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The Origin of Bacterial Resistance to Proflavine

1. Training and Reversion in *Escherichia coli*

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SUMMARY: Several strains from *Escherichia coli* NCTC 8196 were isolated from discrete colonies. They were grown in liquid media in the presence of proflavine, and then in its absence. The distribution of resistance was measured by the number of organisms able to multiply at different concentrations of proflavine. Some strains were also grown in the presence of proflavine on plates, and the resistance of broth cultures from them was measured on gradient plates. The results cannot be explained on the assumption that the resistance was determined solely by specifically induced adaptation.

In this series of five papers we describe experiments designed to elucidate the mechanism by which cultures of *Escherichia coli* may acquire resistance to proflavine. We have chosen this substance because it was from work with it that Hinshelwood (e.g. 1946, 1949) has so strongly pressed the view that acquisition of drug resistance is through specifically induced adaptation rather than through mutation. One of us suggested that a third mechanism, clonal variation, may sometimes be involved (Yudkin, 1953). Many workers have thought that the reconciliation of these views lies in the existence of each of these mechanisms in different instances of acquired drug resistance. There is, however, a second possible way of reconciling the different mechanisms which have been put forward. This is that they may exist together in a single system of bacteria + drug, and that the mechanism which is found depends on the different experimental approaches made by different workers. We believe that we have been able to demonstrate this second possibility in the *Escherichia coli* + proflavine system. The work we describe in these papers brings evidence that, in this system, there is: (1) mutation and selection (Thornley & Yudkin, 1959); (2) a relatively small and reversible increase in resistance due to induction (adaptation) (Sinai & Yudkin, 1959*a*); (3) transformation of sensitive organisms to resistance by extracts containing deoxyribonucleic acid (DNA) from resistant organisms (Sinai & Yudkin, 1959*b*). Whilst we have not been able to demonstrate clonal variation, we believe that some of our results are best explained by assuming the existence also of this mechanism. We also have some evidence, though not yet conclusive, of a large and stable increase in resistance by induction.

In this paper, we describe experiments which were in the nature of a preliminary exploration of the problem. They were concerned with the changes in the resistance of cultures grown for varying periods in the presence of

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proflavine, and subsequently in its absence. They differ from the numerous experiments performed by Hinshelwood and his colleagues in two ways. These workers studied '*Bacterium lactis aerogenes*', and they measured changes in resistance by changes in the lag period in the presence of the drug. Most of our experiments were made with *Escherichia coli*, and in general we used the more usual criterion of resistance, the proportion of bacteria which can multiply in given concentrations of the drug.

METHODS

Organism. We began with a culture of *Escherichia coli* NCTC 8196. It was maintained on nutrient agar slopes kept at 5° and subcultured at intervals of about two months. Several strains were isolated from discrete colonies, and were used in the experiments. Each of these has been given a different reference number.

Media. Two liquid media were used: a peptone broth of 1% (w/v) peptone (Difco) in distilled water, and a nutrient broth containing 1% (w/v) peptone (Difco), 0.5% (w/v) sodium chloride and 0.3% (w/v) 'Yeastrel' (Brewers' Foods Supply Co. Ltd.) in tap water, both at pH 7.0. Nutrient agar was made by adding 2% (w/v) Japanese agar (British Drug Houses Ltd.) to the nutrient broth, and the pH value was adjusted to 7.4.

Incubation. All cultures were incubated at 37°.

Drugs. Proflavine was used as the neutral hemisulphate (British Drug Houses Ltd.); weighed portions were sterilized by heating to 100° for 1 hr., and dissolved in sterile medium. Chloramphenicol was dissolved directly in the medium, or used from concentrated stock solutions, without sterilization. No difficulty with contaminants was encountered.

Assessment of resistance. Resistance was measured in three different ways.

(1) *Dilution counts.* These were always made on overnight cultures grown without aeration at 37° in 1% (w/v) peptone broth with or without proflavine. A series of 1/10 dilutions in tap water was made, then 0.5 ml. portions from appropriate dilutions were transferred to assay tubes containing 4.5 ml. peptone broth, or peptone broth containing different concentrations of proflavine. Each dilution was made into five replicate tubes, and those which gave visible turbidity after 48 hr. incubation were noted. This period was chosen because only occasionally was an additional positive tube obtained on more prolonged incubation, and this did not significantly affect the results. The reading of the number of positive tubes with three different dilutions of the inoculum (i.e. 'significant number') was converted to the 'most probable number'/ml. inoculum by the use of McCrady's tables (Buchanan & Fulmer, 1928). Where the assay tubes contained only broth, this number represented the total viable count of the culture under test. Where drug was included in the assay tubes, the number obtained was the most probable number of organisms/ml. able to multiply at that particular drug concentration. By using a range of about six different concentrations, figures were obtained showing the distribution of

drug-resistant organisms in the original population, where resistance is defined as the ability to multiply at a particular drug concentration. Since the action of proflavine is largely bacteriostatic, bacteria survived at concentrations much higher than those which caused growth inhibition.

