The Origin of Bacterial Resistance to Proflavine

5. Transformation of Proflavine Resistance in *Escherichia coli*

BY JEHUDITH SINAI* AND J. YUDKIN

Department of Nutrition, Queen Elizabeth College, University of London

SUMMARY: We attempted to transform proflavine-sensitive strains of *Escherichia coli* to proflavine resistance by growth in the presence of deoxyribonucleic acid-containing extracts from resistant organisms. Three methods were used to obtain the DNA preparations. Method 1 (Boivin, 1947) did not give active transforming principle, even when a variety of modifications was introduced. Method 2 (McCarty & Avery, 1946) and Method 3 (Mayers & Spizizen, 1954) gave extracts which were active in transformation. With DNA prepared by Method 2, an increase in the number of resistant organisms was found in one of four rough sensitive strains. We concluded that only a small proportion of the organisms of this strain were competent. We were able to increase the proportion of these competent organisms by a method of 'double replica plating'. According to Method 3, organisms were lysed by sodium dodecylsulphate (Duponol) in presence of citrate, and protein removed by sodium acetate. From the supernatant fluid transforming principle was precipitated by acidified ethanol, and then dissolved in saline. The smooth strain of *Escherichia coli* used in most of our experiments served as the recipient strain. The transforming principle from resistant organisms was not active alone, but was active in the presence of the protein precipitate. The activity appeared to be lost when the transforming principle was treated with DNAase. No activity was shown by extracts from sensitive organisms. The activation of transforming principle by the protein precipitate is thought to be due to the Duponol carried with it. Duponol appears to inhibit DNAase, so that it might act by preserving transforming DNA from destruction by the enzyme present in the recipient organisms. Our experiments did not always give positive results. Whilst we believe that we have demonstrated transformation in this system, the low reproducibility of our results makes it necessary to repeat and extend.

Transformation may be defined as the acquisition by an organism from its environment, by non-sexual means, of a genetically active unit directing an inheritable change. The transforming principle behaves as a self-reproducing unit within the transformed organism (Austrian, 1952). The transformation phenomenon was first discovered by Griffith (1928) in *Pneumococcus*, and transformation by soluble transforming material in *Pneumococcus* has been demonstrated by McCarty & Avery (1946), Ephrussi-Taylor (1951) and Hotchkiss (1951); in *Haemophilus influenzae* by Alexander & Leidy (1950, 1951, 1953) and in *Neisseria meningitidis* by Alexander & Redman (1953).

There has, however, been no authenticated instance of transformation in *Escherichia coli*. Boivin (1947) claimed to have transformed *E. coli* from R to S. He reported success in only one out of 400 pairs of organisms which he tested. His effective strain has since been lost, and no further positive results have been reported.

* Present address: Israel Institute for Biological Research, Ness-Ziona, Israel.

We were stimulated to study transformation of proflavine resistance of *Escherichia coli* for the following reasons. (1) In this species of organism, studies of recombination have revealed a structure analogous to chromosomes on which different determinants may be mapped. They suggest that the genetic activity of DNA may ultimately be linked to the organization of chromosomes. The fact that many mutants have been obtained from *E. coli* makes this species particularly useful in studies of the hereditary control of many characters. (2) We are mainly concerned in this work to show that several mechanisms for origin of drug resistance may operate for one system of bacterium and drug. To what extent transformation, if it occurs, may play a role in natural situations in causing the emergence of resistant strains will be discussed later. (3) The demonstration of transformation to proflavine resistance in *E. coli* would show conclusively that in this instance the change was genotypic.

METHODS

Organism. We worked with *Escherichia coli* No. 8196 obtained from the National Collection of Type Cultures (NCTC). *E. coli* strain 36 was a substrain from *E. coli* NCTC 8196 isolated in our laboratories. Strain 36 is a smooth strain, sensitive to 10 μ g. proflavine/ml. Strains R1–R6 are rough substrains isolated from single colonies of strain 36. They were obtained by growing strain 36 in broth containing homologous rabbit antiserum, kindly prepared by Dr F. Himmelweit. They are all similar in morphology and their sensitivity on isolation was similar to that of the parent strain. Strain 22 is a highly resistant strain 'trained' by growing strain 36 on successively increasing concentrations of proflavine in nutrient broth. We assume that it is a multi-step mutant of the original strain. Strain M1 is a proflavine-resistant first step mutant isolated from strain 36 by the replica plating technique (Thornley & Yudkin, 1959). This means that it has never been in contact with the drug. Strain M4 is a second-step mutant isolated from strain 36 by the replica plating technique. This second-step mutant was isolated from a first-step mutant different from strain M1. It, too, was never in contact with the drug.

