The Origin of Bacterial Resistance to Proflavine

4. Cycles of Resistance in *Escherichia coli* and their Bearing on Variations in Resistance in Cultures

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SUMMARY: Small amounts of proflavine were added at intervals of 30 min. to growing cultures of *Escherichia coli*. Additions during the logarithmic phase gave an increase of resistance of two- or threefold in a high proportion of organisms. Additions in the lag or late logarithmic phase gave no such increase, although the resistance of the organisms themselves was higher in these phases. Whether organisms were able to grow in the presence of proflavine, therefore, depended not only on their resistance but on the conditions in the culture medium. The increase in proflavine resistance, which occurred when drug was added to growing cultures, was not accompanied by increase of cross-resistance to other drugs. The resistance was lost on growth in the absence of drug. For these reasons, the increase is held to be a phenotypic adaptation. There was also an increase in the number of organisms with a high resistance, of the order of that of mutants. These organisms showed cross resistance with other drugs.

Partial synchronization of division was achieved by the temporary cooling of cultures. These synchronized cultures showed cycles of division of about 30 min. They also showed cycles of resistance of c. 20 min. Evidence is presented for the view that the organisms undergo cycles of varying adaptability to proflavine resistance. It is suggested that this variation in adaptability can explain the range of resistance found in an ordinary sensitive culture. It can also explain the effect of proflavine additions in raising the resistance of a high proportion of organisms in a growing culture.

Our experiments with '*Bacterium lactis aerogenes*' showed that rapid adaptation to high degrees of proflavine resistance did not occur (Sinai & Yudkin, 1959). There was, however, some small increase in resistance in the presence of the drug. In the present paper we describe experiments which demonstrate that, in appropriate conditions, a similar small increase in resistance occurs with *Escherichia coli*. The results have a bearing on the variation in resistance found in the organisms of a sensitive culture. This variation is seen for example in the usual 'survivor' curves where most organisms are found to be prevented from forming colonies over a fairly narrow range of drug concentration, but some are inhibited by lower concentrations and some only by higher concentrations (Fig. 1; see also Thornley & Yudkin (1959), Figs. 1 and 2).

There is at present no explanation of this phenomenon, which has experimental backing. The proponents of the 'mutation-selection' theory of the origin of drug resistance only speak of 'phenotypic variation' (Cavalli, 1952). The proponents of the adaptation theory speak of two opposing forces, the

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lethal action of the drug and the adaptation of the organism towards resistance. Neither attempt an explanation of why there should be any variation between organisms in either their sensitivity or in their ability to adapt. The theory of clonal variation (Yudkin, 1953) suggests that the variation in resistance in a culture arises from asymmetrical division, but whilst there is evidence that this occurs (Hughes, 1952, 1955), it has not been proved that it is responsible for the continuous curve of variation of resistance in a culture. A possible explanation might lie in a variation in resistance with physiological age of the cells, i.e. from division of the parent cell to division of the daughter cells. Since a growing culture would have organisms of the whole range of physiological age, it would then have the whole range of resistance.

Fig. 1. Distribution of resistance in a sensitive culture of *Escherichia coli* 36. Number of colonies which appear on plates with different concentrations of proflavine.

We have shown that there is a number of mutational steps to increased proflavine resistance in *Escherichia coli* (Thornley & Yudkin, 1959). One can imagine that on this is superimposed a continuous variation of resistance of a narrower range for both the sensitive organisms and for each of the mutations; this would give a much more continuous range of resistance. We confined ourselves therefore to a study of the range of resistance expressed in the normal sensitive culture, and assume that whatever we were able to demonstrate as to its origin probably applies to the mutants of successively higher degrees of resistance. The first series of experiments were designed chiefly to test whether it was possible to increase the resistance of a sensitive culture in a continuous manner. The second series of experiments tested the possibility that the variations in resistance within a culture were due to differences in physiological age of the bacteria constituting the culture.

In experiments on the effect of small additions of proflavine on resistance of a sensitive culture, proflavine was added at intervals to cultures of *Escherichia coli* at different periods of growth. The amounts added were such as not to inhibit growth completely. The intervals were roughly equal to the mean

generation time. By this means, we sought in a relatively short time to produce a culture of significantly higher resistance than the original culture. It was of course necessary to ensure that such an increased resistance, when it occurred, was not due to a multiplication of pre-existing mutants.

