J. gen. Microbiol. (1963), **30**, 173–181
Printed in Great Britain

On the Nature of the 'Lethal Zygote' produced by Crossing Non-Colicinogenic with Colicinogenic Bacteria

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(*Received* 10 *February* 1962)

SUMMARY

When colicinogenic female bacteria are crossed with non-colicinogenic males, all the genes of the male chromosome can enter into the zygote without necessarily killing it. There is a great difference with regard to the number of certain recombinants tested, derived from crosses of the type Hfr col$^-$ × F$^-$col$^+$, as compared to their numbers in crosses of the type Hfr col$^-$ × F$^-$col$^-$. In certain cases this difference was reduced when the zygotes were incubated at 28° instead of the standard temperature of 37°. It was found that resistance to colicines is not always an all-or-none phenomenon. There are different degrees of colicine resistance and those mutants which show complete resistance by the standard tests are still found to be sensitive under more rigorous conditions. In the case of colicine B the sensitivity of these semi-resistant mutants is greater at 37° than at 28°.

INTRODUCTION

An asymmetry in crosses between colicinogenic and non-colicinogenic bacteria was first described by Fredericq & Betz-Bareau (1953). On crossing F$^+$*col*$^+$ × F$^-$*col*$^-$, the *col*$^+$ property was transferred to some of the recombinants, but when the F$^+$ parent was *col*$^-$ and the F$^-$ parent *col*$^+$, the *col*$^-$ property did not appear in any of the recombinants. It was concluded that the colicinogenic factor is not a part of the bacterial chromosome, but a cytoplasmic particle, a plasmagene. Alföldi *et al.* (1957, 1958), demonstrated that the *col* E 1 factor is a part of the Hfr chromosome and is transferred to the recombinants in a normal fashion, when on the Hfr chromosome. However, they claimed that when the Hfr parent was non-colicinogenic, and its chromosome had the *col*$^-$ property, the entry of this factor into recipient bacteria which were *col*$^+$ was lethal for the zygote. They called this phenomenon 'lethal zygosis'. The present paper describes a study of several crosses of the Hfr *col*$^-$ × F$^-$*col*$^+$ type.

METHODS

Organisms. All the strains used were derived from non-lysogenic derivatives of *Escherichia coli* K 12. Strain TR 21 is a derivative of strain Y 20; it requires threonine + leucine + thiamine (*thr leu thi*), it is streptomycin-sensitive (*str-s*), produces colicines E 2 and I (Fredericq, 1955) and is F$^-$. Strain TR 24 is a derivative of strain 112–12, it requires cysteine + histidine (*cys his*), is resistant to streptomycin (*str-r*), produces colicine B (Fredericq, 1955) and is F$^-$. Strain TR 25 is also a derivative of strain 112–12, (*cys his str-r*), produces colicine K (Fredericq, 1955) and is F$^-$. (These

strains were kindly supplied by Professor P. Fredericq, Université de Liège, Belgium.) Strain Hfr C requires thiamine (*thi*) and is streptomycin-sensitive. Strain Hfr P4X6 requires methionine (*met*) and is streptomycin-sensitive. PA417 requires arginine + histidine + proline (*arg his pro*), is streptomycin-resistant and F^-. (These strains were kindly supplied by Dr F. Jacob, Institut Pasteur, Paris.)

Media. Nutrient broth was Difco nutrient broth. Nutrient agar was nutrient broth + 2% Difco agar. Minimal medium had the following composition: Na_2HPO_4, 0·7% (w/v); KH_2PO_4, 0·3% (w/v); NH_4Cl, 0·1% (w/v); NaCl, 0·05% (w/v); $CaCl_2$, 10^{-4}M; $MgSO_4$, 10^{-3}M; glucose, 0·4% (w/v); amino acids (when added) 0·016% (w/v), thiamine 5 μg./ml. Glucose, amino acids, thiamine and $CaCl_2$ were autoclaved separately. Minimal medium agar was prepared by adding 4% (w/v) agar, autoclaved separately, to double-strength minimal medium. When streptomycin was used, 200 μg./ml. was added to the medium. Bacto Penassay Broth was from Difco.

Experimental procedure. Strains were stored on nutrient agar slopes at 4°. Cultures for use were propagated from such stock by overnight growth in liquid medium (nutrient broth + thiamine; minimal medium; or Penassay broth) with aeration at 37°. Samples (0·5 ml.) of each parent culture were diluted the following morning into 4·5 vol. of fresh medium at 37° and incubated at this temperature on a rotary shaker for 3 to 4 hr. until the population density of bacteria reached about 1 to 2×10^8 organisms/ml. To cross the organisms, 1·8 ml. of F^- culture were mixed with 0·2 ml. of Hfr culture, the mixture then incubated at 37° on a very slow rotary shaker for 2 hr., and the culture then diluted and plated on suitable medium to detect selected recombinants. The plates were incubated at 37° for 48 hr. and the colonies counted. Variations from this procedure are mentioned in the text.

RESULTS

The preparation of mutants resistant to various colicines

In order to cross Hfr bacteria with colicinogenic F^- bacteria it was necessary to prepare Hfr mutants resistant to the colicine produced by the F^- parent. To prepare such mutants, Hfr organisms were spread as a uniform layer on nutrient agar plates, on top of which single drops of suspensions of colicinogenic bacteria, sterilized by chloroform, were added. The colicine-resistant mutants were obtained from the few colonies which grew in the inhibition zones thus produced. These were selected, purified twice by re-streaking, checked for purity and used for further work. In the case of colicines B and K, the resistant mutants of strain Hfr C prepared in this way showed only partial resistance. They were only resistant to the colicines in a chloroformed culture which had stood for more than 1 day in the refrigerator, but were not resistant to a completely fresh chloroformed culture, or when colicinogenic bacteria were grown over them. Mutants partially resistant to colicine B were sensitive to this colicine when tested at 37° and much less so at 28°; the original strains were equally sensitive to the colicine at both temperatures. These partially resistant mutants were incubated again with freshly prepared colicines and resistant colonies isolated as before. These new mutants were resistant to the colicines even when tested by growing colicinogenic bacteria over them at 37° and at 28° and were used for further work.

The survival of col$^+$*F*$^-$ *bacteria mixed with an excess of* col$^-$ *Hfr bacteria*

F$^-$*col*$^+$*str-r* bacteria were mixed in nutrient broth with an excess of Hfr *col*$^-$ *str-s* bacteria (1×10^7 F$^-$ bacteria for $1–2 \times 10^8$ Hfr bacteria) and the mixture agitated very gently at 37°. At intervals samples were plated on nutrient agar supplemented with streptomycin. Table 1 gives the number of viable F$^-$ *str-r* bacteria at different times. In each experiment the Hfr organism used was the Hfr mutant resistant to the colicine (S) which the corresponding F$^-$ organism produced. As seen from Table 1, no noticeable killing effect by Hfr *col*$^-$ bacteria was observed with the three colicinogenic strains used with two different Hfr strains.

Table 1. *Survival of F*$^-$col$^+$ *bacteria when mixed with excess of Hfr* col$^-$ *organisms*

Suspensions of different mutants of streptomycin-sensitive HfrC and Hfr P4×6 were mixed in broth with different colicinogenic streptomycin-resistant F$^-$ organisms. The proportion was $1–3 \times 10^8$ Hfr organisms/ml. for $1–3 \times 10^7$ F$^-$ organisms/ml. The mixture was agitated at 37°, samples taken at different times and counted on nutrient agar supplemented with streptomycin.

F$^-$*str-r* strain	Hfr *str-s* strain *col*$^-$	Time (min.)	No. of surviving *str-r* organisms $\times 10^{-7}$/ml.
TR 24 *colB*$^+$	HfrC *colB*^{R} (colicine B-resistant)	0	2·5
		75	2·9
		105	2·7
PA 417 *col*$^-$	HfrC *colB*^{R} (colicine B-resistant)	0	3·2
		75	2·9
		105	3·5
TR 24 *colB*$^+$	Hfr P4X6 *colB*^{R}	0	1
		120	1·5
PA 417 *col*$^-$	Hfr P4X6 *colB*^{R}	0	1·8
		120	3·1
TR 21 *colE 2*$^+$ *& I*$^+$	Hfr P4X6 *col* (E 2 and I)^R	0	2·9
		120	3·9
PA 417 *col*$^-$	Hfr P4X6 *col* (E 2 and I)^R	0	3·8
		120	5·4
TR 21 *col E2*$^+$ *& I*$^+$	HfrC *col* (E 2 & I)^R	0	3·2
		120	4·3
PA 417 *col*$^-$	HfrC *col* (E 2 & I)^R	0	4·3
		120	6
TR 25 *colK*$^+$	HfrC *colK*^{R}	0	2·9
		120	4·3
PA 417 *col*$^-$	HfrC *colK*^{R}	0	3·4
		120	5
TR 25 *colK*$^+$	Hfr P4X6 *colK*^{R}	0	3·6
		120	5·4
PA 417 *col*$^-$	Hfr P4X6 *colK*^{R}	0	3·6
		120	4·9

Crosses of non-colicinogenic Hfr donors and colicinogenic F⁻ recipients producing colicine B

To be sure that all the genes of the chromosome entered the zygotes, we used two different Hfr donor strains, so that the chromosomes entered into the F⁻ recipients from different directions (Jacob & Wollman, 1957, 1958). These donor strains were: strain HfrC, whose chromosome enters the zygote in the order *thr leu pro gal his str arg thi*; strain Hfr P4X6 whose transfer of the genetic characters proceeds in the order *pro leu thr thi arg str his gal*. In all crosses we selected a marker for amino acid independence which is negative in the recipient and positive in the donor, namely *his*. The recombinants thus selected must have been derived from zygotes which received a segment of the donor chromosome containing at least the part from 0 (where it starts to enter the zygote) until the selected marker his⁺. Two *his⁺str-r* recombinants, one from each donor, must derive from two zygotes that together contained at least one complete copy of the Hfr chromosome with all its genes.

Table 2. *A comparison of the numbers of* his⁺str-r *recombinants with colicinogenic (colicine B) and non-colicinogenic recipients, incubated at* 37°

Parent suspensions were prepared in the usual way and mated in broth + thiamine, in defined minimal medium or in Penassay broth. After 2 hr., the mixture of mating organisms was plated and counted on streptomycin minimal media supplemented with the amino acids required by the parents, but omitting histidine.

Parents mated	Mated in	No. of *his⁺str-r* recombinants* 1	2
HfrC (*ColB*^{E} *str-s*) × TR 24 (*cys his str-r colB*⁺)	broth + thiamine	$2{\cdot}7 \times 10^{3}$	$2{\cdot}4 \times 10^{3}$
	defined medium†	$7{\cdot}1 \times 10^{4}$	$4{\cdot}4 \times 10^{4}$
HfrC (*colB*^{R} *str-s*) × PA 417 (*arg his pro str-r col*⁻)	broth + thiamine	1×10^{4}	$2{\cdot}4 \times 10^{4}$
	defined medium	$3{\cdot}7 \times 10^{5}$	$1{\cdot}5 \times 10^{5}$
Hfr P4 × 6 (*colB*^{R} *str-s met*) × TR 24 (*cys his str-r colB*⁺)	broth + thiamine	$1{\cdot}8 \times 10^{4}$	$1{\cdot}1 \times 10^{3}$
	Penassay broth	$5{\cdot}3 \times 10^{4}$	.
Hfr P4 × 6 (*colB*^{R} *str-s met*) × PA 417 (*arg his pro str-r col*⁻)	broth + thiamine	$1{\cdot}5 \times 10^{4}$	$1{\cdot}9 \times 10^{3}$
	Penassay broth	$3{\cdot}8 \times 10^{4}$	.

* Five-fold dilutions of the mating suspensions were plated on 3 plates for each dilution.
† During this work it was found that crosses in defined medium yielded more recombinants so that the crosses towards the end of this work were carried out in defined medium.

As recipient for the first series of experiments the colicinogenic strain TR 24 *cys his colB* was used. Each cross had a control cross with the non-colicinogenic strain PA 417 (*arg his pro col*⁻) as recipient bacteria. The donors used were the mutants of Hfr resistant to colicine B produced by the recipient bacteria. The results of some typical experiments are summarized in Table 2. It can be seen that some *his⁺str-r* recombinants were obtained from crosses of the colicinogenic recipient with each of the Hfr strains. In other experiments of the same sort similar results were obtained. The number of recombinants is smaller by a factor of 4 to 20 in crosses of the HfrC parent with the *col*⁺ recipient, as compared to the crosses of HfrC with the *col*⁻ recipient; no such difference was seen when Hfr P4X6 was used.

The effect of temperature on recombinant frequencies. We changed the usual pro-

cedure for the detection of recombinants by incubation on the selective medium at room temperature for 24 hr. The results of two typical experiments are recorded in Table 3. Incubation of the plates at room temperature first resulted in a significant increase in the number of *his*$^+$*str-r* recombinants of the HfrC *col*$^-$ × *col*$^+$ crosses, but no increase in the number of *his str-r* recombinants in the HfrC *col*$^-$ × *col*$^-$ crosses. The ratio of the number of recombinants at the two temperatures varied in other experiments of the same sort from 1:3 to 1:8 for *col*$^-$ by *col*$^+$ crosses.

Table 3. *Number of* his$^+$str-r *recombinants when incubated for* 24 *hr. at* 23°–24° *and then at* 37°

	Number of *his*$^+$*str-r* recombinants			
	Cross of HfrC *col*$^-$ × TR 24 *colB*$^+$		Cross of HfrC *col*$^-$ × PA 417 *col*$^-$	
Temperature of incubation of the selective plates	Exp. 1*	Exp. 2	Exp. 1	Exp. 2
37° all the time	$1{\cdot}7 \times 10^3$	$6{\cdot}6 \times 10^3$	$1{\cdot}8 \times 10^4$	2×10^5
24 hr. at 23–24° and then at 37°	$6{\cdot}8 \times 10^3$	$4{\cdot}9 \times 10^4$	$1{\cdot}8 \times 10^4$	$2{\cdot}1 \times 10^5$

* In experiment 1 the crosses were made in nutrient broth + thiamine; in experiment 2 in defined medium.

When the effect of other temperatures was examined it was found that pre-incubation at 28° had about the same effect as pre-incubation at room temperature, while pre-incubation at 18° or at 10° had a smaller effect. To determine whether the increase in the number of *his*$^+$*str-r* recombinants formed at room temperature was due to a factor formed at room temperature, or rather to one destroyed at 37°, the selective plates were incubated first at 37° for 24 hr. and then at room temperature, as compared with plates incubated first at room temperature. It was found that initial incubation at 37° destroyed the ability to form more recombinants on subsequent incubation at room temperature.

The inheritance of the col$^+$ (*B*) *character in* col$^-$ × col$^+$ *crosses. his*$^+$*str-r* recombinants from *col*$^-$ × *col*$^+$ (B) crosses were picked from the plates, resuspended in the same selective medium and tested for colicinogeny; 150 recombinants selected at 37° and 250 recombinants selected at 28° were tested; all were colicinogenic. To separate the recombinants from the zygotes and from other segregants so as to decrease the time during which they could be infected with the colicinogenic factor, the plates were respread after incubation for a few hours. A large number of single colonies from plates incubated at 37° and 28° were tested for colicinogeny; all were found positive. Another experiment to separate the segregants was carried out in liquid media; in this case also only colicinogenic recombinants were found.

Crosses of non-colicinogenic Hfr cells and colicinogenic F$^-$ *producing colicine K.* Crosses were carried out between the recipient bacterium TR 25 *cys his* (*colK*) and non-colicinogenic Hfr organisms. Each cross had a control cross with the non-colicinogenic strain PA 417 (*arg his pro*) as recipient. The donors used for these crosses were mutants resistant to colicine K. The results of two typical experiments are summarized in Table 4. It appears that some *his*$^+$*str-r* recombinants were obtained when TR 25 *colK*$^+$ was crossed with HfrC and when TR 25 *colK*$^+$ was crossed with Hfr P4X6. but that there were more recombinants when the cross

was of the type *col*$^-$ × *col*$^-$. The presence of the *colK*$^+$ factor in the F$^-$ parent decreased the yield of recombinants with each of the two Hfr donor strains used. With HfrC the factor of decrease varied from 1:10 to 1:30, and with P4 × 6 the factor varied from 1:1 to 1:4. Crosses between TR 25 *colK*$^+$ and HfrC *colK*^{R} were made, comparing the number of the recombinants for the case in which the zygotes were incubated at 28° with the number found when the zygotes were incubated at 37° (Table 5). When the crosses were of the type *col*$^-$ × *colK*$^+$, incubation of the zygotes at 28° increased very little the number of recombinants. The number of *his*$^+$*str-r* recombinants from the *col*$^-$ × *col*$^-$ cross was much higher, even then. *his str-r* recombinants from *col*$^-$ × *colK*$^+$ crosses were tested for colicinogenicity, 100 recombinants selected at 37° and 90 selected at 28° were tested; all were colicinogenic.

Table 4. *Comparison of the number of* his$^+$str-r *recombinants with colicinogenic recipients (producing colicine K) and non-colicinogenic recipients*

Parents	No. of *his*$^+$*str-r* recombinants. Mating was done in defined medium	
	Exp. 1	Exp. 2
HfrC *colK*^{R} × *colK*$^+$ TR 25	$4{\cdot}9 \times 10^3$	9×10^3
HfrC *colK*^{R} × *col*$^-$ PA 417	$4{\cdot}5 \times 10^4$	$2{\cdot}8 \times 10^5$
Hfr P4X6 *colK*^{R} × TR 25 *colK*$^+$	$1{\cdot}5 \times 10^3$	$3{\cdot}8 \times 10^3$
Hfr P4X6 *colK*^{R} × PA 417 *col*$^-$	$6{\cdot}7 \times 10^3$	$5{\cdot}4 \times 10^3$

Table 5. *The number of* his$^+$str-r *recombinants when the zygotes were incubated at 28° for the first 24 hr.*

Temperature of incubation of the zygotes for the first 24 hr.	No. of *his*$^+$ *str-r* recombinants*					
	Crosses of HfrC *colK*^{R} × TR 25 *colK*$^+$			Crosses of HfrC *colK*^{R} × PA 417 *col*$^-$		
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
37°	3×10^3	9×10^3	$4{\cdot}9 \times 10^3$	1×10^5	$2{\cdot}8 \times 10^5$	$4{\cdot}5 \times 10^4$
28°	8×10^3	$1{\cdot}1 \times 10^4$	6×10^3	9×10^4	$2{\cdot}4 \times 10^5$	$4{\cdot}3 \times 10^4$

* The mating was done in defined medium.

The fate of the donor organisms in crosses of the col$^-$ × col$^+$ *type as compared to their fate in crosses of the* col$^-$ × col$^-$ *type.* Table 6 presents two typical experiments that give the number of viable donor organisms in crosses of the *col*$^-$ × *col*$^-$ type, as compared to their number in crosses of the *col*$^-$ × *col*$^+$ type. The ratios of viable counts of HfrC before and after the crosses ranged as follows: (1) HfrC *colB*^{R} × F$^-$*colB*$^+$ 1:2 to 1:1·3, compared to 1:2·3 to 1:1·5 when crossed with F$^-$*col*$^-$; (2) HfrC *colK*^{R} × F$^-$*colK*$^+$ 1:0·14 to 1:1·4 compared to 1:1·6 to 1:2·5 when crossed with F$^-$ *col*$^-$; (3) HfrC. *col* (E 2 and I)^R × F$^-$*col* E 2$^+$ and I$^+$ 1:2, i.e. the same as when crossed with F$^-$*col*$^-$. It appears that colicinogenic females which produced colicines E 2 and I did not influence the survival and growth of HfrC organisms during the period allowed for mating, and that colicinogenic females producing colicine B did not have much influence on the male survival and growth, whereas colicinogenic females which produced colicine K affected much more the survival and growth of HfrC organisms mated with them.

Table 6. *The survival of Hfr* col$^-$ *bacteria when crossed with* col$^+$ *females compared to their survival when crossed with* col$^-$ *recipients*

Suspension of Hfr organisms (resistant to the colicines produced by the corresponding females) were mixed in defined medium with colicinogenic female bacteria. The proportion was approximately 1 Hfr organism to 5–10 F$^-$ organisms/ml. The mixture was agitated at 37°, samples taken before and after the cross and counted on minimal medium + thiamine. The plates were incubated at 37°.

Hfr strain	F$^-$ strain	Time (hr.)	No. of surviving Hfr organisms/ml. in the mating mixture $\times 10^{-7}$ Exp. 1	Exp. 2
HfrC B^R	TR 24 *colB*$^+$	0	1·8	5·5
		2	3·5	7·1
HfrC B^R	PA 417 *col*$^-$	0	1·8	5·5
		2	4·2	8·2
*HfrC K^R	TR 25 *colK*$^+$	0	2·5	7·2
		2	0·33	7·6
HfrC K^R	PA 417 *col*$^-$	0	2·5	7·2
		2	5·2	17·0
HfrC (E 2 and I)^R	TR 21 *colE 2*$^+$ and *I*$^+$	0	3·9	
		2	7·3	
HfrC (E 2 and I)^R	PA 417 *col*$^-$	0	3·9	
		2	7·1	

* For experiment 1, one mutant of HfrC K^R was used; for experiment 2 another mutant was used.

DISCUSSION

The above experiments show that when recipient bacteria are colicinogenic for colicine B or colicine K, the presumed *col*$^-$ allele, if located on the chromosome of an Hfr bacterium (Alföldi *et al.* 1958), does not always kill the zygote when it enters. This was definitely proved by crossing each colicinogenic F$^-$ bacterium with two different Hfr donors whose directions of gene transfer are opposite to each other. Thus, the *his*$^+$*str-r* recombinants selected in both crosses must have been derived from zygotes which received a segment of the donor chromosome containing at least the part from 0 to *his*$^+$ (Fig. 1).

Although the entry of the *col*$^-$ factor into a zygote does not necessarily kill it, it was found that the number of *his*$^+$*str-r* recombinants in a cross of *col*$^-$ × *col*$^+$ type was decreased as compared with the number in a cross of the *col*$^-$ × *col*$^-$ type. The presence of the *col K* factor in the F$^-$ parent decreased the yield of recombinants with each of the two Hfr donor strains used, as compared to the yield of these recombinants in the presence of *col B* factor in the F$^-$ parent. The difference in the yield of recombinants with the HfrC donor was even bigger when compared to the number of these recombinants derived from an F$^-$*col*$^-$ recipient. It could be argued that the non-colicinogenic and the colicinogenic strains compared as F$^-$ partners do not differ merely by the presence or absence of the *col*$^-$ factor concerned and, therefore, the F$^-$*col* strain (PA 417) is not an adequate control. However, strain 112–12 *col*$^-$ was not available and attempts to cure the colicinogenic strains of their

colicinogenic factors or to infect another K 12 strain (PA 417 *col*$^-$) with any of the colicinogenic factors K or B were unsuccessful. Strain PA 417 used in this work could be regarded as a reference strain and the two 112-12 *col* strains which differ from each other only by the presence of different *col* factors can be compared with this reference.

The number of *his*$^+$*str-r* recombinants in *col*$^-$ × *col*$^+$ crosses increased when the zygotes were incubated at a temperature lower than 37°. In crosses of HfrC × TR 24 (*colB*), the number of *his*$^+$*str-r* recombinants was nearly the same as in the HfrC × *col*$^-$ crosses, when the zygotes were incubated at 28°. The smaller number of

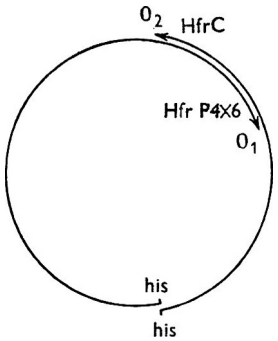

Fig. 1. The parts of the chromosome that are sure to enter the *his*$^+$ recombinants in the two crosses: from 0_1 to *his* in the cross with Hfr P 4X6; from 0_2 to *his* in the cross with HfrC. Two such complementary recombinants contain at least one complete copy of the Hfr chromosome with all its genes.

recombinants from Hfr *col*$^-$ × *col*$^+$ crosses could be explained by the observations that when bacteriocinogenic bacteria become non-bacteriocinogenic, they lose their immunity and become sensitive to the bacteriocin which they have produced (Fredericq, 1948; Hertman & Ben-Gurion, 1959). If a recombinant inherits from the Hfr parent the *col*$^-$, but not the colicine-resistant allele, it should become sensitive to the colicine, since the colicinogenic F$^-$ were not resistant but only immune, as the colicinogenic strains used were prepared from a sensitive 112-12 strain (Professor Fredericq, personal communication). Such a *col*$^-$ recombinant should be sensitive to the colicine that other bacteria produce. This could account wholly or partly for the smaller number of *his*$^+$*str-r* recombinants that come out of such crosses, since some of them could be killed by the colicine. The hypothesis is perhaps supported by the fact that one does get more recombinants when the zygotes are incubated at 28°, as it has been shown in one bacteriocinogenic system that the bacteriocin produced was far more active towards sensitive organisms at 37° than at 28° (Ben-Gurion & Hertman, 1958). With colicinogenic bacteria for colicine B, we have found, using the semi-resistant mutants, an indication that there is a higher sensitivity at 37° than at 28°.

In crosses of HfrC, where the F$^-$ is colicinogenic for K, there are far less *his*$^+$ *str-r* recombinants than with F$^-$ colicinogenic for B. Here we found that although by the standard tests the Hfr mutants were resistant to colicine K, viable counts of the Hfr parents showed that the Hfr organisms were inhibited and some of them

killed by the presence of the F^- colicinogenic bacteria. This could also account, at least partly, for the small number of recombinants that arise from such crosses. The fact that all the recombinants tested were colicinogenic could be explained, perhaps, by the episomal characteristic of colicines. Since the zygotes contained these episomes to start with, it is possible that all the *col*$^-$ segregants were infected. Another fact emerges from the experiments described, namely that resistance to some colicines is not an all-or-nothing phenomenon. We have seen that resistance to colicine K or B could be acquired by two mutational steps and even then some Hfr mutants grew much less in the presence of colicinogenic bacteria than in the presence of non-colicinogenic bacteria, creating the impression that they were still partly sensitive to it.

The author is grateful to Miss Karla Schajevitz for excellent technical assistance.

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J. gen. Microbiol. (1963), **30**, 183–191
Printed in Great Britain

The Conditions which Govern the Adsorption of a Tryptophan-Dependent Bacteriophage to Kaolin and Bacteria

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(*Received* 12 *March* 1962)

SUMMARY

The adsorption of a tryptophan-requiring (tryp$^+$) hydroxymethylcytosine (HMC) phage (Ox 6) to bacteria has been compared with that to kaolin. In a system free from monovalent cations other than tris (2-amino-2-hydroxymethylpropane-1,3-diol) buffer, adsorption in both cases was temperature-dependent and required not only tryptophan but an optimum concentration of NaCl. The adsorption to kaolin came to an equilibrium which was determined by temperature since it depended on a preliminary activation of the phage, which process was temperature-dependent. Elution of phage adsorbed to kaolin took place to a greater or less degree on altering any one of the three requirements, NaCl, tryptophan or temperature. In experiments with bacteria no equilibrium was reached because adsorption was followed rapidly by inactivation; no elution was demonstrable.

INTRODUCTION

It is generally agreed (cf. Luria, 1953) that the adsorption of bacteriophages to bacteria and to inanimate particles requires not only a certain ionic concentration but also in some cases the presence of 'adsorption factors'. Doubt however exists on the validity of some accepted conclusions and it was thought worth while to explore the field by methods different from those generally used. This paper gives details of the factors involved in the adsorption of a single tryptophan-requiring member of the hydroxymethylcytosine (HMC) phage group (Kay & Fildes, 1962*a*) to kaolin and to a susceptible bacterium.

MATERIALS

Tris buffer. All solutions and suspensions were made in 0·011 M-tris (2-amino-2-hydroxymethylpropane-1,3-diol) buffer (pH 7·6) + human serum albumin. The tris solution was sterilized by autoclaving at 120° for 20 min., cooled, and 3 ml. 5% human serum albumin added per litre; exceptions to this were the stocks of phage and kaolin.

The *phage* used was 'Ox 6' (Kay & Fildes, 1962*a*). This was produced in a simple glucose + ammonia medium and purified by differential centrifugation. Stocks were suspended in 0·033 M-phosphate buffer containing thymol; they titrated to about 1×10^{11} particles/ml. and for use in an experiment were normally diluted in tris buffer to final 5×10^3 particles/ml.

The *kaolin* was a suspension of British Drug Houses Ltd. kaolin in 0·011 M-tris buffer containing no albumin. A heavy suspension was made and supernatant suspension removed; more buffer was then added and the procedure repeated several times to obtain a stock suspension of finer particles. This stock was not observed to alter when kept at 2°. In an experiment it was so diluted in 0·011 M-tris albumin that the final reading was 0·300 on a Hilger Spekker absorptiometer, using unfiltered light.

The *bacterium* used for adsorption and lawns was a clone 'M', selected from coli 518(3) referred to by Fildes & Kay (1959). It was grown in nutrient broth for use as a lawn. For adsorption experiments organisms were washed twice in tris buffer + albumin and re-suspended in the same. The suspension showed a gradual loss of activity at 2°.

DL-*tryptophan*. The stock was a 0·01 M-solution in 0·002 M-NaOH with thymol added after autoclaving. Normally this was used in an experiment at a final concentration 10·2 μg. L-tryptophan/ml.

In an *adsorption* experiment the various materials were distributed in one ounce screw-cap bottles. Bottles were held at the required temperature for 30 min. by immersion in water. At zero time the experiment was started by adding phage or recipient, as required, to the other materials. Samples were taken from time to time to check the input and to confirm that the phage was heat stable at the temperature used. Other samples were centrifuged and the supernatant phages assayed. The counts were plotted as log % input at the observed times. When using kaolin there was no necessity to stop adsorption by dilution because adsorption was slow, but with bacteria the input was arranged to allow a 1/500 dilution in the same substrate without bacteria. Dilution took place at the operative temperature, usually 37°.

Phage assay. The assay was made by the drop method (Fildes & Kay, 1957). It was preferable to use nutrient agar plates at least 7 days after pouring and not to heat them on a hot plate to accelerate drying of the drops. With the pipettes used four drops delivered 0·1 ml. Since the input was of the order of 5×10^3 particles/ml., 0·1 ml. contained about 500 phage particles or 125/drop. The nutrient agar contained 1·5 % agar, 1·0 % Difco Bacto nutrient broth, 0·125 M-NaCl, 0·01 M-sodium citrate. Plates were seeded with sufficient coli strain 'M' in Bacto nutrient broth and all excess removed; they dried in a few minutes with the covers off. Counts were made after incubation for 4–5 hr. or overnight; a Zeiss binocular microscope was used at × 10 diam. When assay of a supernatant fluid was required, 4 ml. were centrifuged in an angle centrifuge at 4000 rev./min. for 5 min.; the sample was then removed with a Pasteur pipette from the meniscus on the near side of the tube.

RESULTS

The effect of NaCl *on the adsorption of phage Ox*6 *to kaolin*

Experiment 1. Two series of bottles 1 to 8 and 9 to 16 were used; all contained tryptophan; bottles 2 to 8 and 10 to 16 contained graded amounts of NaCl to final concentrations 0·02 M to 0·10 M. Bottles 9 to 16 contained kaolin. The volumes of fluid were equalized with tris buffer to 9 ml. The phage suspension suitably diluted, and the bottles were heated to 37° for 20 min. Then at zero time, phage suspension

(1·0 ml.) was added to all and the bottles returned to 37°. Supernatant fluids from bottles 1 and 9 were assayed at zero time and all bottles (9 to 16 supernatant fluids) after 60 min. Figure 1 shows that 0·05 M-NaCl gave the maximum degree of adsorption; in the absence of NaCl there was no adsorption even though tryptophan was present.

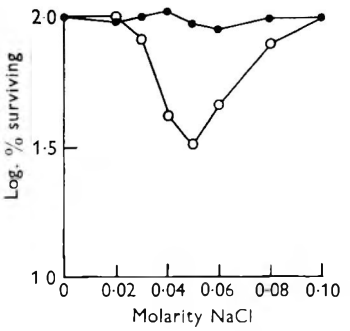

Fig. 1

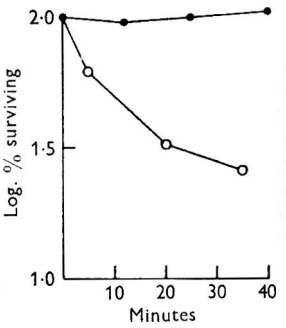

Fig. 2

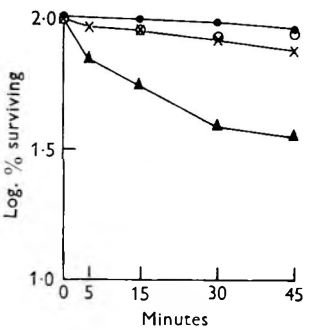

Fig. 3

Fig. 1. Titration of NaCl to show optimum concentration for adsorption of phage Ox6 to kaolin. ●, No kaolin; ○, with kaolin (supernatant phage). Both contain tryptophan.

Fig. 2. Effect of tryptophan on adsorption of phage Ox6 to kaolin at 40°. ●, No tryptophan; ○, with tryptophan. Both contain optimal NaCl and kaolin. Supernatant phage counts.

Fig. 3. Effect of temperature on adsorption of phage Ox6 to kaolin. ●, No kaolin at 18°; ×, with kaolin at 18°; ○, no kaolin at 37°; ▲, with kaolin at 37°. All contain optimal NaCl and tryptophan. Counts with kaolin are on supernatant phage after centrifuging.

The effect of tryptophan on the adsorption of phage Ox6 to kaolin

Experiment 2. Three bottles were used containing: (1) tris buffer + NaCl (0·05 M final) + phage; (2) tris buffer + NaCl (0·05 M final) + kaolin + phage; (3) tris buffer + NaCl (0·05 M final) + kaolin + tryptophan + phage.

Bottle 1 was plated three times during 45 min. to determine the average input and the supernatant fluids of the kaolin-containing bottles were plated three times to show adsorption. Figure 2 indicates the result; in the presence of optimal NaCl without tryptophan there was no adsorption at 40°, but 74 % adsorption in 35 min. in the presence of NaCl + tryptophan.

The effect of temperature on the rates of adsorption of phage Ox6 to kaolin

Activation. There was no measurable adsorption of phage Ox6 to kaolin at 2°, but adsorption was detectable at 15–20°. Figure 3 illustrates the difference of adsorption between 18° and 37°. So marked an effect of temperature was not expected from the physical theory of adsorption and it was assumed that the observations had been complicated by the 'activation' of the phage by tryptophan, a process which is known in the case of coliphage T4 (Anderson, 1948) to be thermally controlled. Since an increasing proportion of the phage particles is activated as the temperature rises, the rates of adsorption also increase, giving an impression that adsorption is directly influenced.

Elution. It will be noticed that the rate of adsorption of phage Ox6 to kaolin declined with time and thus did not follow the course generally described as characteristic of adsorption to bacteria; with bacteria a plot of the log of the number of phage particles remaining free against time is linear. In a purely physical process of adsorption this would not be expected but rather the development gradually of a dynamic equilibrium between adsorption and elution, resulting in a more rapid adsorption at first followed by a gradual slowing. In our experience with kaolin the results are characteristic of a physical process in which, at equilibrium, the rates of adsorption and elution are equal. Only when the environment is changed to one inappropriate to the maintenance of this equilibrium are the phages permanently released.

Change in the ionic environment. In Expt. 3 each of two bottles contained tris buffer, tryptophan, 0·05 M-NaCl and phage; one bottle also contained kaolin. Adsorption was allowed to proceed for 30 min. at 40° and then 20 ml. were removed from the kaolin bottle to another containing 1·0 ml. 6 M-NaCl. In this bottle therefore the NaCl was about 0·35 M. Figure 4 shows the log % phage particles surviving in the kaolin supernatant fluids at various times. On altering the NaCl concentration to a value well above that at which adsorption occurs, all adsorbed phage particles were immediately released. The adsorption therefore had no effect on the plating efficiency of the phage. The same result may be seen merely by plating the whole of a kaolin adsorption mixture in addition to the supernatant fluid. Although the supernatant fluid count may indicate a high degree of adsorption, the whole count is equal to the input. Thus the adsorbed phage particles in the mixture must have been released on the plate.

Change in the temperature. When phage Ox6 was adsorbed at 37° and then cooled to 4°, any phage particles momentarily released would be liable to de-activation so that they would not be adsorbed at 4°. The extent of this risk depends upon the length of the period of elution and the rate of de-activation by lowered temperature. Since the former period is probably much shorter than the latter permanent elution by lowering the temperature is not likely to be marked. Figure 5 indicates that 8 % of the phage particles adsorbed at 40° may have been eluted by lowering the temperature to 2°; this is hardly significant but it is clear that continued adsorption ceased.

Change in the tryptophan concentration. Alteration in the tryptophan concentration is also liable to affect the stability of adsorbed phage. When the phage particles were adsorbed at 37° in the presence of L-tryptophan 10 μg./ml. and then diluted 1/500 in the absence of tryptophan, active phage particles momentarily released would be exposed to L-tryptophan 0·02 μg./ml., a concentration below the effective value for adsorption. Again, depending on rates of de-activation (2 min. according to Anderson, 1948) and of re-adsorption, some of the phage particles will be liable to de-activation and permanent release. As shown in Fig. 6, 34 % of the particles adsorbed at 37° were immediately released by decreasing the concentration of tryptophan to 0·02 μg./ml., i.e. they were de-activated after release, but, as the counts of the whole suspension indicate, not inactivated. This implies that 66 % of the adsorbed phage particles either were not released long enough to become de-activated or that tryptophan was not removable by dilution from the complex (phage + tryptophan + NaCl + kaolin).

These experiments with kaolin, in which the rate of adsorption was always slow, suggest that an experiment designed on the lines of Anderson's work to prove activation of the phage by tryptophan is likely to be vitiated by de-activation during the adsorption phase. This was found to be the case; but reference may be made to Table 1 and Fig. 10 showing experiments with bacteria in place of kaolin.

Comparison between the adsorption to phage Ox6, to kaolin and to bacteria

The effect of NaCl *concentration.* Experiments similar to the above but with kaolin replaced by bacteria showed that NaCl was equally essential for adsorption but that its optimum concentration was higher and the peak concentration less sharply defined, though at about 0·10 M; Fig. 7 may be compared with Fig. 1 in this respect.

Elution. When phage Ox6 is adsorbed to bacteria, the phage particle count of the whole uncentrifuged mixture is about the same as the count of the supernatant fluid after centrifugation, showing that all or most of the adsorbed phage particles are permanently inactivated and cannot be eluted unchanged. This inactivation unbalances the equilibrium observed during adsorption to kaolin and results in the

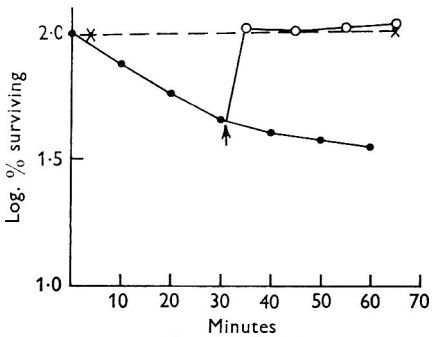

Fig. 4

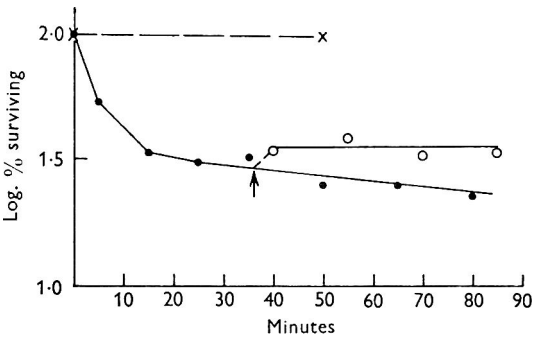

Fig. 5

Fig. 4. Elution of adsorbed phages by adjustment of the NaCl concentration. ×, no kaolin, 0·05 M-NaCl; ●, kaolin with 0·05 M-NaCl; ○ = kaolin with NaCl increased to 0·35 M at the arrow. All contain tryptophan. The kaolin counts are of supernatant phage after centrifuging.

Fig. 5. Effect on phages adsorbed to kaolin at 40°, of decreasing the temperature to 2°. ×, No kaolin, 40°; ●, with kaolin, 40°; ○, with kaolin decreased to 2° at the arrow. All contain optimal NaCl and tryptophan. Counts with kaolin are of supernatant phage.

Table 1. *The effect of tryptophan concentration on the 'activation' of phage Ox6. Details of experimental mixtures*

Activation stage						Adsorption stage				
Bottle	Phage (ml.)	L-Tryptophan (8 μg./ml.)	Tris (ml.)	μg. L-Trypto-phan/bottle	μg. L-Trypto-tophan/ml.	Bottle	Bact. (ml.)	Tris (ml.)	L-Tryptophan (8 μg./ml.)	Final μg./ml. L-tryptophan
A	0·25	0·25	15·5	2	0·125	A¹	4	0	—	0·1
B	0·25	0·25	7·5	2	0·25	B¹	4	8	—	0·1
C	0·25	0·25	3·5	2	0·5	C¹	4	12	—	0·1
D	0·25	0·25	1·5	2	1·0	D¹	4	14	—	0·1
E	0·25	0·25	0·5	2	2·0	E¹	4	15	—	0·1
F	0·25	0·25	0	2	4·0	F¹	4	15·5	—	0·1
G	0·25	0	0·25	0	0	G¹	4	15·5	—	0
H	0·25	0·25	0	2	4·0	H¹	4	5·75	9·75	4·0

linear plot of the log % surviving referred to as characteristic of adsorption to bacteria. Figure 8 illustrates these results.

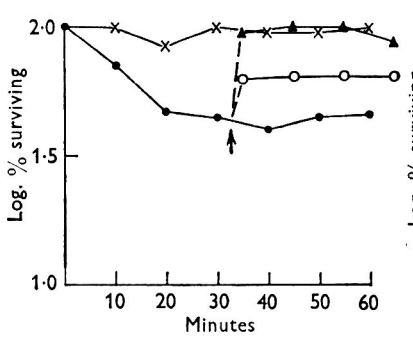

Fig. 6

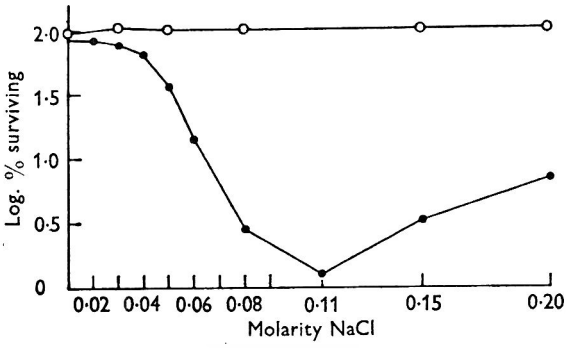

Fig. 7

Fig. 6. Effect on phages adsorbed to kaolin of diluting out tryptophan. ×, NaCl, tryptophan and kaolin (count of whole); ●, NaCl, tryptophan and kaolin (count of supernatant phage). At the arrow tryptophan was diluted out leaving the same concentrations of NaCl and kaolin. ▲, NaCl and kaolin (count of whole); ○, NaCl and kaolin (count of supernatant phage).

Fig. 7. Effect of NaCl concentration on adsorption of phage Ox6 to bacteria. ○, No bacteria; ●, with bacteria (supernatant phage). Both contain tryptophan.

The effect of temperature. The rate of adsorption of phage Ox6 to bacteria was affected by temperature as was adsorption to kaolin (Fig. 9). It has been stated (cf. Adams 1959) that the proportion of T4 coli-phage particles adsorbed to bacteria at low temperatures could be increased to equivalence with the proportion at higher temperatures by raising the tryptophan concentration to 100 μg./ml. An effect of this sort was hardly demonstrable with phage Ox6 (Fig. 9). It seems clear that the effect of temperature on adsorption of phage Ox6 to bacteria is exerted through a temperature effect on tryptophan activation, as in the kaolin experiments, but the various elution effects noted with kaolin cannot occur with bacteria because inactivation rapidly follows adsorption.

Activation of phages by tryptophan

Anderson's (1948) description of the activation of coli phage T4 by tryptophan was largely based on his finding that free phage particles did not produce plaques on a tryptophan-free medium, while adsorbed phage did. It was presumed that the successive bursts on the plate necessary to produce a visible plaque were due to liberation of tryptophan at the first burst. Since all free phages produced plaques on a tryptophan-containing medium, the difference between plaque counts on a tryptophan-containing and a tryptophan-free media was a measure of the number of phage particles adsorbed before plating. His procedure for showing that activation was due to an action of tryptophan on the phage and not on the bacteria was as follows. Equal amounts of coliphage T4 and L-tryptophan were added to each of six tubes but the volumes of the suspensions were adjusted to give a graded series of tryptophan concentration from 0·1 to 4·0 μg./ml. After standing at 37° for 25 min. to activate the phage, suspensions of coli B in tryptophan-free medium were added

to each tube so that the final concentrations of phage particles, bacteria and tryptophan were the same in all. Five minutes were allowed for adsorption to take place and the mixtures were plated on the two media. The counts on the tryptophan-containing medium were the same in all cases, but the counts on the tryptophan-free medium showed a large increase in the number of plaques as the concentration of tryptophan increased in the activation phase of the experiment. 'Since the conditions in the adsorption tubes were identical, the differences in the assays on F agar must have been due to the differences in the *concentration* of L-tryptophan with

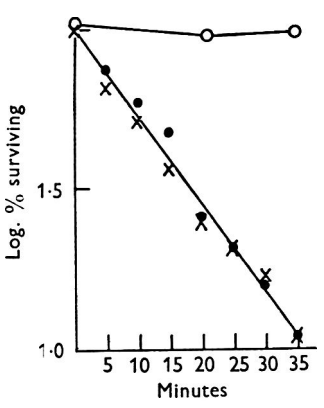

Fig. 8

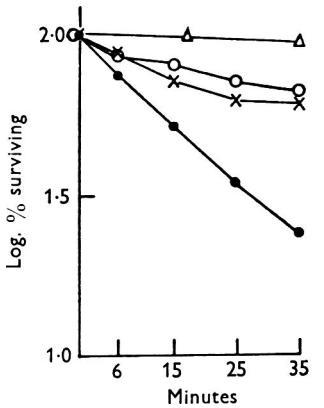

Fig. 9

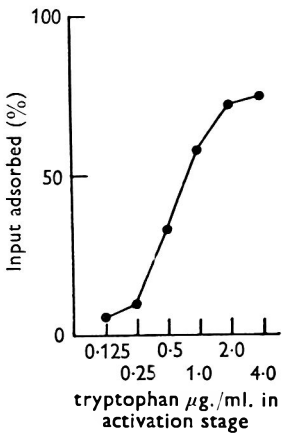

Fig. 10

Fig. 8. Adsorption of phage Ox6 to bacteria at 40°. ○, No bacteria; ●, with bacteria (count of whole); ×, with bacteria (count of supernatant phage). All contain optimal NaCl and tryptophan.

Fig. 9. Effect of temperature and of excess tryptophan on adsorption of phage Ox6 to bacteria. △, No bacteria, L-tryptophan 10 μg./ml., 37°; ●, with bacteria, L-tryptophan 10 μg./ml., 37° (supernatant phage); ○, with bacteria, L-tryptophan 10 μg./ml., 20° (supernatant phage); ×; with bacteria, L-tryptophan 100 μg./ml., 20° (supernatant phage). All contain optimal NaCl.

Fig. 10. To show that phage Ox6 pre-treated with tryptophan is 'activated' and no longer dependent upon tryptophan for adsorption

which the virus has been incubated' (Anderson, 1948, fig. 3). Anderson then went on to show that this activation phenomenon was temperature-dependent with an optimum about 35°, that it took about 2 min. to complete, and about the same time to de-activate after removal of the tryptophan. It will be noted that Anderson's observation of the action of tryptophan on a tryp$^+$ phage which makes it adsorbable is based on inference; it was thought desirable to repeat his crucial experiment to show directly that activation was an essential for adsorption. Table 1 indicates the procedure used; it was based on Anderson's experiments (1948). All materials were dissolved in tris buffer containing 0·1 M-NaCl. The final concentration of bacteria was 3×10^9/ml. Bottles A to H were heated for 20 min at 37° for activation and then the contents of bottles A^1 to H^1 were poured in. After a further 15 min. the supernatant fluids of all bottles were assayed. Figure 10 shows the adsorption in the various bottles as % of the bottle G without tryptophan. As a result of the increasing tryptophan concentration in the activation stage of the experiment the phage

adsorbed rose from a few particles to 75 %. In bottle H the concentration of tryptophan present during the activation phase was maintained at the same value during the adsorption phase and resulted in the phage particles being completely adsorbed. The difference between H and F was clearly due to de-activation of free phage in F by dilution of tryptophan before adsorption could take place. Figure 10 shows the same features as Anderson's (1948) fig. 3.

DISCUSSION

Kay & Fildes (1962*b*) showed that a heat-labile tryp$^+$ phage underwent changes in its tail and loss of DNA under three conditions all of which had to operate simultaneously: (i) presence of tryptophan; (ii) presence of NaCl especially at two optimal concentrations; (iii) an adequate elevation of temperature to control the rate of action. Further (unpublished) work has shown that when similar tests are made in the presence of kaolin, adsorption of the phage takes place (i) in the presence of tryptophan; (ii) in the presence of NaCl at the lower but not at the higher NaCl concentration; (iii) at an increase of temperature above 4° adequate to allow adsorption without heat-inactivation during the period of observation. Kay & Fildes (1962*b*) found that discharge of DNA occurred in proportion to the degree of heat inactivation, but we have found (unpublished) that in the presence of kaolin discharge of DNA does not occur. It may be said that under certain conditions the heat-labile phage undergoes changes in its tail tip which result in loss of DNA, while under the same conditions in the presence of kaolin particles the phage becomes adsorbed and does not lose its DNA. It would thus appear that the site of leakage is the same as that of adsorption and highly probable that the nature of the initial change is the same in each case.

In the present work tests of the heat-stable tryp$^+$ phage, Ox6, have also shown the necessity for the same three conditions to be fulfilled simultaneously before adsorption takes place. Thus the difference between the two phages is that in the heat-labile phage the tail changes in the absence of a recipient go beyond the point of recovery, while in the heat-stable phage the changes are all reversible on removal of any one of the three conditions.

It has been confirmed directly that 'tryptophan-activation' of a tryp$^+$ phage is due to a temperature-dependent reaction between tryptophan and phage. Since this action is only demonstrable by adsorption, it is not yet clear whether the requirement for NaCl is in the activation process, in the adsorption process or in both. If, however, the initial lesion in a labile phage which results in loss of DNA is the same as that which 'activates' a phage for adsorption, it follows that NaCl is necessary in the process of activation.

In the present work the conditions affecting adsorption to kaolin have been compared with those necessary for adsorption to bacteria. The differences between the two types of recipient appear to be: (i) the NaCl requirement for bacteria is less sharply defined at an optimum concentration; (ii) the adsorption to bacteria is irreversible and therefore the various results of elution are not observed. It is generally accepted that adsorption of phage to bacterium takes place in two steps, the first being reversible and the second not. We have not found strong evidence with phage Ox6 of the existence of a reversible stage owing, no doubt, to the

rapidity with which the adsorbed phage is inactivated. We therefore prefer to think that the actual adsorption follows much the same course as with kaolin but that the fate of the adsorbed particle subsequent to adsorption is governed by the complex recipient on the bacterial surface.

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Printed in Great Britain

Spontaneous Mutation in Spheroplasts of *Escherichia coli*

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(*Received* 4 *April* 1962)

SUMMARY

Spheroplast suspensions containing as few as one rod in a thousand were made with *Escherichia coli* (15 *his*$^-$) by the use of penicillin. These spheroplasts could be reverted to the rod state with efficiency approaching 100 %. Spontaneous mutation from histidine requirement to non-requirement was observed in these spheroplasts, in which cell division did not take place.

INTRODUCTION

The mechanism of induced mutation has been investigated profitably along several lines but less is known about spontaneous mutation (Ryan, 1963). Some studies have been made with a histidineless strain of *Escherichia coli* (15 *his*$^-$) in a non-dividing condition (Ryan, 1955, 1959; Ryan & Kiritani, 1959; Ryan, Rudner, Nagata & Kitani, 1959). The method involves distributing a culture into several hundred tubes containing a defined medium without histidine. When the concentration of organisms is about 1×10^7/ml. the cultures are so clear that overgrowth of *his*$^+$ mutants which might be present is easily recognized through the development of turbidity. At 20°, *his*$^+$ mutants, initially present at the time of distribution, overgrow in about 100 hr. Thereafter, for several hundred hours new overgrowths, in tubes that did not receive a pre-existing *his*$^+$ mutant, occur because of *his*$^+$ mutants arising during the stationary phase. Since the medium used does not contain histidine it might be expected that the *his*$^-$ organisms do not grow or divide; in fact, while the total count remains constant, the number of viable bacteria usually decreases by death during a long incubation. This fact may seem to indicate that the mutation occurs in non-dividing organisms. It does not, however, exclude the possibility of a cell turnover in which a cryptic lysis and replacement might balance one another. If this were the case, mutation in non-dividing bacteria could occur as an error in replication of the chromosome (or of DNA) during the cell division required for replacement. Much effort has been expended in examining this possibility by several methods (Ryan, 1959; Ryan, Nakada & Schneider, 1961). These attempts, which included reconstruction and penicillin experiments and the determination of DNA, did not reveal any cell turnover or cell growth in the stationary phase. Each piece of evidence was, however, circumstantial and not critical; together the evidence pointed to the conclusion that the stationary-phase bacteria were not dividing; in fact they did not even seem to be engaged in a turnover of DNA. A system of *his*$^-$ spheroplasts was, therefore, developed in order to investigate the role played by enzymes which digest macromolecules. This system and the evidence that mutations occur in spheroplasts are presented herewith.

METHODS

The media used were: Difco penassay broth, and penassay broth and G & T (Ryan, 1959) medium with 0·05 % glucose, both containing 20 % sucrose and 0·2 % $MgSO_4.7H_2O$. The last two hypertonic solutions were the complete and defined media used for maintenance and enumeration of spheroplasts. To solidify them, 1·0 % agar was added. Nutrient agar or G & T agar supplemented with 0·5 % yeast extract and 0·5 % NZcase (YECA agar) were used for counting the rods. To make spheroplasts, penicillin was used at a concentration of 1000–2000 units/ml. in penassay broth.