All strains were kept on nutrient agar slopes at 5° and transferred every 2 months.

Media and drugs were as given in our previous papers in this series.

Sodium dodecylsulphate (Duponol) was made up as 6% (w/v) solution in distilled water at pH 7 and freshly prepared for each experiment.

Sodium deoxycholate. A stock solution (2%, w/v) of this in distilled water was sterilized by incubation at 37° overnight and stored at 5°.

Assay of transformation of proflavine resistance

(1) *Bacterial counts.* Here we compared the ratio of the proflavine-resistant organisms to proflavine-sensitive organisms in transformation cultures and in control cultures.

(a) The total viable count was made on nutrient agar plates by spreading

0.1 ml. of serial dilutions. This enabled us also to test the purity of the culture and any changes in morphology of the organisms.

(b) Proflavine-resistant organisms were counted on nutrient agar plates containing different amounts of proflavine. Serial dilutions were made and 0.1 ml. spread on plates in duplicate.

(2) *Gradient plates*. The general change in the degree of resistance of the culture was assayed by streaking on gradient plates with different concentrations of drug. This method is described on p. 386 of this number. All plates were incubated at 37° for 24 hr.

Detection of DNA. This was done by the method of Stumpf (1947).

Nomenclature. *Recipient organisms*. Organisms which are grown in the presence of DNA extract. *Donor organisms*. Organisms from which DNA is extracted. *Transforming principle (TP)*. This term is used for the DNA extract whether transformation is successful or not. Similarly, the terms 'transformation medium' and 'transformation culture' do not imply that transformation was necessarily successful.

RESULTS

Use of method of Boivin (1947)

By this means Boivin was able to transform a rough strain of *Escherichia coli* to smooth, though only a small proportion of his experiments were successful. We did the following experiments. Washed donor organisms were killed with toluene, the toluene removed by aeration, and the washed organisms suspended in 0.9% saline. The suspension was heated to 100°, centrifuged and the deposit discarded. The supernatant fluid contained DNA as indicated by the Stumpf test; this was used as TP. Cultures of recipient organisms were made, diluted 1/10, and 0.1 ml. added to 4.5 ml. nutrient broth + 0.5 ml. TP. Controls which did not contain TP were also prepared. Five replicates were made of each mixture, and incubated for 48 hr. Samples of the replicates were pooled, and counts made of sensitive and resistant organisms after 24 and 48 hr. For donor organisms we used strains 22 and M1, and for recipient organisms we used strains 36, R1 and R2. We varied the age and density of the recipient organisms, the time of contact with the toluene, and the time and duration of extraction. In no instance did we observe an increase in the number of resistant organisms.

Use of method of McCarty & Avery (1946)

Our lack of success with experiments with TP prepared by the Boivin method might have been due to a destruction of the DNA by deoxyribonuclease (DNAase) extracted from the organisms at the same time. Perhaps, also, Boivin's success with only one pair of strains was due to an unusually low DNAase content of these strains. In the method which McCarty & Avery (1946) used for *Pneumococcus*, the DNA is protected from destruction by DNAase by inhibiting the action of the enzyme. The organisms are suspended in saline with sodium citrate, which forms a complex with the magnesium ions necessary for the action of DNAase. The organisms are then lysed by sodium

deoxycholate, protein removed by chloroform-amyl alcohol and the DNA precipitated by ethanol. The DNA is dissolved in saline and kept at 5°. This method we applied as follows.

Cultures of *Escherichia coli* strain 22 (resistant) and strain 36 (sensitive) were grown in nutrient broth for 18 hr.; the organisms were centrifuged and washed with normal saline. They were then resuspended in a solution containing sodium deoxycholate 0.1% (w/v), sodium citrate 0.05 M, NaCl 0.1 M, and incubated for 3 hr. at 50°. This produced lysis of the organisms, although not so readily as with pneumococci, which McCarty & Avery found required only 1 hr. at room temperature. To the lysate was added 0.3 vol. of chloroform and 0.1 vol. of amyl alcohol, and the mixture shaken for 30 min. and centrifuged. A protein gel was formed at the interface, which was removed by pipetting. Ethanol (2 vol.) was added, when a granular and fibrous precipitate formed. The fibrous precipitate was lifted out with a glass rod and washed in ethanol. This TP was suspended in 5 ml. saline and stored at 5°.

