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The Origin of Bacterial Resistance to Proflavine

3. The Alleged Rapid Adaptation to Proflavine Resistance in *Bacterium lactis aerogenes* (syn. *Aerobacter aerogenes*, *Klebsiella pneumoniae*)

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SUMMARY: It was reported (Baskett, 1952) that small additions of proflavine made at intervals to a growing culture of *Bacterium lactis aerogenes* (synonyms *Aerobacter aerogenes*, *Klebsiella pneumoniae*), did not prevent continued growth; this was regarded as proof that drug resistance might be acquired by adaptation. This phenomenon does not occur with *Escherichia coli* grown in broth, but we confirm that it occurs with *B. lactis aerogenes* grown in a chemically defined medium containing glucose. Growth of the aerogenes organism ceased when all the proflavine was added at once. Examination of the aerogenes organisms at the end of the experiments showed that a high proportion had become slightly more resistant, and a few had become highly resistant. These changes were not sufficient to account for continued growth of the aerogenes organisms in increasing concentrations of proflavine. In high concentrations of proflavine, growth of sensitive organisms did occur when some filtrate from a drug-free culture was added to the culture medium. It also occurred when the growing culture was kept acid, or when the medium was made acid before inoculation. On the other hand, growth did not occur in the presence of proflavine when the culture was kept neutral. It is concluded that the growth of *B. lactis aerogenes* in cultures to which proflavine is added gradually is chiefly due to a decrease of the inhibitory action of the drug by the acid formed during growth in a glucose + salts medium. The absence of the phenomenon when *E. coli* was grown in broth can be explained by the absence of glucose so that there was no lowering of pH value. There is thus no evidence of a rapid adaptation to proflavine resistance in *B. lactis aerogenes*.

One of the mechanisms suggested for the origin of drug resistance is a phenotypic adaptation induced by the drug (e.g. Hinshelwood, 1949; Dean & Hinshelwood, 1953). However, the evidence presented by Hinshelwood and his colleagues, as well as by other workers, is mostly inconclusive, since the experimental conditions do not eliminate the possibility of mutation and selection. There is, however, a publication by Baskett (1952) from Hinshelwood's laboratory, which seemed to prove that *Bacterium lactis aerogenes* (synonyms *Aerobacter aerogenes*, *Klebsiella pneumoniae*) may become resistant to proflavine by direct induction. Baskett added proflavine in small amounts at intervals to a rapidly growing culture. He found that the culture was still able to grow, though rather more slowly than usual, even when quite high concentrations of drug had been achieved. The rate of growth in presence of the drug depended on the original rate of growth of the culture and on the rate of addition of proflavine. Large inocula transferred to new media containing the drug were able to resume this growth. Small inocula, however,

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were not able to grow in a new medium containing the same high concentration of drug as had been achieved in the experiment. Baskett explained this phenomenon by assuming a rapid adaptation of the organisms, the rate of this adaptation being represented by the maximal rate at which proflavine could be added. That these adapted organisms did not grow when inoculated in small numbers into fresh medium containing proflavine was explained by Baskett by the short time of adaptation, which had consequently not been able to 'impress' itself sufficiently on the organisms to become permanent. The obvious explanation of these results is the change induced in the organisms by the drug, acting in these conditions, so that they become able to grow in concentrations far higher than would permit growth of the original sensitive organisms. This explanation has been accepted by a number of observers (e.g. Schnitzer & Grunberg, 1957). It was clearly important to confirm these experiments. It occurred to us, however, that a confirmation of the phenomenon did not imply a confirmation of Baskett's explanation. Briefly, one can consider that the growth of the organisms in the conditions of the experiment might be due either to changes in the organisms so that they become more resistant, or to changes in the medium so that the inhibitory action of proflavine is decreased.

The experiments to be described were designed to test these possibilities. They fell into three groups: (1) to see whether the phenomenon occurs with *Escherichia coli* and to confirm that it occurs with *Bacterium lactis aerogenes*; (2) to determine whether the organisms show an increased resistance and if so something of its extent and persistence; (3) to determine whether the medium shows changes which decrease the inhibitory effect of proflavin and if so to discover their nature.

METHODS

Organisms. The strain of *Bacterium lactis aerogenes* was that used by Hinshelwood and colleagues, and was a culture kindly supplied by Sir Cyril Hinshelwood.

A culture of *Escherichia coli* NCTC 8196 from the National Collection of Type Cultures was also used in some experiments. *E. coli* 36 was mainly used, which had been isolated in this laboratory from a single colony of *E. coli* NCTC 8196 (see Thornley & Yudkin, 1959).

Media. The chemically defined medium containing glucose and ammonium sulphate was used for *Bacterium lactis aerogenes* as described by Baskett (1952). The components were (g./l.) glucose, 20; disodium hydrogen phosphate, 6.16; potassium dihydrogen phosphate, 1.14; ammonium sulphate, 0.96; $\text{MgSO}_4 \cdot 5\text{H}_2\text{O}$, 0.038.