The bacterium used was *Escherichia coli* 15 *his*$^-$ *col-r* (colicine-resistant and simultaneously sensitive to all T phages; Ryan, Fried & Mukai, 1955; Mukai, 1960) derived from 15 *his*$^-$ *col-s* which has been used for mutation experiments since 1955. This bacterium was grown in penassay broth at 37° overnight with gentle rotation. Following this, 1·0 ml. of the fully grown suspension was introduced into 4·0 ml. of penassay broth and incubated at 37° for 2–3 hr. in the same way, to bring the bacteria into the logarithmic phase. Suspensions thus obtained were used for all experiments.

The number of viable spheroplasts was determined by diluting the suspension in hypertonic penassay broth whereupon 0·5 ml. of a suitable dilution was plated in the same medium containing 1·0 % agar melted and cooled to 45°. The colonies develop from both spheroplasts and rods; by subtracting the number of rods the number of spheroplasts can be calculated.

For enumerating the rods, the suspension was diluted with 0·85 % saline solution and 0·1 ml. of the diluent was plated on nutrient agar medium. This procedure destroyed the spheroplasts but not the rods.

The procedure for making spheroplasts was almost the same as that of Lederberg (1956) and Lederberg & St Clair (1958). The efficiency of forming spheroplasts and their stability was first compared at 37° and at 20°. After introducing 1·0 ml. of cell suspension into 4·0 ml. of hypertonic penassay broth which contained 1000 units of penicillin/ml., the mixture was incubated at both temperatures. Samples were taken at 3 and at 8 hr. and the number of viable spheroplasts and rods was determined. As Fig. 1*a* shows, spheroplasts formed at 20° were more stable than those formed at 37° and the efficiency of their formation (intact spheroplasts/rods) was rather better at 20° than at 37°. Figs. 1(*b*) and (*c*) show data obtained from spheroplast preparations at 20°. The number of viable spheroplasts did not decrease appreciably during 6 hr. of incubation but the proportion of rods decreased rapidly to 10^{-3} during the first 3 or 4 hr. of treatment with penicillin, and then more gradually. In Fig. 1(*b*), one of the two curves for rod number was obtained by diluting and plating the suspension without washing, while the other is based on the behaviour of washed cells. This result shows that there was little influence on the rod count even when penicillin was not washed out of the suspension before plating. Two sugar concentrations, 10 and 20 %, were also compared; Fig. 1(*c*) shows that there is no real difference between them. On the basis of the results described above, incubation was carried out in penicillin and 20 % sucrose for 4 hr. at 20° to create conditions suitable for making spheroplasts.

After the spheroplasts were made the suspension, which had been in penicillin

hypertonic penassay broth at 20° for 4 hr., was centrifuged in a Serval Centrifuge (type SS-1) at about 36,000 *g* for 10 min., the supernatant fluid decanted, and the organisms were resuspended in the same volume of G & T medium containing 20 % sucrose and 0·2 % $MgSO_4.7H_2O$ but no histidine. This procedure was repeated 3 times (two washings) in a 5° cold room and the final suspension diluted tenfold in the same medium. During this procedure there was no appreciable death of spheroplasts.

The cell suspension thus obtained, which contained from 5×10^3 to 1×10^7 spheroplasts/ml. and was clear to the naked eye, was distributed as 2 ml. portions into more than a hundred (usually about two hundred) small tubes and incubated statically at 20°. During this incubation the numbers of viable spheroplasts and rods were counted by sampling one or two clear cultures every day.

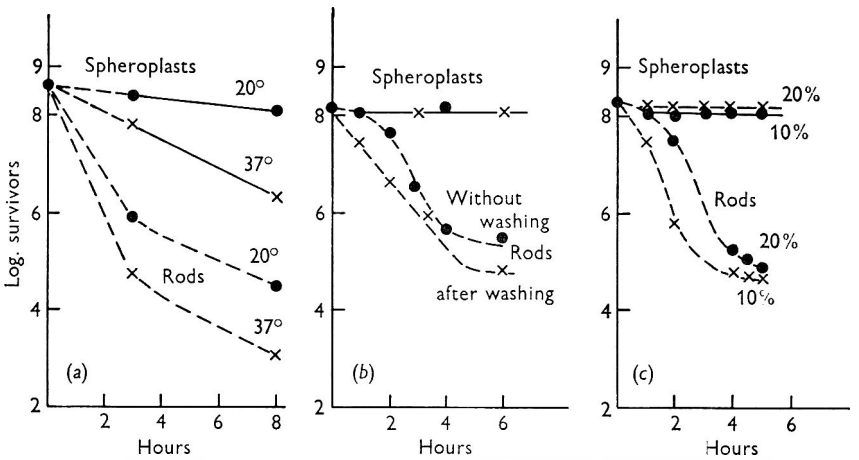

Fig. 1. (*a*) The effect of temperature on spheroplast formation. (*b*) The effect of washing twice with saline on spheroplast formation at 20°. (*c*) The effect of sugar concentration on spheroplast formation at 20°. Solid lines represent the viable spheroplast number and dotted lines the viable rod number.

Recovery of his⁻ *spheroplasts* was achieved by plating washed suspensions on penassay agar plus 20 % sucrose and 0·2 % $MgSO_4.7H_2O$ where the spheroplasts produced rods and visible colonies. The fraction of spheroplasts which converted into rods was calculated from the number of spheroplasts in the suspension plated (enumerated in a Petroff–Hauser counting chamber) and the number of colonies produced; it varied in five different experiments from 56 to 109 %. In the experiment reported in Fig. 1*a* conversion was 109 % at 3 hr. and 80 % at 8 hr.

The method of calculating the mutation rate of *his*⁻ spheroplasts to *his*⁺ was based on the fact that if a spheroplast of *his*⁺ genotype is introduced into medium of high osmotic pressure devoid of both penicillin and histidine it is able to grow and will yield rod-shaped bacteria. The culture will become turbid through the overgrowth of such *his*⁺ mutants, or of whatever *his*⁺ rods were present in the spheroplast population. Consequently, the mean number of mutants, m, can be calculated from the frequency of clear cultures, P_0, and the zero term of the Poisson equation:

$$P_0 = e^{-m}. \qquad (1)$$

Knowing that it takes about 125 hr. for a single *his*⁺ mutant spheroplast to form

rods and overgrow a culture, one can use the P_0 at this time to estimate the average number, m, of *his*$^+$ mutants per culture at the time of distribution. These mutants arose, of course, during previous growth.

Thereafter, while the suspensions are incubated under conditions of histidine starvation, mutations from *his*$^-$ to *his*$^+$ occur among the spheroplasts, and very occasionally among the rods, which result in the continued appearance of overgrowths. These new overgrowths increase the value of m as calculated by equation (1). The rate of mutation/bacterium/hr. (μ) can be calculated (Ryan, 1955, 1959) from the equation:

$$\mu = \frac{km^1}{N_0(1-e^{-kt})}, \tag{2}$$

where m^1 is now the average number of *mutations* (m at time t_2 minus m at time t_1, which is any time after 125 hr., the time required for the overgrowth of pre-existing *his*$^+$ mutants), t equals t_1 minus t_2 in hr., N_0 the number of bacteria present 125 hr. before t_1, and k is the death rate constant calculated from the formulation:

$$N = N_0^1 e^{-kt}, \tag{3}$$

N_0^1 being the number of bacteria at time 0 and N the number at time t in hr. Equation (1) can be used to calculate m because each mutation in the stationary phase gives rise to one new mutant and where no mutations occur, the culture remains clear.

In cases in which death cannot be observed, the viable cell number remaining constant, the formulation

$$\mu = \frac{m^1}{N_0 t} \tag{4}$$

is used.

Usually the frequency of *his*$^+$ mutations in an *his*$^-$ culture is about 2×10^{-7} bacteria/hr. when normal rod-shaped bacteria and not spheroplasts are used. The chance of mutation/bacterium/hr. during growth is usually about 4×10^{-8} and, in a stationary phase with glucose present, about 2×10^{-9} (Ryan, 1955, 1959).

RESULTS

Representative results are shown in Fig. 2. Part (*a*) shows the death curves of spheroplasts and rods. In this case, the rod number is nearly constant but the spheroplast number decreases rapidly in early stages and then gradually. Fig. 2(*b*) shows the behaviour of m with time. The curve rises rapidly for the first 125 hr. and then gradually until 200 hr. The first rapid rise of the curve is caused by the pre-existing *his*$^+$ mutants which have finished overgrowing at about 125 hr. Thereafter the gradual increase in m is due to the overgrowth of *his*$^+$ mutants which arose after the cultures were suspended in medium devoid of histidine. Eventually, when the cultures contained only about 3×10^5 cells/ml., mutations among them became too rare to be detected. The average frequency of pre-existing *his*$^+$ mutants in this experiment was about 2×10^{-8} and the μ for stationary phase cells was about 2×10^{-9}/bacterium/hr. Table 1 shows that these figures are not exceptional. The values calculated on the basis of spheroplast number more closely resemble those calculated for *his*$^-$ bacteria which had never been converted into spheroplasts. If it were supposed that the *his*$^+$ overgrowths observed were due to the small fraction of rods

inevitably present in suspensions of spheroplasts, then their mutation rates would have to be unrealistically high and mutation would unexpectedly cease when the spheroplast number became small but the rod number remained as large as ever (Fig. 2). It is, on the contrary, judged that the *his*$^+$ overgrowths were due to mutations in the spheroplasts. The somewhat smaller values for the initial frequency of

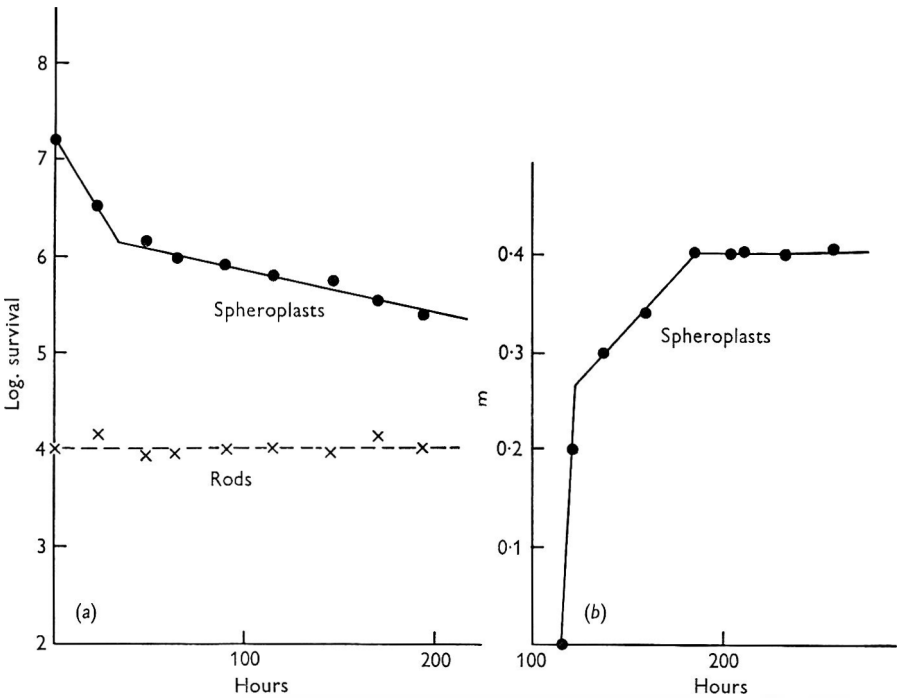

Fig. 2. Stationary phase mutation. Spheroplasts of *Escherichia coli* 15 *his*$^-$ made with penicillin were washed, resuspended, diluted in hypertonic G & T liquid medium without histidine and distributed as 2 ml. samples into 200 small tubes. They were incubated at 20° and 4 determinations were made at each time of the number of surviving spheroplasts and rods and also of the number of clear cultures from which m was calculated. (*a*) The number of viable spheroplasts (solid line) and of viable rods (dotted line). (*b*) The average number of mutations, calculated from P_0, plotted against time. At the first break in the curve, m is equivalent to a frequency of *his*$^+$ back mutants in the original culture of about 2×10^{-8}; thereafter mutation occurred in the stationary phase at a rate, μ, of $1{\cdot}7 \times 10^{-9}$ until it could no longer be detected because of the small number of cells remaining.

his$^+$ mutants calculated on the basis of spheroplast mutation as compared with that obtained for normal *his*$^+$ cells can be attributed to a lower efficiency in the recovery of *his*$^+$ spheroplasts, because of the use of synthetic medium (the *his*$^-$ spheroplasts recovered on penassay agar).

Experiments were made in which spheroplasts were burst in hypotonic penassay broth and then provided with sucrose, while another group was simply suspended from the beginning in the hypertonic medium. If mutations really occur in spheroplasts, one would not expect them in the suspension of burst cells but would expect them in the control suspension. These experiments ended in failure, because in the experimental culture the rods grew on nutrients liberated from the shocked spheroplasts.

Table 1. *Mutation rates from* his⁻ *to* his⁺ *in the stationary phase and frequencies of* his⁺ *mutants among freshly grown* his⁻ *cultures estimated on the basis of the number of spheroplasts and on the basis of the number of rods present*

	Calculated on the basis of spheroplasts		Calculated on the basis of rods	
	Initial frequency of		Initial frequency of	
Expt.	$his^+ = \frac{m \text{ at 125 hr.}}{N \text{ at 0 hr.}}$	$\mu = \frac{km^1}{N_0(1-e^{-kt})}$	$his^+ = \frac{m \text{ at 125 hr.}}{N \text{ at 0 hr.}}$	$\mu = \frac{m^1}{Nt}$
1 (see Fig. 2)	$1{\cdot}8 \times 10^{-8}$	$1{\cdot}7 \times 10^{-9}$	$2{\cdot}9 \times 10^{-5}$	$1{\cdot}5 \times 10^{-7}$
2	$3{\cdot}5 \times 10^{-8}$	$3{\cdot}3 \times 10^{-9}$	$4{\cdot}6 \times 10^{-5}$	$5{\cdot}5 \times 10^{-6}$*
3	$4{\cdot}6 \times 10^{-8}$	$3{\cdot}4 \times 10^{-9}$	$2{\cdot}6 \times 10^{-5}$	$5{\cdot}2 \times 10^{-6}$*
values obtained with normal *his*⁻ cells†	2×10^{-7}	2×10^{-9}	2×10^{-7}	2×10^{-9} 4×10^{-8}*

* In these cases the rods increased in number so that the rate was calculated as the chance per bacterium per generation ($a = (mln2)/N$, Ryan, 1955) and then converted to μ on the basis of a 50 min. generation time.

† Ryan (1955, 1959).

DISCUSSION

Szybalski & Pitzurra (1959) reported mutation to streptomycin-independence in spheroplasts of *Escherichia coli* produced with LiCl. Although they did not prove that the mutants observed did not arise by mutation in the rods present, it seems likely that they took place in the spheroplasts. Their 'spheroplasts' were unlike ours, inasmuch as they did not burst in saline while in 20 % sucrose the mutations occurred infrequently, if at all, even in the presence of chemical mutagens.

The present work with spheroplasts bears out previous experiments (Ryan, 1959) in strongly supporting the conclusion that mutation from *his*⁻ to *his*⁺ occurs in non-dividing bacteria and that the mutated *his*⁺ genotypes manifest their phenotypes in a non-dividing condition to grow in a medium without histidine.

However, the question still remains whether mutation requires DNA replication. The number of DNA-containing bodies could not be observed to increase in the spheroplasts which shrank somewhat during the stationary phase. Experiments to detect the replication of DNA by determining its quantity, and by the use of ^{14}N and ^{15}N with density gradient centrifugation, were negative. (Ryan, *et al.* 1961). Nonetheless, most investigations of induced mutation (Ryan, 1962) suggest that mutation occurs during DNA replication and support the theory that one of the mechanisms of mutation is pairing error. Errors in base pairing might be made not only during the replication of DNA but also by specific exchange when it is not replicating. This possibility is being examined by the use of isotope-marked base analogues and thymidine which may be incorporated into the DNA and induce mutations in non-dividing bacteria. It is also necessary to know whether a turnover of DNA was not observed because of isolation of the pool of DNA precursors, and whether an unusually high mutability characterizes an undetectably small turnover of DNA in non-dividing cells.

It is interesting to note that, although the *his*⁻ gene reverts spontaneously and in response to ultraviolet radiation (Ryan, Fried & Schwartz, 1954) it does not respond

to 2-aminopurine, 5-bromouracil or nitrous acid under conditions where auxotrophs are induced to form (Okada & Ryan, unpublished results). This suggests that base-pair insertions or deletions of the sort discussed by Crick, Barnett, Brenner & Watts-Tobin (1961), unequal sister-strand crossing-over in a compound locus as proposed by Grigg & Sergeant (1961) or transversions as defined by Freese (1961) may be involved. Yet proflavin, which induces auxotrophs, also does not revert the *his*⁻ gene. Moreover, the possibility has not been excluded that the mutable unit under investigation is composed of RNA or protein even though it is located in a DNA-containing body (Ryan & Wainwright, 1954). Not enough evidence is as yet at hand to require rejection of the proposition that some natural mutations may result from copy-errors during the replication of the gene.

This work was supported in part by grants from the U.S. Public Health Service and the National Science Foundation. One of the authors (T. Nagata) was a Public Health Service Predoctoral Trainee in Genetics.

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Printed in Great Britain

High Infectivity of *Salmonella typhimurium* newly infected by the *colI* factor

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(*Received* 13 *April* 1962)

SUMMARY

In 18 hr. incubation of broth inoculated with a majority of bacteria of *Salmonella typhimurium* LT2 *col*$^-$, i.e. non-colicinogenic, and a minority of strain LT2 (*colI*), i.e. carrying colicine factor *colI*, 30–70 % of the *col*$^-$ bacteria acquired *colI*; this increased to 50–90 % after 2 hr. of secondary incubation after tenfold dilution with broth. These cultures, containing a high proportion of bacteria newly infected by *colI*, transmitted *colI* to about 50 % of the bacteria of a *col*$^-$ strain in 1 hr. (whereas pure cultures of LT2 (*colI*) transmitted to only 0·01 %); they are termed HFC (high-frequency colicinogeny-transferring). An inoculum containing 1–3 recently infected bacteria from an HFC preparation of a streptomycin-sensitive strain sufficed to transmit *colI* to a resistant strain in streptomycin broth. We infer that 30–100 % of bacteria newly infected by *colI* are 'competent donors', able to transmit *colI*. By the same test the proportion of competent donors in LT2 (*colI*) strains was only 0·02 %. Inoculum size, ratio of inoculum components, motility, aeration and secondary incubation affected the HFC property of mixed cultures in a way explicable by the need for the epidemic spread of *colI* in the *col*$^-$ component to reach a peak at the time of testing. The rate of loss of the HFC property on further growth indicated that for 3–7 generations the progeny of newly infected bacteria are competent donors. Transmission was associated with clumping and pairing. Bacteria of an HFC preparation pair with 10 % of an acceptor population within 2 min. of mixing; completion of transfer requires $2\frac{1}{2}$–30 min. Non-availability of nutrients and some growth inhibitors interfere with transfer.

We conclude that *colI* multiplies autonomously in newly infected bacteria and their immediate progeny and enables them to conjugate, but does not confer ability to conjugate in established colicinogenic strains; perhaps it is then integrated into the host chromosome.

INTRODUCTION

In a previous paper (Ozeki, Stocker & Smith, 1962) we showed that the genetic factors or episomes *colI*, *colE*2, etc., which determine, respectively, the production of colicines I, E2, etc., could be transferred from standard colicinogenic strains of *Escherichia coli* or *Shigella sonnei* to *Salmonella typhimurium* strain LT2. When a broth culture of *S. typhimurium* LT2 (*colI*)—i.e. an LT2 line producing colicine I—as donor strain was incubated with a broth culture of a non-colicinogenic (acceptor) LT2 strain, < 0·1 % of the acceptor bacteria became colicinogenic during incubation for 1 hr., but after incubation for 18 hr. about 50 % of them had done so. We

showed that in a broth culture of LT2 (*colI*) only about 1 bacterium in 5000 was a 'competent donor', able to transmit the *colI* factor by contact to a non-colicinogenic acceptor bacterium. This accounted for the low proportion of acceptor bacteria which acquired colicinogeny in 1 hr. contact with a donor strain. We attributed the high proportion of acceptor bacteria which acquired *colI* on long incubation of a mixture, to serial transmission of *colI* amongst the acceptor bacteria, initiated by the few acceptor bacteria which acquired the factor during the first hour. We explained this 'epidemic spread' by the hypothesis that many or perhaps all bacteria which newly acquire *colI* become competent donors, able to pair with, and transmit *colI* to, acceptor bacteria.

We were led to this hypothesis by the observation that overnight broth cultures inoculated directly from one particular Dorset egg stock culture of a donor strain, CL114, transmitted *colI* to a considerable proportion (about 10%) of acceptor bacteria in 1 hr. When material from this stock slope was streaked out only about 5% of the *Salmonella paratyphi* B colonies obtained were colicinogenic; broth cultures inoculated from such colicinogenic colonies transmitted colicinogeny no better than did broth cultures of LT2 (*colI*). But broth cultures inoculated with a mixture of the purified colicinogenic and non-colicinogenic components of strain CL114, in the same ratio as was found in the stock slope, transmitted to about 10% of an acceptor culture in 1 hr. These broth cultures inoculated with mixtures of colicinogenic and non-colicinogenic bacteria, in the ratio of 1:20, when streaked out after overnight incubation yield about 50% of colicinogenic colonies, as a result of the acquisition of *colI* by bacteria of the originally non-colicinogenic component inoculated. We therefore surmised that such newly infected bacteria might be much more effective as donors of colicinogeny than bacteria which had carried *colI* for many generations.

The experiments to be described show that in strain LT2 a high proportion (half or more) of bacteria which have just acquired *colI* become competent donors, and that this competence persists in their progeny for between 2 and 7 generations. We also describe the kinetics of transfer of *colI*, show that it is associated with the formation of pairs and clumps and describe the effect of various environmental factors and metabolic inhibitors on the process of transfer.

In all experiments, except the initial ones on the *Salmonella paratyphi* B strain mentioned above, we used *S. typhimurium* strain LT2 carrying the *colI* factor derived from *Shigella sonnei* strain P9 (Ozeki *et al.* 1962). In some experiments the strain inoculated into broth with LT2 (*colI*) was not non-colicinogenic but, instead, LT2 (*colE2*); however, the presence of *colE2* in these bacteria seemed to have no effect on the behaviour of *colI* in them. In a later paper (Smith, Ozeki & Stocker, in preparation) we shall describe the transmission of *colE2*, etc., by such colicinogenic bacteria newly infected by *colI*.

METHODS

In general the strains, media, tests for colicinogeny and other methods used were as described by Ozeki *et al.* (1962). Some additional strains used are listed in Table 1. The major component of a Dorset egg stock culture labelled CL114 was a *Salmonella paratyphi* B strain, of unknown origin because the stock culture concerned purported to be a colicinogenic *Escherichia coli* strain; colicinogenic sublines of this

S. paratyphi B strain (producing colicine I) we designate CL114 ($colI_2$), non-colicinogenic sublines CL114 *col*$^-$. Except where the contrary is indicated the colicine I factor used was that derived from *Shigella sonnei* P9 (Ozeki *et al.* 1962). The colicine I factor of *Salmonella paratyphi* B, strain CL114 is indicated $colI_2$. The colicines determined by these two factors are indistinguishable.

Table 1. *Bacterial strains used**

Laboratory stock no.	Genotype	Remarks
(*a*) Sublines of *Salmonella typhimurium* strain LT2		
SL497	*cysC-7 str-r*	—
SL486	*cysD-36* (*colI*)	—
SL636	*cysD-36* (*colE2*)	—
—	*cysB-12 tryD-10* (*colI*)	—
—	*cysB-12 tryD-10* ($colI_2$)	—
SL536	*adeC-7 proA-46 fla-29* (*colI*)	—
SL747	*adeC-7 proA-46 fla-29 str-r*	—
—	*adeC-7 proA-46 fla-29 str-r* (*colI*)	—
SL724	*athA-4 phe-401 fla-28 str-r*	—
SL541	*athA-4 phe-401 fla*$^+$ *str-r*	Motile revertant of SL724
SL656	*mot*$^-$ *str-r*	Derived from SW1153, a 'paralysed' mutant of LT2 wild type (Iino, 1958)
SL737	*mot*$^+$ *str-r*	Motile revertant of SL656
—	*proA-46*	—
—	*proA-46* (*colI*)	—
(*b*) Sublines of *S. typhimurium* var. copenhagen O = SW541 (Stocker *et al.* 1953)		
SL178	*fla*$^-$ *str-r*	—
SL722	*fla*$^-$ *str-r* cured of an A phage	—
SL723	*fla*$^+$ *str-r*	Non-lysogenic *fla*$^+$ transductant of SL722

Requirements for growth: *ade* = adenine; *ath* = adenine and thiamine; *cys* = cystine; *phe* = phenylalanine; *pro* = proline; *try* = tryptophan.

str-r = streptomycin-resistant (1 mg./ml.).

fla$^-$ or *fla-28*, etc. = non-flagellated, non-motile; *fla*$^+$ = flagellated.

mot$^-$ = flagellated, non-motile ('paralysed'); *mot*$^+$ = not paralysed.

(*colI*) = carrying *colI* factor derived from *Shig. sonnei* P9 (Ozeki *et al.* 1962).

($colI_2$) = carrying *colI* factor derived from *S. paratyphi* B, CL114.

(*colE2*) = carrying *colE2* factor derived from *Shig. sonnei* P9.

Most of the auxotrophic mutants used were obtained from the Department of Genetics, Carnegie Institution of Washington at Cold Spring Harbor (see Demerec *et al.* 1956; Clowes, 1958; Miyake & Demerec, 1960). Non-flagellated, streptomycin-resistant and colicinogenic stocks were then derived by us.

* See also the strains listed in Table 1 of Ozeki *et al.* (1962).

In experiments in which motile and non-motile variants of *Salmonella typhimurium* strains were compared (Table 3) we either used non-motile strains originally isolated as such, and motile sublines obtained from them by mutation or transduction (Stocker, Zinder & Lederberg, 1953); or selected non-motile variants (either non-flagellated or 'paralysed') from motile strains by exposure to phage $\phi\chi$ (Sertic & Boulgakov, 1936; Meynell, 1961). In experiments in which we used non-motility as a phenotypic or genotypic marker, we chose non-motile strains in which motile bacteria arising by phenotypic variation (Quadling & Stocker, 1957) were absent or rare. The semi-solid medium used was that of Stocker *et al.* (1953) with a decreased

agar concentration: i.e. peptone, 10 g.; Difco gelatine, 80 g.; Difco agar, 3 g.; sodium chloride, 5 g.; water, 1000 ml.

Bacteria visibly 'labelled' with formazan granules (Hartman, Mudd, Hillier & Beutner 1953) were obtained by growth in broth containing 2,3,5-triphenyltetrazolium chloride, 0·01 % (w/v); after 24 hr. at 37° the culture was diluted tenfold into broth containing twice this concentration of the tetrazolium compound. After a further 2 hr. incubation period at least 50 % of the bacteria contained visible granules.

High-speed mixing of broth cultures was effected in an M.S.E. blendor as described by Stocker & Campbell (1959). Treatment for 2 min. at full speed (about 12,000 rev./min.) sufficed to break up all clumps.

To see whether *Salmonella typhimurium* newly infected by the *colI* factor developed any new antigen (compare the f+ antigen of *Escherichia coli* described by Ørskov & Ørskov, 1960) a rabbit was given two courses (0·5 ml., then 1 ml. weekly for 3 weeks; later five 1·0 ml. inocula during 2½ months) of intravenous inoculations of a formalinized broth culture of an HFC preparation (see below) of strain LT2 *proA-46*. O-agglutinable suspensions were made by deflagellation (by high-speed mixing) and washing of a broth culture of strain *proA-46*, and of an HFC culture of the same strain.

RESULTS

When material from a Dorset egg stock culture labelled CL114 was plated on nutrient agar, only about 5 % of the *Salmonella paratyphi* B colonies obtained were colicinogenic, producing colicine I; but when broth inoculated from the same slope was streaked out after overnight incubation more than half the *S. paratyphi* B colonies obtained were colicinogenic (Table 2, Expt. 1). A similar increase in the proportion of colicinogenic bacteria occurred during overnight incubation of broth inoculated with an artificial mixture, in the ratio 1:20, of the colicinogenic and the non-colicinogenic *S. paratyphi* B components of stock culture CL114 (Table 2, Expt. 4); or when the inoculum was a similar mixture of genetically labelled LT2 sub-lines, one non-colicinogenic and the other carrying *colI*, derived either from *S. paratyphi* B, CL114 (Table 2, Expt. 7) or from *Shigella sonnei* P9 (Table 2, Expt. 6). In the latter two experiments testing of the nutritional character of the colonies showed that the increase in the proportion of colicinogenic organisms from 5 to 50–80 % resulted from the acquisition of the *colI* factor by bacteria of the originally non-colicinogenic strain, and not from overgrowth by the donor bacteria.

Donor preparations, i.e. broth cultures grown overnight from mixed inocula of colicinogenic and non-colicinogenic bacteria in the ratio 1:20, and cultures inoculated only with the colicinogenic component, were compared in respect of their ability to transmit the *colI* factor to a streptomycin-resistant acceptor strain during 1 hr. contact (Table 2). The pure cultures of colicinogenic strains transmitted to less than 0·2 % of the acceptor bacteria; whereas donor preparations grown from mixed inocula infected 10–21 %. The same result was obtained whether the Salmonella strain used for the donor preparations was *Salmonella paratyphi* B, CL114 or *S. typhimurium* LT2; and whether the *colI* agent was that derived from CL114 or that from *Shigella sonnei* P9. As the behaviour in strain LT2 of the *colI* agent from P9 has been fully investigated (Ozeki, Stocker & Smith, 1962) we used this system in all later experiments. In experiments on the transmission of *colI* by donor prepara-

tions, bacteria of the initially non-colicinogenic component of the donor mixture accepted *colI* during the growth of the donor mixture; when the donor preparation was tested for its ability to transmit *colI* to a third strain (usually marked by streptomycin-resistance) these newly infected bacteria now acted as donors. We shall therefore call the non-colicinogenic strain used as one component of a donor mixture the *intermediate* strain, since it first accepts *colI*, then transmits it.

Table 2. *Behaviour of cultures inoculated with colicinogenic or non-colicinogenic strains, and with mixtures thereof*

Broths inoculated either directly from stock culture slopes, or with about 5×10^4 and 10^6 bacteria/ml. of the indicated strains, were streaked on nutrient agar before and after 18 hr. incubation, and the proportion of colicinogenic colonies determined. To test their transmitting ability the incubated broth cultures were mixed in equal parts with a culture of a streptomycin-resistant, non-colicinogenic acceptor strain (SL178); after 1 hr. incubation the mixture was streaked on steptomycin agar and the resulting acceptor colonies were tested for colicinogeny.

Expt. no.	Inoculum	Colicinogenic bacteria (%) Before incubation	Colicinogenic bacteria (%) After 18 hr. incubation	% *str-r* acceptors acquiring *colI in* 1 hr.
1	CL114 'stock'*	About 5	68	12
2	CL114 ($colI_2$)†	100	100	0·1
3	CL114 *col*-‡	0	0	0
4	CL114 ($colI_2$) and CL114 *col*-, in ratio 1:20	5	60	16
5	LT2 *cys try* (*colI*)	100	100	0·1
6	LT2 *cys try* (*colI*) and LT2 wild-type *col*-, in ratio 1:20	5	78	11
7	LT2 *cys try* ($colI_2$) and LT2 wild-type *col*-, in ratio 1:20	5	54	21

* CL114 'stock': broth inoculated direct from stock slope of *S. paratyphi* B, strain CL114.
† CL114 ($colI_2$): purified colicinogenic component of stock slope CL114.
‡ CL114 *col*-: purified non-colicinogenic component of stock slope CL114.

The preponderance of non-colicinogenic *Salmonella paratyphi* B in the stock culture slope CL114 was found to result from an instability of the colicinogenic state in this strain, since Dorset egg slopes inoculated with CL114 ($colI_2$), i.e. the purified colicinogenic component of the original culture, after several weeks at room temperature yielded a majority of non-colicinogenic colonies, as also did clones of CL114 *col*- re-infected with $colI_2$, or infected with the *colI* factor from *Shigella sonnei* P9. A clone of CL114 (*colI*) (*colE2*) after some weeks gave mainly CL114 *col*- and a few CL114 (*colI*) and CL114 (*colI*) (*colE2*). The instability of colicinogeny for *colI* and *colE2* in *Salmonella paratyphi* B, CL114 contrasts with their stability in *S. typhimurium* LT2.

Incubation of acceptor bacteria for up to 2 days with supernatant fluids from 'donor preparations' (i.e. overnight mixed cultures of colicinogenic and non-colicinogenic strains) sterilized by filtration or chloroform treatment, did not result in any detectable acquisition of colicinogeny; and the bacteria from donor preparations spun down and resuspended in fresh broth transmitted colicinogeny as well as did the original preparations. This is what would be expected if, as we believe, transmission of colicinogeny occurs only by cell conjugation.

Factors affecting the transmitting ability of donor preparations

As we surmised that the high transmitting ability of overnight mixed cultures resulted from the presence of a high proportion of bacteria of the intermediate strain which had very recently acquired the *colI* factor, we sought to obtain donor preparations in which a high proportion of the bacteria of the intermediate strain were colicinogenic; in general such donor preparations transmitted colicinogeny well, i.e. infected a high proportion of the bacteria of the acceptor strain during 1 hr. contact. A method which generally gave satisfactory results was to inoculate 10 ml. of broth with about 5×10^5 donor bacteria and about 10^7 intermediate bacteria, and to incubate at 37° without aeration by shaking for 18 hr., by which time the proportion of colicinogenic bacteria had increased from 5 % to 30–70 %. When this preparation was diluted tenfold in broth and incubated for a further 2 hr., the proportion of colicinogenic bacteria further increased to 50–90 %, presumably as a result of the rapid transmission of the *colI* factor by the recently infected bacteria of the intermediate strain. The donor preparations so obtained transmitted colicinogeny to about 20 % of the bacteria of an acceptor culture during 15 min. contact, and to 50 % in 60 min.; we shall term them HFC (high frequency colicinogeny-transferring) preparations.

Composition of inoculum of donor preparation. To determine the effect of varying the composition of the inoculum, the 10 ml. tube of broth used for growth of a donor preparation received the standard inoculum of intermediate bacteria, i.e. 10^6 bacteria/ml., and graded numbers of donor bacteria. When the inoculum of donor bacteria was < 5000/ml. (and the donor:intermediate ratio was < 1:200) < 1 % of the intermediate bacteria became colicinogenic after 18 hr. and the transmitting ability of the preparation was very low; with inocula of donor bacteria between 5000 and 10^6/ml. (and donor:intermediate ratios between 1:200 and 1:1) a high proportion of the intermediate bacteria became colicinogenic during overnight incubation, and ability to transmit was high; with a donor:intermediate ratio of 10:1 or 100:1, the proportion of intermediate bacteria which acquired colicinogeny after overnight incubation was low (about 3 %). We therefore adopted a donor:intermediate ratio of 1:20 as the inoculum for routine growth of HFC preparations. Tubes inoculated with 10^6, 10^7, 10^8 or 10^9 bacteria/ml. with the donor:intermediate ratio constant at 1:20, gave HFC preparations containing 39–68 % colicinogenic bacteria, and transmitting *colI* to 12–24 % of the acceptor bacteria in 30 min.

Effect of aeration by shaking. When broth inoculated with a mixture of colicinogenic and non-colicinogenic bacteria was shaken during the overnight incubation, transfer of colicinogeny to the non-colicinogenic bacteria was greatly decreased, as compared with an unshaken control (Ozeki *et al.* 1962); such shaken cultures, as might be expected, transferred colicinogeny very poorly during 60 min. contact with an acceptor. By contrast, when a donor preparation, grown by overnight incubation without shaking from a standard inoculum, was shaken during the 2 hr. final incubation stage, there was a further increase in the proportion of colicinogenic intermediate bacteria, e.g. from 63 to 88 %, as compared with an increase to 94 % in an unshaken control; and the HFC preparation so obtained transmitted well.

Effect of medium. HFC preparations could be prepared by incubating donor and intermediate bacteria in a defined glucose + ammonia + salts medium supplemented

with any required growth factors; but the percentage of intermediate bacteria which acquired colicinogeny was more variable than when broth was used. In the defined medium, though not in broth, transfer of colicinogeny to the intermediate bacteria occurred even when the mixture was shaken during the overnight incubation. Such cultures transmitted colicinogeny at high frequency.

Effect of bacterial motility. When the non-colicinogenic (intermediate) component of the mixed inoculum was a non-motile strain, either non-flagellated or 'paralysed' (Stocker *et al.* 1953), the proportion of the intermediate bacteria which acquired colicinogeny during the primary overnight and the secondary 2 hr. incubation was greatly decreased as compared with a control mixture in which the intermediate was a motile form of the same strain; e.g. < 1 % colicinogenic after the 2 hr. incubation as compared with about 70 % in the control (Table 3). However, when secondary incubation was continued for 24 hr. the proportion of colicinogenic intermediate bacteria increased, e.g. to 50 %. When the intermediate component was motile, satisfactory HFC preparations were obtained even when the donor component was non-motile.

Table 3. *Effect of motility or non-motility of intermediate strain on proportion of intermediate bacteria acquiring* colI

Proportion of intermediate bacteria colicinogenic determined on samples plated on streptomycin nutrient agar after 18 hr. primary incubation, and after 2 and 24 hr. secondary incubation subsequent to tenfold dilution in broth.

Donor: LT2 *ade C-7 pro A-46 fla*$^-$ (*colI*).
Intermediate: as indicated, in all cases *col*$^-$ and *str-r*.
Inoculum: 5×10^4 donors/ml., 10^6 intermediates/ml.

Intermediate strain	Percentage in intermediate bacteria colicinogenic after: 18 hr. primary incubation	2 hr. secondary incubation	24 hr. secondary incubation
SL722 (*fla*$^-$)	< 0·5	< 1	13
SL723 (*fla*$^+$ transductant of SL722)	3	16	18
SL656 (*mot*$^-$)	< 0·5	< 0·5	50
SL737 (*mot*$^+$ mutant of SL656)	40	70	80
SL 724 (*fla*$^-$)*	11	13	15
SL 541 (*fla*$^+$ mutant of SL724)	34	83	91

* A very few motile bacteria were seen on microscopy of this culture.

Effect of secondary incubation. In the standard procedure for making an HFC preparation an overnight mixed culture was diluted tenfold in broth and incubated for 2 hr. This secondary incubation resulted in an increase in the proportion of colicinogenic bacteria and in a greater increase in transmitting ability. For instance, in one experiment the proportion of colicinogenic intermediate bacteria increased from 27 to 86 % during secondary incubation; samples taken before and after secondary incubation (and adjusted to the same bacterial count, after blendor treatment to break up clumps) infected 5 and 40 %, respectively, of the bacteria of a streptomycin-resistant acceptor strain in 1 hr. contact. No large increase in the

proportion of colicinogenic intermediate bacteria occurred when the overnight mixture was incubated for a further 2 hr. without addition of fresh broth. When the overnight mixed culture was diluted 1/100 or 1/1000 in fresh broth, the increase in proportion of colicinogenic bacteria during incubation for 2 hr. was less than when the culture was diluted 1/10 as in the standard procedure.

Cell pairs and clumps associated with transfer of the colI *factor*

Microscopy of HFC preparations showed many clumps; e.g. about 60 % of the bacteria in clumps of 2 to 20, average about 4, bacteria. No clumps were seen in broth cultures of non-colicinogenic or of pure colicinogenic strains, nor in mixtures of such cultures incubated for 1 hr. Shaking by hand did not break up the clumps; but after 2 min. treatment in a high-speed blendor less than 0·1 % of the bacteria were in clumps. After 30 min. incubation of the blendor-treated HFC suspension, 14 % of the bacteria were once more in clumps, on an average of three bacteria. To prove that the motile bacteria of an HFC preparation adhered to bacteria of an acceptor strain we mixed such an HFC preparation with a non-motile acceptor grown in tetrazolium broth, so that most bacteria contained a visible formazan granule or granules. Active movement of pairs of bacteria, one containing a formazan granule, proved that donor bacteria became attached to acceptor bacteria. Granule-bearing acceptor bacteria could also be seen attached to the clumps characteristic of HFC preparations. The use of this technique also showed that the bacteria of an HFC preparation adhered about as readily to bacteria of a colicinogenic strain, already carrying *colI* as they did to bacteria of the ordinary non-colicinogenic acceptor strain.

We also used an indirect test to detect the adherence of motile bacteria of an HFC preparation to acceptor bacteria. The acceptor strain used (SL747) was streptomycin-resistant and completely non-motile. After incubation of the HFC preparation for 15 min. with the acceptor culture, loopfuls of the mixture, and of dilutions of it, were plated on semi-solid medium containing streptomycin. There was no growth on control plates inoculated with the streptomycin-sensitive HFC preparation; on control plates inoculated with the acceptor strain there was growth only on the surface. On the plates inoculated with the mixture there appeared, in addition to surface growth, numerous microcolonies beneath the surface, up to 3 mm. below it. We suppose that these colonies developed from streptomycin-resistant non-motile acceptor bacteria carried into the depths of the medium by their motile streptomycin-sensitive partners. Many deep colonies were obtained from inocula of a 1/100 dilution, and a few from a 10^{-4} dilution of test mixtures in which the acceptor strain was either non-colicinogenic or was colicinogenic carrying *colI*. This confirmed our microscopical observation that bacteria of HFC preparations paired as readily with colicinogenic as with non-colicinogenic acceptor bacteria. When a pure culture of a motile, streptomycin-sensitive strain carrying *colI* was mixed with a culture of a non-motile, streptomycin-resistant acceptor, some deep colonies were produced from an inoculum of the undiluted mixture; but none from a 1/100 dilution. We infer that a broth culture of a strain carrying *colI* contains some bacteria able to adhere to acceptor bacteria, but far fewer than are present in an overnight mixed culture or HFC preparation; and too few to detect by microscopy. Presumably these few bacteria able to pair correspond to the about 1/5000

'competent donors' able to transmit the *colI* factor in such cultures (Ozeki *et al.* 1962). No deep colonies were obtained from control mixtures of non-colicinogenic motile streptomycin-sensitive and non-colicinogenic non-motile streptomycin-resistant strains.

Antigenic character of HFC preparations. The adherence of bacteria in HFC preparations to other bacteria presumably results from some alteration of their surface, which might cause some new antigenic specificity. A rabbit given two courses of inocula of an HFC preparation yielded sera with equal titres (1/6400) on O-agglutinable suspensions made from the HFC preparation and from the non-colicinogenic strain. After two absorptions with non-colicinogenic bacteria the sera no longer agglutinated either the HFC or the non-colicinogenic O suspension, even when tested undiluted. Thus in a very limited experiment we did not detect any new antigen in *Salmonella typhimurium* newly made colicinogenic for *colI*.

Treatment of HFC preparations by high-speed blendor; kinetics of transfer of colI *by HFC preparations*

Treatment of an HFC preparation in a high-speed blendor broke up all the clumps originally present; such treatment does not kill *Salmonella typhimurium*, but makes the organisms temporarily non-motile by breaking off their flagella (Stocker & Campbell, 1959). Such deflagellated HFC preparations and untreated controls transmitted *colI* to about the same proportion of the bacteria of a motile acceptor strain in 1 hr. But when the acceptor strain was non-motile the deflagellated HFC preparation transmitted rather less well than did the untreated control. This presumably indicates that the collisions of donor and acceptor bacteria, which would be less frequent when both are non-motile, are rate-limiting under these conditions.

Although a mixture of an HFC preparation and an acceptor culture after 60 min. incubation contained many clumps, the proportion of acceptor bacteria (selected by plating on streptomycin agar) which produced colicinogenic colonies was about the same when the mixture was de-clumped by blendor treatment before plating as when this step was omitted, probably because the spreading of the bacteria over the surface of the agar breaks up clumps about as well as does blendor treatment.

To study the time needed for transmission of *colI*, an HFC preparation and a streptomycin-resistant acceptor culture were mixed and incubated for 2 min. to allow pairing. The mixture was then gently diluted 1/100 or 1/1000 in broth, to preserve pairs already formed but to prevent further pairing; the dilute mixture was then incubated. At intervals samples were treated in the high-speed blendor to break up clumps and pairs, and either plated at once or after further incubation. It appeared (Table 4*a*, *b*): (i) that incubation of the undiluted mixture for 2 min. sufficed for 10 % of the acceptor population to pair with donors as against 30 % in a control mixture incubated without dilution for 22 min.; (ii) that in a small minority of pairs transfer of *colI* was completed (i.e. had reached a stage where separation of the partners did not affect the outcome) within 2 or $2\frac{1}{2}$ min. of mixing the cultures; (iii) that in most pairs transfer was completed by about 20 min. after pair formation.

Table 4. *Kinetics of transfer of* colI *as determined by blendor treatment*

(*a*) An HFC preparation, components *adeC-7 proA-46 fla*$^-$ (*colI*) and *cysD-36* (*colE2*), and an acceptor culture, *athA-4 phe-401 str-r*, were mixed in equal parts, and after 2 min. the mixture was diluted 1/100 in broth. At intervals samples were treated in the blendor and then plated on streptomycin agar. One sample of the mixture was incubated undiluted for 22 min., then treated in blendor and plated.

Sample	Time of blendor treatment, (min.)	Acceptor colonies colicinogenie	
		%	Number
Diluted	2½	0·8	2/252
	5	1·8	3/178
	10	4·1	3/73
	20	7·6	7/92
	35	10	9/90
Undiluted	22	31	27/86

(*b*) An HFC preparation (components as in (*a*) above) was treated in the blendor, then mixed with an acceptor culture as above. 2 min. later the mixture was diluted 1/1000 in broth and at intervals samples were treated in the blendor. All samples were incubated until 75 min. from time of original mixing, then plated on streptomycin agar.

Time of blendor treatment (min.) after	Acceptor colonies colicinogenie	
	%	number
3	2·7	7/259
10	5·6	19/338
25	7·6	31/408
45	8·9	23/258
75	10·1	36/348

Factors affecting the transfer of colI *from an HFC preparation to an acceptor culture*

Acceptor culture. When a suitable HFC preparation was incubated with an acceptor culture, the proportion of acceptor bacteria which acquired *colI* in 60 min. was about the same, regardless of whether the acceptor suspension was from an overnight unshaken broth culture, an overnight shaken broth culture, or an overnight unshaken culture diluted tenfold in broth and incubated for 2 hr. (the more concentrated cultures being diluted, so that all three acceptor suspensions had about the same total count). Thus the physiological state of the acceptor bacteria appears to be unimportant. The proportion of bacteria of a non-motile acceptor strain which acquired *colI* during 1 hr. contact with an HFC preparation (of motile bacteria) was about the same as when the acceptor strain was motile.

Aeration. In an experiment in which the mixture of the HFC preparation and the acceptor bacteria was incubated on the shaker for 1 hr., the proportion of acceptor bacteria which acquired colicinogeny was 50 %, while in an unshaken control it was 32 %.

Medium. In the experiments described above the transfer of *colI* to acceptor bacteria took place in broth, the HFC preparation and acceptor culture being mixed

without alteration of the medium. Fisher (1957*a*, *b*) observed that recombination in *Escherichia coli* K12 was influenced by the medium, being greatly decreased in liquid minimal medium lacking an energy source, and restored to normal by the addition of glucose and aspartate. We therefore looked for such effects on the transmission of *colI*. HFC and acceptor cultures were centrifuged and washed twice in minimal salts solution. Resuspension in fresh broth resulted in as good or nearly as good transfer of *colI* to acceptor bacteria as in control mixtures of the original broth cultures (e.g. 13 % in 30 min., as compared with 18 % in a control). Rather variable results were obtained when the washed HFC and acceptor cultures were resuspended and mixed in a minimal salts medium without a carbon source (minimal medium of Ozeki *et al.* 1962, but with glucose and citrate omitted). In one experiment (HFC strains *cysD-36* (*colI*) and *cysD-36 col*$^-$ and acceptor strain *adeC-7 proA-46 fla*$^-$ *str-r*) only about 0·4 % of the acceptor bacteria acquired *colI* in 30 min. (compared with 13 % when resuspended in broth), and the addition of glucose (0·2 %, w/v) to the final mixture only increased the degree of transfer to 1 %. In this experiment the addition of: aspartate (0·02 %, w/v); or of cystine (0·002 %, w/v), required by the donor strain; or of both adenine and proline (each 0·002 %, w/v), required by the acceptor strain, increased the rate of transfer to 9·5, 10 and 3 %, respectively.

However, in other experiments involving HFC and acceptor cultures twice washed and resuspended in minimal medium lacking citrate and glucose, there was good transfer (e.g. 44 % in 30 min.) when glucose was added to the mixture, and some transfer (e.g. 5 % in 30 min.) in its absence. Previous starvation of one or both components by aeration for 1 hr. in minimal salts solution did not affect the transfer which occurred at a low rate in the absence of glucose but did depress the transfer in the presence of glucose. These results, however, were not consistent enough to permit conclusions to be drawn as to whether starvation of the donor or the acceptor, or of both, caused inhibition of the transfer of *colI*.

Effect of metabolic inhibitors and of acriflavine

Various metabolic poisons and acriflavine (which interferes with multiplication of the F agent in *Escherichia coli*; Hirota, 1960) were tested for ability to prevent the transmission of *colI* from HFC preparations to acceptor cultures (Table 5). The agents were added to HFC and acceptor cultures, separately, to allow them to establish their effect on bacterial metabolism before transmission could begin; after 11 min. equal parts of the treated HFC and acceptor cultures were mixed and the mixture incubated for 30 min.; samples were then treated in the blendor and plated. In a separate experiment an overnight broth culture was diluted 1/10 into tubes of broth containing the various agents. Turbidity measurements were made at intervals for 3 hr. to test the growth-inhibitory effect of the agents under these conditions, in which growth of the control culture is fairly rapid; the turbidity of the control culture increased × 13 in this period. Streptomycin at 1 mg./ml. decreased *colI* transfer only slightly, to 64 % of the control value in a mixture without streptomycin, even though this concentration was rapidly bacteriostatic and less rapidly bactericidal (20 % survival of acceptor bacteria after 1 hr. exposure). Dinitrophenol (M/500) and potassium cyanide (M/250) greatly inhibited growth but had little effect on the transfer of *colI*. Chloramphenicol (10 and 25 μg./ml.) though bacteriostatic only decreased transfer by about half. Sodium azide (M/100)

did not inhibit growth during the first hour (although thereafter it did inhibit growth to some extent) but it did decrease transfer of *colI* to about 5% of the control value. Acriflavine (8 μg./ml.) had no effect on growth and little on transfer of *colI* acriflavine (40 and 80 μg./ml.) were growth-inhibitory and decreased transfer to 16 and 2% of the control value. However acriflavine (40 μg./ml.) was bactericidal and killed bacteria carrying *colI* more rapidly than non-colicinogenic ones (e.g. survivals of 7 and 53%, respectively, after exposure to acriflavine 40 μg./ml. in broth for 2 hr.), so that some of its apparent effect in diminishing the rate of transfer of *colI* may have been spurious. Exposure of the HFC preparation alone to acriflavine (40 μg./ml.), chloramphenicol (10 μg./ml.) or azide (M/200) for 15 or 30 min., before washing and mixing with the acceptor culture, did not diminish the extent of transfer of *colI* as compared with an untreated control. Conversely, addition of the same concentration of acriflavine or chloramphenicol at the time of mixing the HFC preparation with the acceptor culture decreased the transfer of *colI* to about the same extent as when the separate HFC and acceptor cultures were pre-treated for 11 min., but the inhibition of the transfer of *colI* by azide was less when pre-treatment was omitted.

Table 5. *Effect of metabolic inhibitors on transmission of* colI *by HFC preparations*

Components of HFC preparations *adeC-7 proA-46 fla⁻* (*colI*) and *cysD-36* (*colE2*). Proportion of bacteria producing colicine I 21% (expt. 1) and 27% (expt. 2) after primary incubation, and 89% and 80%, respectively, after secondary incubation. Inhibitors were added separately to HFC and acceptor cultures, which were mixed 11 min. later. After 30 min. incubation the mixtures were diluted, treated in blendor, and plated on streptomycin agar, and the proportion of acceptor (streptomycin-resistant) colonies producing colicine I was determined. Acceptor strain: *adeC-7 proA-46 fla⁻ str-r* (Expt. 1); *athA-4 phe-401 str-r* (Expt. 2).

Inhibitor	Concentration	Acceptor colonies colicinogenic, (% of control)
None	—	100*†
Potassium cyanide	M/500	79*
	M/250	45†
Sodium azide	M/200	18*
	M/100	5·4†
Dinitrophenol	M/500	100†
Chloramphenicol	10 μg./ml.	36*
	25 μg./ml.	54†
Streptomycin	1 mg./ml.	64*
Acriflavine	80 μg./ml.	2†
	40 μg./ml.	19*
	8 μg./ml.	40*

* Expt. 1. 47% of acceptor colonies colicinogenic in control, without inhibitor.
† Expt. 2. 37% of acceptor colonies colicinogenic in control, without inhibitor.