We investigated: (i) The degree of increase of resistance. This was done usually by the gradient plate technique, which is very sensitive. Sometimes we also measured the number of organisms of increased resistance, by dilution counts on solidified medium containing proflavine. By 'increased resistance', we mean a resistance intermediate between that of the sensitive cells, and that of first-step mutants. (ii) The degree of inhibition by the drug. This was measured both as an inhibition of the increase of mass of organisms, and as an inhibition of the increase in number of viable organisms. (iii) The number of organisms of high resistance at the beginning and end of each experiment. These were assumed to be mutants. (iv) The stability of the organisms of increased resistance. (v) The resistance to other drugs (cross-resistance).

Experiments were also designed to produce cultures in which divisions were synchronized, and to test the resistance of the organisms at various times. By this means we hoped to detect changes in resistance in organisms of roughly the same physiological age during the cycle from one division to the next.

METHODS

Details of the organism *Escherichia coli* strain 36 and of the drugs are given in the previous papers in this series (Sinai & Yudkin, 1959; Thornley & Yudkin, 1959).

Growth of organisms. The cultures were made in 50 ml. nutrient broth held in 250 ml. Buchner flasks. The inoculum was 0.2 ml. over-night culture in nutrient broth. The flasks were kept in a water bath at 37°. In most instances the cultures were aerated by an air compressor through a manifold. A syringe with a polythene extension was used to take samples and to make drug additions, without disturbing the growing cultures. At the end of each experiment, routine checks were made that the cultures had remained neutral and that they were not contaminated by other organisms.

Measurements of growth. Growth in mass was estimated by the optical density as measured by the Spekker spectrophotometer. Growth in numbers of viable bacteria was measured in agar plates. Serial dilutions of the cultures were made and 0.5 ml. mixed with 9.5 ml. nutrient agar. Plates were poured in triplicate.

Measurement of drug resistance. This was done by dilution counts and by gradient plates (Szybalski, 1952). For dilution counts, proflavine pour-plates proved unsatisfactory since the resistance to proflavine is higher in semi-aerobic conditions. We therefore spread 0.1 ml. of cultures of various dilutions on the surface of a series of plates of solid medium containing proflavine at various concentrations. In different experiments there was appreciable variation in the number of resistant organisms surviving at given concentrations of proflavine. This seems to be due to slight differences in the medium

which affected the inhibitory action of the low concentrations of proflavine which were being used. For this reason, we have only compared the results within each group of experiments in which the same batch of nutrient agar was used.

For measurements with gradient plates we were concerned to avoid the effect of differences in the gradients of drug concentration in different plates. We have therefore always compared the resistance of cultures from streaks on the same plates. If, then, the treated culture gave a streak on this plate 50 % longer than that given by the untreated culture, we call this an increase in resistance by a factor of 1.5.

Definitions of degrees of resistance. Our normal untreated culture of strain 36 was sensitive to about 10 μ g. proflavine/ml., that is, only one or two organisms in 10^6 gave colonies in this concentration. These organisms proved to be the first-step mutants demonstrated by Thornley & Yudkin (1959). Up to a concentration of about 7 μ g. proflavine/ml., there was a continuously decreasing number of organisms able to give colonies. In such a normal culture, then, the majority of organisms did not give colonies on, for example, 5 μ g./ml. Sometimes after treatment a culture was able to give a much higher proportion of colonies at such a concentration of proflavine, but not necessarily a higher number at 10 μ g./ml., at which first-step mutants did. We refer to such a culture as one of 'higher resistance'. In general then this culture had a resistance greater than that of the original culture but lower than that of a culture of first-step mutants.

Synchronization of division. We used the technique of temporary cooling described by Hotchkiss (1954) in his experiments on *Pneumococcus*. Cooling was carried out either rapidly or slowly. In the first method, the culture was placed in iced water until it reached the required temperature, and then in a water bath at that temperature. Slow cooling was carried out by placing the culture directly into a water bath at the required temperature. A variety of times and temperatures of cooling were used, and the cultures were of various ages. No improvement in synchronization was achieved by the second period of cooling. In appropriate conditions, quite good synchronization was achieved. An example of the results is given in Fig. 2. Here cultures were cooled at different intervals after inoculation. They were placed in iced water for 2 min., then held at 12° for 15 min., and finally returned to 37°. Counts were made before and immediately after cooling, and then at intervals of 10 min.

The best synchronization was achieved in this experiment when cooling began 70 min. after inoculation. On returning to 37°, there was a burst of divisions in the next 10 min., followed by a division cycle of 30 min. Half of the divisions occurred in the first 20 min., and the other half in the next 10 min. A second division cycle followed.

