of the recipient strain and sterility of the lysate.

Isolation of mutants. Glycerol kinase negative (*glpK*) mutants were isolated as described by Cozarelli & Lin (1966). Rhamnose negative (*rha*) mutants were isolated after ethylmethane sulphonate mutagenesis (Lin, Lerner & Jorgensen, 1962) and penicillin treatment (Gorini & Kaufman, 1960). Rhamnose non-fermenters were identified on rhamnose-eosin-methylene blue plates.

Assay of enzymes. Triosephosphate isomerase was assayed as described by Anderson & Cooper (1969).

RESULTS AND DISCUSSION

The approximate map position of the *tpi* gene was determined by mating AA200 and PA309. After 120 min. incubation, streptomycin-resistant recombinants which had inherited the ability to grow in the absence of a particular amino acid were selected on appropriately supplemented gluconate minimal plates. A large proportion (30 to 40%) of the *arg*$^+$ recombinants and a smaller proportion (5%) of the *his*$^+$ recombinants were unable to grow on lactate, suggesting that they had inherited the *tpi*$^-$ allele. Such lactate negative recombinants had no detectable TPI activity and one, AA210, was selected for use in further experiments.

Interrupted mating experiments between the Hfr donor strain P10 (Schwartz, 1966) and AA210 showed that the *tpi*$^+$ allele entered about 1 min. after the *argHBCE* cluster, thus placing *tpi* at about 76 min. on the *Escherichia coli* linkage map (Taylor & Trotter, 1967). Thus *tpi* should be cotransducible with one or more of the amino acid markers *argHBCE*, *metBF*, *metE* and *ilv*. The results of various cotransduction studies are shown in Fig. 1 and Table 2. When AA210 was the recipient there was no detectable cotransduction between *tpi* and *ilv*. There was no linkage of *tpi* to *metE* but *tpi* was readily cotransducible with both *argHBCE* (8 to 15%) and *metBF* (38%). In the reciprocal cross (Table 2, Expt. 4, 5) the *tpi* lesion from the donor showed extremely low linkage of *tpi* to *metE* (< 0·3%) but significant, albeit reduced, linkage to both *argHBCE* (3%) and *metBF* (20%) was again observed.

From the values deduced by Taylor & Trotter (1967) to relate cotransduction frequency with P1 to map distance, these linkage values suggest that *tpi* is 0·5 min. from *metBF*, 1 min. from *argHBCE* and 2 min. from *metE*. Each of these values places *tpi* at 76·3 min. on the *Escherichia coli* map (Taylor & Trotter, 1967), a region which contains markers for the metabolism of rhamnose (*rha*) (Power, 1967) and glycerol (*glpK*) (Cozzarelli & Lin, 1966).

To locate *tpi* more precisely a *glpK* mutant, K1.17, was isolated and subsequently

further mutated to yield a *rha* mutant, KI.17.18 (see Methods). Both were used in cotransduction studies with *tpi*. Since the absence of TPI renders organisms unable to grow on rhamnose or glycerol (Anderson & Cooper, 1969) the selection in such three-factor crosses must be for *tpi*$^+$ to allow unambiguous determination of the various phenotypes. As is shown in Fig. 1 and Table 2, Expt. 6, 7, there was a very high linkage of *tpi* to both *glpK* (81%) and *rha* (55%). Essentially identical linkage values were found for two further *tpi* mutants, AA201 and AA202 (Table 2, Expt 8, 9).

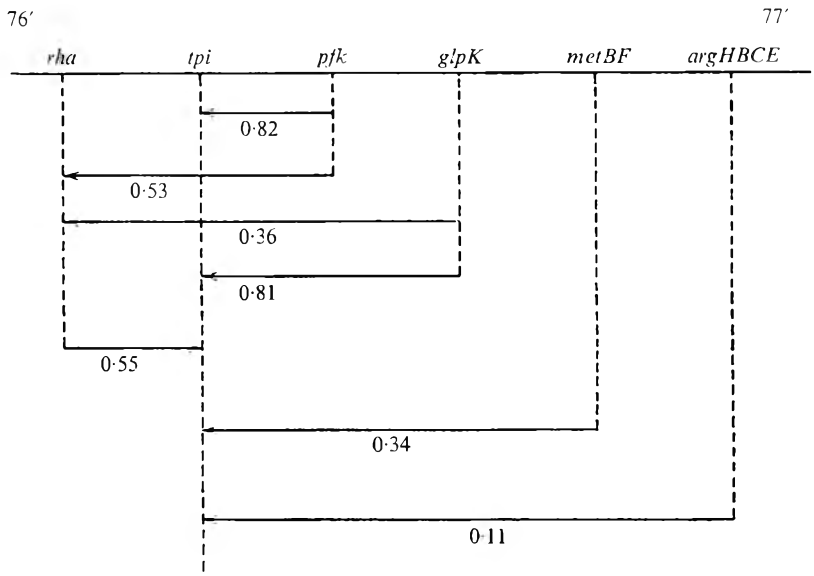

Fig. 1. Genes at 76 to 77 min. on the *Escherichia coli* linkage map (modified from Taylor & Trotter, 1967). Cotransduction frequencies from Table 2 are given as the fraction of the selected recombinants which have inherited the unselected marker. The selected marker is at the head of the arrow and the unselected marker at the tail.

If we assume that transductants requiring a minimum of four crossovers will occur significantly less frequently than those requiring a minimum of two crossovers, the frequencies of the various classes of recombinants shown in Table 2 are consistent with a gene order *rha tpi glpK*. In particular in Expt. 6, the least frequent of the four possible phenotypes is that expected for the order *tpi glpK metBF*, whilst in Expt. 7 the failure to find a single phenotype of especially low frequency is consistent with the gene order *rha tpi glpK*.

When the location of a *pfk* gene was reported very recently (Morrissey & Fraenkel, 1969) its position between *rha* and *glpK* indicated that it should be readily cotransducible with *tpi*. Since a similar *pfk* mutant was available in this laboratory, we have measured the cotransduction of *tpi* and *pfk* and the anticipated high linkage (82%) was found. Thus the *tpi* and *pfk* genes appear to be very close together on the *Escherichia coli* chromosome. The frequencies of the various phenotypes observed in this experiment are shown in Table 2, Expt. 10, and are consistent with the gene order *tpi pfk metBF*. Since the properties of the Pfk mutant used in these studies (H. L. Kornberg, personal communication) appear to be identical to those described

Table 2. *Transduction of tpi and pfk*

The selection and scoring of nutritional markers was on the appropriately supplemented gluconate minimal plates since both *tpi* and *pfk* mutants grow normally on gluconate. The selection and scoring of the other markers involved the use of the following appropriately supplemented minimal plates: $GlpK^+$ on glycerol, Rha^+ on rhamnose, Tpi^+ on lactate and Pfk^+ on mannitol.

Expt.	Donor	Recipient	Selected transductants	No. scored	Distribution of unselected markers in the transductants (%)			
1	AB 2297 (*ilv*)	AA 210 (*tpi-1*)	Tpi^+	305	Tpi^+ Ilv^- 0	Tpi^+ Ilv^+ 100		
2	AT 2444 (*metE*)	AA 210 (*tpi*–1, *argHBCE*)	Arg^+	330	Met^- Tpi^- 0	Met^- Tpi^+ 0	Met^+ Tpi^- 90	Met^+ Tpi^+ 10
			Tpi^+	148	Met^- Arg^- 0	Met^- Arg^+ 0	Met^+ Arg^- 92	Met^+ Arg^+ 8
3	K 1 (*metBF*)	AA 210 (*tpi-2, argHBCE*)	Arg^+	518	Met^- Tpi^- 24	Met^- Tpi^+ 6	Met^+ Tpi^- 67	Met^+ Tpi^+ 3
			Tpi^+	364	Met^- Arg^- 27	Met^- Arg^+ 11	Met^+ Arg^- 58	Met^+ Arg^+ 4
4	AA 200 (*tpi-1*)	PA 374 (*metE, argH*)	Arg^+	327	Met^- Tpi^- 4	Met^- Tpi^+ 96	Met^+ Tpi^- 0	Met^+ Tpi^+ 0
			Met^+	288	Arg^- Tpi^- < 1	Arg^- Tpi^+ > 99	Arg^+ Tpi^- 0	Arg^+ Tpi^+ 0
5	AA 200 (*tpi-1*)	AB 1911 (*metBF, argHBCE*)	Arg^+	576	Met^- Tpi^- 0	Met^- Tpi^+ 64	Met^+ Tpi^- 3	Met^+ Tpi^+ 33
			Met^+	506	Arg^- Tpi^- 10	Arg^- Tpi^+ 32	Arg^+ Tpi^- 10	Arg^+ Tpi^+ 48
6	K 1.17 (*glpK, metBF*)	AA 210 (*tpi-1*)	Tpi^+	182	Met^- Glp^- 43	Met^- Glp^+ 1	Met^+ Glp^- 38	Met^+ Glp^+ 18
7	K 1.17.18 (*rha, glpK*)	AA 200 (*tpi-1*)	Tpi^+	234	Rha^- Glp^- 46	Rha^- Glp^+ 9	Rha^+ Glp^- 34	Rha^+ Glp^+ 11
8	K 1.17.18 (*rha, glpK*)	AA 201 (*tpi-2*)	Tpi^+	335	Rha^- Glp^- 49	Rha^- Glp^+ 10	Rha^+ Glp^- 29	Rha^+ Glp^+ 12
9	K 1.17.18 (*rha, glpK*)	AA 202 (*tpi-3*)	Tpi^+	231	Rha^- Glp^- 39	Rha^- Glp^+ 10	Rha^+ Glp^- 40	Rha^+ Glp^+ 11
10	K 1.1.5^c.16 (*pfk, metBF*)	AA 200 (*tpi-1*)	Tpi^+	313	Pfk^- Met^- 26	Pfk^- Met^+ 56	Pfk^+ Met^- 4	Pfk^+ Met^+ 14
11	K 1.1.5^c.16 (*pfk, metBF*)	K 1.17.18 (*rha, glpK*)	Rha^+	180	Pfk^- Glp^- 21	Pfk^- Glp^+ 32	Pfk^+ Glp^- 43	Pfk^+ Glp^+ 4

by Morrissey & Fraenkel (1969) and the *pfk* allele is located in the same region (see Table 2, Expt. 11), the overall results suggest the order *rha tpi pfk glpK metBF*, as shown in Fig. 1.

The results reported in this paper mean that the chromosomal locations of four genes, *pgi*, *pfk*, *fda* and *tpi* (Böck & Neidhart, 1966; Fraenkel, 1967; Morrisey & Fraenkel, 1969; this paper), specifying four enzymes which act in sequence in the glycolytic pathway, are now known and of these only *tpi* and *pfk* are closely linked. The significance of this close linkage is not yet known.

We wish to thank Professor H. L. Kornberg, F.R.S., Professor A. L. Taylor and Professor N. Glansdorff for providing several of the bacterial strains used in these studies, and Dr C. B. Brice for a gift of P1*kc* and details of its propagation and use. We are especially indebted to Professor Kornberg for providing the *pfk* mutant and for his advice. A.A. is in receipt of a Studentship from the Science Research Council.

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Biochemical and Genetical Studies on Ribose Catabolism in *Escherichia coli* K12

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SUMMARY

A gene specifying ribokinase has been located on the *Escherichia coli* chromosome close to the reported position of an uncharacterized ribose*-negative mutation. This uncharacterized mutant has been shown to lack ribose permease and thus the genes for two enzymes of ribose catabolism are close together on the chromosome.

INTRODUCTION

Two mutants of *Escherichia coli* K12 unable to grow on ribose as their sole carbon source have been reported in the literature (Taylor & Trotter, 1967; Anderson & Cooper, 1969) but on the information available it was not possible to decide whether the two were biochemically identical. In this paper we show that one of them (Taylor & Trotter, 1967) has a defect in ribose permease and that the chromosomal location of ribokinase is very close to the reported position of ribose permease.

METHODS

Bacteria. The two ribose-negative mutants used in these studies were AT715 (Taylor & Trotter, 1967) derived from AB259 (HfrH) and AA100 (formerly called R-1) (Anderson & Cooper, 1969) derived from PA309 (F^-). Strain AT2614 (*phoS1*, *ilv-12*, Sm^R, F^-) was used as recipient in the phage transduction experiments.

Growth of bacteria. Bacteria were grown in liquid minimal medium or nutrient broth as described by Ashworth & Kornberg (1966).

Uptake experiments. To measure ribose uptake, the cells were grown aerobically to mid-exponential phase (about 0·34 mg. dry wt/ml.) in either Oxoid nutrient broth or, to induce the enzymes of ribose catabolism, in nutrient broth supplemented with 10 mM ribose. Organisms grown in the presence of ribose were harvested, washed with 2 vol. of nutrient broth and resuspended in nutrient broth to the original volume before use. Nutrient broth-grown cells were used directly. The cell suspension was cooled to 25° and 2 ml. added to a flask containing, in 30 μl., 1 μCi of [1-^{14}C]ribose and 0·2 μmoles of ribose ($7{\cdot}5 \times 10^6$ c.p.m./μmole ribose). Samples (0·2 ml.) were withdrawn at known times and treated as described by Morgan & Kornberg (1969) but using 10 ml. of scintillation fluid.

Assay of enzymes. Cell-free extracts were prepared as described previously (Anderson & Cooper, 1969). Ribokinase was assayed at 30° by the method of Horecker (1967)

* All sugars are D-isomers unless otherwise specified.

and protein was determined by the Folin–Ciocalteu method (Lowry, Rosebrough, Farr & Randall, 1951) using crystalline bovine serum albumin as the standard.

Fructose 6-phosphate (F6-P) formation from ribose 5-phosphate (R5-P) was measured in a reaction mixture containing (per ml.): 50 μmoles of tris-HCl, pH 7·5; 5 μmoles of $MgCl_2$; 0·25 μmoles of NADP; 2μg. of crystalline glucose 6-phosphate dehydrogenase; 4 μg. of crystalline phosphoglucose isomerase; 2·5 μmoles of R5-P and 400 μg. of crude bacterial protein. The change in absorbance at 340 nm. was measured at 30° on a recording spectrophotometer.

Genetic techniques. Phage P1*kc* transduction experiments were carried out by standard techniques (Glover, 1962). The phage lysate was added to recipients (multiplicity = 1) and suitable dilutions were spread on to appropriately supplemented glucose minimal medium. The transductants were streaked on to the appropriate plates to determine the inheritance of the unselected markers. To determine the inheritance of the PhoS marker, the transductants were grown aerobically for 36 h. in nutrient broth + 0·1 M-sodium phosphate, pH 7·5. A sample (0·5 ml.) of the suspension was toluenized for 10 min. at 37° then 0·2 ml. of 0·5 M-tris-HCl buffer, pH 8·6, and 0·2 ml. of 0·05 M-*p*-nitrophenyl-phosphate were added. After 5 min. incubation at 37° the recation was stopped by the addition of 1 ml. of 1 N-NaOH. The alkaline phosphatase constitutive ($PhoS^-$) transductants gave a bright yellow colour whilst the repressible ($PhoS^+$) transductants remained colourless.

RESULTS AND DISCUSSION

Although the mutant AT715 failed to grow on ribose as sole carbon source, it grew normally on other pentoses such as xylose and L-arabinose, and on glycolytic and gluconeogenic compounds such as glucose and lactate. This suggested that the defect in AT715 was in an enzyme specific to ribose catabolism. Since AT715 grew normally on uridine which is catabolized to yield ribose 5-phosphate (R5-P) (Kammen, 1967), it was probably in one of the enzymes which leads to the formation of internal R5-P from external ribose; that is, either ribose permease or ribokinase. The experiments reported here have been carried out to investigate these possibilities.

When ribose uptake by nutrient broth-grown organisms was measured, AB259 (the parental strain of AT715) incorporated [1-^{14}C]ribose at a fast and constant rate, but no incorporation of radioactivity could be detected when AT715 was used (Fig. 1). When the organisms were grown in the presence of ribose, the rate of [1-^{14}C]ribose incorporation by AB259 was increased threefold but again no uptake could be detected with AT715. In contrast, a spontaneous revertant of AT715 which had regained the ability to grow on ribose was found to incorporate radioactivity to the same extent as did AB259. Thus the inability of AT715 to grow on ribose appears to be due to the absence of a functional ribose permease.

In support of the view that the AT715 lesion affects a ribose permease, we find that the mutant can still synthesize ribokinase. It can be seen from Table 1 that nutrient broth-grown AT715 contains ribokinase at about half the specific activity of its parent, AB259. This ribokinase produced R5-P since crude extracts of AT715 form F6-P as a result of the non-oxidative reactions of the pentosephosphate pathway when either R5-P or ribose + ATP were supplied as substrate. Although growth in the presence of ribose led to an increase in the ribokinase activity of AB259, that of AT715 was unchanged suggesting that external ribose could not be converted to the internal inducer

in this mutant. Thus the ribose-negative AT715 differs from the ribose-negative AA100, which is unable to grow on ribose because it lacks ribokinase (Anderson & Cooper, 1969). This view is further supported by the observation that the ribokinase-negative mutant can take up ribose (Fig. 1). The initial rate of ribose uptake by AA100 was identical to that of its parent, PA309, but subsequently the rate decreased rapidly in the mutant whilst that of the parental strain remained constant. It is likely that this

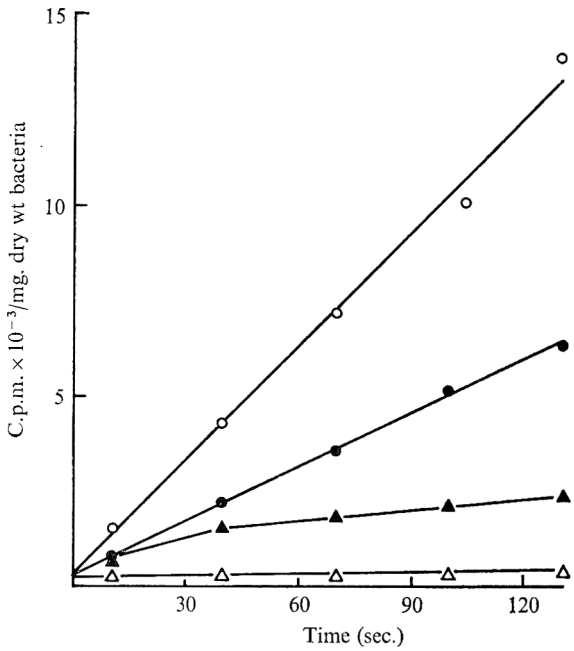

Fig. 1. Accumulation of [1-^{14}C]ribose by nutrient broth-grown *Escherichia coli* strains AT715 (△), AA100 (▲), PA309 (●), AB259 (○). For experimental details, see text.

Table 1. *Ribokinase activity in AT715 and AB259*

Ribokinase activity was measured according to Horecker (1967) as the ATP-dependent removal of ribose. The reaction mixture at 30° contained in 1 ml.: tris-HCl buffer, pH 8·0 (100 μmoles); KCl (50 μmoles); $MgCl_2$ (5 μmoles); ATP (5 μmoles); *Escherichia coli* protein (20 to 1000 μg.) and ribose (1 μmole).

	Ribose phosphorylated (μmoles/mg. protein per min.)	
Growth medium	AB259	AT715
Nutrient broth	0·038	0·016
Nutrient broth + ribose	0·166	0·018

Table 2. *Order of the ilv, rbsK and phoS loci*

P1 donor...	AA100	+	*rbsK*	+
Recipient...	AT2614	*ilv-12*	+	*phoS*1

Percentage of Ilv$^+$ transductants that are:

Rbs$^-$ Pho$^-$	Rbs$^-$ Pho$^+$	Rbs$^+$ Pho$^-$	Rbs$^+$ Pho$^+$
53	20	20	7

difference is due to the inability of AA100 to phosphorylate the internal ribose and thereby further metabolize it. When PA309 and AA100 were grown on nutrient broth + ribose there was a threefold increase in the rate of [1-^{14}C]ribose incorporation by PA309 but, surprisingly, no increase for AA100. The reason for this is not clear at the moment, but it could be that in the absence of ribokinase the organisms are unable to form the inducer for ribose permease synthesis. An alternative explanation, that the permease and kinase are functionally linked for the uptake of ribose seems unlikely, since we have other mutants which also lack ribokinase but which take up ribose very rapidly to a high level since they form ribose permease constitutively (R. A. Cooper, unpublished observations).

These results show that the two ribose-negative mutants AT715 and AA100 are biochemically distinct. Since the genetic location of the AT715 lesion, identified in the present work as ribose permease (*rbsP*), is known (Taylor & Trotter, 1967) it was of particular interest to determine the chromosomal location of the ribokinase gene (*rbsK*) to see whether the two genes were closely linked.

Preliminary studies on the location of *rbsK* by interruption of conjugation experiments indicated that it was close to 74 min. on the *Escherichia coli* linkage map (Taylor & Trotter, 1967) and cotransduction studies showed appreciable linkage of *rbsK* to *ilv* (73%) and a lower linkage to *metE* (2·5%). The position of the *rbsK* locus was determined more precisely by a three-factor cross infecting strain AT2614 with P1*kc* grown on the Rbs$^-$ donor. Ilv transductants were selected and subsequently analysed for the inheritance of the unselected markers Rbs$^-$ and Pho$^+$. Among 250 Ilv$^+$ transductants 73% had inherited the Rbs$^-$ marker and 27% the Pho$^+$ marker. As Table 2 shows, the Rbs$^+$Pho$^+$ recombinants were the least frequent category among the four possible phenotypes. If we assume that those transductants formed as a result of a minimum of four crossovers will occur significantly less frequently than those transductants formed as a result of a minimum of two crossovers, these results suggest that *rbsK* is situated between *phoS* and *ilv*. Since *rbsP* is also located between *phoS* and *ilv*, and the high linkage of *rbsK* to *ilv* (73%) is identical to the reported linkage of *rbsP* to *ilv* (Taylor & Trotter, 1967), *rbsK* and *rbsP* must be very close together on the *Escherichia coli* chromosome.

Considering the physiological roles of ribose permease and ribokinase it seems unlikely that their close chromosomal relationship is fortuitous. It is possible that they belong to a single unit of transcription and this possibility is being investigated at the present time.

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Inducible Resistance to Tetracycline in *Staphylococcus aureus*

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SUMMARY

When staphylococci, resistant to 4 μg. tetracycline/ml., were grown in nutrient media at subinhibitory levels of the drug, phenotypical resistance increased until the cocci grew with 160 μg. tetracycline/ml. Resistance increased most rapidly at the highest concentration of tetracycline which did not significantly inhibit growth. Increase in resistance was also obtained by pre-incubation with β-apo-5-oxy-tetracycline. Increase in resistance could be prevented by chloramphenicol and actinomycin D, but not by nalidixic acid. When a highly resistant culture was transferred to tetracycline-free medium, phenotypical resistance decreased gradually; after four transfers on nutrient agar it returned entirely to the original level.

INTRODUCTION

Williams (1967) has drawn attention to the resistance to mercury salts (Moore, 1960) of tetracycline-resistant strains of *Staphylococcus aureus*. Resistance to tetracycline was in general correlated with resistance to mercury; in mercury-sensitive strains, however, high-level resistance to tetracycline could be obtained by 'training', i.e. growth in peptone water containing 3 μg. antibiotic/ml.

METHODS

Bacterial strains. The strain studied, *Staphylococcus aureus* 111 (Sompolinsky, Yiflah & Aboud, 1968), was susceptible to mercury salts (Moore, 1960) and resistant to tetracycline (Tc) by virtue of a plasmid that could be eliminated by growth at 44° (May, Houghton & Perret, 1964). The eliminated susceptible strain grew on nutrient agar containing 0·2 μg. Tc/ml.; on agar containing 0·4 μg. Tc/ml., colonies did not develop from an inoculum of 10^8 staphylococci. With the uneliminated culture, identical colony counts were obtained on plain agar and on agar containing 4·0 μg. Tc/ml. At concentrations of up to 160 μg. Tc/ml., some colonies developed but growth did not occur on nutrient agar containing 320 μg. Tc/ml. Three types of resistant cultures have been used: (*a*) a culture transferred daily on plain nutrient agar; only 5 to 10% of the viable cocci from this culture produced colonies on agar containing 10 μg. Tc/ml.; (*b*) a highly resistant culture subcultured daily on nutrient agar containing 160 μg. Tc/ml.; and (*c*) cultures with intermediate levels of resistance obtained by growth of type (*a*) culture for a short period in nutrient broth containing tetracycline at different concentrations.

Chemicals. Tetracycline hydrochloride was a gift from Lederle Laboratories, American Cyanamid Co., Pearl River, N.Y.; β-apo-5-oxy-tetracycline was from Chas. Pfizer & Co., Groton, Conn., U.S.A.; chloramphenicol from Abic Ltd, Ramat Gan, Israel; nalidixic acid from Sterling–Winthrop Research Institute, Rensselaer, New York, U.S.A., through the courtesy of Dr Nachod; actinomycin D (Dactinomycin) was obtained from Merck Sharp & Dohme, West Point, Pa, U.S.A.

Saline buffer was prepared as described by Sompolinsky, Ernst-Geller & Segal (1967).

Culture media. Difco nutrient broth (NB) and nutrient agar (NA) were used. All cultures were incubated at 37°, NB cultures were grown in a water bath and aerated by shaking.