(2) *Spread plate counts.* Like the dilution counts, these were always made on overnight 37° cultures in liquid medium. Plates of nutrient agar, alone or with added proflavine, were prepared and thoroughly dried. Serial 1/10 dilutions of the culture in tap water were made, and 0.1 ml. portions of the appropriate dilutions were transferred by a capillary blood pipette to each plate, and spread over the surface with a bent glass rod. Colony counts were made after various periods of incubation, but unless otherwise stated the counts recorded were those at 48 hr. Little or no increase in numbers took place after this time. The colonies were of fairly uniform size, so that there was no difficulty in counting them.

(3) *Gradient plates.* These were prepared according to the method of Szybalski (1952). Volumes of 10 ml. very hot nutrient agar were poured into a series of plates. This was allowed to set with one edge of the plates supported on a glass rod. In order to ensure an even slope and a correct direction of the gradient, a line was drawn $\frac{1}{2}$ in. from one side of the plate, and on this line the plate was supported on the glass rod. The nutrient plates were now placed flat on the bench, and 10 ml. nutrient agar, containing the drug at the required concentration, poured on to the slope. To allow for diffusion of the drug and the drying of the agar, the plates were incubated overnight at 37° over CaCl_2 . The plates so formed thus had a high concentration of drug at one end, which fell continuously across the plate to the other end, where it was zero.

From each broth culture to be tested was taken a standard loopful, and one streak made across the plate from the side with the highest concentration of drug. Up to 7 streaks, of different samples, were made on each plate. The plates were incubated overnight or for 48 hr. The usual appearance after incubation was solid growth part of the way across the plate, from the side with the lowest drug concentration. Beyond this, there was sometimes very thin dispersed growth, and near the side with the highest concentration there appeared a few large colonies. The resistance of a culture assessed in this way was determined from the length of the streak of solid growth, since this represented the resistance of at least a high proportion of the organisms. Thus, if on a plate containing 100 μg . proflavine/ml. solid growth occurred across one-third of the plate, the culture may be said to be resistant to *c.* 33 μg ./ml.

The three methods were used for different types of experiment. In those concerned with training and reversion in liquid media, we desired to estimate the ability to multiply in liquid media. For these experiments therefore we used dilution counts. Similarly, for training on solid media, spread plate counts were used. Gradient plates were used for a rapid but less detailed assessment of the resistance of a large number of cultures.

The resistance measured by spread plate counts was apparently higher than that measured by dilution counts. This difference was some 20% with the

sensitive strain assayed at low concentrations, but increased to some three-fold for the strains of high resistance. It is possible that this may have been associated with a different solubility of proflavine in broth than in agar at high concentrations. The reproducibility of the spread-plate method is indicated in the next paper (Thornley & Yudkin 1959, figs. 1 & 2).

RESULTS

Training and reversion in liquid media

Each experiment was begun by plating the stock culture on to nutrient agar, incubating overnight at 37°, picking off a discrete colony into 5 ml. broth and again incubating overnight. One loopful of this culture was then transferred into 5 ml. broth without or with a particular concentration of drug. Daily transfers were continued into broth with or without the drug for as long as was required for each experiment. At intervals, resistance was estimated by dilution counts on an 18 hr. culture.

Training. The resistance of sensitive strain 23 was tested immediately after isolation from a discrete colony, that is, after one growth period in broth, and again after 7 and 12 subcultures in broth (Fig. 1). In this and similar experiments, there was a tendency in later subcultures for an increase in numbers of the more resistant organisms, which would grow for example in 10 µg. proflavine/ml.

The effect of training was studied after one subculture in broth. An inoculum was made into broth with 5 µg. proflavine/ml., and dilution counts made after 1, 7 and 13 subcultures in the same concentration (Fig. 2). There was a considerable increase in numbers of resistant organisms, for example to 25 µg./ml., even after 1 subculture in drug, and a progressive increase with further subcultures.