It may be added that we have frequently determined viable counts, also at 10 min. intervals, of cultures which had not been cooled. None of these experiments showed the step-wise increase in numbers shown, for example, in Fig. 2, B, C and Fig. 8, A.

Fig. 2. Growth of a synchronized culture of *Escherichia coli* 36. Synchronization of division by cooling to 12° for 15 min., begun at different times after inoculation. Growth measured by viable counts. Cooling started at min. after inoculation: A, 120; B, 90; C, 70; D, 60. Time from returning the flasks to 37° after cooling.

RESULTS

Effect of small additions of proflavine on the resistance of a sensitive culture

Several experiments were performed in which proflavine was added in small amounts at 30 min. intervals to a growing sensitive culture. The first additions usually brought the concentration of proflavine to 1 $\mu\text{g./ml.}$ Subsequent additions were constant in each experiment, but differed in different experiments from amounts which increased the concentration by 0.15 $\mu\text{g./ml.}$ with each addition, to amounts which increased it by 0.4 $\mu\text{g./ml.}$ We give details of one such experiment.

Experiment 1. Fifty ml. nutrient broth in a 250 ml. Buchner flask were inoculated with 0.2 ml. of a 17 hr. culture. After incubation for 2 hr., the culture was divided into two parts, and incubation continued. To one flask, proflavine was added to 1 $\mu\text{g./ml.}$ At 30 min. intervals, further additions of proflavine were made, each increasing the concentration by 0.3 $\mu\text{g./ml.}$, so that a final concentration of 3.7 $\mu\text{g./ml.}$ was achieved after 270 min. The second flask had no added proflavine, and served as control. Samples were taken for measurements of mass growth by optical density, and for measurements of resistance. The latter was done by streaks on gradient plates containing 13 $\mu\text{g.}$ proflavine/ml., and by counts of the number of organisms which gave colonies on plates containing proflavine at 6.6, 10 and 20 $\mu\text{g./ml.}$ The

results of this experiment are shown in Table 1. The results of a similar experiment are also shown in Fig. 3, in which proflavine was added in different amounts to two flasks.

Table 1 shows that by the end of the first experiment the resistance as measured on gradient plates increased by a factor of 3 (i.e. an increase of 200 %) when proflavine was added, and by a factor of 1.3 (an increase of 30 %) in the control culture without proflavine. This increased resistance was shown

Fig. 3. Effect of addition of proflavine to a growing culture of *Escherichia coli* 36 on increase of resistance. Curve 1. Proflavine added gradually. First addition to 1 $\mu\text{g./ml.}$, subsequent additions every 30 min. increasing concentration by 0.25 $\mu\text{g./ml.}$ to final 2.75 $\mu\text{g./ml.}$ Curve 2. Proflavine added gradually. First addition to 1 $\mu\text{g./ml.}$, subsequent additions every 30 min. increasing concentration by 0.4 $\mu\text{g./ml.}$ to final 3.8 $\mu\text{g./ml.}$ Curve 3. No proflavine added. A, Growth, measured by turbidity; B, resistance measured by gradient plates. Time from 120 min. after inoculation.

also by the number of organisms which gave colonies on plates with 6.6 $\mu\text{g.}$ proflavine/ml. At the beginning of the experiment, there were 30 such colonies from 1 ml. of culture. At the end, the culture with proflavine gave 3200 normal sized colonies and 2×10^6 small colonies; the control culture gave 1000 normal sized colonies, but no small colonies.

The counts on the plates with 10 and 20 $\mu\text{g./ml.}$ suggest that the organisms which gave these small colonies could not do so at the higher concentration of proflavine. The colonies which did appear at these concentrations were of normal size. We believe that these arose from the multiplication of pre-existing first-step mutants. Their number was about five times greater from the culture to which proflavine had been added from the control culture. This was probably due to an advantage in growth of the mutant organisms in the presence of proflavine.

The increased length of streaks on the gradient plate, and the appearance of large numbers of organisms which gave colonies on plates with 6.6 $\mu\text{g.}$ proflavine/ml., showed that the addition of small amounts of proflavine to a growing culture of *Escherichia coli* in the logarithmic phase gave an increase in resistance to a degree below that of the first-step mutant. We believe, in fact, that the increased length of the streaks was caused chiefly, if not entirely, by the organisms with increased resistance.