Proportion of competent donors in HFC preparations

In broth cultures of stock LT2 (*colI*) sublines only one bacterium in about 8000 is a competent donor able to transmit *colI* to an acceptor bacterium (Ozeki *et al.* 1962). To test the hypothesis that all or many bacteria which have just acquired *colI* can transmit, we determined the proportion of competent donors in HFC preparations in which a large fraction of the bacteria had acquired *colI* during the final 2 hr. of secondary incubation. The method used was like that used to determine the proportion of competent donors in broth cultures of established LT2(*colI*) stocks (Ozeki *et al.* 1962). Graded numbers of bacteria from an HFC preparation of a streptomycin-sensitive strain were added to tubes containing 1 ml. of broth culture of a streptomycin-resistant acceptor strain (7×10^8 bacteria). After 20 or 30 min. streptomycin (to 1 mg./ml.) was added, to prevent any growth of the streptomycin-sensitive donor bacteria, and the tubes after dilution $2\frac{1}{2}$ hr. later with 4 ml. streptomycin broth were incubated for 18 hr. The tubes were then diluted in streptomycin broth and incubated for a further 24 hr., and thereafter tested for the presence of streptomycin-resistant (acceptor) bacteria producing colicine I. As in the experiments on stock colicinogenic strains (Ozeki *et al.* 1962) either 25–50 %, or apparently none, of the acceptor bacteria were colicinogenic when the incubated tubes of streptomycin broth were plated. In one experiment all tubes which received an average of 2·3 colony-forming units or more from the HFC preparation contained colicinogenic acceptor bacteria. Of five tubes receiving an average of 0·7 colony-forming units only one tube contained *colI str-r* bacteria. Therefore an average of 2·3 colony-forming units sufficed to initiate the spread of *colI*. In this experiment the HFC preparation was not treated in the blendor to break up clumps before dilution, and the concentration of colony-forming units was determined by seeding drops on nutrient agar (Miles & Misra, 1938) without spreading. It is therefore possible that an inoculum of 2·3 colony-forming units represented a larger number of viable bacteria. In another experiment, in which the strains used were those shown in Table 6, the HFC preparation was treated in the blendor immediately before it was diluted. Inocula of the dilute HFC preparation were added to two series of tubes containing acceptor bacteria, so that the average number of bacteria inoculated per tube was 0·33 in one series and 0·83 in the second series, as determined by a subsidiary experiment in which 4/14 and 8/14 tubes of broth which received the same inocula gave bacterial growth. After the addition of streptomycin broth and incubation as in the preceding experiment 4/42 (0·09) tubes of the first series and 5/18 (0·28) tubes of the second series yielded colicinogenic acceptor bacteria. The average number of competent donor bacteria inoculated per tube calculated from these proportions is 0·09 for the first series and 0·33 for the second series. That is, about one bacterium in three (0·09/0·33 and 0·33/0·83) was a competent donor. In this experiment the proportion of colicinogenic bacteria in the donor preparation increased from 21 to 83 % during the secondary incubation, so that about 62 % of the bacteria of the HFC preparation had acquired *colI* during the final 2 hr. If only these newly-infected bacteria transmitted, then rather more than half of them must have been competent donors.

Duration of high infectivity after acquisition of colI

When an HFC preparation was incubated without addition of fresh broth for 18 hr., or was stored at about 4° for 18 hr., its transmitting ability decreased somewhat (22 and 16 %, respectively, of acceptor bacteria acquiring *colI* in 60 min. contact, as compared with 57 % for the fresh HFC preparation). But when the HFC preparation was diluted with broth even as little as 1/5 and then incubated for 18 hr., the HFC property was lost (< 1 % acceptor bacteria made colicinogenic in 1 hr.). Broth cultures comprising all or most of the $1–2 \times 10^8$ progeny of the 27th generation of single bacteria newly infected with *colI* were obtained, either (i) by inoculating broth with small acceptor colonies, later shown to have grown from acceptor bacteria which had acquired *colI* from an HFC preparation; or (ii) by incubating until just turbid 1 ml. samples of broths, each seeded with an inoculum containing an average of < 1 bacterium from an HFC preparation. None of 23 cultures so obtained transmitted at high frequency. Thus under conditions which permitted rapid growth the high infectivity of bacteria newly infected with *colI* was not shown by their about 27th generation progeny.

Table 6. *Duration of high infectivity of an HFC preparation allowed to grow*

An HFC preparation (components *adeC-7 proA-46 fla⁻* (*colI*) and *cysD-36* (*col E2*)) was diluted to about 10^4 bacteria/ml. and further diluted during incubation so as to keep the concentration between 10^4 and 10^5 bacteria/ml. At intervals 0·5 ml. inocula of graded numbers of bacteria were added to tubes containing 1 ml. of broth culture of a streptomycin-resistant acceptor culture (*athA-4 phe-401 str-r*). Streptomycin to 1 mg./ml. was added after 20 min and 5 ml. streptomycin broth at 2 hr. Tubes were tested for presence of streptomycin-resistant colicinogenic bacteria after overnight incubation; negative tubes were re-tested after further dilution and incubation in streptomycin broth.

Time of incubation of diluted HFC preparation (min.)	No. of* generations since dilution	Samples tested for ability to transmit: Mean no. bacteria/sample	Samples tested for ability to transmit: Proportion of tubes positive	Calculated† proportion of competent donors
0	0	670	5/5	$\frac{1·6}{6·7} = 0·24$
		67	5/5	
		6·7	4/5	
95	3	460	5/5	$\frac{0·5}{4·6} = 0·11$
		46	5/5	
		4·6	2/5	
255	7·5	4800	2/5	$\frac{0·5}{4,800} = 0·0001$
		960	0/5	
		96	0/5	
		9·6	0/5	

* Calculated from multiplication, as determined by viable counts.

† Calculated from sample size giving some but not all positive tubes; mean no. of competent donors/sample calculated by Poissonian method from proportion of negative tubes, then divided by mean no. of bacteria/sample.

The number of generations for which high infectivity persists under conditions of rapid bacterial growth was determined as follows (Table 6). An HFC preparation after treatment in a high-speed mixer was diluted to about 10^4 bacteria/ml., so that the frequency of collisions would be negligible, and incubated at 37°. This culture was further diluted at intervals, so as to keep the concentration between 10^4 and

10^5 bacteria/ml. At various times graded inocula from the dilute culture were tested for ability to initiate the epidemic spread of the *colI* factor in a streptomycin-resistant acceptor culture. At time zero about one bacterium in four was a 'competent donor'. After 3 generations of growth the proportion of competent donors had decreased from one in four to one in nine; thus about half the third generation progeny of the originally competent donors were themselves competent donors. After $7\frac{1}{2}$ generations of growth the proportion of competent donors was only 0·01 %, which is about the proportion found in a broth culture of a stock colicinogenic strain. Thus most of the eighth generation clones (about 200 bacteria) derived from the originally competent donors did not include even a single competent donor.

DISCUSSION

The experiments described above show that cultures grown from suitable mixtures of colicinogenic and non-colicinogenic bacteria, that is HFC preparations, transmitted *colI* to a high proportion of the bacteria of an acceptor culture during 20–60 min. of contact, and that inocula of as few as 1 to 3 bacteria of a streptomycin-sensitive HFC culture sufficed to initiate the spread of *colI* amongst a streptomycin-resistant population in the presence of streptomycin. These observations and others show that a high proportion (between 30 and 100 %) of bacteria which had recently acquired *colI* were competent donors of *colI*, and that this competence persisted amongst their progeny for several generations (probably between 3 and 7) and then was abruptly lost. The only sort of culture able to transmit *colI* at high frequency will thus be one containing a high proportion of bacteria which have acquired *colI* within the last few generations, that is ones in which the 'epidemic' spread of *colI* is reaching or has just reached its peak.

The conditions required for the production of an HFC culture can then be understood in terms of the epidemiology of a contagious condition affecting the bacteria, and of the known changes in population density during incubation of broth cultures, unaerated or aerated. Presumably the spread of *colI* occurs to a significant extent only when the population density is high enough to permit frequent contacts of infective and susceptible bacteria. Transmission of *colI* by the small proportion of effective donors which occur in an established colicinogenic culture will then occur, and the newly infected recipient cells will become infectious and initiate epidemic spread amongst the susceptible population. On this hypothesis the function of the colicinogenic component of the mixed inoculum is to provide sufficient competent donors to initiate the spread of *colI* amongst the non-colicinogenic bacteria when the bacterial population density becomes high enough for collisions to be frequent. With a suitable constant ratio of colicinogenic to non-colicinogenic bacteria, variation in the absolute number of bacteria inoculated would be expected to have little effect on the progress of the *colI* epidemic, since in any event transmission would only occur at a significant rate after the bacterial population density reached a high level. Similarly, the number of colicinogenic bacteria inoculated together with a fixed number of non-colicinogenic bacteria, provided that the former were in a minority, would affect only the time of onset of the epidemic. We found that colicinogenic:non-colicinogenic inoculum ratios between 1:200 and 1:1 gave satisfactory HFC preparations. Presumably the failure to obtain HFC preparations

when the proportion of colicinogenic bacteria in the inoculum was still smaller resulted from a delay in the onset of the epidemic. When the inoculum contained more colicinogenic bacteria than non-colicinogenic ones, HFC preparations were not obtained, and the proportion of the non-colicinogenic component which acquired *colI* was very small; in these circumstances the majority of collisions of infective donors would be with bacteria already carrying *colI*, which would not themselves become effective donors (see Ozeki *et al.* 1962). In epidemiological terms the high proportion of immunes in the population will decrease the population density of susceptibles and thus impede the progress of the epidemic.

Mixed inocula of non-motile colicinogenic and motile non-colicinogenic bacteria gave satisfactory HFC preparations; but when the colicinogenic component was motile and the non-colicinogenic was non-motile, the usual incubation procedure did not give HFC preparations (Table 3). In these two situations the frequency of collision of effective donors of the colicinogenic component with non-colicinogenic bacteria would be the same, but in all of many subsequent cycles of infection in the initially non-colicinogenic strain the average time before an effective donor collided with a susceptible bacterium would be increased when the non-colicinogenic strain was non-motile; this would account for the delayed spread of *colI* in this situation observed when secondary incubation was prolonged (Table 3).

The 2 hr. 'secondary incubation' of a 1/10 dilution of an overnight mixed culture provides a period in which population density is high and metabolism active, so that conditions are favourable for infection of a large proportion of the remaining susceptibles (usually about 70 % of the bacteria of the initially non-colicinogenic strain).

The high transmitting ability of broth cultures inoculated directly from old stock slopes of a *Salmonella paratyphi* B strain carrying *colI* we attribute to the instability of colicinogeny in respect of *colI* in this strain. The accumulation of non-colicinogenic bacteria during storage results in the presence of a high proportion of bacteria susceptible to the epidemic spread of *colI* when material from the stock slope is incubated in broth. The failure of the non-colicinogenic bacteria to become re-infected in the stock slope itself presumably results from conditions there being unsuitable for the transmission of *colI*, perhaps because of lack of available nutrients (compare the absence of spread of *colI* in mixtures of fully grown aerated donor and acceptor cultures; Ozeki *et al.* 1962).

Our experiments in which inocula of graded numbers of streptomycin-sensitive bacteria from HFC preparations were tested for their ability to transmit in the presence of streptomycin indicate that of bacteria infected by *colI* within the previous 2 hr. about one-half to one-third can transmit. These experiments presented various technical difficulties. In particular we could not prepare suspensions containing bacteria all of which had carried *colI* for a known short period. We suspect that in consequence the proportion of newly infected bacteria which are competent donors was underestimated, and may in fact well be 1, or near it, rather than 0·5 or 0·3, as estimated. Our attempts to measure the duration of high infectivity when newly infected bacteria were allowed to multiply were made difficult by the same experimental limitations. However, the data obtained (Table 6) seem to establish that for several generations a high proportion (probably at least half and perhaps more) of the immediate progeny of a competent newly infected

bacterium are themselves competent donors; and that this high infectivity persists for 3 to 7 generations. The observed decrease in the proportion of competent donors from about 1 in 4 at the time of dilution of an HFC preparation to 1 in 9 three generations after dilution, and to about 0·01 % after $7\frac{1}{2}$ generations, permits some inference about the mechanism of the loss of competence. These data indicate that of the 8 third-generation progeny of a competent donor, an average of at least 4 are competent; but that an average of fewer than 1 (about 0·08) of the 200 seventh- to eighth-generation progeny are competent donors. If the possession of at least one stable but non-replicating particle synthesized only by newly infected bacteria (e.g. a non-chromosomal *colI* particle) sufficed to confer competence, then the clone produced by a newly infected bacterium, since it contains 4 competent donors and therefore 4 particles at the 8 bacterium stage, should contain at least 4 competent donors amongst the 200 descendants after 7 to 8 generations; but in fact the average number of competent donors at this stage was 0·08. Therefore the hypothesis stated above is excluded. It seems more likely that at least half, perhaps all, the descendants of a competent newly infected bacterium are competent donors, but that after 3 to 7 generations every one of the progeny changes over to the non-competent state characteristic of established colicinogenic cultures.

The clumps seen by microscopy in HFC preparations and the way in which non-motile acceptor bacteria are carried through a semi-solid medium by motile bacteria of an HFC culture show that in cultures which contain a high proportion of competent donors many bacteria adhere to other bacteria with which they have collided. The kinetic experiments show that blendor treatment (which breaks up such clumps and pairs) given immediately after pair formation has been permitted, prevents the transfer of *colI*. Pair formation is very rapid (about one-third completed in 2 min., when one or both strains are motile) and transfer of *colI* in a very few pairs is completed (i.e. has reached a stage unaffected by blendor treatment) within $2\frac{1}{2}$ min.; but in most pairs the transfer is not completed until at least 20 min. after pair formation. Perhaps a variable time is required for the formation of a conjugation canal linking the interior of the donor and recipient bacteria, or for the movement of a *colI* particle through such a canal. The adherence of newly infected bacteria suggests that their surface may be altered; but in a very limited experiment we did not detect any new antigenic specificity in such bacteria. Observations on clump formation and on the carriage of streptomycin-resistant non-motile acceptor bacteria by their HFC partners away from the site of inoculation indicate that competent donor bacteria pair with bacteria of established colicinogenic strains carrying *colI* about as readily as they do with non-colicinogenic bacteria, and the extent of clumping in HFC preparations suggests that newly infected bacteria also adhere to each other.

Our experiments on the effect of various factors on the extent of transfer of *colI* by HFC preparations show that the physiological state of the acceptor bacteria is unimportant; this suggests that their role in transfer is a passive one. Aeration by shaking did not interfere with transmission; this supports our conclusion (Ozeki *et al.* 1962) that the inhibitory effect of aeration by shaking on the transfer of *colI* during the prolonged incubation of mixtures of donor and intermediate strains in broth is a result not of a direct effect on pair formation or transfer, but an indirect one, through shortening the period of growth at a high bacterial concentration. The

failure of aeration to inhibit such transfer in a defined medium may have been due to the longer generation time in such a medium, so that even in aerated cultures there is a longer period of growth at a high bacterial concentration to allow transfer of *colI*. Our experiments on the environmental requirements for transfer showed that bacteria which had been infected by *colI* during growth in broth could transfer *colI* to some extent when resuspended in a defined medium (unlike the transfer of the F agent in *Escherichia coli*; Cavalli, Lederberg & Lederberg, 1953) but that there was only slight transfer when the defined medium contained no energy source. Furthermore, our ability to obtain HFC preparations by growth in defined medium shows that newly infected bacteria become competent donors of *colI* in a defined medium, as well as in broth. Our results with prior starvation of one or the other partner did not establish whether it is only the donor strain which requires an energy source (external or internal), as in the case of chromosomal transfer from *E. coli* Hfr to F^- (Fisher, 1957*b*).

Our experiments on the effect of metabolic inhibitors, etc., on the transfer of *colI* showed there was little correlation between their ability to inhibit growth and to prevent transfer. Thus cyanide, dinitrophenol and streptomycin at growth-inhibitory concentrations had little effect on transfer, whereas azide (M/100) almost entirely prevented transfer without slowing growth. The failure of cyanide and dinitrophenol to prevent transfer of *colI* in *Salmonella typhimurium* to any major extent contrasts with their ability, at lower concentrations, to prevent chromosomal recombination in *Escherichia coli* K12 (Fisher, 1957*a*); but it is not clear whether this difference results from a species difference or from a difference in the susceptibility of chromosomal transfer or of episomal transfer (there being no data on the transmission of F in *E. coli* in the presence of inhibitors). Only growth-inhibitory concentrations of acriflavine suppressed transfer and we have some data suggesting that it preferentially kills colicinogenic bacteria; thus we have no evidence for a specific effect on the multiplication of *colI*, comparable to the effect of acriflavine on the multiplication of the F factor in *E. coli* (Hirota, 1960). Streptomycin 1 mg./ml. only diminished transfer by 36%; we observed that *S. typhimurium* remained motile for several hours in this concentration, which suggests that it leaves unaffected at least some energy-yielding mechanisms. In our experiments on the proportion of competent donors in stock colicinogenic strains (Ozeki *et al.* 1962) and in HFC preparations, the time available for transfer of *colI* by any competent donors in the inoculum of streptomycin-sensitive bacteria was therefore longer than the 20 or 30 min. before streptomycin was added. But since streptomycin 1 mg./ml. immediately arrests multiplication of strain LT2 the inferences drawn as to the number of competent donors are unaffected.

We have above assumed that broth cultures of stock colicinogenic strains carrying *colI* are heterogeneous, containing a very few competent donor bacteria able to pair and transmit *colI*, amidst a large majority unable to do so; and that of the newly infected bacteria present in HFC preparations a large fraction (e.g. 0·2–0·5) or perhaps all, can pair and transmit, the remainder, if any, being unable to do so. Our data however do not exclude the hypothesis that each of these bacterial populations is, on the contrary, homogeneous, every newly infected bacterium having the same probability of pairing and transmitting *colI* (under the conditions of our experiments, see above, between 0·3 and 1), the corresponding probability for

every bacterium in a stock colicinogenic strain being only about 10^{-4}. However, the rare competent donors in stock colicinogenic cultures behave, so far as we have tested, just like the frequent competent donors in HFC preparations; for instance, in usually transmitting, as well as *colI*, any other colicine factor they may possess (Ozeki *et al.* 1962; Smith, Ozeki & Stocker, in preparation). For this reason we prefer the hypothesis of two possible phenotypes for bacteria carrying *colI*: the effective donors, having a probability of one, or near one, of mating and transmitting under suitable conditions; and other colicinogenic bacteria, with zero probability of doing so. Our uncertainty as to the homogeneity or heterogeneity of transmitting ability in our bacterial populations does not, we think, affect the validity of the rest of our argument.

From the rapid spread of *colI* during the growth of HFC cultures it is evident that in such cultures the *colI* factor is multiplying much more rapidly than the bacteria, and therefore that in newly infected bacteria *colI* multiplies autonomously. As the large majority of bacteria in stock colicinogenic strains do not transmit *colI* we have no evidence that in them *colI* is multiplying more rapidly than its bacterial host. These non-transmitting bacteria do not adhere to other bacteria, as shown by the absence of clumping and by the rarity of satellite colonies when a mixture of a streptomycin-resistant non-motile acceptor culture and a stock streptomycin-sensitive motile colicinogenic culture is plated on semi-solid streptomycin medium. The failure of such bacteria to adhere to other bacteria with which they collide seems a sufficient explanation for their failure to transmit *colI*. We do not know why the *colI* factor in most newly infected bacteria manifests the F-like property of causing its bacterial host to conjugate, but does so in only a very small minority of bacteria of strains which have long harboured it. Perhaps the *colI* factor when first introduced multiplies freely in the cytoplasm but after some generations is nearly always present only as a single particle attached to the chromosome; either a 'dose-effect' or a 'position-effect' might then account for the usual failure of the attached *colI* factor to confer 'maleness' on its host. If this be so, *colI* in newly infected *Salmonella typhimurium* would resemble the non-integrated F factor in *Escherichia coli* F^+ strains; and the *colI* agent in established *S. typhimurium* colicinogenic strains would resemble the chromosomally attached F agent in *E. coli* Hfr strains (though the integrated F episome continues to confer the male phenotype on its Hfr host). However, there is as yet no evidence that *colI* (or any other colicine agent so far investigated) can be integrated into the chromosome of *S. typhimurium*. The failure to transmit which sets in between 3 and 7 generations after invasion by *colI* may result not from integration of the episome into the chromosome but from some other change causing loss of ability to conjugate. However preliminary experiments (Dubnau & Stocker, unpublished) indicate that when an Hfr subline of *S. typhimurium* LT2 (Zinder, 1960) made colicinogenic for *colI* transfers the *ile*$^+$ gene to a non-colicinogenic *ile*$^-$ *str-r* LT2 acceptor strain most *ile*$^+$ *str-r* recombinants do not acquire *colI*; whereas in the recombination experiments of Ozeki & Howarth (1961), in which HFC preparations were used as gene donors, the majority of recombinants did acquire *colI*. This argues against the autonomous multiplication of *colI* in the cytoplasm of bacteria of stock colicinogenic strains.

In *Escherichia coli* F^- strains bacteria newly infected by the F agent or by colicine

factors have not been shown to differ from stock F^+ or colicinogenic strains. In *Salmonella typhimurium*, however, the immediate progeny of bacteria newly infected by the temperate phage PLT22 differ in two respects from bacteria of established lysogenic strains carrying prophage PLT22: (1) They produce a high proportion of non-lysogenic descendants which after a delay revert to phage sensitivity (Luria, Fraser, Adams & Burrous, 1958). (2) All or most of them manifest the phage-determined antigenic specificity called Factor 1, attributable to the presence of a glucose-α-1,6-galactose-mannose side-chain in the polysaccharide of their O antigen (Stocker *et al.* 1960), whereas in established lysogenic strains the expression of Factor 1 is subject to form-variation (Kauffmann, 1940), so that many lysogenic bacteria are totally unaffected by Factor 1 serum (Stocker & de Margerie, unpublished). We do not know why bacteria recently infected by *colI* or by prophage PLT22 should behave differently from bacteria of established colicinogenic or lysogenic strains. In each case the two states, that is of newly infected and of established strains, are distinct from the situation when the episome becomes more active, either spontaneously or after ultraviolet irradiation, and directs the synthesis of either colicine or infective phage, with the consequent death of the bacterial host. (See Ozeki, Stocker & de Margerie (1959) for production of colicine, spontaneously or after induction, by individual bacteria in cultures of colicinogenic *S. typhimurium* LT2 sublines.)

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Printed in Great Britain

The Relationship Between the Nature of the Cell Wall and the Gram Stain

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(*Received* 1 *May* 1962)

SUMMARY

None of the classes of major chemical constituents of the walls of bacteria and yeasts is exclusive to organisms which give a Gram-positive reaction. Walls of Gram-positive bacteria are richer in mucopeptide than are those of Gram-negative bacteria. The latter are characterized by high lipid contents. Yeast walls are rich in polysaccharide complexes. There appears to be a broad correlation between the Gram reaction and the decreased leakage of ^{32}P compounds from labelled cells on exposure to increasing ethanol concentrations in the range 50–100% (v/v) ethanol. The data on the release of ^{32}P compounds are in accord with the cell-wall 'permeability' mechanism for the Gram reaction and imply quantitative rather than all or none differences in Gram behaviour. Mechanical rupture of the cell wall or digestion of the wall with lysozyme rendered organisms which had been previously Gram stained in suspension by the technique of Wensinck & Boevé (1957), susceptible to decolorization with 96% (v/v) ethanol in water.

INTRODUCTION

Although the Gram reaction is probably the most widely used staining procedure in bacteriology, there has been no wholly acceptable explanation of the mechanism of its reaction despite the great proliferation of theories advanced since the description of the technique by Christian Gram in 1884. Many of the earlier views of the mechanism of the Gram reaction were discussed in some detail by Bartholomew & Mittwer (1952). As pointed out by Salton (1961*a*) it is now virtually impossible to propose any new theory to explain the Gram reaction, for the most likely possibilities have been covered at some time or other and all of the major classes of cellular constituents have been implicated. The theories propounded from the studies of the mechanism of the Gram reaction fall into two major groups: (i) those which involve the presence of a particular substance or class of substances which confer Gram positivity; (ii) those which invoke a difference in the 'permeability' of the cells to the dyes.

Much effort in the past has been concentrated on searching for specific bacterial substances which may account for the Gram-positive reaction. Thus a positive response to the Gram stain has been claimed to be due to lipoprotein (Stearn & Stearn, 1924, 1930), lipids (Eisenberg, 1910; Schumacher, 1928), nucleoproteins and nucleic acids (Deussen, 1921; Dubos & MacLeod, 1938; Henry, Stacey & Teece, 1945; Henry & Stacey, 1946; Webb, 1948); carbohydrate (Webb, 1948) and

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glycerophosphate substances (Schumacher, 1928; Mitchell & Moyle, 1950, 1951, 1954). Fischer & Larose (1952) suggested a similarity in chemical structure between highly degraded wool and the cytoplasmic membrane of Gram-positive bacteria. Other chemical constituents believed to be related to the Gram reaction in some unknown way include the polyamines found in greatest amounts in the Gram-negative group of bacteria (Herbst, Weaver & Keister, 1958). Evidence that the Gram reaction was related to Mg-ribonucleate in Gram-positive bacteria was presented by Henry *et al.* (1945) but an explanation of the stain reaction based on this proved less satisfactory when Mitchell & Moyle (1954) made a thorough investigation of the ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) contents of various bacteria. Further doubts that the Gram reaction was due to a Mg-ribonucleate + basic protein complex (Stacey, 1949) also came from the studies of Shugar & Baranowska (1957), reinforcing the earlier conclusion of Lamanna & Mallette (1950) that much of the nucleic acid can be extracted from yeast cells without loss of a Gram-positive reaction.

As an alternative to the mechanism of the Gram reaction based on a nucleic acid + basic protein complex as proposed by Stacey (1949), Mitchell & Moyle (1950, 1951, 1954) suggested a correlation between Gram positivity and the presence of phosphate esters ('XSP'). These phosphate esters were subsequently identified as mixed glycerol and ribitol phosphate polymers (Mitchell & Moyle, 1958), substances now well known as the teichoic acids of bacterial walls (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958). The polyglycerophosphate of *Staphylococcus aureus* was given the name 'positic acid' by Mitchell & Moyle (1958). However, Jones, Rizvi & Stacey (1958) showed that most organisms (both Gram-positive and Gram-negative) contained 'XSP'. Thus it now seems unlikely that 'positic acid' could be directly responsible for Gram positivity.

The opponents of the idea that a specific cellular substance is responsible for the Gram reaction have sought other explanations, and 'permeability' factors have long been suggested as the basis of the differential staining observed. Thus, Burke & Barnes (1929) concluded that the Gram reaction was due to differences in cell-wall permeability. Further support of the 'permeability' theories have come from the studies of Kaplan & Kaplan (1933) and more recently from Bartholomew, Cromwell & Finkelstein (1959). The studies of Wensinck & Boevé (1957) on the pattern of extractibility with ethanol, of the crystal violet + iodine (CVI) complex from bacteria, provide convincing evidence that some 'permeability' effect is involved.

That the cell wall is involved in some manner seemed inescapable, especially from the loss of Gram positivity following mechanical crushing (Benians, 1920), lysozyme treatment of heated cells (Webb, 1948) or protoplast formation (Gerhardt, Vennes & Britt, 1956). Although Chelton & Jones (1959) have shown that disintegrated yeast cells can, under certain conditions, give a positive Gram reaction, it has been widely established that loss of Gram reaction occurs concomitantly with loss of cellular integrity (Bartholomew & Mittwer, 1952). Since many of the theories of the mechanism of the Gram reaction were proposed before the more recent studies of the chemistry of bacterial cell walls, it seemed worth while to re-examine the problem in terms of present knowledge of the surface structure of bacteria. In this paper, details of the composition of bacterial cell walls from the present investiga-

tion and from earlier studies (Salton, 1953*a*; Salton, 1958; Salton & Pavlik, 1960) have been considered in relation to the Gram reaction and a possible method for studying the mechanism of the Gram differentiation is presented.

METHODS

Organisms and growth conditions. Unless otherwise specified, the following organisms used were laboratory strains: *Alcaligenes faecalis* (NCTC 8764); *Bacillus brevis* (NCTC 7577;) *B. cereus* P2; *B. megaterium* KM; *Candida pulcherrima* (NCYC 373); *Chromobacterium prodigiosum*; *Clostridium perfringens* (*C. welchii*); *C. sporogenes*; *Corynebacterium hoffmanni*; *Corynebacterium xerosis*; *Escherichia coli* B; *E. dispar* (NCTC 4169); *Klebsiella aerogenes* (*Aerobacter aerogenes*) A 30; *Lactobacillus arabinosus* 17/5; *Leuconostoc mesenteroides* P60; *Micrococcus lysodeikticus* (NCTC 2665); *Neisseria catarrhalis* (kindly provided by Dr S. E. Hartsell); *Proteus vulgaris*; *Pseudomonas fluorescens*; *Pseudomonas* sp. (kindly provided by Dr J. M. Shewan); *Saccharomyces cerevisiae* (NCYC 366); *Salmonella gallinarum*; *Spirillum serpens*; *Staphylococcus aureus* Duncan; *Streptococcus faecalis* (NCTC 6782); *Vibrio metchnikovii* (NCTC 8443).

The organisms were grown on the following media: Clostridia on Robertson's cooked meat medium at 37°; *Lactobacillus arabinosus* and *Leuconostoc mesenteroides* on 2 % Bacto Casitone + 0·1 % Marmite + 1 % glucose + 2 % agar at 30°; *Spirillum serpens* on 2 % Bacto Casitone + 0·1 % Marmite + 1 % glucose broth at 25°–28°; yeasts on malt agar at 25°; all other organisms were cultivated on nutrient agar or nutrient broth at 30° or 37°. The yeasts and *Spirillum serpens* were harvested after incubation for 2 days and all other organisms after 24 hr. Cultures of the spore-forming Bacillus species were harvested after incubation for 12–18 hr. The harvested organisms were generally washed three times with distilled water on the centrifuge. Organisms which showed any tendency to lyse under these conditions were harvested and washed in physiological saline.

For studying the leakage of ^{32}P compounds from organisms, these were grown under exactly the same conditions as specified above, except that the media were supplemented with ^{32}P as orthophosphate (Radiochemical Centre, Amersham, Buckinghamshire, England) in sterile saline solutions; 0·1 ml. sterile ^{32}P solution containing about 10^7 counts/min. was added to 20 ml. portions of agar or liquid medium. The ^{32}P-labelled organisms were harvested and washed as described above.

Gram reaction. Smears were stained by the method modified by Hucker (1921). All smears were blotted dry before decolorizing with 96 % (v/v) ethanol in water. The Gram method applied to bacterial suspensions by Wensinck & Boevé (1957) was used as described by these authors with the modification of dissolving 0·9 g. Gurr's crystal violet in 100 ml. 0·067 M-phosphate buffer (pH 7).

Leakage of ^{32}P compounds from micro-organisms. The effect of ethanol concentration on the leakage of ^{32}P compounds from labelled organisms was studied in the following way. Washed suspensions of organisms were pipetted into 1 cm. × 7 cm. test tubes and 1 ml. water or saline (when bacteria suspended in this fluid) added to each tube and the organisms packed by centrifugation. The volume of washed suspension added was adjusted so that each tube contained about 3–5 mg. dry weight organism. After centrifugation, the supernatant liquid was carefully sucked

off with a pump so that the packed organisms were undisturbed, leaving a minimum of residual liquid in the tube (usually less than 0·05 ml.). Packed organisms were suspended in 1 ml. water or aqueous ethanol solution (concentrations of ethanol ranging from 25–100 %, v/v) and allowed to stand at room temperature (about 20°) for specified periods. Organisms were deposited by centrifugation for 10 min. at room temperature and the supernatant liquids further clarified when necessary by centrifugation. The leakage of ^{32}P compounds was determined with a Geiger counter by measuring the radioactivity of 0·2 ml. samples of supernatant fluid dried on planchettes as described by Roberts, Abelson, Cowie, Bolton & Britten (1955).

The time course of leakage in 96 and 100 % (v/v) ethanol was determined by preparing a series of test tubes containing ^{32}P-labelled organisms as described above, adding the ethanol and filtering off the organisms on Oxoid bacteriological membrane filters. Filtration was rapid (10 sec. for about 1 ml. cell suspension) thus permitting samples to be taken at times much shorter than would have been possible by using centrifugation for removal of the extracted organisms. The ^{32}P contents of the filtrates were determined in the usual way.

Isolation and composition of cell walls. Cell walls were isolated as described by Salton & Horne (1951). Analytical procedures used were those given in earlier studies (Salton, 1953*a*; Salton & Pavlik, 1960). 'Total lipid' was determined by extraction with ether after preliminary hydrolysis with 6 N-HCl for 2 hr. at 100° (Salton, 1953*a*). Amino sugar contents were estimated by the Rondle & Morgan (1955) method after previous hydrolysis of cell walls for 2 hr. with 2 N-HCl at 100°.

RESULTS

Chemical composition of walls of Gram-positive and Gram-negative organisms

Extensive investigations of the chemistry of bacterial cell walls (Salton, 1961*b*) and yeast walls (Falcone & Nickerson, 1956; Kessler & Nickerson, 1959; Northcote & Horne, 1952) have been made during the past 10 years; analyses have been

Table 1. *Major classes of cell-wall constituents and the Gram reaction of certain micro-organisms*

Gram-positive organisms	Major chemical components of cell walls
Yeasts	
Saccharomyces cerevisiae	Polysaccharide, protein, lipid
Candida spp.	Polysaccharide, protein
Bacteria	
Micrococcus lysodeikticus	Mucopeptide
Staphylococcus aureus	Mucopeptide, teichoic acid
Streptococci	Mucopeptide, teichoic acid, polysaccharide
Corynebacterium	Mucopeptide, polysaccharide
Gram-negative organisms	
Escherichia coli *Aerobacter aerogenes* *Pseudomonas* spp. *Spirillum serpens*	Protein, polysaccharide, lipid, mucopeptide

performed on a large enough variety of Gram-positive and Gram-negative species to give a comprehensive picture of the major chemical constituents of walls from both groups. The principal types of chemical constituents found in walls of Gram-

positive and Gram-negative organisms are summarized in Table 1. One of the most conspicuous differences between the walls of Gram-positive and Gram-negative bacteria observed in the earlier studies (Salton, 1953*a*) was the higher lipid contents of walls of the latter group. The amino sugar contents of the walls of Gram-positive bacteria were generally higher than those of Gram-negative organisms, such a difference probably being a reflexion of the total amount of mucopeptide (peptido-polysaccharide or glycopeptide) component in the wall. Thus, as suggested by Salton (1958) a 'spectrum' of lipid and amino sugar contents exists and quantitative results for the walls of a variety of Gram-negative bacteria are compared with the range observed for Gram-positive organisms (Salton & Pavlik, 1960) in Table 2.

Table 2. *Amino sugar and lipid contents of cell walls of Gram-negative and Gram-positive micro-organisms*

	Amino sugar (%)	Total lipid (%)
Gram-negative		
*Vibrio metchnikovii**	1·8	11·2
Aerobacter aerogenes	2	14·6
Chromobacterium prodigiosum	2	12·8
*Escherichia coli**	3	20
Salmonella gallinarum	3·5	22
Proteus morganii	4·1	—
P. vulgaris	5·1	17·6
Escherichia dispar	5·0	12·6
*Spirillum serpens**	6·8	—
Alcaligenes faecalis	7·6	—
Neisseria catarrhalis	8·1	12·2
Bacillus brevis	14·5	2·7
Gram-positive		
Candida pulcherrima	1·2	5·4
*Saccharomyces cerevisiae**	1–2	8·5–10
Staphylococcus aureus	17	—
Bacillus megaterium	18	0
*Micrococcus lysodeikticus**	16–22	0
*Bacillus cereus**	31–32	0

* Data from earlier studies: Northcote & Horne (1952); Salton (1953*a*, 1958); Salton & Pavlik (1960); Salton & Shafa (1958).

In considering the chemical nature of the cell wall in relation to the Gram reaction (Table 1), it became apparent that Gram positivity could not be correlated with the presence of one particular type of substance in the cell wall. Thus, the teichoic acids (Armstrong *et al.* 1958) are present in only certain Gram-positive bacteria, and similarly, polysaccharide components are found in some and not in others (Salton, 1961*b*). All of the Gram-positive bacteria contain mucopeptides (glycopeptides) and all of the yeast walls are rich in polysaccharides and polysaccharide complexes (Northcote & Horne, 1952; Kessler & Nickerson, 1959). Although the walls of Gram-negative bacteria were rich in lipids, the lipid content of yeast walls may be as high as 10 % and as low as 1 % (Kessler & Nickerson, 1959). These considerations of the chemistry of cell walls led to the conclusion that the Gram reaction is not due to the presence of any specific substance in the wall and that several types of polymeric substances may serve equally well as wall structures for Gram-positive organisms.

Influence of ethanol concentration on the leakage of ^{32}P compounds across the walls of Gram-positive and Gram-negative bacteria

In their analysis of the Gram reaction Wensinck & Boevé (1957) showed that the amounts of crystal violet and iodine taken up was similar for Gram-positive and Gram-negative organisms. The main divergence in behaviour was found in the extractability of the CVI complex from the stained organisms washed in aqueous ethanol solutions ranging from about 90–100 %, v/v. Thus under these conditions the CVI in Gram-positive organisms remained largely unextractable despite the fact that the CVI complex formed by mixing solutions of the Gram reagents was completely solubilized in such concentrations of aqueous ethanol. If the CVI is not rendered alcohol-insoluble by being bound to specific chemical constituents present in only Gram-positive organisms then other explanations of the results of Wensinck & Boevé (1957) would have to be sought. As an alternative to the possession of a specific 'Gram-positive' chemical entity it seemed conceivable that the Gram differentiation brought about by decolorizing with 96 % ethanol may result from a dehydration of the wall structures and consequent decrease in pore size, thus impeding the passage of small molecules across the wall and rendering the CVI complex inaccessible to extraction. Any information on the passage of small molecules across the walls (release from inside as well as passage from the external medium into the cell) when organisms are suspended in ethanol concentrations used in Gram differentiation may lead to a greater understanding of the mechanism of the Gram reaction.

To test the possibility that the passage of small molecules across the outer envelope is impeded when Gram-positive organisms are exposed to ethanol concentrations which bring about the Gram differentiation, the release of ^{32}P compounds from organisms suspended in aqueous ethanol solutions (25–100 %, v/v) was investigated. The influence of ethanol concentration on the leakage of ^{32}P compounds from a variety of Gram-positive and Gram-negative bacteria was studied; the results for two Gram-positive organisms (*Saccharomyces cerevisiae* and *Streptococcus faecalis*) and two Gram-negative bacteria (*Escherichia coli* and *Proteus vulgaris*) are illustrated in Figs. 1 and 2, respectively.

It was conceivable that the decreased leakage of ^{32}P compounds from Gram-positive organisms might have been due simply to a smaller fraction of the cells having had their plasma membranes damaged by the treatment. That this explanation of the type of results given in Figs. 1 and 2 was unlikely was shown by the results of the following experiments. ^{32}P-labelled *Staphylococcus aureus* was treated with ethanol solutions, the organisms deposited by centrifugation and the supernatant fluids removed. The organisms were then suspended in distilled water and the release of residual ^{32}P compounds determined after 10 min. at room temperature. *Escherichia coli* was treated in the same manner. The results for *S. aureus* and *E. coli* are presented in Figs. 3 and 4, respectively; they show that the larger fraction of ^{32}P material 'trapped' inside *S. aureus* treated with ethanol concentrations between 80–100 % readily leaked out when the organisms were subsequently suspended in water.

To test the possibility that the differences in the patterns of ^{32}P leakage in ethanol solutions were not due to a slower release from Gram-positive bacteria, the

time course of leakage from Gram-positive and Gram-negative bacteria was studied. Organisms of *Escherichia coli* and *Staphylococcus aureus* were dispersed in 100 % ethanol and filtered as described in the Methods, at intervals of time up to 20 min. The time course for the release of ^{32}P compounds from *S. aureus* and *E. coli* are shown in Fig. 5. Although a smaller fraction of the total ^{32}P content leaked out from *S. aureus* in 100 % ethanol, the fraction was released at about the same speed as that observed for *E. coli*.

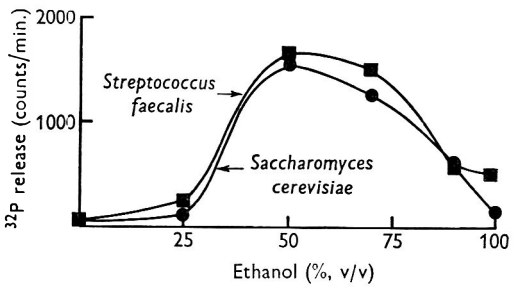

Fig. 1

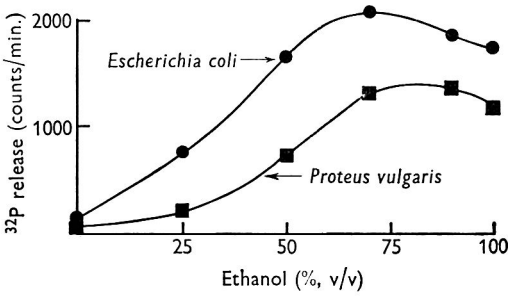

Fig. 2

Fig. 1. Leakage of ^{32}P compounds from *Streptococcus faecalis* and *Saccharomyces cerevisiae*. Organisms suspended in ethanol solutions for 10 min. at room temperature.

Fig. 2. Leakage of ^{32}P compounds from *Escherichia coli* and *Proteus vulgaris*. Organisms suspended in ethanol solutions for 10 min. at room temperature.

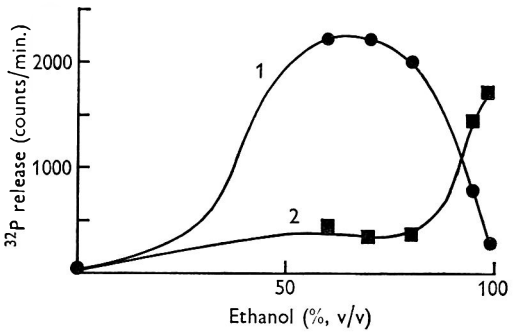

Fig. 3

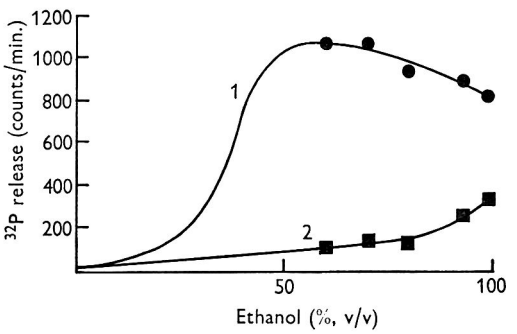

Fig. 4

Fig. 3. Leakage of ^{32}P compounds from *Staphylococcus aureus* suspended in ethanol solutions for 10 min. at room temperature (curve 1) and the subsequent release of ^{32}P compounds into distilled water when deposits of cocci from curve 1 samples were suspended in distilled water for 10 min. at room temperature (curve 2).

Fig. 4. Leakage of ^{32}P compounds from *Escherichia coli* suspended in ethanol solutions for 10 min. at room temperature (curve 1) and the subsequent release of ^{32}P compounds into distilled water when deposits of organisms from curve 1 samples were suspended in distilled water for 10 min. at room temperature (curve 2).

Experiments were performed with a collection of Gram-positive and Gram-negative organisms to determine the release of ^{32}P compounds in 100 % ethanol. The leakage in 100 % ethanol was determined in relation to the maximum leakage occurring at lower ethanol concentrations (usually at 50–75 %, v/v, ethanol in

water) and has been expressed as a percentage of the maximum release in the results summarized in Table 3.

These differences between the Gram-positive and Gram-negative organisms only establish that the passage of certain small molecules (inorganic phosphate, nucleotides, etc.; Salton, 1951) across the wall or wall-membrane of Gram-positive bacteria is impeded when they are suspended in high concentrations of ethanol, whereas many of the Gram-negative bacteria were affected to a lesser degree. This finding in itself gives not more than a clue about a likely mechanism of the Gram reaction and suggests that the crystal violet+iodine complex is 'trapped' inside the organisms when the 'permeability' of the outer wall is decreased on treatment with concentrations of ethanol exceeding 90 %.

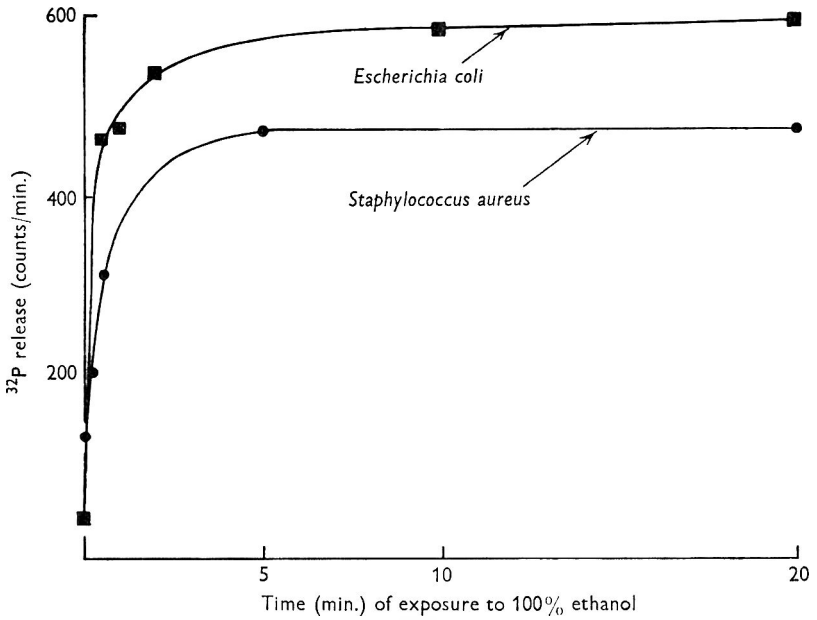

Fig. 5. The time course for the release of ^{32}P compounds from *Escherichia coli* and *Staphylococcus aureus* suspended in 100 % ethanol at room temperature.

Unfortunately the role of mordanting with iodine in the Gram reaction could not be investigated in relation to the leakage of ^{32}P at different ethanol concentrations, as pretreatment of the bacteria with the iodine solution resulted in an immediate loss of intracellular ^{32}P compounds from Gram-positive and from Gram-negative bacteria. It seems likely that a more successful approach to the problem of the role of mordanting with iodine might be to study the penetration of small molecules into organisms suspended in ethanol before and after treatment with iodine solutions.

Effects of mechanical rupture and wall removal on extractability of crystal violet+iodine complex from suspensions of Gram-stained organisms

If, as the above results and the iodine permeability effects reported by Kaplan & Kaplan (1933) and Bartholomew *et al.* (1959) suggest, the pore size of the wall of Gram-positive bacteria is decreased by mordanting with iodine and dehydration

with 95% ethanol, thereby trapping the crystal violet+iodine (CVI) complex within its boundary, then mechanical rupture or enzymic removal of the wall after Gram staining should then render the CVI complex accessible to extraction.

Table 3. *The relationship between the Gram reaction and the leakage of* ^{32}P *compounds from labelled bacteria exposed to about* 100% *ethanol*

Gram reaction	Organism	^{32}P released (%) relative to maximum*
−	*Pseudomonas* sp.	96
−	*Proteus vulgaris*	90
−	*Escherichia coli*	84
−	*Alcaligenes faecalis*	78
−	*Vibrio metchnikovii*	75
−	*Salmonella gallinarum*	75
−	*Aerobacter aerogenes*	75
−	*Chromobacterium prodigiosum*	69
−	*Neisseria catarrhalis*	65
−	*Pseudomonas fluorescens*	60
−	*Spirillum serpens*	56
−	*Escherichia dispar*	54
−	*Bacillus brevis*	54
+	*Leuconostoc mesenteroides*	43
+	*Clostridium perfringens*	36
+	*Micrococcus lysodeikticus*	35
+	*Candida pulcherrima*	34
+	*Streptococcus faecalis*	33
+	*Clostridium sporogenes*	30
+	*Lactobacillus arabinosus*	30
+	*Staphylococcus aureus*	26
+	*Bacillus megaterium*	22
+	*Corynebacterium hoffmanni*	17
+	*C. xerosis*	13
+	*Bacillus cereus*	11
+	*Saccharomyces cerevisiae*	10

* Maximum ^{32}P released from organism usually occurred in 50–75% (v/v) ethanol in water solution.

Heated washed suspensions of *Bacillus megaterium*, *Micrococcus lysodeikticus* and *Saccharomyces cerevisiae* were Gram stained as described by Wensinck & Boevé (1957). The Gram-positive organisms were washed several times with 96% ethanol (no counter stain used), the supernatant solutions decanted off and the packed organisms drained before dispersing in distilled water. These suspensions were mixed with Ballotini beads and shaken in the Mickle apparatus under the conditions normally used for cell-wall isolation (Salton & Horne, 1951). The beads were separated by allowing them to settle and after several washes with distilled water the fluid containing the disintegrated organisms was centrifuged. The deposits from disintegrated organisms and untreated Gram-positive organisms were suspended in 96% ethanol and centrifuged again. On extraction with 96% ethanol the control organisms not submitted to mechanical disintegration still remained strongly Gram-positive whereas the material from the ruptured organisms was decolorized.

Salton (1953*b*) showed that the walls of heated *Bacillus megaterium* and *Micrococcus lysodeikticus* were removed on incubation with egg-white lysozyme, leaving

the coagulated protoplasmic bodies. Similar experiments were performed with suspensions of organisms Gram stained by the method of Wensinck & Boevé (1957). After decolorizing by washing several times with 96 % ethanol on the centrifuge, the packed organisms were drained and suspended in 0·067 M-phosphate buffer (pH 7) and incubated with 50 μg. lysozyme/ml. for 1 hr. at 37°. Control preparations without lysozyme were incubated under the same conditions. Lysozyme-treated and untreated organisms were then deposited by centrifugation, the supernatant fluids decanted, the deposits washed with distilled water and the washing fluid drained off. The deposits were then dispersed in 96 % ethanol and the organisms sedimented by centrifugation. Under these conditions only residual amounts of crystal violet remained in the deposits from the lysozyme-treated preparations; the organisms not exposed to lysozyme action were still strongly Gram-positive.

DISCUSSION

Burke & Barnes (1929) suggested that the Gram reaction could be explained by the impermeability of the walls of Gram-positive bacteria to the crystal violet + iodine complex. Both before and since the proposal of this mechanism, attempts have been made to relate the Gram reaction to specific chemical compounds in Gram-positive bacteria. Convincing correlations between the presence of certain cellular constituents and a positive Gram reaction have ultimately broken down and as pointed out by Shugar & Baranowska (1957) the demonstration of certain substances in Gram-positive organisms 'does not necessarily mean that this component is responsible for Gram staining, but only that it may be one of the manifestations of the differences in physiological and physico-chemical characteristics between Gram-positive and Gram-negative organisms'. Indeed, a wide variety of simple and polymeric substances can behave as Gram-positive material (Shugar & Baranowska, 1957).

Strong support for the 'permeability' mechanism of the Gram reaction came from the earlier studies of Kaplan & Kaplan (1933) and more recently from the work of Bartholomew *et al.* (1959). A clearer interpretation of the mechanism of the Gram reaction has only been possible as a result of the studies of Wensinck & Boevé (1957) who were the first to provide convincing quantitative information about the sequence of steps in the Gram reaction and the points of departure in behaviour between the Gram-positive and Gram-negative organisms on decolorization with ethanol. The similarity between their patterns of extractability of crystal violet + iodine (CVI) complex and the release of ^{32}P compounds at different ethanol concentrations shown in the present investigations (Figs. 1, 2), is striking. Thus, the ethanol concentration used as the differentiating step in the Gram stain decreases the accessibility of the CVI complex and the same concentrations have been shown to impede the passage of intracellular ^{32}P compounds across the walls of Gram-positive organisms. These results together with the observations on disrupted Gram-stained organisms involving either simple mechanical damage or enzymic removal of wall, strongly support the view that a Gram-positive reaction is due to the CVI complex being 'trapped' within the organisms owing to dehydration and diminution in pore size of the walls of Gram-positive organisms. It also appears likely that mordanting with iodine could have a twofold effect of forming a CVI complex as well as an

association between iodine atoms and OH groups of polysaccharide (composed of sugars and/or amino sugars) components of the walls. This might further decrease the pore size on dehydration of the structure in ethanol, in a similar manner to the decrease in the internal diameter of the starch helix which accompanies the association of the large iodine atom with this polysaccharide (Greenwood, 1956).

If the Gram reaction is thus due to the physico-chemical state of the wall during differentiation with 96 % ethanol, then many of the conflicting results of earlier investigation can be readily understood. Any breach in the mechanical integrity of the wall either before or after Gram staining would render the CVI complex more accessible to extraction. Gerhardt *et al.* (1956) indeed attempted such a conclusive experiment with *Bacillus megaterium* but their results were not entirely satisfactory and not as clear cut as the data obtained from washed Gram-stained suspensions. The loss of Gram positivity on disintegration, autolysis and digestion of heated organisms with lysozyme (Webb, 1948) and even the effects of ageing, are all readily understandable if an intact rigid wall is needed as a barrier for the retention of the CVI complex. The presence of cell-wall degrading enzymes in bacteria has been well established (Mitchell & Moyle, 1957; Salton, 1956) and could account for the conversion of bacteria to the Gram-negative state.

It is now generally agreed that the Gram reaction is not an 'all or none' phenomenon, for gradations between Gram-positive and Gram-negative extremes have long been recognized (Neide, 1904; Churchman & Siegel, 1928; Shugar & Baranowska, 1958). Some of these quantitative differences may well be accounted for by the presence of more mucopeptide component in the walls of organisms within the Gram-negative group. The amino sugar content of the walls is probably a good index of the amount of cell-wall mucopeptide and it is of interest to note that several of the organisms intermediate between the extremes of ^{32}P leakage in ethanol (Table 3) also contain appreciable quantities of amino sugar in the walls (Table 2). Thus the total amount of mucopeptide and polysaccharide complex in the walls of bacteria and yeasts may have an important bearing on the quantitative aspects of the Gram reaction. The physicochemical state of the wall mucopeptides and polysaccharides may also be of considerable importance in determining the response in the Gram reaction. It appears likely that the mucopeptide polymers in the walls of Gram-positive bacteria form thick continuous sheets, whereas in a Gram-negative organism such as *Escherichia coli* the mucopeptide component responsible for the rigidity of the wall is present as a very thin layer (Weidel, Frank & Martin, 1960). Gram-variable properties may thus be explicable on the basis of mucopeptide layers of thickness intermediate between those found for the two extremes of Gram-positive and Gram-negative bacteria. More information about the physico-chemical structure of microbial cell walls is obviously needed before a clearer picture will emerge of the effects of the differentiation with ethanol which leads to a Gram-positive or Gram-negative reaction; and the role of iodine and the possible groups in the wall with which it may associate must be further investigated.

I wish to thank Mr J. G. Pavlik for preparing cell walls and carrying out some analyses.

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J. gen. Microbiol. (1963), 30, 237–244
Printed in Great Britain

The Adansonian Taxonomy of some Yellow Pigmented Marine Bacteria

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(*Received* 2 *May* 1962)

SUMMARY

An Adansonian analysis of the features of 62 yellow pigmented marine bacteria, all but one of which were identified as Flavobacterium species by the workers who isolated them, revealed that 34 fell into one of two large pleista or groups; the rest fell into smaller pleista, or were the sole representative of their pleiston present. The possibility that these pleista approximate to various established genera is discussed.