Viable counts were determined by plating appropriate dilutions of the bacterial suspension on NA; four replicate plates were prepared for each dilution and a total of 100 to 400 colonies were recorded on each plate.

Turbidity of bacterial cultures was determined with a Klett–Summerson photoelectrical colorimeter, using a red filter (660 nm.) for NB cultures. Five hundred Klett units = extinction (E) of 1·00.

RESULTS

When an exponentially growing culture of *Staphylococcus aureus* 111 was inoculated into nutrient broth to give an initial turbidity of 0·032 E, growth continued at an exponential rate; addition of 10 μg. Tc/ml. caused a temporary decrease in the growth rate which then recovered to that of the antibiotic-free control. When 40 μg. Tc/ml. was added with the inoculum, no increase in turbidity occurred for 1 h., after which growth occurred at a rate slightly less than that of the control (Fig. 1). This type of growth curve in which the lag period varies with the drug concentration is seen when an inhibiting drug is inactivated by the culture (Sompolinsky & Samra, 1968). However, no clear evidence for inactivation was found experimentally (Sompolinsky, Zaidenzaig, Ziegler-Schlomowitz & Abramova, 1970).

Other possible explanations for this pattern of growth in 40 μg. Tc/ml. NB might be 'adaptation' to the drug during the lag period (Williams, 1967) or outgrowth of a few bacteria with initial resistance to 40 μg. Tc/ml. To examine these possibilities, further growth studies were performed by determining viable counts simultaneously on NA and on 40 μg. Tc/ml. NA (Fig. 2). The rate of growth demonstrated by counts on 40 μg. Tc/ml. NA soon approximated to that shown by viable counts on NA from cultures in NB and 10 μg. Tc/ml. NB. Furthermore, after 3 to 5 h. the difference between the number of colony-forming units on NA and 40 μg. Tc/ml. NA was relatively constant, corresponding approximately to the original inoculum size (3×10^7 cocci/ml.). These data might suggest that the population was initially heterogeneous in ability to grow on medium containing Tc, that only a few organisms (4×10^6 out of 3×10^7) were able to grow at 40 μg. Tc/ml. and that these eventually outnumbered the sensitive non-proliferating organisms.

However, selection from a heterogeneous bacterial population was not the only cause of development of tetracycline resistance. After growth in non-inhibitory concentrations of Tc (0·1 or 1·0 μg./ml.), significant increase in the proportions of cocci growing on 10 to 40 μg. Tc/ml. was observed (Table 1). Resistance was also produced (Table 2) by growth with 10 μg. apo-oxy-tetracycline/ml. which is virtually non-inhibitory. In these experiments, practically the whole bacterial population increased

in resistance through contact with Tc. The apparent discrepancy between these and earlier results could be explained by the assumption that resistance depends on the synthesis of a specific protein which is induced by Tc at a rate dependent on the concentration of antibiotic. Apo-oxy-tetracycline would, according to the results in Table 2, be a less effective inducer. Development of resistance was inhibited by chloramphenicol as would be expected for such induction.

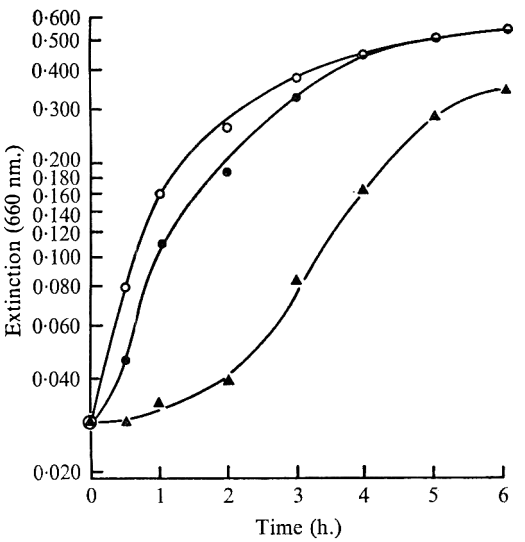

Fig. 1

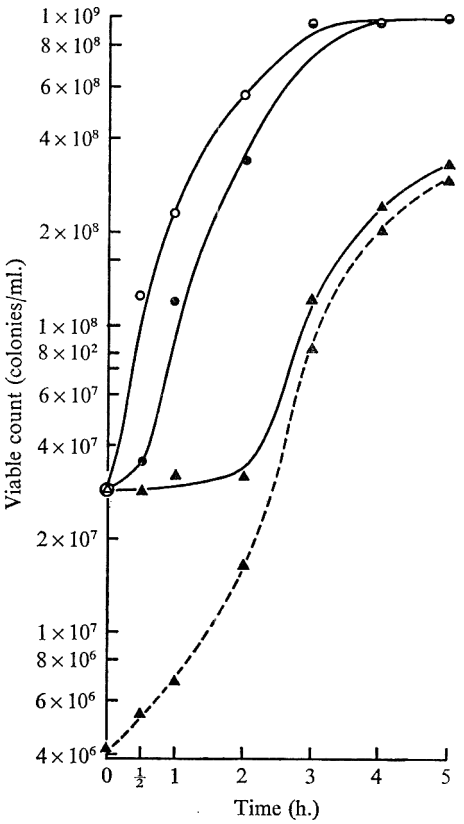

Fig. 2

Fig. 1. Growth of *Staphylococcus aureus* 111 in nutrient broth (NB) (○—○); tetracycline (Tc) 10 μg./ml. NB (●—●); and 40 μg. Tc/ml. NB (▲—▲). Determined by measurement of extinction (*E*) at 660 nm.

Fig. 2. Growth of *Staphylococcus aureus* 111 in nutrient broth (NB) (○—○); 10 μg. tetracycline (Tc)/ml. NB (●—●); and 40 μg. Tc/ml. NB. (▲—▲). Determined by viable counts on nutrient agar (NA). The culture in 40 μg. Tc/ml. NB was also counted on 40 μg. Tc/ml. NA. (▲---▲).

When an inhibitor of protein synthesis is also an inducer, no simple kinetics of induction can be expected (Shaw & Brodsky, 1968). Figure 3*a* shows that growth of *Staphylococcus aureus* 111 in NB+Tc was independent of the drug concentration in the range 5 to 10 μg./ml. and that growth after 4 h. was little influenced by 20 μg./ml., but considerably by 40 μg./ml. If Tc is an inducer and induction is concentration-dependent, it would be expected that the highest concentration of the drug with no

Table 1. *Increase in phenotypical resistance to tetracycline in Staphylococcus aureus III during growth in nutrient broth with subinhibitory concentrations of the drug*

Time (h.)	Viable cocci on plain NA	Cocci resistant to (μg. Tc/ml.)			
		5	10	20	40
Growth in Tc-free NB					
0	$2{\cdot}0 \times 10^7$	84·7*	79·2	3·1	nt
2	$9{\cdot}5 \times 10^7$	66·5	25·1	0	nt
4	$3{\cdot}3 \times 10^8$	58·5	9·9	0	nt

Time (h.)	Viable cocci on plain NA	Cocci resistant to (μg. Tc/ml.)					
		5	10	20	40	80	160
Growth in 0·1 μg. Tc/ml. NB							
0	$2{\cdot}0 \times 10^7$	84·7	79·2	3·1	nt	nt	nt
2	$9{\cdot}6 \times 10^7$	88·2	77·8	12·9	0·9	0	0
4	$3{\cdot}1 \times 10^8$	104·8	99·2	21·0	0	0	0
Growth in 1·0 μg. Tc/ml. NB							
0	$2{\cdot}0 \times 10^7$	84·7	79·2	3·1	nt	nt	nt
2	$9{\cdot}2 \times 10^7$	89·0	80·8	58·3	9·2	1·6	0
4	$2{\cdot}9 \times 10^8$	99·6	101·2	100·0	24·6	10·6	0

Abbreviations: NA = nutrient agar; NB = nutrient broth; Tc = tetracycline; nt = not tested.
* Resistant cocci, determined by viable count on NA + indicated concentrations of Tc, expressed as % of viable count on NA.

Table 2. *Influence of pre-incubation with tetracycline or apo-oxy-tetracycline on resistance of Staphylococcus aureus III to tetracycline*

Pre-incubation conditions (3 h.)	Cocci resistant to tetracycline (μg./ml.)			
	20	40	80	160
No Tc	33·4*	0	nt	nt
Tc 10 μg./ml. NB	99·6	79·0	66·8	56·8
apo-oxy-Tc 10 μg./ml. NB	65·8	32·3	4·9	0
Tc 10 μg./ml. + chloramphenicol 200 μg./ml. NB	6·7	0	0	0

Abbreviations: Tc = tetracycline; NB = nutrient broth; nt = not tested.
* Resistant cocci, determined by viable count on NA + indicated concentrations of Tc, expressed as % of viable count on NA.

significant inhibitory effect would be most rapidly effective in induction. This was confirmed by the experiments reported in Fig. 3*b*.

In a similar experiment, the appearance of cocci resistant to 160 μg. Tc/ml. during growth in 20 μg. Tc/ml. NB was depicted at shorter time intervals (Fig. 4); at the time of positive growth acceleration there was a rapid increase in the proportion of highly

resistant bacteria from 8 to 60% of the whole population. Development of phenotypical high-level resistance seemed therefore to be associated with other vital metabolic activities of the organisms. Further experiments (Tables 2, 3; Fig. 5*a* to *c*) indicated that protein synthesis was involved, since no highly resistant cocci developed in the presence of chloramphenicol, and substitution of NB by saline buffer caused a striking delay in the appearance of high-level resistance. This is in accordance with the observation that inhibitory concentrations of Tc inhibit induction (Fig. 1).

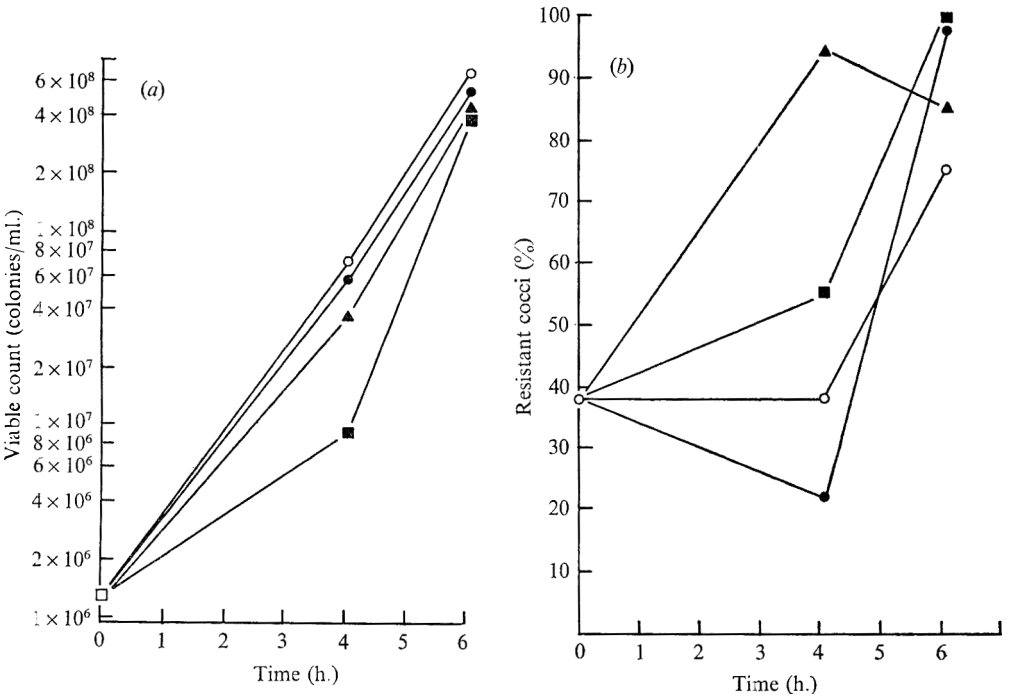

Fig. 3*a*. Growth of *Staphylococcus aureus* 111 in nutrient broth (NB) with 5 μg. tetracycline (Tc)/ml. (○—○); 10 μg. Tc/ml. NB (●—●); 20 μg. Tc/ml. NB (▲—▲); and 40 μg. Tc/ml. NB (■—■). Determined by viable counts on nutrient agar (NA).

Fig. 3*b*. Change in frequency of bacteria resistant to 160 μg. Tc/ml. in the cultures of Fig. 3*a*. Determined by viable counts on 160 μg. Tc/ml. NA and calculated as % of viable count on NA.

In a Tc-susceptible (eliminated) culture of *Staphylococcus aureus* 111, growth in 0·05 μg. Tc/ml. NB had no influence on resistance; the strain remained resistant to 0·2 μg. Tc/ml. and susceptible to 0·4 μg. Tc/ml.

The increased resistance of the non-eliminated cultures achieved by incubation with subinhibitory concentrations of Tc might be due either to induction of resistance through derepression of the synthesis of a specific 'resistance protein' or to the development of a higher multiplicity of 'resistance genes' (plasmids) per coccus. In the latter case, induction of increased resistance would require DNA synthesis, and therefore be susceptible to nalidixic acid, whereas induction by repressor inactivation would be susceptible only to drugs that inhibit transcription (actinomycin D) or translation (chloramphenicol) of the resistance gene. In experiments with nalidixic acid at 200 μg./ml., significant increase in viable count of a culture of *Staphylococcus aureus* 111

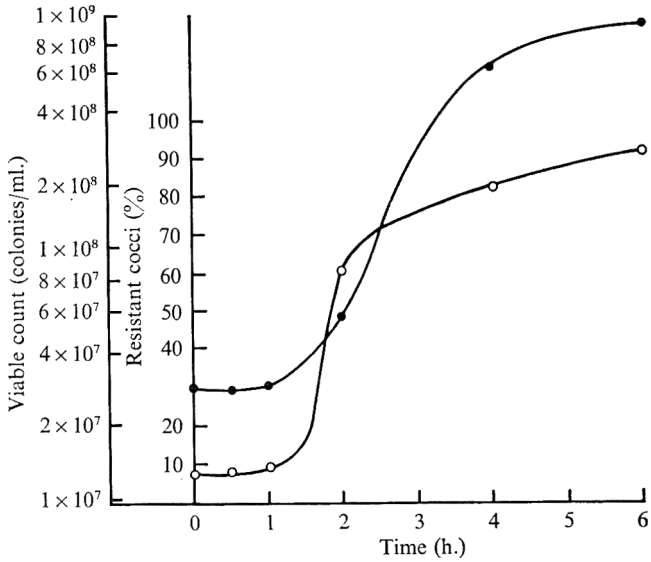

Fig. 4. Kinetics of increase in resistance of *Staphylococcus aureus* 111, growing in nutrient broth (NB) containing 20 μg. tetracycline (Tc)/ml. Growth curve of broth cultures by viable counts on nutrient agar (NA) (●—●), and % resistant cocci by viable count on 160 μg. Tc/ml. NA as percentage of viable count on NA. (○—○).

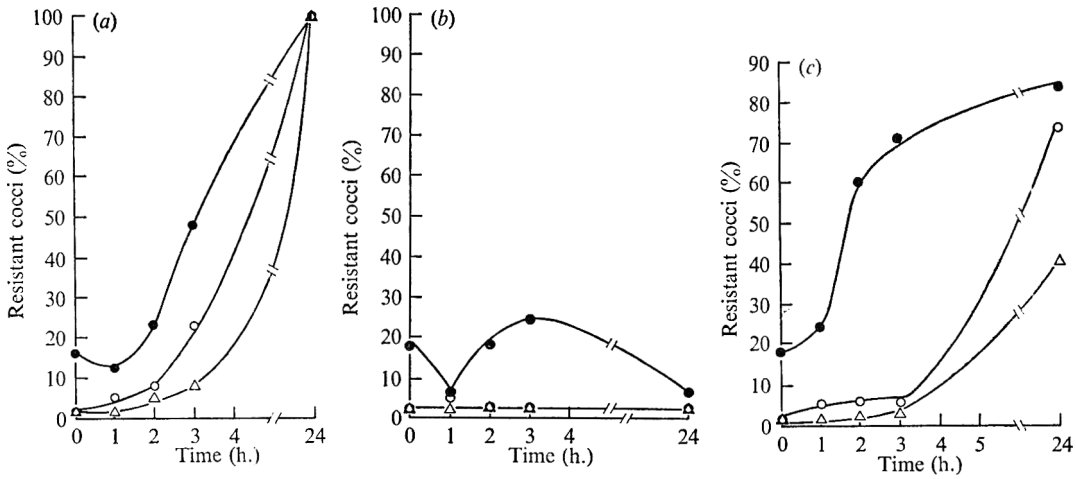

Fig. 5. Frequency of colony-forming staphylococci (*Staphylococcus aureus* 111) from nutrient broth (NB) culture, developing on 20 μg. tetracycline (Tc)/ml. nutrient agar (NA) (●—●); 40 μg. Tc/ml. NA (○—○); and 80 μg.Tc/ml. NA (△—△); as percentage of viable count on NA. Cultures incubated during the time indicated, in (*a*) 20 μg. Tc/ml. NB, (*b*) 20 μg. Tc/ml. NB + 200 μg. chloramphenicol/ml., (*c*) 20 μg.Tc/ml. saline buffer. Inoculum size was in all cases $1{\cdot}6 \times 10^7$ bacteria/ml. After 24 h., viable counts on plain NA were (*a*) $3{\cdot}9 \times 10^8$/ml., (*b*) $2{\cdot}3 \times 10^6$/ml. and (*c*) $3{\cdot}2 \times 10^5$/ml. The bacteria incubated with chloramphenicol were washed twice in saline buffer before plating.

during 2 h. incubation, was prevented, whereas 256 μg./ml. caused an extensive decrease in the number of colony-forming units. Table 3 shows that 1·0 μg. Tc/ml. caused a rapid increase in resistance of a previously entirely uninduced culture, in

Table 3. *Induction of tetracycline resistance in Staphylococcus aureus III in presence of nalidixic acid*

An exponential-phase culture of *S. aureus* III, in nutrient broth (NB) (O.D. = 0·100), was diluted in NB + nalidixic acid to a calculated O.D. of 0·008; the other indicated drugs were added and the cultures incubated with aeration. Samples of the cultures were centrifuged, the organisms washed once in saline buffer, suspended and diluted in saline buffer and viable counts determined on NA and NA + Tc.

Drugs in incubation medium (in μg./ml.)	Incubation (37°) in h.	Cocci resistant to tetracycline (μg./ml.)			
		10	20	40	80
Nil	0	16·2*	0·9	< 0·01	< 0·01
Nalidixic acid 200·0	1	7·8	0·3	< 0·01	< 0·01
	2	9·2	0·3	< 0·01	< 0·01
	3	nt	0·4	0·02	< 0·01
Nalidixic acid 200·0 + tetracycline 20·0	1	12·9	0·01	< 0·01	< 0·01
	2	nt	0·01	< 0·01	< 0·01
	3	12·4	0·2	< 0·01	< 0·01
Nalidixic acid 200·0 + tetracycline 5·0	3	22·5	2·8	0·7	0·2
Nalidixic acid 200·0 + tetracycline 1·0	1	106·8	60·2	5·7	< 0·01
	2	91·0	60·3	22·9	1·6
	3	92·3	68·6	28·6	1·5
Nalidixic acid 200·0 + tetracycline 0·1	3	85·6	23·6	7·0	0·6
Nalidixic acid 200·0 + chloramphenicol 200·0 + tetracycline 1·0	1	17·3	1·1	< 0·01	< 0·01
	2	12·4	2·2	0·02	< 0·01
Nalidixic acid 200·0 + actinomycin D 1·5 + tetracycline 1·0	1	13·5	7·6	0·5	0·07
	2	7·8	3·0	0·3	0·12

nt = Not tested.

* Resistant cocci, determined by viable count on NA + indicated concentrations of Tc, expressed as % of viable count on NA.

Table 4. *Loss of phenotypical high-level resistance to tetracycline after growth on drug-free nutrient agar*

	Fraction* of cocci resistant to 160 μg. Tc/ml.
Original strain →	$1{\cdot}1 \times 10^{-7}$
↓	
After several transfers on 160 μg. Tc/ml. NA →	1.00
↓	
After two transfers on drug-free NA →	$4{\cdot}3 \times 10^{-6}$
↓	
After two more transfers on drug-free NA →	$3{\cdot}1 \times 10^{-7}$

Abbreviations: Tc = tetracycline; NA = nutrient agar.

* Determined by colony counts on plain agar and on nutrient agar containing 160 μg. Tc/ml. NA.

spite of the addition of nalidixic acid. Chloramphenicol and actinomycin D, added to nalidixic acid + Tc, strongly inhibited induction of increased resistance. In these experiments, 20 μg. Tc/ml. inhibited induction almost entirely, while 5·0 and 0·1 μg. Tc/ml. were less effective than 1·0 μg. Tc/ml.

When *Staphylococcus aureus* 111 was grown in NB + Tc until the whole population was phenotypically of high-level resistance, and the bacteria were subcultured on plain NB or NA, high-level resistance was gradually lost. Table 4 shows that after four subcultures the frequency of high-level resistant cocci was as in a culture that has had no previous contact with the drug. If high resistance depends on induction of synthesis of a specific protein, then return to lower levels of resistance would be explicable simply as dilution of this protein by cell division and re-repression of its synthesis *de novo*.

DISCUSSION

Resistance of staphylococci to penicillin (Richmond, 1965) and chloramphenicol (Shaw & Brodsky, 1968) is due to inactivation of the drugs by enzymes synthesized after induction, the respective drugs serving as inducers. Our results indicate that in Tc resistance, although resistance is probably not due to inactivation, an inducible genetic system is involved. This has not been shown for low-level resistance; either the coccus produces constitutively a small amount of the resistance protein, or it has sufficient time to produce such small amounts, protecting against low concentrations of Tc, before uptake inhibits further protein synthesis. High resistance of a bacterial population, however, depended under our experimental conditions on pre-incubation with subinhibitory concentrations of the drug. This induction of high resistance was inhibited by both chloramphenicol and actinomycin D, but was not dependent on growth since it occurred in cultures inhibited by nalidixic acid during the period of contact with tetracycline.

It may seem curious that an inhibitor of protein synthesis is also an inducer for the development of resistance by a procedure that depends on protein synthesis. Pre-incubation with low concentration of the drug in order to induce high-level resistance is probably an entirely artificial laboratory procedure with no biological importance. However, it is possible that in the natural environment of the staphylococcus Tc molecules might penetrate even more slowly than in laboratory media, due to differences in the metabolic state of the organism; under such conditions the organism might be induced to develop a high level of resistance before phenotypical expression is inhibited by the inducer itself.

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Mechanism of Tetracycline Resistance in *Staphylococcus aureus*

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SUMMARY

Resistance of *Staphylococcus aureus* III to tetracycline was due to an extrachromosomal genetic unit (plasmid) that could be eliminated by growth at 44°. The susceptible (eliminated) strain actively concentrated tetracycline from the nutrient medium by an energy-dependent transport system. The resistant culture accumulated the drug to a much lesser degree than the susceptible culture, both according to the *E* 380 of the bacterial extract and to its radioactivity after incubation with tritiated tetracycline. Accumulation of tetracycline was low and independent of the external concentration until this reached a level corresponding approximately to the minimal inhibitory concentration.

Pre-incubation with tetracycline at low concentrations decreased ability to accumulate the drug. This pre-incubation effect was not prevented by nalidixic acid but was by actinomycin D.

INTRODUCTION

In a previous communication we reported that subinhibitory amounts of tetracycline cause a temporary lag in the growth of an exponentially growing culture of *Staphylococcus aureus* III, but thereafter growth continues at the original rate. The duration of growth inhibition depends on the drug concentration. This pattern of inhibition can be due to (i) inactivation of a bacteriostatic drug (Sompolinsky & Samra, 1968), (ii) selection of resistant bacteria from a heterogeneous population, (iii) development of a higher level of resistance by induction. Both selection of resistant cocci and induction contributed to tetracycline resistance (Sompolinsky, Krawitz, Zaidenzaig & Abramova, 1970). The present study deals with the mechanism of tetracycline resistance of *S. aureus* III. Two possible mechanisms were investigated, inactivation of the drug and decreased uptake by the resistant cells.

METHODS

Bacterial strains. The strain used, *Staphylococcus aureus* III, has been described by Sompolinsky *et al.* (1970). For bioassay of tetracycline (Tc) we used the same strain of *Shigella boydii* as was used for assay of chloramphenicol (Sompolinsky & Samra, 1968).

Chemicals. The reagents used, with the exception of those described below, were the same as in the previous paper (Sompolinsky *et al.* 1970). The following were also

used: [^{14}C]D-threo-chloramphenicol (methylene-^{14}C) of specific activity 6·81 mCi/mmole, obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England, and [^{3}H]tetracycline-7-^{3}H(N)HCl (specific activity 114 mCi/mmole) from New England Nuclear Corporation, Boston, Mass., U.S.A. Chemicals for chromatography (see below) were of the highest purity obtainable commercially.