The recipient organisms used were prepared from strains R1, R2, R3 and R4. A 3-hr. culture was made of each, diluted 1/10, and 0.1 ml. used for inoculation into transformation medium. This consisted of 4.5 ml. nutrient broth + 0.5 ml. TP preparation. The control medium for each culture consisted of 5 ml. nutrient broth. Each test and control culture was replicated five times and incubated for 18 hr. Bacterial counts on pooled samples of the five replicate tubes were made for total viable organisms and for organisms resistant to 10 µg. proflavine/ml.

Only one experiment showed a slight increase in resistant organisms by a factor of 4. This was strain R1 grown in TP prepared from the resistant strain 22. None of the other preparations showed any increase in number of resistant organisms.

This experiment suggested that the method of McCarty & Avery in the conditions used by us did not give active transforming principle. There was, however, a slight increase in resistance of one of the recipient strains, which might indicate the presence of a relatively few organisms in this strain which had competence to undergo transformation.

Isolation of a competent substrain

We tried to isolate a competent substrain from the *Escherichia coli* strain R1, which had shown a slight increase in resistance in the previous experiment. A culture was made of strain R1. After 18 hr. incubation, 0.1 ml. was spread on a nutrient agar plate and incubated for 6 hr. This master plate was replicated by velveteen mounted on a rubber bung on to a nutrient agar plate containing TP from resistant *E. coli*, together with 0.05 M-sodium citrate to inhibit the action of DNAase. The master plate was kept in the refrigerator, and the TP plate incubated for 24 hr. This was then replicated on to a plate containing 10 µg. proflavine ml., which was in turn incubated for 24 hr. At this time, 10 clusters of resistant colonies were detected on the proflavine plate. Subcultures were made from the corresponding areas on the master plate. The subcultures contained organisms which had not been in contact

with TP or proflavine, and presumably contained either pre-existing proflavine-resistant mutants, or organisms which were capable of undergoing transformation to proflavine resistance. Their competence to undergo transformation was then tested.

TP was prepared from resistant strain 22 and from sensitive strain 36. Each of the ten cultures after incubation for 1 hr. was inoculated into medium containing 4.5 ml. nutrient broth + 0.5 ml. TP preparation, and also into control medium containing no TP. Incubation was continued for 18 hr. Bacterial counts were then made in the absence and in the presence of 10 μ g. proflavine/ml. One of the ten strains tested showed an increased number of resistant organisms when incubated in presence of TP from the proflavine-resistant strain 22, but not when incubated with TP from the sensitive strain 36. The number of resistant organisms increased from 20 to 4000/ml. This apparently transformable strain we called *Escherichia coli* R1T. None of the remaining nine cultures showed this increase.

This experiment was repeated twice with successive broth cultures of strain R1T. Each time there was an increase in number of resistant organisms by 200 times when grown with extracts from resistant organisms, and no increase when grown with extracts from sensitive organisms. The transformed organisms retained this resistance when subcultured once in broth. After two further subcultures of R1T in broth, a repetition of the experiment gave a negative result.

That we were able three times to show an increased resistance in strain R1T when it was grown with extracts from resistant organisms, but not with extracts of sensitive organisms, suggests that we were dealing with an instance of true transformation. The apparent loss of competence of strain R1T on subculture may have been due to overgrowth with non-competent organisms, since we did not proceed further in our isolation than an area from the master plate which almost certainly consisted of mixed organisms. The results were nevertheless encouraging as a possible method of isolating competent organisms.

A further experiment was carried out with a different pair of organisms to see whether the phenomenon was more general. For a competent strain we chose strain R5; for the preparation of TP we used a proflavine-resistant mutant of strain M1, obtained by indirect selection. By replica plating as in the previous experiment, eleven areas were picked off from the master plate. Subcultures from them were tested with a preparation of TP from strain M1 and one from strain 36. An increase in number of resistant organisms occurred in one of the eleven strains when it was grown in the presence of TP from resistant organisms, but not when it was grown in TP from sensitive organisms. The increase in number of resistant organisms was of the order of 50 times. This strain we called *Escherichia coli* R5T. Like strain R1T, strain R5T seemed to lose its competence after a few subcultures in nutrient broth. We should stress here that, throughout the four years in which we have worked with this strain, we have never observed a significant increase in the number of resistant organisms in untreated cultures.

Use of method of Mayers & Spizizen (1954)

At this stage in our work, Mayers & Spizizen (1954) published their work on the preparation of active DNA from viruses and from *Escherichia coli*. The principle of this method is to dissociate the nucleoprotein with a detergent and then to salt out the protein.