INTRODUCTION

The genus *Flavobacterium* and the genus *Cytophaga* are placed in different Orders of the Class Schizomycetes by *Bergey's Manual* (1957). This would lead one to expect that it would be easy to distinguish the members of either genus from the members of the other. In fact, several workers have commented on the difficulty that is encountered in making this distinction. For example, Stanier (1947) found that a number of soil cytophagas formed short elements which morphologically closely resembled true bacteria. He commented that inspection of cultures with a predominance of short cells 'does not suggest that the organisms are myxobacteria; flexibility is not evident, and the motility of the individual cells is hard to detect; the only indicative feature which remains being the weak refractility of the cells. The picture both in wet mounts and in stained preparations is strongly suggestive of a small, non-motile, Gram-negative true bacterium.' Gibson (1955) noted that certain soil organisms, closely resembling the cytophagas in many respects, did not move by means of the gliding motility which is held to be diagnostic of the myxobacteria. This difficulty was also experienced by one of us (P.R.H.) when making a detailed study of 62 marine bacteria, all but one of which had been labelled Flavobacterium by the workers who isolated them. By using orthodox taxonomic methods, it was concluded that some of the test organisms were probably cytophages, though others had been properly assigned to the flavobacteria (Hayes, 1962). However, these conclusions were far from being unequivocal and it was thought that the application of Adansonian methods might help to solve this problem and, at the same time, provide an interesting comparison between different taxonomic techniques.

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METHODS

The sixty-two isolates used in this work were of marine origin, and all except NCMB 11 had been identified as Flavobacterium species by the workers who isolated them; isolate NCMB 11 was labelled as a presumptive Cytophaga. All the isolates were examined for a large number of characters; details of the methods used were reported previously (Hayes, 1962).

The data on the characters of the organisms were resolved into 104 features, and put in a form suitable for feeding into a computer. The features ranged over morphology, cultural characters, biochemical reactions and nutrition. The data were processed by an Elliot 402 computer, using Sneath's (1957) programme.

RESULTS

A diagrammatic representation of the sorted S values is shown in Fig. 1. It is clear that in terms of overall similarity as determined by the formula $S = n_s/n_s + n_d$, the organisms examined fell into two large groups or pleista (A, G), several smaller pleista (F, T, L) while the remaining isolates differed widely from each other in their S values, that is, they were the sole representative of their pleiston present. Pleiston G consists of an inner cluster and an outer array of somewhat less similar organisms. The position of the bottom boundary of pleiston G has been somewhat arbitrarily drawn between NCMB 287 and 277 (see Fig. 1). It could also have been drawn so as to include one or more organisms lower down the list. If the view is taken that boundaries of this type are not facts of nature, but merely convenient devices (Floodgate, 1962) then it follows that the best line will be the one which serves the greatest number of bacteriological purposes. At the moment, it is not possible to state which of the possible boundary lines is the most satisfactory, hence an arbitrary decision has to be made. The term pleiston (pl. pleista), referring to a cluster of similar organisms, was coined by Sneath (1957) and is preferred to 'groups' or 'species' because both these terms have specialized taxonomic meanings laid down in the *International Code of Bacterial Taxonomy and Nomenclature* (*Judicial Commission*, 1958).

Description of the pleista and diagnostic features

To identify a new isolate as a member of pleiston A or G with certainty it would be necessary to calculate its S value with respect to the established members of the pleiston. However, if the isolate possesses all or most of the characters of either A or G, the probability of it belonging to the one or the other pleiston is very high. The characters of both pleista and the frequency of their occurrence are set out in Table 1, and those which occur most frequently can be thought of as diagnostic. In so far as they possess most of the diagnostic characters NCMB 245 and NCMB 262 can be said to be typical strains of pleiston A and G respectively. Furthermore, the average of their similarity (S) values to each of the other strains within the pleiston is very high, suggesting that they are very similar to the others in very many respects.

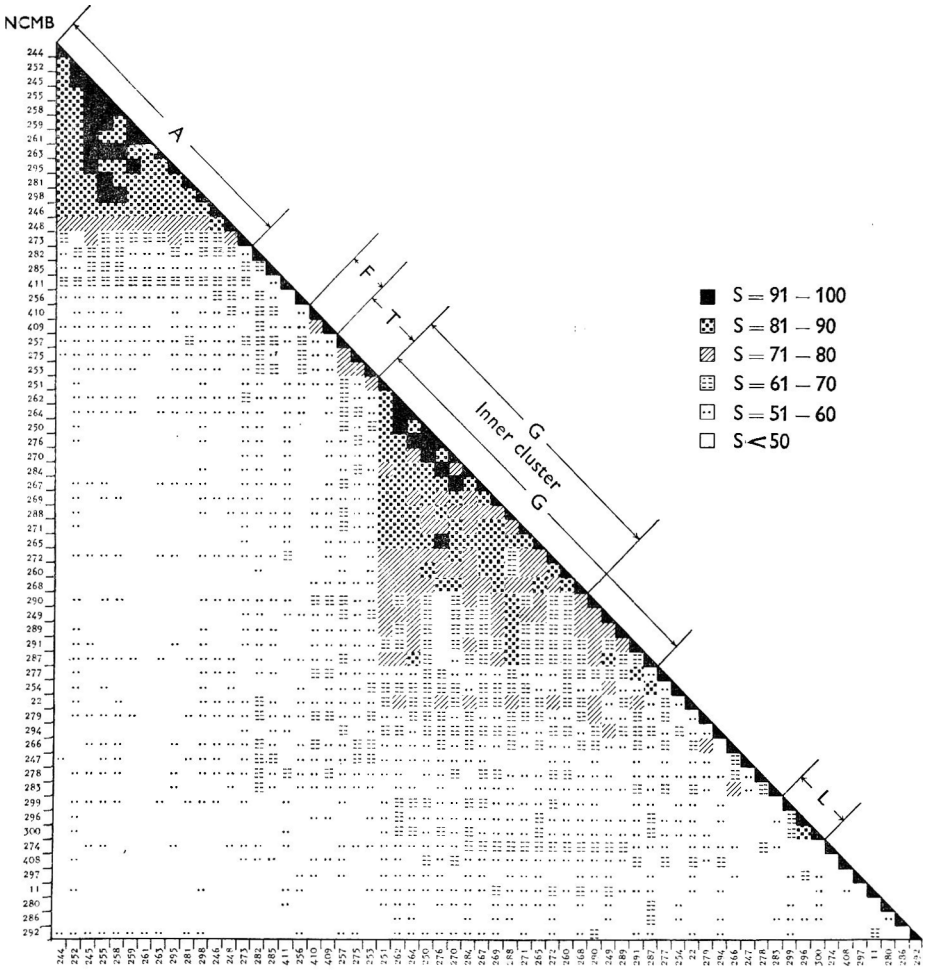

Fig. 1. Diagrammatic representation of sorted S values.

DISCUSSION

Having established the pleista, it is natural to inquire whether any of them resemble any well established bacterial genera or species although a pleiston ought not to be equated to any other taxon. The possibility that the organisms which make up pleiston A or G would also fall into the genus *Flavobacterium* of *Bergey's Manual* (1957), the genus *Pseudobacterium* of Krassilnikov (1949) or to the genus *Cytophaga* described in both these works, is discussed below. It must be emphasized that each of these classifications is based upon a different taxonomic principle so that it would be surprising if each method should lead to the same arrangement of the organisms. Krassilnikov (1949) classified bacteria by what he believed to be their phylogeny. *Bergey's Manual* (1957), being concerned with bringing together and co-ordinating the work of a large number of bacteriologists, reflects several taxonomic viewpoints, whereas pleista A and G are comprised of organisms which are alike in their over-all similarity. Fortunately, however, the concept of similarity, although not

expressed mathematically, is frequently used by the authors of *Bergey's Manual* (1957), while phylogenetically close organisms are often similar to each other; whence some approximation between the pleista and the genera of *Bergey's Manual* (1957) or Krassilnikov may exist. When *Bergey's Manual* (1957) is consulted, pleiston G is found to resemble the genus *Flavobacterium*, in that the cultures consist of medium sized rods, forming an orange or yellow pigment, and attacking sugars either feebly or not at all. This pleiston can be further associated with Section I of the key to the genus *Flavobacterium* since they are non-motile, modify litmus milk and liquefy gelatin. Thereafter, when division is largely made on the kind of reaction to litmus milk, the key becomes unworkable. Three pleiston G organisms did not change milk, 16 reduced the indicator, 10 formed alkali or peptonized the casein, 2 took on a slightly acid reaction and 1 formed a clot. Most organisms carried out several of these reactions simultaneously. The fact that these very similar strains differed in their reaction to litmus milk suggests that a division into several species by means of this test is not advisable. The nomenclatural type species of the genus *Flavobacterium*, *F. aquatile* ATCC 11947 was not included

Table 1. *Characters of pleista A and G*

Characters of organisms			Number of strains possessing the characters in: Pleiston A (total 14 strains)	Pleiston G (total 20 strains)
Sides of organisms	Parallel		14	8
	Bulging		0	11
	Irregular		0	1
Regularity of morphology*	Monomorphic		14	0
	Pleomorphic		0	20
Insoluble pigment	Yellow		5	6
	Green-yellow		9	0
	Orange-yellow		0	14
Growth in broth	Granular ring		12	9
	Pellicle		2	0
	Granular, easily dispersed deposit		12	0
	Flocculent deposit, dispersed with difficulty		2	15
Lowest temperature permitting growth	0°		14	13
	5°		0	3
	10°		0	4
Highest temperature permitting growth*	30°		13	1
	25°		1	19
Optimum temperature	20°		0	19
	21–24°		14	0
	25°		0	1
Heat resistance*	55°	No. killed in 15 min.	9	20
		No. surviving 15 min. but not 30 min.	5	0
	45°	No. killed in 15 min.	0	8
		No. surviving 15 min. but not 30 min.	0	9
		No. surviving 30 min. but not 45 min.	0	2
		No. surviving 45 min.	14	2
	37°	No. killed in 24 hr.	0	16
		No. surviving 24 hr. but not 48 min.	0	2
		No. surviving 48 hr.	14	2

Table 1 (*cont.*)

Characters of organisms		Number of strains possessing the characters in: Pleiston A (total 14 strains)	Pleiston G (total 20 strains)
NaCl tolerance	No. tolerating 2 % but not 4 %	0	1
	No. tolerating 4 % but not 6 %	1	0
	No. tolerating 6 % but not 10 %	13	18
	No. tolerating 10 %	0	1
Penicillin sensitive	—	14	9
Streptomycin sensitive	—	14	16
Vibriostat sensitive*	—	14	1
Gelatin liquefaction*	Incomplete liquefaction in 2–4 weeks	12	1
	Complete liquefaction in 2–3 weeks	0	15
Ammonia from peptone*	—	0	20
Acid from	Xylose*	14	0
	Rhamnose*	12	0
	Glucose*	14	0
	Fructose*	14	0
	Mannose*	14	0
	Galactose	7	0
	Sucrose*	14	0
	Maltose*	12	0
	Mannitol*	13	0
	Salicin	9	0
Litmus milk	Reduction of indicator	1	16
	Acid reaction	3	2
	Alkaline reaction	1	6
	Peptonization	0	4
	Clot	0	1
'Oxidase'	—	0	9
Starch hydrolysis	—	1	5
Tributyrin agar	—	6	1
Nutrition*	Requiring unknown factors in sea-water broth	12	4
	Growth in defined media plus growth factors	2	10
	Growth in defined media plus amino acids	0	4
	Growth in defined media plus purines	0	2
'Swarming'*	Swarm at peptone concentrations of 0·25 %	7	0
	Swarm at peptone concentrations of 0·05 %	10	0
	Swarm at peptone concentrations of 0·01 %	12	0
	Did not swarm	2	20

The cells of pleiston A strains measured 1–5 $\mu \times 0{\cdot}4$–$0{\cdot}6\,\mu$ with most strains 1–3 $\mu \times 0{\cdot}4$–$0{\cdot}6\,\mu$; the cells of pleiston G strains measured 1–> 10 $\mu \times 0{\cdot}4$–$1{\cdot}0\,\mu$ with most strains 1–5 $\mu \times 0{\cdot}5$–$0{\cdot}8\,\mu$.

All strains were composed of Gram negative, non-motile rods. All colonies were circular, smooth, shiny, translucent and with an entire edge when grown on sea-water nutrient agar, all grew with a uniform turbidity in broth; all were sensitive to chloramphenicol and terramycin; none produced nitrite from nitrate; all produced H_2S; none formed acid from arabinose, lactose, trehalose, raffinose, starch, insulin, dulcitol, sorbitol or inositol. All were catalase positive and none used citrate as sole carbon source.

* Indicates those characteristics which distinguish the pleista and can be used for identification.

in the computer analysis because sufficient data on this strain were not available. However, some of the morphological, cultural and biochemical characters of the type strain have been examined by one of us (P.R.H.), and found to be such as to suggest that this strain might well fall into pleiston G. This is further evidence for the proposition that the genus *Flavobacterium* and pleiston G are conformable.

The only other genus with which pleiston G is at all likely to be associated is the genus *Alcaligenes*. However, this is not as plausible as associating pleiston G with the flavobacteria because although Alcaligenes species have some biochemical properties similar to those of members of pleiston G, the production of yellow pigments by Alcaligenes is not very common. Furthermore, the organisms of pleiston G tend to be orange-yellow rather than brown or grey-yellow as the Alcaligenes group are.

It is difficult to find a taxon among the true bacteria in the scheme of *Bergey's Manual* (1957) which resembles pleiston A. They are yellow pigmented, but the pigments tend to be greenish yellow rather than yellow or orange. Acid was formed from a high percentage of the carbohydrates examined, and the organisms were not very proteolytic. These characteristics would not necessarily dissociate them from the genus *Flavobacterium*, particularly as several of the species listed in *Bergey's Manual* (1957) also form acid from a number of sugars.

When the organisms of pleista A and G are compared with Krassilnikov's classification (1949), both can be associated with the genus *Pseudobacterium*. These strains are described by Krassilnikov as having rod-shaped cells of varying size, sometimes pleomorphic and occasionally decomposing into short elements of coccoid forms; they are non-motile and may be Gram-positive or Gram-negative; the colonies are colourless, red, yellow or orange. Physiologically they are heterogeneous. Clearly pleista A and G could be associated with this genus.

The question remains whether either pleiston A or G can be allied to the genus *Cytophaga*. The main characters of this genus as described by *Bergey's Manual* (1957) are briefly as follows: weakly refractile flexible rods; reproduction by binary transverse fission; absence of fruiting bodies; movement is by a gliding motion over a surface giving rise to a swarm or pseudoplasmodium of a flat, thin, spreading mass of vegetative organisms. The type species of the genus is *Cytophaga hutchinsonii*, a culture which is not now available. The attributes of the 62 isolates examined in the present work with regard to the first three of these characters were not available for inclusion in the Adansonian analysis; they were referred to in a previous paper (Hayes 1962). The ability to swarm was, however, included in the analysis. Hayes (1962) confirmed the observation of Stanier (1947) that there was a relationship between peptone concentration and motility, and showed that a number of the strains under study could be induced to form a swarm when the peptone concentration was lowered sufficiently. He suggested that the formation of a swarm on a peptone-depleted agar medium constituted a sensitive method of demonstrating a gliding motility which is too slow to be observed microscopically in a wet film preparation. When pleiston G was examined it was found that none of its constituent isolates showed any sign of swarming at any of the peptone concentrations used, while all but 2 of the 14 strains of pleiston A did so. It would be reasonable then to consider pleiston A as bearing a resemblance to the genus *Cytophaga*.

Unfortunately some other swarmers did not fall into pleiston A, including the two most vigorous ones, NCMB 11 and 292, both of which were also seen to move by gliding when observed under the microscope. Indeed these two organisms have a low S value when compared to almost all the other cultures, although they are similar to each other ($S_{11-292} = 69\,\%$). Other swarmers were NCMB 247 and 285, and NCMB 253, 257 and 275, the latter three forming pleiston T. The pair NCMB 247 and 285 are also fairly similar to each other ($S_{247-285} = 63\,\%$), but did not fit into any of the pleista. It is well established that gliding motility is exhibited by many widely differing organisms, including blue-green algae, a fact which suggests that this kind of motility has arisen several times during the evolution of micro-organisms. There is no *a priori* reason for believing that a similar development could not occur among the true bacteria, giving rise to organisms that would in this respect closely resemble the cytophagas.

A comparison can be made between the groups formed by Hayes (1962) by traditional taxonomic methods, and the pleista formed by Adansonian techniques. Pleiston A corresponds exactly to Hayes group I. Pleiston G is composed of Hayes group 2 + group 3 except NCMB 254 which fell just outside the pleiston G boundary as now drawn. Also included in pleiston G are two organisms NCMB 288 and 290 which Hayes could not place in any of his groups. Three of the five members of Hayes group 4 make up pleiston T, the exceptions being NCMB 247 and 285, neither of which fell into any category in the computer analysis. NCMB 409 and 410 which formed pleiston F were not classified by Hayes, while NCMB 296 and 300 which he assigned to the genus *Flavobacterium* came together as pleiston L in the Adansonian analysis. However, these organisms are very unlike to the organisms of pleiston G which are associated with the genus *Flavobacterium*. It is also interesting to note that NCMB 280 and 299, both of which Hayes (1962) was able to place in the genus *Corynebacterium* as described in *Bergey's Manual* (1957), are very unlike each other when all their properties are taken into account ($S_{280-299} = 54\,\%$). Similarly, NCMB 286 and 294, which can be assigned to the genus *Pseudomonas* by using the criteria of Shewan, Hobbs & Hodgkiss (1960) have a low over-all similarity value to each other ($S_{286-294} = 52\,\%$).

The work described in this paper was carried out as part of the programme of the Department of Scientific and Industrial Research.

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J. gen. Microbiol. (1963), **30**, 245–248
Printed in Great Britain

Vitamin Requirements of Root Nodule Bacteria

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(*Received* 8 *May* 1962)

SUMMARY

The vitamin requirements of 63 strains of *Rhizobium* and 18 of *Agrobacterium* were examined. *R. trifolii*, *R. leguminosarum* and *R. phaseoli* responded in most cases to thiamine, biotin, and calcium pantothenate. Strains of *R. meliloti* and the slow-growing rhizobia sometimes responded to biotin, but not to the other vitamins. An unknown growth factor, active for the slow-growing rhizobia, was detected. Three strains of *Agrobacterium* required thiamine.

INTRODUCTION

Species of root nodule bacteria differ in their vitamin requirements. Biotin is essential for growth of strains of *Rhizobium leguminosarum*, *R. trifolii*, *R. phaseoli*, *R. meliloti* and *R. lupini* (West & Wilson, 1939*a*, *b*, 1940). In some instances it is required only to initiate growth (Allen & Allen, 1950). According to Jordan (1952), some strains of *R. meliloti* require neither biotin nor any other of the B group of vitamins. Biotin is apparently not required by cowpea or soybean rhizobia or by the common nodule contaminant *Agrobacterium radiobacter* (West & Wilson, 1939*a*, 1940). Thiamine and pantothenic acid stimulate growth of some strains of *R. trifolii* (West & Wilson, 1939*a*; Lilly & Leonian, 1945; Ljunggren, 1961), but in others they are either not required for growth (McBurney, Bollen & Williams, 1935; Jordan, 1952) or have not been tested (Allen & Allen, 1950). Synthesis of large amounts of vitamin B_{12} by the rhizobia (Burton & Lochhead, 1952; Levin, Funk & Tendler, 1954) has been considered in relation to leghaemoglobin production in the nodule (Levin *et al.* 1954). A cobalt requirement has been established for both rhizobial growth (Lowe, Evans & Shaukat, 1960) and symbiotic fixation (Shaukat & Evans, 1961).

The experiments reported here form part of an investigation of rhizobial taxonomy and were suggested by Dr C. A. Parker and Mr A. E. Oakley following their development of a defined growth medium for strains of *Rhizobium trifolii*.

METHODS

Organisms. The organisms used in routine work were maintained on yeast extract mannitol (YM) agar and were subcultured at monthly intervals. They were preserved by the lyophilization method described by Annear (1956) and the ampoules were stored at 4°.

Media. The defined basal medium used throughout contained (g./l. distilled water): $MgSO_4.7H_2O$, 0·25; $CaSO_4.2H_2O$, 0·03; KH_2PO_4, 0·55; $CuSO_4.5H_2O$,

0·00008; $ZnSO_4.7H_2O$, 0·00016; $FeSO_4.7H_2O$, 0·0035; NaCl, 0·25; $Na_2HPO_4.12H_2O$, 1·20; $MnSO_4.4H_2O$, 0·0004; H_3BO_3, 0·0005; urea, 0·5; glucose, 10·0; sucrose, 10·0; tyrosine, 0·015; aspartic acid, 0·015; lysine, 0·015; histidine, 0·015; glutamic acid-HCl, 0·10; arginine, 0·015. The medium (minus the phosphates) was sterilized by autoclaving for 35 min. at 5 p.s.i. Solutions and the phosphates were sterilized separately and added before use.

This medium, with thiamine-HCl (0·004 g.), calcium pantothenate (0·004 g.) and biotin (0·001 mg.) per litre added, had previously been developed for two strains of *Rhizobium trifolii*. It supported growth to about 2×10^{10} organisms/ml. (Dr Parker and Mr Oakley, personal communication). In the present work it was used either as a broth or solidified with 1·5 % agar previously washed for 3 weeks in repeated changes of distilled water.

Experimental procedure. Ten vitamins were tested. Requirements for thiamine, calcium pantothenate, nicotinic acid, riboflavin, inositol, folic acid, *p*-aminobenzoic acid, vitamin B_{12} and pyridoxin were studied by the agar disk method described by Ford & Hutner (1957). Tubes of basal agar medium, each containing 25 ml., were liquefied by autoclaving, allowed to cool to 45° and inoculated with a washed suspension of the appropriate organism; they were then agitated and poured. Sterile filter paper disks, each impregnated with 1 μg. of one of the vitamins under test, were placed on the agar plates. Plates were examined for the presence or absence of growth stimulation after 3 days at 28°. Biotin requirements could not be studied by this method because the biotin content of the agar, even after repeated washing, was sufficient to supply the needs of the organisms. Instead, liquid medium (20 ml.) was used in 100 ml. Erlenmeyer flasks loosely plugged with biotin-free cotton wool and covered with a 50 ml. beaker. The flasks were then inoculated with a washed suspension as before; duplicate flasks and positive controls were used. They were incubated for 3 days at 28° in a reciprocal shaking machine. After three successive transfers in the basal medium the flasks were examined for differences in growth.

RESULTS

There was no growth response by any of the cultures to nicotinic acid, pyridoxin, folic acid, *p*-aminobenzoic acid, inositol, vitamin B_{12} or riboflavin. Strains of *Rhizobium trifolii*, *R. leguminosarum* and *R. phaseoli* were alike in their requirement for biotin, thiamine and calcium pantothenate (Table 1). Of the 31 strains of these 3 species, 5 responded to both biotin and calcium pantothenate, 8 to thiamine and calcium pantothenate and 10 to thiamine, biotin and calcium pantothenate. Only 5 of the 31 strains did not require calcium pantothenate for maximum growth. In several instances both thiamine and calcium pantothenate were essential to initiate growth (Fig. 1). Biotin was also required by strains of *R. lupini* and *R. meliloti*, but the frequency with which it was required appeared to be much less than that suggested by earlier workers (Allen & Allen, 1950). Thiamine was also required by one of 7 strains of *A. radiobacter* and 2 of 11 strains of *A. tumefaciens*.

Strains of *R. lupini*, *R. japonicum* and the cowpea rhizobia did not appear to require most of the vitamins studied in this work, although some unknown growth factor present in the yeast extract mannitol medium was absent from or deficient in the defined one. On transfer from YM agar medium these bacteria grew adequately

Table 1. *Vitamin requirements of Rhizobium and Agrobacterium species*

	Species								
	R. trifolii	*R. leguminosarum*	*R. phaseoli*	*R. lupini*	*R. japonicum*	Cowpea complex	*R. meliloti*	*A. radiobacter*	*A. tumefaciens*
No. strains tested	16	8	7	7	6	9	10	7	11
Vitamin required									
Biotin	—	1	—	3	—	2	4	—	—
Thiamine	—	—	—	—	—	—	—	1	2
Ca pantothenate	2	—	—	—	—	—	—	—	—
Biotin + thiamine	—	—	—	—	—	—	—	—	—
Biotin + Ca pantothenate	3	1	1	—	—	—	—	—	—
Thiamine + Ca pantothenate	3	2	3	—	—	—	—	—	—
Biotin, thiamine and Ca pantothenate	8	2	—	—	—	—	—	—	—

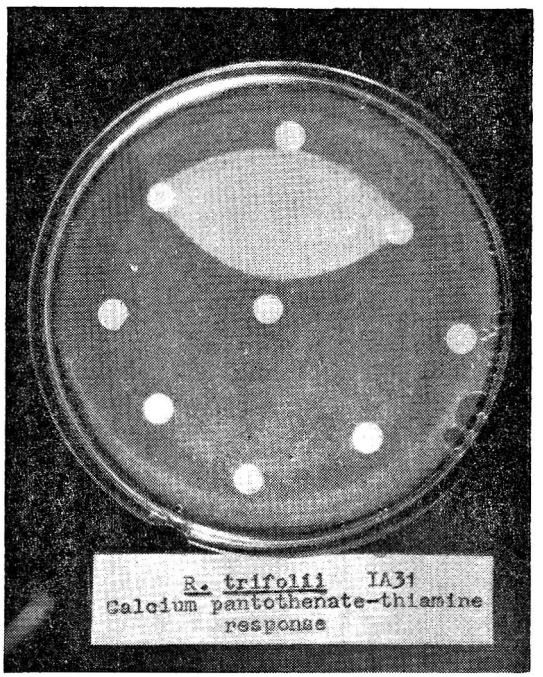

Fig. 1. Stimulation of the growth of *Rhizobium trifolii* strain 1 A 31 by thiamine (central disk) and calcium pantothenate (centre, top). Note the absence of growth unless both vitamins are supplied.

at first but in subsequent transfers were much diminished in growth. Certain contaminating fungi overcame this deficiency, but adding L-glutamic acid, DL-tryptophan, DL-methionine, DL-valine, DL-phenylalanine, DL-threonine, L-citrulline, L-cysteine HCl, or L-serine singly or in combination to the medium did not replace

the requirement. Guanine, adenine, thymine, uracil, choline, thioctic acid and ascorbic acid were similarly inactive in making the defined medium like the YM agar.

Bergersen (1961) considered that near-maximal growth of root nodule bacteria can be achieved in a medium in which the only growth-promoting substances are thiamine, biotin and sodium glutamate. It would appear that at least two additional substances, namely, pantothenic acid and an unknown growth factor(s) are important in the nutrition of some of these bacteria. Bergersen's results indicate the necessity for using numerous representative strains in work on Rhizobium.

Vitamin and amino-acid requirements have been used in the separation and classification of various soil-borne bacteria (Lochhead, 1952) and in Adansonian classification (Sneath & Cowan, 1958). The present results suggest that they could be equally valuable in classifying the root nodule bacteria. Further taxonomically useful characters are being sought.

The author thanks Dr C. A. Parker and Mr A. E. Oakley for their advice, and Miss R. Ambrose for technical assistance. The work was done during the tenure of the Bank of New South Wales Research Fellowship in Agriculture, and was financed from Wheat Industry Research Grants.

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J. gen. Microbiol. (1963), 30, 249–260
Printed in Great Britain

Heterozygous clones in *Streptomyces coelicolor*

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(*Received* 10 *May* 1962)

SUMMARY

Certain of the colonies that arise when spores from a mixed culture of two complementary auxotrophic strains of *Streptomyces coelicolor* A3(2) are plated on a selective medium give rise to a mixture of spores with parental and recombinant genotypes. These heterogeneous colonies (*heteroclones*) probably develop from spores containing heterozygous nuclei which segregate during the development of the colonies. The heterozygous nuclei probably undergo a number of equational divisions before segregating. The markers in two linkage groups segregate independently. Large imbalances in the allele ratios and in the frequencies of complementary genotypes have been attributed to terminal chromosome deletions which affect both chromosome pairs. Absence (or homozygosity of the markers) of one whole chromosome is observed very often in heteroclones selected on media which make one chromosome dispensable. The heteroclones provide an efficient system for studying the genetics of the organism without recourse to selective methods.

INTRODUCTION

The actinomycete *Streptomyces coelicolor* is a filamentous organism which grows in the form of a mycelium and produces regular chains of uninucleate haploid spores as the terminal stage in the life-cycle. There is no morphological evidence of a sexual cycle (Hopwood, 1960), but genetic recombination occurs; it was discovered independently by Sermonti & Spada-Sermonti (1955) and by Hopwood (1957). When two strains of the organism, each having one or more biochemical deficiencies, are grown together in mixed culture for a few days and spores then harvested and plated on a medium lacking at least one of the growth-factors required by each parent strain, 1 in 10^2 to 10^5 of the plated spores gives rise to a colony. These colonies are of two kinds: haploid recombinants and segregating heterozygotes. The relative proportions of the two kinds of colonies vary according to the particular combination of nutritional requirements against which the medium selects. The haploid recombinant colonies are homogeneous, breeding true on subculture. The relative frequencies of the different genotypes of recombinants can be used to determine the linkage relations of the markers, and two linkage groups each represented by half a dozen loci were recognized by Hopwood (1959). The segre-

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gating heterozygous colonies, on the other hand, contain in their spores a mixture of different genotypes, and these colonies have therefore been called heteroclones (Sermonti, Mancinelli & Spada-Sermonti, 1960). The evidence suggests that they arise from single spores (or, more precisely, single 'plating-units') each containing a heterozygous nucleus which allows them to grow on the selective medium.

The heteroclones make up a significant proportion of the total colonies which arise on a selective medium only when the nutritional requirements (at least one in each parental strain) which the medium does not satisfy are determined by closely linked mutants. Under these conditions, only a small proportion of the total haploid recombinants in the plated spore suspension are able to give rise to colonies, while the number of heterozygotes that can grow is unaffected by the stringency of selection. When the two markers selected against are about five recombination units apart, heteroclones represent about 10 % of the total colonies, and the proportion rises to nearly 100 % when the selected markers are less than one unit apart.

The heterozygous nuclei segregate during the growth of the heteroclones, and nearly all the spores to which they give rise are haploid. Since the heterozygous nuclei contain two closely linked nutritional mutants, one in each parental genome, the great majority of the haploid spores have one or other growth requirement and are unable to grow on the selective medium. This provides a ready means of recognizing the heteroclones. Replica plates are prepared from the original selective plates, on a medium of the same composition, by using a velvet pad (Lederberg & Lederberg, 1952); the homogeneous recombinants are perpetuated on the replica plates, while the heteroclones are not (Hopwood & Sermonti, 1962).

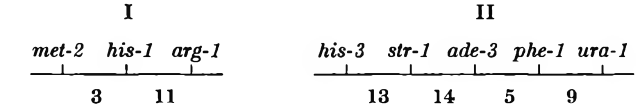
The interest of the heteroclones of *Streptomyces coelicolor* lies primarily in the fact that in them the heterozygous condition of the nucleus is prolonged enough for its segregation to be studied. In eubacteria, on the other hand, the heterozygous condition produced by transfer of genetic material from a donor to an acceptor cell is generally very short-lived. When transfer is by Hfr-mediated conjugation (Hayes, 1960), transformation (Ephrussi-Taylor, 1960) or 'general' transduction (Hartman & Goodgal, 1959), the transferred fragment appears not to reproduce itself, or to do so to a very limited extent, before its incorporation in a recombinant genome. Thus the acceptor cell does not produce a clone of heterozygous cells. Only when genetic transfer is mediated by episomes (Jacob & Wollman, 1958) do clones of heterozygous cells occur regularly, as in 'special' transduction (Morse, Lederberg & Lederberg, 1956*a*) and in 'sexduction' (Jacob & Adelberg, 1959), where attachment of a fragment of bacterial chromosome to an episome apparently enables it to reproduce with the acceptor cell.

The persistent heterozygotes of *Escherichia coli*, discovered by Lederberg (1949), represent an exception. In the presence of a mutant gene (*Het*), the zygotes were able to undergo several divisions before segregation took place. These heterozygotes have contributed little to the picture of the genetic system of *E. coli*; other lines of investigation have been exploited instead (Wollman, Jacob & Hayes, 1956), and no recent work on the heterozygotes appears to have been published. The heterozygotes of *E. coli* seem to have some features in common with those of *Streptomyces coelicolor*. However, in *S. coelicolor* they have not only facilitated the formal genetic analysis of the organism, but have also provided some information on the genetic

system involved in the formation of recombinants. In this paper we shall describe the analysis of the heterozygotes and discuss the information about the genetic system that can be obtained from them.

METHODS

Mutant strains. All the auxotrophic and streptomycin-resistant mutants used in this work have been derived from the same wild-type strain of *Streptomyces coelicolor* (Hopwood, 1959). The origins, characteristics and linkage relations of the various mutants were recently summarized by Hopwood & Sermonti (1962). The markers mentioned in the present paper are located in the two linkage groups as follows, the distances being expressed as % recombination as deduced from the analysis of heteroclones:



The symbols for the markers are as follows: *ade*, *arg*, *his*, *met*, *phe*, *ura* = requirement for adenine, arginine, histidine, methionine, phenylalanine or uracil, respectively; *str* = resistance to streptomycin.

Media. For details of the complete and minimal media and of the supplements added to minimal medium, see Hopwood & Sermonti (1962).

Preparation of mixed cultures and plating on selective media. Two pairs of closely linked nutritional markers were used for the selection of heteroclones: either *met-2* and *his-1* in linkage group I, or *ade-3* and *phe-1* in linkage group II. Various other markers were incorporated in the parent strains, depending on the scope of the experiment. Inocula of the two parent strains were mixed together on a slope of complete medium. After incubation of the culture for 3–5 days at 30°, a suspension of spores was prepared in sterile water, filtered through cotton wool to remove large mycelial fragments, and spread on plates of selective medium at a density of about 10^5 to 10^6 spores per plate. The selective medium lacked the growth factors corresponding to the pair of closely linked markers, and might or might not contain growth factors corresponding to other markers.

Recognition and study of the heteroclones. When the recombinant colonies were sporulating on the selective medium (about 4 days), replica plates were prepared on a medium of the same composition. Those colonies which did not give growth on the replica plates after incubation for 2 days were identified as heteroclones. The heteroclones were usually, though not invariably, smaller than the homogeneous recombinant colonies. Spores were isolated from a heteroclone in a loopful of water and plated on complete medium at suitable dilutions to obtain isolated segregant colonies. Sometimes spores were also plated on selective media for the recovery of second-order heteroclones. After growth for 3 days the segregant colonies were inoculated to master plates of complete medium, and were characterized by replicating to plates of diagnostic media (Hopwood & Sermonti, 1962).

RESULTS

Quantitative analysis of heteroclones

Table 1 shows the genotypes of the segregants obtained from a single heteroclone that was isolated on unsupplemented minimal medium. Six markers were segregating, two in linkage group I and four in linkage group II. In a sample of 166 segregants, 23 of the 64 possible genotypes were found, six of them being represented only once. This indicates that many more genotypes would have been recovered by isolating a larger sample of segregants. The results are presented as a contingency table in which the relative frequencies of the four combinations of markers in linkage group I are shown in association with each combination of markers in group II. There is no evidence of heterogeneity in the table, since G calculated by

Table 1. *Numbers of segregants of each genotype in a heteroclone*

1st parent: *met-2* + + + *ade-3* +

Regions: 1 2 3 4

2nd parent: + *arg-1* *his-3* *str-1* + *phe-1*

Linkage groups: I II

Markers in linkage group II				Markers in linkage group I					Cross-over in region
				+ *arg*	*met* +	+ +	*met arg*	Total	
+	+	*ade*	+	39	32	6	2	79	—
his	*str*	+	*phe*	6	10	2	2	20	
+	*str*	+	*phe*	7	7	3	1	18	
his	+	*ade*	+	2	0	1	0	3	2
+	+	+	*phe*	21	13	4	2	40	
his	*str*	*ade*	+	0	0	0	0	0	3
+	+	*ade*	*phe*	0	0	1	0	1	
his	*str*	+	+	0	0	0	0	0	4
+	*str*	*ade*	+	0	1	0	0	1	
his	+	+	*phe*	1	0	0	0	1	2, 3
+	+	+	+	2	1	0	0	3	
his	*str*	*ade*	*phe*	0	0	0	0	0	3, 4
+	*str*	+	+	0	0	0	0	0	
his	+	*ade*	*phe*	0	0	0	0	0	2, 4
+	*str*	*ade*	*phe*	0	0	0	0	0	
his	+	+	+	0	0	0	0	0	2, 3, 4
			Total	78	64	17	7	166	
Cross-over in region:					—		1		

Of the 166 segregants, 157 (those in rows 1, 2, 3 and 5) have been used to test the independence of marker combinations in the two linkage groups (see text).

the method of Woolf (1957) is equal to 6·09 with 9 degrees of freedom; this corresponds to a probability of 0·75. Thus marker combinations of the two linkage groups are associated at random. This indicates the absence, in this heteroclone, of preferential associations of unlinked markers such as result in some heteroclones from contamination by an extraneous clone, or from the presence of a fast-growing sector originated within the heteroclone. The independence test is used routinely

to recognize heteroclones in which segregations are disturbed by such effects, and only those segregations which show no preferential combinations of unlinked markers are analysed quantitatively. In Table 1, the proportions of the various pairs of complementary genotypes are in general those predictable from the linkage relations of the markers. However, complementary genotypes are not equally numerous, those containing the allele *his-3*$^-$ being in the minority. Such inequalities in the frequencies of complementary genotypes are characteristic of the heteroclones; their interpretation and their effects on the estimation of linkage will now be considered.

Table 2. *Segregation of markers in linkage group II in a heteroclone*

Constitution of the heterogenote:*	+	+	*ura-1*
	1	2	*y*
	str-1	*ade-3*	+
Allele ratios:	88	98	103
	43	33	28

(*a*) *Numbers of segregants of each genotype*

Genotypes of segregants			Observed numbers	Cross-over in regions
+	+	*ura*	77	—
str	*ade*	+	19	*y*
str	+	*ura*	16	1
+	*ade*	+	4	1, *y*
str	*ade*	*ura*	8	2
+	+	+	5	2, *y*
+	*ade*	*ura*	2	1, 2
str	+	+	0	1, 2, *y*

(*b*) *Recombination between pairs of loci*

	str, ade			*ade, ura*			*str, ura*		
Parental combinations:	+	+	82	+	*ura*	93	+	*ura*	79
	str	*ade*	27	*ade*	+	23	*str*	+	19
	str	+	16	*ade*	*ura*	10	*str*	*ura*	24
Recombinant combinations:	+	*ade*	6	+	+	5	+	+	9
% recombination:			17			12			25

* The dotted line indicates a deleted region.

In this segregation, inequalities in the frequencies of complementary genotypes are explained by a single terminal deletion (contrast Table 3): χ^2 (3 D.F.) testing deviation from equality of ratios within the four pairs of complementary genotypes = 0·79 (P = 0·85).

Table 2 shows the data for the segregation of three loci in linkage group II from another heteroclone. Complementary genotypes do not have equal frequencies, genotypes including the allele *ura-1*$^+$ always being less frequent. The diminished frequencies of the markers in coupling with *ura-1*$^+$, namely *ade-3*$^-$ and *str-1*$^-$, can be attributed to the primary disturbance at the *ura-1* locus; they do not require independent explanations. This is shown by the fact that none of the ratios between

the frequencies of complementary genotypes differs significantly from the allele ratio at the *ura-1* locus, that is 103:28.

Other heteroclones were found in which the magnitude of the disturbance in the allele ratio at the *ura-1* locus varied widely from one heteroclone to another, one allele being occasionally completely absent. Sometimes *ura-1*$^+$ and sometimes *ura-1*$^-$ was the less frequent. Various other loci were found to show primary disturbances in allele ratios in different heteroclones, and like *ura-1* these were always terminal loci of one or other linkage group. The simplest explanation of these findings (Hopwood, Mancinelli, Sermonti & Spada-Sermonti, 1961) is that the units from which the heteroclones arise are 'heterogenotes' (Morse, Lederberg & Lederberg, 1956*b*) in which one or more terminal segments of chromosome are lacking. For an allele in coupling with a deletion to be inherited by viable segregants, a cross-over must occur between the locus and the point of deletion, assuming the deficiency to be lethal in the haploid condition; this accounts for the decreased frequency of the allele.

Table 3. *Segregation of markers in linkage group II in a heteroclone*

Constitution of the heterogenote*:	+	+	*ade-3*	
	x	1	2	*y*
	his-3	*str-1*	+	
Allele ratios:	142	127	84	
	24	39	82	

(*a*) *Numbers of segregants of each genotype*

Genotypes of segregants			Observed numbers	Cross-over in regions
+	+	*ade*	80	*y*
his	*str*	+	20	*x*
+	*str*	+	18	1
his	+	*ade*	3	*x*, 1, *y*
+	+	+	43	2
his	*str*	*ade*	0	*x*, 2, *y*
+	*str*	*ade*	1	1, 2, *y*
his	+	+	1	*x*, 1, 2

(*b*) *Recombination between pairs of loci*

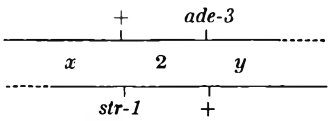
	str, ade			*his, str*			*his, ade*		
Parental combinations:	+	*ade*	83	+	+	123	+	*ade*	81
	str	+	38	*his*	*str*	20	*his*	+	21
Recombinant combinations:	+	+	44	+	*str*	19	+	+	61
	str	*ade*	1	*his*	+	4	*his*	*ade*	3
Total recombinants:			45			23			64
% recombination (*str–ade* = 14)			(14)			7			20

* Dotted lines indicate deleted regions.

In this segregation, disturbances are explained by two terminal deletions in *trans* (contrast Table 2): χ^2 (3 D.F.) testing deviation from equality of ratios within the four pairs of complementary genotypes = 17·10 ($P \ll 0{\cdot}01$). The data come from Table 1, the locus *phe-1*, closely linked to *ade-3*, being disregarded.

When, as in the heteroclone in Table 2, the departure from equality of complementary genotypes is attributable to a single disturbance (a single deficiency on the hypothesis of terminal deletions), the estimation of linkage between pairs of loci presents no difficulty (Table 2*b*). The observed proportion of segregants showing the recombinant genotypes can be taken as a reliable estimate of recombination, since the decreased frequency of a single marker affects proportionately the frequencies of parental and recombinant segregants. This situation can be recognized by means of a 2×2 χ^2 which compares the ratios within parental and recombinant classes. When this chi-square is not significant, as for the three pairs of loci in Table 2*b*, the linkage estimates can be accepted as reliable to the degree of accuracy with which we are concerned here. Several pairs of markers were studied in this way in many heteroclones and, when estimates of recombination were accepted only from data giving a non-significant chi-square, an entirely consistent picture of the linkage relations of the markers resulted (Hopwood & Sermonti, 1962). Moreover, the linkage map of Hopwood (1959), which was based on the much less direct method of selective analysis, was very largely confirmed.

Often a single disturbance will not explain the irregularities in the segregation of the markers in a linkage group. An example involving three loci in linkage group II is presented in Table 3. As before, the frequencies of complementary genotypes are different, but this time the ratio is not the same in all pairs of genotypes. Two disturbances have to be postulated, and they can be represented as deletions at opposite ends of the two homologous chromosomes (that is in the *trans* configuration). The situation is most clearly illustrated if we consider the segregation of the alleles at two loci. For example, considering only the loci *str-1* and *ade-3*, a model for this heterogenote is as follows:



The four classes of segregants would be produced as follows:

			Cross-over in	Observed no.
Parental classes:	+	*ade*	*y*	83
	str	+	*x*	38
Recombinant classes:	+	+	2	44
	str	*ade*	*x*, 2, *y*	1

The greater inequality in the frequencies of the two recombinant classes as compared with the parental pair is explained by the necessity of a triple cross-over for the formation of the type *str ade*, as compared with single cross-overs for the other three classes. Moreover, the observed proportion of recombinants between the two loci, 45/166, is an overestimate of the true frequency of recombination. This is because the need for a cross-over in either interval *x* or *y* to produce the parental classes diminishes the frequencies of both of these classes, while only one of the recombinant classes suffers a decrease in frequency, this time a compound one caused by the double cross-over in *x* and *y*; the net result of a reduction of each of the parental classes is proportionately greater than that of a compound reduction of only one of the recombinant classes.

Even in the presence of two disturbances in one linkage group, estimates of recombination can be made. The situation of a pair of loci included between two deletions in *trans* is formally equivalent to the four-point bacterial cross with selection for wild-type alleles in *trans* at the two outside loci considered by Bailey (1951), and so on for larger numbers of loci As pointed out by Bailey, a method of calculating recombination percentages which uses the frequencies of the classes requiring multiple cross-overs suffers from the severe drawback that these values are very small and easily biased. The safest approach is to calculate unknown recombination values in proportion to another distance in the same linkage group that is already known (from the analysis of other heteroclones). When, for example, we take the distance between *str-1* and *ade-3* to be 14 units (the mean value from the data in Hopwood & Sermonti, 1962), the other two recombination percentages in the heteroclone of Table 3 can be calculated by simple proportionality. The estimates obtained in this way are only first approximations, since no account is taken of multiple crossing-over, and a more complex mathematical treatment is needed to obtain more precise values. However, the correction is negligible when the various intervals are small and of comparable length.

We have seen that the data from heteroclones in which the segregation of markers in a linkage group is affected by a single disturbance or by two disturbances in the *trans* configuration can be used to estimate the recombination percentages between pairs of loci, and the various distances show reasonable additivity. The order of the loci on the linkage map can also be readily determined by considering the segregation of three loci, and identifying the least frequent complementary pair as that produced by double cross-over. Thus the data in Table 2 indicate the order *str-1–ade-3–ura-1* for these three loci, while those in Table 3 give the order *his-3–str-1–ade-3*; these combine to give the following order for the four loci: *his-3–str-1–ade-3–ura-1*. Many more data are summarized by Hopwood & Sermonti (1962).

The range of heteroclone types

The heteroclones just considered were recovered on unsupplemented minimal medium, on which a number of wild-type alleles were selected, besides those at the two closely linked loci which are the minimum requirement for the recovery of heteroclones. When each parent strain carries selected markers in each linkage group, the heteroclones are normally heterozygous for markers in both linkage groups. They always show terminal deletions in at least one member of both chromosome pairs. Both linkage groups have shown the same kinds of deletions, and no relationship has been found between the deletions in the two linkage groups. Single deletions (Table 2) and double deletions at opposite ends of the two homologous chromosomes (that is in the *trans* configuration; Table 3) are common. Occasionally, the allele ratios suggest two deficiencies at opposite ends of the linkage group, but affecting the markers contributed by the same parent (that is in the *cis* configuration). However, in such cases it turns out that there has been a change in the coupling of the markers, such that the two chromosomes in the plating unit that gave rise to the heteroclone must have been non-parental. This means that the two deficiencies are on different chromosomes, so that the deletions are in the *trans* configuration. In some heteroclones, one allele at a terminal locus is absent. Sometimes this can be explained by the inclusion of this allele in a deletion, because the segregation of

the other markers shows an appropriate disturbance. Sometimes, however, a terminal marker is missing, but the segregation of the other markers shows no disturbance attributable to a deletion including the missing allele. The simplest explanation is that the lack of one allele in such heteroclones is due to homozygosity, rather than hemizygosity, of the homologous allele.

When various supplements are added to the medium on which the heteroclones are recovered, so that the number of selected markers is reduced, a much wider range of heteroclone types is possible and a great variety of segregation patterns has been found amongst the several hundred heteroclones that have been studied. When the selective conditions are relaxed in this way, many heteroclones show an excess of one or two genotypes. The genotype or genotypes in excess are usually able to grow on the selective medium, and their presence is probably due to the origin, within the growing heteroclone, of recombinants which then multiply clonally. Heteroclones showing this kind of bias are the more common the fewer are the selected markers, when the probability of cross-over events that can give rise to recombinants capable of growth on the selective medium is greater. When the alleles of at least one locus in each linkage group are heterozygous, heteroclones of this kind can be recognized by the test of independence between the two linkage groups (see Table 1), which turns out to be significant.

When the plating medium is selective for heterozygosity of the markers of only one linkage group, the other linkage group is very often represented by a single set of markers, usually those of one of the parents, but occasionally a recombinant set. It seems probable that the plating units which give rise to heteroclones of this type contained an aneuploid nucleus, in which one linkage group was present in the diploid condition, and the other in the haploid condition. However, we cannot exclude the possibility that the markers of the second linkage group are also present in the diploid condition, but homozygous.

A special kind of heteroclone, which gives a highly significant independence χ^2, is made up almost exclusively of the two parental genotypes, sometimes together with rare recombinants. These colonies are interpreted as heterokaryons, and they are particularly numerous in certain crosses.

Higher-order heteroclones

Second-order heteroclones are obtained by sowing spores from a heteroclone on a medium selective for closely linked markers in repulsion. They are much more numerous when the inoculum consists of fragments of the mycelium of the original heteroclone, instead of spores. A single colony can give rise to several thousand second-order heteroclones. The few second-order heteroclones that have so far been analysed have often shown a pattern of segregation resembling that of the parent heteroclone. However, a high proportion of them have shown greater deficiencies of markers, when the selective medium has allowed the recovery of incomplete heteroelones.

The classification of the heteroelones as first, second, or higher order merely indicates the number of transfers on selective media that have preceded their isolation. The first-order heteroelones are not necessarily derived from plating-units containing primary zygote nuclei, since their nuclei may already have undergone several multiplications in the mixed culture. The problem of isolating the zygotes

has not yet been resolved, and so we cannot say whether they are complete or incomplete. However, comparison of the segregation patterns of first and second-order heteroclones clearly indicates the occurrence of post-zygotic losses of markers.

DISCUSSION

From a mixed culture of two suitably marked strains of *Streptomyces coelicolor* strain A3(2) it was possible to isolate heterozygotes containing the marker alleles of both parents. These heterozygotes gave rise to mixed clones, the heteroclones, in which the parental and numerous recombinant genotypes were present. Therefore the heterozygotes represent a stage in the pathway of production of recombinants. We cannot, however, exclude the existence of alternative pathways of recombinant formation. The variety of recombinant genotypes recoverable from a single heteroclone is large, probably being limited only by the number of markers and the size of the sample of segregants analysed. This indicates that the number of independent events giving rise to recombinants, and so the number of heterozygous nuclei undergoing segregation in any heteroclone, is large, probably several thousand. This contrasts with the normal situation in *Escherichia coli* (Lederberg, 1957; Anderson, 1958), where a single zygote produced not more than four recombinant genotypes (omitting the characters of motility and morphology).

The occurrence of numerous segregational events need not necessarily imply that the heterozygous nucleus in the heteroclone multiplies equationally for many generations. Another possibility, suggested by Lederberg (1957) to account for the presence of several recombinant genotypes amongst the progeny of a single zygote of *Escherichia coli* is a 'recycling of meiosis', that is an alternation of haploid and diploid nuclear generations. This hypothesis has also been used to explain recombination in bacteriophage (Visconti & Delbrück, 1953). Clearly such a process would lead to an increase in apparent recombination frequencies, which would approach 50 % if the process were repeated many times. However, the two linkage groups of *Streptomyces coelicolor* turn out to be particularly short (Hopwood & Sermonti, 1962), and this is not due to parental contamination, because the two linkage groups always segregate independently. Within the two linkage groups, the original coupling of the markers is preserved, with rare exceptions, in all heteroclones analysed quantitatively, whether first or higher order. Thus an extensive recycling of meiosis seems improbable.

Anderson (1958) put forward another hypothesis to account for the occurrence of repeated segregational events in the descendents of the zygotes of *Escherichia coli*, without equational divisions of the zygote nucleus. He suggested that the fragment of donor chromosome did not multiply, but was inherited unilinearly, segregants being produced at intervals by a copy-choice mechanism. A consequence of this hypothesis is that not more than one heterogenote could occur amongst the descendents of a single zygote; this is certainly not true in the heteroclones of *Streptomyces coelicolor*, each of which can give rise to several thousand segregating subclones.

These considerations suggest that the heterozygous nucleus multiplies for a number of generations, probably at least a dozen, during the development of the heteroclones of *Streptomyces coelicolor*. At sporulation, nearly all the spores in the heteroclone contain haploid nuclei. Crossing-over has taken place, but we cannot yet say whether this occurs only at the time of reduction to haploidy, or during the

equational divisions of the heterozygous nuclei, nor whether it occurs simultaneously in the two linkage groups. Heteroclones heterozygous for markers in one linkage group and with the other represented by a single set of markers are very common, the latter occasionally representing a non-parental arrangement, but these markers could be present in the homozygous or hemizygous condition.

Complementary segregant classes always have different frequencies in the heteroclones, even when the markers of a single linkage group are considered. These disturbances can be attributed to a factor that reduces the frequency of one of the terminal alleles of a chromosome, or sometimes to two factors that affect two alleles at opposite ends of the two homologous chromosomes. From the point of view of the formal genetic analysis, the nature of these factors is unimportant. However, the hypothesis that they are haplo-lethal chromosome deletions appears to be the most reasonable, especially in view of the variety of deficiencies observed in different heteroclones from the same cross and in higher-order heteroclones derived from the same heterogenotic nucleus. The fact that the alleles with the lowest frequencies (or absent altogether) are always terminal ones is in favour of the hypothesis of terminal deletions.

Some rearrangements do occur during the equational divisions of the heterozygous nuclei, as shown by occasional homozygosity, and rare changes in coupling of the markers. These rearrangements could arise by mitotic crossing-over at a four-strand stage, and this could also account for the disappearance of terminal deletions and for the transfer of a deletion from one chromosome to its homologue, phenomena that are observed when certain higher-order heteroclones are compared with the parent heteroclone. The extent of these phenomena has still to be determined.

The incompleteness of the heterogenotes from which the heteroclones originate appears to be due largely to post-zygotic changes, since higher-order heteroclones show more extensive deficiencies than the heteroclones from which they originated. Completeness or incompleteness of the primary zygotes has still to be determined. Study of the heteroclones has thrown little light on the early stages of sexual reproduction, that is conjugation and the transfer of genetic material, apart from confirming the conclusion from the selective analysis of recombination that large parts of the parental genomes, if not whole genomes, must be transferred (Hopwood, 1959).