Bioassay of tetracycline was performed, essentially in the same way as for chloramphenicol (Sompolinsky & Samra, 1968), on samples of nutrient broth (NB)+160 μg. Tc/ml. inoculated with a highly resistant culture of *Staphylococcus aureus* 111

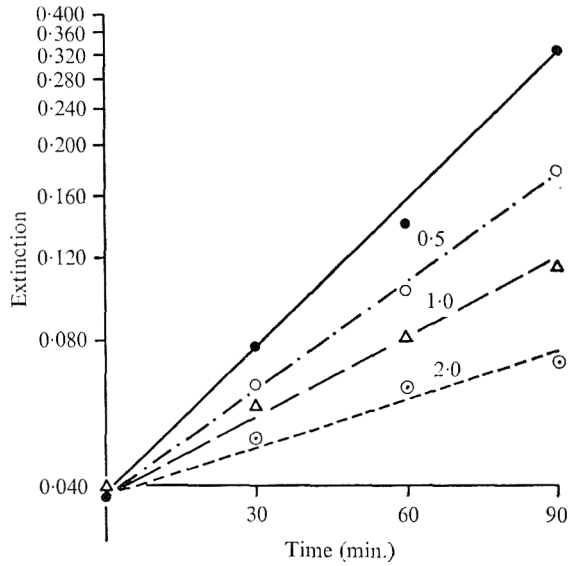

Fig. 1. Tetracycline assay using *Shigella boydii* as test organism. An exponential culture of the test organism was divided into four portions, the indicated concentrations of tetracycline were added to three of the samples, which were incubated at 37°. Extinction of the samples was measured at 660 nm.

aerated at 37°. The control for spontaneous inactivation was uninoculated NB+160 μg. Tc/ml. incubated in the same way. Freshly prepared dilutions of Tc served as references. The experimental samples were cooled immediately in ice, centrifuged at 2°, and the clear supernatant fluid stored at −20° until titration. Addition of 0·25 ml. of this fluid to 20 ml. of an exponential culture of the assay organism (*Shigella boydii*) would give a final concentration of 2·0 μg./ml. if full activity was preserved. The concentration of active Tc was estimated from the generation time compared with cultures inhibited by freshly prepared Tc solutions at 0, 0·50, 1·0 and 2·0 μg./ml. (Fig. 1). The growth rate was fairly constant during the assay period (90 min.) but not entirely proportional to the drug concentration. In spite of this, more reproducible results were obtained by this method than by diffusion assay (Garrod & O'Grady, 1968). Ten independent assays of a solution of 0·75 μg. Tc/ml. gave values between 0·615 and 0·779 μg./ml. (mean 0·712 μg./ml., $\sigma = \pm 0{\cdot}047$).

Uptake of tetracycline or β-apo-5-oxy-tetracycline was examined essentially as indicated by Franklin (1967). Usually, 160 μg. Tc/ml. was added to the staphylococcal culture in exponential growth (extinction 0·120 to 0·140 at 660 nm.) and incubation

at 37° was continued for 1 h. Thereafter, samples (10 ml.) were cooled, centrifuged, the organisms washed once with 40 ml. 0·85% NaCl and resedimented. (When cultures of lower turbidity were examined, as in Fig. 5*a*, a correspondingly greater sample volume was used.) The cocci were suspended in 4·0 ml. cold perchloric acid (0·5M), incubated 10 min. at room temperature, neutralized with 4·0 ml NaOH (1·0M), centrifuged, and the extinction (380 nm.) of the supernatant determined. Standard solutions of the drug, treated similarly with perchloric acid and NaOH, were used as references. Efficacy of the extraction method was checked by repeating the extraction on the treated bacterial sediment; the tetracycline of the second extract never accounted for more than 5% of the first extract.

An extinction (*E*) of 0·240 at 380 nm. in a Zeiss absorption spectrophotometer corresponded to 50 μg. Tc in the bacterial pellet. By extraction of bacteria not treated with tetracycline, extinction values of −0·03 to +0·02 were obtained (six experiments). No correction for these blank values were made.

Protein content of the bacterial extracts was examined according to Lowry, Rosebrough, Farr & Randall (1951); 10 ml. culture of extinction 0·12c (660 nm.) contained about 0·23 mg. protein. Values of 50 μg. Tc/mg. bacterial protein were based on direct spectrophotometer readings of 0·06. Low values of uptake calculated by this method should therefore be accepted with due precaution and interpreted as 'low' or '< 35 μg. Tc/mg. protein', but for practical reasons the calculated values are indicated under Results.

Uptake of [^{3}*H*]*tetracycline.* Some of the uptake experiments, particularly those expected to give low values, were performed with [^{3}H]Tc diluted with nonradioactive Tc. Chloramphenicol (10 μg./ml.) was used to inhibit growth of the cultures, since uptake of the susceptible and resistant cultures would not be entirely comparable if the resistant culture grew in presence of Tc, whilst the susceptible one did not. Preliminary experiments with sufficient Tc to inhibit growth of the resistant culture showed that 10 μg. chloramphenicol/ml. had no influence on Tc accumulation.

In the experiments with [^{3}H]Tc, the culture incubated with Tc contained about 40 μg. bacterial protein. The radioactivity of Tc (40,000 to 230,000 c.p.m./ml.) was chosen so that the samples with lowest uptake yielded at least 250 c.p.m. After incubation at 37° for 1 h. the samples were centrifuged at 2° and the pellet was washed twice with 2 ml. NB and once with 2 ml. distilled H_2O. The supernatant of the last washing contained less than 80 c.p.m./ml. To the pellet was added 0·3 ml. 5% *n*-butanol (Egan & Morse, 1965) and, after 10 min., the content of the test-tube was transferred to a scintillation vial together with 15 ml. of the following scintillation fluid: 16 g. 2·5-diphenyloxazole, 0·8 g. 1,4-bis-2(4-methyl-5-phenyloxazolyl)-benzene and 240 g. naphthalene in a mixture of *p*-dioxane 2800 ml., methanol 700 ml. and ethylene glycol 160 ml. The vials were counted in a Packard Tricarb liquid scintillation spectrometer, model 3203, and quenching was determined by external standardization. No correction for radioactivity of the intercellular fluid was attempted.

Uptake of [^{14}*C*]*chloramphenicol* was examined after incubation in 10 μg. chloramphenicol/ml. NB containing 500,000 c.p.m. of [^{14}C]chloramphenicol. Cocci were prepared for liquid scintillation as for [^{3}H]Tc.

In all examinations of radioactivity, the data quoted are means of three parallel samplings.

Chromatography or Tc (or ^{3}H-Tc) was performed by the ascending technique on

Eastman-Kodak thin-layer silica gel sheets with: butyl acetate + methyl isobutyl ketone + *n*-butanol + water (5 + 15 + 2 + 22 by vol.) with 2 vol. formic acid added before use as solvent. Ascending chromatography on Whatman no. 1 paper was performed with 10% citric acid as solvent. The paper was cut into strips of 0·5 cm. each which were counted in 10 ml. toluene scintillation fluid (2,5-diphenyloxazole, 4 g. plus 1,4-bis-2(4-methyl-5-phenyloxazolyl)-benzene, 300 mg. in 1 l. toluene).

Absorption spectrum of tetracycline was examined with a Unicam SP 800 automatic recording spectrophotometer in the range 200 to 450 nm.

RESULTS

Inactivation of tetracycline (Tc). A high-level resistant strain of *Staphylococcus aureus* 111 was incubated with aeration at 37° in nutrient broth (NB) and in 160 μg. Tc/ml. NB from about 10^7 cocci/ml. After 24 h., heavy growth was obtained in both cultures (extinction > 0·350). Simultaneously, a susceptible culture was suspended in NB and in 160 μg. Tc/ml. NB at an initial extinction of 0·300 and also incubated. After 24 h. all four cultures were sedimented by centrifugation, and the clear supernatant fluid separated from the bacterial pellet.

This supernatant was diluted in 0·11 N-HCl (1:8), and the absorption spectrum recorded, using the diluted supernatants of the corresponding drug-free cultures as blanks. The absorption spectrum was compared with that of freshly prepared 160 μg. Tc/ml. NB also diluted 1:8 in 0·11 N-HCl. Figures 2*a*, *b* show that incubation with susceptible or resistant staphylococci had no influence on the absorption maxima at 356, 273 and 220 to 225 nm. due to drug. The exact positions and relative heights of the absorption peaks varied when different nutrient media were used (NB, Bacto penassay, Bacto tryptose phosphate broth) and even with different batches of the same medium. This was especially marked in the short wave u.v. because of the high absorption of the nutrient broth blank in the 200 to 230 nm. region. The absorption spectrum of Tc in 0·1 N-HCl demonstrated a well-defined peak at 220 nm. This peak was not so apparent in a nutrient broth solution probably as a result of interference by the broth (Fig. 2*a*, *b*).

Five ml. of the supernatant samples were lyophilized, redissolved in 0·25 ml. H_2O and chromatographed with paper and thin-layer silica gel techniques. The Tc spots on silica gel were easily recognized by their golden fluorescence (u.v. short-wave lamp); after a few days the spots oxidized in the air and turned dirty golden. On paper, the Tc spots were visualized after spray with HCl/N and heating to 50° for 30 min. Comparable R_F values were obtained repeatedly on all samples, on thin layer 0·48 to 0·51 and on paper 0·70 to 0·75. Anhydrotetracycline prepared by acid treatment of Tc (Kelly, Peets & Hoyt, 1969) had R_F of 0·18 on thin layer and 0·23 on paper.

The sedimented cocci from the same cultures were extracted with perchloric acid and the extract neutralized with NaOH, as described under Methods, before examination for uptake of Tc. The extract of the susceptible culture incubated with Tc had the same absorption spectrum as solutions of Tc. In this case, the blank was an extract of bacteria grown in drug-free NB. A lyophilized sample of the former extract (susceptible cocci incubated in NB + Tc) when examined chromatographically showed a spot with the same R_F values as pure Tc. Resistant cocci treated in the same manner showed no characteristic absorption, possibly due to the inadequate amount

of the drug present in this extract. The Tc of this extract could not be visualized by chromatography on paper with citric acid as solvent.

If the resistant staphylococci overcome the inhibiting effect of Tc by inactivating it intracellularly, this might be demonstrated more easily when the drug concentration is moderate than with high concentrations, since a greater fraction of the drug should be metabolized.

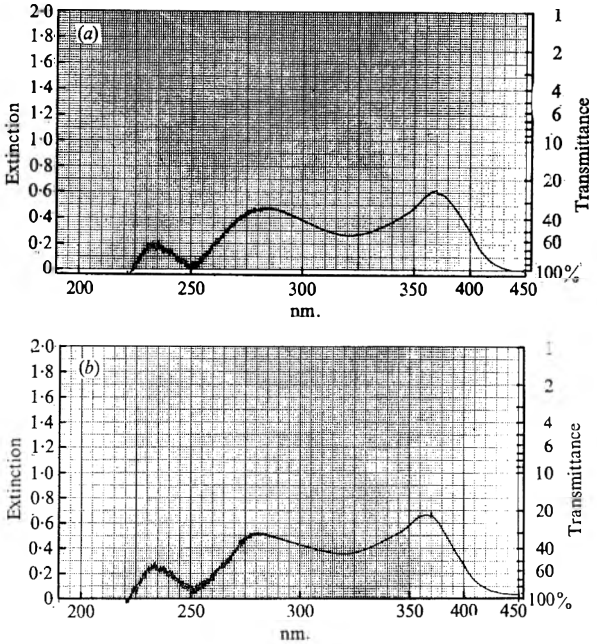

Fig. 2. Absorption spectrum of tetracycline in the range 200 to 450 nm. (*a*) Tetracycline (Tc) 160 μg./ml. in nutrient broth (NB) diluted 1:8 with H_2O. Blank: drug-free NB diluted 1:8 with H_2O. (*b*) A highly resistant strain of *Staphylococcus aureus* 111 was grown in NB+160 μg. Tc/ml. and in drug-free nutrient broth. After 24 h. the cultures were centrifuged, and the supernates diluted in H_2O 1:8. The absorption spectrum of the tetracycline sample was examined with the drug-free sample in the blank cuvette.

A similar study was therefore undertaken with the high-level resistant strain incubated during 24 h. (37°) in 20 μg. Tc/ml. NB containing [^{3}H]Tc (500,000 c.p.m./ml.) After 24 h. lyophilized samples of the supernatant fluid and control solutions of Tc were chromatographed on paper, which was cut into 0·5 cm. pieces and the radioactivity determined by liquid scintillation. The radiograms showed a peak at R_F 0·72 (Fig. 3). A radiogram from the cocci extracted with 4 ml. perchloric acid, lyophilized and again dissolved in 0·1 ml. H_2O also yielded a peak at R_F 0·72.

The biological activity of Tc in a resistant culture in 160 μg. Tc/ml. NB was assayed periodically during 11 days; the amounts demonstrated were comparable to those found in NB+Tc incubated in the same way (Fig. 4).

No evidence was thus obtained indicating inactivation or metabolism of Tc during growth of a resistant culture of *Staphylococcus aureus* 111 in NB+Tc.

Accumulation of tetracycline. Figure 5*a* depicts variations in the ability to accumulate Tc intracellularly of organisms from three cultures of *Staphylococcus aureus* 111: (i) a susceptible (eliminated) strain grown in NB; (ii) a highly resistant strain grown in

NB; and (iii) this latter strain grown in 4·0 μg. Tc/ml. NB. The high-level resistant strain was maintained by daily transfer on 160 μg. Tc/ml. NA. Uptake was examined after addition of 160 μg. Tc/ml. to samples of the cultures and incubation at 37°

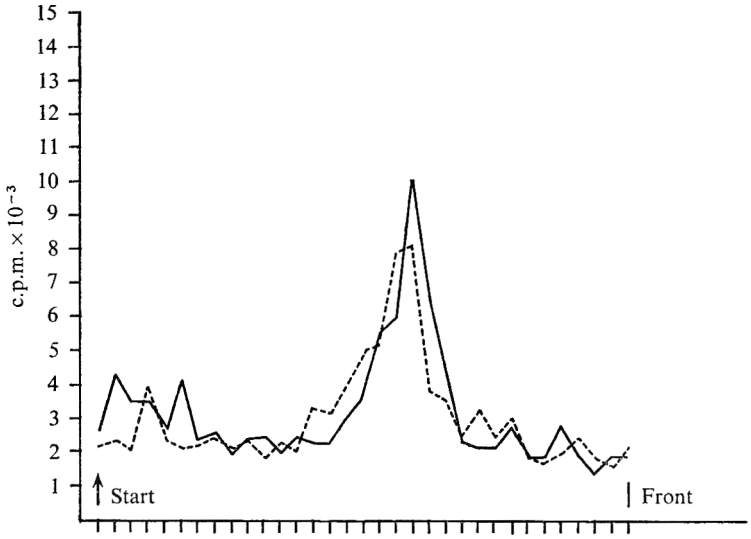

Fig. 3. Radiograms of [³H]tetracycline. A 24 h. culture of a high-level resistant strain of *Staphylococcus aureus* 111 in NB + 20 μg. Tc/ml. (500,000 c.p.m. of ³H) was cleared by centrifugation and the supernatant chromatographed on paper. The paper was cut in strips of 0·5 cm. width and counted by liquid scintillation. The radiogram (——) was constructed from the c.p.m. of each strip. For the sake of comparison, uninoculated tritiated tetracycline in nutrient broth was treated similarly and chromatographed (. . . . .).

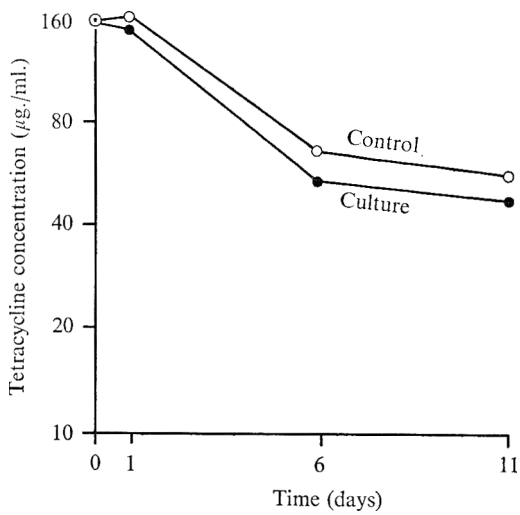

Fig. 4. Stability of tetracycline during growth of *Staphylococcus aureus* 111 (a high-level resistant culture) at 37° in nutrient broth with 160 μg. Tc/ml. (●—●—●). Inoculum 10^7 cocci/ml.; extinction at 660 nm., at 24 h. 0·400. At the indicated times, residual Tc was assayed biologically (see Methods). As a control for spontaneous decomposition of the drug, uninoculated nutrient broth with 160 μg. Tc/ml. was incubated and examined simultaneously (○—○—○).

during 1 h. In the susceptible strain, concentrating ability of the cocci reached a maximum towards the end of the exponential growth phase, and decreased thereafter rapidly. Maximal accumulation was 338 μg. Tc/mg. bacterial protein (a more than 100-fold concentration). The resistant strain grown in 4·0 μg. Tc/ml. NB concentrated the drug to a low degree at all stages of growth. When this strain was grown in NB alone, accumulating ability was minimal during the first half hour, but thereafter it gradually approached that of the susceptible culture.

From these results it is evident that a resistant culture either accumulates Tc to a far lesser degree than a susceptible one, or else that the drug is altered after accumulation in a resistant culture so that it no longer absorbs at 380 nm. Accumulation was therefore measured using tritiated Tc. In this study, 10 μg. chloramphenicol/ml. was added to all the samples during incubation with 160 μg. Tc/ml. in order to ensure that no growth occurred in the resistant cultures; the experimental conditions for the susceptible and resistant culture were therefore more strictly comparable. The results shown in Fig. 5*b* are similar to those of Fig. 5*a*. The accumulation of [^{14}C]chloramphenicol by the susceptible (eliminated) strain also varied with the growth phase (Fig. 5*b*). Uptake of chloramphenicol has been shown to depend on both the energetic state of the bacteria (Vazquez, 1963) and the number of ribosomes (Hurwitz & Braun, 1967). Uptake of [^{14}C]chloramphenicol by the resistant *Staphylococcus aureus* 111 growing in 4·0 μg. Tc/ml. NB was measured only at 90 min. and was virtually identical to that of the susceptible strain at that time, showing that decreased uptake of Tc was not a non-specific effect of that drug.

Table 1. *Uptake of* [^{3}*H*]*tetracycline by Staphylococcus aureus 111*

S. aureus 111 was grown in nutrient broth to the exponential growth phase (E = 0·160) and then diluted 1:5 in nutrient broth containing the indicated drugs. After 1 h. at 37° (aeration), 20 μg./ml. tetracycline containing 175,000 c.p.m. [^{3}H]tetracycline was added. Uptake was measured by liquid scintillation as described under Methods.

Pre-incubation condition (1 h.)	Accumulation of tetracycline in μg/mg. bacterial protein
Nalidixic acid 200 μg./ml.	54·8
Nalidixic acid 200 μg//ml. + tetracycline 1·0 μg./ml.	31·7
Nalidixic acid 200 μg./ml. + tetracycline 0·1 μg./ml.	26·4
Nalidixic acid 200 μg./ml. + actinomycin D 1·5 μg./ml. + tetracycline 1·0 μg./ml.	51·8

Figures 5*a*, *b* show that a decreased accumulating ability of the high-level resistant strain was maintained by subculturing with 4·0 μg. Tc/ml. In a Tc-susceptible (eliminated) culture of *Staphylococcus aureus* 111, growth in 0·05 μg. Tc/ml. NB had no effect on subsequent uptake of [^{3}H]Tc when compared with uptake by organisms grown in plain NB. It was therefore assumed that a lowered uptake depends on the presence of a gene on the resistance plasmid and that the synthesis of 'resistance protein' is generally repressed but may be derepressed by the drug (Sompolinsky *et al.* 1970). If repression in this system is effected on the level of transcription, addition of actino-

mycin D should inhibit the synthesis of the resistance protein. Table 1 shows that induction occurred in the presence of an inhibitor of DNA replication (nalidixic acid), but not when DNA-dependent RNA synthesis was inhibited by actinomycin D. The study reported in this table was performed with an entirely uninduced resistant culture that was maintained by frequent transfers on plain NA. In this strain, pre-incubation with 0·1 μg. Tc/ml. was sufficient to reduce accumulation of the drug by 50% after challenge with 20 μg. Tc/ml.

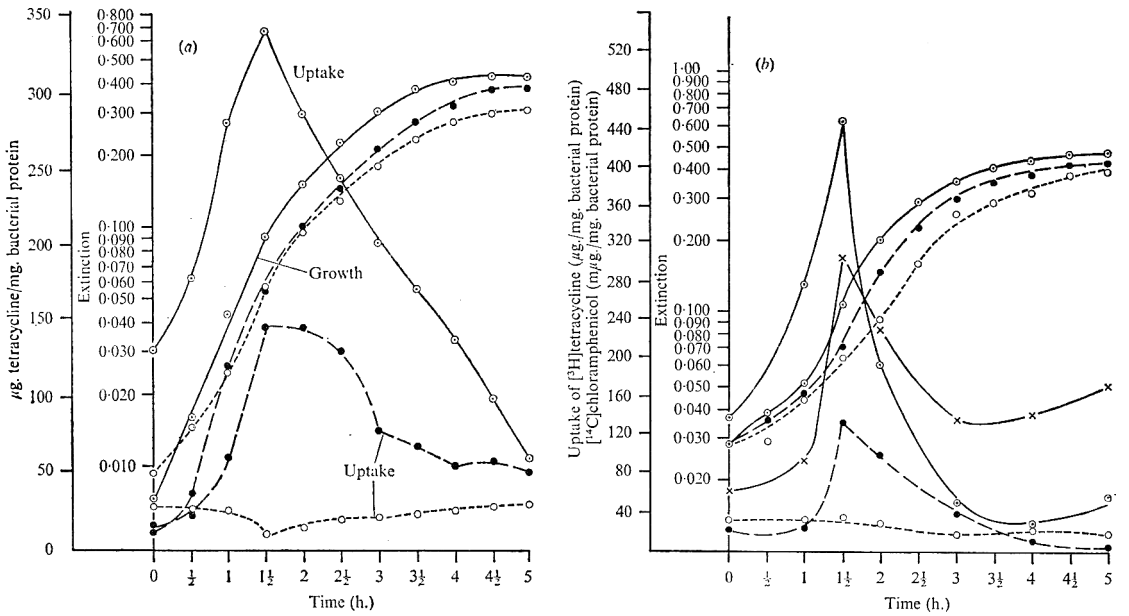

Fig. 5*a*. Uptake of tetracycline by *Staphylococcus aureus* 111 during different growth phases. ⊙——⊙, Growth and uptake by a susceptible (eliminated) strain after growth in nutrient broth; ●——● indicates a high-level resistant strain after growth in nutrient broth, and ○.....○ after growth in 4·0 μg. Tc/ml. NB. Uptake was determined as *E* 380 nm. of bacterial extracts after incubation with 160 μg./ml. tetracycline (see Methods).

Fig. 5*b*. Uptake of tetracycline by *Staphylococcus aureus* 111 during different growth phases measured as radioactivity after incubation with 160 μg. Tc/ml. containing tritiated Tc 150,000 c.p.m./ml.). Legends as for Fig. 5*a*. Uptake of chloramphenicol (× — × — ×) examined in the susceptible strain, after incubation with chloramphenicol 10 μg./ml. containing 500,000 c.p.m./ml. [¹⁴C]chloramphenicol.

Table 2. *Uptake of tetracycline by Staphylococcus aureus 111 under various conditions*

Sodium azide (0·01 M) was added to the samples simultaneously with the tetracycline.

	μg. Tetracycline/mg. bacterial protein		
Incubation conditions	Susceptible strain in NB	Resistant strain in NB	Resistant strain in 4·0 μg. Tc/ml. NB
37°	320	158	11
0°	25	7	nt
37°, with 10^{-2} M-sodium azide	38	15	7

Abbreviations: NB = nutrient broth; Tc = tetracycline; nt = not examined.

Table 2 shows that uptake was much less at 0° and the presence of sodium azide (0·01 M). Similar results were obtained when uptake was examined using [^{3}H]Tc (160 μg. Tc/ml. + 10 μg. chloramphenicol/ml.).

The connexion between uptake and resistance became particularly evident when uptake was studied as a function of external drug concentration (Fig. 6). For the susceptible strain in the exponential growth phase, the uptake was more or less proportional to the drug concentration in the medium, in the range 40 to 320 μg./ml., and had then reached a maximum of 468 μg./mg. protein. In the highly resistant strain,

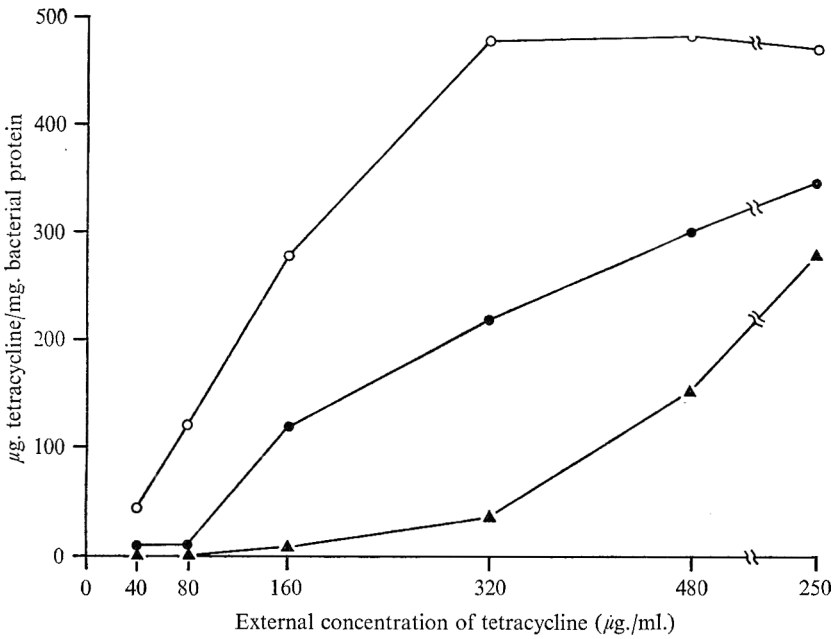

Fig. 6. Uptake of tetracycline by *Staphylococcus aureus* 111 at different external concentrations of the drug. O——O, A susceptible (eliminated) culture grown in nutrient broth; ●——●, a high-level resistant culture grown in nutrient broth; ▲——▲, a high-level resistant culture grown in nutrient broth + 4·0 μg. Tc/ml. The drug was added to samples of exponential growing cultures (37°).

derepressed through growth in plain NB during 2 h., subsequent uptake was not affected by increase in external Tc concentration from 40 to 80 μg./ml. but increased strongly when the drug concentration was further increased. When the resistant organism was grown in 4·0 μg. Tc/ml NB, subsequent uptake was little influenced by variations in external Tc concentrations of 160 to 320 μg./ml., but thereafter the slope in the uptake curve increased steeply. We did not succeed in the examination of uptake at higher concentrations, since the cultures underwent gross lysis. We therefore do not know if maximal uptake is identical in resistant and susceptible cultures. Uptakes by a non-induced resistant and by a susceptible strain at low external drug concentration were compared through the use of tritiated Tc (Fig. 7). The uptake by the resistant strain was influenced by the external concentration only when the Tc concentration exceeded 5 μg./ml.