Nutrient broth (Difco) was used for experiments with *Escherichia coli*.

Nutrient agar (Difco) was used with both organisms for counts, sterility tests and drug gradient plates.

Proflavine hemisulphate (British Drug Houses Ltd.) was sterilized dry by heating for 1 hr. at 100°. A fresh stock solution was made up in the appropriate sterile medium for each experiment.

Growth of organisms. The procedure was designed to allow for the inoculation of cultures into the medium, for the aeration of the medium kept at constant temperature, for the addition of solutions of proflavine, and for the withdrawal of samples. The experiments were carried out in 250 ml. Buchner flasks containing 50 ml. medium. The inoculum was 0.5 ml. of an overnight culture of organism in nutrient broth. The flasks were kept in a bath at 40° for *Bacterium lactis aerogenes* or at 37° for *Escherichia coli*. Aeration was effected through a manifold by a compressor. Additions were made and samples withdrawn under sterile conditions through a syringe to which was attached a narrow tube of polythene.

Measurements of relative growth were made by measuring changes in optical density with a Spekker spectrophotometer. The use of filter H508 eliminated changes in optical density through changes in proflavine concentration. The curve of readings plotted against dilution was linear up to an optical density of 0.4; densities above this were determined by previous dilution with medium.

Additions of proflavine. The solution of proflavine was added always at intervals of 10 min. to give concentrations in the medium increasing at each addition by 5 µg./ml. for *Bacterium lactis aerogenes* and by 2 µg./ml. for *Escherichia coli*.

Measurement of relative growth was made by optical density and expressed as log₂ Spekker reading. In this way, 1 unit = 1 cell division.

Culture filtrates were prepared usually after 5 hr., i.e. at the end of the logarithmic growth phase. The samples were centrifuged for 30 min. at 1500 rev./min. and then filtered through sintered glass of porosity 5. The filtrates were usually kept in a refrigerator overnight before being used in the experiments.

pH values. Preliminary readings were made by British Drug Houses Ltd. universal indicator; more precise measurements were made with a pH meter.

Tests for contamination. At the end of all experiments cultures were routinely tested for contamination by plating on nutrient agar and staining by Gram's method.

RESULTS

Experiments were made to confirm the observations of Baskett (1952) with *Bacterium lactis aerogenes*, and also to see whether the phenomenon occurs with *Escherichia coli*.

Experiment 1. Bacterium lactis aerogenes. Four flasks of glucose ammonia medium were inoculated at the same time. They were treated as follows:

Flask 1. Two hr. after inoculation, when turbidity was just visible, additions of proflavine solutions were begun, and continued at 10 min. intervals until 24 additions had been made, i.e. for 4 hr. The final concentration of proflavine, allowing for dilution by the additions and for taking out of the samples, was 97 µg./ml.

Flask 2. Additions of proflavine were begun after 250 min. of incubation and were continued at 10 min. intervals for 4 hr.

Flask 3. After 250 min. of incubation without proflavine, the drug was added in one amount to give a final concentration of 97 $\mu\text{g./ml.}$

Flask 4. No addition of proflavine was made to this flask.

From all flasks, samples were withdrawn 2 hr. after inoculation, and thereafter at 2 hr. intervals.

The results are given in Fig. 1. Gradual addition of proflavine allowed the growth of *Bacterium lactis aerogenes*, the growth rate being quite high (Fig. 1, curves 1 and 2). The optical density of the culture at the beginning of proflavine addition affected the resulting growth rate; that is, when the optical density was low (curve 1), the growth rate was lower. Addition of the whole amount of proflavine inhibited the growth almost completely. These results confirm the observations of Baskett.

Fig. 1. Effect of proflavine on growth of *Bacterium lactis aerogenes* in glucose ammonia medium. 1, Proflavine added gradually from 120 min. after inoculation; 2, proflavine added gradually from 250 min. after inoculation; 3, proflavine all added at 250 min. after inoculation; 4, no proflavine added. ---- = Proflavine added gradually; — = no proflavine added; = proflavine added at once 97 $\mu\text{g./ml.}$ Time from 120 min. after inoculation. Growth measured by turbidity.

Experiment 2. Escherichia coli. This was carried out in a similar manner to Expt. 1 but with *E. coli* in nutrient broth instead of *Bacterium lactis aerogenes* in glucose ammonia medium. Since *E. coli* is more sensitive to proflavine, lower concentrations of it were used, the final concentration being 20 $\mu\text{g./ml.}$ Unlike the previous experiment, however, very little growth occurred after addition of proflavine, although the experiment was repeated several times with various modifications. Thus Baskett's phenomenon of 'rapid adaptation' did not occur with *E. coli* under these conditions.

Changes in resistance of organisms

These experiments were designed to determine what changes in resistance and morphology occur in the organisms during the Baskett 'adaptation'.