The heteroclones show striking similarities to the semi-stable heterozygous strains of *Escherichia coli* K12 isolated by Lederberg (1949). These, like the heteroclones of *Streptomyces coelicolor*, were derived from heterogenotes whose nuclei divided equationally for a certain number of generations before segregating haploid products, they regularly lacked one or more chromosome segments derived from one or other of the parents, or occasionally from both, and they sometimes showed homozygosity of certain markers (Lederberg, Lederberg, Zinder & Lively, 1951; Nelson & Lederberg 1954). Unfortunately the heterozygotes of *E. coli* have not been fully exploited. They were used as an argument in support of the theory of post-zygotic elimination of chromosome segments in *E. coli* (Nelson & Lederberg, 1954), and when pre-zygotic elimination was elegantly demonstrated (Wollman, Jacob & Hayes, 1956) they appear to have been abandoned, and have even been considered to be an example of genetic teratology (Jacob & Wollman, 1961). However, pre-zygotic and post-zygotic elimination are not mutually exclusive; on the contrary, they may be different expressions of the same tendency towards fragmentation of the chromosome that

seems to be a general feature of all bacterial genetic systems. The heteroclones of *Streptomyces coelicolor* should help to throw more light on this aspect of microbial genetics.

This work was aided by travel grants from the Italian Comitato Nazionale per l'Energia Nucleare (C.N.E.N.).

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J. gen. Microbiol. (1963), **30**, 261–269
Printed in Great Britain

On the Mode of Action of 11-deoxycorticosterone on the Metabolism of *Trichophyton rubrum*

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(*Received* 14 *May* 1962)

SUMMARY

The mechanism by which 11-deoxycorticosterone (DOC) inhibits the growth of *Trichophyton rubrum* was studied by examining the effects of this steroid upon metabolism by the intact mycelium. This compound inhibited the rate of endogenous respiration and the rate of uptake of glucose from the medium, but no evidence was found for the release of intracellular components from the mycelium. Incubation of mycelium in the presence of [^{14}C]-glucose resulted in the incorporation of [^{14}C] into 37 components within 3 min. in aqueous ethanol extracts of mycelium, although 98 % of the [^{14}C] in these extracts was contained in fructose, alanine, phosphorylated compounds and an unidentified compound. Incubation in the presence of 11-deoxycorticosterone resulted in a marked change in distribution, with decrease of the labelling of the phosphorylated compounds and fructose and increases in the labelling of the alanine and unidentified component. The results show considerable similarity to the effects of agents such as 2,4-dinitrophenol on yeast metabolism and suggest that the uncoupling of phosphorylation from oxidation may be one result of the action of 11-deoxycorticosterone on *T. rubrum*.

INTRODUCTION

Previous work (Chattaway, Townsley & Barlow, 1959, 1962) showed that 11-deoxycorticosterone (DOC) was one of the most potent of a group of steroids which inhibited the growth of *Trichophyton rubrum*, strain OS. The present paper describes studies designed to throw light upon the mechanism by which DOC exerts its inhibitory action on the growth of this fungus. A preliminary report of this work has been published (Townsley, Chattaway & Barlow, 1961).

METHODS

Organism. The organism used was *Trichophyton rubrum*, strain OS, and mycelial pads were grown at 33° on Sabouraud liquid medium or on the defined medium previously described (Chattaway *et al.* 1959).

Relation of DOC concentration to mycelial weight. The previously reported growth studies showed that 50 % inhibition of growth of *Trichophyton rubrum*, strain OS, was given by 0·15 mg. DOC/10 ml. medium. This was the inhibition of the growth of an inoculum equivalent to 1 mg. dry weight organism or 6 mg. wet weight (i.e.

mycelium dried between sheets of filter paper), giving a ratio of weight of DOC required to cause 50 % inhibition of growth to that of mycelial wet weight of 1:40. In the following experiments on the effect of DOC on glucose uptake, endogenous respiration, release of cell components and metabolism of glucose this ratio was maintained.

Uptake of glucose. For studies of the uptake of uniformly labelled [^{14}C]-glucose by mycelium, the fungus was grown in Sabouraud medium for 7 days, washed three times with water and dried between sheets of filter paper; 0·15 g. portions of this mycelium were then suspended in water (4·5 ml.) with the addition of DOC (final concentration 4·5 mM) as required. The DOC was added to the sterile flasks in chloroform ethanol (1 + 1 by vol.) solution and the solvent evaporated before addition of the medium. The mycelial suspensions were incubated for 1 hr. at room temperature and then 1·0 μc. [^{14}C]-glucose (Radiochemical Centre, Amersham, Buckinghamshire) added (final glucose concentration 0·053 M).

Respiration determinations were made by the conventional Warburg techniques, the organism being grown on the defined medium for 8 days, washed twice with 0·0133 M-phosphate buffer (pH 6·8) and suitable amounts suspended in the buffer. Oxygen uptake was measured for 1 hr. and then glucose (to final 40 mM) and/or DOC (to final 0·76 mM) in 0·5 ml. phosphate buffer added. Oxygen uptake was followed for 1 hr. further.

Metabolism of [^{14}C]-glucose. This was studied with mycelial pads grown on Sabouraud medium for 7 days, then washed three times with water, dried between filter papers and weighed portions (0·5 g.) suspended in 1·0 ml. water or aqueous DOC (7·6 mM.) suspension. After incubation for 1 hr. at room temperature the contents of each flask were transferred to a Pyrex sintered glass filter funnel (No. 4, 3 cm. diameter) and connected to, but shut off from, a filter pump. A solution containing 300 μl. of [^{14}C]-glucose (33·3 μc., 18 μmole, final glucose concentration, 1·8 mM) was added to each filter funnel and the funnel closed by a rubber bung pierced by a glass tube fitted with a tap. After the required incubation period the rubber bung was removed, the supernatant fluid removed by suction and the funnel rapidly transferred to another filter flask and boiling 80 % (v/v) aqueous ethanol (5 ml.) poured on to the mycelium, the bung then being replaced. The incubation time was taken to be that period from the addition of the glucose until the addition of ethanol. Extraction was continued for 90 min., the extract sucked off and two more extractions for 60 min. periods made with 5 ml. portions of boiling 20 % (v/v) aqueous ethanol. The combined extracts were evaporated to dryness *in vacuo* at 40° and when required the supernatant fluids from the initial incubation with [^{14}C]-glucose were similarly concentrated. The residues were washed three times with ether (5 ml.) to remove residual DOC since this was found to cause streaking on chromatograms; the final ether-free residue was dissolved in 2·5 ml. 50 % (v/v) aqueous ethanol.

Paper chromatography. The radioactive substances in the concentrated cell extracts and supernatant fluids were analysed by two-dimensional descending paper chromatography on 46 × 57 cm. sheets of Whatman No. 4 filter paper. The phenol + formic acid water system of Kornberg (1958) was used in the first dimension and *n*-butanol + propionic acid + water (46·8 + 22·0 + 30·1 by vol.) in the second (Benson *et al.* 1950). Of the final 2·5 ml. of concentrated extract, 0·2 ml. was chromato-

graphed and the radioactive substances on the chromatogram located by radioautography with Kodirex X-ray film (Kodak Ltd.). The radioactivity in each spot was counted directly by means of a G.E.C. Geiger–Müller tube, type E.H.M. 25 operating at 1480 V.

Radioactive spots on the chromatograms were identified provisionally by their chromatographic positions in the above solvent systems. This was confirmed by co-chromatography with authentic materials in these systems and in an ethyl acetate + water + pyridine system (4 + 4 + 1, by vol.; see Jermyn & Isherwood, 1949) and the *n*-propanol + ethyl acetate + water (7 + 1 + 2, by vol.) system of Baar & Bull (1953) for carbohydrate materials. For ninhydrin-reacting substances *n*-butanol + acetone + diethylamine + water (10 + 10 + 2 + 5, by vol.; Hardy, Holland & Naylor, 1955) and ethanol + ammonia (0·880) + water (180 + 10 + 10; by vol.; Smith, 1960) systems were used. The presence of orthomonophosphate esters was shown by treatment of chromatogram eluates with alkaline phosphatase (Light & Co.) by the method of Heppel (1955).

Chemical estimations. Amino acids present in mycelial supernatant fluids were estimated by determination of total α-amino nitrogen by the method of Moore & Stein (1954). Inorganic phosphate was estimated by the method of Fiske & Subba Row (1925).

RESULTS

Effect of 11-*deoxycorticosterone on endogenous respiration*

DOC has been shown to inhibit the respiration of baker's yeast in the presence of glucose (Shacter, 1949) and of germinating conidia of *Neurospora crassa* in the presence of sucrose (Lester, Stone & Hechter, 1958); in both cases endogenous respiration was either stimulated or unaffected. Many workers have demonstrated the high endogenous respiration rate of dermatophytes and the virtual absence of response in respiration rate to added substrates. In common with these findings the mycelium of *Trichophyton rubrum* used in this work showed no response to added glucose, and the endogenous respiration rate was linear for at least 2 hr. The effect of DOC on the endogenous respiration is shown in Fig. 1, the DOC being added after 1 hr. incubation of the mycelium in phosphate buffer. The rate of respiration is seen to be inhibited 50 %.

Effect of DOC on the release of cytoplasmic contents

Fatty acids and certain detergents are known to be fungistatic and to inhibit fungal respiration by action on the cell wall, with resulting loss of intracellular components. Since certain steroids exhibit surface activity, the possibility that DOC exerted its inhibitory effect on growth and respiration of *Trichophyton rubrum* by this means was examined by comparing the release of inorganic phosphate and amino acids from the mycelium into the medium in the presence of cetyltrimethylammonium bromide (CTAB) or DOC, with that produced in the presence of water alone. The results, Fig. 2, show that CTAB released appreciable amounts of both materials, while DOC promoted no release beyond that which occurred with water alone. Thus DOC did not appear to exert its inhibition by virtue of a disrupting action on the fungal wall.

The effect of 11*-deoxycorticosterone on glucose metabolism*

It appeared possible that the effects of DOC on growth, glucose uptake and endogenous respiration by *Trichophyton rubrum* might be explicable in terms of an effect on energy availability; therefore its overall effect on the metabolism of [^{14}C]-glucose was studied. The experiments were similar to those in which Moses (1958) studied the effects of starvation, ammonia and azide on the incorporation pattern of [^{14}C]-glucose in *Zygorhynchus moelleri* and in which Moses & Smith (1960) investigated the effects of uncoupling agents on glucose metabolism in baker's yeast.

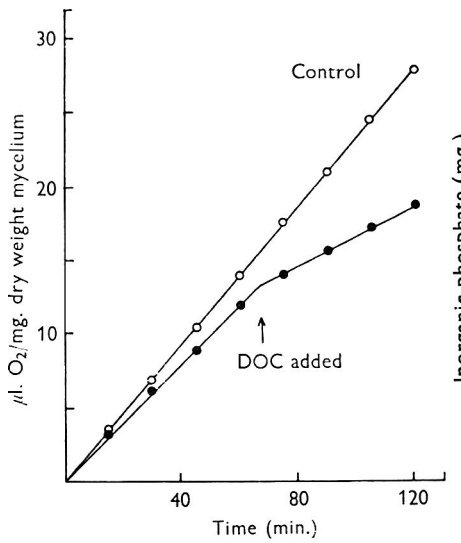

Fig. 1

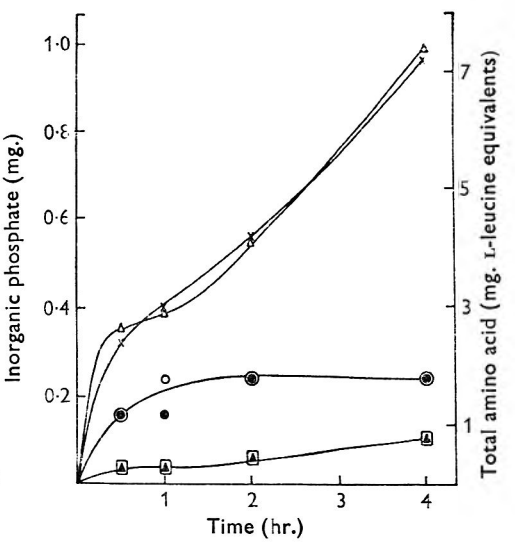

Fig. 2

Fig. 1. Inhibition of endogenous respiration of *Trichophyton rubrum* by 11-deoxycorticosterone (DOC). Washed mycelium was suspended in 0·0133 M-phosphate buffer (pH 6·8). DOC suspension in phosphate buffer (0·5 ml.) was added as shown, to final concentration 0·76 mM.

Fig. 2. Release of intracellular components from *Trichophyton rubrum* by 11-deoxycorticosterone (DOC) and by cetyltrimethylammonium bromide (CTAB). Washed mycelium (0·4 g. suspended in 60 ml. of either 1·0 mM-DOC suspension, 0·27 mM-CTAB or water), was incubated for 4 hr. at room temp. Samples (4 ml.) were withdrawn at intervals. Amino acids released in presence of DOC (▲), CTAB (△), water (□). Phosphate released in presence of DOC (●), CTAB (×), water (○).

The incorporation of [^{14}C]-glucose into the aqueous ethanol fraction was determined as described in Methods. The incorporation of [^{14}C] into this fraction after exposure for 30 min. to [^{14}C]-glucose was decreased by 50 % by the presence of DOC (Fig. 3). A typical chromatogram showing diagrammatically the pattern of incorporation into compounds of the aqueous ethanol extract from normal mycelium of *T. rubrum* is shown in Fig. 4. This was obtained after exposure of mycelium to [^{14}C]-glucose for 3 min. and shows that radioactivity was present in 37 compounds besides the glucose. Similar experiments were carried out to determine the effect of DOC on the pattern of incorporation after exposure of mycelium to [^{14}C]-glucose for 10,

60, 180 and 1800 sec. The radioactivity of each spot was determined in the resulting chromatograms and the values expressed as a percentage of total [^{14}C] other than glucose incorporated into the aqueous ethanol soluble fraction (Table 1).

Table 1. *Incorporation of* [^{14}C] *from* [^{14}C]-*glucose by* Trichophyton rubrum *strain OS in the absence and presence of* 11-*deoxycorticosterone* (*DOC*)

Values given are percentage of total [^{14}C] (other than glucose) incorporated into the aqueous ethanol soluble fraction of the organism during the incubation periods shown. Spot nos. refer to Fig. 4.

	Time of incubation (sec.)							
	10		60		180		1800	
Spot no.	Control	DOC	Control	DOC	Control	DOC	Control	DOC
	Incorporation of ^{14}C (%)							
1	0·72	0·00	0·95	0·00	0·88	0·00	0·45	0·60
2	0·46	0·00	0·00	0·00	0·81	0·00	0·52	0·00
3	0·00	0·00	0·00	0·00	0·00	0·00	1·48	0·00
4	8·84	0·59	9·32	3·23	17·75	1·94	11·94	1·28
5	0·59	0·00	0·00	0·00	0·81	0·00	0·58	0·00
6	0·00	0·00	0·00	0·00	0·54	0·00	0·58	1·75
7	0·00	0·59	0·00	0·00	0·47	0·00	0·00	0·00
8	0·00	0·00	1·01	0·00	0·95	0·00	0·71	0·00
9	0·00	0·98	0·00	0·00	0·00	0·00	0·00	0·00
10	0·00	3·14	0·00	0·00	0·54	0·00	0·00	0·00
11	0·00	0·00	0·61	0·92	0·00	1·61	0·00	0·00
12	0·39	1·11	0·00	0·99	0·00	0·74	0·00	0·00
13	1·24	2·29	1·16	3·90	0·47	0·00	0·00	0·00
14	4·77	3·73	0·61	9·24	0·74	13·50	0·45	0·00
15	0·00	4·57	0·41	0·00	0·47	0·00	0·00	0·00
16	5·55	8·56	0·88	2·38	1·08	2·28	0·00	0·00
17	1·50	2·88	1·29	0·00	0·68	3·01	0·84	0·00
18	1·24	2·42	0·00	1·52	0·00	0·87	0·00	0·00
19	1·24	4·44	0·75	0·00	0·41	0·67	0·00	0·00
20	20·30	3·72	0·00	0·00	0·00	0·00	0·00	0·00
21	0·00	0·00	0·43	0·00	0·00	0·00	0·00	0·00
22	0·65	2·29	0·00	0·00	0·00	0·00	0·00	0·00
23	11·63	13·70	3·13	5·28	2·64	2·16	0·00	0·00
24	9·34	7·06	37·55	31·40	55·20	28·10	67·70	51·40
25	1·63	3·14	0·00	0·92	0·47	0·67	0·00	0·00
26	4·44	6·21	1·77	7·07	1·01	7·63	0·00	0·00
27	0·85	0·79	0·00	0·92	0·00	0·42	0·00	0·00
28	0·00	0·00	0·43	0·40	0·00	0·00	0·00	0·00
29	4·12	6·92	0·82	2·44	1·76	2·01	0·00	0·00
30	1·31	1·44	0·54	5·51	0·00	2·68	0·00	0·00
31	2·02	2·48	10·80	6·08	6·42	20·97	13·10	27·00
32	1·44	1·96	0·88	2·31	0·68	2·07	0·00	0·00
33	1·05	1·44	0·00	0·00	0·00	0·74	0·00	0·00
34	1·50	1·83	0·00	0·00	0·47	1·34	0·00	0·00
35	11·70	11·50	26·95	13·48	4·66	6·75	1·03	16·90
36	1·60	0·39	0·00	0·00	0·00	0·00	0·00	0·00
37	0·00	0·00	0·00	2·24	0·00	0·00	0·45	0·81

Identification of [^{14}C] *labelled compounds*

The identification of labelled compounds in the aqueous ethanol extracts was attempted by a study of sugars, amino acids and phosphates present in extracts from unlabelled mycelium, prepared under conditions identical with those used in

the [^{14}C] incorporation experiments, by using the chromatographic techniques described. The only labelled carbohydrate compounds detected were glucose (spot 0, Fig. 4) and fructose (spot 24). Seventeen compounds which reacted with ninhydrin were present, of which only one appeared to be labelled; this was identified as alanine (spot 31). A consideration of the chromatographic maps of Bassham & Calvin (1957) suggested that spots 1–8 represented phosphorylated compounds, due allowance being made for the fact that acidic compounds show increased R_F values

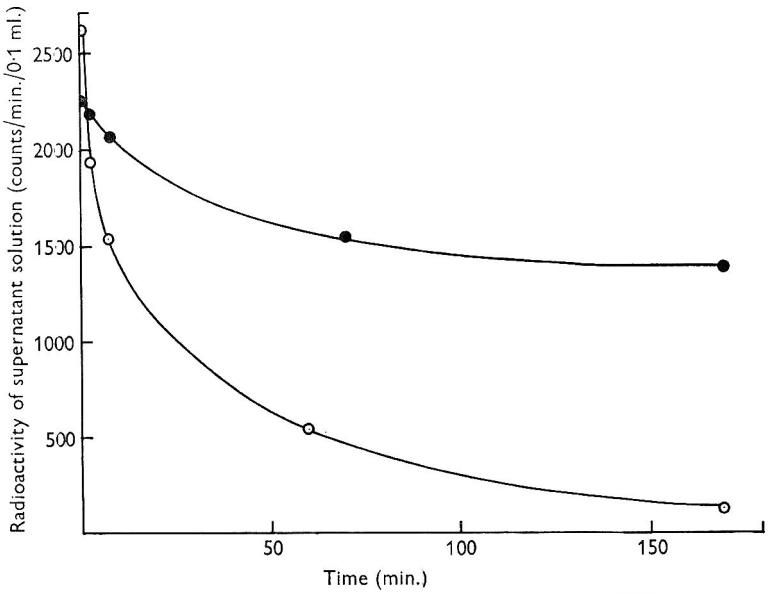

Fig. 3. Inhibition by 11-deoxycorticosterone of the uptake of [^{14}C]-glucose by *Trichophyton rubrum*. Two portions of washed mycelium (0·15 g) suspended in 4·5 ml. water, one containing DOC (7·5 mg.) in suspension, were incubated for 1 hr. at room temp. [^{14}C]-glucose (0·5 ml. containing 1 μc.; final concentration 0·053 μM) was added to each flask. Samples (0·1 ml.) of the suspending medium were withdrawn at intervals for determination of radioactivity. Mycelium with DOC (●), control (○).

in the phenol + water + formic acid solvent system of Bassham & Calvin. This was confirmed for spots 4 and 6, by showing them to be orthomonophosphate esters by elution and treatment with alkaline phosphatase followed by further study of the chromatographic properties of the products.

Effect of DOC on [^{14}C] *distribution*

It can thus be seen from Table 1 that in normal mycelium after 30 min. exposure to [^{14}C]-glucose 98 % of the [^{14}C] incorporated was present in fructose (67·7 %), alanine (13·1 %), phosphorylated compounds (16·3 %) and an unidentified compound spot 35 (1·0 %). After exposure to DOC the above compounds still accounted for over 98 % of the [^{14}C] incorporated but the distribution of radioactivity changed appreciably, being fructose (51·4 %), alanine (27 %), phosphorylated compounds (3·6 %) and spot 35 (16·9 %). The effect on the phosphorylated compounds was marked since after exposure to DOC only 3 such compounds were labelled and the decrease in percentage incorporation into this fraction was apparent from the

shortest time of exposure to the [^{14}C]-glucose. Since it was shown above that DOC did not bring about the release of intracellular compounds it appears unlikely that the effect on the uptake of glucose was the result of a direct action of DOC on the permeability of the cell wall; this conclusion was supported by a chromatographic study of the radioactivity of compounds in the supernatant solutions.

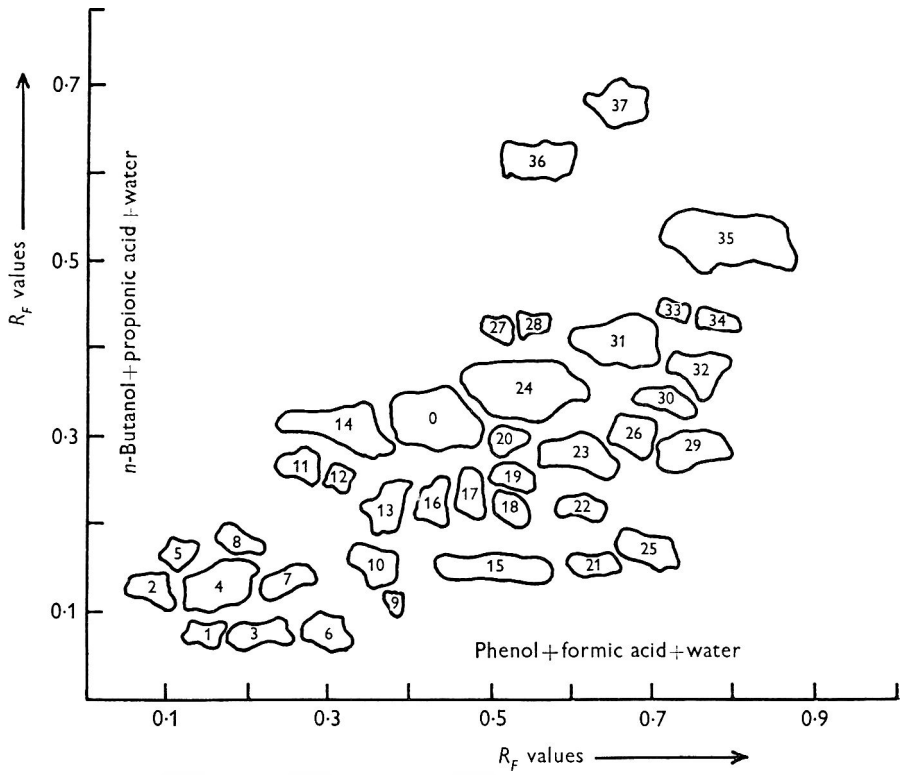

Fig. 4. Incorporation of [^{14}C] into cytoplasmic components of *Trichophyton rubrum*. Diagrammatic representation of labelled areas (number 1 to 37) on a 2-dimensional chromatogram obtained from the aqueous ethanol extract of normal mycelium (0·5 g) exposed for 3 min. to [^{14}C]-glucose (33·3 μc; 18 μmole, final glucose concentration 1·8 mM). Spot 0 = glucose.

DISCUSSION

The inhibitory effects of DOC on growth, endogenous respiration and uptake of glucose by *Trichophyton rubrum* and its effect upon the pattern of distribution of [^{14}C]-glucose within the mycelium show results similar to those obtained by Moses & Smith (1960) in their study of the effects of uncoupling agents on yeast metabolism. Thus they found that 2,4-dinitrophenol (DNP) inhibited endogenous respiration and respiration in the presence of exogenous substrates, and also decreased the incorporation of [^{14}C]-glucose by over 50 %; incorporation of [^{14}C] into phosphorylated compounds and free sugars (primarily trehalose) was decreased but incorporation into amino acids (mainly glutamic acid and alanine) was increased. A similarity between the action of DNP and DOC was shown by Conner (1957, 1959) with *Tetrahymena piriformis*; both compounds inhibited growth of this organism

and the inhibition was annulled by stigmasterol. DOC has been reported to inhibit the NADH-cytochrome *c* reductase of heart muscle (Mahler, 1955) and of *Saccharomyces fragilis*, *Escherichia coli* and *Bacillus subtilis* (Yielding & Tomkins, 1959), an effect which would impede the formation of ATP and thus interfere with energy-requiring reactions.

There is thus evidence which suggests a similarity between the action of DOC in *Trichophyton rubrum* and that of uncoupling agents in other organisms, suggesting that one result of the action of DOC on *T. rubrum* is the uncoupling of oxidative phosphorylation. This does appear to be contrary to the findings of Lester *et al.* (1958) and of Lester & Hechter (1959) in their studies of the action of DOC on *Neurospora crassa*. They found that growth, oxygen uptake in the presence of exogenous substrate, and uptake of sugars, amino acids and rubidium were inhibited, but that endogenous respiration was not affected; the distribution of sodium within the cell was also affected by DOC. They postulated that the effect of DOC was upon the binding of ions within the cell and not upon the energy-requiring reactions associated with uptake of substrates.

We are grateful to the Wellcome Trust for a maintenance grant to one of us (J.D.T.) and a contribution towards the cost of this work; also to Messrs E. R. Squibb for a grant towards the cost of radioactive materials.

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J. gen. Microbiol. (1963), **30**, 271–280
Printed in Great Britain

Immunological Assay of some Immobilizing Antigens of *Paramecium aurelia*, Variety 1

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(*Received* 14 *May* 1962)

SUMMARY

Quantitative methods are described for estimating the activity of antisera against *Paramecium aurelia*, variety 1, and purified preparations of the immobilizing antigen derived from the organisms. Confirmation was obtained that the relation between the serum concentration and the reciprocal of the immobilization time is approximately linear, for times between 3 and 10 min. Titration of antigen preparations by a method based on inhibition of the immobilization reaction gave somewhat higher values than did titration by a gel-diffusion method. Cross-reactions between antigens controlled by genes at different loci and their various specific antisera were in all cases very small. The cross-reactions between antigens controlled by three genes at the D locus and their antisera varied according to the method used to measure them. Extensive cross-reactions were observed when the antisera were absorbed with heterologous paramecia and when precipitin formation in agar gel was followed. Only slight cross-reactions were observed by two methods based on the immobilization reaction.

INTRODUCTION

The immunological specificity of the immobilization antigens of *Paramecium aurelia* is controlled by series of multiple alleles at several genetic loci (Sonneborn, 1951; Beale, 1952, 1954, 1957). In a given organism at a given time only the antigens controlled by genes at a single one of these loci can be detected, as a general rule. *P. aurelia* is, however, a diploid organism and in a heterozygote two antigens controlled by different alleles may be detected simultaneously. A combination of environmental, cytoplasmic and genetic influences determines which of the genetic loci is expressed; by careful regulation of the conditions of culture it is possible to produce large clones in which nearly every organism expresses the same locus. When the specificities of two antigens are controlled by genes at different loci, the antigens are said to differ in 'type', and when specific antigen differences are controlled by alleles the antigens are said to differ in 'sub-type' (Beale, 1954, p. 113).

McClung (1943) showed that when a suspension of Paramecium was agitated without breaking the organisms, the supernatant fluid remaining after the animals were centrifuged off was capable of eliciting immobilizing antibody in the rabbit. Dr G. H. Beale (unpublished experiments) has shown that a water-soluble extract of homogenized *Paramecium aurelia* inhibits the immobilization of homologous organisms by immune serum; by using a technique of precipitin formation in agar gel Finger (1956) demonstrated that a water-soluble extract of *P. aurelia* contained several antigens, one of which was the immobilization antigen. Preer (1959*b*) and

Bishop (1961) developed methods of purifying the immobilization antigens, and showed that they were at least 95 % protein in composition. A method of starch gel electrophoresis was used by Bishop & Beale (1960) to measure the relative mobilities of several antigens. It was found that antigens of the same type were electrophoretically more similar than were antigens of different types. The present paper describes titration techniques used to assay immobilization antigens and antisera, and measurements of the cross-reactions between different antigens by these methods. These cross-reaction measurements are estimations of the immunological affinities of the antigens, and may be usefully compared with the electrophoretic observations.

METHODS

Stocks. The organisms were from stocks, 60, 90, and 103 of *Paramecium aurelia*, variety 1, maintained by Dr G. H. Beale. Serotypes D, G and S of stock 60 and serotype D of stocks 90 and 103 were used.

Culture medium. A 7·5 % (w/v) suspension of dried grass was boiled for 15 min., filtered, and the filtrate autoclaved (20 min., 120°). Two days before use, it was diluted with 50 volumes of de-ionized water and seeded with a growing culture of *Aerobacter aerogenes*. The culture medium prepared in this way was mixed with an equal volume of a cleared Paramecium culture. When the new culture had itself cleared (i.e. when the paramecia had removed all the bacteria), half of the medium was removed and the organisms were harvested, while an equal volume of fresh culture medium was added to the remaining half.

Antisera. The paramecia were collected from cleared cultures by a modification of the electromigration method of Van Wagtendonk, Simonsen & Zill (1952). The suspension was then centrifuged at 3000 *g* for 5 min., resuspended at a concentration of 10^6 organisms/ml. in solution A (0·013 M-NaCl, 0·003 M-KCl, 0·003 M-$CaCl_2$, 0·004 M-phosphate buffer, pH 6·8) and homogenized by ejection from a hypodermic syringe. Rabbits (9 months old) were injected intravenously twice weekly for 3 weeks, 1 ml. of homogenate being given at each injection. Ten days after the last injection, 30 ml. of blood was taken from the marginal ear vein. The blood was allowed to clot, the serum drawn off, heated at 60° for 30 min. and dialysed overnight at 2° against 3 l. of solution A.

Antigen. Purified antigen was prepared as described elsewhere (Bishop, 1961). About 95 % of the protein of purified antigen was immobilization antigen; carbohydrate and nucleic acid formed only about 2 and 0·5 %, respectively, of the total organic material.

Absorption of antisera by intact paramecia. The intact organisms, concentrated by electromigration, were centrifuged (3000 *g* for 5 min.) and resuspended in the appropriate antiserum. The mixture was maintained at 32° for 60 min. and the organisms then centrifuged down.

Precipitin formation in agar. Antiserum, diluted with solution A, was mixed with an equal volume of 2 % (w/v) agar (purified by the technique of Feinberg, 1956) and maintained at 60°. The mixture was pipetted into agar-coated capillary tubes (Oudin, 1952), inside diameter 2 mm.; further steps were as described by Oudin (1952). Serial dilutions of purified antigen were pipetted on top of the agar layer, the tubes sealed with wax and incubated at 25°. At intervals of 1 day, the distance from the agar-antigen interface to the leading edge of the precipitin band was

measured with a pair of dividers, and the equivalence value of antigen and antiserum was calculated as described by Oudin.

Estimation of immobilization time. Measurements of immobilization time were used in the assay of antiserum and in the assay of antigen. In the former case, a known volume (usually 0·05 ml.) of antiserum was pipetted into the three depressions of a depression slide. Next, the same volume of a suspension of paramecia (200 organisms/ml. solution A) was added to each depression and a stopwatch was started. Each depression was examined at every 30 sec. under a binocular microscope, and the number of organisms not moving noted. The number which stopped in each 30 sec. period was later deduced from this cumulative record. The mean immobilization time was readily calculated for the organisms in each depression. The mean of means for the three depressions was the estimated immobilization time. When antigen was to be assayed the procedure was identical except that a small known volume of antigen in solution A was added to each depression after the antiserum, and 2 min. before addition of organisms. A control slide was run in which an equal volume of solution A was added to each depression in place of the antigen.

Counting the organisms. The organisms were counted by making serial dilutions into solution A, and directly counting the number of organisms in ten 0·1 ml. samples of the final dilution.

RESULTS

Estimation of the activity of antisera and solutions of antigen

The immobilization of living paramecia by rabbit antiserum prepared against paramecia provides a ready means of estimating the immobilizing antibody content of different sera. According to the method of Sonneborn (1950) the immobilizing activity of an antiserum is measured by making a series of dilutions of the serum and finding the greatest dilution which will immobilize a small number of paramecia in the space of 2 hr. The same technique, with minor modifications, was used by Preer (1959*a*). It is, however, a very insensitive method in its present forms, as is any titration method which involves serial twofold dilutions.

Beale (1948) showed that the time taken by a given dilution of antiserum to immobilize paramecia (the immobilization time) was directly proportional to the dilution; that is, the greater the dilution, the greater the immobilization time. It seemed likely, therefore, that an accurate assay of antiserum activity could be developed, based on measurement of the immobilization time. To test the linearity of the dependence of immobilization time upon antiserum dilution, ten dilutions of 60D antiserum were made, each in triplicate, and the mean immobilization time of 30 homologous paramecia found for each of the thirty dilutions. When the reciprocal of the immobilization time ($1/t$) was plotted against the antiserum concentration (A) it was seen that the line obtained was slightly but significantly curvilinear (Fig. 1). For the purpose of routine observations, this deviation from linearity was ignored, and the activity of an antiserum, in arbitrary units (a)/ml., was taken as equal to $1/tA$, where t was the immobilization time in minutes and A the fraction of the final reaction mixture constituted by undiluted antiserum. Where the purpose of the assay is to compare different antisera, the error introduced by the non-linearity of the relationship between antiserum concentration and time will be smallest when the difference in immobilization time is smallest. Accordingly serum dilutions which

produced immobilization between 3 and 10 min. after contact were used. The maximum error encountered, measured as the maximum deviation of the points from the straight line in Fig. 1, was then about 5 %, comparing favourably with the errors encountered with the older methods. Where more accurate determinations were desired, a series of dilutions of antiserum (calculated from a pilot experiment to give immobilization times of between 3 and 10 min.) was made, and a was calculated as the slope of the regression of A upon $1/t$.

This method has the advantages of great sensitivity and economy of material. It also provides a basis for the standardization of antisera produced against different types and subtypes of antigen, if it be assumed that the mechanism of immobilization is the same in each case. It suffers the disadvantage, however, of being dependent upon the physiological state of the paramecia used in the test. When using paramecia collected from cleared cultures by overnight electromigration into solution A, the method gave highly reproducible results when the same antiserum was assayed on different occasions. However, paramecia collected directly by centrifugation from cleared, or uncleared, cultures were highly variable in their response to antiserum.

Antiserum concentration, a, as measured by this method, can be used to obtain an approximate value for the antiserum titre as defined by Sonneborn (1950) by taking $1/120a$ as the titre. When this was done, the denominator was invariably 10–20 % greater than the denominator of the value obtained by direct titration by the Sonneborn method. This is doubtless related to the slight deviation from linearity observed in the relationship between antiserum concentration and the reciprocal of immobilization time (Fig. 1).

Assay of antigen by the immobilization reaction

Essentially the same reaction was used to assay solutions of antigen, by using the immobilization reaction to estimate how much antibody activity was lost upon contact with antigen. Figure 2 shows the relationship between the amount of antigen added (in arbitrary units) and the % of initial antibody activity lost, measured as $100(1-t_0/t)$, where t and t_0 are the immobilization times in the presence and absence of antigen, respectively. Over the initial part of the curve, where the amount of added antigen is low compared with the amount of antibody, the slope is constant. The amount of antigen in a given solution, in units of antigen/ml. (g), can be calculated from $g = 1/G\,(1/t_0 - 1/t)$, where G is the fraction of the final reaction mixture constituted by undiluted antigen solution. Units of antigen, g, so defined, are entirely equivalent to the units of antibody inactivated when antibody concentration is given by $a = 1/tA$ as described above. Measurements could be made only within certain limits of antigen and antibody concentration. To satisfy the conditions necessary for antiserum assay, of which this is a modification, times between 3 and 10 min. had to be used. Secondly, to avoid the second part of the curve shown in Fig. 2, dilutions of antigen had to be chosen such that t was less than $2t_0$. In practice, a series of determinations was made within the range permitted by these two limitations and the value of g was obtained as the slope of the regression of $-G$ upon $1/t$.

The validity of this assay of the concentration of immobilizing antigen in solution depends upon the existence of linear relationships between antiserum concentration

and immobilization time on the one hand, and between antigen concentration and amount of antibody inactivated on the other. The near linearity of the initial part of the curve of Fig. 2 is an indication of how closely these conditions are met over this range of concentration. It may be asked what relationship exists in this region between the amount of antigen added and the amount of antibody removed, in terms of molar ratios. The gel diffusion technique of Oudin (1952) provides a method of determining the equivalence concentrations of antibody and antigen solutions, and may readily be applied to the immobilizing antigen and antibody system. The same antigen can then be titrated against the same antiserum by the immobilization method. The concentration of antiserum equivalent to the given concentration of antigen under the conditions of the immobilization reaction is $A/G(1-t_0/t)$ where the symbols have the same meaning as before. Comparison of the values for the equivalence concentration of antiserum obtained by these two methods should reveal how close the immobilization method comes to measuring true immunological equivalence. Such tests were applied to solutions of purified antigen of two types, type 90D and type 60D. The results (Table 1) show that the equivalence concentration of antiserum as measured by the immobilization method was 25 % greater than the serological equivalence concentration, as measured by the gel-diffusion method. That is, a greater amount of antiserum was bound by a given amount of antigen as measured by the immobilization method. This suggests that the serological equivalence point may be found at the change of slope in Fig. 2, which represents a smaller antiserum to antigen ratio than does the initial slope. It may be noted (Table 1) that the true serological equivalence concentrations of both serotypes examined bear the same relationship to the equivalence concentrations measured by the immobilization reaction.

Table 1. *Equivalence concentrations of stock antisera against* Paramecium aurelia, *variety* 1, *for test antigen solutions, measured by the immobilization and gel-diffusion methods*

The equivalence concentration of antiserum is given as the proportional concentration of the stock antiserum which is equivalent to the initial concentration of the test antigen.

Serotype of antiserum and antigen	Equivalence concentration of antiserum		Ratio of immobilization to gel-diffusion equivalence
	Immobilization method	Gel-diffusion method	
90D	0·0120	0·0089	1·3
60D	0·0097	0·0081	1·2

Cross-reactions of antisera with heterologous antigens

Immunological cross-reactions of antisera with heterologous antigens were used as a measure of the structural similarities between the antigens. In particular, it was of interest to determine whether antigens of the same type but different sub-type differ from each other more than do antigens of different types. Previous work on the relationships between different immobilizing antigens of *Paramecium aurelia*, variety 1 (Beale, 1952), was based on a comparison of the titres obtained when a given antiserum was titrated against both homologous and non-homologous paramecia by the serial dilution method of Sonneborn (1950). Only very slight cross-reactions between

antigens of different types were observed by this method. Within type D seven sub-types were detected (Beale, 1954) and several more sub-types have since been found (Dr G. H. Beale, unpublished data). Three of these, namely sub-types 60D, 90D and 103D were found to cross-react only to a very limited extent, to the same extent, in fact, as did the different types. These findings were re-examined by using the modified immobilization method described above, and were completely confirmed. The greatest extent of cross-reaction observed between any pair of antigens examined was 3·5 %.

Cross-reactions between antisera and heterologous antigens were also examined by investigating the inhibition of the immobilization reaction by heterologous antigen. For example, the extent to which the immobilization of 60D paramecia by 60D antiserum is inhibited by 60G antigen is a measure of the cross-reaction between 60D antiserum and 60G antigen. This method revealed no cross-reactions between different antigenic types and only very slight cross-reactions between the antigenic sub-types of type D. In the latter case a slight cross-reaction was obtained at very high antigen concentrations; but the effects observed with heterologous antigen were unlike those observed with homologous antigen. The effect of adding homologous antigen in the immobilization reaction could not be distinguished from the effect of diluting the antiserum. On the other hand, when a high concentration of heterologous (different sub-type) antigen was added, in addition to a slight increase in immobilization time, an effect was observed which appeared very similar to the effect of adding a toxic concentration of saline. Addition of the heterologous antigen in the absence of antiserum did not produce such an effect. If the aberrant behaviour of the paramecia can be ignored, and only the immobilization time taken into consideration, cross-reactions between antigenic sub-types were always less than 10 % of the homologous reaction when measured by this method.

A third useful method of studying the cross-reactivity of antisera with heterologous antigens was described by Finger (1957). Homologous or heterologous paramecia are incubated with the antiserum and then removed by centrifugation. The amount of antibody removed or neutralized by this means is measured by the decrease in antiserum titre which results, the antiserum being titrated against homologous paramecia by an immobilization method. The absorption of 90D antiserum by increasing amounts of 90D paramecia is illustrated in Fig. 3. The amount of antibody remaining after absorption for 1 hr. was measured by the immobilization method described in the present paper. When a sufficiently large number of paramecia was used in the absorption step (in this case about 12,000 paramecia/ml. antiserum) no residual immobilizing activity remained. When heterologous 60D paramecia (i.e. differing in sub-type) were used to absorb the antiserum, a point was reached (Fig. 3) at which an increase in the number of absorbing paramecia did not further the activity of the antiserum against homologous paramecium. The number decrease of heterologous paramecia (60D) required to reach this point was the same as the number of homologous paramecia required to exhaust the antiserum (in this case 12,000/ml. antiserum). In the reciprocal experiment, namely the absorption of 60D antiserum by 60D and 90D paramecia, the same phenomenon was observed (Table 2). On the other hand, when antiserum was incubated with paramecia of a different type (as in the absorption of 60D antiserum by 60G paramecia) no significant decrease in titre was observed. These results are summarized in Table 2, where

the cross-reaction of each antiserum with heterologous paramecia is expressed as % decrease in antiserum activity obtained with excess of absorbing paramecia. The cross-reactions observed between the D sub-types by this method are thus greater by an order of magnitude than the cross-reactions observed by the immobilization methods.

Another method, more commonly used in immunological work, of quantitating cross-reactions, is the gel-diffusion method (Oudin, 1952). Where the same concentration of antigen is allowed to diffuse into a series of dilutions of the antiserum (mixed with agar gel) the equivalence concentration of the antiserum is found by extrapolating to the antiserum dilution which gives zero rate of migration of the

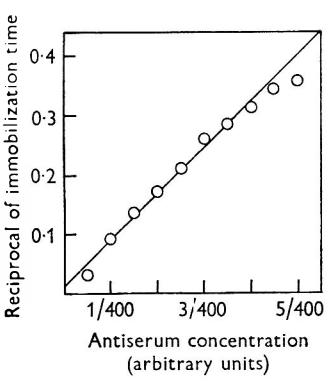

Fig. 1

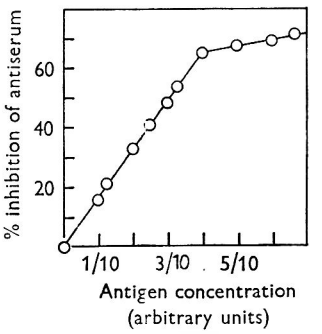

Fig. 2

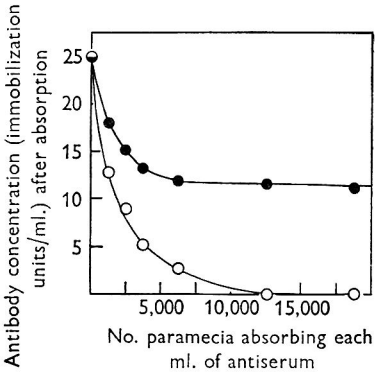

Fig. 3

Fig. 1. Dependence of the reciprocal of the immobilization time of *Paramecium aurelia*, variety 1 (serotype 60D), upon the antiserum concentration.

Fig. 2. Inhibition of the immobilization of *Paramecium aurelia*, variety 1 (serotype 60D), by increasing concentrations of purified homologous antigen. The antiserum concentration in the untreated control was 0·32 immobilization units/ml. The percentage of immobilizing activity lost in the presence of antigen was calculated as described in the text.

Fig. 3. Loss of immobilizing activity from 90D antiserum upon incubation with increasing numbers of 90D (○) and 60D (●) paramecia.

Table 2. *Cross-reactions between antisera against* Paramecium aurelia, *variety* 1, *and immobilizing antigens measured by the serum-absorption and gel-diffusion methods*

Cross-reactions are presented as % of the value for the homologous reaction, as described in the text. A dash indicates that that particular cross-reaction was not measured.

		Antigen			
Method	Antiserum	60D	90D	103D	60G
Serum absorption	anti-60D	100	54	—	5
	anti-90D	52	100	—	—
	anti-60G	5	5	—	100
Gel-diffusion	anti-60D	100	47	—	5
	anti-90D	55	100	—	5
	anti-103D	39	60	100	5
	anti-60G	5	5	—	100
	anti-60S	5	5	5	5

precipitin band. The cross-reactivity of the immobilizing antigens and antisera was measured as follows. The equivalence concentrations of homologous and heterologous antisera for a given concentration of antigen were determined by parallel gel-diffusion experiments. The antisera were then titrated, each against its own homologous serotype paramecia, by the immobilization method. The equivalence concentration of each antiserum for the antigen used in the gel-diffusion experiments could then be expressed in terms of units of immobilizing activity. The percentage cross-reaction was calculated as the ratio of the homologous to the heterologous equivalence concentrations, multiplied by 100. The immobilization method was thus utilized as a means of standardizing the concentrations of different serotype antisera. If the method is to be valid it is required that the mechanism of the immobilization reaction is the same for all serotypes. That this requirement is satisfied is indicated by: (*a*) the relationship between the immobilization and gel-diffusion titrations is the same for different antigens (Table 1); (*b*) the immunological cross-reactions between the antigens as measured by this composite method are internally consistent, as will be seen below.

The results of the gel-diffusion cross-reaction measurements are presented in Table 2. In agreement with the cross-reactions measured by the immobilization methods, cross-reactions between different types of antigens and antisera were again very slight. In contrast to the immobilization results, but in agreement with the results obtained by absorbing antiserum with heterologous paramecia, the gel-diffusion method showed strong cross-reactions between the three antigens controlled by allelic genes at the D-locus. It may be noted, too, that the cross-reaction of 60D antigen with 90D antiserum as measured by this method is quantitatively not significantly different from the cross-reaction of 90D antigen with 60D antiserum. The internal consistency of the method thus lends veracity to the assumption that the mechanism of immobilization is the same for different serotypes of Paramecium. Quantitatively, the cross-reaction between 60D antigen and 90D antiserum was greater than the cross-reaction between 60D antigen and 103D antiserum (Table 2). At the same time the cross-reaction between 90D antigen and 103D antiserum was greater than that between 90D antigen and 60D antiserum. Taken together, these results suggest the possibility that 90D antigen is an intermediate antigenic sub-type between sub-types 60D and 103D.

DISCUSSION

An investigation of the electrophoretic mobilities of these antigens in starch gel (Bishop & Beale, 1960) showed that antigens 60D, 60G and 60T (i.e. of different types) had different mobilities at pH 9·6, whereas antigens 60D, 90D and 103D (i.e. sub-types of type D) had the same mobility. This agrees well with the immunological relationships revealed by the serum-absorption and gel-diffusion methods: antigens of different types showed negligible cross-reactions, while the three type D sub-types showed cross-reactions ranging from 25 to 50% of the homologous reactions. This, in turn, is not surprising in view of the genetic relationships of the antigens. It is to be expected that immobilizing antigens controlled by genes at different loci will differ from each other chemically and immunologically to a greater extent than will antigens controlled by allelic genes.

Since the different antigenic types differ significantly in electrophoretic mobility, it may be supposed that the virtual absence between them of immunological cross-reaction is a consequence of differences in primary structure. It is possible that the immunological differences between the type D sub-types are also consequent upon differences in primary structure, differences however which do not result in charge differences at pH 9·6 or which lead to charge differences too small to detect by the electrophoretic method employed.

The cross-reactions measured by the immobilization methods between the type D sub-types complicate the otherwise straightforward interpretation of the data. When antiserum was applied to heterologous paramecia (of different sub-types) little cross-reaction was observed as measured by the immobilization time of the paramecia. The heterologous paramecia were capable, however, of absorbing up to half of the immobilizing activity of the antiserum (Table 2). This observation, that paramecia absorb heterologous antiserum of different sub-type but are not readily immobilized by it, may be explained if it is supposed, simply, that the complex of antigen and heterologous antibody is a weak agent in inducing immobilization. This might occur, for example, if the complex were built up more slowly than the complex of antigen and homologous antibody. Since the immobilization method is a measurement of the rate of immobilization the heterologous antiserum would then give a weaker reaction.

Again, heterologous antigen, differing in sub-type, only slightly inhibited the immobilization of paramecia by homologous antiserum. Measured by the gel-diffusion method, however, the heterologous antigen had up to half as much affinity for immobilizing antibody as did the homologous antigen. This effect, that although heterologous antigen combines with antibody it does not significantly prevent the antibody from causing immobilization, could result, for example, if homologous antigen were capable of displacing heterologous antigen from the antigen + antibody complex.

Although these explanations are very tentative, the basic difference between methods which show a high degree of cross-reaction and methods which show a low seems to be that the former measure the extent of complex formation between the heterologous antigen and antibody while the latter depend upon the extent of the immobilization reaction in the presence of such a complex. Similar discrepancies between different methods of titration have previously been reported by Finger (1956) and by Balbinder & Preer (1959). In these cases, too, the greater cross-reactions were observed when the extent of complex formation was measured. At this time, then, all indications point to the mechanism of immobilization as the source of such discrepancies.

This work was supported by a grant to Dr G. H. Beale from the Medical Research Council. I am indebted to Dr Beale for much helpful advice and encouragement.

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J. gen. Microbiol. (1963), **30**, 281–287
Printed in Great Britain

Dose-Mutation Relationships in Ultraviolet-Induced Reversion from Auxotrophy in *Escherichia coli*

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(*Received* 1 *June* 1962)

SUMMARY

Curves for the absolute yield of revertants as a function of ultraviolet (uv) radiation dose were obtained after post-irradiation incubation in saline and after exposure to reactivating light. The curves exhibited plateau regions and final slopes which were equal to the slopes of the survival curves for the auxotrophic *Escherichia coli* parent. These features may be due to a saturation of the mutagenic effect at low doses. This saturation would be the result of (*a*) heterogeneity in the unirradiated population with respect to the amount of protein and RNA synthesis which can occur before DNA replicates in the irradiated organisms, combined with (*b*) limitation of the observable yield by the selection method. The observable yield would be derived from the fraction of the population most resistant to the lethal effect of radiation.

INTRODUCTION

The mechanism by which ultraviolet (u.v.) radiation produces mutations to prototrophy in amino acid-requiring strains of bacteria has been the subject of many recent investigations (Witkin, 1958, 1961; Lieb, 1960; Weatherwax & Landman, 1960; Doudney & Haas, 1958, 1960*a*, *b*; Haas & Doudney, 1959; Doudney, 1961). Briefly, u.v.-radiation produces a large number of potential prototrophs. The fraction which is realized (the yield) is determined by the amount of protein and RNA metabolism which takes place in the irradiated auxotrophic organisms during the period before DNA synthesis. During this critical period, the yield can be decreased by treatments which inhibit protein or RNA synthesis. Treatments which prolong this critical period increase the yield.

Two hypotheses have been advanced to explain these findings. The first suggests that the initial effect of u.v.-radiation is some unknown kind of damage to DNA and that in the absence of adequate protein and RNA metabolism, the damage is repaired. Failure of repair results in the stabilization of the damage in the form of a mutation when DNA replicates (Witkin, 1961). The second hypothesis is that the initial damage is not to DNA but to a precursor of RNA. When this modified precursor becomes part of RNA, it causes a mistake in the structure of DNA (Doudney, 1961).

The following report concerns the relationship between the u.v. dose and the yield of mutations. The effects on this relationship of various post-irradiation treatments and of the method of scoring mutants will be described.

METHODS

Bacterial strains. The experiments were performed with tryptophan-requiring strains of *Escherichia coli* strain B/r, mostly with a strain which will be referred to as WP2. This strain had been passed from Dr E. M. Witkin to Professor F. J. Ryan, to Dr S. B. Greer before we obtained it. A few experiments were done with a strain obtained directly from Dr Witkin and therefore designated as WWP2. Both strains are apparently blocked at the same point, namely, a step before anthranilic acid in the synthesis of tryptophan. Neither strain can utilize shikimic acid. The growth of these strains in anthranilic acid is restricted. The maximum rate is obtained with 0·5–1 μg./ml. and the rate decreases with concentrations greater than this. Inhibition of the growth of an auxotroph by an excess of the same metabolite which is required was pointed out by Soboren & Nyc (1961). Although WP2 and WWP2 seem to be identical in their growth behaviour, their response to u.v. irradiation is different. The yield for WWP2 is 4–5 times that for WP2.

Irradiation. The techniques for growing cultures, irradiating and determining the survival of the parent and the yield of revertants were those described by Witkin (1958). Unless stated otherwise, stationary-phase cultures were used. Unfractionated doses were always given. Plating was done on SEM (minimal medium + 5 % nutrient broth, solidified with unwashed agar) and/or, MM (minimal medium E solidified with washed agar; Vogel & Bonner, 1956).

The apparatus used for u.v.-irradiation and for photoreversal was described by Hill & Simson (1961). Post-irradiation incubation was carried out by simply transferring the saline suspension from the Petri dish in which it had been irradiated to a test tube and immersing the test tube in the 37° water-bath of the photoreversal apparatus for 1 hr. In experiments in which the effect of incubation, with and without exposure to the light, was to be determined, the irradiated culture was divided into two test tubes. One of these was wrapped in aluminium foil before immersion in the water-bath. Although the effect of the reversing light was actually the combined effect of the light and the incubation, no correction was made. Since it was not feasible to obtain entire dose/mutation curves with and without post-treatments in a single experiment, limited sections of dose range were used and the results normalized to the data for overlapping doses used in different experiments.

RESULTS

Figure 1 shows the effects of incubation in saline and of exposure to light as functions of u.v. dose when the yield was measured on SEM. With both types of post-treatment, the absolute yield of revertants was decreased in the low dose range, in agreement with other reports (Doudney & Haas, 1960*a*; Lieb, 1960). In the very high dose range, the revertant yield seemed to decline at the same rate as the auxotrophs were killed. To determine whether this was true, repeated experiments were done in this range. No difference in rate was detected. It may be concluded that either the mutagenic effect continued at very high doses but the increase in surviving revertants was too small to be detected, or the mutagenic effect did not continue at high doses. In either case, the final slope represents a killing effect only.