From these results, it seems justifiable to assume that the highest concentration of Tc

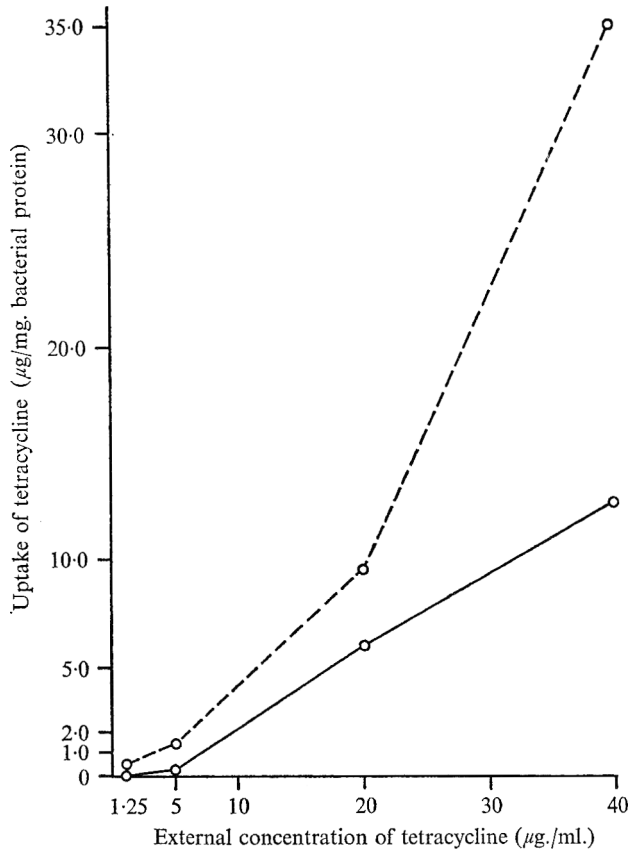

Fig. 7. Uptake of [^{3}H]tetracycline by a susceptible (eliminated) and a low-level resistant (non-induced) culture of *Staphylococcus aureus* III as a function of outer drug concentration. Legends as for Fig. 6, but uptake calculated by radioactivity of the cocci measured by liquid scintillation.

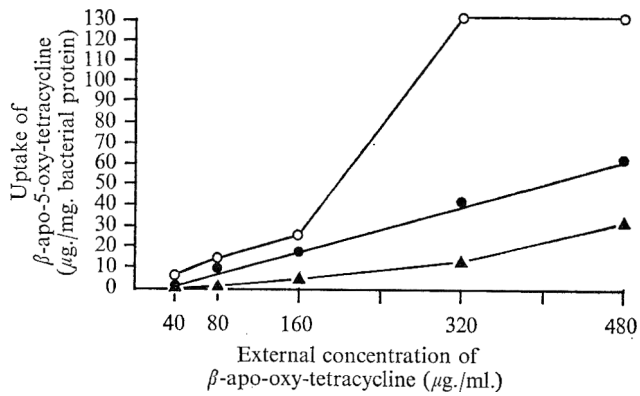

Fig. 8. Uptake of β-apo-5-oxy-tetracycline by *Staphylococcus aureus*. Legends as for Fig. 2.

which does not cause an increase in uptake by non-induced organisms reflects the level of their resistance. Similar observations were recently reported for *Escherichia coli* (DeZeeuw, 1968). It should also be mentioned that β-apo-5-oxy-tetracycline, which is only very slightly inhibitory, was accumulated to a relatively slight degree, even by the susceptible culture (Fig. 8).

DISCUSSION

Development of bacterial resistance to a chemotherapeutic drug which is associated with an extrachromosomal genetic unit is most likely, albeit not necessarily, due to the occurrence of an entirely new cell product not present in the susceptible strain. An inactivating enzyme would be an attractive candidate for such a product, and indeed inactivation of penicillin (Datta & Kontomichalou, 1965), chloramphenicol (Shaw, 1967) and aminoglycoside antibiotics (Okamoto & Suzuki, 1965) has been described in bacteria that owe their resistance to extrachromosomal DNA.

If inactivation were the way in which *Staphylococcus aureus* III overcomes the inhibiting action of tetracycline, the drug could either be entirely inactivated so that the concentration in the growth medium decreases below the tolerated level of the susceptible culture lacking a resistance plasmid, or it could be altered intracellularly so that the affinity to the site of action decreases but the altered molecules still remain bound to the permease system, thereby preventing further entrance of active drug from the surrounding medium.

In our case, no evidence for inactivation was borne out experimentally, since the drug in the nutrient medium after full growth of a resistant strain remained unaltered both in its spectrophotometric and chromatographic characteristics, and also biological activity could be demonstrated in the range comparable with an uninoculated control sample. In addition, the tetracycline extracted from resistant organisms after growth in Tc-containing medium was recognizable by its characteristic R_F on paper chromatography. Furthermore, we have demonstrated a decreased uptake of the drug by the resistant strain. If this was due partly to alteration of the drug intracellularly, with alteration of extinction at 380 nm. and with excretion to the surrounding medium of the [^{3}H]-containing fragment from the tetracycline-7-^{3}H (N), then extensive alteration of the molecule would have occurred, and Tc molecules from the medium would probably rapidly replace those metabolized. No new radioactive product was found by chromatography of the [^{3}H]-Tc containing medium after growth of the resistant strain.

We conclude, therefore, that resistance to Tc in *Staphylococcus aureus* III results from a decreased accumulation of the drug, as was demonstrated for Tc resistance in *Escherichia coli* (Izaki, Kiuchi & Arima, 1966; Franklin, 1967; DeZeeuw, 1968).

Decreased uptake due to a resistance plasmid might be due to (i) a gene product functioning as a repressor on the chromosomal operon coding for the active uptake system; (ii) a product combining with and inactivating the uptake system; or (iii) a product which facilitates transport of Tc molecules out of the cell, thereby competing with the uptake system. Further studies will be necessary to decide between these possibilities.

Decreased uptake of the Tc proved inducible, as was the case with phenotypical resistance (Sompolinsky *et al.* 1970), and in both cases the drug served as inducer. Since it has recently been suggested that induction of penicillinase synthesis occurs at the translation level (Csanyi, Jacobi & Straub, 1967), we felt it important to verify in

an uptake experiment our previous claim that induction is inhibited by actinomycin D (Sompolinsky *et al.* 1970), thereby proving that at least for Tc resistance in *Staphylococcus aureus* 111 induction occurs on the level of transcription.

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Effect of *Prymnesium parvum* Toxin, Cetyltrimethylammonium Bromide and Sodium Dodecyl Sulphate on Bacteria

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SUMMARY

At low concentrations (0·03 to 3 μg./10^9 cells), purified prymnesium toxin lysed penicillin and lysozyme-EDTA spheroplasts of *Escherichia coli* and *Pseudomonas fluorescens* and protoplasts of *Micrococcus lysodeikticus* and *Bacillus subtilis*. Intact *E. coli* and *P. fluorescens* were unaffected by the toxin (3 3 μg./ml.), but *E. coli* B was lysed by prymnesium toxin in the presence of EDTA. The activities of selected detergents against spheroplasts and EDTA-treated *E. coli* B were compared with the action of prymnesium toxin.

INTRODUCTION

The toxins produced by the phytoflagellate *Prymnesium parvum* have a wide range of biological activity, including lysis of various mammalian erythrocytes (Yariv & Hestrin, 1961; Bergmann & Kidron, 1966) and nucleated cells such as Ehrlich ascites cells (Dafni & Shilo, 1966; Dafni, 1969), HeLa cells, and normal liver and amnion cells (Shilo & Rosenberger, 1960). Common to all these toxin effects is damage to the cytoplasmic membrane and one result of this is leakage of intracellular constituents into the medium. Prymnesium extracts, including the highly purified preparations, lead to changes in the potassium and sodium content of Ehrlich ascites cells with very rapid leakage (within 20 sec.) of most of the intracellular potassium (Dafni, 1969). Furthermore, the primary effect of the prymnesium ichthyotoxin on immersed fish seems to be the loss of selective permeability of the gill epithelial cells towards various substances, including macromolecules such as trypan blue or radio-iodinated albumin (Ulitzur & Shilo, 1966).

This study describes the effects of prymnesium toxin on bacteria (intact organisms, protoplasts, spheroplasts, and also species of Mycoplasma). The similarity of chemical and physical properties of various detergents (cetyltrimethylammonium bromide and sodium dodecyl sulphate) to those of prymnesium toxin (Ulitzur & Shilo, 1966, 1970) made their testing in this experimental system of interest.

METHODS

Prymnesium parvum toxin. The intracellular toxic principle ('toxin B') was extracted and purified as described previously (Ulitzur, 1969; Ulitzur & Shilo, 1970). The purified 'toxin B' preparation had a specific activity of 3000 haemolytic units (h.u.)/μg., and was dissolved in methanol. In the experimental systems, the final methanol

concentration was never greater than 0·1 %; this concentration did not affect the bacteria tested. For alkaline treatment of the toxin, 0·5 N-NaOH (in methanol) was mixed with toxin and incubated for 30 min. at 20° and then neutralized by addition of 1 N-NaCl (in methanol). For several experiments, 'toxin B' was separated into six components by thin-layer chromatography (Ulitzur & Shilo, 1970).

Bacterial strains and growth conditions. The bacterial strains used were *Escherichia coli* B, *E. coli* K12, *E. coli* ML35 (a lactose permeaseless mutant with constitutive β-galactosidase ($i^-z^+y^-$)), *Pseudomonas fluorescens* MD9 (an isolate described by Rosenberger & Shilo, 1961), *Bacillus subtilis* W168, and *Micrococcus lysodeikticus* (all from the collections of the Departments of Bacteriology and Microbiological Chemistry of the Hebrew University-Hadassah Medical School, Jerusalem). Bacteria were grown in nutrient broth (Difco) at 37° with shaking and harvested at the logarithmic phase of growth by centrifugation at 10,000 *g* for 15 min. at 20°. They were washed and resuspended in 0·05 M buffer (pH 7·6).

Mycoplasma capri and *M. laidlawii* (obtained from S. Razin, Department of Clinical Bacteriology, Hebrew University-Hadassah Medical School, Jerusalem) were grown as described by Razin (1963).

Preparation of spheroplasts and protoplasts. Lysozyme-EDTA spheroplasts of *Escherichia coli* and *Pseudomonas fluorescens* were prepared as described by Repaske (1958). Protoplasts of the Gram-positive organisms were obtained by incubation of 2×10^{10} bacteria/ml. in buffer (0·01 M-tris, 0·05 M-NaCl and 1 M-sucrose at pH 8) with the addition of 100 μg. lysozyme (Fluka) and 20 μg. DNase (Calbiochem) per ml. After incubation of this mixture for 30 min. at 30°, phase contrast microscopy showed that at least 90 % of the organisms were converted into protoplasts. The protoplasts were centrifuged at 5000 *g* for 20 min. at 4° and resuspended in the buffer. Penicillin spheroplasts of *E. coli* and *P. fluorescens* were prepared by growth in nutrient broth containing 0·6 M-sucrose. When the bacterial population reached 5×10^7 organisms/ml., 1000 u. penicillin G/ml. (potassium salt; Rafa, Jerusalem) were added. Microscopic examination showed that, after 2 to 3 h. incubation, more than 90 % of the organisms were converted into spheroplasts. Spheroplasts and protoplasts were stored at 4°.

Determination of lytic effect of prymnesium toxin on intact and osmotically sensitive bacteria. Assay mixtures containing 10^9 spheroplasts, protoplasts, or mycoplasma organisms/ml. in 6 ml. of various isotonic buffers and different concentrations of prymnesium toxin or detergents were incubated at 35° for 1 h. or at 8° for 17 h. The degree of lysis was determined by following the decrease in turbidity in a Klett–Summerson photometer (filter 42). Untreated cell suspensions were used as controls. Complete (100 %) lysis was obtained by adding 9 ml. water to 1 ml. of bacterial suspension (10^{10} cells) and incubating the diluted suspension for 30 min. at 20°. This system was also used for testing the lytic effects of prymnesium toxin, sodium dodecyl sulphate (SDS, Fluka) and cetyltrimethylammonium bromide (CTAB, Fluka) on intact *Escherichia coli* B and *E. coli* K12 in the presence or absence of 2×10^{-4} M-sodium ethylenediaminetetra-acetate (EDTA).

Viable count of bacteria. The bacterial suspension was incubated for 3 h. at room temperature in 0·05 M-tris buffer (pH 7·6) with prymnesium toxin. The control contained the same mixture with suitable concentrations of methanol instead of toxin. After incubation the bacteria were diluted and plated on nutrient agar (Difco). Colonies were counted after incubation for 24 h. at 37°.

Assay of β-galactosidase activity. Hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) by *Escherichia coli* ML35 was carried out as described by Wallenfels, Lehman & Malhotra (1962).

RESULTS

The effect of prymnesium toxin on bacterial spheroplasts and protoplasts and on Mycoplasma. Prymnesium toxin lysed penicillin and lysozyme-EDTA spheroplasts of *Pseudomonas fluorescens* and *Escherichia coli* as shown in Fig. 1. The minimal dose which lysed 50% of the organisms was found to be independent of the method used for preparing the spheroplasts. The toxin also lysed protoplasts of *Bacillus subtilis* and

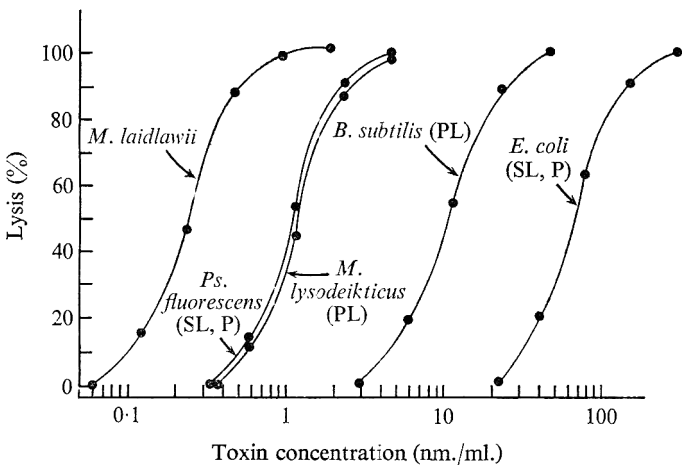

Fig. 1

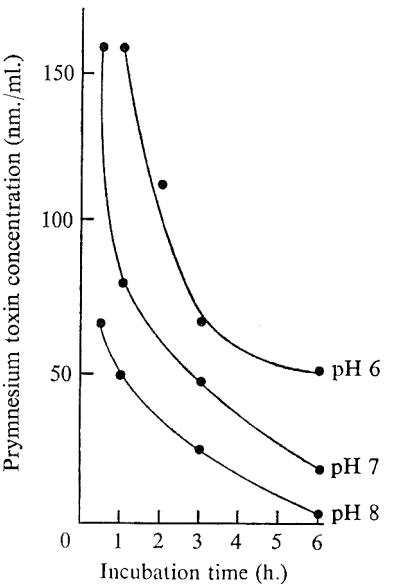

Fig. 2

Fig. 1. The lytic activity of prymnesium toxin on spheroplasts, protoplasts and mycoplasma organisms. *Escherichia coli* and *Pseudomonas fluorescens* spheroplasts were suspended in 0·3M-sucrose in 0·06M-tris buffer (pH 8). *Bacillus subtilis* and *Micrococcus lysodeikticus* were suspended in 1M-sucrose and 0·005M-NaCl in 0·01M-tris buffer (pH 8), and the mycoplasma organisms were suspended in 0·04M-sucrose and 0·25M-NaCl in 0·02M-tris buffer (pH 8). The degree of lysis was determined after 1 h. at 35° as described in Methods. PL = protoplasts prepared with lysozyme; SL = spheroplasts prepared by the lysozyme-EDTA method; P = spheroplasts prepared with penicillin.

Fig. 2. The effect of pH on the lytic activity of prymnesium toxin. The incubation time which gave lysis of 50% of *Pseudomonas fluorescens* lysozyme-EDTA spheroplasts was determined in the presence of different toxin concentrations in a solution of 0·3M-sucrose at different pH values (in 0·02M-sodium phosphate buffer) at 35°.

Micrococcus lysodeikticus and *Mycoplasma laidlawii* organisms (Fig. 1). The sensitivity of mycoplasma organisms to the toxin was more than 500 times that of the *E. coli* spheroplasts. *Micrococcus capri* was even more sensitive: 0·05 nm. toxin/ml. lysed 50% of the *M. capri* population under the same conditions. The sensitivity of *Escherichia coli* spheroplasts to prymnesium toxin was more than 10 times greater than their sensitivity to CTAB and 500 times greater than their sensitivity to SDS on a weight basis.

Figure 2 shows that the lytic activity of the toxin on *Pseudomonas fluorescens* sphero-

plasts increased progressively when the pH of the system increased from 6 to 8. Figure 3 shows that toxin pretreated with NaOH had practically no lytic effect on *P. fluorescens* spheroplasts when incubated at 35°. However, such pretreated toxin retained some of its original lytic activity when tested at 8°. Tabor, Tabor & Rosenthal (1961) showed that divalent cations (Mg^{2+} and Ca^{2+}) and polycationic substances (spermine) stabilized osmotically sensitive spheres in hypotonic media. We found that spermine or 3′,3-diaminodipropylamine markedly protected *Mycoplasma laidlawii* against the lytic activity of the prymnesium toxin. It was found that, while 0·5 μg. toxin/ml. gave 50% lysis in the absence of these bases, 25 μg./ml. were required in the presence of spermine (0·001 M) and 30 μg./ml. in the presence of 3′,3-diaminodipropylamine (0·0015 M).

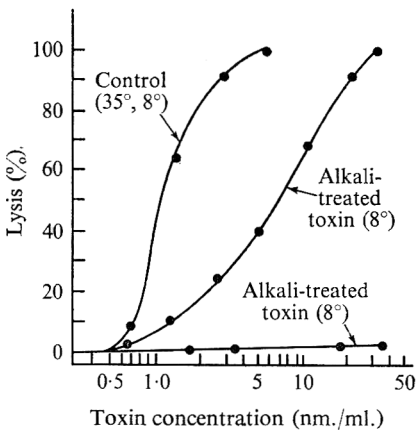

Fig. 3

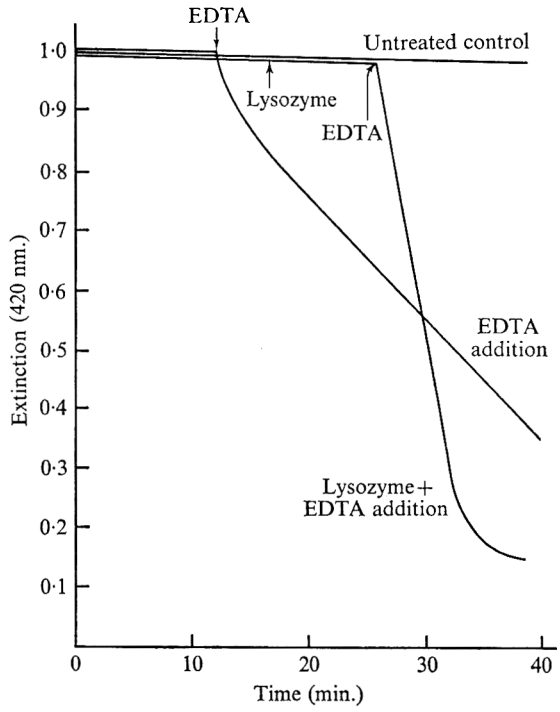

Fig. 4

Fig. 3. The lysis of *Pseudomonas fluorescens* spheroplasts by NaOH-treated prymnesium toxin. The lytic activity was determined before (control) and after the toxin had been treated with NaOH (as described in Methods); the extent of lysis was determined at 35° after 60 min. and at 8° after 17 h., a time after which no further lysis occurred.

Fig. 4. The effect of prymnesium toxin on EDTA-treated *Escherichia coli* B. At a final concentration of 10^9 organisms/ml. *E. coli* B were suspended in 0·3 M-sucrose in 0·05 M-tris buffer (pH 8) containing 0·66 μg. prymnesium toxin/ml. EDTA (2×10^{-4} M) and lysozyme (10 μg./ml. were added to the mixture as indicated by arrows. The rate of lysis at 22° was determined in a Perkin–Elmer spectrophotometer (Model UV 137) at 420 nm. against a cuvette containing only tris–sucrose buffer.

The lytic activity of four of the six haemolytic components separated by thin-layer chromatography (t.l.c.) of 'toxin B' (Ulitzur & Shilo, 1970) was tested on *Pseudomonas fluorescens* spheroplasts. All four components showed lytic activity, and for each the ratio of lytic to haemolytic activity was similar.

The effect of prymnesium toxin on intact cells. Untreated logarithmic-phase *Escherichia coli* ML35 and *Pseudomonas fluorescens* bacteria were resistant to prymnesium toxin. No decreases in viable counts occurred when these bacteria were treated with 3·3 μg. toxin/ml. at 35° for 3 h. This concentration of toxin is 100 times that sufficient to lyse spheroplasts of *E. coli*. Incubation of *E. coli* ML35, a strain cryptic for β-galactosidase, with 3·3 μg. toxin/ml. under similar conditions did not result in any detectable cell membrane damage as determined by the ability of such cells to hydrolyse ONPG.

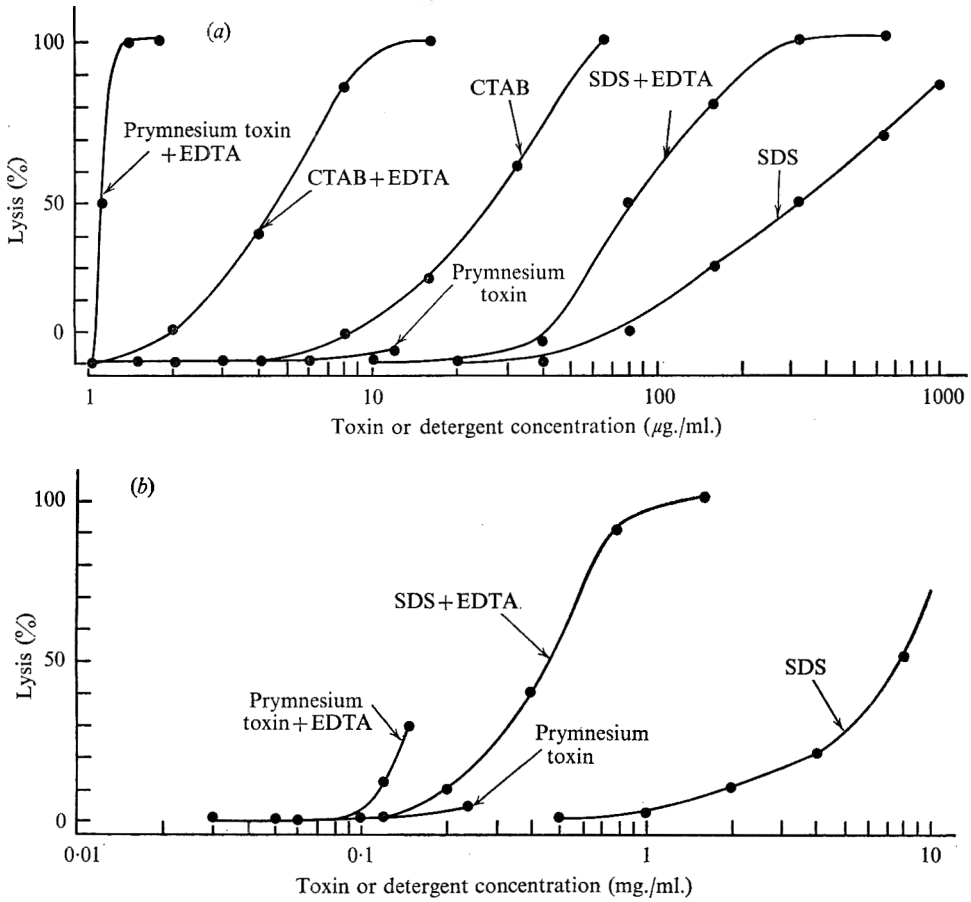

Fig. 5. The lytic effect of prymnesium toxin, SDS, and CTAB on EDTA-treated *Escherichia coli* B (*a*) and *E. coli* K12 (*b*). Both strains of *E. coli* were studied in systems without and with EDTA (2×10^{-4} M) and lysis measured after 60 min. at 35°. *E. coli* B was suspended in sucrose-tris buffer as described in Fig. 4, and *E. coli* K12 was suspended in 0·05 M-tris buffer (pH 8).