Preparation of TP. An 18 hr. culture of the organisms was centrifuged and washed once with saline containing 0.2 M-sodium citrate. To the packed organisms was added 6% (w/v) sodium dodecylsulphate (Duponol) at pH 7. Lysis began immediately and was completed after stirring at 500 rev./min. for 45 min. A 3 M solution of Na acetate at pH 7 was added, equal to one-ninth of the original volume, the mixture warmed to 60° and stirred for 15 min. It was then cooled to 5° and centrifuged.

The supernatant fluid was added to 2.8 vol. acidified alcohol (95%, v/v, ethanol, 5%, v/v, methanol, with 1 part conc. HCl to 175 parts mixture). The strings of DNA were removed by a glass rod to a Buchner funnel and washed with 100 ml. ethanol. The DNA was placed in a centrifuge tube with some ethanol and left overnight at 5° to sterilize it. It was centrifuged and the deposit suspended in one-fortieth of the original volume of saline; it dissolved with great difficulty. It was stored at 5° and constituted our DNA preparation TP; it was Stumpf positive. This and all subsequent preparations of TP were routinely tested for sterility by plating on agar with and without proflavine.

The precipitate was washed twice with 3 M-sodium acetate, sterilized with ethanol as above and redissolved in saline to one-fortieth of the volume of the original culture. This protein fraction was also stored at 5° and constituted the protein preparation Pr.

Testing for transformation. A culture of *Escherichia coli* strain 36, as recipient strain, was taken 3 hr. after inoculation, and 0.1 ml. inoculated into four sets of tubes each in duplicate and each containing 4 ml. nutrient broth with: (i) 1 ml. saline (control); (ii) 0.5 ml. TP + 0.5 ml. saline; (iii) 0.5 ml. Pr + 0.5 ml. saline; (iv) 0.5 ml. TP + 0.5 ml. Pr. After incubation for 18 hr. counts were made on agar plates for total viable organisms and on proflavine plates for organisms resistant to proflavine.

Tests for transformation

Protein fraction and TP from resistant and sensitive organisms. The resistant organisms were from a culture of *Escherichia coli* M4. This was a second-stage mutant isolated by replica plating (Thornley & Yudkin, 1959). Table 1 shows that the TP from the resistant organisms did not by itself increase the number of resistant organisms. The protein fraction alone increased the number of resistant organisms in 10^7 viable organisms from less than 10 to about 10,000 resistant to 20 μ g. proflavine/ml., and from 0 to 500 organisms resistant to 50 μ g. proflavine/ml. The colonies to which the organisms gave rise were small. The TP + protein fraction (PrM4) together gave a 20 to 30-fold further increase in number of resistant organisms, and these gave rise to normal-sized colonies. The protein fraction from strain M4, alone or with the TP, decreased the viable count to 10% of that of the control.

Table 1. *Transformation of sensitive Escherichia coli strain 36 with extracts of resistant (mutant) organisms*

Culture	Fraction present	Organisms/ml. resistant to proflavine ($\mu\text{g./ml.}$)		Organisms in 10^7 total viable organisms resistant to proflavine ($\mu\text{g./ml.}$)	
		20	50	20	50
		No. org./ml.		No. org./ 10^7 viable	
1	—	1.5×10^3	0	7	0
2	TPM4	3.0×10	0	1.5	0
3	PrM4	2.0×10^4 s	1×10^3 s	1×10^4 s	5×10^2 s
4	TPM4 + PrM4	6.0×10^5	4×10^4	3×10^5	2×10^4
5	TP36	1.0×10^3	0	5	0
6	Pr36	3.2×10	0	1.2	0
7	TP36 + Pr36	3.6×10	0	4.6	0

Fraction TPM4 = DNA-containing fraction } From second-step proflavine-resistant
 Fraction PrM4 = protein fraction } mutant
 Fraction TP36 = DNA-containing fraction } From proflavine-sensitive *E. coli*
 Fraction Pr36 = protein fraction } strain 36
 s = small colonies

The experiment was carried out five times, each time with new preparations from *Escherichia coli* M4. On three occasions preparations from the resistant organisms gave positive results as described, on the other two occasions no increase in the number of resistant organisms was observed. We also carried out two similar experiments with preparations made from *E. coli* M1, a first-stage mutant isolated by replica plating. One of these gave, in 10^7 viable organisms, 10,000 organisms resistant to 50 $\mu\text{g.}$ proflavine/ml. A second experiment gave no increase in resistant organisms. In each experiment, controls containing preparations from the sensitive strain 36 were also used. In none of these was there an increase in the number of resistant organisms, nor did the protein fraction from the sensitive strain decrease the viable count. The resistant organisms produced in the presence of the protein fraction alone always gave small colonies; we do not know why.