An interesting feature of the mutant yield curves obtained after saline incubation

and after exposure to light is the rather extended plateau which connects the dose ranges which correspond to an increasing and decreasing yield. Again, alternative explanations would be either that the plateau is the result of an exact balance between the mutagenic and lethal effects as modified by post-treatment or that the plateau is due to a saturation of the mutagenic effect at low doses combined with a very broad shoulder on the survival curve for the organisms which become revertants.

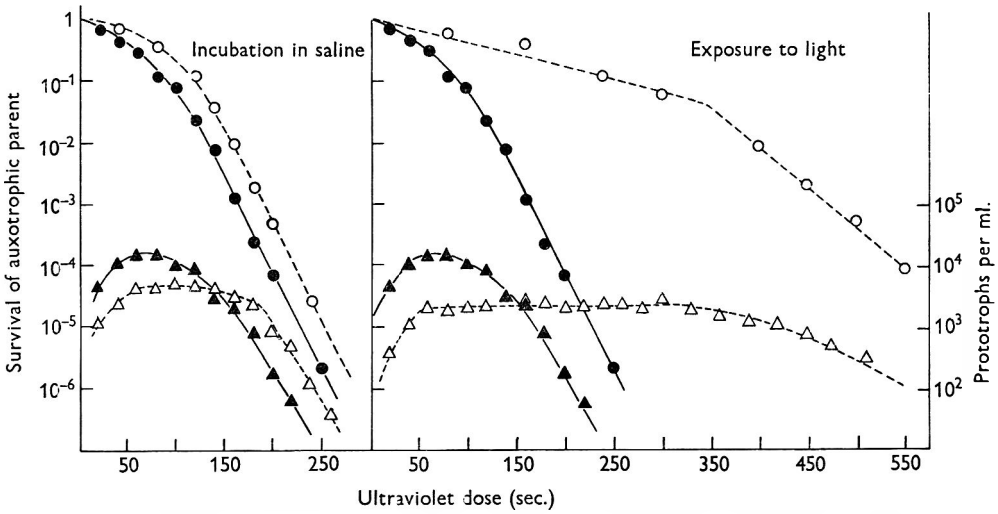

Fig. 1. Survival curves for the parent tryptophan-requiring strain *Escherichia coli* WP2 and absolute yields of prototrophs. Solid curves: no post-irradiation treatment; broken curves: post-irradiation incubation in saline and post-irradiation exposure to light. Circles = parent survival values; triangles = yields of prototrophs. All platings on SEM.

When irradiated organisms were plated on MM instead of SEM, only a small number of revertant colonies appeared. These originated from organisms which were capable of sufficient protein and RNA synthesis for fixation of the mutation and for phenotypic expression, without the necessity of supplying a pool of amino acids (Haas & Doudney, 1959). Figure 2 shows the dose-mutation curves for this small 'zero-point' fraction of the total yield, as obtained by plating on MM immediately after irradiation and after 1 hr. of incubation in saline or 1 hr. of exposure to light. Plateaus appear in all of these cases. However, in contrast to the results for the major part of the yield, saline incubation appeared to increase the 'zero-point' yield after all doses of u.v.-radiation.

Further details concerning the latter effect are given in Table 1. In the case of *Escherichia coli* strain WP2, the increased yield obtained by saline incubation before plating was also obtained by simply plating a smaller number of bacteria. This dilution effect is usually ascribed to the existence of competitive suppression (Grigg, 1958). Since the plating was on MM, it is probable that the potential revertants competed with other surviving auxotrophic bacteria for nutrients released by non-survivors (Ryan, 1954). Presumably this release of nutrients also occurred during the incubation in liquid medium before plating. Therefore the 'zero-point' yield is probably not truly zero-point but derives from bacteria whose requirement

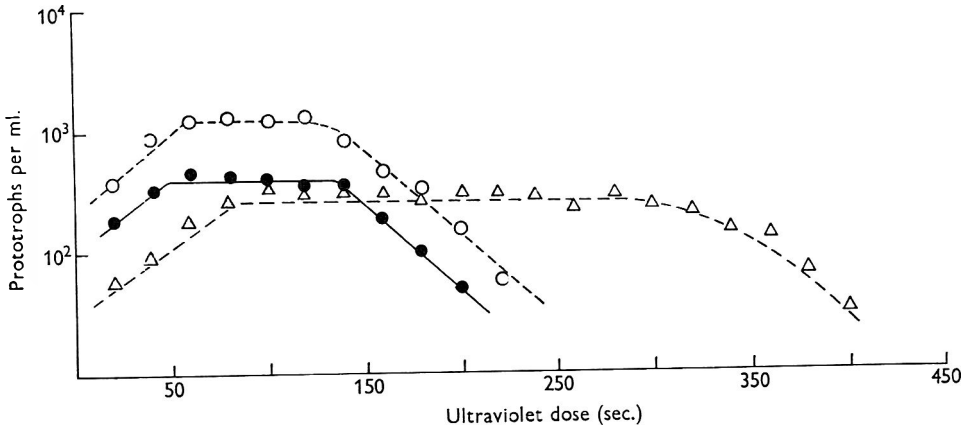

Fig. 2. Dose mutation curves obtained by plating on MM. Solid curve: no post-irradiation treatment; open circles = post-irradiation incubation in saline; open triangles = post-irradiation exposure to light.

Table 1. *Effect of incubation in saline after u.v.-radiation on the zero-point yields of strains* WP2 *and* WWP2

The strains were grown in broth to a concentration of 2×10^9 per ml., centrifuged twice and re-suspended in saline. u.v. dose = 80 sec. Volume plated = 0·1 ml. Plating on MM. Colonies counted after 3 days incubation at 37°.

Time of incubation in saline (hr.)	Dilution plated	No. of zero-point revertants per ml. of irradiated suspension	
		WP2	WWP2
0	10^{-1}	1300	515
	10^{-2}	2550	800
1	10^{-1}	2230	845
	10^{-2}	2800	1800

Table 2. *Effect of prolonged aeration in broth before irradiation on revertant yield*

An overnight broth culture of WP2 grown without aeration, was diluted to give 10^3 cells per ml. in a total volume of 30 ml. This culture was grown by aeration. At various times, 3 ml. were removed, centrifuged and re-suspended in saline. One tenth of an ml. was diluted appropriately to determine the density of WP2 cells. The remaining 3 ml. were irradiated and plated. u.v. dose = 80 sec. (450 ergs per mm²).

Time of sampling (hr.)	Density of WP2 cells ($\times 10^{-9}$)	Revertants/ml. appearing after u.v.-irradiation on		% MM/SEM
		SEM	MM	
18	0·97	7,000	280	3·9
20	1·1	10,800	320	3·0
22	1·3	8,300	640	7·6
24	2·0	9,150	960	10·5
26	2·2	8,200	900	12·5
28	2·4	9,000	1,280	14·3
30	2·4	9,200	1,500	16·3
43	2·2	3,500	1,100	29·6

of an external supply of nutrients for fixation and phenotypic expression of the mutation was much smaller than the requirement of the rest of the population.

In the case of strain WWP2, the dilution effect was not completely eliminated by incubation in saline. This probably means that release of nutrients by non-survivors started during the 1 hr. incubation period in saline, and for this strain continued after plating.

A 'zero-point' yield of revertants is not obtained when the auxotrophic bacteria are irradiated while in the logarithmic phase of growth (Lieb, 1958). Table 2 shows that the 'zero-point' yield increased as the bacteria to be irradiated entered the late logarithmic phase. This yield reached a maximum at a later time than the rest of the yield and declined more slowly as the culture aged.

DISCUSSION

It has been suggested that the 'zero-point' yield may be due to storage of intracellular precursor material, thereby making the bacteria less dependent upon an external supply (Lieb, 1958). Since the total yield is not 'zero-point', it would seem that, if this suggestion is correct, the unirradiated stationary-phase population must be heterogeneous with respect to the size of the intracellular pool of precursors. This raises some interesting questions. If the yield obtained by plating directly on MM after irradiation comes from bacteria which have the largest pool, then the size of this small part of the yield is already determined *before* irradiation. This is in agreement with the second of the two possible interpretations of the dose/mutation curves mentioned previously, i.e. the mutagenic effect is saturated at low doses. A similar suggestion was proposed for the case of colour variants of *Escherichia coli* strain B/r appearing after u.v.-irradiation (Newcombe & Whitehead, 1951).

The major part of the yield appears only on supplemented MM, increasing with the amount of supplement to a maximum when the broth concentration is about 2·5–5 % (Witkin, 1956). By extending the case of the 'zero-point' yield to the remainder of the yield, it would appear that the number of revertants appearing on *any* given level of enrichment may also be pre-determined by the number of unirradiated cells which have a corresponding-sized pool of precursor material.

Why does the number of revertants not continue to increase when the broth concentration is increased to 100 %? The obvious answer is that the medium loses its selectivity since the surviving auxotrophic bacteria will undergo more and more divisions. In other words, the number of revertants that can be detected is limited by the method of detection.

Witkin showed that the critical period following irradiation, during which the yield can be influenced by treatments affecting protein (and RNA) synthesis, was proportional to the u.v. dose in the low dose-range. There is apparently a maximum dose in the low dose-range such that further irradiation no longer increases the sensitive period, presumably because there is no further delay in DNA synthesis (Witkin, 1958). Since (*a*) it is the amount of protein synthesis which takes place before DNA synthesis which determines the observed yield, (*b*) the relative amount of protein synthesis which takes place in a given bacterium is determined before irradiation, and (*c*) the method of detection limits the amount of protein synthesis which can lead to an *observable* mutation, an apparent saturation of the mutagenic effect is readily understandable.

These considerations also explain the appearance of plateaus in the dose/mutation curves. It was suggested above that if the mutagenic effect is saturated at a low dose of u.v. radiation, then the plateau might represent a broad shoulder on the survival curve of the bacteria which become revertants. The preceding analysis indicates that the fraction of potential prototrophs which is actually detected must arise in those auxotrophic bacteria which have a larger intracellular pool of precursors in comparison with those which are not detected. The size of this pool increases as the bacteria enter the stationary phase of the growth cycle. Gillies & Alper (1960) found that the survival curves for *Escherichia coli* B/r (of which strain WP2 is a mutant) had the same final slopes when the bacteria were u.v.-irradiated in the logarithmic and stationary phases. However, when the bacteria were in the logarithmic phase, the survival curve was a single exponential; when they were in the stationary phase, a shoulder appeared and the extrapolation number increased to about 3. Evidently, the more 'stationary phase' the bacteria are, the broader the shoulder on the survival curve. Thus the plateau on the dose/mutation curve may simply be a reflection of the greater radiation-resistance of the most 'stationary phase' bacteria, i.e. the bacteria with the largest intracellular pools, the bacteria responsible for the observed yield.

Support for the above hypothesis might be obtained from investigation of the effect on the yield of mutations of experimental conditions, other than the use of bacteria in different phases of the growth cycle, which broaden the shoulder of the u.v. survival curve. Preliminary results obtained by growing cultures before irradiation in medium supplemented with a high concentration of glucose (Stapleton & Engel, 1960) have already shown that the broadening of the shoulder of the survival curve is accompanied by a marked increase in the absolute yield of revertants.

The author wishes to express gratitude to Dr H. H. Rossi for frequent helpful discussions and Miss Barbara Potter for technical assistance. Based on work performed under Contract AT-(30-1)-2740 for the U.S. Atomic Energy Commission.

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J. gen. Microbiol. (1963), **30**, 289–297
With 1 *plate*
Printed in Great Britain

The Stability of Spontaneous and Ultraviolet-Induced Reversions from Auxotrophy in *Escherichia coli*

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(*Received* 1 *June* 1962)

SUMMARY

Spontaneous and u.v.-induced revertants were found to be unstable in serial subculture. Instability was manifested by (*a*) replacement of the original revertant by revertants with slower growth rates on minimal medium, and (*b*) the appearance of auxotrophic organisms which overgrew in some cases but not others. In one case, the instability was found to be more pronounced when the organisms were maintained in the logarithmic phase of growth. This evidence, along with the previous radiological evidence, was interpreted according to the following hypothesis. The auxotrophic organism has an episomic element located at the suppressor locus. The damage inflicted by radiation consists in the detachment of this episome. The prototrophic cell may again become auxotrophic by reattachment.

INTRODUCTION

Previous investigation (Hill, 1962) of the u.v. dose-mutation relationship for the reversion of *Escherichia coli* strain WP2 from tryptophan-dependence to independence suggested that the observed revertants might not have been produced at random in the population of irradiated auxotrophic organisms but rather might have been produced in a select fraction. This fraction would consist of organisms which had a greater degree of stationary phase metabolism before irradiation and, associated with it, a greater resistance to the lethal effect of u.v.-irradiation. The logical extension of this concept leads to the possibility that if the unirradiated population of auxotrophic organisms was uniform and had the maximum possible degree of stationary phase metabolism before irradiation, *every* surviving cell would be prototrophic. It seemed to us that this conclusion implied something unique about the mutation from auxotrophy to prototrophy in WP2. To determine whether this might be true, a study of the revertants was made. The results are reported here.

METHODS

Bacterial strains. The revertants used in these experiments were derived from the tryptophan-requiring strains, *Escherichia coli* WP2 and WWP2, described previously (Hill, 1962). Spontaneous and u.v.-induced revertants were used.

Culture conditions. Cultures of the revertants were maintained by serial transfer in nutrient broth. One-tenth ml. of each culture was diluted into 2 ml. of broth for transfer and the dilution incubated without aeration at 37° overnight. This allowed

about 4–5 divisions to occur in each subculture. In one set of experiments, to be described in more detail later, transfer was also made by diluting the overnight culture into 10 ml. broth and growing with aeration at 37°. When a culture was to be studied for its content of organisms, after dilution into fresh broth, the remainder of the culture was centrifuged, re-suspended in physiological saline solution, diluted in saline and 200–300 organisms plated in duplicate on MM and on SEM agar. As described previously, MM is minimal medium E solidified with washed agar and SEM is the same but supplemented with 5% broth. In experiments where the requirement of secondary auxotrophs arising among the prototrophs was to be determined, MM was supplemented with 40 μg. tryptophan/ml. or with 20 μg. phenylalanine or tyrosine. These last two amino acids were used to determine whether the secondary auxotrophs were less specific in their requirement than the primary auxotrophs WP2 and WWP2. In all cases, the secondary auxotrophs were found to require tryptophan specifically.

Isolation of revertants. Spontaneous and u.v.-induced revertant colonies were obtained by plating saline suspensions of unirradiated and irradiated WP2 and WWP2 on SEM or on MM. To be reasonably certain that the mutant colonies appearing when irradiated cultures were plated were not of spontaneous origin, the volume plated on SEM was chosen to give about 100 mutant colonies. The background of spontaneous mutant colonies, arising during the growth of the auxotrophic parent on SEM was usually 6–15. On MM this precaution was unnecessary since the parent strains cannot grow and the frequency of spontaneous mutants capable of forming colonies on MM did not exceed about one per 10^8 organisms. A very small portion of each revertant colony was carefully scraped from the surface with a needle and suspended in 1 ml. of saline. Several dilutions of this suspension were placed in duplicate on SEM and on MM so as to observe the initial characteristics of the revertant. These plates were incubated at 37° and examined daily to determine the rate of growth on MM and any changes that might appear on SEM. After 3 days of incubation, scrapings from one or two colonies were put into 2 ml. broth. The original saline suspension was also diluted as 0·1 ml. into 2 ml. of broth. Both sets of broth cultures—one set inoculated from the revertant colony when it first appeared and the other set inoculated with a subclone derived from it—were then maintained by serial subculture (the cultures were refrigerated between subcultures). After every few subcultures, the cultures were studied as described above.

Terminology. Various authors have reported that revertants arising among amino acid-dependent organisms vary in their ability to grow on unsupplemented minimal medium (Smith-Keary, 1958; Stadler & Yanofsky, 1959; Strauss & Okubo, 1960; Bacon & Treffers, 1961). The revertants are usually classified according to the time required for the colony size on MM to reach the size produced by the wild type after 24 hr. of incubation at 37°. Thus they may be F (fast) meaning that the growth rate is indistinguishable from that of the wild-type; S48, (slow, 48 hr. required); S72, etc. The same terminology is used in the present study.

RESULTS

Fourteen independent spontaneous revertants and 18 subclones derived from them were studied first. Of the 14, four were fast types when first examined and remained fast. This was also characteristic of the subclones derived from them.

The rest of the 10 original cultures and the 15 subclones derived from them were of the S48 type initially. However, after various numbers of subcultures, all of these 25 began to show signs of instability. The instability was manifested in the following ways:

(*a*) Gradual overgrowth by a cell type which gave a colony on SEM with the same morphology as that produced by WP2 (small, grey and translucent) and which did not form a colony on MM. Whenever this colony type appeared, the colony was tested and invariably found to be tryptophan-dependent (Pl. 1, fig. 1).

(*b*) Marked variation in the sizes of the colonies on MM (Pl. 1, fig. 2, 3). In some cases, this feature preceded overgrowth by auxotrophic organisms; in others, it persisted for some time and then disappeared, only to appear again at a later time. In the latter cases, an occasional or a few auxotrophic colonies appeared but the culture was not overgrown. A detailed study of one such case will be presented later.

(*c*) In some cases, as the percentage of S48 types decreased, they were replaced not only by auxotrophic organisms but also by a very much slower type of revertant which did not produce a visible pinpoint-size colony on MM until after 3–5 days of incubation (Pl. 1, fig. 4). Sometimes the culture became stabilized for many subcultures with a mixture of auxotrophs and very very slow revertants. Eventually the very very slow type declined in favour of the auxotrophic type.

Although the four cultures and their subclones which were classified as fast remained fast, an occasional slight variation in colony size on MM was observed. Also an auxotrophic colony appeared occasionally.

An interesting finding was that cultures of subclones did not always show exactly the same pattern of instability as the colony from which they were isolated. For example, in one case, the original culture became a mixture of 10 % auxotrophic cells and 90 % very very slow revertants by the twenty-first subculture. It then remained stable at this ratio through the next nineteen subcultures. On the other hand, cultures of two subclones from this parent were overgrown by auxotrophic cells by the 9th and 11th subcultures. In one, the overgrowth seemed to be gradual and complete, i.e. no revertants were found when 10^6 organisms were plated; in the other, a ratio of 1 % S48 revertants and 99 % auxotrophs was maintained through six subcultures.

Revertants induced by u.v.-irradiation were studied next. After u.v.-irradiation of WP2 and WWP2, more than 95 % of the revertant colonies were slow in appearing when plating was done on MM. Bacon & Treffers (1961) reported that, in the case of spontaneous reversion from ornithine-dependence, the growth rate of the colony, as it first appeared on MM, did not indicate its characteristic rate. Colonies which did not reach full size until 96 hr. of incubation initially, often turned out to be fast types after subculture. However, this was not observed with irradiated WP2 and WWP2. Colonies which were slow in appearing initially were also slow after subculture.

The series of u.v.-induced revertants used for study included equal numbers of S48 and S72 types. A small fraction of types appearing even more slowly on SEM and MM were found, but these were not used. Ten of the revertants were obtained from WP2 and eight from WWP2. No difference due to the auxotrophic parent was observed. Both the S48 and S72 types and subclones derived from them

exhibited the same patterns of instability as the spontaneous revertants. Instability did not appear to depend on whether the revertant was S48 or S72.

One u.v.-induced revertant was studied in some detail. This mutant was originally S48. It began to show variations in colony size on MM by the 19th subculture. By the 34th subculture, this variation was still observed (Pl. 1, fig. 2) and in addition a small percentage of the colonies appearing on SEM were tryptophan-dependent. When the smaller-sized colonies on MM were picked and re-plated, they turned out to be S72 and S96 types. The 34th subculture was done on 17 August 1961. In spite of bi-weekly subculture by 1/20 dilution in broth since then, there has been no change in the percentage of auxotrophic organisms and the colony size variation on MM has persisted until the time of writing—April 1962. However, a way to change this picture was discovered.

In September 1961, the following experiment was performed. It was repeated twice in February 1962, with some modifications of procedure but essentially the same results. The experiment consisted simply of (*a*) growing by aeration in broth, (*b*) sampling frequently, and (*c*) comparing the properties of the organisms when the culture was allowed to complete one growth cycle with the properties when the culture was maintained in the logarithmic phase. Details of the experiment and the results are shown in Tables 1 and 2.

Table 1. *Instability of u.v.-induced revertant no.* 103 *as a function of the growth cycle*

An overnight broth culture grown without aeration was diluted 1/1000 into fresh broth and aerated at 37°. Two ml. samples were removed every 2 hr., the organisms washed in saline, diluted and plated. After 8 hr., the culture was refrigerated overnight. The next day, it was diluted 1/4000 and the experiment repeated.

Time of sampling (hr.)	Organisms/ml. calculated from nos. of colonies appearing after 48 hr. incubation of plates containing				Nos. of cols. on MM × 100, / Nos. of prototroph cols. on SEM when MM plates were incubated for	
	MM + 40 μg. tryptophan per ml.	SEM				
		Total	% proto.	% auxo.	48 hr.	72 hr.
0	$2{\cdot}9 \times 10^5$	$2{\cdot}6 \times 10^5$	96·7	3·3	100	100
2	$5{\cdot}9 \times 10^6$	$6{\cdot}3 \times 10^6$	97·0	3·0	54	89
4	$1{\cdot}1 \times 10^8$	$1{\cdot}0 \times 10^7$	95·6	4·4	84	100
6	$6{\cdot}1 \times 10^8$	$5{\cdot}6 \times 10^8$	92·5	7·5	85	104
8	$1{\cdot}1 \times 10^9$	$9{\cdot}2 \times 10^8$	91·6	8·4	92	100
0	$3{\cdot}0 \times 10^5$	$2{\cdot}9 \times 10^5$	95·5	4·5	72	88
2	$2{\cdot}6 \times 10^6$	$2{\cdot}5 \times 10^6$	96·0	4·0	75	88
4	$4{\cdot}1 \times 10^7$	$4{\cdot}0 \times 10^7$	91·0	9·0	63	71
6	$3{\cdot}9 \times 10^8$	$3{\cdot}2 \times 10^8$	88·4	11·6	65	92
8	$1{\cdot}7 \times 10^9$	$1{\cdot}6 \times 10^9$	84·7	15·3	83	100
24	$2{\cdot}3 \times 10^9$	$2{\cdot}0 \times 10^9$	80·0	20·0	100	100

At the beginning of the experiment, the culture contained about 3 % of auxotrophic organisms. Although there was a variation in the size of the colonies formed on MM, all of the prototrophic organisms produced colonies on this medium which were visible after 48 hr. of incubation. As shown in Table 1, during the logarithmic phase of the growth cycle, the percentage of auxotrophic organisms increased slightly. More importantly, the colonies formed by many of the prototrophic organisms on MM took longer to appear. Both of these effects became more pronounced when the culture

was put through a second cycle of growth. When the culture entered the stationary phase, the rate of growth of the prototrophic organisms on MM increased again. Refrigeration of the culture when in the late logarithmic stage of growth seemed to cause a decrease in both the percentage of auxotrophic organisms and the rate of growth of prototrophic organisms on MM but this phenomenon was not investigated.

Table 2. *Instability of u.v.-induced revertant no.* 103 *when kept in the logarithmic growth phase*

These experiments were performed simultaneously with those described in Table 1 and the same overnight culture was used initially. This was diluted 1/100 and aerated. After 2 hr., a 1/100 dilution was made; the dilution was then aerated another 2 hr.; the remainder of the original culture was sampled in the same manner as the accompanying culture (see Table 1). This procedure was repeated every 2 hr. The dilution made after a total time of 8 hr. was allowed to aerate overnight. The next day, this was sampled, diluted 1/1000 initially and the procedure of the previous day repeated. The 1/1000 dilution was also sampled.

Time of dilution (hr.)	Organisms/ml. calculated from nos. of colonies appearing after 48 hr. incubation of plates containing				Nos. of cols. on MM × 100 / Nos. of proto. cols. on SEM when MM plates were incubated for	
	MM + 40μg. tryptophan per ml.	SEM				
		Total	% proto.	% auxo.	48 hr.	72 hr.
0	$3{\cdot}0 \times 10^8$	$3{\cdot}0 \times 10^8$	96·7	3·3	95	100
2	$5{\cdot}6 \times 10^7$	$4{\cdot}2 \times 10^7$	96·0	4·0	91	100
4	$1{\cdot}1 \times 10^7$	$1{\cdot}1 \times 10^7$	93·5	6·5	76	88
6	$2{\cdot}3 \times 10^8$	$2{\cdot}1 \times 10^6$	91·4	8·6	88	95
8	$3{\cdot}4 \times 10^5$	$3{\cdot}2 \times 10^5$	86·5	13·5	79	90
24	$3{\cdot}4 \times 10^9$	$3{\cdot}1 \times 10^9$	69·8	30·2	47	70
24	$3{\cdot}5 \times 10^8$	$3{\cdot}4 \times 10^6$	70·9	29·1	38	77
26	$4{\cdot}3 \times 10^7$	$4{\cdot}6 \times 10^7$	70·8	29·2	34	57
28	$1{\cdot}0 \times 10^7$	$1{\cdot}3 \times 10^7$	52·8	47·2	18	41
30	$3{\cdot}0 \times 10^8$	$3{\cdot}9 \times 10^6$	49·4	50·6	12	48
32	$8{\cdot}3 \times 10^5$	$9{\cdot}0 \times 10^5$	38·0	62·0	19	67

Table 2 shows the results of keeping the culture in the logarithmic phase for an extended time. There is a more pronounced decrease in the rate of growth of the prototrophic organisms on MM and a more rapid increase in the fraction of auxotrophic organisms.

DISCUSSION

It was suggested previously (Hill, 1962) that the number of u.v.-induced revertants which can be detected is limited by the selective medium, since those requiring higher broth concentrations would be masked by the increased growth of auxotrophic organisms. A second source of limitation is indicated by the present observation that, after irradiation of the auxotrophic parent, some revertants do not appear on SEM or MM until after 4 or more days of incubation. If even slower ones were present, they might produce only microcolonies. Still another source of limitation may be the phenomenon of instability. If instability happens to occur within the first few divisions after the newly induced prototroph starts to grow on agar, the prototrophic organisms remaining in such a clone might not produce a visible colony.

The finding of instability as a characteristic of both spontaneous and u.v.-induced

reversions from tryptophan dependence in *Escherichia coli*, WP2 and WWP2, is in agreement with observations made on spontaneous leucine-independent revertants in *Salmonella typhimurium* by Smith-Keary (1958). As in the case of the revertants derived from WP2 and WWP2, the slow types derived from *S. typhimurium leu*⁻ showed different degrees of instability manifested as variations in the fraction of auxotrophic organisms and in the sizes of colonies formed by the prototrophic organisms on MM. In addition, revertant clones which appeared initially to be stable slow types frequently became unstable after subculture (Dawson & Smith-Keary, 1962). Although instability was reported only for the slow types, our results suggest that the faster types are probably unstable also.

The slow types in the Salmonella case were shown by genetic analysis to be due to mutation at a suppressor locus (Smith-Keary, 1960). Although genetic analysis of *Escherichia coli* B—the wild-type parent of WP2 and WWP2—has not been done, many genetic studies of *E. coli* K12 have been reported. In this strain, suppressor mutations in tryptophan auxotrophs are common and most of the revertants obtained by u.v.-irradiation are the result of this type of mutation (Stadler & Yanofsky, 1959). It seems safe to assume that this is true for *E. coli* B also.

Dawson & Smith-Keary (1962) showed that the most probable explanation for the instability of the leucineless prototrophs is the attachment of an episomic element (Jacob & Wollman, 1961) to the suppressor locus. Their data also indicate that the episome which attaches to the leucine suppressor locus can also attach to the proline suppressor locus. Furthermore, instability at the leucine suppressor locus was found to be more frequent when this locus was transduced into an arabinose-negative strain, which presumably had more episomes available for attachment than the original leucineless strain. This ubiquity and non-specificity of attachment of this type of episome accounts for the observation that instability may appear at any time. A reasonable hypothesis capable of explaining all of the present findings can be made, if it be assumed that the instability of the tryptophanless revertants studied here, is due similarly to the presence of episomes.

According to Dawson & Smith-Keary, the attachment of an episome to the mutated suppressor gene causes an increased rate of mutation to auxotrophy. Thus the variations in colony size on MM are due to mutation during the growth of the prototrophic clones on minimal agar. The experiment with the unstable tryptophanless revertant (Tables 1 and 2) shows that the rate of mutation to auxotrophy during growth on minimal agar is increased if the organisms were in the logarithmic phase in broth before transfer to minimal agar. Conversely, when the cells are transferred from the stationary phase, the rate of mutation to auxotrophy on the plate is decreased. This phenomenon is the reverse of what is observed when the mutation to prototrophy from auxotrophy is induced by u.v.-irradiation. In the latter case, the number of revertants appearing on minimal agar increases as the auxotrophic organisms go from the logarithmic to the stationary phase in complete medium, before irradiation and subsequent plating on agar (Hill, 1962). If the change from prototrophy to auxotrophy is due to the attachment of an episome to the suppressor locus, it seems likely that the reverse change from auxotrophy to prototrophy in these strains is due to detachment of an episome from the suppressor locus.

Dawson & Smith-Keary take the view that the original leucineless strain becomes prototrophic by mutation of the suppressor locus (when the mutation is clearly not

at the *leu*⁻ locus) and that the attachment of an episome increases the probability that the mutated suppressor locus will back-mutate to its original inactive state. The present findings suggest that the mutation of the suppressor locus causing prototrophy is the detachment of an episome from this locus and that the back-mutation to auxotrophy is the attachment *itself* rather than something which is caused by the attachment. An interesting corollary of this hypothesis is that the reason why the suppressor locus is ordinarily inactive is that it is masked by having an episome attached to it.

According to our hypothesis, the effect of u.v.-radiation on WP2 and WWP2 is then the same as the effect of radiation on inducible lysogenic strains of bacteria (Lwoff, Siminovitch & Kjeldgaard, 1950). There are at least three features which are common both to the induction of prototrophy and the change from the prophage to the vegetative state:

(1) There is an optimal dose of u.v. in the low dose-range which gives a maximum effect (Jacob & Wollman, 1953).

(2) In the case of *Escherichia coli* K12 (λ), the optimal dose is about 80 sec. for a u.v.-lamp of the same type as the one used in the present investigation and a lamp to sample distance of 80 cm. (Weigle & Delbrück, 1951). For a lamp to sample distance of 56 cm., which was used by us, the optimal dose would be 40 sec. The dose at which the mutagenic effect for the change from auxotrophy to prototrophy seems to be saturated is about 60 sec. (Hill, 1962). The agreement in doses, to a first approximation, is to be expected since the radiosensitivities of *E. coli* K12 and *E. coli* B/r are similar (Zelle, 1955).

(3) Following irradiation, a period of incubation in rich medium is necessary to obtain the maximum yield of induced lysogenic bacteria (Weigle & Delbrück, 1951) and the maximum yield of revertants. Furthermore, chloramphenicol treatment after irradiation decrease the yield of revertants (Witkin, 1956). According to our hypothesis, a decreased yield means either a failure of the episome to detach from the suppressor locus or immediate re-attachment. Chloramphenicol treatment has been reported to increase the probability that the phage type of episome will be attached (Bertani, 1957; Christensen, 1957).

Cells containing unattached episomes can be 'disinfected' by treating with acridine dyes (Hirota & Iijima, 1957; Watanabe & Fukasawa, 1961). This effect is attributed to prevention of multiplication of the episomes while they are cytoplasmic, followed by dilution amongst daughter cells during division. The same dyes increase the yield of u.v.-induced reversions from auxotrophy (Witkin, 1961). If reversion consists of the detachment of an episome, the action of the dyes would be to prevent re-attachment. If the revertant is now grown in the presence of the dye, the presumed episome, and any others which are capable of replacing it at the suppressor locus and are cytoplasmic, might be diluted out. The revertant clone would then be stable. Similarly, if the auxotrophic strain is treated with the dye before irradiation, the induced revertants should be stable. Experiments to determine whether these predictions are correct are now in progress.

The removal of an episome as the mechanism responsible for the change to prototrophy in the tryptophanless strains studied here may have general validity for other nutritionally-deficient strains in which suppressor mutations are common. It remains to be seen whether this type of reversion occurring in other strains shows

the kind of instability reported by Dawson & Smith-Keary for a leucineless strain and confirmed above for tryptophanless strains. If our hypothesis be correct, then strains in which suppressor mutations have not been detected (Rudner, 1961) may give only stable revertants after irradiation. It would also be interesting to see whether in those strains for which u.v.-radiation is not a mutagen for a particular marker, the spontaneous revertants correspond only to a change at the original locus (Demerec & Cahn, 1953). In those strains where spontaneous mutations at both the original locus and at a suppressor locus are possible (Stadler & Yanofsky, 1959) is u.v.-radiation an effective mutagen for both types or only for the mutation at the suppressor locus?

Finally, if the effect of u.v.-irradiation is the detachment of an episome, one may ask, as Lwoff has, whether this may justifiably be called mutation (Lwoff, 1953). Until the discovery of episomic elements, mutation was usually regarded as the result of a stable change in the sequence of bases in DNA. Superficially, the question may seem to be simply a matter of whether one wishes to stretch this definition. However, there is a more important consideration. If it should turn out that u.v.-radiation induces change to prototrophy only by an effect at a suppressor locus and the removal of an episome becomes more than a hypothetical explanation, then the mechanism responsible for other reported types of u.v.-induced mutagenesis would require more extensive investigation. Thus mechanisms other than a change of base in DNA may turn out to be more common than is usually assumed. For example, differential killing and the relief of competitive inhibitory effects are obvious mechanisms by which the mutant fraction of a population would be increased. Unfortunately their possible roles in many reported cases of radiation-induced mutagenesis in bacteria have not been studied.

The author wishes to express gratitude to Dr F. J. Ryan for suggesting the possible presence of an episome, Dr P. F. Smith-Keary for a lively exchange of correspondence and for permission to quote results before publication, and Misses Barbara Potter and Rosanne Levitt for technical assistance. Based on work performed under Contract AT-(30-1)-2740 for the U.S. Atomic Energy Commission.

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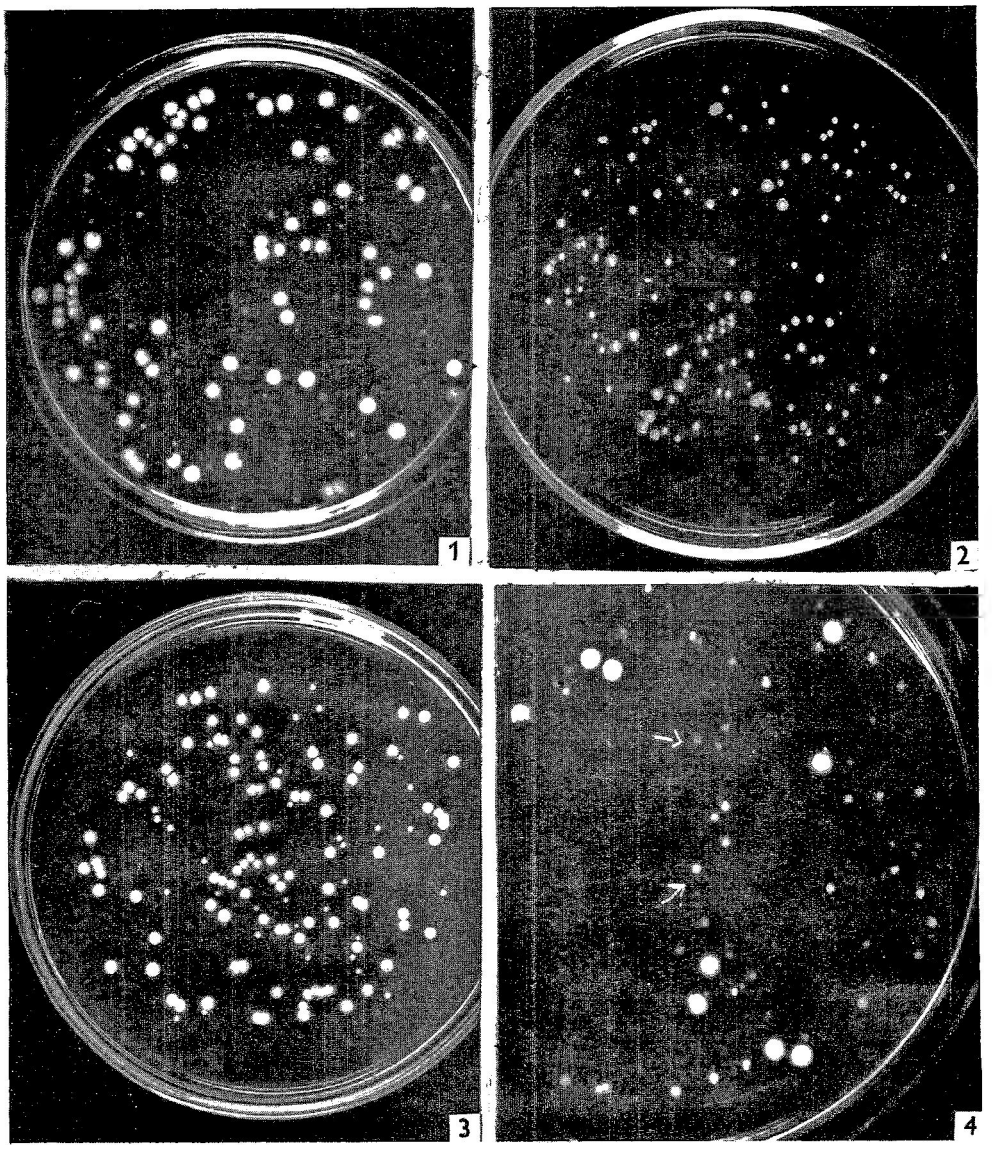

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EXPLANATION OF PLATE

PLATE 1. Colony types produced by u.v.-induced revertants after subculture.

Fig. 1. Auxotrophic and revertant colonies formed on SEM by a subclone of no. 101 after 10 subcultures.

Fig. 2. Variations in size of colonies formed on MM by no. 103 after 19 subcultures.

Fig. 3. Variations in size of colonies formed on MM by a subclone of no. 107. This revertant was overgrown by auxotrophic cells by the fifth subculture.

Fig. 4. Colonies of auxotrophs, S48 and S > 96 revertants formed on SEM by no. 106 after 11 subcultures.

All plates incubated for 48 hr at 37°.

J. gen. Microbiol. (1963), 30, 299–306
Printed in Great Britain

Penicillinase and Ampicillin Resistance in a Strain of *Escherichia coli*

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(*Received* 4 *June* 1962)

SUMMARY

A penicillinase-positive variant was isolated from a penicillinase-negative strain of *Escherichia coli* during 'training' towards ampicillin resistance. The penicillinase activity of the variant was increased tenfold when the organisms were disrupted in a Milner press or treated with toluene, suggesting the presence of a permeability barrier. The enzyme released by disrupting the organisms was not precipitated by centrifugation nor removed by filtration. The penicillinase activity of toluene-treated organisms was easily removed by centrifugation. Disruption of the organisms released enzyme into solution whereas it remained intracellular in toluene-treated organisms. The variant strain, although resistant to over 200 μg. ampicillin/ml., did not appreciably destroy it; of other penicillins tested only penicillins G and V were readily destroyed. The enzyme was a 'penicillinase' (β-lactamase) since the product was penicilloic acid. The enzyme was not inducible: exposure of organisms to 6-amino-penicillanic acid or phenoxybenzylpenicillin produced a threefold increase in penicillinase activity not due to true induction but to an increased permeability. The penicillinase of this strain of *E. coli* thus differs qualitatively and quantitatively from other penicillinases.

INTRODUCTION

Penicillinase produced by Gram-negative and other bacteria was first described by Abraham & Chain (1940). However, until recently the interaction between penicillin and Gram-negative bacteria did not receive much attention because penicillin G was not highly active against such organisms. Much greater activity against these is shown by ampicillin (α-aminobenzylpenicillin; Penbritin, BRL 1341), a new semi-synthetic penicillin described by Rolinson & Stevens (1961). The present paper describes a study of resistance and penicillinase in a strain of *Escherichia coli*.

METHODS

Penicillins. Penicillin G was obtained from Glaxo Laboratories Ltd. (Greenford, England). Penicillin V, phenethecillin ('Broxil'), methicillin ('Celbenin'), ampicillin ('Penbritin') 5-methyl-3-phenyl-4-isoxazolyl-penicillin (BRL 1400, P 12) and 6-aminopenicillanic acid from Beecham Research Laboratories (Brockham Park, Surrey), phenoxypropyl penicillin from Pfizer Ltd. (Folkestone, Kent) and phenoxybenzyl penicillin (Penspek) from Distillers Co. Ltd. (Biochemicals) (Speke, Liverpool).

Measurement of antibacterial activity. One drop (0·02 ml.) of a 4 hr. infusion broth culture was inoculated into 2 ml. of infusion broth containing serial dilutions of antibiotic. The results were read after incubation overnight at 37°.

Measurement of penicillin destruction. (*a*) Production of acid from destruction of penicillin G was determined qualitatively by the method of Knox & Smith (1961*a*). Organisms were grown overnight on cellulose acetate membranes (Oxo Ltd.) on nutrient agar and transferred to a pad of three filter-papers moistened with a solution of penicillin G containing 25,000 units/ml. in Andrade's indicator (2 %). After incubation at 37° for 2–3 hr. penicillinase-producing colonies developed a pink colour due to production of penicilloic acid. Acid is also produced by amidase-catalysed destruction of penicillin G giving 6-aminopenicillanic acid, but in practice a positive result indicates penicillinase activity.

(*b*) Microbiological activity of penicillin was estimated by determining the zone size given by 0·1 ml. of various dilutions of the antibiotic in holes cut in agar plates. The test organism used was *Sarcina lutea* (ATCC 934).

(*c*) Destruction of penicillin by amidase was tested for by the method of Batchelor, Chain, Richards & Rolinson (1961). This method relies on the fact that at pH 2·0 penicillin G can be extracted by butyl acetate, leaving any 6-aminopenicillanic acid, when present, in the aqueous phase. The latter was then estimated by the hydroxylamine method of Boxer & Everett (1949).

(*d*) Destruction of penicillin by penicillinase was followed at a penicillin concentration of 2 mg./ml. at 37° and pH 7·4 in infusion broth. Cultures were grown overnight at 37° in infusion broth and the organisms harvested and washed twice in infusion broth by centrifugation at 2000 *g* for 10 min. Sodium *p*-chlormercuribenzoate was added to 0·001 M to prevent induction of penicillinase during the experiment (Steinman, 1961*a*, *b*). The penicillin remaining was determined by the hydroxylamine method of Boxer & Everett (1949).

Tests of penicillinase induction. Cultures were grown overnight in infusion broth at 37° containing various concentrations, up to 1000 μg./ml. of the penicillin being tested for inducing ability. The organisms were then centrifuged down, washed twice with 0·001 M-*p*-chlormercuribenzoate in infusion broth and resuspended at similar optical densities by using an EEL nephelometer. The rate of destruction of penicillin G was then determined by the hydroxylamine method.

Organisms. The organism used in this study was a strain of *Escherichia coli* isolated from a urinary-tract infection, laboratory reference no. 214. This organism (strain O) was sensitive to 7·5–10 μg. ampicillin/ml. by the tube dilution method and did not destroy penicillin G when tested qualitatively by the membrane technique. When strain O was trained to become resistant to ampicillin by serial subculture on solid ampicillin-containing media a variant was isolated which was resistant to 230 μg. ampicillin/ml. This variant (strain T) destroyed penicillin G when tested by the membrane technique.

Strains O and T grew at similar rates and gave the same biochemical reactions: acid and gas were produced in glucose, mannitol, dulcitol, lactose or maltose, and no reaction was observed in adonitol, inositol, sucrose, gelatin, urea, arabinose, phenylalanine or salicin. Both strains were indole-positive, Voges-Proskauer- and malonate-negative and were motile. They did not produce H_2S and did not grow when ammonium citrate was the sole carbon source; but they did produce acid and gas in MacConkey broth at 44°. Rabbit antisera prepared against each strain exhibited cross-agglutination at similar titres (1/1000). Each strain completely absorbed antibodies from both heterologous and homologous antisera.

RESULTS

Antibacterial activity of penicillins against Escherichia coli *no.* 214 *strains O and T*

The minimum inhibitory concentrations of various penicillins using the tube dilution method are shown in Table 1. It can be seen that strain T was more resistant than strain O to all the penicillins tested.

Table 1. *Minimum inhibitory concentrations obtained with various penicillins*

Penicillin	*Escherichia coli* strain O (μg./ml.)	*Escherichia coli* strain T (μg./ml.)
Ampicillin	10	250
6-aminopenicillanic acid	60	90
G	62·5	1,000
V	200	1,000
Phenoxybenzyl-	300	1,000
5-Methyl-3-phenyl-4-isoxazolyl-	500	1,000
Phenoxypropyl-	600	1,000
Phenoxyethyl-	700	1,000
Methicillin	2,500	10,000

Table 2. *Rates of destruction of penicillins as percentages of rate obtained with penicillin G*

Penicillin	*Staphylococcus aureus* strain E3	*Bacillus cereus* NRRL 569	*Escherichia coli* strain T
G	100 (696)	100 (560)	100 (2)
V	110	117	82
Phenoxyethyl-	100	103	7
Phenoxypropyl-	71	56	4
Ampicillin	198	156	3
Phenoxybenzyl-	167	91	5
Methicillin	1	1	7
5-Methyl-3-phenyl-4-isoxazolyl-	1	1	2
6-Aminopenicillanic acid	1	3	5

Figures in parentheses indicate the absolute values for the rates of hydrolysis of penicillin G in μg./ml./min./10^9 organisms.

Destruction of penicillins by Escherichia coli *no.* 214 *strain T*

The ability of strain T to destroy penicillins was investigated and it was found to destroy penicillin G when tested by the hydroxylamine assay method. The product was identified as benzylpenicilloic acid by paper chromatography. The presence of amidase was tested for by the method of Batchelor *et al.* (1961) and the organism was found not to possess this type of enzyme. About 30 % of the penicillinase activity was found in the culture medium; thus it seems that the penicillinase was mainly intracellular.

The results (Table 2) show the activity of the penicillinase of an intact untreated suspension of strain T against various penicillins; also included are results with a suspension of *Staphylococcus aureus* strain E3 (Knox, 1960; Knox & Smith, 1961*b*) grown overnight in 0·5 μg./ml. of methicillin as an inducing agent and the super-

natant fluid from a culture of *Bacillus cereus* NRRL 569 grown overnight in 5 μg. methicillin/ml. as an inducing agent. It can be seen that the penicillinases from these two organisms are fairly similar as regards hydrolysis of various penicillins except for the rates of destruction of 6-aminopenicillanic acid and phenoxybenzyl/penicillin. Both types of penicillinase destroyed ampicillin much more rapidly than they destroyed penicillin G and their absolute activity was much greater than the penicillinase of strain T; however, the latter organisms were not induced.

The penicillinase of *Escherichia coli* strain T had a much more restricted pattern of activity in that it only destroyed penicillins G and V to any appreciable extent. It had a very low but significant activity against ampicillin, which was so readily destroyed by the other two enzymes, when tested by the hydroxylamine and microbiological assay techniques. No increase in the rate of destruction of ampicillin was found at substrate concentrations as high as 15 mg./ml. (about M/25).

Induction of penicillinase in Escherichia coli *no.* 214 *strain T*

Induction of the penicillinase in strain T was investigated. The results are shown in Table 3. It can be seen that induction with all the penicillins tested was of a low order; staphylococci can be induced to increase their rate of enzyme production 50 times (Knox & Smith, 1962) and *Bacillus cereus* can possess several hundred-fold more enzyme when induced (Pollock, 1952; Steinman, 1961*a*, *b*).

Table 3. *Inducing ability of different penicillins*

Penicillin	Concentration of penicillin tested for induction (μg./ml.)	Ratio of induced to basal penicillinase activity of intact organisms
Ampicillin	100*	1·00
5-Methyl-3-phenyl-4-isoxazolyl-	1000	1·00
V	1000	1·42
G	500*	1·00
Phenoxyethyl-	1000	1·64
Phenoxypropyl-	1000	1·64
Methicillin	1000	1·64
6-Aminopenicillanic acid	30*	3·00
Phenoxybenzyl-	500*	3·45

* The organism did not grow well enough in higher concentrations of these antibiotics to test for induction.

Penicillinase activity of disrupted Escherichia coli *no.* 214 *strain T*

The effect of disrupting the organisms of strain T in a press was investigated. A suspension of strain T was taken and disrupted in a press described by Milner, Lawrence & French (1950) and modified by Wimpenny (1962), at 4° and 3000 lb./sq.in. The broken organisms were then tested for destruction of various penicillins. The broken organisms destroyed penicillin G at a rate about 10 times the rate obtained with intact organisms but did not destroy ampicillin to any appreciable extent. The enzyme released by breaking strain T in the press was further investigated. The enzyme was not precipitated when centrifuged at 33,000 *g* at 2° for

90 min.; it passed through an Oxoid filter membrane of mean pore size of 0·5–1·0 μ; it was destroyed by boiling for 10 min. or by incubation at 37° overnight in 0·2 % trypsin.

Penicillinase activity of toluene-treated Escherichia coli *no.* 214 *strain T*

Since the penicillinase activity of strain T was increased tenfold by disruption of the organisms the effect of toluene treatment was investigated. Organisms were treated with toluene, samples broken in the press and the activity of both suspensions tested against penicillin G. Both suspensions were about 10 times more active than untreated organisms. The toluene treatment did not lyse the organisms (as judged by nephelometry and microscopy), whereas disruption of the organisms with the press causes the optical density to fall by some 60 %. It was found that the penicillinase activity of toluene-treated organisms was precipitated by centrifuging at 2000 *g* for 10 min.

Penicillinase activity of disrupted induced Escherichia coli *no.* 214 *strain T*

The effect of disrupting induced organisms in the press was examined. Strain T was grown overnight in infusion broth and infusion broth containing 30 μg. 6-aminopenicillanic acid/ml. The organisms were centrifuged down, washed twice in 10^{-3} M-*p*-chlormercuribenzoate in broth and resuspended at similar opacities by using an EEL nephelometer. Portions of 6-aminopenicillanic acid-treated and of untreated organisms were broken in the press at 3000 lb./sq.in. at 4° and all suspensions were tested for destruction of penicillin G. The rates of destruction are shown in Table 4.

Table 4. *Penicillinase activity of intact and disrupted 6-aminopenicillanic-acid-treated organisms*

	Destruction of penicillin G, μg./ml./min./mg. dry weight bacteria
Intact untreated strain T	3·84
Intact 6-aminopenicillanic acid-treated strain T	12·24
Broken untreated strain T	42·00
Broken 6-aminopenicillanic acid-treated strain T	42·00

It can be seen that the intact 6-aminopenicillanic acid-treated organisms possessed about 3 times the activity of intact untreated organisms. However, both treated and untreated organisms, when broken, possessed identical activities. Thus treatment with 6-aminopenicillanic acid had not caused any increase in total enzyme content. Similar results were obtained with organisms grown in 500 μg. phenoxybenzylpenicillin/ml. and with the toluene treatment in place of disruption of the organisms in the press.

Penicillinase activity of disrupted Escherichia coli *no.* 214 *strain O*

The presence of penicillinase in strain O was investigated. Strains O and T were grown overnight in infusion broth. The organisms were centrifuged down and washed

in 10^{-3} M-*p*-chlormercuribenzoate in broth and resuspended at similar opacities by using an EEL nephelometer. A sample of each suspension was taken and disrupted in the press and the activity of the broken and intact strain O organisms compared with similarly treated strain T organisms. These results are shown in Table 5. It can be seen that disrupted strain O organisms showed a small amount of enzyme but not the intact ones. The total amount of enzyme in the strain O was about one-fiftieth of the total amount of enzyme in strain T.

Table 5. *Penicillinase activity of intact and disrupted organisms*

	Destruction of penicillin G, μg./ml./min., per mg. dry weight of bacteria
Intact strain O	0
Broken strain O	0·84
Intact strain T	4·22
Broken strain T	42·44

The penicillinase activity of strain O released by disruption was found to be similar to the enzyme possessed by strain T; it was heat- and trypsin-labile, not precipitated by centrifugation nor removed by filtration through an Oxoid filter membrane. The penicillinase activity was exposed by either breaking the organisms in a press or by toluene treatment. The activity of toluene-treated suspensions was sedimented by centrifugation at 2000 *g* for 10 min. The enzyme of strain O destroyed penicillins G and V at similar proportional rates to those found with enzyme of strain T and was relatively inactive against any other penicillins.

DISCUSSION

As a result of 'training' *Escherichia coli* no. 214 strain O to resist the antibacterial activity of ampicillin, strain T was selected, and this strain possessed a total content of about 50 times more penicillinase than the parent strain. Both strains O and T gave similar biochemical reactions and serologically could not be separated. Therefore it seems likely that strain T was a mutant of strain O. Further attempts to select a mutant from strain O with the characteristics of strain T have so far failed; therefore it seems that such a change is a rare event. Although the enzyme of strain T was relatively inactive against ampicillin, as compared with its activity against penicillin G, it did destroy some. If the enzymes of both strain O and T are identical the fact that strain T possessed much more enzyme than strain O may explain the resistance of the former to ampicillin. Indeed the penicillinase activity of strain O was only demonstrable when the cells were disrupted or treated with toluene, whereas a tenth of the total penicillinase activity of the variant strain could be detected in intact cells. Thus the variant strain could have some sort of advantage over the parent strain in the presence of ampicillin.

Another possible way of explaining the increased resistance of the variant strain would be if the penicillinase had some essential metabolic function to the cell. The finding that the variant strain had about 50 times more enzyme might mean that this strain would have an advantage over the parent strain if the enzyme were also

a site for antibiotic action. Indeed Abraham & Chain (1940) stated that the number of bacteria found to contain an enzyme acting on penicillin points to the possibility that it may have a function in their metabolism.

Of course resistance to penicillin is not always due to penicillinase. Staphylococci trained to become resistant *in vitro* do not destroy penicillin G but become tolerant to it, whereas resistant strains isolated from clinical specimens possess penicillinase (Spink & Ferris, 1945). The strain of *Escherichia coli* used here was trained to ampicillin resistance *in vitro* and it is quite possible that the ampicillin-resistant variant was resistant by a mechanism similar to that seen in staphylococci made resistant to penicillin G *in vitro* (Knox & Smith, 1961*b*).