Table 1. *Effect of addition of proflavine on resistance of Escherichia coli 36*

Comparison of increased resistance as measured on proflavine gradient plates, and by dilution counts on proflavine plates. Flask 1: gradual proflavine additions, to final concentration of 3.6 $\mu\text{g./ml.}$ Flask 2: no proflavine added.

Flask	Time* (min.)	Proflavine present ($\mu\text{g./ml.}$)	Viable count, (colonies/ ml.)	On proflavine (count/ml.)			Factor increase in resistance (gradient plates)
				6.6 $\mu\text{g./}$ ml.	10 $\mu\text{g./}$ ml.	20 $\mu\text{g./}$ ml.	
1	0	0	3×10^7	3×10	< 10	< 10	—
	120	2	2×10^8	1×10^8 3×10^8 s	3.6×10^8	2.5×10^8	—
	270	3.6	1×10^8	3.2×10^8 2×10^8 s	1.5×10^8	1.5×10^8	3
2	0	0	2.5×10^7	3×10	< 10	< 10	—
	120	0	6.4×10^8	2.5×10^8	1.5×10^8	1.4×10^8	—
	270	0	2×10^9	1×10^8	3×10^8	2.8×10^8	1.8

* Time from 135 min. after inoculation.

s = small colonies.

The inhibition of growth by the added proflavine was to some extent an inhibition of increase in bacterial mass and to a greater extent an inhibition of division. Thus this experiment showed that, between 120 min. and 270 min., there was a 21% inhibition of growth in mass, and a twofold decrease in viable count division; examination of smears showed many organisms forming filaments.

Experiment 2. Growth in this experiment was measured by optical density and by viable counts. Resistance was measured both by gradient plates with 14 $\mu\text{g.}$ proflavine/ml. and by plates with proflavine 6.6, 10 and 20 $\mu\text{g./ml.}$

A culture 135 min. after inoculation was divided into two flasks. One acted as control. To the other was added 1 $\mu\text{g.}$ proflavine/ml., and further 30 min. additions of 0.33 $\mu\text{g./ml.}$ Samples were taken just before the first addition, after 2 hr. and after 4.5 hr.

In the control, the growth rate decreased after 4.25 hr. from inoculation. By 6.75 hr., the number of generations was 4.2 as judged by optical density, and 6.6 as judged by viable count.

In the flask with added proflavine, the number of generations at 6.75 hr. was 3.1 from optical density and 2.7 from viable count. In the period from 4.25 to 6.75 hr., the optical density increased twofold, but the viable count

decreased. By the end of the experiment, the ratio of increase in mass to increase in number of viable organisms was 0.23 in the absence of proflavine and 2.7 in its presence. This suggests that proflavine inhibited cell division more than increase in mass. Microscopical examination showed that the organisms in the control culture became smaller during incubation, whilst those in the presence of proflavine became much larger.

Gradient plates showed again an increase of resistance by 30 % in the absence of proflavine, and by 200 % in its presence. On plates with 6.6 μ g. proflavine/ml., the control culture gave about 1000 normal sized colonies/ml.; the culture with added proflavine gave 3000 such colonies, but also 2×10^6 small colonies. On plates with proflavine 10 or 20 μ g./ml., only normal sized colonies appeared: about 250/ml. from the control culture and 1200/ml. from the culture with added proflavine. We shall refer later to the possible origin of the organisms which gave rise to small colonies on the proflavine plates.

Effect of physiological age of culture

Experiment 3. In this experiment eight cultures were used. To five of them proflavine additions were made in the way already described; the other three cultures acted as controls. The proflavine additions were begun at different times after inoculation, from 30 min to 5.25 hr. Thus they covered the range from the lag phase to the late logarithmic phase.

As in our earlier experiments, proflavine additions begun early in the logarithmic phase (flask 3) caused an immediate rise in resistance, as measured by streaks on gradient plates (Fig. 4). Proflavine added in the late logarithmic phase (flasks 4 and 5) did not cause any increase in resistance.

When proflavine additions were begun in the lag phase there was no increase in resistance for the first 4 hr. (flasks 1 and 2). After 4.25 hr. there was a rapid rise in resistance (flask 2). In flask 3, where additions were begun after 2.5 hr., there was an immediate rise in resistance. Thus by 4.5 hr. after inoculation there was an increase in resistance with additions begun at 2.5 hr., but not with additions begun at 45 min. At this time, however, there was about the same concentration of proflavine in both flasks, and the rate of growth of the culture was also very similar. Yet when, after the delay, the resistance began to rise in flask 2, it soon reached that in flask 3.