Substitution of the protein fraction. The activity of the protein fraction alone may be explained by its content of TP; the protein fraction showed a weakly positive Stumpf reaction. According to Mayers & Spizizen (1954) about 30 % of the DNA comes down with the protein fraction. Since, however, the TP was itself not active, it seems as if some other fraction necessary for transformation was present in the protein fraction. This might be due to some unspecific action of the protein. It might also be due to the Duponol, since some of this was precipitated with the acetate and was subsequently found in the protein fraction. We attempted to substitute a variety of other substances for the protein fraction, to see whether they would also activate the TP. We used rabbit serum (as an unspecific protein), Duponol + acetate mixture, and deoxycholate (as an alternative surface-active agent). They were used in the following quantities: rabbit serum, 0.1 ml. of a 1/20 dilution; Duponol-acetate, 0.1 ml. of a mixture of 1 ml. Duponol 6% (w/v) and 5 ml. 3M-sodium acetate; deoxycholate, 0.1 ml. 0.1% (w/v) solution. Duplicate cultures were set up

in the usual way. Each contained 5 ml. fluid, with or without TP from the sensitive or resistant (strain M4) organisms, and with or without the protein fraction or the substitute for it. They were inoculated with the sensitive strain 36, incubated for 18 hr., and counts on pooled duplicate samples made of total viable organisms and of resistant organisms on plates with proflavine 20 and 50 $\mu\text{g./ml.}$ Cultures which showed increased resistance were subcultured overnight in nutrient broth without proflavine. These were then tested on gradient plates with 100 $\mu\text{g.}$ proflavine/ml. and for cross-resistance to chloramphenicol on gradient plates containing 15 $\mu\text{g.}$ chloramphenicol/ml.

Table 2. *Transformation of sensitive Escherichia coli 36 with extracts of resistant (mutant) organisms*

Culture	DNA* fraction	Protein fraction or substitute	Proflavine ($\mu\text{g./ml.}$)		Proflavine ($\mu\text{g./ml.}$)	
			20	50	20	50
			Organisms/ml. resistant to proflavine		Organisms / 10^7 total viable resistant to proflavine	
1	—	—	2.0×10^2	0	1.0×10	0
2	—	Duponol	2.0×10^2	0	1.0×10	0
3	—	Serum	1.0×10^2	0	5	0
4	—	Deoxycholate	1.3×10^2	0	6	0
5	—	PrM4	5.0×10^4 s	1×10^3 s	2.5×10^4 s	5×10^2 s
6	—	Pr36	4.0×10^2	0	2.0×10	0
7	TPM4	—	1.1×10^2	0	5	0
8	TPM4	Duponol	5.2×10^5	4×10^4	2.6×10^5	2×10^4
9	TPM4	Serum	8.0×10	0	4	0
10	TPM4	Deoxycholate	1.0×10^2	0	5	0
11	TPM4	PrM4	6.0×10^5	1×10^4	3.0×10^5	5×10^4
12	TP36	—	1.2×10^2	0	6	0
13	TP36	Duponol	3.0×10^2	0	1.5×10	0
14	TP36	Serum	2.4×10^2	0	1.2×10	0
15	TP36	Deoxycholate	1.5×10^2	0	7.5	0
16	TP36	Pr36	4.5×10^2	0	2.3×10	0

s = small colonies.

* See Table 1.

Table 2 shows that no increase in resistant organisms occurred with TP or with protein fraction, or with both together, from the sensitive organisms (cultures 6 and 12–16). As in previous experiments, the TP from the resistant organisms was also not active by itself (culture 7), but the protein fraction was active (culture 5). Still greater activity was shown by a mixture of TP and the protein fraction from the resistant organisms (culture 11). In this experiment, too, there was a decrease of the viable count by about 90 % in cultures containing the protein fraction (cultures 5 and 11). Neither serum nor deoxycholate replaced the protein fraction (cultures 9 and 10). On the other hand, Duponol replaced the protein fraction and caused a similar increase in the number of resistant organisms (culture 8). Duponol alone was not active. We do not know why there was a decreased viable count only in those cultures which contained protein fraction from resistant organisms or Duponol.

This experiment was carried out eleven times. In four, with three different

preparations, results were obtained similar to those described in Table 2. The other seven experiments, with four different preparations, gave no increase in the number of resistant organisms.

Specificity of TP. The only instances where sensitive organisms were made to give resistant organisms were those in which TP was prepared from resistant organisms. Two strains of these were used; both were isolated by replica plating and had never been in contact with proflavine. In no instance were extracts of sensitive organisms active. In four of the experiments described above, additional culture tubes were included in which TP was replaced by commercial DNA. It was supplied at 500 $\mu\text{g./ml.}$, and we purified it in the same way as we prepared TP from *Escherichia coli*. When acid ethanol was added, a granular precipitate was formed instead of the stringy precipitate formed from bacterial extracts. In two of the four experiments, transformation occurred with the TP from resistant organisms, but not with commercial DNA + Duponol. In the other two experiments no transformation occurred even with TP from resistant organisms.