It is likely that intact bacteria are resistant to prymnesium toxin because cell-wall components block access of the toxin to the bacterial membrane. EDTA treatment of *Escherichia coli* has been shown to destroy cell-wall barriers towards certain antibiotics, such as actinomycin D (Leive, 1965*a*), polymyxin B and novobiocin, as well as towards detergents, such as cetylpyridinium bromide and sodium tetradecyl sulphate, and towards complement (Muschel & Gustafson, 1968). EDTA-treated cells were therefore tested for their sensitivity to prymnesium toxin. When suspended in

0·05M-tris buffer (pH 5), *E. coli* B organisms, but not *E. coli* K12 were lysed in the presence of 2×10^{-4}M-EDTA. The sensitivity of EDTA-treated *E. coli* B organisms to prymnesium toxin was therefore tested in the presence of stabilizing concentrations of sucrose (0·3M). Figure 4 shows that prymnesium toxin alone had no effect on intact *E. coli* B suspended in the sucrose–tris buffer solution. When EDTA (2×10^{-4}M) was added to these toxin-treated bacteria, the turbidity decreased. Addition of lysozyme to toxin-treated *E. coli* B had no effect, but subsequent addition of EDTA, which converted the cells into spheroplasts, caused rapid lysis.

The two detergents (SDS and CTAB) tested for their lytic effect on *Escherichia coli* B showed a four- to fivefold increase of activity in the presence of EDTA (Fig. 5*a*), similar to the action of prymnesium toxin. *Escherichia coli* K12, on the other hand, was found to be more resistant to the lytic activity of prymnesium toxin and SDS even in the presence of EDTA (Fig. 5*b*).

DISCUSSION

The highly purified prymnesium toxin preparation has been shown to be one of the most active lysins described, having a specific haemolytic activity more than 3000 times that of digitonin or lysolethicin (Ulitzur & Shilo, 1970). It has been suggested that the strong affinity of the toxin towards biological membranes results from its chemical structure which, as with synthetic detergents and lysophospholipids, consists of lipid (fatty acids) and polar (protein and phosphate) moieties (Ulitzur & Shilo, 1970). Unlike most of the ionic detergents which exert lytic activity on intact bacteria (Baker, Harrison & Miller, 1941; Hotchkiss, 1946; Salton, 1951), prymnesium toxin only acts upon bacteria whose cell walls are partially removed and thus resembles some non-ionic detergents and antibacterial agents (such as actinomycin D and complement) which are not capable of affecting certain intact bacteria (Leive, 1965*a*; Muschel & Gustafson, 1968).

The resistance of intact bacteria to lysis by prymnesium toxin may be related to the impermeability of the cell wall to the large micelles of the toxin existing in aqueous solutions. On the other hand, it is known that some detergents can affect the metabolism of an organism without affecting its viability (Hotchkiss, 1946). The fact that *Escherichia coli* ML35 treated with prymnesium toxin did not become permeable to ONPG indicates that, if indeed it occurs at all, toxin damage to the intact cell membrane was small.

Treatment of bacteria with lysozyme-EDTA or growth in the presence of penicillin, treatments which expose the cell membrane or part of it, rendered the bacteria susceptible to lysis on subsequent exposure to prymnesium toxin. However, this lytic effect on the osmotically sensitive bacteria was very disparate, in contrast to the consistent effect on different sorts of vertebrate erythrocytes; for instance, mycoplasma organisms are highly sensitive to prymnesium toxin, while *Escherichia coli* spheroplasts are more resistant.

Malamy & Horecker (1964) have shown that after spheroplast formation by treatment with lysozyme-EDTA, but not by growth in the presence of penicillin, the alkaline phosphatase bound in the periplasm is liberated. In spite of the differences in the nature of spheroplasts formed by lysozyme-EDTA and penicillin treatment, no noticeable differences in sensitivity were observed between the two kinds of spheroplasts towards prymnesium toxin. It appears that the resistance of intact bacteria to

lysis by prymnesium toxin must depend on the presence of a 'barrier' in the cell wall against the toxin, and not on any particular stability of the cell membrane. This would explain the sensitivity of *Escherichia coli* B to toxin (as well as to SDS and CTAB) in the presence of EDTA. As is known, EDTA treatment of *E. coli* liberates a considerable portion of the cell wall lipopolysaccharide (Leive, 1965*b*), rendering the cell penetrable to a number of substances which cannot enter the intact organism (or hardly so). Muschel & Gustafson (1968) have found that EDTA-treated *E. coli* showed higher sensitivity to ionized detergents, polymyxin B, and complement.

The findings described here indicate that both the haemolysin and the lysin of osmotically sensitive bacteria could be the same toxic principle. This suggestion is based on the similar response to alkaline treatment of both activities, in contrast to the ichthyotoxic activity which is not affected by alkaline treatment (Shilo, 1967). Furthermore, it was found that four out of six of the haemolysins separated by t.l.c. showed lytic activity towards Pseudomonas spheroplasts corresponding to their haemolysin content, while no fixed relationship was observed between their haemolytic and ichthyotoxic activities (Ulitzur & Shilo, 1970).

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Mutants of *Escherichia coli* with Altered Surface Properties which are Refractory to Colicin E2, Sensitive to Ultraviolet Light and which can also Show Recombination Deficiency, Abortive Growth of Bacteriophage λ and Filament Formation

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SUMMARY

Mutants refractory to colicin E2, previous phenotypic symbol RefII, have now been divided into two major phenotypic groups. CetC mutants in distinction to CetB mutants show increased sensitivity to u.v. and to a detergent (DOC). CetC mutants may also show other properties, including abortive growth of bacteriophage λ and defective division. CetB mutants are refractory to E2 at 30° but largely sensitive at 37°. CetC mutants are also refractory at 30° but may be either sensitive or completely refractory at 37°. In contrast to Rec⁻ mutants, CetC strains are not sensitive to gamma rays but two mutants show enhanced DNA breakdown after u.v. irradiation. CetC mutants seem defective in a specific membrane component which renders them insensitive to E2 and alteration of which can affect several aspects of DNA metabolism.

INTRODUCTION

One approach to the study of the functional organization of the bacterial membrane appears to be through the use of certain protein antibiotics, colicins. The general properties of colicins (see Review, Nomura, 1967) suggest that their primary site of action is the cytoplasmic membrane although they do not appear to induce detectable permeability changes in sensitive cells (Nomura, 1964). It is assumed, therefore, that colicins induce subtle changes in the physical properties of the cell membrane, which in turn induce the intracellular changes specific to each colicin. To establish the nature and normal functional role of colicin-specific sites in the cell surface, efforts have therefore been made to isolate mutants, resistant to colicin, with altered membranes. The isolation of mutants possibly of this type, which still adsorb colicin normally but remain refractory to its effect, has been described previously (Hill & Holland, 1967). The mutants included a class which were specifically refractory to colicin E2 at 30° but largely sensitive at higher temperatures. Further studies confirmed that E2 refractivity in one mutant, ASH 101, was temperature-dependent and that the mutant growing at 40° immediately became resistant to E2 after a shift of temperature to 30° (Holland, 1968). At 30° inhibition of cell division and induction of rapid degradation of DNA, characteristic effects of E2 on sensitive cells, were not observed in this mutant. Studies of other mutants refractory to E2 have shown that a small proportion were also

u.v.-sensitive, although this property was not apparently temperature-dependent (Holland, 1967).

In all previous communications E2-refractory strains were designated as Ref11 mutants. In accordance with the recommendations of Clark, Demerec, Adelberg & Hartman (1966), E2-refractory mutants are now identified by the symbol Cet (Colicin E-two). For convenience, mutants of normal u.v. sensitivity are designated CetB, whilst u.v.-sensitive, E2-refractory mutants are designated CetC. In this paper additional CetC mutants are described and the basis of their u.v. sensitivity further examined. The temperature dependence of E2-refractivity in these mutants has been found to be more complex than that of CetB mutants and they have acquired, in addition to u.v. sensitivity, several other mutant characteristics including altered cell surface properties. The properties of these mutants suggest that colicin E2 may normally interact with a part of the cell membrane which is involved in the regulation of cell division and in some aspects of DNA metabolism.

METHODS

The general properties of strains used in this study are shown in Table 1. Mutant strains ASH 101, 110, 111, 112 (Holland, 1967, 1968) and ASH 102, 113 (Holland & Threlfall, 1969) have been studied previously. Strains ASH 114, 115 and 116 are described for the first time. E2-refractory strains ASH 101, 111 and ASH 114 were derived from parental strain ASH 1 (HfrB11, *thi⁻ met⁻ thy⁻ leu⁻ str^r λ⁻*) whilst ASH 102, 112, 113, 115 and 116 were derived from the parental strain ASH 10 (F⁻ *thi⁻ met⁻ thy⁻ leu⁻ lac⁻ str^r λ⁺*). The E2-refractory strain ASH 110 was derived from HfrH (*thi⁻ thy⁻ lac⁻ str^r λ⁻*). The isolation and initial characterization of the mutants has also been described previously (Holland & Threlfall, 1969).

Table 1. *Summary of phenotypic properties of CetB and CetC mutants*

Strain		Sex	Response to E2		Response to				Recombination proficiency	Filament formation (+/−)
			25°	37°	u.v.	X-ray	DOC	λ		
Parental	ASH 1	Hfr	S	S	R	R	R	S	/*	−
	ASH 10	F⁻	S	S	R	R	R	S	+	−
CetC	ASH 110	Hfr	R	R	S	/*	S	S	/	Fil⁺
	ASH 111	Hfr	R	S	S	R	S	S	Rec⁻	Fil⁺
	ASH 114	Hfr	R	R	S	R	S	R	Rec⁻	−
	ASH 112	F⁻	R	R	S	R	S	R	Rec⁻	−
	ASH 113	F⁻	R	S	S	R	S	S	Rec⁻	−
	ASH 115	F⁻	R	S	S	/	S	S	+	−
	ASH 116	F⁻	R	S	S	/	S	R	+	−
CetB	ASH 101	Hfr	R	S	R	/	R	S	/	−
	ASH 102	F⁻	R	S	R	/	R	S	+	−

R denotes resistance, or refractivity; S denotes sensitivity. The response to colicin E2 was determined in liquid-grown cultures as in Fig. 1. With phage λ, three strains, ASH 114, 112 and 116, showed increased but not complete resistance to the phage (see text); * denotes strains not tested for this character.

The RecA strain KMBL 239 (*rec* 34) was obtained from Professor A. Rörsch, and the RecB mutant, JC 4457, was kindly provided by Dr A. J. Clark. Culture methods and production and assay of colicin E2 (P9) obtained from *Salmonella typhimurium* LT2 906 were described previously (Hill & Holland, 1967).

Irradiation procedures. Ultraviolet irradiation. The source of u.v. was a low-pressure mercury lamp (Hanovia Ltd) used without filter and with an incident dose rate of 6·5 ergs/mm². at 46 cm. For quantitative determination of the u.v. sensitivity of mutant strains, the procedure described previously was used (Holland, 1967). Exponential cultures of various strains grown in tryptone broth (TB) were harvested, washed and resuspended in 0·07 M-phosphate buffer at 10^6 bacteria/ml. Appropriate dilutions of bacteria were then plated on tryptone (1 % Oxoid tryptone, 0·5 % w/v NaCl) agar plates, irradiated and incubated in the dark for 14 h. at 37° before counting.

Gamma irradiation. Exponential cultures (5×10^8 bacteria/ml.) of mutants in TB media were harvested, washed and resuspended in 0·07 M-phosphate buffer at 10^8 bacteria/ml.; 2 ml. aliquots in $1\frac{1}{4}$ in. $\times \frac{3}{8}$ in. glass vials were then irradiated in a Gammacell 200 (Atomic Energy of Canada, Ltd) output 0·13 megarads/h. Irradiated samples were diluted, plated on TB plates, and survival determined after 14 h. at 37°.

Determination of u.v.-induced DNA breakdown. Exponentially growing cultures of strains in Difco nutrient broth (NB) were labelled with H^3 thymine (16 μCi/μg. thymine/ml.) in the presence of 100 μg./ml. deoxyuridine, for five generations and harvested when the density reached 3×10^8 bacteria/ml. Labelled cells were washed twice and finally resuspended (2×10^7 bacteria/ml.) in 0·07 M-phosphate buffer pH 7·2 plus 25 μg./ml. cold thymine; 8 ml. samples were irradiated in a thin layer (2 mm.) and then diluted with an equal volume of NB and reincubated in dim light at 37°. Samples (0·5 ml.) were removed at intervals, mixed with 0·5 ml. 10 % cold trichloroacetic acid and cold and hot acid soluble counts analysed as described by Howard-Flanders & Theriot (1966). Full details of labelling procedures and methods of the determination of E2-induced DNA breakdown have been described previously (Holland, 1968).

Growth of bacteriophage λ. Production and assay of λ and λgv (Jacob & Wollman, 1953) have been described previously (Holland, 1967). One-step growth experiments were carried out by the standard technique based upon that described by Ellis & Delbrück (1939) using *Escherichia coli* C600 as indicator. The medium used in one-step growth experiments consisted of an M9 basal medium (Anderson, 1946) with 1·4 % (w/v) maltose as carbon source and supplemented with 1 % (w/v) Casamino acids (Difco), 40 μg./ml. thymine and 10 μg/ml. vitamin B1. For the assay of intracellular phage, 0·5 ml. aliquots of infected cultures were removed, mixed with an equal volume of 10 % (v/v) chloroform in phage buffer (0·02 M-tris pH 7·2, 0·01 M-$MgSO_4$, 0·005 % gelatin) and blended vigorously on a Vortex mixer. Finally, the suspension was incubated 10 min. at 37° to complete lysis, and, after cooling, 0·1 ml. samples were assayed for free phage.

RESULTS

Response of Cet mutants to colicin E2

In the initial characterization of E2-refractory mutants (Hill & Holland, 1967), refractivity to colicin was determined in cross-streak tests on nutrient broth agar. The majority of the mutants, although pregrown at 37°, were refractory to E2 at 25° but completely sensitive at 37°. In one mutant, ASH 112, some growth in the presence of E2 was observed at 37° and so sensitivity in this strain was not complete at high temperature. Similar results were again obtained in cross-streak tests with all the mutants examined in this study. When sensitivity to colicin was determined in liquid culture, although all strains grown and tested at 30° were still refractory to E2, the response at 37° varied

widely in different mutants. As shown in Fig. 1, some strains, including ASH 112, were completely refractory to E2 whilst others, e.g. ASH 111 were quite sensitive. Strains ASH 101 and ASH 102, which appeared to be typical of the majority of Cet mutants, showed, as described previously (Holland, 1968), an intermediate sensitivity at 37°. The induction of DNA breakdown by colicin E2 at 37° was also examined in all the mutants. As found previously and as shown in Fig. 2, the extent of E2-induced DNA breakdown at 37° closely parallels the E2-sensitivity of each strain at this temperature.

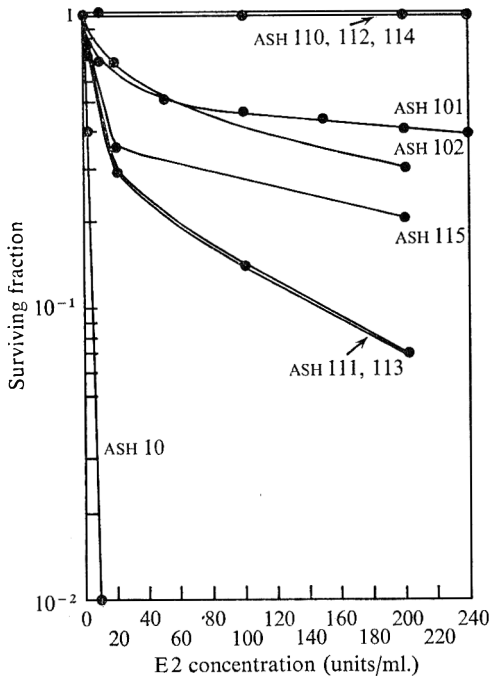

Fig. 1

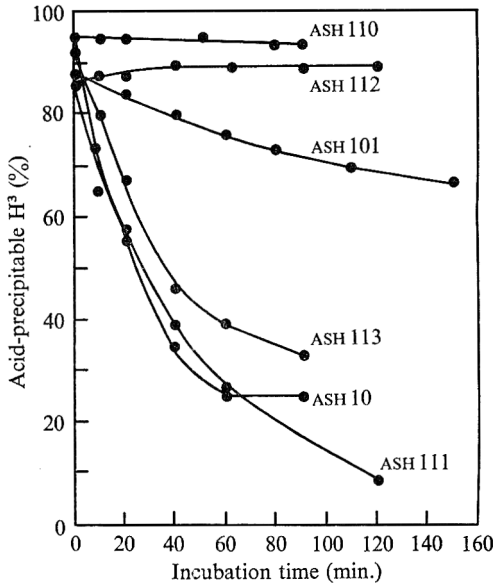

Fig. 2

Fig. 1. Sensitivity of CetC mutants to colicin E2 at 37°. Exponentially growing cultures in NB medium of various CetC mutants, two CetB mutants (ASH 101 and 102) and a wild-type strain, ASH 10, were adjusted to 10^8 bacteria/ml. After treatment with different concentrations of colicin E2 for 40 min. at 37°, the surviving fractions were determined by plating on NB agar plates.

Fig. 2. Colicin-induced DNA breakdown in CetC mutants at 37°. Bacteria were labelled by growth in NB media plus H^3 thymine as described in Methods, harvested, washed and resuspended in NB at 2×10^7 bacteria/ml. E2 was added and the cells reincubated with shaking at 37°. Samples (1·0 ml.) were removed at intervals, mixed with 4 ml. 10 % ice-cold TCA and the content of acid-soluble and acid-precipitable radioactivity determined as described previously (Holland, 1968). The colicin concentrations used were 1000 units/ml. for ASH 110, 112; 400 units/ml. for ASH 101, 113 and 111; 100 units/ml. for ASH 10.

Ultraviolet-sensitive Cet mutants. When further properties of Cet mutants were examined they were found to be divisible into two major groups. CetB mutants, e.g. ASH 101 and ASH 102, apparently differ from the wild-type only in their refractivity to colicin E2. CetC mutants, e.g. ASH 112, in contrast have acquired a varied range of additional properties (Table 1). However, CetC mutants, which contribute about 15 %

of all Cet mutants, i.e. seven out of about 50 tested, have one property in common: they are all u.v.-sensitive. The u.v. sensitivity of several CetC mutants at 37° is shown in Fig. 3. The mutants again show a wide range of response with those mutants showing maximum refractivity to E2 at 37° (ASH 112 and ASH 114) being the least sensitive to u.v. The u.v.-sensitivity of ASH 111 varied somewhat in different experiments (see also Holland, 1967) but was usually similar to that shown in Fig. 3.

It was previously reported (Holland, 1967) that CetC mutants ASH 110 and ASH 113, like Rec⁻ mutants, are still capable of host-cell reactivation (i.e. they are Hcr⁺). However, unlike Rec⁻ mutants, CetC mutants are not particularly sensitive to gamma-irradiation (Fig. 4).

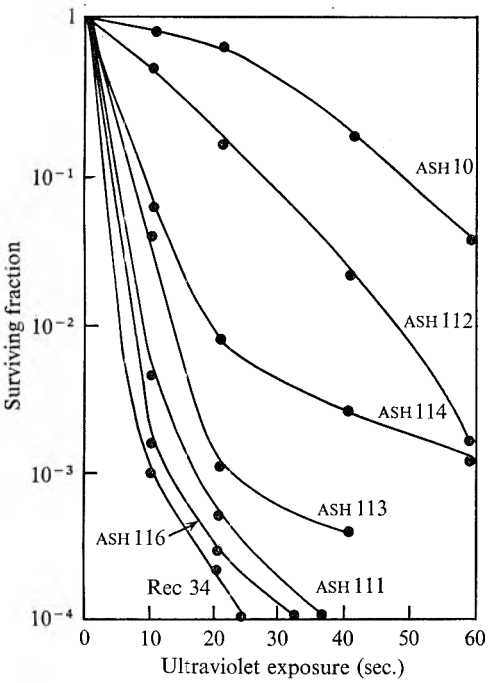

Fig. 3

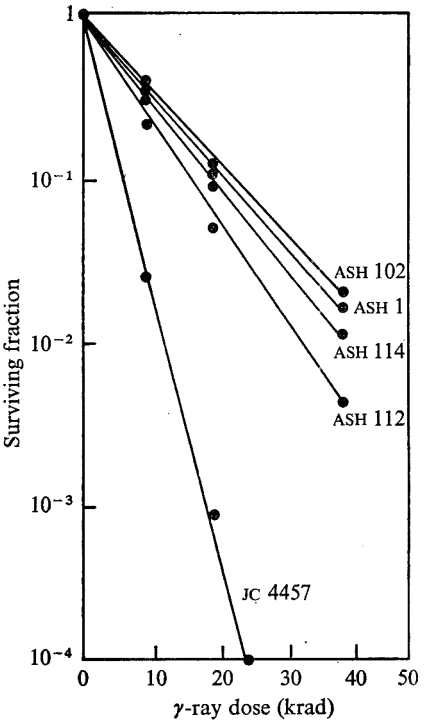

Fig. 4

Fig. 3. Ultraviolet sensitivity of CetC mutants at 37°. The *cet*⁺ strain ASH 10 and the *rec*A mutant *rec* 34 were included as controls.

Fig. 4. Gamma-ray sensitivity of Cet mutants. The *rec*B mutant, JC 4457, an X-ray sensitive strain, was included as a control.

Ultraviolet-induced DNA breakdown in CetC mutants. Howard-Flanders & Theriot (1966) and Willetts & Clark (1969) have shown that whilst at least some RecB and RecC mutants show a 'cautious' (less than wild-type) DNA breakdown, RecA mutants show 'reckless' DNA breakdown after u.v. irradiation. The CetC mutant ASH 111 was previously found to show enhanced DNA breakdown after u.v. irradiation (Holland, 1967) and several other CetC mutants have now been tested for this property. The u.v.-induced DNA breakdown patterns obtained (Fig. 5) demonstrate that none of the mutants fall clearly into the two categories originally defined by Howard-Flanders & Theriot (1966). Although ASH 114 and ASH 111 and possibly ASH 113 showed increased

breakdown over the parental type after large doses of u.v., they could still be distinguished from typical RecA mutants which characteristically show extensive spontaneous DNA breakdown, have large intracellular nucleotide pools and rapidly degrade their DNA after small doses (50 ergs) of u.v. (Howard-Flanders & Theriot, 1966). With other CetC mutants DNA breakdown after irradiation was similar to the parental strain ASH 10 or the CetB mutant ASH 101.

Detergent sensitivity of CetC mutants. De Zwaig and Luria (1967) reported that some mutants refractory to both colicins E2 and E3 showed increased sensitivity, under certain cultural conditions, to sodium deoxycholate (DOC) and to EDTA. They

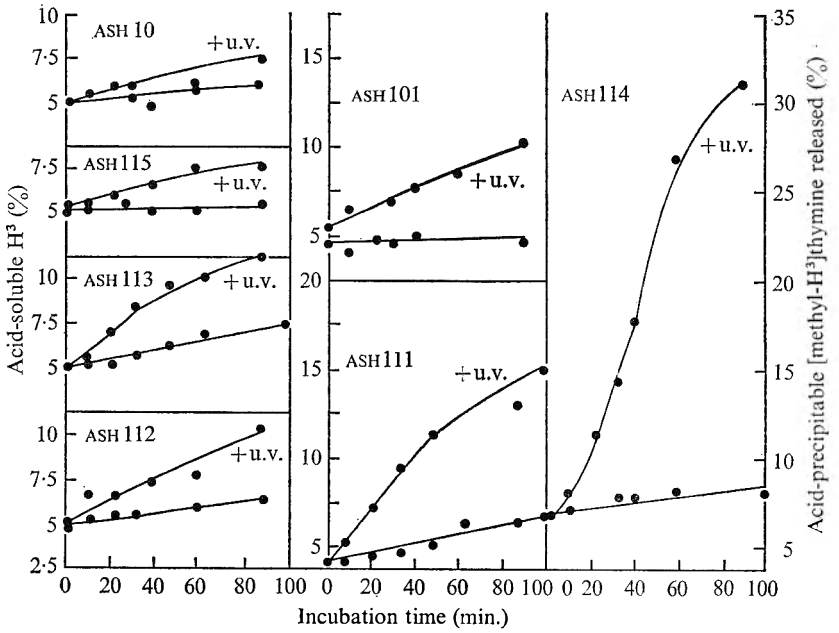

Fig. 5. DNA breakdown in CetC mutants after 400 ergs/mm.² u.v. Content of acid-soluble H^3 thymine as a percentage of total radioactivity of the culture is plotted against time. The control culture, ASH 10 (*cet*⁺), is isogenic with the CetC mutants ASH 112, 113 and 115 whilst the CetB mutant ASH 101 is shown as an isogenic control for CetC mutants ASH 111 and ASH 114.

suggested that this and other properties of the mutants indicated that refractivity to colicin was associated with a change in properties of some component of the cytoplasmic membrane. When grown in nutrient broth, all CetC mutants showed greatly increased sensitivity to DOC whilst CetB mutants were largely unaffected (Fig. 6). For these tests the bacteria were first grown at 37° and then tested for DOC-sensitivity at either 25° or 37°. The CetC strains were almost equally sensitive at both temperatures as with their response to u.v. As in other tests the mutants showed a wide range of response to the treatment with, to some extent, the mutants most sensitive to u.v. being least sensitive to the detergent. Some CetB mutants also showed slight sensitivity to DOC (e.g. ASH 102) but the effect was much less than that observed with CetC strains.