Training in higher concentrations of proflavine was carried out after 1 subculture in broth, 2 subcultures in 5 µg./ml., and then repeated subcultures in 33 µg./ml. Measurements of resistance after 1, 7 and 15 subcultures in the higher concentration showed as before a great increase in numbers of highly resistant organisms, and progressive increase with further subcultures (Fig. 3). As well as strain 23, other strains were tested similarly and gave the same general picture but with differences in detail. In all instances, even one subculture in proflavine 5 µg./ml. or 33 µg./ml. gave rise to a high proportion of organisms able to grow at much higher concentrations than these.

Reversion after short training in 5 µg. proflavine/ml. Strain 22 was grown once in 5 µg. proflavine/ml. and then repeatedly in broth. After 5 subcultures there was reversion to a low resistance only a little higher than that of the original sensitive strain (Fig. 4). Very little further reversion occurred after 80 further subcultures.

Strain 20 was studied in a similar way. This gave a higher degree of resistance after 1 subculture in 5 µg. proflavine/ml. than did strain 22 after 2 subcultures. Subsequent growth in the absence of drug also gave much less reversion than was given by strain 22 (Fig. 5). There was slight reversion after

Fig. 1

Fig. 2

Fig. 1. Distribution of resistance in sensitive culture of *Escherichia coli* 23, measured by dilution counts. These gave number of organisms/ml. capable of growing at given proflavine concentrations in broth. ---- = 1 subculture; — = 7 subcultures; = 12 subcultures.

Fig. 2. Effect of growth in 5 µg. proflavine/ml. on resistance of *Escherichia coli* 23, measured by dilution counts. ---- = 1 subculture in proflavine; — = 7 subcultures in proflavine; = 13 subcultures in proflavine.

Fig. 3

Fig. 4

Fig. 3. Effect of growth in 5 µg. proflavine/ml. for 2 subcultures, and then 33 µg./ml. for varying number of times, on resistance of *Escherichia coli* 23, measured by dilution counts. ---- = 1 subculture in 33 µg. proflavine/ml.; — = 7 subcultures in 33 µg. proflavine/ml.; = 15 subcultures in proflavine 33 µg./ml.

Fig. 4. Reversion after training. The resistance, measured by dilution counts, of *Escherichia coli* 22 grown once in 5 µg. proflavine/ml., then in broth. ---- = 1 subculture in proflavine; — = 1 subculture in proflavine, then 5 times in broth; = 1 subculture in proflavine, then 85 times in broth.

Fig. 5. Reversion after training. The resistance, measured by dilution counts, of *Escherichia coli* 20 grown once in 5 µg. proflavine/ml., then in broth. ---- = grown in broth (original sensitive culture); ---- = 1 subculture in proflavine; — = 1 subculture in proflavine, then 4 times in broth; = 1 subculture in proflavine, then 15 times in broth.

4 subcultures, and a little more after 15, but the resistance was still much higher than that of the original sensitive strain 20. Thus, complete reversion did not always occur on subculture in broth even when only 1 subculture in the presence of 5 μg . proflavine/ml. had been used for 'training'.

Reversion after prolonged training in 5 μg . proflavine/ml. Strain 22 was subcultured 12 times in 5 μg . proflavine/ml. and then repeatedly in its absence. There was a gradual decline in resistance (Fig. 6). Nevertheless, reversion was far from complete after 232 subcultures in the absence of drug. A very similar picture emerged after training for 84 subcultures in 5 μg . proflavine/ml. and subsequent subculturing in the absence of drug (Fig. 7).

Fig. 6

Fig. 7

Fig. 6. Reversion after prolonged training. The resistance, measured by dilution counts, of *Escherichia coli* 22 grown 12 times in 5 μg . proflavine/ml., then in broth. ---- = 12 subcultures in proflavine; — = 12 subcultures in proflavine, then 42 times in broth; = 12 subcultures in proflavine, then 232 times in broth.

Fig. 7. Reversion after prolonged training. Resistance, measured by dilution counts of *Escherichia coli* 22 grown 84 times in 5 μg . proflavine/ml., then in broth. ---- = 84 subcultures in proflavine; — = 84 subcultures in proflavine, then 24 times in broth; = 84 subcultures in proflavine, then 84 times in broth.

Reversion after training in 33 μg . proflavine/ml. Strain 22 was grown for 3 subcultures in 5 μg . proflavine/ml. and then once in 33 μg /ml. Subsequent subculture in the absence of drug gave a gradual decrease in the number of highly resistant organisms, but even after 175 subcultures without drug, the degree of reversion was small (Fig. 8). After more prolonged training, for 14, 29 or 84 subcultures in 33 μg . proflavine/ml., the pattern of reversion was similar to that seen after only one subculture in this concentration (e.g. Fig. 9).