Again, as in previous experiments, those flasks which showed an increase in resistance measured by length of streaks on gradient plates also showed, when compared with the controls, about a fivefold increase in the number of organisms which gave rise to colonies on plates with 10 μ g. proflavine/ml. Flasks 4 and 5, which showed no increase in resistance on gradient plates, also showed no relative increase in number of these organisms.

This experiment emphasizes that the ability of organisms to multiply in the presence of proflavine is due to a combination of two factors: the inherent resistance of the organisms, and the conditions in the medium. Thus organisms transferred from medium at the late logarithmic phase consistently gave streaks about 30 % longer than those given by organisms in the lag phase. The organisms were therefore more resistant in the late logarithmic phase.

Yet proflavine added to cultures showed no inhibition of division in the lag phase, but considerable inhibition in the late logarithmic phase. Thus, in presence of their culture medium, the organisms showed a decreased resistance, though their inherent resistance was higher.

Fig. 4. Effect of addition of proflavine to cultures of *Escherichia coli* 36 at different phases of cultural growth, on increase of resistance as measured by gradient plates. Curve 1. Additions start in lag phase, 30 min. after inoculation. Curve 2. Additions start in lag phase, 45 min. after inoculation. Curve 3. Additions start in early logarithmic phase, 2.5 hr. after inoculation. Curve 4. Additions start in late logarithmic phase, 4 hr. after inoculation. Curve 5. Additions start in late logarithmic phase, 5.25 hr. after inoculation. Curve 6. Control for 1. Curve 7. Control for 2, 3, 4 and 5. Curve 8. Control for 4 and 5. - - - - = Proflavine additions; ——— = no proflavine.

The stability of the increased resistance

Experiment 4. From several of the experiments where proflavine was added to growing cultures, samples of 0.05 ml. were inoculated into 5 ml. broth at the beginning and end of the experiment. After overnight incubation the resulting cultures were streaked on proflavine gradient plates. No difference in length of streak was found between cultures made at the beginning or end of the experiment, from either the control cultures or those with added proflavine. Thus, the increase in resistance found after the addition of proflavine to cultures in the logarithmic phase did not persist after subculture in the absence of drug.

At the end of one of the earlier experiments, inocula were taken from the beginning and from the end of the streak made on a gradient plate with 10 μ g. proflavine/ml., both from the control culture and from the culture with added proflavine. In this experiment, the streak from the control culture showed during the experiment an increase in resistance by a factor of 1.2, and that from the culture with proflavine of 2.1. The inocula were streaked on to gradient plates with 20 μ g. proflavine/ml.

Inocula from either end of the streaks made with the culture with added

proflavine now gave streaks which grew right across the plate containing 20 μg . proflavine/ml. With the control culture there was an appreciable difference in the streaks made from inocula from the beginning (low proflavine concentration) and end (high proflavine concentration) of the original streak. Those from the beginning of the streak grew right across the plate. But those from the end of the streak gave only a short solid streak, and a few scattered colonies towards the end of high concentration.

A similar experiment was made with the small and the normal sized colonies which had appeared on a plate with 6.6 μg . proflavine/ml., with samples of the culture to which proflavine had been added. They were streaked on a gradient plate with 50 μg . proflavine/ml. The small colonies gave a thin dispersed growth across the plate; the normal sized colonies gave a thick solid growth across the plate.

The simplest explanation of these results is that small amounts of proflavine allowed an adaptation to a low degree of resistance. Unadapted organisms, from the control culture, can adapt to grow at a low concentration of the drug, but not to grow at a high concentration. Thus, the growth at the end of the streak represented organisms that were of poor viability, for when streaked again they give poor growth. Adapted organisms from the culture with proflavine can go on growing at a higher concentration. In either instance, the growth on the low or the high concentration in plates gave rise to a large number of highly resistant 'mutant' organisms within the colony. As would be expected, there were fewer of these in the small colonies than in the large, so that there was a difference in the streaks found on 50 μg . proflavine/ml. We believe that the organisms which gave small colonies on proflavine plates had become adapted in the presence of the drug and retained their resistance so long as the drug continued to be present. The increase in resistance which occurred when proflavine was added to growing cultures thus seems to be in the nature of an adaptation.