The behaviour of the enzyme found in the *Escherichia coli* strain T was different from that of penicillinases belonging to other species of micro-organisms which have been previously described.

(1) The penicillinase activity of strain T was increased by about tenfold by disrupting the cells or by toluene treatment, indicating the presence of a permeability barrier. Geronimus & Cohen (1958) found that the cell membrane of penicillinase-producing staphylococci did not present a barrier between penicillin G and the penicillinase, and Pollock (1961) obtained similar results with *Bacillus subtilis*. On the other hand, Novick (1962), using staphylococcal penicillinase, found that the extracellular enzyme had a higher affinity for penicillins G and V than the cell-bound enzyme, which could suggest that a permeability barrier may be present.

(2) When the organisms were broken in the press the penicillinase activity was not precipitated by centrifugation nor removed by filtration and therefore seems to be 'soluble'. Saz, Lowery & Jackson (1961) found that the intracellular penicillinase of staphylococci was particulate as it was removed by centrifuging at 144,000 *g* for 15 min. Sabath & Finland (1962) found that the penicillinase from disrupted staphylococci could be removed by filtration through a bacteria-proof sintered glass filter.

(3) When the enzyme of either strain O or T was exposed by toluene treatment it was easily precipitated by centrifuging down the cells, whereas the penicillinase activity of disrupted cells remained in the supernatant. Thus the penicillinase may be attached to the cell by a mechanism which is not associated with the permeability barrier, or toluene treatment may damage the permeability barrier sufficiently to allow access of penicillin G to the penicillinase but not sufficiently to allow the enzyme out.

(4) The specificity pattern of the *Escherichia coli* penicillinase was much narrower than that of *Staphylococcus aureus* or *Bacillus cereus* penicillinases. It is interesting that it destroyed penicillin V twelve times more rapidly than phenoxyethylpenicillin which differs from the former in that it has one more methyl group in the sidechain.

(5) The penicillinase of the *Escherichia coli* strain T was not inducible. Treatment of the organisms with 6-aminopenicillanic acid or phenoxybenzylpenicillin did not give rise to a real increase in total enzyme content but caused a larger proportion (3/10) of enzyme to be accessible to the substrate than the proportion (1/10) of unmasked enzyme in untreated cells. Whether this is due to damage of the permeability barrier allowing the substrate easier access to the enzyme or due to an increased active transport mechanism, such as permease, across the permeability barrier is unknown.

Grateful thanks are due to Beecham Research Laboratories for supplies of phenethicillin, α-aminobenzylpenicillin, 5-methyl-3-phenyl-4-isoxazolylpenicillin and 6-aminopenicillanic acid, to Pfizer Ltd for phenoxypropylpenicillin, to Distillers Company Ltd for phenoxybenzylpenicillin and to Miss B. Whittamore for valuable technical help. I am particularly grateful to Professor R. Knox for encouragement and constructive criticism, and to Dr J. Mandelstam for suggesting toluene treatment.

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Printed in Great Britain

Anaerobic Growth as a Factor Influencing Radiosensitivity

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(*Received 7 June* 1962)

SUMMARY

Damage to living organisms by X-rays (but not by ultraviolet radiation) is generally enhanced by the presence of oxygen, which probably acts at a physico-chemical level. Sensitivity to both u.v.- and X-radiation may also be related to the presence of oxygen acting as a metabolic factor during the growth of micro-organisms before or after irradiation.

Anaerobic growth of *Escherichia coli* strain B after u.v. or X-rays decreased lethal damage, the treatment being most effective after u.v.- and more effective after X-rays delivered in the absence than in the presence of oxygen. In this respect anaerobic growth corresponded with a variety of inhibitory conditions after irradiation, and rescue by anaerobic growth was about as effective as chloramphenicol treatment; but when these two treatments were used together, the effectiveness of each was decreased.

The effects of u.v.- and X-radiation on *Escherichia coli* B/r were unchanged by anaerobic growth after irradiation; but anaerobic growth beforehand resulted in survival curves which were much more sigmoid in shape than those pertaining to aerobically grown organisms. This tendency was noted with all strains tested. Anaerobic growth before X-irradiation did not alter the effect of oxygen during irradiation.

INTRODUCTION

Various lines of evidence support the view that oxygen, which enhances the effectiveness of ionizing radiation when present during irradiation, acts physico-chemically, rather than by affecting metabolism. With some strains of micro-organism, effects of radiation on colony-forming ability may be greatly modified by changing the conditions of growth, both before and after irradiation. Modification of this type is clearly related to effects on the biochemical processes which lead to cell division, and the presence or absence of oxygen during growth of the organisms may affect the end result. The mechanisms of this modifying action of oxygen are easily distinguishable from those involved in the part which oxygen plays by intervening in the 'metionic reaction', i.e. the chemical reaction immediately following an ionizing event (Alper, 1956).

It was reported by Alper & Gillies (1960*a*) that *Escherichia coli* strain B organisms when kept anaerobic after X- or u.v.- irradiation behaved like those to which other metabolic inhibitors were applied in that their colony-forming ability was much less affected than when they were grown in optimal conditions. Effects of anaerobic growth on this strain, and on its mutant *E. coli* B/r, have now been studied in greater detail.

METHODS

Organisms. The organisms used in most of the experiments were *Escherichia coli* strains B and B/r, as used in previous work in this laboratory. A few comparative experiments were done with *E. coli* strains K12(λ) and K12S, and with *Salmonella typhimurium* strain LT2.

Media. Oxoid blood-agar base was used as plating medium and for slopes. Oxoid nutrient broth no. 2 was used for broth cultures.

Preparation of organisms. Logarithmic-phase broth cultures were prepared by inoculating 0·2 ml. of an overnight broth culture into 5 ml. of fresh broth which was incubated for 90 min. at 37° with a moderate degree of aeration. A further dilution of this culture was made and aeration continued for 105 min. The organisms were then centrifuged down and washed thrice in 0·067 M-phosphate buffer (pH 7·0) and finally resuspended in this buffer.

The most convenient way of attaining maximally aerobic conditions was to grow the organisms on slopes for a short time. Anaerobically grown cultures were prepared by using slopes incubated in a McIntosh & Fildes anaerobic jar so that the conditions of growth were the same in all but this one respect. All aerobically grown slope cultures were harvested after growth for 4–4·5 hr.; anaerobic cultures were usually grown for the same time, but in a few experiments they were left long enough to enter the stationary phase. The organisms were harvested by washing off in phosphate buffer, and were then washed twice more.

For ultraviolet- (u.v.-) irradiations the organisms were spread very thinly on cellophan carriers supported on 1·5 % agar in distilled water, a technique described by Alper & Gillies (1958). Suspensions of organisms were exposed to X-rays or to 8 MeV. electrons in specially designed vessels through which air or oxygen-free nitrogen was continuously bubbled (Alper, 1955).

Sources of radiation. (1) Ultraviolet-irradiations were carried out by using a water-jacketed Hanovia 'Germicidal' lamp, 90 % of the energy being emitted at 2537 Å.; the dose rate was about 200 ergs/mm.^{2}/min. (2) For X-irradiations we used a Solus Schall 300 kV. constant potential X-ray unit, operating at 250 kV. and 10 mA., with no added filtration. The dose rate in the vessels was 1050 rads./min. as determined by ferrous dosimetry. (3) In certain experiments we used the 8 MeV. linear accelerator of the M.R.C. Radiotherapeutic Research Unit (Batchelor, Bewley, Morrison & Stevenson, 1959). The arrangements for irradiation were as described by Alper (1959).

Post-irradiation treatment. All nutrient agar plates were warmed to 37° before transferring to the surface either samples of suspensions of organisms, or cellophan carriers.

Anaerobic conditions were obtained by using a McIntosh & Fildes anaerobic jar, evacuating and refilling with pure hydrogen twice. The jar was maintained at 37° throughout. After overnight incubation air was admitted and the incubation continued until the colonies were large enough to count (about 6 hr.) Since we wished to establish comparative rates of growth on plates, under aerobic and anaerobic conditions, the technique described by Alper & Gillies (1960*a*) was used. Organisms were seeded on to strips of cellophan supported on the surface of nutrient agar, removed after suitable intervals and shaken in MacCartney bottles containing phosphate buffer. Viable counts were made on the suspensions so obtained.

Chloramphenicol treatment. In general we followed the methods described by Gillies & Alper (1959). Cellophan carriers on which the organisms had been irradiated were laid on the surface of nutrient agar chloramphenicol and left overnight in aerobic or anaerobic conditions. The carriers were then transferred to normal nutrient agar and incubated until the colonies were large enough to count.

In one experiment air was admitted to chloramphenicol agar plates which had been incubated anaerobically overnight and the organisms incubated aerobically for a few hours on chloramphenicol agar, in case the anaerobiosis had prevented the chloramphenicol from exerting its full effect. The final result was not affected.

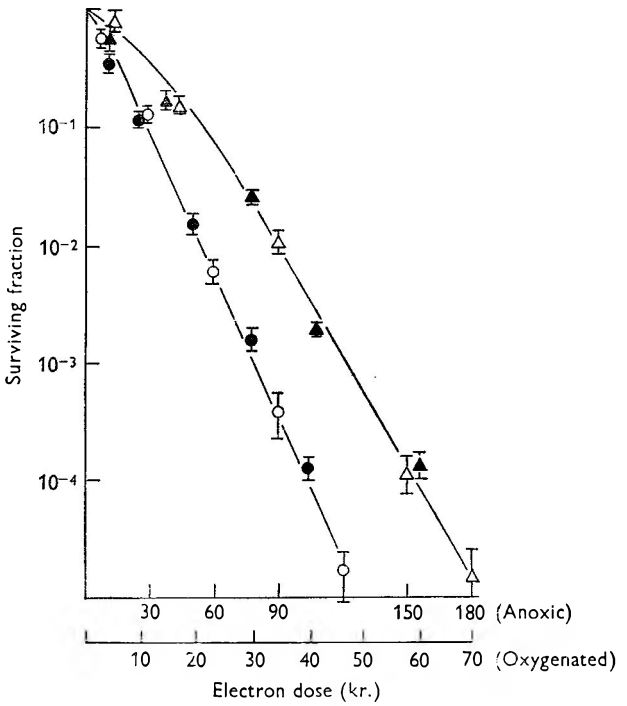

Fig. 1

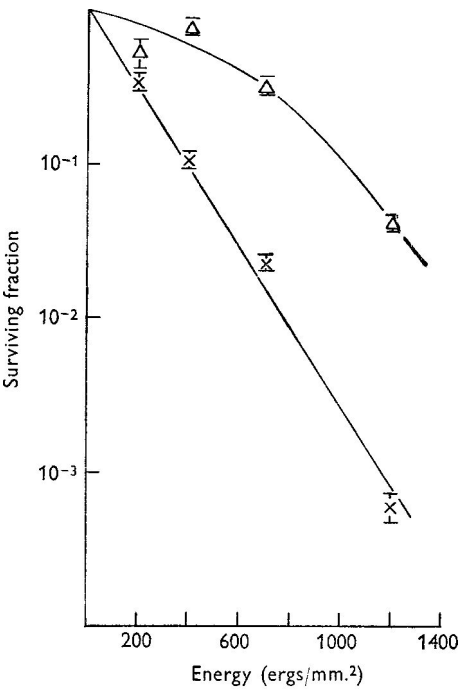

Fig. 2

Fig. 1. Survival of *Escherichia coli* strain B/r (harvested from a slope growing aerobically or anaerobically) after exposure in buffer solution to X-rays under oxygenation or anoxia. Subsequent incubation aerobic. ●, Grown aerobically, irradiated under oxygen; ○, grown aerobically, irradiated under anoxia; ▲, grown anaerobically, irradiated under oxygen; △, grown anaerobically, irradiated under anoxia. Dosage scales have been selected so that survival curves for irradiations with and without oxygen are superimposed. 95 % confidence limits on every point are indicated.

Fig. 2. Survival of *Escherichia coli* strain B/r (harvested from slopes grown aerobically or anaerobically) after exposure in buffer suspension to u.v.-radiation. Subsequent incubation aerobic. ×, Grown aerobically before irradiation; △, grown anaerobically before irradiation.

Chloramphenicol (5 μg./ml.) was used in the experiments reported by Gillies & Alper (1959); but in the present series we found a loss of viability in unirradiated organisms which had been left in contact with chloramphenicol (5 μg./ml.) over-

night. We cannot account for this increase in toxicity. Chloramphenicol at 1 μg./ml. had as much rescuing effect as was found previously with 5 μg./ml.; the lower concentration was used in the present experiments.

RESULTS

Effect of anaerobiosis before harvesting organisms for irradiation

Escherichia coli B/r. Young cultures of *E. coli* strain B/r grown on nutrient agar slopes yielded exponential or very nearly exponential survival curves, while those of the same age grown anaerobically yielded curves of equal slope, but with an initial

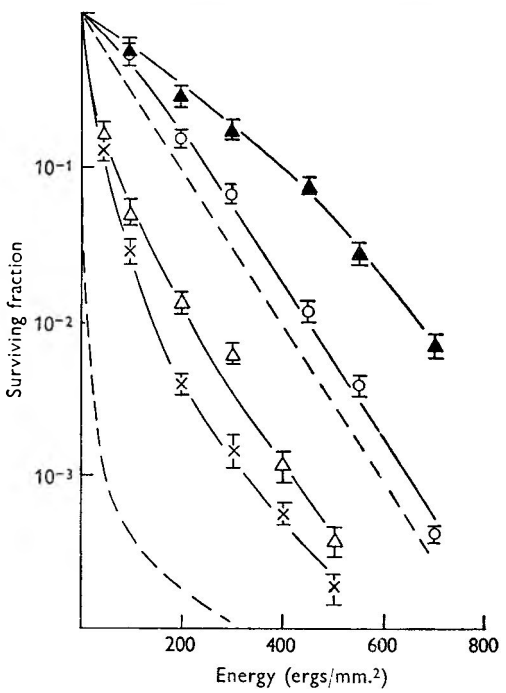

Fig. 3

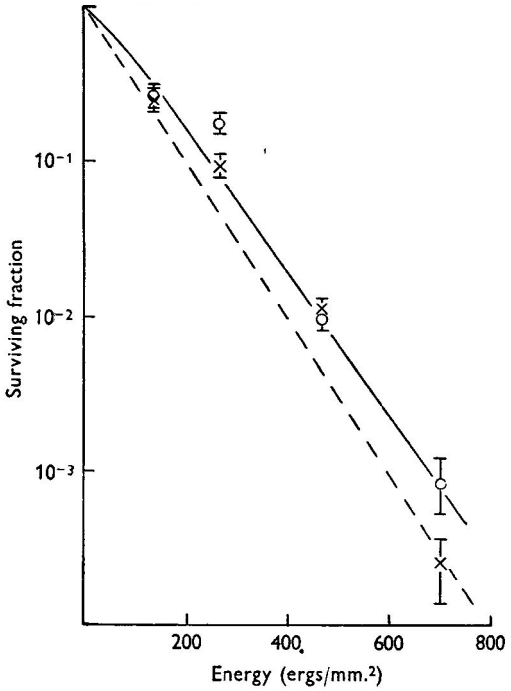

Fig. 4

Fig. 3. Survival of *Escherichia coli* strain B (harvested from slopes grown aerobically or anaerobically) after exposure on cellophan carriers to u.v.-radiation. ×, Grown aerobically, incubated aerobically; ○, grown aerobically, incubated anaerobically; △, grown anaerobically, incubated aerobically; ▲, grown anaerobically, incubated anaerobically. The dashed curves indicate the survival of broth-grown logarithmic phase organisms grown aerobically or anaerobically after u.v.-irradiation (see Fig. 5).

Fig. 4. Survival of *Escherichia coli* strain B/r (logarithmic phase, broth-grown) after exposure on cellophan carriers to u.v.-radiation. ×, Aerobic incubation; ○, anaerobic incubation. The dashed curve indicates the survival of *E. coli* B grown under similar conditions and incubated anaerobically after u.v.-irradiation.

shoulder. Survival curves pertaining to anaerobically grown organisms had extrapolation numbers (Alper, Gillies & Elkind, 1960) as high as 8 (Figs. 1, 2). The final slope of a survival curve with stationary-stage anaerobically grown organisms was only slightly less than that observed with logarithmic-phase organisms grown aerobically (see Fig. 1). Figure 1 shows that pairs of survival curves for the two

populations, each irradiated in the presence and absence of oxygen, could be superimposed by using the same dose-multiplying factor (2·6 in the conditions of the experiment illustrated). These results are in agreement with those reported by Hollaender, Stapleton & Martin (1951), Howard-Flanders & Alper (1957) and Sargent (1961). Figure 2 shows the difference in response to u.v.-irradiation of aerobically and anaerobically grown organisms of the same age.

Escherichia coli B. Organisms of this strain grown aerobically on nutrient agar slopes were not so sensitive to u.v.- or X-irradiation as were broth-grown organisms; those grown anaerobically were still less sensitive (Fig. 3).

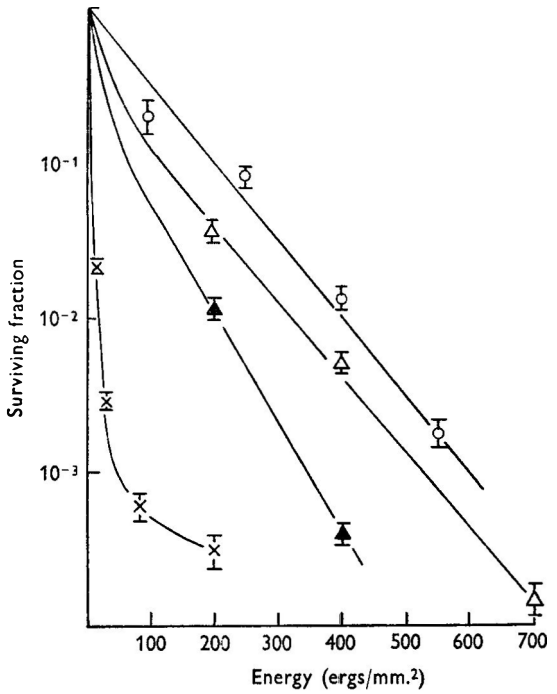

Fig. 5

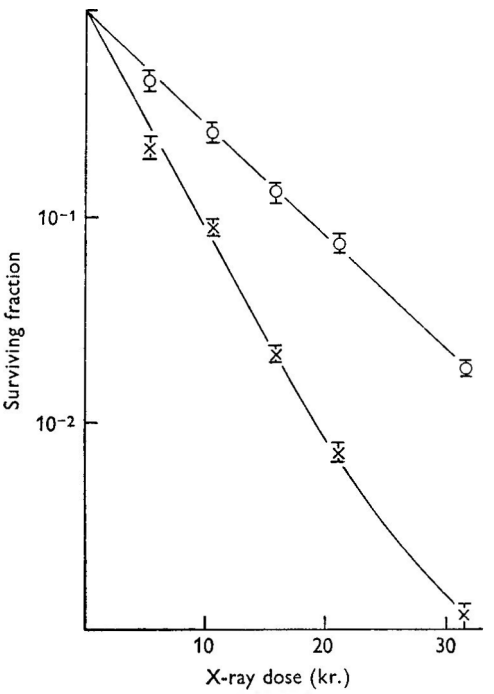

Fig. 6

Fig. 5. Survival of *Escherichia coli* B (logarithmic phase, broth-grown) after exposure on cellophan carriers to u.v.-radiation. ×, Aerobic incubation on Oxoid agar; ○, anaerobic incubation on Oxoid agar; △, aerobic incubation on Oxoid agar + 1 μg. chloramphenicol/ml. followed by incubation on nutrient agar; ▲, anaerobic incubation on Oxoid agar + 1 μg./chloramphenicol/ml., followed by incubation on nutrient agar.

Fig. 6. Survival of *Escherichia coli* strain B (logarithmic phase, broth-grown) after exposure in buffer suspension to X-rays under anoxia. ×, Aerobic incubation after X-irradiation; ○, anaerobic incubation after X-irradiation.

Salmonella typhimurium LT2. This strain showed the same characteristics as did *Escherichia coli* B/r: survival curves of anaerobically grown organisms showed an initial shoulder not seen with aerobically grown organisms.

Anaerobiosis after irradiation

Escherichia B/r. This organism showed no effect on the survival curve after u.v.- (Fig. 4) or X-irradiation.

Escherichia B. Anaerobiosis was more effective in rescuing the organisms after

u.v.-irradiation than after X-irradiation; irradiation was more effective when the organisms were X-irradiated in the absence of oxygen than in its presence (Figs. 3 and 5–7). Up to the time of the first cell division, increasing duration of anaerobiosis permitted more X- or u.v.-irradiated organisms to give colonies; conversely, increasing periods of aerobic growth before anaerobiosis was started decreased the final colony counts until the subsequent imposition of anaerobiosis no longer affected the number of colonies formed. Figure 8 shows how the colony-forming ability varied when the organisms exposed to 200 ergs/mm.^{2} of u.v.-radiation were transferred successively from an anaerobic to an aerobic environment and vice

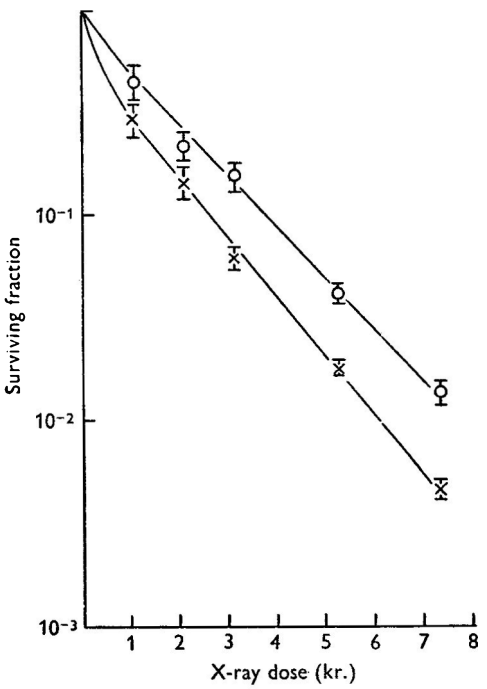

Fig. 7

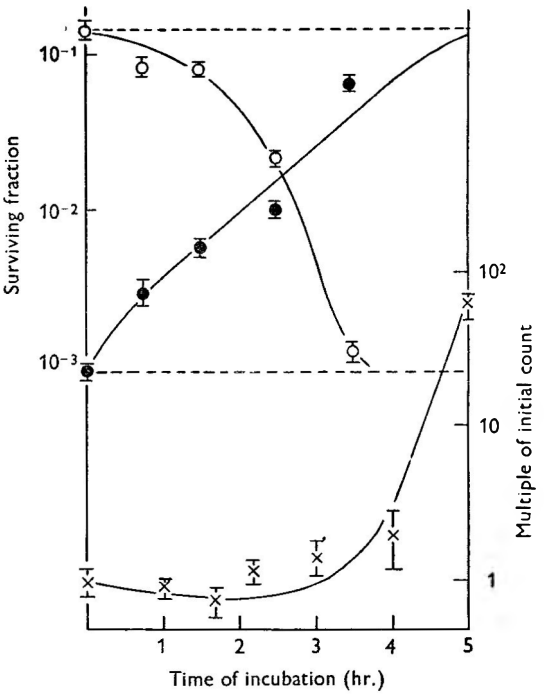

Fig. 8

Fig. 7. Survival of *Escherichia coli* strain B (logarithmic phase, broth-grown) after exposure in buffer suspension to X-rays under oxygen. ×, Aerobic incubation after X-irradiation; ○, anaerobic incubation after X-irradiation.

Fig. 8. Survival and growth of *Escherichia coli* strain B (logarithmic phase, broth-grown) after exposure on cellophan carriers to 200 ergs/mm.^{2} u.v.-radiation. ○, Transferred from aerobic to anaerobic incubation at the time indicated on abscissa; ●, transferred from anaerobic to aerobic incubation at the time indicated on abscissa; ×, viable counts, on u.v.-irradiated organisms growing aerobically, as multiple of initial count.

versa. The growth of the u.v.-irradiated organisms in aerobic conditions is shown. There was a similar dependence of survival on duration of aerobic or anaerobic growth after X-irradiation. The dose of X-rays required to give the same survival as 200 ergs/mm.^{2} u.v.-radiation imposed a shorter lag period, and the period within which change occurred was correspondingly shortened.

Other organisms. Anaerobiosis had no effect on *Escherichia coli* K 12(λ) or K 12S after u.v.-irradiation. *Salmonella typhimurium* was affected like *E. coli* B, but to a lesser extent.

Effect of anaerobiosis on growth of Escherichia strains B and B/r (unirradiated)

Escherichia coli B/r had the same lag phase and generation time whether grown aerobically or anaerobically. With *E. coli* B, however, the lag phase and the generation time were increased (Fig. 9).

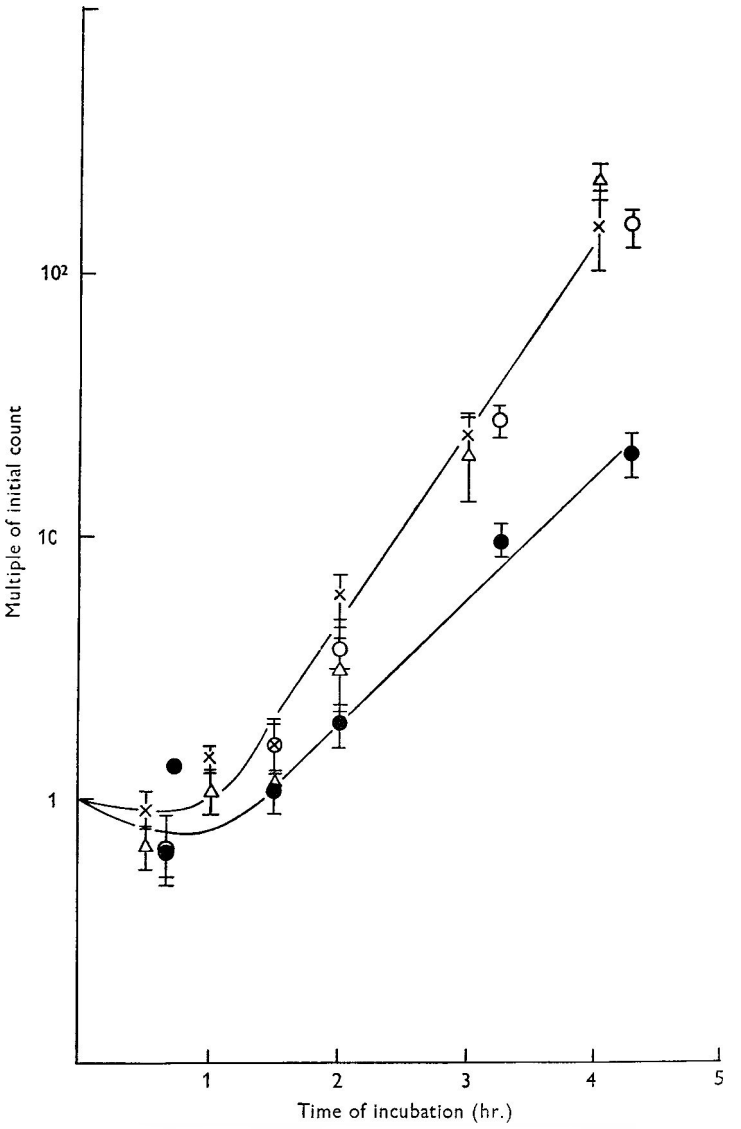

Fig. 9. Growth of non-irradiated *Escherichia coli* strains B and B/r (harvested in logarithmic phase from broth). ×, *E. coli* B growing aerobically; ●, *E. coli* B growing anaerobically; △, *E. coli* B/r growing aerobically; ○, *E. coli* B/r growing anaerobically.

Interaction of anaerobiosis with chloramphenicol treatment after irradiation

Anaerobiosis as a mechanism for rescuing irradiated *Escherichia coli* B had slightly more effect than had chloramphenicol treatment when the latter was applied immediately after plating. As reported by Gillies & Alper (1959), chloramphenicol treatment applied to u.v.- or X-irradiated organisms after short incubation on nutrient agar allowed more organisms to form colonies. When the counts were expressed as a dose-effect curve, the higher counts were seen to be due to the appearance of an initial shoulder on the survival curve. Chloramphenicol treatment in anaerobic conditions brought about much less rescue than anaerobic growth alone, or chloramphenicol treatment in aerobic conditions (Fig. 5).

DISCUSSION

Anaerobiosis as a rescuing treatment for u.v.-irradiated Escherichia coli *B*

It was suggested by Alper (1961, 1962) and by Gillies (1961) that the lethal effect of X- or u.v.-radiation on micro-organisms might occur as a result of damage in at least two sites, such that treatment after irradiation might act differentially on these. As observed with other treatments which rescue irradiated organisms of this strain of *Escherichia coli* there seems to be a minimum degree of damage which anaerobiosis is unable to affect; much the same dose-effect curves are seen when u.v.- or X-irradiated *E. coli* B is treated by keeping the organisms anaerobic, by chloramphenicol, by specific inhibition of protein synthesis (Gillies, 1961) or simply by holding the organisms out of contact with nutrients (Roberts & Aldous, 1949; Charles & Zimmermann, 1956; Gillies, 1961). It seems probable that all such treatments enable the organisms to by-pass damage to a particular site, but the mechanism may very well be different with different treatments. Certainly the kinetics of rescue by chloramphenicol or by puromycin (Dr N. E. Gillies, personal communication) differ from those observed with other treatments we have used. As shown in Fig. 8, preliminary aerobic incubation decreased the effectiveness of subsequent anaerobiosis: similarly, a preliminary period of normal protein synthesis decreased the effectiveness of subsequent specific inhibition thereof (Gillies, 1961). The reverse is true of treatment by chloramphenicol and puromycin, their overall effectiveness being greater after a preliminary period of vigorous normal metabolism. But when chloramphenicol or puromycin was applied immediately, and for short periods, to u.v.- or X-irradiated organisms, particularly when these were in the logarithmic stage of growth, there was a decrease in the final colony count, not an increase (Gillies & Alper, 1959; Dr N. E. Gillies, personal communication). Not only was there no additivity in the rescuing effect of chloramphenicol treatment and anaerobiosis, but these two treatments in fact counteracted each other (Fig. 5); this suggests that two different pathways of rescue from radiation damage may interfere with each other.

Anaerobiosis before u.v.- or X-irradiation. In all three strains tested, anaerobic growth decreased the effect of radiation on colony-forming ability. Anaerobic growth conferred on *Escherichia coli* B/r and on *Salmonella typhimurium* the ability to absorb a dose of radiation before the killing became exponential with dose, while with *E. coli* B the slope of the survival curve was decreased. When *E. coli* B was

grown anaerobically after irradiation, as well as before, the survival curve showed the same characteristics as did that pertaining to anaerobically grown *E. coli* strain B/r (Fig. 3). It was inferred by Alper & Gillies (1960*a*, *b*) that the two strains of *E. coli* as normally grown (e.g. in nutrient broth) do not differ in the targets which they present to radiation, but rather in the effect which damage to these targets has on the biochemical pathways which lead to cell division. Like other treatments, anaerobiosis after u.v.-irradiation has the effect on *E. coli* B of making its response to u.v.-radiation similar to that of its resistant mutant B/r, the growth curve of which is exactly the same in aerobic and in anaerobic conditions. It is noteworthy that anaerobic conditions, without affecting generation time, affect *E. coli* B/r so profoundly that the organisms acquire the ability to absorb a 'quasi-threshold dose' (Alper *et al.* 1962) which is about twice that required to produce an average of one lethal event per organism, once the curve has become exponential.

We are grateful to Dr N. E. Gillies for permission to quote some of his unpublished results.

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Printed in Great Britain

Induction of Forward Mutants in the *pyr*-3 Region of Neurospora

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(*Received* 7 *June* 1962)

SUMMARY

Forward mutations at the *pyr* locus in Neurospora, and back mutations at *arg*, were scored by using the 'suppressor method'. This method is efficient and well suited for mutagen screening. Nitrous acid, ethylmethane sulphonate and ultraviolet radiation were effective as mutagens; 8-ethoxycaffeine yielded inconclusive results. The same pattern of forward mutations at *pyr*, but different frequencies of back mutations at the *arg* locus, were obtained with the three agents. In this Neurospora system, nitrous acid appeared to induce mutations which affected simultaneously both DNA strands.

INTRODUCTION

To facilitate studies in mutagenesis it is necessary to have screening methods, both simple and thorough, for the selection of forward and back mutants at specific loci (Westergaard, 1960). A particularly promising forward selection method is available for the *pyr*-3 (*pyr*) locus of Neurospora. This method was described in the first paper of this series (Reissig, 1960), and will be henceforth called the suppressor method. It can be outlined as follows: uninucleate conidia (microconidia) from an arginine-dependent strain of Neurospora are plated on medium supplemented with pyrimidine, but lacking arginine. Arginine-dependence in this strain is determined by the *arg* gene. The arginine-independent colonies which grow on the screening plates result from any of the following events: (i) back mutation at *arg*; (ii) forward mutation from pyr^+ to *pyr*, having the pleiotropic effect of suppressing the arginine requirement while creating a requirement for pyrimidines (mosaic pyr^+/pyr colonies also grow, because the suppressor effect of *pyr* is dominant); (iii) forward mutation from pyr^+ to $pyr^{su\text{-}arg}$ alleles which suppress *arg*, but do not create a pyrimidine requirement. The main purpose of the present study was to test the usefulness of the suppressor method for: (*a*) screening mutagens; (*b*) analysing patterns of mutagen specificity; (*c*) determining how many of the chains in the DNA duplex are altered per mutagenic hit. It was of particular interest to test (*c*) for nitrous acid, since Tessman (1959) and Vielmetter & Wieder (1959) showed that nitrous acid produced single-chain mutagenic hits in bacteriophage. Contrary to those results, the data to be presented here suggest that nitrous acid produces mutations in Neurospora by simultaneously altering both chains in the DNA molecule.

The following abbreviations are used: *pyr* for *pyr*-3; *arg* for *arg*-2.

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METHODS

Neurospora strains. Strain ED416–1a carries the *arg* (Srb & Horowitz, 1944), *cot* (Mitchell & Mitchell, 1954), *pe*^{m} and *fl* (Barratt & Garnjobst, 1949) markers. In consequence it is arginine-dependent, colonial at temperatures above 31°, and microconidial. Microscopic observation by using Giemsa staining showed that 98 % of the microconidia were uninucleate and 2 % binucleate.

A tester strain carried the following markers: *co* (Mitchell & Mitchell, 1954), *arg* and *pyr* (Reissig, 1959).

Media. The medium of Westergaard & Mitchell (1947) with lower sugar content (0·2–1 %) and supplemented as required was used for crosses. The solid medium of Fries (Beadle & Tatum, 1945), supplemented as required, was used in all other cases. Hydrolysed ribonucleic acid (RNA; 0·5 g./l.) was used as pyrimidine source; L-arginine HCl was added to 0·1 g./l.

Screening. Microconidia were harvested from cultures of ED416–1a grown at 20° for a fortnight on arginine-supplemented medium. This low temperature of incubation was required to obtain good microconidial viability (F. J. de Serres, personal communication; Reissig, unpublished observations). In the experiments to be reported, the viability of untreated microconidia ranged from 18 to 61 %, average 31 %.

The microconidial suspension was filtered through sintered glass (average pore diam. 50 μ) and washed twice with water by centrifugation. Suspensions containing 10^8 microconidia/ml. were incubated at 25° with agitation under the conditions indicated below.

For treatment with ultraviolet (u.v.) radiation, an aqueous suspension of microconidia was exposed to a Hanovia Germicidal Unit for various times. The u.v. dose in ergs was determined by using the dosimeter of Latarjet. For treatment with ethylmethane sulphonate, the microconidia were incubated for 11 hr. in the pH 9 buffer of Teorell & Stenhagen (1938) + ethylmethane sulphonate. This procedure was developed by H. Malling (personal communication). For treatment with nitrous acid, the microconidia were incubated for 35 min in 0·05 M-potassium tartrate + sodium nitrite. The nitrite was added at time zero to the incubation mixture as a freshly made, sterile filtered, aqueous solution. For treatment with *p*-benzoquinone, the microconidia were incubated for 2 hr. in half strength pH 6·5 Teorell & Stenhagen buffer + *p*-benzoquinone. Treatment with 8-ethoxycaffeine required incubations for 5 days in liquid minimal medium (i.e. minimal medium minus agar) + 8-ethoxycaffeine. In every experiment an untreated series was incubated in a similar fashion, but without the mutagen.

The screening plates contained minimal medium supplemented as follows: bottom layer (12 ml.) with hydrolysed RNA; middle layer (3 ml.) like the bottom layer, but containing the microconidial suspension; top layer (12 ml.) with hydrolysed RNA + L-canavanine sulphate (0·15 g./l.) + L-lysine HCl (0·05 g./l.). In some experiments, an aqueous microconidial suspension was substituted for the middle layer. The top layer was required to inhibit residual growth, and was added 18–25 hr. after plating the microconidia. The plates were incubated at 32°, but cooled to 25° for some hours on the day before scoring. Scoring was done on the 8th day, unless otherwise specified. Viability was reckoned from platings on minimal medium + arginine.

Low plating densities and premature addition of the top layer resulted in low recoveries of mutants (Reissig, 1960; and unpublished). Since some mutagenic treatments alter the value of those parameters, each experiment was run in six series, plating microconidia at densities of 2×10^7 and 10^8/plate, and adding the top layer 18, 21 and 25 hr. after plating. Only the results of the highest-yielding series are reported. Analysis of the complete results, and reconstruction experiments, indicated that recovery was essentially quantitative for treatments with low doses of mutagens, but was occasionally incomplete at higher doses.

Other procedures. For further study, mutants were picked on to slopes supplemented with arginine + pyrimidines. Filtered microconidial suspensions from each of those cultures, at concentrations of 10^3–10^4 viable microconidia/plate were plated on three different media: arginine supplemented, pyrimidine supplemented, and unsupplemented. These plates were used for purification of the strains, preliminary classification of nutritional requirements, and classification of the clones as pure or mosaic. Further experimental details are presented in an earlier publication (Reissig, 1960).

RESULTS

Dose effects of mutagens

Figure 1*a* shows that u.v. radiation, ethylmethane sulphonate and nitrous acid were effective mutagens. The curves presented relate mutant frequency among survivors to the log of the surviving fraction. They are roughly equivalent to dose dependence curves, since the log of the surviving fraction is approximately a linear function of dose (Fig. 2).

Each microconidium receives on the average one lethal hit when the survival is 37 % (Lea, 1947). The probability of a scorable mutation at this survival value can be read off Fig. 1*a*. It is 2×10^{-5} with radiation or ethylmethane sulphonate, and 10^{-5} with nitrous acid. Therefore, the ratios of the probability of a lethal hit to the probability of a scorable mutation are $1 : 2 \times 10^{-5}$ or $1 : 10^{-5}$, according to the mutagen used. Such ratios can be taken to represent the relative size and perhaps sensitivity, of the targets involved in killing (the whole genome) and in mutation (genes *pyr* and *arg*). Yet this can be at best an approximation since the mutation frequency curves for uv radiation and ethylmethane sulphonate are non-linear. Treatment with *p*-benzoquinone kills microconidia, but induces no mutations. The data are included in Fig. 1 to show that mere killing will not simulate the induction of mutants (Grigg, 1952; Kølmark & Westergaard, 1952). Figure 1*b* is similar to Fig. 1*a*, except that mutant frequency was calculated per treated microconidium instead of per surviving microconidium. Net increases in the number of mutants were observed, thus ruling out selection of pre-existing mutants as a significant factor in the results presented. In other experiments, treatment with 8-ethoxycaffeine increased mutant frequency among survivors, without effecting a net increase over the spontaneous value. Survivals were (%) 39, 17, 10, 7, 3 and 2 under the conditions used, which involved incubation for 3–5 days with concentrations of 8-ethoxycaffeine up to saturation, at temperatures ranging from 12 to 32°. Controls were incubated under the same conditions, but without 8-ethoxycaffeine.

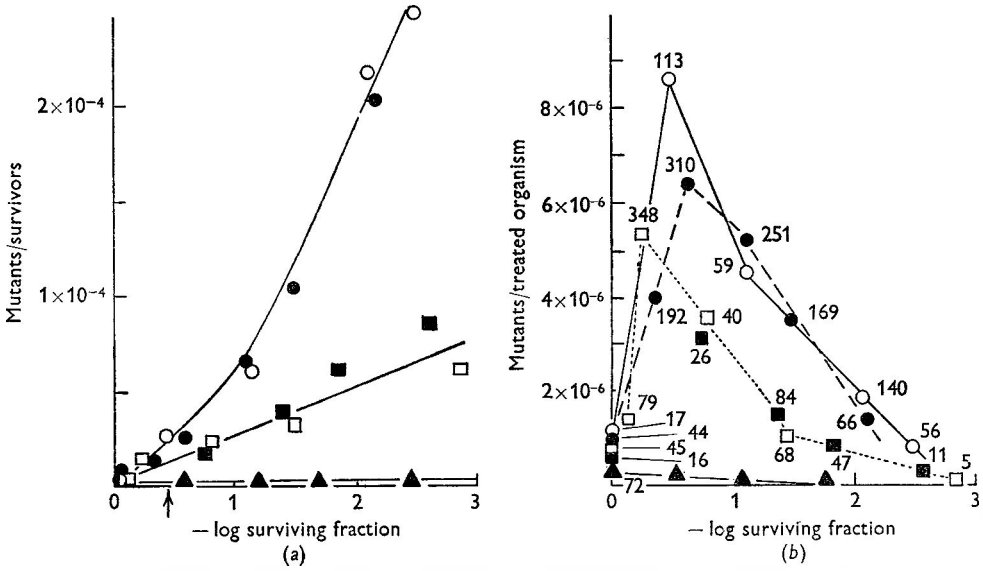

Fig. 1. Relation between mutant frequency and surviving fraction after several treatments. (*a*) Neurospora mutants calculated/survivor. The arrow indicates the 37 % survival level. (*b*) Mutants calculated/microeonidium viable before treatment. Treatments: ●, u.v. radiation; ○, ethylmethane sulphonate; ■, nitrous acid, pH 3; □, nitrous acid, pH 4; ▲, *p*-benzoquinone. Actual colony counts are indicated by the numbers in the body of (*b*).

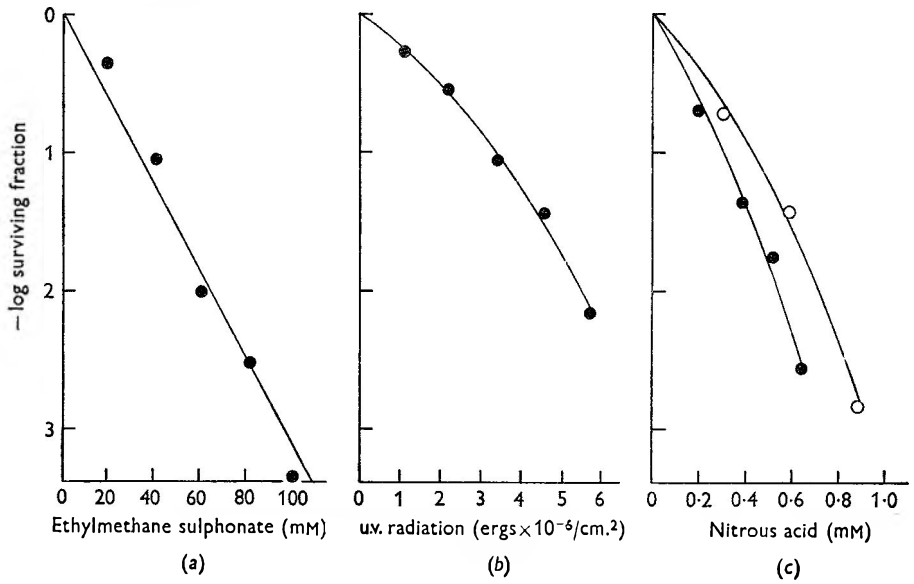

Fig. 2. Survival of Neurospora microconidia as a function of dose. Treatments: (*a*) ethylmethane sulphonate; (*b*) u.v. radiation; (*c*) nitrous acid. In (*c*) two pH values were used: ●, pH 3; ○, pH 4; and the plotted molarity is that of the undissociated acid, calculated from the dissociation constant of nitrous acid (= $5{\cdot}8 \times 10^{-4}$; Sneed & Brasted, 1956), the actual pH value, and the amount of added nitrate. Data from the same experiments as in Fig. 1.

Mutagen specificity

The suppressor method yields a variety of mutant types: pyrimidine-independent types, including back mutations at *arg* and mutations to $pyr^{su\text{-}arg}$; and pyrimidine-dependent types, comprising five different complementation classes and whole range of degrees of dependence (Reissig, 1963). The question arose whether different mutagens give the same spectrum of mutant types. This is so within the pyrimidine-dependent group, where the three mutagens tested yielded the same distribution of complementing types (Table 1). Furthermore, comparison of the frequency of types exhibiting residual growth among mutants induced by u.v. radiation, ethyl methane sulphonate or nitrous acid, did not reveal any mutagen-specific differences.

Table 1. *Mutations in Neurospora representing the different complementation groups, classified according to origin*

Methods as described in Reissig (1962)

	No. mutants in complementation groups				
Origin	Alpha	Beta	Gamma	Delta	Non-complementing
Spontaneous*	4	1	0	0	0
U.v. radiation†	24	9	0	1	4
Ethylmethane sulphonate	30	4	0	0	1
Nitrous acid	97	24	3	0	2
Total	155	38	3	1	7

* Independent events.
† Including data reported previously (Reissig, 1959).

Table 2. *Dependent and independent mutants of Neurospora classified according to origin*

Only mutants scored on the 6th day are tabulated.

	No. of mutants		
Origin	Pyrimidine-independent	Pyrimidine-dependent	Pyrimidine-independent (%)
U.v. radiation	89	32	74
Ethylmethane sulphonate	41	24	63
Nitrous acid	100	53	65

P that the difference between u.v. mutants and the others is due to chance alone = 0·1.

On the other hand, relating pyrimidine-dependent to independent mutants, u.v. radiation seems to produce more of the latter than either ethylmethane sulphonate or nitrous acid (Table 2). A closer examination of this difference demands classification of the mutants into the two expected genotypes: arg^+pyr^+ and $arg\ pry^{su\text{-}arg}$, by recombination tests (Reissig, 1960). Briefly, the unknown strain is crossed to a *co arg pyr* tester, and the ensuing ascospores are plated on arginine medium. The few colonial recombinants obtained are then tested for arginine dependence. If the unknown parent was $arg^+\ pyr^+$, most *co* pyr^+ recombinants (35 out of 47 in the

experiment reported below) will be arginine dependent (*co arg pyr*$^+$) because the *arg-pyr* distance is longer than *co-arg*. On the other hand, if the unknown was *arg* $pyr^{su\text{-}arg}$ all pyrimidine-independent segregants will carry $pyr^{su\text{-}arg}$ and be thus arginine-independent. The results of such tests are reported in Table 3.

The probability of misclassification of back mutants as a result of recovering only cross-overs in the *co-arg* region is slight. Adequate numbers of colonial recombinants (ranging in each cross from 3 to 21, with an average of 8) were tested for 77 of the 89 mutants classified as $pyr^{su\text{-}arg}$.

It is clear the u.v. radiation induced back mutations at *arg*, relative to $pyr^{su\text{-}arg}$ events, more frequently than ethylmethane sulphonate or nitrous acid. On the basis of this result, the larger yield of prototrophs after u.v. irradiation (Table 2) can also be understood.

Table 3. *Pyrimidine-independent mutants of Neurospora tested genotypically, classified according to origin*

Origin	No. mutants	
	arg$^+$ *pyr*$^+$	*arg* $pyr^{su\text{-}arg}$
Spontaneous*	0	3
U.v. radiation†	13	6
Ethylmethane sulphonate	0	39
Nitrous acid	5	43

* Independent events.

† Including 10 *arg*$^+$ *pyr*$^+$ and 2 *arg* $pyr^{sua\text{-}rg}$ from a previous study (Reissig, 1960).

Mechanism of action of nitrous acid

According to current ideas, nitrous acid produces mutations by chemical modification of only one of the two chains of the DNA duplex. In the present system such one-chain hits should yield mosaic clones (mutational mosaics) containing *arg pyr* and *arg pyr*$^+$ nuclei derived respectively from the mutated and the unmutated strand. The expectation that these heterokaryons would be detectable by the usual screening method is based on the following observations: (*a*) the suppressor effect of *pyr* is dominant over *pyr*$^+$ (Reissig, 1958); (*b*) mutations decreasing *pyr*$^+$ activity to one half are readily scored by the present method (Reissig, 1963); (*c*) *arg pyr*/*arg pyr*$^+$ mosaics originated by mutation of one nucleus in multinucleated macroconidia were in fact recovered (Reissig, 1960); (*d*) when plating microconidia at high concentration, a sizable proportion of the mutant colonies analysed were *arg pyr*/*arg pyr*$^+$ mosaics, probably as a result of contamination with the background microconidia.

The frequency of mosaics after nitrous acid treatment was determined. As a control to estimate the frequency of mosaics originating by contamination (contamination mosaics), microconidia from a *pyr* strain of an unusual complementation type were mixed at a concentration comparable to the level of induced mutants expected. Colonies involving the unusual *pyr* types were classified as control series, since almost all mosaics among them must be contamination mosaics. Colonies of other complementation types make up the test series, and mosaics in this series could be originated by mutation or by contamination. The occurrence of muta-

tional mosaics might have been inferred if the frequency of mosaics in the test series had been significantly larger than in the control. This was not the case (Table 4).

Before disallowing the theory that nitrous acid produces mutations in this system by means of single-chain hits on the DNA duplex, we must consider the possibility that the absence of mutational mosaics is due to concurrent induction of single-chain recessive lethals which made the DNA molecule effectively single-stranded for information transfer. Now, if killing were due to single-chain recessive lethals the killing curve ought to have been typical multi-hit, with a wide shoulder and extrapolating at zero dose to 2^m (on the usual log scale), where m is the total number of essential genes (Atwood & Norman, 1949). This is not compatible with Fig. 2*c*. Another model postulates that each chain is essentially a single target for lethal hits which destroy the capacity of the chain to replicate. A 2-hit killing curve is expected, and this might be consistent with the data depicted in Fig. 2*c*. However, such a mechanism could not have prevented the detection of mosaics in the experiment reported in Table 4. There, nitrous acid killed 36 % of the organisms, and the probability of a lethal hit/strand would be the square root of this, or 60 %. Forty % of the strands would have survived, and thus 40 % of the mosaics would have been recovered. On the other hand, if killing by nitrous acid were a more complex phenomenon, combining features of the two simple models described above and possibly involving also dominant lethals and double-chain hits, then it would become impossible to decide what proportion of mosaics can be recovered.

Table 4. *Frequency of* pyr/pyr$^+$ *mosaics, among mutants of Neurospora induced by nitrous acid (test series) or pre-existing (control series)*

A 10^6:1 mixture of *arg* and *pyr* (type beta, much residual growth) Neurospora microconidia, treated with nitrous acid at pH 4 and plated at a density of 10^7 microconidia/plate in a solid middle layer. Survival: 64 %. Frequency of induced mutants: 8×10^{-6}/survivor. Colonies carrying the added *pyr* type are tabulated in the control series. Clones without > 10 % *pyr*$^+$ nuclei are classed as mosaics.

	No. tested mutant clones	
Series	Not mosaic	Mosaic
Test	99	4
Control	73	6

DISCUSSION

The suppressor method appears very suitable for mutagen screening, being efficient and simple. Its sensitivity is only limited by the usual value of spontaneous mutants (of the order of 10^{-7}/live microconidium), and by recovery difficulties encountered when using highly toxic treatments (survival below 0·1 %). Contrary to the clear-cut results obtained with u.v. radiation, ethylmethane sulphonate and nitrous acid it was not possible to decide whether or not 8-ethoxycaffeine was mutagenic. If 8-ethoxycaffeine induces mutations in the present Neurospora system, they are not frequent enough in relation to lethal events to produce a net increase in the number of mutants. Under such conditions, reconstruction experiments would be required to rule out selection as the cause for the increase in mutant

frequency. Selective effects were particularly suspect in the present experiments because very long incubation times in minimal medium were demanded by the low solubility of 8-ethoxycaffeine. On the whole, the results obtained with this system are not unlike those reported for Ophiostoma by Zetterberg (1960), who claimed a slight mutagenic effect for 8-ethoxycaffeine on the basis of the results of reconstruction experiments.

The curves of the present work which relate mutant frequency/survivor to lethal hits ($-\log$ of surviving fraction) after treatment with u.v. radiation or ethylmethane sulphonate, depart from the linearity which simple theory predicts. They are, however, typical of the dose curves obtained for u.v.-induced prototrophy in Neurospora (Giles, 1951) and bacteria. A plausible explanation for this effect was furnished by Witkin (1959) who worked with a bacterial system.

The suppressor method, being essentially a forward-mutation system, is probably more reliable than the usual back-mutation method for the screening of potential mutagens. Forward mutation is the summation of events at a considerable number of mutons and the specificities of their reaction average out. Back mutation, on the other hand, involves a restricted number of mutons being altered in specific ways; therefore different alleles respond specifically to different mutagens. This question was reviewed by Westergaard (1960). The contrasting behaviour of forward and back mutation is again brought out by the data here presented. The relative frequencies of *pyr-α*, *pyr-β*, $pyr^{su\text{-}arg}$ mutations and lethal events were the same whether uv radiation or ethylmethane sulphonate were used as mutagens; yet u.v. radiation is far more effective than ethylmethane sulphonate as an inducer of back mutations at *arg* relative to the above-mentioned events. The obvious interpretation is that back mutation of the *arg* allele is mutagen specific, while forward mutation at *pyr* is not.

The results obtained with nitrous acid strongly suggest that this compound does not induce mosaics in Neurospora. The possibility that mosaics occur, but are not detectable, was examined; no evidence in its favour was found. Further proof would need to come from reconstruction experiments with microconidia known to be mosaic for both DNA chains, but this material is not available. Thus, on the basis of the available evidence, the absence of mosaics contradicts the hypothesis that nitrous acid produces mutations by deaminations which affect only one DNA chain/hit. Geiduschek (1961) showed that *in vitro* treatment of DNA by nitrous acid produced cross-linking between both chains of the molecule. Should such events lead to mutation, they may be expected to yield pure mutant clones. Tessman's (1959) results with phage, which confirmed the single-chain hit hypothesis, might in fact be more consistent with the hypothesis that nitrous acid produces both single-chain hits (deamination) and double-chain hits (cross-linking). About one-third of the clones obtained by Tessman after nitrous acid treatment were pure, and two-thirds were mosaic. Pure clones did not result from concomitant inactivation of the other DNA strand, because their proportion did not decrease with increasing doses of nitrous acid. C. M. Wieder (personal communication) has found comparable results with coliphage T_2. Therefore, either the DNA of one-third of the particles is effectively single stranded for information transfer at the locus studied (for which there is no evidence), or both single- and double-strand mutagenic hits occur when treating phage with nitrous acid.