Training on solid medium

In these experiments, broth cultures of sensitive strain 36 were spread on to plates containing varying concentrations of proflavine, over a range which prevented the growth of most of the organisms. From the colonies formed after incubation, inocula were transferred into broth, the tubes incubated overnight, and a loopful of the culture streaked on gradient plates.

Experiment 1. The sensitive strain was spread on 4 plates containing proflavine 3.3, 4, 5 and 6.7 μg /ml., respectively. The number of colonies which grew on each was noted at 24 and 48 hr. There was only a slight increase in numbers

between these times (Table 1A). Both at 24 hr. and at 48 hr., 7–10 colonies were picked off each plate, grown separately overnight in broth, then streaked on gradient plates containing proflavine 10 $\mu\text{g./ml.}$ or 67 $\mu\text{g./ml.}$

Most of the colonies grew either one-third and one-half across the plate with 10 $\mu\text{g./ml.}$ (low resistance), or right across the plate with 67 $\mu\text{g./ml.}$ (high resistance). One colony showed an intermediate resistance, growing about one-half across the plate with 67 $\mu\text{g./ml.}$ Finally, some colonies gave growth across the plate with low proflavine concentration but only scattered colonies on the plate with high concentration. These are called colonies of unclassified resistance, and will be discussed below.

Fig. 8

Fig. 9

Fig. 8. Reversion after training. The resistance, measured by dilution counts, of *Escherichia coli* 22 grown 3 times in 5 $\mu\text{g. proflavine/ml.}$, then once in 33 $\mu\text{g./ml.}$, then in broth. ---- = 1 subculture in 33 $\mu\text{g. proflavine/ml.}$; — = 1 subculture in 33 $\mu\text{g. proflavine/ml.}$, then 22 times in broth; = 1 subculture in 33 $\mu\text{g. proflavine/ml.}$, then 175 times in broth.

Fig. 9. Reversion after prolonged training. The resistance, measured by dilution counts, of *Escherichia coli* 22 grown once in 5 $\mu\text{g. proflavine/ml.}$, then 84 times in 33 $\mu\text{g./ml.}$, then in broth. ---- = 84 subcultures in 33 $\mu\text{g. proflavine/ml.}$; — = 84 subcultures in 33 $\mu\text{g. proflavine/ml.}$, then 24 times in broth; = 84 subcultures in 33 $\mu\text{g. proflavine/ml.}$, then 84 times in broth.

After 24 hr. of incubation on the gradient plate, 19 colonies showed low resistance and 11 high resistance (Table 1B). After 48 hr. the proportion of colonies of low resistance fell, there being 6 of these, 1 of intermediate resistance and 30 of high resistance. Since very few new colonies had developed between 24 and 48 hr., the increased proportion of resistance amongst the colonies must have developed during the further period of incubation. This change in proportion is discussed below.

Experiment 2. A sensitive culture was spread on to plates containing 10 $\mu\text{g. proflavine/ml.}$ The colonies formed represented 1 in 2×10^6 of the organisms of the inoculum and of these colonies 79 were picked off and grown overnight in broth. Streaks were then made on gradient plates containing 100 $\mu\text{g. proflavine/ml.}$ After 24 hr. of incubation, 76 of the cultures gave streaks representing resistance to more than 100 $\mu\text{g. proflavine/ml.}$, and the other 3 gave streaks representing resistance to about 80 $\mu\text{g./ml.}$

Table 1. *Effect of training of Escherichia coli 36 on solid medium containing proflavine*

A. Number of colonies appearing on spread plates containing varying concentration of proflavine, inoculated from broth culture.

Proflavine concentration on which colonies were grown ($\mu\text{g./ml.}$)	Colony count/ml. inoculum after incubation for	
	24 hr.	48 hr.
0	7.8×10^8	7.8×10^8
3.3	8.1×10^7	8.2×10^7
4	4.4×10^7	4.5×10^7
10	3.6×10^2	4.4×10^2

B. Between 7 and 10 colonies growing on each proflavine concentration at 24 and 48 hr. were tested on gradient plates with proflavine 10 and 67 $\mu\text{g./ml.}$

Proflavine concentration from which colonies obtained ($\mu\text{g./ml.}$)	Number of colonies of indicated resistance*							
	24 hr.				48 hr.			
	3-5 $\mu\text{g./ml.}$	33 $\mu\text{g./ml.}$	> 67 $\mu\text{g./ml.}$	Un-classified	3-5 $\mu\text{g./ml.}$	33 $\mu\text{g./ml.}$	> 67 $\mu\text{g./ml.}$	Un-classified
3.3	7	0	0	3	2	0	7	1
4	7	0	1	2	4	0	4	1
5	5	0	1	1	0	1	9	0
10	0	0	9	0	0	0	10	0
Total	19	0	11	6	6	1	30	2

* Expressed as approximate concentration of proflavine at end of streak on gradient plate.