Cross-resistance

Experiment 5. We found that mutants selected by replica plating for resistance to proflavine were also resistant to chloramphenicol, Erythromycin, Aureomycin and Terramycin. The resistance to proflavine and to these drugs of a series of cultures to which proflavine had been added in the manner described in earlier experiments was tested on gradient plates with proflavine 10 μg ./ml., chloramphenicol 4 μg ./ml., Erythromycin 12 μg ./ml., Aureomycin 75 μg ./ml. or Terramycin 15 μg ./ml. As in earlier experiments the gradual addition of proflavine increased the length of streak on proflavine plates, up to a maximum of three times the original length. The length of the streaks on the other drugs, however, fell. There was a rough proportionality between the increase in resistance to proflavine and the decrease in resistance to the other drugs. Together with the rapid loss of resistance on subcultivation in the absence of proflavine, we take these findings to support the view that the increased proflavine resistance when proflavine was added to growing cultures was different from the mutational change which gave proflavine resistance in cultures isolated by replica plating.

Proflavine resistance in relation to division cycles; cycles of resistance in a sensitive culture

Experiments were made to test the hypothesis that the continuous range of resistance in a sensitive culture, in which there were organisms of every stage of the division cycle, was due to differences in resistance at different stages of the division cycle. If there be such a cycle of resistance, two consequences follow which should be demonstrable in a synchronized culture. First, the proportion of organisms which survive at a given concentration of proflavine (i.e. the proportion of resistant organisms) should vary during the division cycle. Secondly, the period out of the whole division cycle during which organisms will be able to survive a given concentration of proflavine should be inversely proportional to that concentration. We should find, that is, that organisms from a synchronized culture are able to grow in relatively high concentrations of drug during only a small part of the cycle; they should grow in lower concentrations during a greater part of the cycle. The general plan of the experiments was to synchronize a culture by cooling (see Methods). After returning the flasks to 37°, samples were withdrawn at intervals of 5 or 10 min. and measured for total viable count and for the number of colonies which would grow on plates with one or more concentrations of proflavine.

Experiments 6-8. The results of three experiments are shown in Figs. 5-7. In general, we found cycles of division lasting 30 min., and cycles of resistance varying, in different experiments, from 15 to 30 min. Thus, Fig. 5 shows two division cycles, in each of which half the divisions occurred in the first 20 min. and half in the next 10 min. There were four complete cycles of resistance, each of 20 min. duration. During part of each resistance cycle, there were 10^5 or more organisms/ml. which formed normal sized colonies on 5 μ g. proflavine/ml., and during the rest of the cycle about 10^3 such organisms. Fig. 6 shows that a lower degree of synchronized division, compared with Figs. 5 and 7, was associated with less pronounced and less regular cycles of resistance. After three or four resistance cycles, there was a gradual decrease in the degree of synchronization and of cycles of resistance.

We have no explanation for the difference between the periodicity of cycles of resistance and of division. Hotchkiss (1954) found a similar difference with *Pneumococcus*, with division cycles of 30 min. and transforming cycles of 40 min.

In conformity with our expectation, the amplitude of the cycles of resistance depended on the degree of resistance, i.e. on the concentration of proflavine used to test it. Thus Fig. 6 shows that there was no detectable periodicity in the number of organisms forming colonies on 4 μ g. proflavine/ml., whilst the number which formed colonies at 5.5 μ g./ml. showed a clear periodicity. The maximum variation of numbers here was by a factor of nearly 1000. Again, as predicted, the duration of the peak of resistance was shorter with higher concentrations of proflavine (Figs. 6, 7). Occasionally, though not always, the same number of organisms occurred at the peaks of the cycles when different degrees of resistance were being measured. Thus, in Fig. 7, this occurred for resistance to proflavine at 5 μ g./ml. and at 5.9 μ g./ml.

Fig. 5

Fig. 6

Fig. 5. Changes in number of resistant organisms within the division cycles in a synchronized culture of *Escherichia coli* 86. Synchronization was obtained by cooling a culture after 90 min. from inoculation at 12° for 15 min. ○ = Total viable count expressed as number of generations; ● = number of organisms/ml. forming colonies on agar containing 5 µg. proflavine/ml., expressed as log₁₀. Time from returning to 37° at end of cooling.

Fig. 6. Changes in number of resistant organisms within the division cycles in a synchronized culture of *Escherichia coli* 36. Synchronization was obtained by cooling a culture after 70 min. from inoculation at 17° for 15 min. Number of generations derived from viable counts. Number of organisms forming colonies on proflavine agar. ○—○ = Total viable count; ●—● = resistant to 4 µg. proflavine/ml.; +—+ = resistant to 4.5 µg. proflavine/ml.; ×—× = resistant to 5.5 µg. proflavine/ml. Time measured from end of cooling.