This work was performed during the tenure of a fellowship from the Rask Ørsted Foundation and was supported by grants from the Carlsberg Foundation and the Rockefeller Foundation. I am most grateful to Professor M. Westergaard for his generous hospitality, encouragement, and for many discussions; to Mrs Joan Kjemtrup for her able technical assistance; and to Mr Arne Holm (Chemical Laboratory, University of Copenhagen) for the preparation of canavanine sulphate.

This is paper II in the series on forward and back mutation in the *pyr*-3 region of Neurospora.

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J. gen. Microbiol. (1963), **30**, 327–337
Printed in Great Britain

Spectrum of Forward Mutants in the *pyr*-3 Region of Neurospora

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(*Received* 7 *June* 1962)

SUMMARY

About 200 forward mutants of Neurospora obtained as suppressors of *arg* were studied. All fell in the *pyr* region, and all appeared deficient in aspartic transcarbamylase (ATC) to various degrees: extreme deficiencies in ATC (*pyr-N* alleles) resulted in pyrimidine-dependence as well as suppression of *arg*, while partial ATC deficiencies ($pyr^{su\text{-}arg}$ alleles) led only to suppression. Five different complementation groups were represented among the *pyr-N* alleles studied. Evidence is presented which suggests that *pyr-N* and $pyr^{su\text{-}arg}$ alleles affect ATC via different mechanisms.

INTRODUCTION

The genetics and the complementation pattern of mutants at the *pyr*-3 (*pyr*) region of Neurospora was studied by Mitchell & Mitchell (1956), by Suyama, Munkres & Woodward (1959) and by Woodward (1962). Davis (1960), investigating the enzymic defects in the same mutants, showed that the *pyr* region is functionally heterogeneous: it comprises mutants which lack aspartic transcarbamylase (ATC) activity, as well as mutants with normal values of the same activity and presumably deficient in a previous step (Davis, 1961, 1962). The former or ATC-less mutants will be designated *pyr*-3*N* (*pyr-N*), and the latter *pyr*-3*M* (*pyr-M*). The results just outlined were obtained with mutants isolated by the usual methods for the selection of auxotrophs. A special screening method, which will be referred to as the suppressor method, is also available for the isolation of *pyr* mutants (Reissig, 1963). This method takes advantage of the fact that *pyr* mutants (*pyr-N* mutants, as will be seen) suppress the arginine requirement of a strain carrying the *arg*-2 (*arg*) mutant gene. Even partial decreases in the activity of the normal pyr^+ allele result in suppression of *arg* (Reissig, 1960). Consequently the suppressor method screens for a whole spectrum of mutant alleles, ranging from the subthreshold $pyr^{su\text{-}arg}$ (no pyrimidine requirement) to *pyr* alleles allowing no trace of growth in the absence of pyrimidines. With two basically different methods available for the selection of mutants at *pyr*, it seemed profitable to attempt a more thorough assessment of the genetic information contained in this region.

The following abbreviations are used: *arg* for *arg*-2; *pyr* for *pyr*-3; ATC for aspartic transcarbamylase; Rg_{arg} for growth in the absence of arginine relative to growth in its presence; Rg_{ud} for growth in the absence of uridine relative to growth in its presence.

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METHODS

General methods were described in the preceding paper (Reissig, 1962). Additional details are given in this section.

Strains. Several *pyr* mutants obtained by auxotroph selection following treatment with u.v.-radiation, were kindly provided by K. D. Munkres (strains prefixed KS; Suyama *et al.* 1959) and by M. B. Mitchell; representing types N and M (Davis, 1960; and personal communication): KS36a, KS36A, 70007–45502–32723a (*pyr-3d co*) and 45502–1507–1A (*pyr-3d T*) all type N; and KS138a, KS138A and ED354–1a (a re-isolate of 37301) all type M. The St Lawrence wild types 74A and 73a were also used.

Measurement of growth responses. Growth was measured as in the usual tube method (Ryan, Beadle & Tatum, 1943); but by using 15 × 150 mm. test tubes with a notch near the open end, laid horizontally so that the notch retained the medium (5 ml.). Each strain was tested on four different media: unsupplemented, supplemented with arginine (0·1 g./l.), supplemented with uridine (0·05 g./l.), and supplemented with arginine + uridine.

Strain ED416–1a, the source of all mutants screened, is arginine-dependent, but can grow slowly on arginine-free medium. This slow growth is characterized by a long lag phase (5–6 days instead of the usual 1 or 2) followed by growth at a constant and normal rate. Therefore, linear growth rate is useless as a measure of growth response to arginine: a meaningful growth index must take into account the duration of the lag phase. The growth index used was defined as the reciprocal of the time taken by the mycelial front to travel 11 cm. from the point of inoculation. Measurements were discontinued on the 14th day, and extrapolated whenever necessary.

The growth of each mutant in the absence of arginine, relative to its growth in the presence of arginine, was called Rg_{arg} and calculated from equation (1):

$$Rg_{arg} = \frac{\text{index on minimal} + \text{index on uridine}}{\text{index on arginine} + \text{index on arginine} + \text{uridine}}. \quad (1)$$

Similarly, relative growth without uridine (Rg_{ud}) is given by equation (2):

$$Rg_{ud} = \frac{\text{index on minimal} + \text{index on arginine}}{\text{index on uridine} + \text{index on arginine} + \text{uridine}}. \quad (2)$$

The measurement of response to uridine does not present complications of the type discussed in connexion with arginine response: uridine-stimulated mutants respond to this metabolite by shortening the lag phase and increasing the linear growth rate. However, for the sake of uniformity, the growth index was defined throughout as previously indicated. It should be noted that substitution of rates of linear growth for the growth indexes in equation (2), leaves Rg_{ud} values practically unchanged. Therefore, the choice of growth index is not imposing any arbitrary distortion upon the description of the experimental data. A difference in Rg_{arg} or in Rg_{ud} larger than 0·2 is probably significant. However, statistical controls were not included in the experiments to be reported: therefore only the major features of these results can be discussed.

Complementation tests. Unless otherwise specified, mutants were tested in pair-

wise combinations for their ability to complement (i.e. to form a prototrophic heterokaryon) on plates of minimal medium + arginine in the manner described by de Serres (1956). Unsupplemented plates yielded similar results. Two weeks were allowed whenever possible before classing a test as negative, but positive reactions were in evidence between the 2nd and 4th day except when unrelated strains were used. Complementation tests between *arg* and *pyr* (or *arg pyr*) strains were made on plates supplemented with orotic acid (1 g./l.). This supplement inhibits the residual growth of *arg* and stimulates only slightly *pyr* strains.

RESULTS

The frequency distribution of Rg_{arg} values among 190 mutants obtained with the suppressor method, is shown in Fig. 1. Clearly, partially arginine-dependent mutants (Rg_{arg} in the 0·4–0·8 range) were also obtained. This is so with the usual scoring schedule; but by picking 2 days earlier, only fully arginine-independent mutants are recovered.

The distribution of Rg_{nd} values among the same 190 mutants is strikingly bimodal (Fig. 2). Before attempting to interpret these results we must ascertain the genetic basis of the mutations examined. This is particularly important because it is known from previous work that the screening method used yields back-mutants at *arg* as well as mutants at *pyr*. The genotype of the prototrophs was ascertained by recombination analysis, as reported in the preceding paper of this series (Reissig, 1962); the results are indicated in Fig. 2: $pyr^{su\text{-}arg}$ strains by vertical hatching; and back mutants by dotted areas. Pyrimidine-dependent mutants were tested for allelism by complementation criteria (see section on allelism), and all found to be allelic as indicated by horizontal hatching in Fig. 2.

Mutants with low Rg_{arg} values ($< 0{\cdot}85$) could not easily be tested by the recombination method, because the segregation of partial arginine-dependence interferes. Unshaded areas in Fig. 2 correspond to mutants of this type. Preliminary tests performed with some of them favour the hypothesis that they are predominantly $pyr^{su\text{-}arg}$.

It is clear that the distribution of mutants at *pyr* is bimodal. There is no reason to suspect that this may be due to an artefact of the selection method. Bimodality points, rather, to a duality in function, as will be considered in the discussion.

Complementation pattern

In a previous study (Reissig, 1959) 9 *pyr* mutants obtained by the suppressor method were tested for complementation in all possible pairwise combinations. This disclosed two complementation groups or cistrons: alpha, represented by 5 mutants, and beta, represented by 4 mutants. Complementation testing was now extended to 204 new mutants of independent origin, induced by u.v.-radiation, ethylmethane sulphonate or nitrous acid (Reissig, 1963). Testing in all possible combinations was not attempted; instead, 1 alpha tester and 3 beta testers were chosen at random, and their complementation reactions with all other strains were determined. The results of these and some additional tests are shown in Table 1. Four complementation groups (alpha, beta, gamma, delta) were revealed, such that any member of one group complemented with any member of another, but did not complement with members of the same group. A fifth group (non-complementing group) comprises

mutants unable to complement with representatives of any of the other 4 groups.

The failure of this fifth group to complement could be due to relationships intrinsic to the *pyr* region; or to extrinsic factors, such as additional mutations at loci which determine barriers to the formation of heterokaryons. To determine whether the ability to form heterokaryons was impaired in the 7 non-complementing mutants, heterokaryons were attempted between them and the *arg* strain

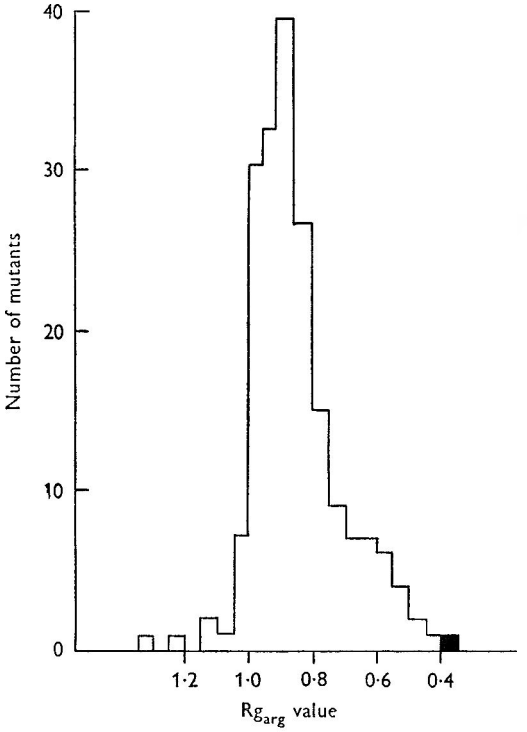

Fig. 1

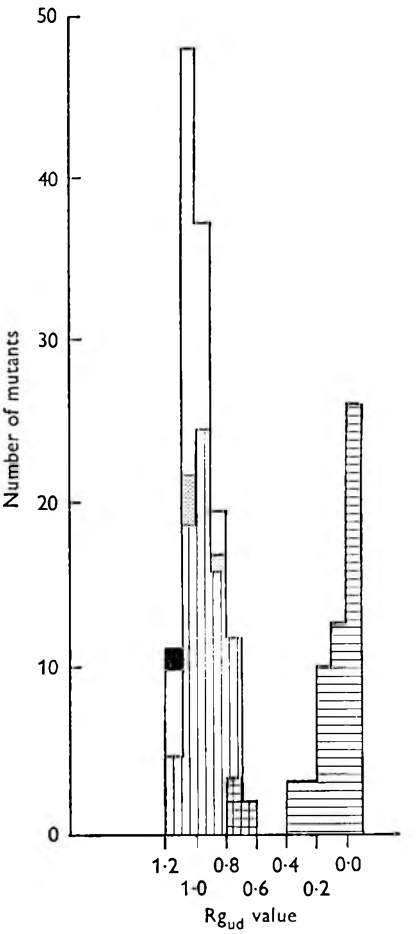

Fig. 2

Fig. 1. Distribution of Rg_{arg} values among Neurospora mutants obtained by the suppressor method. ■, Original *arg* strain. For definition of Rg_{arg}, see Methods.

Fig. 2. Distribution of Rg_{ud} values among Neurospora mutants obtained by the suppressor method. ■, Original *arg* strain; ▨, back mutants (*arg*$^+$); □ not tested. Forward mutations at *pyr*: ▤, as established by complementation; ▥, as established by recombination; ▦, asestablished by complementation and recombination. For definition of Rg_{ud}, see Methods.

from which all *pyr* mutants derived. Positive responses were obtained in every case, and the true heterokaryotic nature of the resulting prototrophic mycelium was confirmed by the isolation of hyphal tips, subcultivation, and plating of microconidia

on appropriately supplemented media. Therefore failure to complement was not due to incompatibility barriers, and was most likely related to the type of functional alteration at *pyr*. A clear correlation was noted between failure to complement, and failure to grow at all on minimal medium (Table 2).

Table 1. *Complementation among pyrimidine-dependent mutants of Neurospora obtained by the suppressor method*

Mutants of Neurospora were classified in 5 complementation groups. The complementation reactions of all members of each group with representative strains from the same and from other groups are indicated in the table: +, complementation; 0, no complementation. The figures in parentheses indicate the number of different pairs tested in each case.

No. of mutants	Complementation group	Alpha	Beta	Gamma	Delta	Non-complementing
155	Alpha	0 (444)	.	.	.	.
38	Beta	+ (267)	0 (41)	.	.	.
3	Gamma	+ (12)	+ (15)	0 (3)	.	.
1	Delta	+ (4)	+ (5)	+ (3)	0 (1)	.
7	Non-complementing	0 (28)	0 (28)	0 (21)	0 (7)	0 (1)
204						

Table 2. *Correlation between failure to grow on pyrimidine free medium, and failure to complement*

Growth on pyrimidine free medium*	No. of *pyr* mutants	
	Complementing†	Non-complementing
Yes	101	0
No	39	10

* Visible growth by the 14th day on arginine supplemented medium.
† Either in alpha, beta, gamma or delta complementing groups.

Comparison with mutants obtained by auxotroph selection

Two types of mutants are known to occur at the *pyr* region (Davis, 1960): *pyr-N* mutants lacking ATC; and *pyr-M* mutants with normal levels of ATC. In first analysis, *pyr-N* and *pyr-M* mutants can be identified with two complementation groups, which will be called, respectively, *n* and *m*. New complementation groups have appeared in more recent work (Woodward, 1962; see also Mitchell & Mitchell, 1956), but the earlier identification of *pyr-N* and *pyr-M* with two complementation groups is still useful as a preliminary criterion for comparative studies. The results just described refer to mutants obtained by auxotroph selection. It is pertinent to ask whether the same picture applies to mutants obtained by the suppressor method. The data in Table 3 show that this is not so: suppressor selection appears to yield only mutants of the *n* complementation type. But before attempting an interpretation of the data in Table 3, it was essential to find out whether the negative complementation tests might result from incompatibility factors extraneous to the *pyr* locus. Incompatibility factors were ruled out by three different criteria.

First criterion. Two mutants, respectively alpha and beta type, were crossed to

74A, a strain highly compatible with KS strains. Eighteen segregants (11 alpha, and 7 beta) were tested for complementation with *m* (KS138A, KS138a, and ED354–1a) and with *n* (KS36a, KS36A, *pyr-3d co*, *pyr-3d T*) strains. Although the speed and intensity of the positive reactions were much improved in most cases, all reactions reported negative in Table 3 were again negative.

Table 3. *Complementation between pyrimidine-dependent mutants of Neurospora obtained by the suppressor method and by auxotroph selection*

Suppressor mutants		Complementation reaction against auxotroph selection mutants			
		Group m mutants		Group *n* mutants	
No. tested	Group	KS138a	ED354–1a	KS36a	*pyr-3d co*
5	Alpha	+	+	0	0
6	Beta	+	+	0	0
3	Gamma	+	+	0	0
1	Delta	+	+	0	0
10	Non-complementing*	+	+	0	0

* That is, non-complementing when tested against suppressor mutants (Table 1).

Second criterion. An alternative method for testing complementation involves crossing both auxotrophic strains and determining whether prototrophic pseudo-wild type ascospores appear. This test is more sensitive than the usual heterokaryon test (de Serres, 1960) and eliminates incompatibility barriers (Mitchell & Mitchell, 1956). A total of 10,300 viable ascospores was examined in crosses of $n \times \alpha$; 7700 ascospores in crosses of $n \times \beta$; and 1030 ascospores in crosses of $n \times$ non-complementers. In every case the results were negative. Control crosses of the same to *m* strains gave an average of 0·2 % prototrophs.

Third criterion. The strain from which all suppressor mutants derive is arginine-dependent. It was a simple matter to test whether this strain (ED416–1a) is heterokaryon-compatible with KS36a, one of the strains used as *n* tester in the experiment of Table 3. Mixed inoculation allowed growth on orotic acid supplemented plates. Isolation of hyphal tips, cultivation on minimal medium slopes, and plating of microconidia on appropriately supplemented media, proved that a true heterokaryon had formed. Therefore, no barriers existed which prevented heterokaryon formation.

Conclusion. The complementation results shown in Table 3 reflect functional relations at the *pyr* locus, and are not distorted by extrinsic factors. Pyrimidine-dependent mutants representative of the different types obtained as suppressors of *arg*, are all of the *n* complementing type, and thus presumably deficient in ATC.

Allelism of suppressor mutants

In this section we shall consider all mutants obtained by the suppressor method, with the exception of back mutants at *arg*. Allelism between $pyr^{su\text{-}arg}$ and *pyr* was demonstrated to within ±0·1 map units in two instances in a previous publication (Reissig, 1960). Recently 89 new $pyr^{su\text{-}arg}$ mutations were identified by genetic tests (Reissig, 1962). These tests involved crossing *arg* $pyr^{su\text{-}arg} \times$ *arg pyr*. If $pyr^{su\text{-}arg}$ and

pyr were not allelic, recombination would yield *arg* + + strains, arginine-dependent but pyrimidine-independent. Yet no arginine-dependent strain was found among 748 pyrimidine-independent segregants isolated from crosses involving the 89 new strains. Thus most if not all mutants described as $pyr^{su\text{-}arg}$ are alleles of *pyr*, or closely linked to this locus.

Allelism of all pyrimidine-dependent mutants is most simply demonstrated by a functional criterion: they all can be classified in five complementation groups, such that one of the groups fails to complement with the other four (α, β, γ, δ). Genetic evidence for allelism between an alpha mutant and a beta mutant was presented earlier (Reissig, 1959). Five additional mutants representing the various complementation groups were crossed to a sixth *pyr* mutant; again no pyrimidine-independent recombinant was detected among several thousand ascospores from each cross.

Having demonstrated that the major mutations screened with the suppressor method are allelic, the possibility should be considered that concomitant mutations at modifier genes might be responsible for the minor phenotypic differences observed. This seemed unlikely, since it would require simultaneous mutation of two genes (*pyr* and modifier) affecting the same character, but nevertheless was tested. Four *pyr* mutants with Rg_{ud} = 0·6–0·7 ($pyr_{0\cdot6\text{-}0\cdot7}$) were crossed to a *pyr* strain with Rg_{ud} = 0·0 ($pyr_{0\cdot0}$) marked with *co* (colonial), about 3 units away from *pyr*. Both parents carried also the *cot* (colonial at 32°) allele. When ascospores from such a cross are incubated at 32°, all growing clones make small colonies. The morphological difference between *co* and co^+ is masked at this temperature, and size differences are related to growth requirements only. Cooling then to 25° the difference between *co* and co^+ can be clearly appreciated in a few hours. Ascospores from crosses of co^+*arg* $pyr_{0\cdot6\text{-}0\cdot7}$*cot* × *co arg* $pyr_{0\cdot0}$ *cot* were plated on minimal medium with and without pyrimidines. On the former medium the colonies formed were of normal size, half *co* and half co^+. On medium lacking pyrimidines, the colonies which appeared were small, and most (695 out of 702) were co^+. This result shows that the factor allowing residual growth is closely linked to *co*.

In another experiment, ascospores from the cross *co arg* $pyr_{0\cdot2}$ *cot* × co^+ *arg* $pyr_{0\cdot0}$ *cot* were plated on minimal medium. Seven days later, microcolonies could be distinguished and transferred to pyrimidine supplemented medium. On cooling, all 174 colonies were found to be *co*. Control platings on pyrimidine medium yielded half *co* and half co^+. Control platings on minimal of crosses involving $pyr_{0\cdot0}$ × $pyr_{0\cdot0}$ gave no microcolonies. Thus, linkage between the residual growth factor and *co* was again demonstrated. In all likelihood this means that residual growth is an attribute of the *pyr* alleles characteristic of each mutant, and that additional modifier mutations are not involved.

ATC activities

Dr R. H. Davis, at the University of Michigan, has assayed some of our suppressor mutants for ATC activity and very kindly made available his results for insertion in the present publication (Table 4). It is clear that *pyr* mutants belonging to the five complementing groups are all deficient in ATC. Mutants with low Rg_{ud} have no demonstrable ATC activity, where 5 % of the normal value would have been detectable; and mutant KØ493–50a, capable of appreciable growth in the absence

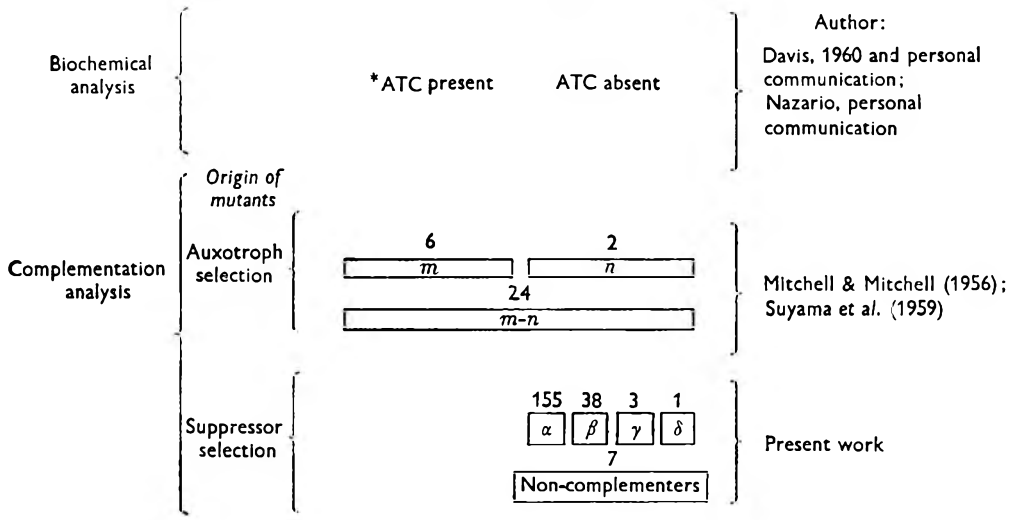

Fig. 3. Summary of comparisons between pyrimidine-dependent mutants of Neurospora obtained by auxotroph selection and by the suppressor method. The numbers indicate numbers of mutants found in each class. Failure to complement is signified when bars overlap. The scheme is based on the complementation map of Suyama *et al.* (1959). The more recent results of Woodward (1962) differ by the finding of complementation among certain '*m*' mutants, and in some combinations of '*m*–*n*' with '*n*' or '*m*'. *ATC = aspartic transcarbamylase.

Table 4. *Aspartic transcarbamylase activities in Neurospora mutants isolated by the suppressor method, and in controls*

Enzyme assays performed by Dr R. H. Davis (personal communication). Methods are described elsewhere (Davis & Woodward, 1962). Cultures in liquid minimal medium, supplemented with arginine and uridine (each 50 mg./l.). All strains derived from ED416-1a by one-step mutation, except 73a.

Source of extract				Specific ATC activity in extract (μmole ureido-succinate/mg. protein/hr.
Strain number	Genotype	Complementation type	Rg_{ud}	
Mutants:				
KØ493–14a	*arg pyr*	Alpha	0·18	0·0
KØ493–54a	*arg pyr*	Beta	0·02	0·0
KØ556–185a	*arg pyr*	Gamma	0·00	0·0
KØ545–122a	*arg pyr*	Delta	0·00	0·0
KØ545–31a	*arg pyr*	Non-complementing	0·00	0·0
KØ493–50a	*arg pyr*	Alpha	0·60	0·55
KØ493–40a	*arg* $pyr^{su\text{-}arg}$	.	1·05	0·43
Controls:				
ED416–1a	*arg* pyr^{+}	.	1·17	1·66
KØ493–56a	arg^{+} pyr^{+}	.	1·01	1·39
73a	arg^{+} pyr^{+}	.	*ca.* 1	1·72

of uridine (Rg_{ud} = 0·6), had about one-third of the activity in the controls. Mutant KØ493–40a, a typical $pyr^{su\text{-}arg}$ strain, also displayed decreased ATC activity; while KØ493–56a, a back-mutant of ED416–1a, had the same activity as ED416–1a or an unrelated wild strain.

Dr M. Nazario (personal communication; Instituto Nacional de Microbiología, Buenos Aires) has confirmed the absence of ATC activity in strains KØ493–14, KØ493–54, KØ545–122 and KØ545–31, by using a method capable of detecting 0·3 % of the normal activity. He measured incorporation of ^{14}C carbamyl phosphate in the acid-stable fraction, using extracts of strains cultured under conditions of de-repression, i.e. with limiting uridine.

The biochemical data reported confirm the conclusions arrived at in previous sections on the basis of genetic and complementation data alone, namely that suppressor mutants are deficient in ATC (Fig. 3); and that *pyr*$^{su\text{-}arg}$ is an allele of *pyr*.

DISCUSSION

The mutants screened by the suppressor method fall into two groups: back mutations at *arg* and forward mutations at *pyr*. The latter involve drastic (*pyr-N*) or partial (*pyr*$^{su\text{-}arg}$) deficiencies in ATC activity. Since selection is based on the suppression of the *arg* gene, it could be concluded that this suppression entails in every case a total or partial block in reaction (1):

$$\text{carbamyl phosphate} + \text{aspartate} \xrightarrow{\text{ATC}} \text{ureidosuccinate} \quad (1)$$

The available evidence (Reissig, 1960; Davis, 1962) indicates that *arg* has a partial (cf. Fig. 1) block in the synthesis of carbamyl phosphate, a common precursor of arginine and pyrimidines. From carbamyl phosphate onwards, the arginine and pyrimidine biosynthetic pathways diverge via reactions catalysed, respectively, by ornithine transcarbamylase (OTC) and by ATC (reaction 1). Thus, OTC and ATC compete for carbamyl phosphate. Why a block in carbamyl phosphate synthesis should result in a mutant responding to arginine, but not to pyrimidines, has not been explained with certainty; but this might easily result from irreversibility of certain reactions, channelling, or prevailing regulatory systems. At any rate, suppression of *arg* by a second mutation creating a deficiency for ATC, is readily understood in terms of a diminished competition for carbamyl phosphate. Diminution of ATC activity to about one-third its normal value suffices for full suppression of *arg* (Table 4). Other mutations in the *pyr* region cause only partial suppression of *arg* (Reissig, 1960, and present paper), presumably as a result of smaller decreases in ATC activity.

There are two types of genes which control protein synthesis: regulatory and structural (Jacob & Monod, 1959). In the present study, the frequency distribution of ATC-deficient mutants is clearly bimodal when they are classified according to the extent of their pyrimidine dependence (Fig. 2), and presumably also when classified by ATC activity (Table 4). This can be most simply explained on the hypothesis that most pyrimidine-dependent types (*pyr-N*) are due to mutations at a structural gene, and most prototrophs (*pyr*$^{su\text{-}arg}$) to mutations at a regulatory gene. Of course, the evidence for the existence of both regulatory and structural genes in this system is not compelling, and is indirect. Alternative but less plausible explanations are possible. For instance, the structural relations in the ATC molecule may be such that amino acid substitution would most often lead to two discontinous activity states. Or discontinuity may follow from the peculiar effects of chromosomal sites of high mutability ('hot-spots', in the terminology of Benzer & Freese, 1958). The

latter alternative is rendered improbable by the fact that the same distribution of mutant types can be obtained with different mutagens (Reissig, 1963).

An interesting feature of the mutants discussed here is the fact that mutants defective in the same enzymic activity are able to complement in heterokaryons. It was noted (Table 2) that there is a positive correlation between residual growth of the various mutants and their ability to complement. This correlation should be kept in mind when comparing mutants obtained by the suppressor method with mutants obtained by the usual methods for the selection of auxotrophs, since the latter methods discriminate against mutants which exhibit residual growth, while the suppressor method does not. A possible theoretical basis for the correlation between residual growth and complementation is provided by the observation (Yanofsky & St Laurence, 1960; Lacy & Bonner, 1961) that tryptophan synthetase mutants which possess CRM (an immunologically related protein) complement more frequently than those which lack it. In turn, possession of CRM might be a condition for residual growth.

This work was performed during the tenure of a fellowship from the Rask Ørsted Foundation, and supported by grants from the Carlsberg Foundation and the Rockefeller Foundation. I am grateful to Professors M. Westergaard, O. Maaløe and N. H. Giles for many useful discussions; to M. B. Mitchell, K. D. Munkres and V. W. Woodward for access to their mutant strains and unpublished results; and to R. H. Davis and M. Nazario for generously facilitating their unpublished results for inclusion in this paper, and for many useful comments.

This is paper 3 in the series on forward and back mutation in the *pyr*-3 region of Neurospora.

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Printed in Great Britain

Ampicillin Inactivation and Sensitivity of Coliform Bacilli

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(*Received* 14 *June* 1962)

SUMMARY

Ampicillin sensitivity tests and tests for ampicillin inactivation were made with 148 strains of coliform bacilli. A correlation was found in strains of *Proteus mirabilis* and Klebsiella between ampicillin resistance and inactivation. No correlation was obtained with *Escherichia coli*, whether sensitive or resistant to ampicillin, although small amounts of penicillinase were produced. Ampicillin-sensitive strains of *P. mirabilis* showed even lower amounts of penicillinase. The strains of *Pseudomonas pyocyanea* (*aeruginosa*) were resistant to ampicillin, but also showed low penicillinase levels. No evidence of induction of penicillinase was obtained with *E. coli* or Klebsiella in the presence of methicillin, ampicillin or cephalosporin C. The ampicillin-inactivating enzyme is a β-lactamase, not an amidase. Resistance of ampicillin-sensitive strains of *E. coli* was increased rapidly by serial subcultures but was not associated with higher penicillinase values. Ampicillin was inactivated less rapidly than was benzylpenicillin by penicillinase from an *E. coli* strain.

INTRODUCTION

Many strains of coliform bacilli have been shown to be sensitive to low concentrations of the new penicillin, ampicillin 6(D(−)α-aminophenylacetamido): penicillanic acid ('Penbritin; Rolinson & Stevens, 1961; Stewart, Coles, Nixon & Holt, 1961; Brumfitt, Percival & Carter, 1962). Nevertheless, in early studies with benzylpenicillin, it was shown that most species of coliform bacilli produced a 'penicillinase' (Abraham & Chain, 1940; Harper, 1943; Woodruff & Foster, 1945; Bondi & Dietz, 1944; Czekalowski, 1950) and ampicillin has been shown to be fully sensitive to the penicillinase produced by penicillin-resistant staphylococci. It was therefore decided to investigate the relationship between penicillin inactivation and ampicillin sensitivity in coliform bacilli.

The terminology of penicillin-inactivating enzymes now needs some clarification since the enzymes may attack the penicillin molecule in at least two different sites, i.e. by splitting the β-lactam ring or by removing the side chain. In the present paper the term penicillinase will be reserved for enzymes splitting the β-lactam ring and the term amidase used for enzymes removing the side chain. When a penicillinase acts on penicillin and splits the β-lactam ring, penicilloic acid is formed. Although penicilloic acids derived from benzylpenicillin and ampicillin differ, they both reduce iodine to the same extent and are therefore referred to in the text as 'penicilloic acids'.

METHODS

Ampicillin sensitivity. Coliform type organisms (148 strains) were identified and tested for sensitivity to ampicillin. The sensitivity of the organisms was determined by the ditch plate method, the ditches containing 50 μg. ampicillin/ml. A standard strain of *Escherichia coli* sensitive to 3 μg. ampicillin/ml. was used as a control. The results were recorded as sensitive, partially resistant or resistant as compared with the control organism. Six strains each of ampicillin-sensitive *E. coli* and *Proteus mirabilis* were tested by the broth dilution method to determine the range of minimum inhibitory concentrations of ampicillin-sensitive strains.

Ampicillin and benzylpenicillin inactivation

Cup-plate assay. A screening test for ampicillin inactivation was performed on the organisms tested for ampicillin sensitivity. Ampicillin was added to 5 ml. of a 6 hr. shaken broth culture to a final concentration of 100 μg./ml. After a further 18 hr. of incubation at 37° without shaking, the cultures were examined for residual ampicillin by the cup-plate assay method. The results were recorded as complete inactivation, partial or no inactivation. This test did not detect low degrees of ampicillin inactivation and some of the organisms which showed no inactivation were further tested by incubation with a lower concentration of ampicillin (20 μg./ml.). Samples were removed at 1, 3, 6 and 18 hr. and again tested for residual ampicillin by the cup-plate method. Similar tests were performed on three strains of *Pseudomonas pyocyanea* (*aeruginosa*) and four strains of ampicillin-sensitive *Proteus mirabilis*. A strain of Klebsiella and broth containing antibiotic only were used, as positive and negative controls respectively.

Iodometric method. Penicillinase was estimated by the iodometric method of Perret (1954). This test depends on the hydrolysis of the various penicillins to their respective penicilloic acids by penicillinase. Iodine is reduced by penicilloic acids and approximately eight equivalents iodine are equivalent to 1 mole penicilloic acid. In the test samples (0·5–2 ml.) of an overnight shaken broth culture were added to 5 ml. of a benzylpenicillin solution (0·25 g. of benzylpenicillin in 100 ml. of phosphate buffer, pH 6·8) and shaken at 37° for 1–5 hr., depending on the rate of formation of penicilloic acid by the organism concerned. Six strains of *Escherichia coli*, two strains of *Citrobacter freundii*, six strains of Klebsiella, fourteen strains of Proteus, also a strain of a penicillinase-producing staphylococcus and a non-penicillinase-producing staphylococcus were tested by this method.

Effect of pH *values on penicillinase activity.* Penicillinase activity was estimated by the iodometric method, using 6 hr. shaken broth cultures of a strain each of Klebsiella, *Proteus vulgaris* and *Escherichia coli*. Two ml. of broth culture from each organism were incubated with penicillin in a series of phosphate buffers of pH range 4·5–8·0.

Penicillinase induction. The strains of coliform bacilli examined were shaken in broth for 6 hr. at 37° to which ampicillin, methicillin or cephalosporin C had been added to a final concentration of 2 μg./ml. Penicillinase was estimated by the iodometric and cup-plate assay methods.

Penicillin amidase production. Penicillin amidase removes the side-chain from the penicillin molecule and leaves the β-lactam ring intact. The product is 6-amino-

penicillanic acid (6-APA) and was tested for by a modification of the hole-plate method described by Uri & Sztaricskai (1961). Phenylacetyl chloride and sodium carbonate were added to the test fluid in a Heatley cup placed on a nutrient agar plate seeded with a culture of the Oxford staphylococcus, and incubated overnight at 30°. When 6-APA is present it is converted into benzylpenicillin by the phenylacetyl chloride and a zone of inhibition is shown. Controls without phenylacetyl chloride were set up, and also a control containing a known amount of 6-APA. 109 broth cultures of coliform bacilli were tested by this technique.

Since penicillin amidase is mainly intracellular, and has an optimum activity at pH 7·5–8·5 (Rolinson *et al.* 1960) further tests were made with two strains of an ampicillin-resistant *Escherichia coli*, two strains of an ampicillin-sensitive *E. coli*, a strain of ampicillin-resistant Klebsiella and a control organism known to produce the amidase. These organisms were grown in shaken broth cultures in a water bath at 28–30° for 24 hr. The cultures were then centrifuged and the deposited organisms resuspended in phosphate buffer (pH 8·0) and incubated with benzylpenicillin for periods from 15 min. to 24 hr. at 30° or 37°. Tests were made for 6-APA by the above method.

Development of resistance of Escherichia coli *to ampicillin.* Six strains of ampicillin-sensitive *Escherichia coli* were subcultured on ampicillin ditch plates containing 50–200 μg. ampicillin/ml. to determine the rate of development of resistance. The number of subcultures and the corresponding minimum inhibitory concentrations were recorded. A strain of *E. coli* was also subcultured on nutrient agar plates containing ampicillin at 2·5–100 μg./ml. Further tests were made to detect the presence of ampicillin-resistant mutants by using large inocula in broth. Nine ml. ampicillin-containing broth were added to 1 ml. of a 24 hr. broth culture of an ampicillin-sensitive strain of *E. coli*; ampicillin was used at 10, 25, 50 and 100 μg./ml., with 10 tubes for each concentration. The cultures were examined at 2 and 5 days for increased growth, the presence of resistant mutants and ampicillin inactivation. A known non-penicillinase-producing staphylococcus was used as a negative control.

The effect of inoculum size on growth in ampicillin. Dilutions of 18 hr. broth cultures were made in sets of 8 tubes containing 5 ml. broth + 500 μg. ampicillin. The dilutions of the broth cultures were 10 fold, ranging from 1/10 to $1/10^8$. The organisms tested were *Proteus vulgaris*, *P. rettgeri*, *Escherichia coli* and a strain of penicillin-resistant *Staphylococcus aureus*. Control tubes containing 5 ml. broth only were inoculated in a similar manner with the staphylococcus. The cultures were incubated for 48 hr. at 37° and the number of tubes which showed growth recorded. Residual ampicillin was assayed by the cup-plate method after incubation for 24 hr.

Comparison of ampicillin and benzylpenicillin as a substrate for penicillinase. The iodometric method was used to determine the relative amounts of penicilloic acid formed from ampicillin and benzylpenicillin when incubated with broth cultures of coliform bacilli or *Staphylococcus aureus*. Samples (0·5–2 ml.) of an 18 hr. shaken broth culture were incubated with 5 ml. of similar concentrations of ampicillin or benzyl penicillin (0·25 g. in 100 ml. phosphate buffer, pH 6·5). Three estimations were made on cultures of the *Escherichia coli* strain. Ampicillin and benzylpenicillin inactivation by the same strain of *E. coli* was also tested by the cup-plate assay

method with antibiotic concentration 20 μg./ml. and an 18 hr. broth culture of organism.

Culture media. A beef digest broth (pH 7·4) was used in all experiments solidified with 1·4 % agar (Oxoid no. 2) when required.

RESULTS

Ampicillin sensitivity tests. The number of strains tested by the ditch-plate method which were sensitive to ampicillin is shown in Table 1. This shows that 34/41 strains of *Escherichia coli* and 26/38 strains of *Proteus mirabilis* were sensitive to ampicillin. All other organisms tested were resistant apart from two strains of Klebsiella which were isolated from sputum (*Klebsiella pneumoniae*).

Ampicillin inactivation. The results of the screening test on ampicillin inactivation are also shown in Table 1. It can be seen that most of the organisms fall in one of two main groups; one group was able to inactivate ampicillin and the other group showed no detectable inactivation by this method. In the smaller, third group of organisms were eight strains of *Proteus morganii* and two strains of *Citrobacter*

Table 1. *Screening test for ampicillin inactivation*

Organism	Total strains	No. of strains sensitive to ampicillin	Inactivation of ampicillin (100 μg./ml. in 5 ml. of broth culture)		
			Positive	Partial	Negative
Escherichia coli I and II	41	34	0	0	41
Citrobacter freundii	6	0	4	2	.
Klebsiella I and II	21	2	19	0	2
Proteus morganii	12	0	2	8	2
P. mirabilis	38	26	12	0	26
P. rettgeri	9	0	5	4	0
P. vulgaris	10	0	10	0	0
Providence	3	0	3	0	0
Pseudomonas pyocyanea	8	0	0	0	8

Positive = complete inactivation of ampicillin. Partial = partial inactivation of ampicillin. Negative = no detectable inactivation.

freundii, resistant or partially resistant to ampicillin and which showed partial inactivation of ampicillin. Seven of the *Escherichia coli* strains were resistant or partially resistant to ampicillin and showed no detectable inactivation. There were 26 strains of *Proteus mirabilis* and 2 strains of Klebsiella sensitive to ampicillin and all showed no detectable inactivation. Of 71 organisms (excluding *Pseudomonas pyocyanea*) which showed no detectable inactivation, 9 were resistant to ampicillin, whereas all strains which showed complete or partial inactivation were resistant to ampicillin. The results indicate that none of the *E. coli* or ampicillin-sensitive strains of *P. mirabilis* actively destroyed ampicillin whereas most of the Klebsiella and ampicillin resistant strains of Proteus did.

The tests made with three strains of *Escherichia coli* incubated with smaller amounts of ampicillin showed that *E. coli* inactivated ampicillin slowly as compared with a strain of Klebsiella. The results of three tests for each organism are shown in Table 2. The strains of *E. coli* usually inactivated very little ampicillin after incubation for 6 hr. but completely inactivated 20 μg. ampicillin/ml. (in 5 ml. broth)

after incubation for 18 hr. There were three exceptions: one test showed complete inactivation after 6 hr. and in two others there was little inactivation after 18 hr. A strain of Klebsiella inactivated 20 μg. ampicillin/ml., in one hr. The four strains of ampicillin-sensitive *Proteus mirabilis* tested by the same method showed no clear evidence of inactivation as compared with a non-penicillinase-producing staphylococcus. This may have been due to the test not being sufficiently sensitive. Inactivation of ampicillin by *Pseudomonas pyocyanea* was also difficult to detect by the cup-plate assay method, as a zone of inhibition of the Oxford staphylococcus occurred with the control organism without added antibiotic. Similar experiments with *Ps. pyocyanea* by the iodometric method gave inconsistent results owing to clumping of the organisms. On several occasions there was evidence of ampicillin inactivation at 24 hr., but very little at 6 hr.

Table 2. *Inactivation of ampicillin*

(20 μg./ml. in 5 ml. of broth culture.)

	Zone diameters in mm.			
	1 hr.	3 hr.	6 hr.	18 hr.
Escherichia coli (1)	28	26	22	0
	28	22	20	0
	22	24	24	16
E. coli (2)	26	23	20	0
	26	26	23	15
	22	22	18	0
E. coli (3)	26	22	21	0
	24	13	12	0
	23	23	0	0
Klebsiella	0	0	0	0
Controls (1)	26	—	—	20
(2)	26	—	—	23
(3)	22	—	—	20

* Controls contained nutrient broth and ampicillin only.

Results of penicillinase estimations by the iodometric method. The relative amounts of penicillinase produced in overnight shaken broth cultures and estimated by the iodometric method are shown in Table 3. The results show the amounts of penicilloic acid formed by 1 ml. broth culture from benzylpenicillin in 1 hr. when shaken at 37°. The results mainly confirm the cup-plate assay results. The *Escherichia coli* strains produced low amounts of penicillinase, and the ampicillin-sensitive strains of *Proteus mirabilis* (which are more sensitive to ampicillin than *E. coli*) produced just detectable amounts of penicillinase. The three strains of *Proteus rettgeri* and the two strains of *Citrobacter freundii* showed no inactivation or partial destruction by the screening test and by this test also seemed to show lower amounts of penicillinase than did the other ampicillin-resistant penicillinase producers. Apart from these strains, the ampicillin-resistant strains in general produced more than 1 μmole penicilloic acid/ml. culture/hr. under these conditions. A culture of a penicillinase-producing staphylococcus of comparable optical density gave a result two to three times higher than did the penicillinase-producing coliform organisms. The penicillinase produced

under these conditions appears to be mainly intracellular. An overnight shaken broth culture of an ampicillin-resistant Klebsiella produced 1·9 μmole penicilloic acid/ml. broth culture/hr. and the supernatant fluid showed 0·4 μmole/ml./hr. An ampicillin-sensitive strain of *Escherichia coli* gave 0·6 μmole penicilloic acid/ml. broth culture/hr. and the supernatant fluid 0·17 μmole/ml./hr.

Effect of pH *value on penicillinase cultures.* Maximum penicillinase activity occurred at pH 6·0 with the strains of Klebsiella, *Proteus vulgaris* and *Escherichia coli* tested. Activity decreased slightly between pH 6·0 and 7·0 but decreased rapidly between pH 6·0 and 5·0.

Table 3. *Penicillinase (iodometric method) activity*

Organism	No. of strains	Ampicillin sensitivity	Penicillinase activity μmole penicilloic acid/ml. of broth culture 1 hr
Escherichia coli	5	Sensitive	0·55, 0·34, 0·42, 0·45, 0·46
E. coli	1	Resistant	0·47
Citrobacter	2	Resistant	1·15, 0·75
Klebsiella	6	Resistant	1·6, 1·8, 2·6, 2·4, 1·6, 2·0
Proteus mirabilis	3	Sensitive	0·16, 0·18, 0·23
	2	Resistant	3·15, 2·16
P. rettgeri	3	Resistant	0·7, 0·9, 0·63
P. morganii	3	Resistant	3·05, 2·35, 1·9
P. vulgaris	3	Resistant	1·7, 2·9, 1·3
Staphylococcus aureus	1	Resistant	9·4
S. aureus	1	Sensitive	0·08, 0·05, 0·06, 0·2, 0·11, 0·14

Table 4. *Induction of penicillinase*

		Ampicillin sensitivity	Inducing agent (2 μg./ml.)	μmole/penicilloic acid/ml. broth culture/hr.
Escherichia coli	(1)	Sensitive	Nil	0·54
			Ampicillin	0·55
			Methicillin	0·55
E. coli	(1)	Resistant	Nil	0·51
E. coli	(2)	Sensitive	Nil	0·36
			Cephalosporin C	0·34
Klebsiella	(1)	Resistant	Nil	2·9
			Ampicillin	3·5
			Methicillin	3·9
Klebsiella	(2)	Resistant	Nil	1·7
			Cephalosporin C	1·8
Staphylococcus aureus		Resistant	Nil	6·0
			Methicillin	80·0

Induction of penicillinase. The results of penicillinase values obtained with 6 hr. shaken broth cultures to which had been added ampicillin, methicillin or cephalosporin C are shown in Table 4. The optical density of the cultures was about the same and, in the results shown, the values had been corrected to the same optical density for each organism examined. The cultures were incubated for 5 hr. with benzylpenicillin for the *Escherichia coli* strains and for 1 hr. for the Klebsiella strains and the strain of *Staphylococcus aureus*. A penicillinase-producing strain of *S. aureus* was included to show the effect of methicillin on the penicillinase level under similar cultural conditions. The results show that there was no significant increase in penicillinase

values in the strains of Klebsiella, or *E. coli* examined in the presence of the three possible inducing agents tested. This was in marked contrast to the large increase in penicillinase when the penicillin-resistant staphylococcus was grown in presence of methicillin.

Amidase production. In tests at 37° none of the 49 strains which inactivated ampicillin gave positive evidence of the production of penicillin amidase; this suggests that the inactivating enzyme was a β-lactamase, which was confirmed by the iodometric method. The 60 ampicillin-sensitive strains also showed no evidence of amidase production at 37°. When organisms of broth cultures grown at 28–30° were incubated with penicillin at pH 8·0 similar negative results were obtained with all strains apart from the known amidase-producing strain of *Escherichia coli.*

Development of resistance of Escherichia coli *to ampicillin.* Resistance developed rapidly when six strains of ampicillin-sensitive *Escherichia coli* were subcultured on nutrient agar plates containing ampicillin. There was a 20–40-fold increase in resistance after 6–8 subcultures. No difference in ampicillin inactivation was seen as between the sensitive and resistant strains by the cup-plate method; this was confirmed by the iodometric method. The result with one strain is shown in Table 4 (*E. coli* (1)), ampicillin-sensitive and resistant).

When a heavy inoculum of a strain of ampicillin-sensitive *Escherichia coli* was grown on ampicillin-containing agar plates, the minimum inhibitory concentration after incubation for 24 hr. was 2·5–5·0 μg. ampicillin/ml. After several days at room temperature large and small colonies appeared on the plate containing 5·0 μg. ampicillin/ml. Although both types of colony grew normally on nutrient agar, the minimum inhibitory concentration for the larger colonies was 12·5 μg. ampicillin/ml., whereas the resistance of the small colonies was unchanged. After 5 subcultures of the larger colonies, the resistance increased to 100 μg. ampicillin/ml. This confirms that resistance develops rapidly in a step-wise manner.

The tests with heavy inocula in broth cultures containing ampicillin showed no evidence of any highly resistant single-step mutants when subcultured on ampicillin plates; growth occurred in 10 to 50 μg. ampicillin/ml., after incubation for 5 days. This appeared to be due to slow inactivation of ampicillin, since ampicillin activity was only detected in the tubes of broth containing 100 μg. ampicillin/ml., and there was no increased growth at this concentration. There was no comparable inactivation in cultures of a non-penicillinase-producing staphylococcus, under similar cultural conditions.

The effect of inoculum size on growth in ampicillin

The results of growth with different inoculum sizes in broth containing 100 μg. ampicillin/ml. are shown in Table 5. The approximate penicillinase activity is shown in the last column. The strain of *Staphylococcus aureus*, although apparently producing more penicillinase than the Proteus or Klebsiella strains, did not grow when the inoculum was diluted $1/10^5$. The Klebsiella and Proteus strains grew when the inoculum was diluted $1/10^8$; no residual ampicillin was detected at this dilution with these organisms after incubation for 24 hr. There was no significant inactivation of ampicillin in the tube containing *S. aureus* at dilutions of inocula from $1/10^5$ to $1/10^8$. There was no detectable inactivation with the *E. coli* strain at any dilution of inoculum.

Penicillinase activity on ampicillin and benzylpenicillin. The amounts of penicilloic acid formed by coliform bacilli and by *Staphylococcus aureus* from ampicillin and benzylpenicillin are shown in Table 6. Penicilloic acid formed from ampicillin by *Proteus mirabilis*, Klebsiella and *S. aureus* was about the same or rather more than from benzylpenicillin. The three tests made with ampicillin-sensitive *Escherichia coli* showed that less penicilloic acid was formed from ampicillin than from benzylpenicillin. The cup-plate assay result also confirmed that ampicillin was inactivated less rapidly than benzylpenicillin by *E. coli*.

Table 5. *The effect of inoculum size on growth in ampicillin*

Organism	Ampicillin sensitivity	Dilution of inoculum showing growth in broth containing 100 μg./ml. ampicillin after 48 hr. incubation	Penicillinase activity μmole penicilloic acid/ml. of broth culture/hr.
Klebsiella	Resistant	10^{-8}	2·9
Proteus vulgaris	Resistant	10^{-8}	2·9
P. rettgeri	Resistant	10^{-8}	0·7
Escherichia coli	Sensitive	$< 10^{-1}$	0·5
Staphylococcus aureus	Resistant	10^{-4}	8·2

Table 6. *Penicillinase activity on ampicillin and benzylpenicillin*

Organism	Ampicillin sensitivity	Penicillinase activity μmole penicilloic acid/ml. of broth culture/hr.	
		Ampicillin	Benzyl-penicillin
Proteus mirabilis	Resistant	1·5	0·86
Klebsiella	Resistant	1·6	1·4
Escherichia coli	Sensitive	0·16	0·6
		0·11	0·54
		0·1	0·4
Staphylococcus aureus	Resistant	9·0	4·2

DISCUSSION

By the not very sensitive cup-plate method used as a screening test for ampicillin inactivation, there was a clear correlation between resistance to ampicillin and its inactivation by most of the strains tested Citrobacter, Klebsiella and the Proteus groups. This correlation was not seen with the strains of *Escherichia coli*, none of which showed ampicillin inactivation by this test, although 7 of the 41 strains tested were resistant to ampicillin. None of the strains of *Pseudomonas pyocyanea* gave positive evidence of ampicillin inactivation although all strains of this species are resistant to ampicillin. Similar results were obtained by Trafford *et al.* (1962) with Klebsiella and a smaller number of strains of *E. coli*, but they described four strains of ampicillin-resistant *Proteus mirabilis* which did not produce penicillinase. By the iodometric method, however, it was found that the strains of *E. coli* did in fact produce some penicillinase, whether they were sensitive to ampicillin or not, although the values were lower than those of the ampicillin-resistant strains of

Proteus, Klebsiella or penicillin-resistant *Staphylococcus aureus*. Ampicillin-sensitive strains of *P. mirabilis* seemed to show even lower values of penicillinase than did the *E. coli* strains.

Ampicillin was inactivated as rapidly, or more rapidly than benzylpenicillin by the ampicillin-resistant strains of Proteus, Klebsiella and *Staphylococcus aureus*. However, it was also shown that ampicillin was more resistant than benzylpenicillin to penicillinase produced by a strain of *Escherichia coli*. This suggests that the penicillinase produced by this *E. coli* strain differs from that produced by the strains of Proteus, Klebsiella or *S. aureus*. This difference in resistance of the two antibiotics to penicillinase corresponds to the difference in sensitivity of *E. coli* to ampicillin and benzylpenicillin.

The experiments described here suggest that there is no marked increase in penicillinase production by coliform bacilli grown in presence of low concentrations of ampicillin, methicillin or cephalosporin C. This contrasts with other penicillinase-producing organisms such as *Staphylococcus aureus* (Geronimus & Cohen, 1957) and *Bacillus cereus* (Pollock, 1950). No evidence of penicillin-amidase production was found for the strains examined under the conditions described. Some degree of amidase production cannot be excluded since many of the organisms also produce penicillinase which splits the β-lactam ring. The resistance of naturally-occurring strains of *S. aureus* to penicillin is considered to be almost entirely due to penicillinase production. In the experiments here described with inocula of different sizes, the strain of *S. aureus* was inhibited by 100 μg. ampicillin/ml. when the inoculum was diluted $1/10^5$, whereas with the strains of Proteus and Klebsiella, although they apparently produced less penicillinase, an inoculum diluted $1/10^8$ grew in this concentration of ampicillin. It therefore seems unlikely that the resistance of these strains to ampicillin depends solely on penicillinase production.

I wish to thank Beecham Research Laboratories Ltd., for a grant which made this research possible, Dr Mary Barber and Dr R. Novick, for advice and criticism and Mrs J. Filbey for technical assistance.

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