Stability and cross-resistance of transformed organisms. In successful experiments cultures containing resistant organisms were subcultured three times in broth. They still gave a considerable increase in the number of colonies growing on proflavine plates when tested after this time. After one transfer in broth the cultures were tested on gradient plates containing 100 $\mu\text{g.}$ proflavine/ml. They gave streaks about half way across the plate as compared with streaks right across the plates which were given by the donor organisms of strain M4 which were used in transformation. The streaks were similar to those given by the first-step mutant M1 when diluted 1/100 with sensitive organisms. They also gave streaks on plates containing 15 $\mu\text{g.}$ chloramphenicol/ml., indicating a resistance to this drug about 60 % of that of strain M4.

Synchronized cultures. Hotchkiss (1951) showed that transformation of pneumococci occurred only during a short period of the division cycle. We therefore attempted transformation to proflavine resistance in synchronized cultures of sensitive *Escherichia coli* strain 36, by extracts of resistant strain M4. Four experiments were carried out, in each of which synchronization was attempted by cooling (see previous paper). In two of these, there was an increase in the number of resistant organisms at one interval after the cooling, in the presence of TP from resistant organisms + Duponol. In the other cultures, at different intervals or without Duponol + TP from resistant organisms, there was no such increase. These results suggest that transformation may occur at particular times of the division cycle, but the lack of reproducibility of transformation, which is a feature of all this work, makes it impossible at present to be definite about this.

Inactivation of TP by DNAase. In the four synchronization experiments, tubes were included in which the TP had been treated with a commercial preparation of DNAase, purified according to the method of McCarty & Avery (1946). In none of these experiments, including the two in which transformation appeared, was there an increase in number of resistant organisms in the tubes containing the treated TP.

Role of Duponol. TP from resistant organisms was only active when Duponol was present. We considered the following ways in which Duponol might act.

(a) Selective action of Duponol. McCarty & Avery (1946) postulated that the demonstration of transformation requires the presence of an agent which will favour the growth of the transformed organisms in the presence of those which have not been transformed. In our experiments we found that Duponol alone did not increase the number of proflavine-resistant organisms, nor did it do so in presence of TP from sensitive organisms, commercial DNA or TP from resistant organisms treated with DNAase. If Duponol acts as a selective agent, we should have to assume that it does so only on newly transformed organisms. We showed by gradient plates that proflavine-resistant organisms were cross-resistant to Duponol; but the converse is not true. With the Duponol-sensitive strain, 0.5 ml. Duponol+acetate added to 10 ml. agar decreased the length of streak by about one-third. There was no effect with 0.1 ml., and a decrease by two-thirds of the length with 1.0 ml. The nine Duponol-resistant strains tested, and the three proflavine-resistant strains, all gave streaks right across the plates with the highest Duponol concentration. The amount of Duponol+acetate in the transformation experiments was equivalent to 0.2 ml./10 ml. medium.

(b) Action of Duponol on the bacterial surface. Austrian (1952) pointed out that there is a correlation between the transformability of some strains of *Pneumococcus* and their high solubility in bile salts. We might then envisage that the low concentration of Duponol, which in higher concentrations is lytic, might increase the permeability of the organisms to the transforming principle. We found, however, that organisms sensitized by Duponol were not transformable, and the deoxycholate did not act like Duponol. Further experiments, however, would be needed to rule out this possibility altogether.

(c) Inhibition of DNAase by Duponol. If the recipient organisms produce sufficient DNAase there might be a destruction of the transforming principle before it had time to act. We tested this possibility. Cultures of *Escherichia coli* strain 36 were grown in pour plates with and without 0.2 ml. Duponol+acetate mixture. Before incubation, just before the agar had set, some of the fibrous TP from *E. coli* strain M4 was placed in the agar. There was significantly less digestion of the TP in the plate containing Duponol. These results support the view that the mechanism by which Duponol activates the transforming principle is by inhibiting the digestion of the DNA therein by DNAase from the recipient organisms.