Fig. 7. Changes in number of organisms resistant to proflavine (µg./ml.) 5, 5.9, 6.6; in a partly synchronized culture within the division cycles. Synchronization was obtained by cooling a culture 90 min. from inoculation at 12° for 5 min. and then at 17° for 20 min. ○—○ = Resistant to 5 µg. proflavine/ml.; △—△ = resistant to 5.9 µg. proflavine/ml.: normal sized colonies; ▲—▲ = resistant to 5.9 µg. proflavine/ml.: small colonies; □—□ = resistant to 6.6 µg. proflavine/ml. Note: at 5 µg./ml. and 6.6 µg./ml., only normal-sized colonies occurred. Time from end of cooling.

Cooling did not affect the viability of the organisms but it did affect the degree of resistance. Immediately after cooling there was a decrease in the number of resistant organisms proportional to the degree of resistance tested. This is seen in Fig. 6, where there is a decrease of 1 log. unit in the number of organisms resistant to 4 μg . proflavine/ml. and of 3 log. units in those resistant to 5.5 μg ./ml.

The presumed first-step mutants differed in two ways from the rest of the organisms in synchronized cultures. First, there was no change in the number of organisms resistant to, for example, 6.6 μg . proflavine/ml. after cooling. Secondly, there was no cycle of resistance, for example, in the number of organisms resistant to 6.6 μg ./ml. Thus we believe that, in Fig. 7, the normal sized colonies formed on 5.9 μg . proflavine/ml., and the colonies on 6.6 μg ./ml. which were all normal in size, were formed from organisms presumed to be first-step mutants. These findings were confirmed in a pure culture of the first-step mutant. Although synchronized division was produced there were no cycles of resistance when the culture was tested at a concentration of 10 μg . proflavine/ml. Control cultures which were not cooled for synchronization showed neither cycles of growth nor cycles of resistance (Fig. 8).

Fig. 8. Growth and resistance of non-synchronized cultures of *Escherichia coli* 36. A, Number of generations derived from viable counts. Time from inoculation. B, Resistance measured by number of organisms forming colonies on proflavine agar. Time from inoculation. ○—○ = Resistant to 4.5 μg . proflavine/ml.; ●—● = resistant to 5 μg . proflavine/ml.; △—△ = resistant to 5.7 μg . proflavine/ml.; ×—× = resistant to 6.6 μg . proflavine/ml.

DISCUSSION

The degree of synchronization we achieved was not complete and was rather less than that achieved with *Pneumococcus* by Hotchkiss (1954), who also used the cooling technique. On the other hand, it was about the same as that achieved by Maruyama & Yanagita (1956) with *Escherichia coli*, although they used a mechanical method less likely to injure the organisms. It is possible therefore that *E. coli* cannot be made to divide entirely

synchronously. One puzzling fact was the difference between the period of the division cycles (about 30 min.) and the period of resistant cycles, usually 20 min., occasionally 15 or up to 35 min. Hotchkiss (1954) also found a difference, although in his experiments the cycles of transformability were always longer than the cycles of division. One possible explanation is that cooling inhibited more than one reaction concerned with division.

An important conclusion may be derived from a consideration of the experimental results and especially of the techniques used to demonstrate varying resistance during the division cycles. This is, that it is not the resistance of the organism which varies during the cycle, but its ability to remain resistant, i.e. its adaptability. Let us imagine the fate of an organism placed on solid medium containing drug. If this is done when the 'resistance' is low, it does not form a colony. When done when the 'resistance' is high, it does form a colony. But in so doing, it must have divided and passed through a period of a division cycle when, in the absence of proflavine, its resistance would have been low. Nevertheless, it was able to grow, repeatedly divide, and eventually give a colony in which the parent organisms would normally many times have passed through periods of low resistance. The only reasonable explanation of this is that it is the adaptability, not the resistance, of the organism which varies with the cycle; once an organism has adapted to increased resistance in the presence of the drug, it retains this resistance through all stages of the cycle, so long as the drug continues to be present.