Other u.v.-sensitive mutants of *Escherichia coli* K 12 including RecA, RecB and Uvr⁻ strains and an Hsp⁻ (host specificity) mutant were also tested for DOC sensi-

tivity and found to be resistant. Increased DOC-sensitivity therefore appears to be a specific change associated with the CetC phenotype.

Effect of temperature shift on E2-induced DNA breakdown in CetC mutant ASH 113

Mutants refractory to colicin E2 should conceivably arise through a change in the activity of the nuclease normally activated by the presence of the colicin. Furthermore, since particular strains, e.g. ASH 111 and ASH 113, are very sensitive to E2 at high temperature but refractory at low temperature, it was of interest to determine whether such mutants produced a cold-sensitive nuclease. A culture of ASH 113 was first pre-

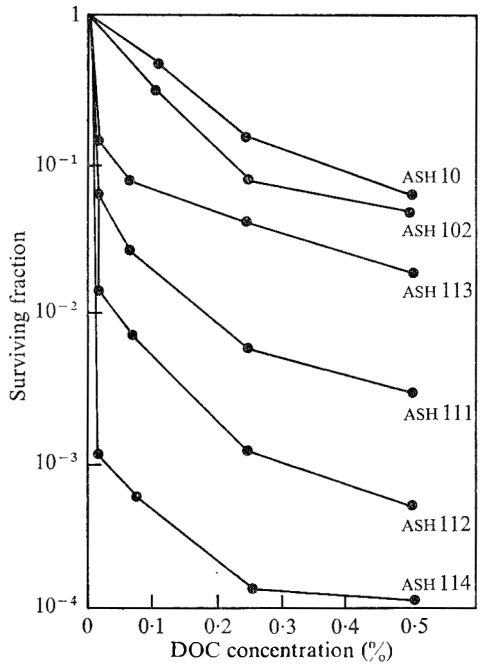

Fig. 6. Sensitivity of CetC mutants to sodium deoxycholate (DOC). Strains were grown to 5×10^8 bacteria/ml. in NB medium at 37°. The bacteria were then plated, after appropriate dilution, onto NB agar plates containing various concentrations of the detergent. Plates were incubated at 25° for 24 h. and the fraction surviving at each DOC concentration calculated. Similar results were obtained when plates were incubated at 37°.

labelled with H^3 thymine at 37° and colicin E2 was added to several different samples. At various times treated cultures were shifted to 30° and the effect on DNA breakdown determined at intervals. As shown in Fig. 7*b*, temperature shift, even at time zero, did not prevent E2-induced DNA breakdown. The observed reduction in the rate of breakdown is compatible with the reduced activity of a normal enyzme operating at 30° rather than at 37° and in no way indicates instability of a nuclease at low temperature. Moreover, cultures shifted to 30° for at least 20 min. before addition of E2 still showed considerable DNA breakdown upon subsequent addition of E2 (Fig. 7*a*). Similar results were obtained with the CetC mutant ASH 111. Neither of these strains therefore produce an E2-specific nuclease which is unstable at low temperature. The results do not, however, exclude the possibility that the mutants produce an altered

nuclease in the form of an aggregate, functioning at both 37° and 30°, which can be assembled at 37° but not at 30°.

The results shown in Fig. 7*a* also indicate a further possible difference between CetB and CetC mutants. Previous studies with CetB mutant ASH 101 (Holland, 1968) showed that this strain produced an altered cellular constituent which, after temperature shift from high to low temperature, immediately underwent a change which rendered the cells insensitive to the bacteriocidal effects of E2. In the present study the

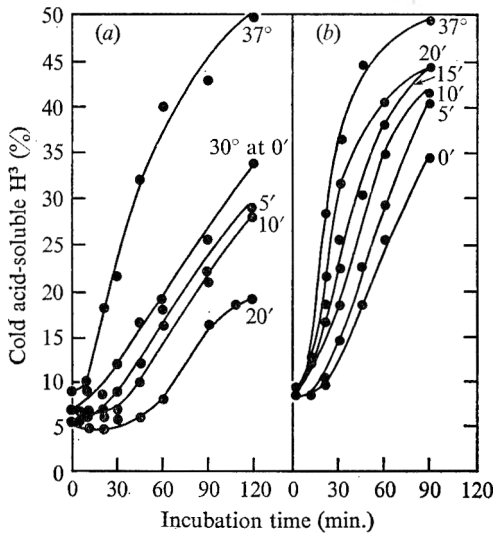

Fig. 7

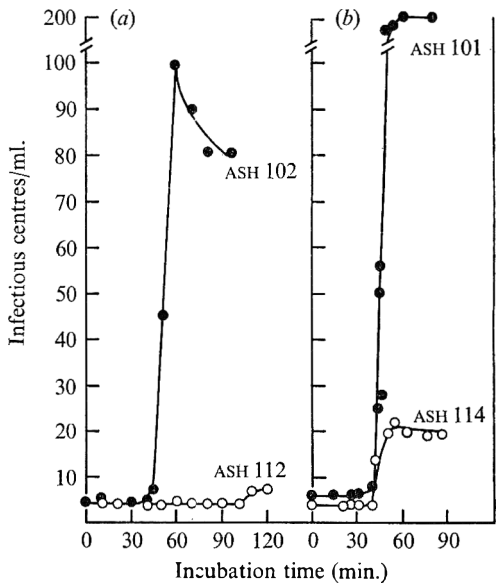

Fig. 8

Fig. 7. Effect of temperature shift on E2-induced DNA breakdown in CetC mutant ASH 113. Strain ASH 113 was labelled with H^3 thymine during growth at 37° as described in Fig. 2 and Methods. The labelled bacteria were washed, resuspended at 2×10^7 bacteria/ml. in NB medium and kept at 37° for 10 min. before use.

(*a*) At time zero E2 (50 units/ml.) was added to each of two cultures, one was incubated at 37° and the other immediately shifted to 30°. E2 was also added to other cultures after 5, 10 and 20 min. incubation at 30° respectively. Release of H^3 thymine as cold acid-soluble material was determined, as for Fig. 2, following temperature shift and the addition of colicin. In a control culture (not shown) growing at 30° throughout, addition of colicin E2 produced no increase in the release of soluble H^3 thymine over a 2 h. period.

(*b*) Colicin E2 (50 units/ml.) was added to several cultures of ASH 113 and one culture shifted to 30° at each of the times indicated by the curves. One culture was maintained at 37° throughout.

Fig. 8. Abortive growth of bacteriophage λ in some CetC mutants. The number of infectious centres obtained in one-step growth experiments is plotted against time for each phage–bacterial system. (*a*) Growth of *λgv* on the lysogenic strains ASH 102 (CetB) and ASH 112 (CetC). (*b*) Growth of λ on non-lysogenic strains ASH 101 (CetB) and ASH 114 (CetC).

bacteriocidal effects of E2 under conditions of temperature shift were not measured. Nevertheless, the results shown in Fig. 7*a* indicate that in this CetC mutant extensive growth at low temperature is required before E2-refractivity is expressed. The cold-sensitive lesion leading to E2 refractivity in CetB mutants and in at least some CetC mutants may therefore be different.

Increased resistance to bacteriophage λ amongst CetC mutants. Since the replication of λ and ØX174 DNA has been reported to take place at specific membrane sites (Knippers & Sinsheimer, 1968; Salivar & Sinsheimer, 1969), the sensitivity of various Cet mutants to phage λ was also determined. So far, out of 30 strains tested, three CetC mutants (Table 1) displayed increased resistance of some kind of λ or its virulent derivative *λgv*. This was reflected in the formation of minute plaques and reduced plating efficiencies. All three strains (ASH 112, 114 and 116) nevertheless appeared to adsorb λ with an efficiency equal to that of wild-type *cet*$^+$ strains. When one-step growth experiments were carried out with ASH 112 and ASH 114, infectious centres were formed at normal frequencies indicating that both adsorption and injection of λ DNA were normal under these conditions. As shown in Fig. 8*b*, however, the average burst-size for λ or *λgv* in ASH 114 was only 5. This compares with burst sizes of 30 to 35 in the CetB mutant ASH 101 or in Cet$^+$ parental strains. Growth of *λgv* in ASH 112 was found to be even more restricted with a maximum burst size of 1 to 2 phage particles per bacterium compared to 25 to 30 in the CetB control ASH 102. Liberation of mature phage was also greatly delayed in the ASH 112 host, but the low burst was not apparently due to lysis inhibition since premature disruption of infected cells with chloroform did not increase the yield. The reason for the restriction of λ multiplication in these CetC mutants is as yet unknown, but further experiments will be carried out to determine which particular step in the growth cycle is blocked.

Other properties of CetC mutants. At least two out of the seven CetC mutants so far studied show a further mutant phenotype, filament formation. These strains, ASH 110 and ASH 111, which both plate λ normally, produce, especially on solid nutrient agar media, large numbers of filaments up to 30 μm. in length, unlike all the other CetC mutants so far studied. With one exception (ASH 116) CetC mutants do not form mucoid colonies on minimal or nutrient agar. In these respects they are clearly distinct from u.v.-sensitive mutants of the Lon-type (Howard-Flanders, Simson & Theriot, 1964) in which u.v. sensitivity has been attributed to defective control of cellular polysaccharide synthesis (Walker & Pardee, 1967). CetC mutants may be further distinguished from Lon-type mutants by their recombination deficiency which is described in the accompanying paper (Threlfall & Holland, 1970).

Table 1 summarizes the main properties of CetC mutants described so far, but a further important characteristic of the mutants should be noted. CetC strains, in contrast to CetB mutants, grow more slowly than wild-type strains in both nutrient and minimal media, and in some cases give reduced (40 to 50 %) colony counts when plated on agar. These characteristics are probably a reflexion of surface defects which may be clearly seen by phase contrast microscopy, particularly with stationary phase cultures. Many free spheroplasts, organisms showing extrusion of almost free spheroplasts and even branched organisms are present. These characteristics have not been observed in CetB, Uvr$^-$, RecA, RecB and RecC mutants examined by us.

DISCUSSION

Adsorption of colicin E2 is normally followed by rapid degradation of DNA and the inhibition of division. Cet mutants, refractory to E2, have now been isolated from several *Escherichia coli* K 12 strains. These strains still adsorb E2 but remain completely refractory to its effect at 30° although the majority are largely sensitive at 37°. All Cet

mutants so far studied show normal sensitivity to colicin E1, bacteriophage BF23 and to colicin E3, a colicin which appears to utilize the same initial receptor as E2 (Maeda & Nomura, 1966; Hill & Holland, 1967). The mutants also appear unchanged in their sensitivity to colicin K and to bacteriophage T1 (Naysmith, 1967). Colicin E2 refractivity appears therefore to be associated with a highly specific surface or intracellular change rendering interaction with this colicin ineffective.

Mutants refractory to E2 can be divided into two phenotypic groups: those, CetB, which have acquired E2-refractivity only, and those, CetC, which are also u.v.- and DOC-sensitive. Most CetC mutants also appear to be recombination-deficient in some way (Holland & Threlfall, 1969; Threlfall & Holland, 1970). In addition, two CetC mutants show filament formation under normal growth conditions whilst three other mutants support only poor growth of bacteriophage λ. Despite these wide variations in phenotype, genetic studies have shown that *cet*B and *cet*C loci are very closely linked. Furthermore, all the additional characteristics associated with CetC refractivity appear to be the pleiotropic effects of a single gene closely linked to, if not identical with, the *cet*C locus, and not the result of several independent mutations (Threlfall & Holland, 1970). Despite the close linkage of *cet*B and *cet*C loci, the radical differences in the properties of these mutants indicate that at least two distinct cistrons may be involved, and complementation studies are being carried out to test this.

Since colicins appear to exert their effects at the level of the cytoplasmic membrane, some mutants refractory to their action might be expected to have altered membranes. Both the increased detergent sensitivity of the CetC mutants and the microscopic evidence of surface abnormality strongly suggest that the mutants do have altered surfaces. Moreover, preliminary experiments have shown that membrane preparations from strains ASH 111 and ASH 114 also have altered protein patterns on acrylamide gels when compared to wild-types (Samson & Holland, unpublished results).

The complex pleiotropy of CetC mutants may be best explained on the basis of either of the following two models. The first model assumes that the *cet*$^+$ gene constitutes part of an operon concerned with DNA metabolism and cell surface formation. Mutations in this gene might then arise having strong polar or pseudopolar effects upon adjacent genes (cf. Fink, 1966; Giles, Case, Partridge & Ahmed, 1967; Zipser, 1969). The second model supposes that the *cet*$^+$ gene determines the synthesis of a component of the cell membrane, malformation of which can directly or indirectly affect u.v. sensitivity, recombinant formation, cell division and, in some cases, growth of λ. Different mutations in the *cet*C gene would then give rise to the pleiotropic effects observed. Further genetic analysis of CetC mutants is now being carried out to test the validity of the first model. To test the second model, a qualitative study of the proteins present in membrane preparations from CetB and CetC mutants and their revertants is in progress. Further investigation of the molecular basis of the u.v. sensitivity of CetC mutants may also aid the understanding of CetC pleiotropy. Results obtained so far indicate that the u.v. sensitivity of these mutants differs from that of all Uvr, Rec and Lon mutants so far described. It is still not clear, however, whether CetC mutants are defective in repair of DNA or in some aspect of cell division which prevents normal recovery from u.v.irradiation.

The mode of action of colicin E2 and the properties of Cet mutants indicate that E2 normally interacts with a unique membrane site. This could be the part of the membrane which binds the DNA and which has been implicated in the cell division

process (Jacob, Brenner & Cuzin, 1963; Ryter, 1968). Comparison of the properties of this membrane fraction which can perhaps be isolated with its attendant DNA (see Tremblay, Daniels & Schaechter, 1969), with those of the remainder of the cell membrane in wild-type and in Cet mutants, should provide a crucial test of our hypothesis.

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Co-transduction with *ser*B of a Pleiotropic Mutation Affecting Colicin E2 Refractivity, Ultraviolet Sensitivity, Recombination Proficiency and Surface Properties of *Escherichia coli* K12

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SUMMARY

A *cet*B locus, determining E2 refractivity at low temperature, has been mapped at minute 89 on the *Escherichia coli* K12 chromosome and is 94 % linked to the *hsp* loci. A second locus, *cet*A, which controls expression of *cet*B in some way, was found to be 70 % co-transducible with and to the right of *thr*. Both *cet*B and *cet*C, another locus which determines E2 refractivity, are 21 % co-transducible with *ser*B and the *cet*C locus therefore also probably lies close to *hsp*. Mutations of the *cet*C locus appear to be pleiotropic and the additional characteristics of these mutants, u.v. sensitivity, detergent sensitivity, recombination deficiency, filament formation, and abortive λ growth, are all fully co-transducible with E2-refractivity. In addition, u.v.-resistant revertants of CetC mutants simultaneously revert to all wild-type characteristics including E2 sensitivity. The nature of the recombination deficiency of some CetC mutants has been examined and both episomal and chromosomal gene transfer to the mutants found to be normal. P1 transduction frequencies with CetC mutants are, however, also normal and it is suggested that the mutants may be deficient in some recombination step unique to Hfr × F^- crosses.

INTRODUCTION

Mutants of *Escherichia coli* which still adsorb colicin E2 but which are, nevertheless, refractory to its effects, particularly at low temperature, are designated Cet. These mutants may be divided into two phenotypic groups, those, CetC, which are u.v.-sensitive and show other pleiotropic effects and those, CetB, in which mutation to E2 refractivity is not pleiotropic. The additional characteristics of CetC mutants include reduced recombination proficiency, inhibition of the growth of bacteriophage λ and filament formation. The general properties of these mutants including evidence for altered cell surfaces have been described previously and in the accompanying paper (Holland, 1967, 1968; Holland, Threlfall, Holland, Darby & Samson, 1970). Previous genetic analysis of Cet mutants using interrupted mating techniques indicated that the *cet*B locus mapped 1–2 min. counter clockwise to *thr*. In addition it was also previously shown that the u.v.-sensitivity associated with the E2-refractivity of CetC mutant ASH 112 also mapped close to *thr* (Holland & Threlfall, 1969).

In this paper we describe further genetic analysis of CetC mutants and also present additional evidence for the recombination deficiency of some CetC mutants. By P1 transduction *cet*B and *cet*C loci have been mapped very close to the *hsp* loci (host

specificity, see Taylor & Trotter, 1967) at minute 89 on the *E. coli* K12 chromosome. Moreover in all cases tested so far, the various mutant characteristics of CetC strains were always found to be 100 % co-transducible with the E2-refractivity of the mutants.

METHODS

Organisms. CetC mutants ASH 111, 112, 113, 114, 115 and 116 and the CetB mutant ASH 102 have all been described in the preceding paper (Holland *et al.* 1970). ASH 5 is an F⁻ strain (*thi*⁻ *met*⁻ *arg*⁻ *thr*⁻ *leu*⁻ *gal*⁻ *trp*⁻ *his*⁻ *str*ʳ *tsx*ʳ); Hfr P10, obtained from Professor Hans Kornberg, transfers *arg*G and *str* as early markers in a clockwise direction; strain HfrH was that used previously (Holland & Threlfall, 1969); strain 4K (*ser*B⁻ *hsp*⁻ *thr*⁻ *leu*⁻ *str*ʳ) was kindly provided by Dr S. Glover. Strain 4K is defective in both restriction and modification processes. The F′ factor KLF-1 (Low, 1968) which covers the *ser*B-*leu* region of the chromosome was provided by Dr Brooks Low. In the studies described here *E. coli* C600 (*thr*⁻ *leu*⁻ *λ*⁻ *lac*⁻ *str*ˢ) was always used as F′ donor. The *rec*B mutant JC 4457 and its *rec*⁺ parent AB 1157 were kindly provided by Dr A. J. Clark. The male specific phage *μ* was obtained from Professor R. H. Pritchard and the virulent phage *λgv* was described previously (Holland, 1967).

The cultural conditions used, the source and assay of colicin E2 (P9) and colicin E3 (CA38) have all been described previously. Methods for linkage analysis of CetC mutants and plate tests for colicin sensitivity determination have also been described elsewhere (Holland & Threlfall, 1969).

Transduction studies with temperate phage P1. Lysates, which usually contained 10^{10}–10^{11} p.f.u./ml., of laboratory stocks of P1 assayed and used as described by Glover (1962). Recipient bacteria, grown in Oxoid tryptone broth (TB) to about 2×10^8 bacteria/ml., were resuspended in 1/5 volume of phage buffer (Glover, 1962) containing 10^{-3} M-$CaCl_2$ and an equal volume of phage was added (m.o.i. of 5 to 10 P1 particles per cell). Adsorption was completed by incubating 90 min at 25°, and the mixture was centrifuged and the pellet resuspended in 0·07 M-phosphate buffer pH 7·2 containing 0·5 % sodium citrate to prevent re-infection on the plate. Aliquots (0·1 ml.) were diluted where necessary and plated on selective plates also containing 0·5 % sodium citrate.

Selection of E2-refractory transductants. The P1 bacterial adsorption mixture described above was incubated for a further 90 min. in nutrient broth (Oxoid no. 2) at 37° before plating in order to allow expression of E2 refractivity in the transductants. Aliquots of the mixture were then plated on nutrient broth (NB) agar plates plus 5×10^3 units of colicin E2 and the plates incubated 24 h. at 25°. Refractory colonies were then replica-plated to plates containing E2 and to plates containing E3 (5×10^3 units in total) and incubated at both 25° and 37° to confirm the E2 refractivity phenotype. Scoring for auxotrophic markers amongst refractory transductants was completed by replica plating from pattern grids of the original *cet*B or *cet*C colonies. In reciprocal crosses *ser*B transductants were replica plated directly to colicin lawns on NB plates to determine the proportion of the unselected *cet* marker. For final confirmation of the presence of the *cet* allele, transductants were picked, purified and tested for E2 and E3 sensitivity in cross-streak tests against each colicin at both 37° and 25°, as described previously (Hill & Holland, 1967).

Scoring for hsp⁺. Cell suspensions of test strains were spotted, in duplicate, on NB

agar plates; the dried spots were half overlayered with a loopful of phage λgv (10^4 p.f.u./ml) previously grown in the *hsp*$^-$ host 4K. After 14 h. at 37°, *hsp*$^-$ mutants showed a clear zone of lysis whilst *hsp*$^+$ strains showed confluent growth.

Scoring for λ '*resistance*'. Test strains were spotted on NB agar as before and half overlayered with λgv (10^8 pfu/ml.) previously grown on a wild-type host. After 14 h. at 37°, wild-type strains showed an area of lysis whilst resistant mutants like ASH 112 showed confluent growth.

Scoring for u.v.-sensitive transductants. The u.v. source was a low pressure mercury lamp (Hanovia Ltd) with an incident dose rate at room temperature of 6·5 ergs/mm.^{2}/sec. at 46 cm. Transductants to be tested were grown to late exponential phase in NB medium, diluted to about 2×10^7 bacteria/ml. and streaked in duplicate on tryptone broth plates. A stepwise irradiation of successive portions of the inoculated streaks was then carried out; different sections of the streaks received, 60, 40, 20, 15, 10, 5 and 0 sec. of u.v. The plates were incubated in the dark for 14 h. at 37°; sensitive strains were usually inhibited by doses of 5 to 15 sec. whilst wild-type strains were resistant to 40 sec. u.v.

Scoring for detergent sensitivity. Transductants to be tested were grown to late exponential phase (10^9 cells/ml.) in NB medium and streaked in duplicate on each of five NB agar plates containing 2·5, 1·0, 0·5 0·1 and 0 % sodium deoxycholate (DOC) respectively. Wild-type *cet*$^+$ strains were resistant to 1 % or higher whilst cetC mutants were completely inhibited by 0·5 % DOC.

Isolation of u.v.-resistant revertants. The procedure used was adapted from that described by Ganesan & Smith (1968). Cultures of CetC mutants were grown to stationary phase in TB medium, diluted to 10^7 bacteria/ml. and 0·1 ml. spread on TB agar plates; the plates were exposed to a u.v. dose of 130 ergs/mm.^{2} and incubated in the dark at 37° for 14 h. The plates containing approximately 100 colonies were replica-plated to duplicate TB plates and immediately exposed to a u.v. dose of 520 ergs. After incubation at 37°, colonies which appeared u.v.-resistant were picked from the original plate, purified and u.v.-resistance confirmed by the plate test described above. Only revertants obtained from different cultures of the original CetC mutants were considered to be of independent origin.

F' transfer. F' and F$^-$ strains were grown to 5×10^7 and 5×10^8 bacteria/ml. respectively in M9 minimal medium (Anderson, 1946) supplemented with required amino acids. Equal volumes were then mixed and shaken slowly at 37°. After 15 or 30 min., streptomycin (100 μg./ml.) was added to kill the male cells and to stop further transfer. The cultures were immediately diluted and plated on appropriate media for the selection of prototrophic heterozygotes. To confirm the F status of heterozygotes, cultures were spotted on NB agar plates, half over-layered with RNA phage μ (about 10^{10} p.f.u./ml. and incubated at 37° for 8 h.; F$^+$ strains showed a half-moon zone of inhibited growth whereas F$^-$ strains showed confluent growth.

Determination of β*-galactosidase synthesis.* Induction of β-galactosidase synthesis by isopropyl-thio-β-D-galactoside (IPTG) and assay of the enzyme were carried out as described by Pardee, Jacob & Monod (1959). The enzyme substrate was *O*-nitrophenol-β-D-galactoside (ONPG) and enzyme activities in toluene extracts of cells were calculated as μmoles of *O*-nitrophenol released/min./ml. of cells suspension. (IPTG and ONPG were obtained from the Sigma Chemical Co., St Louis, Missouri, U.S.A.)

RESULTS

A second gene required for expression of the Cet phenotype

Previous reports (Hill & Holland, 1967; Holland & Threlfall, 1969) indicated that only certain K12 strains are capable of giving rise to Cet mutants after 2-amino purine treatment. These same strains have now been found to be transducible for the Cet character whilst other strains are not. In the course of genetic analysis of various CetB mutants it was observed that a gene potentiating the expression of the *cet*B locus segregated with *thr* in genetic crosses. Mapping studies with transducing phage P1 have now shown that this gene, tentatively designated *cet*A, is 70 % linked to *thr* and probably lies to the right of *thr* (Table 1). We have quite arbitrarily designated those strains which can potentiate the expression of E2 refractivity arising from mutations at the *cet*B locus as mutant, i.e. phenotype CetA, whilst other strains are wild-type. Whether this assignment is correct must await clarification of the role of the *cet*A locus in determining E2 refractivity. We have not yet determined whether the *cet*A locus influences the expression of mutations at the *cet*C locus. The Cet status of various wild-type and mutant strains is summarized in Table 2.