Note. Colonies grown in absence of proflavine always give streaks indicating resistance of 3-5 $\mu\text{g./ml.}$

DISCUSSION

The experiments of training and reversion in liquid media were similar to those reported by Hinshelwood and his colleagues, except that we measured the resistance of the resulting populations more exactly than by Hinshelwood's method of lag periods. Hinshelwood reported two main conclusions: (i) the effect of training was to give a resistance of about that represented by the concentration of drug to which the culture was trained; (ii) the rate of reversion was proportional to the period of training, being very rapid after short periods of training, and slower but still ultimately (nearly) complete after prolonged periods of training.

We cannot confirm either of these conclusions. In our experiments, a considerable proportion of organisms reached a resistance much greater than that represented by the concentration of drug used for training. Further, even after only 1 subculture in drug, reversion was not always complete after 14 or 15 subcultures in absence of drug. After a few transfers in the presence of only 5 $\mu\text{g.}$ proflavine/ml., 232 subcultures in the absence of drug did not give complete reversion. Similarly, after 1 subculture in the presence of 33 $\mu\text{g./ml.}$,

175 subcultures in the absence of drug did not give complete reversion. There was almost no difference in the rate of reversion after 12 subcultures in the presence of drug and after 84 subcultures. According to Hinshelwood's model, one would expect a shift in enzyme balance, characteristic of either sensitive or of resistant (adapted) organisms, to take place with similar ease in either direction. We have seen, however, that only short periods in the presence of drug give relatively high resistance, whilst subsequent quite long periods in its absence do not give complete reversion to sensitivity.

One could more readily explain the results on the assumption that spontaneous mutation to drug resistance occurs, and the mutant organisms are then selected in the presence of drug. We can then also explain the variation which we have observed in the behaviour of different strains, or more strictly different one-colony isolates from the parent strain, *Escherichia coli* NCTC 8196. First, the mutants will appear at random times. Secondly, different strains may well give different rates of mutation. Thirdly, the sensitive and the mutant organisms may have different growth rates. We shall in subsequent papers show that spontaneous mutations do occur, and that other mechanisms may also play a part in the overall behaviour of cultures grown in the presence and absence of the drug.

In three experiments on training on solid media such as we have described, 170 strains were examined from colonies grown on different amounts of proflavine in agar. Of these, 26 had a resistance similar to that of the original strain, 132 a very much higher resistance, and 12 gave scattered colonies. These last are discussed below. According to Hinshelwood's view, we should have expected that most of the organisms would have become adapted to about the degree of resistance represented by the concentration of proflavine on which the colonies had been grown. Against this view there are the following facts: (i) about 15 % of the colonies showed no increase of resistance; (ii) no colonies gave streaks indicating resistance to the concentration of proflavine on which they had been grown; (iii) most of the colonies gave streaks indicating an increase of resistance of at least ten times. It might be supposed that these streaks were produced by a combination of a large number of organisms adapted in the Hinshelwood manner, and a small number of highly resistant organisms derived, for example, from mutants. If this were so, the proportion of highly resistant organisms must be more than 10 %, since we have found that a smaller proportion gives a streak with scattered colonies. Thus, even if adaptation occurs, its effect is masked by the presence of at least 10 % of highly resistant organisms.

The few streaks which showed scattered colonies almost certainly represented a mixture of sensitive and resistant organisms. An exactly similar type of streak is produced from an artificially prepared mixture of organisms from sensitive and resistant strains. Such a mixture would occur if a mutation took place on the drug plate during the growth of the original colony before it was picked off for testing. As these colonies were developing in the presence of proflavine, it would be expected that the mutant organisms would in due course outgrow the sensitive organisms of the colony. This would account for

our finding that the proportion of colonies showing high resistance increased between 24 and 48 hr. of incubation on the drug plate.

It is apparent that experiments of this nature do not allow any certain conclusions to be drawn as to the origin of the resistant organisms which arise during training. We feel, however, that we can perhaps draw one negative conclusion, which is that the results cannot easily, if at all, be explained solely on the basis of the Hinshelwood model of specifically induced adaptation. Our subsequent investigations therefore were designed to examine under more critical experimental conditions the possible modes of origin of proflavine resistance in *Escherichia coli*.

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