DISCUSSION

We summarize here the experiments which we carried out in attempting to demonstrate transformation to proflavine resistance by DNA preparations made according to the method of Mayers & Spizizen. Of the ordinary cultures, we have had a total of 221 in which transformation would not be expected. In none of these was there an increase in the number of resistant organisms. We had a total of 45 cultures in which transformation might have been expected. In 19 of these cultures there was a considerable increase in the

number of resistant organisms. Of the synchronized cultures, we had a total of 68 in which transformation would not be expected, and was not found. We had 13 cultures in which, apart from the time from synchronization, the appropriate materials were present which might have given transformation. Two of these 13 cultures showed a considerable increase in the numbers of resistant organisms. Since we expected to find transformation only at certain times after synchronization, we could not expect as many positive results here as in the experiments in which we used non-synchronized cultures.

We have tested the resistance of the sensitive *Escherichia coli* strain 36 on a large number of occasions over 4 years. In no instance did an untreated culture show the presence of resistant organisms in anything like the number which we have reported as occurring in our successful transformation experiments.

We have clearly not yet determined the factors necessary for successful transformation. In two experiments, preparations which caused transformation did not do so after being stored 3 or 4 days in the refrigerator. On the other hand, a preparation which did not cause transformation when freshly prepared did so after 2 days in the refrigerator.

One factor may be the concentration of Duponol. In none of the four preparations in which 1% (w/v) Duponol was used, as in the experiments of Mayers & Spizizen (1954), was transformation achieved. In all the experiments which were successful, 6% (w/v) Duponol had been used.

All workers have stressed that not all strains or all individual organisms are capable of undergoing transformation. Our results may contribute something toward the understanding of some of the factors involved. If a culture is taken to consist of several substrains, of which only a small proportion is competent, then we feel that our technique of 'double replica plating' helps to isolate them. It offers a far less laborious method than the testing of hundreds of individual strains and we hope that other workers will be encouraged to try this method. In addition to competence of such strains, competence may depend on the period of the division cycle in which transformation is attempted. Our own few experiments suggest that this may be true for *Escherichia coli*, as Hotchkiss (1954) had found for *Pneumococcus*.

The evidence we offer that proflavine sensitivity may be transformed to proflavine resistance may be summarized as follows. (a) A large number of organisms from sensitive cultures have been made resistant after being brought into contact with preparations containing DNA from resistant organisms. One such preparation was made from a strain 'trained' to proflavine resistance; the other two preparations were from independently isolated mutants which had never been in contact with proflavine. (b) Commercial DNA, and preparations containing DNA from sensitive organisms, did not produce this effect. (c) Active extracts from resistant strains were no longer active after treatment with DNAase. (d) The change to resistance in the recipient organisms is heritable. In view of the difficulty we have experienced in reproducing our results, some of these findings need to be confirmed. We believe, nevertheless, that we have demonstrated true transformation to proflavine resistance and that this shows

the genetic nature of the resistance in the organisms used as donors. These were both a 'trained' strain, and two strains isolated by replica plating. Our studies give support to the suggestion of a multistep pattern of proflavine resistance. A second-step mutant, derived without contact with drug, transformed to a lower resistance of about that of the first-step mutant. This recalls the similar observation of Hotchkiss (1951) with transformation to penicillin resistance in *Pneumococcus*. Our experiments support the use we have made of studies in cross-resistance as evidence of genetic change. Organisms transformed to proflavine resistance were cross-resistant to chloramphenicol in the same way as proflavine-resistant organisms which originated by mutation. Since it is generally held that transformation occurs of only one character at a time, this observation implies that the same genetic determinant can be responsible for resistance to both drugs.

Whilst we have worked with only one organism + drug system, it seems reasonable to imagine that the phenomena we have studied are not unique. Let us then consider the ways in which a sensitive culture of bacteria may develop resistance when challenged with a toxic agent such as a drug. We believe we have demonstrated five factors which may determine whether bacteria are able to grow in the presence of a drug: (1) alteration in toxicity of the drug due to environmental changes such as in pH; (2) the stage of development of the culture, which we have called the cultural phase; (3) a small increase in resistance of the organisms by adaptation; (4) spontaneous development of resistant mutants; (5) transformation of sensitive organisms to resistance by extracts of resistant organisms. We also have evidence, though not so strong, of clonal variation and of adaptation (induction) to high degrees of resistance. Excluding these, however, we can begin to see that there are many inter-related factors, occurring together or in sequence, which will determine whether a bacterial culture can grow in the presence of a drug. First, there are the factors related to the environment rather than to the bacteria. A given concentration of drug will prevent growth in particular stages of cultural growth, but not in others. In our experiments, we showed that one concentration of proflavine is inhibitory in the late logarithmic phase but not in the lag phase. Again, an inhibitory concentration of drug may be made non-inhibitory simply by increasing the acidity of the culture. In appropriate conditions, then, even sensitive bacteria may be able to survive and perhaps divide, giving an increasing chance of the organisms developing resistance.