Table 1. *Mapping of the cetA locus by P1-transduction*

P1 donor	Recipient	Selected marker	No. tested	Unselected marker % *leu*⁻	Unselected marker % *cet*A	Possible transductant classes	No. obtained	%
HfrH (*cet*A)	4K (*cet*⁺)	*thr*⁺	60	6·5	70	*thr*⁺ *leu*⁺ *cet*A	4	6·7
						thr⁺ *leu*⁺ *cet*⁺	0	0
						thr⁺ *leu*⁻ *cet*⁺	18	30
						thr⁺ *leu*⁻ *cet*A	38	63

Probable gene order and linkage *thr*——*cet*A———*leu*
← 70 % →
←—— 6·5 % ——→

The *thr*⁺ transductants were replica plated to minimal agar to determine the proportion of the *leu*⁺ marker inherited. To determine inheritance of *cet*A *thr*⁺, transductants were picked, purified and exponential cultures of each clone crossed with a P1 donor grown on the *cet*B strain ASH 102. The proportion of the original *thr*⁺ transductants able to receive and express the *cet*B marker was taken as a measure of the frequency of *thr*⁺ *cet*A transductants.

Table 2. *Cet status of various Escherichia coli K12 strains and refractory mutants*

Strain	CetA	CetB	CetC
HfrH	−	+	+
ASH 5	−	+	+
4K	+	+	+
ASH 6	−	+	+
ASH 10	−	+	+
ASH 102	−	−	+
ASH 111	−	+	−
ASH 112	−	+	−
ASH 113	−	+	−
ASH 114	−	+	−
ASH 115	−	+	−
ASH 116	−	+	−

Wild-types are denoted + and mutant phenotypes as −.

Linkage analysis of CetC mutants

Previous analysis of 21 CetB mutants derived from the parental F⁻ strain ASH 10 (Holland & Threlfall, 1969) demonstrated that E2 refractivity in all mutants mapped to the left of *thr* and *leu* and was closely linked to this region. As described in Table 3, linkage analysis of several CetC mutants derived from ASH 10, in crosses with an HfrH male, showed that E2 refractivity in all these mutants was also linked to *leu* and probably to the left of this locus.

Table 3. *Linkage analysis of CetC mutants*

	Recombinants			
CetC recipient	Selected marker	Input male (%)	No. tested	E2-sensitive (*cet*⁺) (%)
ASH 112	*leu*⁺ *str*ʳ	0·4	72	73
	*ton*ʳ *str*ʳ	0·12	24	59
	lac⁺ *str*ʳ	0·09	96	32
ASH 113	*leu*⁺ *str*ʳ	0·005	46	68
	*ton*ʳ *str*ʳ	—	—	—
	lac⁺ *str*ʳ	0·002	21	30
ASH 115	*leu*⁺ *str*ʳ	2·7	112	78
	*ton*ʳ *str*ʳ	1·6	25	51
	lac⁺ *str*ʳ	0·8	165	34
ASH 116	*leu*⁺ *str*ʳ	3·0	90	70
	*ton*ʳ *str*ʳ	1·3	17	53
	lac⁺ *str*ʳ	0·8	28	40

				Proportion of unselected marker (%)		
				*ton*ʳ	*leu*⁺	*cet*⁺
ASH 102	*lac*⁺ *str*ʳ	—	103	85	75	59
ASH 112	*lac*⁺ *str*ʳ	—	202	90	81	26
ASH 113	*lac*⁺ *str*ʳ	—	83	79	64	53
ASH 115	*lac*⁺ *str*ʳ	—	84	—	76	44

An HfrH strain (*cet*⁺ *ton*ʳ *λ*⁻ *str*ˢ) was crossed with various CetC derivatives of ASH 10. Exponential cultures (10^8 cells/ml.) of each strain were mixed and incubated 90 min. at 37°; selection was then made for appropriate recombinant classes, and subsequently the proportion of unselected markers including E2-sensitivity (*cet*⁺) amongst the recombinants was determined by replica plating.

Analysis of CetB and CetC mutants by P1 transduction

For transduction mapping of CetB and CetC mutations, *Escherichia coli* strain 4K was used as recipient. This strain has convenient markers to the left of *leu* in the order *hsp*⁻ *ser*B⁻ *thr*⁻ *leu*⁻ (Glover & Colson, 1969; S. W. Glover, personal communication). Before strain 4K could be used, however, it was first necessary to introduce the *cet*A allele into this strain. This was achieved by crossing strain 4K with a P1 lysate of HfrH (*thr*⁺ *cet*A) and selecting a *thr*⁺ transductant which also carried the *cet*A allele. The new derivative of strain 4K obtained in this way was designated ASH 6.

Bacteriophage P1 was grown on strain ASH 102 (CetB) and the lysate used to infect cultures of ASH 6. E2-refractory transductants were selected directly by plating infected cultures on E2 lawns incubated at 25°. After confirming the E2 refractivity of

the transductants by replica plating to plates containing colicin E2 or E3 at both 25° and 40°, the inheritance of the unselected marker *ser*B$^+$ was determined. The results obtained (Table 4) showed *cet*B and *ser*B to be 21 % co-transducible. When the same P1 donor was used, but with *ser*B$^+$ as the selected marker, *cet*B was found to be 22 % linked to *ser*B. Furthermore, when P1 grown on the CetC mutant ASH 112 was used, 21 % of ASH 6 *ser*B$^+$ transductants also inherited the *cet*C locus (Table 4). Since previous studies (Holland, & Threlfall, 1969) indicated that both *cet*B and *cet*C loci were 1 to 2 minutes to the left of *thr*, it appeared probable that the *cet* loci were also to the left of *ser*B and close to *hsp*. To confirm the relative order of the *cet* and *ser*B loci, three factor crosses were carried out with primary selection for either *ser*B$^+$ or E2 refractivity and using the *hsp* locus as outside marker. This experiment has so far only been carried out with P1 grown on the CetB mutant ASH 102. The increased λ resistance of the CetC mutant ASH 112 made scoring for inheritance of the phenotypically similar *hsp*$^+$ marker impossible. The results obtained (Table 5) showed an anticipated 19 to 20 % linkage of *ser*B$^+$ and *cet*B$^+$ and also showed *cet*B to be 93 to 94 % linked to

Table 4. *Co-transduction of cetB and cetC with serB$^+$*

	P1 donor	Recipient	Selected marker	Transductants per 10^6 P1	No. scored	Unselected marker: *thr*$^+$	*leu*$^+$	*ser*B$^+$
Expt. 1	HfrH (*cet*$^+$)	4K	*ser*B$^+$	9·8	200	44	0·5	—
			thr$^+$	6·8	200	—	6·5	47
			leu$^+$	1·8	41	6	—	0
						*ser*B$^+$	*cet*B	*cet*C
Expt. 2	ASH 102 (*cet*B)	ASH 6	*ser*B$^+$	1·7	150	100	22	—
			*cet*B$^+$	1·8	150	21	100	—
Expt. 3	ASH 112 (*cet*C)	ASH 6	*ser*B$^+$	4·4	435	100	—	21

Transduction procedures and analysis of unselected markers as described in Methods and in Table 2. ASH 6 is a *thr*$^+$ *cet*A transductant of strain 4K obtained from a cross between P1 HfrH and 4K (*thr*$^-$ *cet*$^+$).

Table 5. *Analysis of cetB linkage to hsp$^+$ and serB$^+$ by P1 transduction*

P1 donor	Recipient	Selected marker	Transductants per 10^6 P1	No. scored	Unselected marker (%): *ser*B$^+$	*cet*B	*hsp*$^+$
ASH 102	ASH 6	*ser*B$^+$	3·2	250	100	21	18·5
		*cet*B	2·1	100	—	100	93

Linkage and possible gene order *hsp*——*cet*B——*ser*B
←— 93 —→←—21—→
←———— 18·5 ————→

Procedures as described in Tables 2 and 3.

the *hsp* loci. The *hsp*$^+$ *ser*B$^+$ linkage was, however, only 17 to 19 %, and these results clearly established the gene order as *cet*B *ser*B *thr leu* with *cet*B and *hsp* extremely closely linked. Examination of the minority classes of transductants obtained in the crosses shown in Table 6 unfortunately still leaves the relative order of *cet*B and *hsp* ambiguous. Selection for *ser*B$^+$, with scoring for the unselected markers *hsp*$^+$ and *cet*B,

Table 6. *Analysis of three-factor crosses involving hsp, serB and cetB*

	P1 donor	Recipient	Selected marker	Transductants per 10^6 P1	No. scored	Unselected marker (%) *ser*B$^+$	*cet*B	*hsp*$^+$	*leu*$^+$	Possible transductant classes			No. obtained	%
Expt. 1.	ASH 102	ASH 6	*ser*B$^+$	0·7	166	—	18·5	17·5	0	*ser*B$^+$	*hsp*$^-$	*cet*$^+$	134	80·7
										*ser*B$^+$	*hsp*$^-$	*cet*B	3	1·8
										*ser*B$^+$	*hsp*$^+$	*cet*B	28	16·8
										*ser*B$^+$	*hsp*$^+$	*cet*$^+$	1	0·6
								Indicated gene order		*hsp*$^+$—*cet*B—*ser*B$^+$				
										←—18·5—→				
										←—17·5—→				
Expt. 2	ASH 102	ASH 6	*cet*B	3·4	160	19	—	94	0	*ser*B$^+$	*hsp*$^+$	*cet*B	30	19
										*ser*B$^+$	*hsp*$^-$	*cet*B	1	0·7
										*ser*B$^-$	*hsp*$^-$	*cet*B	8	5·0
										*ser*B$^-$	*hsp*$^+$	*cet*B	121	76·5
								Indicated gene order		*cet*B—*hsp*$^+$—*ser*B$^+$				
										←—94—→	←— 19 —→			

Procedures as described in Tables 2 and 3.

Table 7. *Co-transduction with cetC of additional phenotypic characters of CetC mutants*

	P1 donor	Recipient	Selected marker	Transductants per 10^6 P1	No. scored	No. of transductants tested and scored as: E2 refractory (*cet*C)	*doc*^{s}	*uv*^{s}	*rec*$^{-(a)}$	*fil*$^-$	λ
Expt. 1	ASH 112	ASH 6	*cet*C	0·3	19	19	19	19	19	—	—
	ASH 113	ASH 6	*cet*C	0·4	38	38	12/12$^{(b)}$	12/12	38	—	—
Expt. 2	ASH 112	ASH 6	*ser*B$^+$	4·4	435	103	103	103	—	—	—
Expt. 3	ASH 112	ASH 5	*cet*C	3·2	14	14	14	14	1/1$^{(b)}$	—	14
	ASH 113	ASH 5	*cet*C	1·9	4	4	4	4	4	—	—
	ASH 114	ASH 5	*cet*C	2·1	14	14	14	14	1/1	—	14
	ASH 111	ASH 5	*cet*C	2·3	14	14	14	14	1/1	14$^{(c)}$	—

Transduction procedures as described in Tables 2 and 3. Scoring for unselected markers as described in Methods.

(a) To determine recombination deficiencies purified clones were crossed with HfrH (Expt. 1) and the deficiency of *leu*$^+$ *str*^{r} recombinants determined, or with Hfr P10 (Expt. 3) when deficiency of *arg*$^+$ *str*^{r} recombinants was measured.

(b) This indicates that 12 out of 12, or 1 out of 1, transductants tested displayed the mutant phenotype.

(c) On initial isolation all showed filament formation, after nutrient broth subculture only three were still found to be filament-forming.

indicated a gene order *hsp cet*B *ser*B. When selection was made for *cet*B the frequencies of unselected markers indicated an order *cet*B *hsp ser*B. Further crosses, with examination of considerably more transductants will be required before the final order can be established.

Genetic analysis of other phenotypic characters of CetC mutants

In a previous study (Holland & Threlfall, 1969) the mutation to u.v. sensitivity in the CetC mutant ASH 112 was also mapped close to the *cet* locus. This indicated that u.v.-sensitivity may be a pleiotropic effect of the CetC mutation in this strain and not the expression of an independent gene.

To establish whether u.v. sensitivity in this and in other CetC mutants was co-transducible with E2 refractivity, bacteriophage P1 was first grown on ASH 112 and ASH 113, and the lysates obtained were used to infect the recipient strain ASH 6. E2-refractive transductants were selected directly and, after purification and confirmation of the Cet phenotype, were scored for u.v. sensitivity. Since the donor strains ASH 112 and 113 are detergent-sensitive and recombination deficient, these characters were also scored amongst CetC transductants. The results presented in Table 7 show that all three unselected markers, u.v. and detergent sensitivity and recombination deficiency, for both donor strains were 100 % co-transducible with E2 refractivity. With ASH 112 as donor, primary selection was also made for *ser*B$^+$ and of 103 *ser*B$^+$, *cet*C transductants obtained all were also u.v.- and detergent-sensitive.

Other phenotypes associated with CetC mutants include filament formation (ASH 111) and increased resistance to bacteriophage λ (ASH 112 and ASH 114). The results for the transduction of these markers, shown in Table 7, demonstrate that they also are fully co-transducible with *cet*C. In the recipient strain ASH 5 used in these studies, the *fil* character was, however, unstable and upon subsequent subculture of the majority of the transductants, filament formation was lost although the other mutant characteristics of the ASH 111 donor were retained. The transduction studies with the male CetC mutants ASH 111 and 114 also revealed that these strains carry a *rec* marker. This is more clearly illustrated in Table 10.

The results described above give a strong indication that the E2 refractivity and u.v. and detergent sensitivity of CetC mutants derives from a mutational event at a single gene, the *cet*C locus. These studies also indicate that the recombination deficiency, filament formation and increased λ resistance of the mutants are determined by one or more loci closely linked to, if not identical with, the *cet*C locus (Fig. 1).

Isolation of u.v.-resistant revertants of ASH 112 and ASH 113

To further clarify the relationship between E2 refractivity and the other phenotypic changes characteristic of CetC mutants, u.v.-resistant revertants of ASH 112 and ASH 113 were selected by the method described by Ganesan & Smith (1968). Six revertants isolated from each of the two strains have now been studied. All were also sensitive to colicin E2 and resistant to the detergent DOC. Moreover, when two revertants from each group were tested for growth characteristics, all showed normal growth rates in liquid culture, in contrast to the reduced growth rates of the CetC parental strains. Similarly, as shown in Table 8, the revertants tested for recombination proficiency all proved to be much more fertile than the parental mutant strains. One revertant,

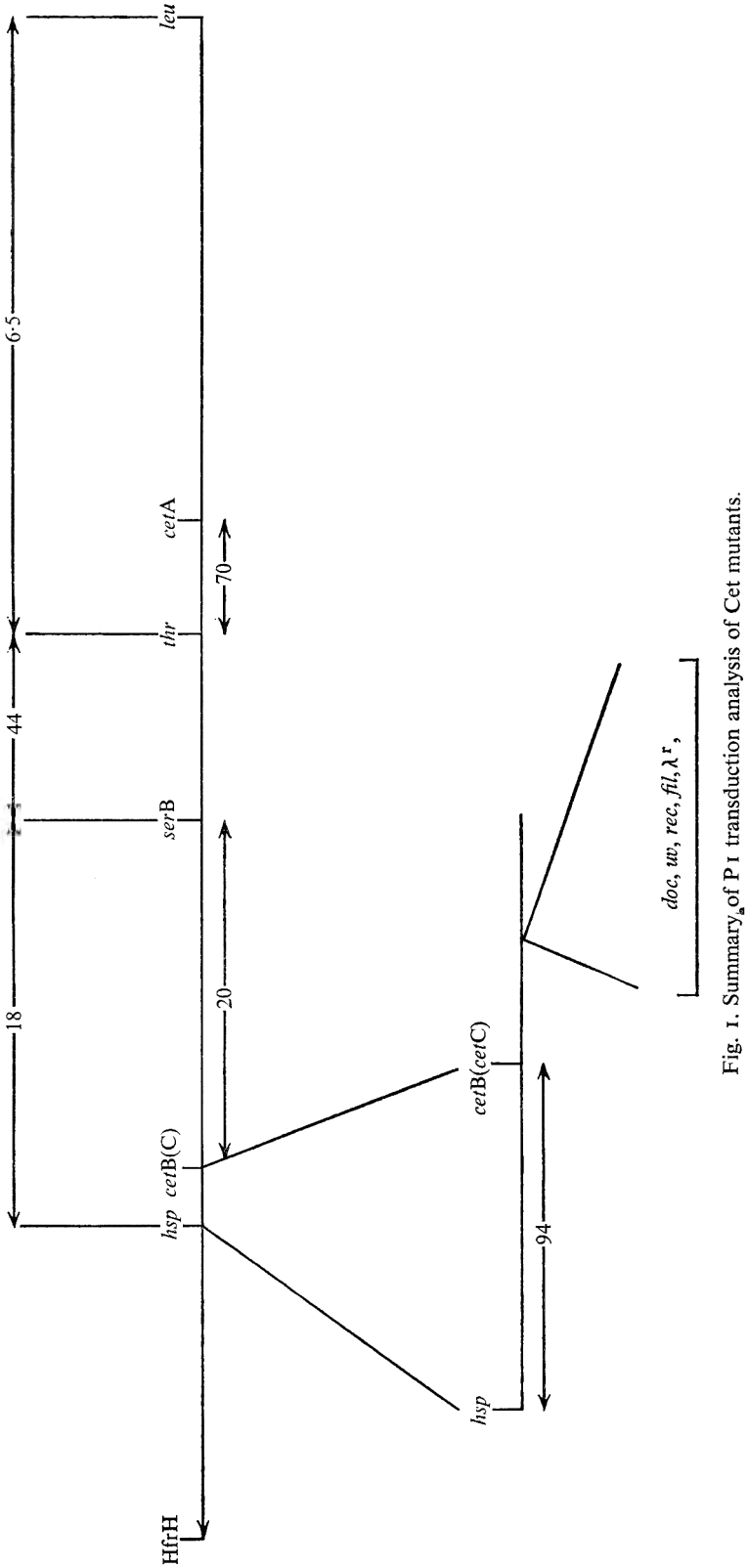

Fig. 1. Summary of P1 transduction analysis of Cet mutants.

ASH 13-R2 although not as recombination-proficient as wild-type *cet*$^+$ strains, nevertheless, yielded ten times more recombinants than the original strain ASH 113.

A P1 transduction analysis of revertant strain ASH 112-R1 was carried out to determine whether the *cet*C allele was still present but suppressed in some way. Table 9 shows the results of crosses between P1 donors, ASH 112 and ASH 112-R1 and the recipient strain ASH 6. Selection was made for *ser*B$^+$ and the transductants scored for inheritance of the *cet*C marker. With the mutant strain as donor, 20% of *ser*B$^+$ transductants were also E2 refractory. In contrast, with the revertant strain as P1 donor none of the 320 *ser*B$^+$ transductants tested were refractory to E2. The site of reversion to u.v. resistance (and to E2 sensitivity) in ASH 112-R1 must therefore reside within the *cet*C locus or be very closely linked to it. Thus the properties of the u.v.-resistant revertants so far studied further support the hypothesis that u.v. sensitivity and the other characteristics of CetC mutants, including E2 refractivity, are the pleiotropic expression of a single gene.

Table 8. *Recombination proficiency of some CetC mutants and their u.v.-resistant revertants*

Donor	Recipient	Recombinants % input male *lac*$^+$ *str*^{r}	Recombination deficiency
HfrH	ASH 10 (*cet*$^+$)	1·5	—
HfrH	ASH 112 (*cet*C)	0·18	8
HfrH	ASH 113 (*cet*C)	0·025	60
HfrH	ASH 112-R1	1·7	—
HfrH	ASH 113-R1	1·0	1·7
HfrH	ASH 113-R2	0·25	6·8

Mating conditions as described in Table 1.
Recombination deficiency index is calculated from ratio of recombinants formed in control cross to that obtained with CetC recipient strain. ASH 112-R1 is a u.v.-resistant revertant of ASH 112, ASH 113-R1 and 113-R2 are u.v.-resistant revertants of ASH 113.
The method of isolation of revertants is described in Methods.

Table 9. *Linkage of serB$^+$ and cetC loci in ASH 112 and ASH 112-R1*

P1 donor	Recipient	Transductants per 10^6 P1	Selected marker	No. scored	E2-refractive transductants (%)
ASH 112 (*cet*C)	ASH 6	4·5	*ser*B$^+$	430	21
ASH 112-R1	ASH 6	5	*ser*B$^+$	320	0

Transduction procedure as described in Tables 2 and 3.
ASH 112-R1 is a u.v.-resistant revertant of ASH 112 (see text).

The nature of the recombination deficiency of CetC mutants

Recombination frequencies obtained in crosses with Hfr males

Characteristic feature of some CetC mutants is the formation of reduced numbers of recombinants in crosses with male strains (Table 10). With Hfr P10, a male strain transferring the *cet*$^+$ locus late, recombination deficiencies of 100 and 400 were obtained with various CetC mutants. Similar results have been obtained previously with RecB mutants (Emmerson, 1968; Willetts & Clark, 1969). Unlike Rec mutants,

however, ASH 113 still showed a large recombination deficiency in crosses with a male strain (HfrH) which transfers the *cet*$^+$ locus early. Nevertheless, as shown below, *cet*C is recessive to *cet*$^+$. These results therefore indicate that expression of the dominant *cet*$^+$ allele in the F$^-$ recipient is delayed and that this delay is sufficient to allow an abortive recombination event to occur.

Table 10. *Recombination deficiency of CetC strains and their derivatives in crosses with Hfr strains*

	Donor	Recipient	Recombinants as % of input male			Recombination deficiency index
			leu$^+$ *str*^{r}	*ton*$^+$ *str*^{r}	*lac*$^+$ *str*^{r}	
Expt. 1	HfrH	ASH 10 (*cet*$^+$)	2·2	1·9	0·7	—
	HfrH	ASH 112 (*cet*C)	0·33	0·17	0·09	7
	HfrH	ASH 113 (*cet*C)	0·005	—	0·002	440
	HfrH	ASH 102 (*cet*B)	2·3	1·7	0·6	—
			arg$^+$ *str*^{r}	*his*$^+$ *str*^{r}	*leu*$^+$ *str*^{r}	
Expt. 2	HfrP10	ASH 5 (*cet*$^+$)	1·3	0·5	0·04	—
	HfrP10	ASH 5-112 (*cet*C)	0·007	0·0014	0·0005	180
	HfrP10	ASH 5-113 (*cet*C)	0·003	0·0007	0·0001	430
	HfrP10	ASH 5-111 (*cet*C)	0·012	0·002	0·0007	110
	HfrP10	ASH 5-114 (*cet*C)	0·006	0·004	0·0003	220

Mating conditions as described in Table 1.

The recombination deficiency index was determined from the frequency of *leu*$^+$ (HfrH) or *arg*$^+$ (HfrP10) recombinants obtained in each cross relative to that obtained with the parental strains ASH 10 or ASH 5. The *cet*C derivatives of ASH 5 were obtained by crossing P1-*cet*C donors ASH 112, 113, 111 and 114 with ASH 5 and selection made for E2 refractory transductants as described in Methods.

Table 11. *Episome transfer to CetC mutants*

Donor episome	Recipient	Frequency of transfer (% input male)
F′ *lac*$^+$	ASH 10 (*cet*$^+$)	1·6
	ASH 102 (*cet*B)	2·2
	ASH 112 (*cet*C)	2·1
	ASH 113 (*cet*C)	1·0
	ASH 115 (*cet*C)	1·9
	ASH 116 (*cet*C)	0·06
KLF-1	ASH 102 (*cet*B)	3·7
F′ *ser*B$^+$-*leu*$^+$	ASH 112 (*cet*C)	1·8
	ASH 115 (*cet*C)	2·4
	ASH 116 (*cet*C)	0·1

Cultures of F′ and F$^-$ strains grown in M9 supplemented medium to 5×10^7 and 5×10^8 cells/ml. respectively; cultures mixed and shaken slowly for 15 min. before plating to determine frequency of episome transfer. Episome donor strain in each case was C 600 (*thr*$^-$ *leu*$^-$ *lac*$^-$ *str*^{s}).

Efficiency of gene transfer to CetC mutants

Since recombination deficiency in F$^-$ recipient strains could result from incorrect pairing with the male donor, it was necessary to determine whether gene transfer to CetC mutants was normal or otherwise. The efficency of episome transfer was first tested and, as shown in Table 11, the transfer of F′*lac*$^+$ or episome KLF-1 to most CetC mutants, including the recombination deficient strains ASH 112 and 113, was

almost normal. Conjugal pairing with male strains appears therefore to be unimpaired for the majority of CetC mutants. Although not previously found to be deficient in recombination with HfrH, one strain, ASH 116, did show a 25-fold reduction in transfer of either episome. The reason for this is not clear, and the properties of this strain will require further study.