The results give some information about the range of the cyclical variation in adaptable resistance, which can be compared to the resistance of first-step mutants. Throughout the whole, or most, of the cycle the organisms of a sensitive culture are able to form colonies on plates with 4 μ g. proflavine/ml. We may then say that this concentration or a trifle less represents the inherent resistance of the organisms. On plates containing more proflavine, only organisms plated out at one part of the cycle can grow, and the higher the concentration of drug, the shorter is the period during the cycle when the organisms can go on growing. The maximal concentration of drug which seems to allow the organisms to grow during the part of the cycle when they had the highest degree of adaptability was about 7 μ g. proflavine/ml. Above this concentration, only first-step mutants will grow. These seem to have an inherent resistance to about 10 μ g. proflavine/ml. These mutant organisms show no fluctuation in sensitivity during the division cycle, in the same sort of way that the sensitive organisms show no fluctuation in the numbers at a concentration of 4 μ g. proflavine/ml.

We also found that, immediately after cooling, the organisms seemed to have minimal adaptability to resistance. Thus at this stage only a small proportion of the sensitive organisms gave colonies on a drug concentration higher than about 5 μ g./ml. whilst the unsynchronized culture before cooling contained a higher proportion of organisms which were able to do so. We have therefore two methods of distinguishing organisms at the stage of highest adaptability during the division cycle from those which pre-exist as mutants. First, the number of the former but not of the latter, which can grow on intermediate

concentrations of drug, decreased immediately after cooling. Secondly, the former but not the latter show during the division cycle fluctuations in the numbers which are resistant to these intermediate concentrations.

We can now explain some of the results obtained when proflavine was added to growing (unsynchronized) cultures of *Escherichia coli*. Some of the organisms in these cultures would have been in the non-adaptable stage of the division cycle, and these would not then have continued to grow in the presence of the added proflavine. There is thus, as we found, a partial inhibition in growth. Other organisms would have been in the adaptable stage of the cycle, and so would adapt and continue to grow and divide. Transference from these liquid cultures on to plates with proflavine allows the formation of colonies from adapted cells. Transference on to media without proflavine results in a loss of the adaptation. Whilst therefore the increased resistance continues for as long as the organisms or their progeny are in contact with proflavine, it is lost in its absence. It is this last fact which suggests that the type of resistance with which we are here concerned conforms to the usual conception of phenotypic adaptation. This is supported by a lack of the cross-resistance shown by mutant organisms.

As to the degree of the phenotypic increase in resistance, we have seen that this is a function of the concentration of drug achieved during additions to the growing cultures. It would then seem possible to continue the additions so as to achieve a high degree of resistance. However, the possibility of killing the organisms limits the rate at which the drug can be added in the logarithmic phase. Moreover, the additions cannot be continued to give a high concentration because before this the culture enters the stationary phase when the organisms become sensitive to the drug. The appropriate conditions might be achieved in a culture kept continuously in the logarithmic phase in a chemostat, and such experiments would be worth doing.

In the conditions of some of our experiments, it seems that many of the organisms had become adapted so that they grew, though not readily, on a concentration of about 6 μg . proflavine/ml. The result was a smaller colony than usual. This we believe to be the explanation of the small colonies we found on such concentrations of proflavine.

The continuous curve of resistance found in an ordinary sensitive culture is, we suggest, mainly an expression of the variable adaptability of organisms at different stages of their division cycle. That is, since an ordinary culture comprises organisms of all stages of the division cycles, there is a continuous range of adaptability which is measured as a continuous range of resistance. It is this, or chiefly this, which, in the mutation-selection theory, is referred to as a phenotypic variation of a fixed genotype. We have not ruled out the possibility of a clonal variation due to asymmetrical division. If this exists, it would be a mechanism of continuous variation superimposed on that of a variable phenotypic adaptation in the division cycle. We believe that the degree of phenotypic variation is probably governed by the genotype. In our experiments, we have demonstrated a maximal adaptability to a resistance below that of the first-step mutant. It seems likely that in certain conditions

the range may extend to or beyond that of the next genotype. It is possible that this and the succeeding steps of mutations have similar ranges of phenotypic adaptability. We suppose that there is in fact, in this and in other examples of multi-step mutation, a continuous range of resistance, or rather of phenotypic adaptability, bridging the intervals between the levels representing inherent genotypic resistance, from the most sensitive organism to the most highly resistant.

As well as the slight increase in resistance of a large proportion of the organisms, there was a considerable increase in the number of organisms able to grow on 10 μ g. proflavine/ml. We have referred to these as if they were first-step mutants. It is, however, possible that some of these organisms are produced by an adaptation but of a higher degree and of a permanent nature.

Our results have a bearing on those described by Hughes (1952, 1955). He described slight differences in resistance of sister organisms separated after two or three divisions from a single organism. One possible explanation of these differences is that the organisms whose resistance was being measured were tested at different stages of the cycles of resistance.

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(Received 21 October 1958)