This brings us to the second series of factors, related to the bacteria themselves—adaptation, mutation and transformation. If there are enough of the bacteria present, there is a chance that some spontaneously-occurring resistant mutants are also present. These might then grow in the presence of drug so as to give a new and resistant population. It is possible, however, that none, or only a few, are originally present. In these conditions, the phenomenon of adaptation may be of importance. Some of the bacteria, perhaps in particular stages of their division cycle, may be able to divide in small or moderate concentrations of the drug. Though, as we have seen, such resistance may be temporary, in the sense that it is lost when the drug is removed, it persists

so long as the drug is present. The longer the bacteria go on dividing, the greater the chance that spontaneous mutation to resistance will occur.

The existence of the phenomenon of transformation gives a bacterial population an additional chance of survival. For bacteria are constantly dying and being lysed by a variety of conditions not necessarily related to the presence of the toxic agent. If then a population happens to contain a moderate number of genotypically-resistant organisms, there is some chance that their death does not involve the complete loss of the property of resistance. The transfer of this property to sensitive organisms means that the population as a whole continues to contain some individual cells which can withstand the drug.

We are clearly not yet able to describe in any precise way the events which may lead to the preservation of a population of bacteria which is challenged by a drug. More especially, it would at this stage be premature to extend these considerations to natural situations, where different micro-environments may exist, and where other species of bacteria and their products would undoubtedly influence the interaction between bacteria and toxic agent. Nevertheless, the multiple factors determining resistance which we know to exist make it possible to begin to visualize these events in a way which is not possible in terms of the concept of a single factor-determining resistance, which has often been suggested for at least single bacteria + drug systems.

This paper completes the series. A preliminary account was given at a Ciba Foundation Symposium ('Drug Resistance in Micro-organisms', Churchill, London, 1957).

REFERENCES

- ALEXANDER, H. E. & LEIDY, G. (1950). Transformation type specificity of *H. influenzae*. *Proc. Soc. exp. Biol.*, N.Y., **73**, 485.
- ALEXANDER, H. E. & LEIDY, G. (1951). Determination of inherited traits of *H. influenzae* by desoxyribonucleic acid fractions isolated from type-specific cells. *J. exp. Med.* **93**, 345.
- ALEXANDER, H. E. & LEIDY, G. (1953). Induction of streptomycin resistance in sensitive *Haemophilus influenzae* by extracts containing desoxyribonucleic acid from resistant *Haemophilus influenzae*. *J. exp. Med.* **97**, 17.
- ALEXANDER, H. E. & REDMAN, W. (1953). Transformation of type specificity of Meningococci. *J. exp. Med.* **97**, 797.
- AUSTRIAN, R. (1952). Bacterial transformation reactions. *Bact. Rev.* **16**, 31.
- BOIVIN, A. (1947). Directed mutation in *Colon bacilli* by an inducing principle of desoxyribonucleic nature: its meaning for the general biochemistry of heredity. *Cold Spr. Harb. Symp. quant. Biol.* **12**, 7.
- EPHRUSSI-TAYLOR, H. (1951). Transformations allogenes du pneumocoque. *Exp. Cell. Res.* **2**, 589.
- GRIFFITH, F. (1928). The significance of pneumococcal types. *J. Hyg., Camb.* **27**, 113.
- HOTCHKISS, R. D. (1951). Transfer of penicillin resistance in pneumococci by the desoxyribonucleate derived from resistant cultures. *Cold. Spr. Harb. Symp. quant. Biol.* **16**, 457.
- HOTCHKISS, R. D. (1954). Cyclical behaviour in pneumococcal growth and transformability occasioned by environmental changes. *Proc. nat. Acad. Sci., Wash.* **40**, 49.

- MCCARTY, M. & AVERY, O. T. (1946). Studies on the chemical nature of the substance inducing transformation of pneumococcal types. *J. exp. Med.* **83**, 89, 97.
- MAYERS, V. L. & SPIZIZEN, J. (1954). The isolation of desoxyribonucleic acid from bacteriophages by an improved method. *J. biol. Chem.* **210**, 877.
- STUMPF, P. K. (1947). A colorimetric method for the determination of desoxyribonucleic acid. *J. biol. Chem.* **169**, 367.
- THORNLEY, M. & YUDKIN, J. (1959). The origin of bacterial resistance to proflavine. 2. Spontaneous mutation to proflavine resistance in *Escherichia coli*. *J. gen. Microbiol.* **20**, 365.

(Received 21 October 1958)