A direct measurement of chromosomal gene transfer from Hfr males to CetC mutants was also carried out by measurement of β-galactosidase synthesis in merozygotes. CetC mutants ASH 112 and 113 are both *lac*$^-$ (*z*$^-$) and make insignificant levels

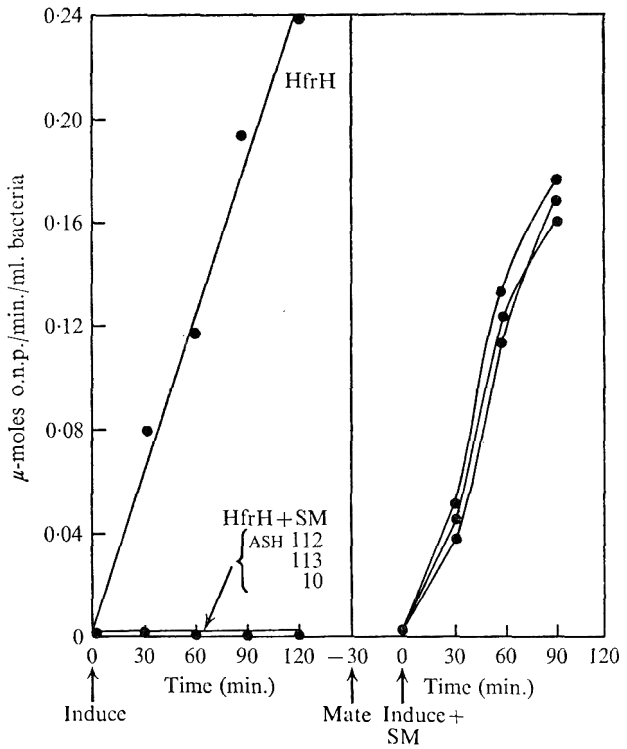

Fig. 2. β-Galactosidase formation in merozygotes. (*a*) Male (HfrH) and female strains ASH 112, 113 and ASH 10 were grown in NB media at 37° to 2×10^8 and 4×10^9 cells/ml. respectively. At time 0 β-galactosidase synthesis was induced by the addition of IPTG (0·25 mM) and samples removed at intervals and enzyme content determined as described in Methods. In the case of strain HfrH (*lac*$^+$), induction was carried out either in the absence or presence of streptomycin (100 μg./ml.) added at −10 min. (*b*) Cultures of male and female strains at 4×10^8 and 2×10^7 cells/ml. respectively were mixed and shaken slowly at 37° for 30 min. Streptomycin (SM) was then added and the culture blended vigorously for 60 sec. to terminate mating. The cultures were then centrifuged and resuspended in 1/20 volume. Inducer IPTG was then added and samples removed at intervals to determine enzyme content as before.

of β-galactosidase in the presence of lactose. As shown in Fig. 2, however, the capacity for β-galactosidase synthesis by these mutants, after 30 min. mating with an HfrH (*lac*$^+$) male, was identical to that of *cet*$^+$ females in similar crosses. Thus the transfer of chromosomal *lac*$^+$ genes by a male donor to both ASH 112 and 113 is quite normal. The results shown in Fig. 2 also make it unlikely that transferred DNA is rapidly

destroyed in the recombination deficient mutants since β-galactosidase synthesis appears to continue at the normal rate for at least 90 min. These strains thus appear to be recombination-deficient as a result of a defect in the molecular mechanism of recombination, including chromosomal pairing, or through the failure of potential recombinants to grow, i.e. as a result of a lethal zygosis of some kind.

Formation of transductants by CetC mutants

Luria & Hertman (1967) showed that a RecA mutant formed transductants at the same low frequency ($< 10^{-3}$) as for recombinant formation after conjugation. RecB strains, in contrast, appear to yield transductants at frequencies as high as 5 to 25 % of wild-type *rec*$^+$ strains (Emmerson & Howard-Flanders, 1967; A. J. Clark, personal communication). The formation of *leu*$^+$, *met*$^+$ and *thy*$^+$ transductants in the CetC mutants ASH 112 and 113 occurs with almost the same frequency as that obtained with *cet*$^+$ strains (Table 12). The RecB mutant JC 4457 included as a control in this experiment gave an anticipated four- to seven-fold reduction in transduction formation. This result, together with the physiological evidence presented in the preceding paper (Holland *et al.* 1970), clearly indicates that the basis of the recombination deficiency of CetC mutants in crosses with Hfr males is quite different from that found in either RecA and RecB mutants.

Table 12. *Transduction frequencies obtained with CetC mutants*

		Transductants per 10^6 P1 particles			
P1 donor	Recipient	*thy*$^+$	*leu*$^+$	*his*$^+$	*arg*$^+$
HfrH	ASH 102 (*cet*B)	6·2	1·8	—	—
HfrH	ASH 112 (*cet*C)	4·9	1·2	—	—
HfrH	ASH 113 (*cet*C)	6·0	1·0	—	—
HfrH	AB 1157 (*rec*$^+$)	—	2·1	5·5	4·4
HfrH	JC 4457 (*rec*B$^-$)	—	0·5	0·8	0·7

Transduction procedure as described in Tables 1 and 2.

Properties of strains heterozygous for CetB or CetC

The F′ factor KLF 1 (Low, 1968) includes the region containing *cet*B and *cet*C and probably also the *cet*A locus, since the factor spans the region from the origin of HfrH to a point beyond *leu*. F′ KLF-1 was originally derived from HfrH, a strain which, using the criteria indicated above, we have designated *cet*A, and is it assumed that the KLF 1 factor also carries the mutant allele *cet*A rather than the wild-type *cet*$^+$ allele.

Transfer of the KLF 1 factor to either ASH 102 or ASH 112 was readily achieved by selection for *leu*$^+$ *str*^{s} exconjugants in crosses between these strains and strain C600 carrying the F′ factor. The presence of the episome in the heterozygotes was confirmed by tests for sensitivity to the male specific phage μ and by secondary transfer of the F′ factor to other recipients. When tested for colicin sensitivity, both *cet*B and *cet*C heterozygotes proved to be sensitive to E2. Thus the chromosomally located *cet*B or *cet*C alleles are both recessive to the *cet*$^+$ allele on the F′ factor.

DISCUSSION

Cet mutants are readily divisible into two major phenotypic groups; those which are u.v.-sensitive and those which are not. A *cet*B locus has now been mapped close to the *hsp* loci at minute 89 on the *Escherichia coli* chromosome. Like *cet*B, *cet*C is 21 % linked to *ser*B and therefore this locus also probably lies close to *hsp*. It is not yet clear whether *cet*B and *cet*C loci are in fact distinct cistrons, but complementation studies are in progress which should clarify this point. In view of the extremely close linkage between *cet*C and *hsp*, a locus controlling chemical modification and degradation of DNA (Arber, 1968; Meselson & Yuan, 1968) the response of *hsp*$^-$ mutants to E2 has also been tested. Such mutants are not refractory to E2 (Holland, 1967) and, moreover, E2-induced DNA breakdown proceeds normally (unpublished results). Thus the *cet* and *hsp* loci are quite distinct although the frequency of co-transduction indicates, according to the formula of Wu (1966), that these genes are only 0·04 minutes or 3 to 4 genes apart.

The results presented here further support in at least three ways the hypothesis (Holland, 1967) that mutations at the *cet*C locus are pleiotropic: (i) all the mutant phenotypes exhibited by CetC mutants, in particular u.v.-sensitivity, DOC sensitivity and recombination deficiency, are fully co-transducible with E2 refractivity; (ii) Ultraviolet resistant revertants simultaneously revert to all the *cet*$^+$ wild-type characteristics; (iii) 2-aminopurine, the mutagen used to isolate the mutants, has not so far been reported to induce multisite mutations.

An unusual property of CetC mutants, not incompatible with pleiotropic gene action, is the way in which various characteristics including E2 refractivity, u.v.-sensitivity and filament formation, can be temporarily and independently lost whilst other characteristics are retained. Such changes are not due to reversion and, indeed, despite the reduced growth rate of some CetC strains the mutants are quite stable. Most, if not all, of the mutant phenotypes displayed by CetC mutants are therefore conditional in an as yet unknown way, upon some aspect of cellular growth and physiology. It is also clear that expression of the CetB and probably CetC, mutants is dependent upon the activity of the *cet*A locus and clarification of the function of this gene may be required before the properties of the mutants are properly understood. In addition, the Cet mutants have now been shown to carry the amber or an ochre suppressor (unpublished results), and this also may profoundly affect the mutant phenotype. Efforts are now being made to eliminate this suppressor from the mutants and also to construct partial diploids, homozygous for *cet*C, in the hopes that this will lead to some stabilization of the properties of the mutants.

Although the reason for the recombination deficiency of CetC mutants is still not clear, the genetical evidence presented here and the biochemical evidence presented previously, which indicates that the majority of mutants show no major alteration in nuclease functions (Holland, 1967; Holland *et al.* 1970), clearly distinguishes these strains from previously studied Rec mutants. Despite the obvious cell surface alterations of CetC mutants, the formation of conjugal pairs and the process of gene transfer appear to be quite normal. Whilst some non-specific effect, leading for instance, to killing of the CetC recipient as a result of conjugation, cannot yet be ruled out, it appears more likely that chromosomal pairing or an enzymic step in the recombination process itself is defective in the mutants. In contrast to Rec$^-$ mutants

including a RecB strain tested in this study, CetC recipient strains give practically normal transduction frequencies with phage P1. This may indicate a deficiency in the mutants unique to some step in HfrH × F⁻ crosses, e.g. chromosomal pairing, which has no effect upon recombination processes involving small transducing fragments. It should be noted, however, that in the studies reported here, P1 multiplicities of infection of 10 were normally used. Under these conditions multiple infection is likely to occur and complementation by normal phage genomes of some defective host recombination function may take place. Since temperate P1 phage was used, normal transductants could form and survive in the lysogenic cell by this mechanism. This possibility will be explored in future studies. To establish whether or not the mutants are truly defective in a specific DNA recombination step, attempts will also be made to measure β-galactosidase synthesis in merozygotes under conditions in which enzyme could only be formed following recombination of male and female DNA.

As discussed in the preceding paper, the pleiotropic effects observed in CetC mutants could either arise from polar mutations or from mutations affecting an important functional site in the cell membrane. Since the varied characteristics of CetC mutants have as a common basis alteration of surface properties and disturbance of DNA metabolism, an attractive candidate for an altered membrane component is that normally concerned with replication and segregation of DNA (Ryter, 1968; Sueoka & Quinn, 1968). Comparison of the constitution of membranes from wild-type and mutant strains is now being carried out to test this hypothesis.

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SHORT COMMUNICATION

Factors Affecting Phosphate Uptake in the Marine Fungus *Dermocystidium* sp.

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(*Accepted for publication 18 May 1970*)

INTRODUCTION

The basis for the obligately marine nature of certain lower Phycomycetes remains unclear. However, the littoral biflagellate fungus, *Thraustochytrium roseum*, has a specific Na^+ requirement for phosphate uptake (Siegenthaler, Belsky & Goldstein, 1967*a*). We report below comparable studies on a related primitive marine fungus, *Dermocystidium* sp. (Goldstein & Moriber, 1966).

METHODS

The fungus used in this study was *Dermocystidium* sp. (Vishniac isolate WH49), a non-filamentous, obligately marine phycomycete. The methods were essentially those used in earlier studies of phosphate uptake by this organism (Siegenthaler, Belsky, Goldstein & Menna, 1967*b*). At the conclusion of the incubation period, organisms were removed from incubation mixtures and a portion of the supernatant was analysed for uptake of glucose, inorganic phosphate or glutamate. The glutamate assay was carried out using glutamate dehydrogenase (2 units, Sigma type 1), nicotinamide adenine dinucleotide (NAD), 0·05 M-potassium phosphate buffer (pH 8·5). Initial rates of NAD reduction were calculated from extinction measurements at 340 nm.

Disappearance of inorganic phosphate in nitrogen and in air was measured using organisms starved for 3 days in phosphate-free medium. Air was bubbled for 6 h. through 70 ml. of a suspension of organisms in 0·01 M-tris-0·4 M-sodium chloride-1 mM-potassium dihydrogen phosphate (pH 7·8) mixture in a 200 ml. flask. Nitrogen was bubbled for 4 h. through a duplicate suspension and a sample was removed for measuring oxygen consumption. The remaining organisms in the flask were then aerated for 2 h. Samples for phosphate analyses were removed periodically from both flasks.

Glucose uptake in the presence of various solutes was measured colorimetrically by the method of Nelson (1944). Since arsenate interfered with the colorimetric determination of phosphate, disappearance of ^{32}P from the supernatant of incubation mixtures with and without arsenate was measured with a gas-flow planchet counting system. Preliminary experiments had established a linear relationship between concentration of fungus and protein at the levels of cellular material used.

RESULTS

Figure 1 shows that phosphate taken up was proportional to NaCl concentration whereas KCl was ineffective. Oxygen consumption was stimulated approximately 50% by 0·4M-NaCl. Considerable glucose (Fig. 2) and glutamate utilization (Table 1) occurred without sodium chloride. There was less oxygen uptake with a mixture of 0·2M-sodium chloride and 0·2M-potassium chloride than with 0·2M-sodium chloride alone. With 0·5 to 1·0mM-potassium dihydrogen phosphate the rate of phosphate disappearance was independent of phosphate concentration. Phosphate below a concentration of 0·1mM disappeared too rapidly for determination of initial uptake

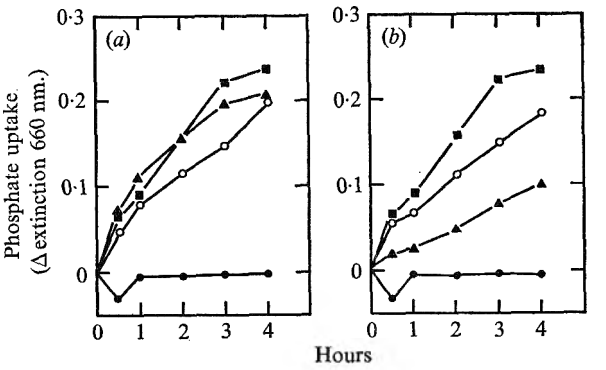

Fig. 1

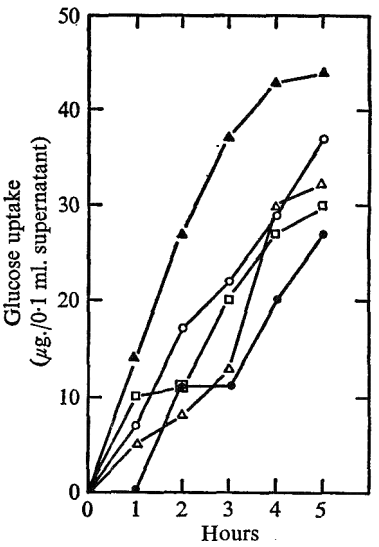

Fig. 2

Fig. 1. Effect of NaCl on phosphate uptake by *Dermocystidium* sp. (*a*) ●, no additions; ○, 0·1M-NaCl; ▲, 0·2M-NaCl; ■, 0·4M-NaCl. (*b*) ●, no additions; ▲, 0·1M-NaCl+0·3M-KCl; ○, 0·3M-NaCl+0·1M-KCl; ■, 0·4M-NaCl. Fungus was grown for 5 days and starved 3 days in phosphate-free growth medium. Basic reaction mixture: tris (pH 7·3); and thalli, 1·3 mg. protein/ml. 1 μmole inorganic phosphate ≡ Δ extinction of 0·37 at 660 nm.

Fig. 2. Effect of additions to incubation medium on glucose uptake by *Dermocystidium* sp. ●, no additions; △, 0·4M-mannitol; ▲, 0·4M-sucrose; ○, 0·2M-NaCl; □, 0·2M-KCl. Fungus was grown for 5 days in complete medium, centrifuged and resuspended in minimal reaction mixture containing 0·05% glucose, 0·1M-tris (pH 8·0), and additions. The concentration of fungus was equivalent to 2·9 mg. protein/ml.

Table 1. *Influence of sodium ion on glutamate uptake by suspensions of Dermocystidium sp.*

Additions	Moles/l.	Glutamate uptake (μmoles/5 h.)
None	—	0·21
Sodium chloride	0·2	0·36
Potassium chloride	0·2	0·00
Sucrose	0·4	0·06

The incubation mixture contained tris-HCl (0·1M, pH 8·0), L-glutamic acid (10^{-2}M), and fungal thalli (2·4 mg. protein/ml.)

rates. Arsenate was a potent inhibitor of phosphate uptake; the degree of inhibition depended on the ratio of arsenate to phosphate. Azide and phenylmercuric acetate abolished phosphate uptake. Indeed, phenylmercuric acetate caused leakage of phosphate from the organism. An initial inhibition of phosphate uptake by 2,4-dinitrophenol disappeared after 4 h. A 20% increase in oxygen consumption and a marked inhibition of phosphate uptake occurred with 0·2 mM-2,4-dinitrophenol. In *Thraustochytrium roseum* (Siegenthaler *et al.* 1967*b*), puromycin abolished the lag period associated with phosphate uptake. In *Dermocystidium* sp., phosphate uptake was enhanced but to a lesser extent than in *T. roseum*.

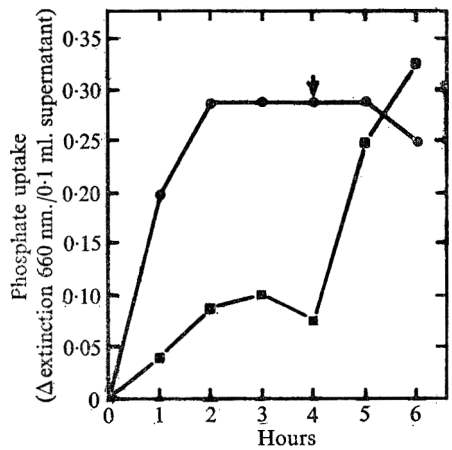

Fig. 3. Phosphate uptake by *Dermocystidium* sp. in a nitrogen atmosphere and in air. ■ Indicates nitrogen for 4 h. followed by introduction of air; ●, air only. The fungus was grown for 5 days and starved 3 days in phosphate-free medium. The reaction mixture contained 0·1 M-tris (pH 8·0), 0·4 M-NaCl, 1·0 mM-KH_2PO_4, and fungus equivalent to a concentration of 5·0 mg. protein/ml. (■) and 4·8 mg. protein/ml. (●), 1 μmole inorganic phosphate ≡ Δ extinction 660 nm. of 0·37.

No significant phosphate uptake occurred under nitrogen; this inhibition was completely reversed by aeration (Fig. 3). Glucose (a component of the growth medium) stimulated phosphate and oxygen uptake whereas a variety of Krebs cycle intermediates stimulated phosphate uptake alone.

DISCUSSION

That Na^+ is required absolutely for phosphate uptake but not for oxygen, glucose or glutamate metabolism implies direct participation of Na^+ in phosphate transport; Na^+ stimulation of other phenomena may be indirect responses to Na^+-dependent reactions. As with *Thraustochytrium roseum*, the obligately marine nature of ocean-inhibiting lower phycomycetes is related to a need for Na^+ for phosphate transport at the concentration of Na^+ in littoral waters. Goldstein, Belsky & Chosak (1969) showed that sucrose or other osmotically active substances permitted optimal growth with as little as 0·1% sodium chloride compared with 2·4% needed in their absence. No growth occurred without sodium chloride. Shapiro (1967) reported that mixed cultures of diverse micro-organisms release a large part of their inorganic phosphorus

into the medium in anoxic conditions. *Dermocystidium* sp. did not; neither phosphate release nor uptake occurred under nitrogen. Uptake could subsequently be induced by aeration, indicating that neither viability nor the phosphate transport system was permanently impaired by anoxia. These data and the effects of the electron-transport inhibitors phenylmercuric acetate and azide support the hypothesis that phosphate uptake in *Dermocystidium* sp. is an active, energy-requiring process. Shapiro's results imply that phosphate retention is also energy-dependent, but this does not apply to Dermocystidium. His use of mixed cultures hampers interpretation since one particular strain in the mixture of micro-organisms could account for the phosphate liberation.

The need for concentrations of inhibitors greater than those which elicit comparable effects on isolated mitochondria probably results from our use of intact organisms (Siegenthaler *et al.* 1697*b*). At effective concentrations these metabolic inhibitors affect respiration and transport in *Dermocystidium* sp. as they do in plant and animal mitochondria. A specific function of Na^+ in physiological systems is emerging. Christensen, Liang & Archer (1967) have reported a distinct Na^+ requirement for amino acid transport in Ehrlich ascites cells, and Shah & Wedding (1968) have shown that 1·0 mM-sodium chloride stimulates threefold phosphate esterification at one of the phosphorylation sites associated with succinate oxidation by turnip root mitochondria. Their findings are consistent with the observation that Na^+ is required for dinitrophenol stimulation of mitochondrial respiration (Siegenthaler *et al.* 1967*b*). Both reports support the hypothesis that Na^+ regulates the formation of a non-phosphorylated high-energy intermediate during oxidative phosphorylation. We have not studied mitochondria from *Dermocystidium* sp. because they have proved difficult to isolate.

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Books Received

Progress in Immunobiological Standardization. Volume 4. Proceedings of the Eleventh International Congress for Microbiological Standardization, Milan, 1968. Edited by R. H. REGAMEY, A. DE BARBIERI, W. HENNESSEN, D. IKIČ and F. T. PERKINS. Published by S. Karger AG, Arnold-Böcklin-Strasse 25, CH-4000 Basel 11, Switzerland. 712 pp. Price SFr. 80 (£8; $19.20). British Agents: Academic Press, Berkeley Square House, Berkeley Square, London, WIX 6BA. American Agents: Albert J. Phiebig, Inc., P.O. Box 352, White Plains, New York 10602.

Antimicrobial Agents and Chemotherapy—1969. Proceedings of the Ninth Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington D.C., 27–29 October 1969. Edited by GLADYS L. HOBBY. Published by The Williams and Wilkins Company, 428 E. Preston Street, Baltimore, Maryland 21202, U.S.A. 510 pp. Price $15.

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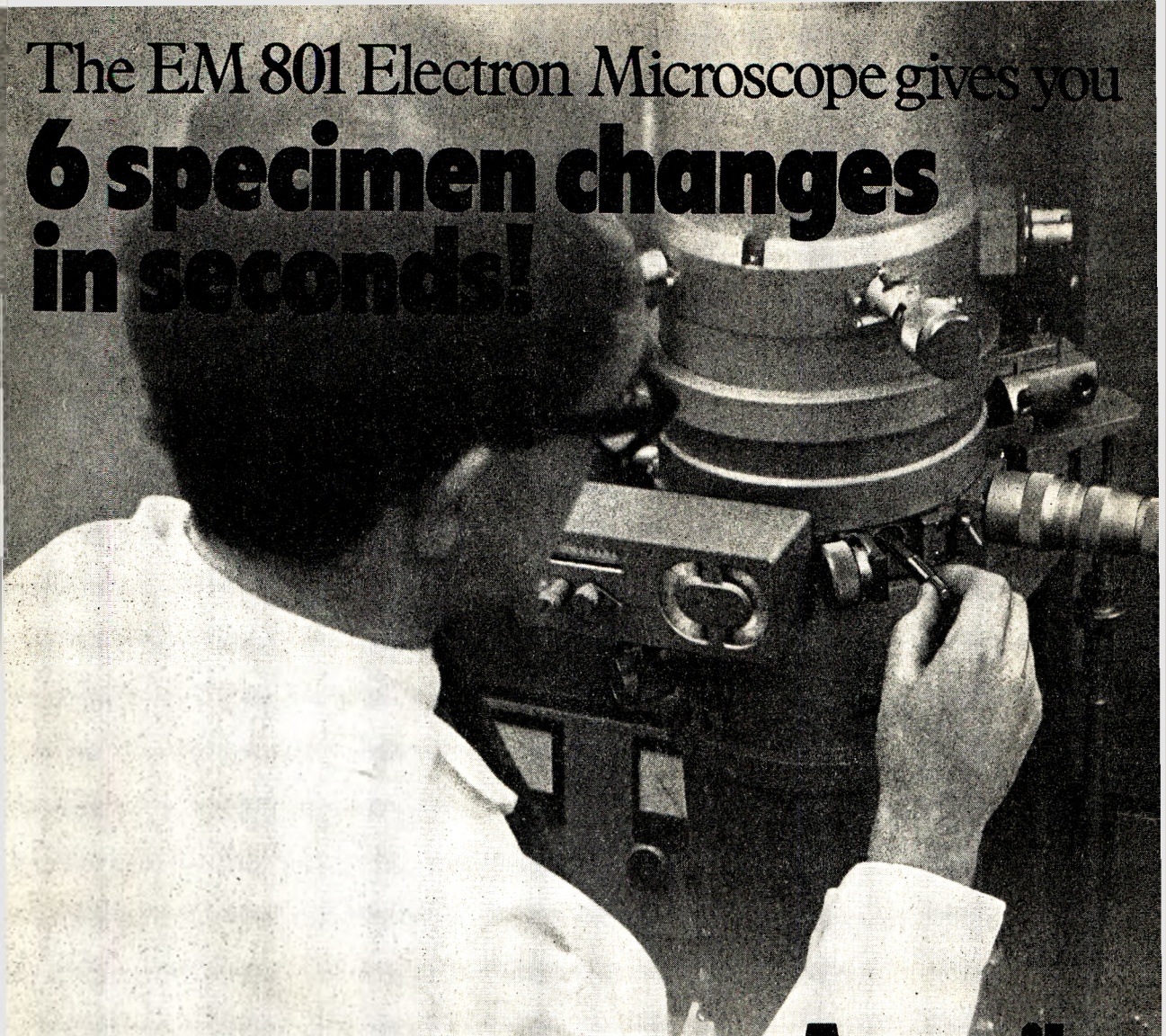

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