Experiment 3. Changes in resistance. The *Bacterium lactis aerogenes* organisms from Expt. 1 were streaked on to a gradient proflavine plate containing 100 $\mu\text{g./ml.}$ (for details of this technique see Thornley & Yudkin, (1959). The bacteria from the control to which no proflavine had been added grew to the middle of the plate; this then represents the normal resistance of *B. lactis aerogenes*. The bacteria from the culture to which gradual additions of proflavine had been made showed a solid streak about 30 % longer than the control; in addition, several colonies of higher resistance appeared up to the end of the plate. Bacteria from the culture to which the whole of the proflavine had been added at once did not grow at all on the gradient plate; a very few colonies grew on a drug-free plate. Thus, the high concentration of proflavine added at once rendered most of the organisms nonviable. Gradual additions, however, led to a slight increase in resistance in a high proportion of organisms and to the emergence of several highly resistant mutants. The slight increase in resistance, which incidentally might easily have been overlooked on ordinary serial proflavine plates, has implications which are discussed later. But neither this nor the emergence of the relatively few highly resistant mutants is able in itself to explain the high growth rate of the organisms which occurred in increasing concentrations of proflavine.

Experiment 4. Changes in morphology. At the beginning and end of Expt. 1, viable counts were made on the cultures which had been grown with and without proflavine. The increase in number of viable organisms was 50 times in the control without proflavine, as compared with twice in the presence of proflavine added gradually (flask 1). Gram-stained films of the cultures showed that the organisms growing in the presence of proflavine were mostly in the form of long filaments. Thus the addition of proflavine had inhibited division more than growth in mass.

Changes in medium

In this series of experiments we examined whether changes occur in the medium during the Baskett type of 'adaptation' which might enable the organisms to grow in the presence of proflavine.

Experiment 5. The purpose of this experiment was to see whether the interaction of a bacterial filtrate and proflavine, in the conditions of the Baskett experiments, resulted in the medium now being able to support the growth of sensitive organisms. The drug was added at intervals, at 40° with aeration, to a mixture of fresh medium + filtrate of a culture which had not been in contact with proflavine. At the same time, an inoculation of sensitive *Bacterium lactis aerogenes* organisms was made into fresh medium, and after 3.5 hr., the organisms from this culture were added to the proflavine medium and incubation continued. Parallel with this, the usual Baskett

type of experiment was carried out. There were thus four flasks, as follows:

Flask 1. This contained 10 ml. fresh glucose ammonia medium + 40 ml. sterile filtrate from a 5 hr. culture of sensitive organisms in similar medium. Proflavine 5 $\mu\text{g./ml.}$ was added every 10 min. at 40° for 3.5 hr. with constant aeration until the concentration reached 66 $\mu\text{g./ml.}$ The medium was inoculated with packed washed *Bacterium lactis aerogenes* from 50 ml. of a culture of sensitive organisms in the middle of the logarithmic phase of growth. The incubation was then continued.

Flask 2. Glucose ammonia medium (50 ml.) was inoculated in the usual way with 0.5 ml. of an overnight culture of *Bacterium lactis aerogenes*. After 3.5 hr., proflavine was added in steps to a final concentration of 66 $\mu\text{g./ml.}$

Flask 3. This was set up as flask 2, but after 3.5 hr. all the proflavine was added at once.

Flask 4. This was set up as flask 2, but no proflavine was added.

The results are given in Fig. 2. The admixture of sterile filtrate of a sensitive culture (which was never in contact with proflavine), and proflavine added gradually at 40° with aeration, resulted in a medium that supported growth of *Bacterium lactis aerogenes* in a normally completely inhibitory proflavine concentration, i.e. 66 $\mu\text{g./ml.}$ The growth rate increased during the experiment from 16 to 23 % normal (flask 1).

In some experiments, we measured the pH value of the glucose ammonia medium during growth. We found a gradual decline from pH 7.2 to pH 4.0–4.5 in medium with or without proflavine. It seemed possible, therefore, that the antagonistic action of the culture filtrate on proflavine inhibition was due to its acidity.

Experiment 6. We therefore studied the effect of different pH values on growth in culture filtrate with proflavine. To four flasks was added filtrate from a 5 hr. culture of sensitive *Bacterium lactis aerogenes*. In three flasks, 4 parts of filtrate were diluted with 1 part of fresh medium; the fourth contained undiluted filtrate. The pH value of 2 flasks was adjusted with dilute NaOH to pH 6.8 and 6.0; the third had a pH of 5.5 and the fourth (undiluted filtrate) a pH value of 4.0. All flasks contained a total volume of 50 ml. of diluted or undiluted filtrate. Proflavine was added gradually in the usual way to a final concentration of 61 $\mu\text{g./ml.}$ At this stage, packed washed *B. lactis aerogenes* from a 3.5 hr. culture in 50 ml. glucose ammonia medium was added to each flask, and incubation with aeration continued.

The results are illustrated in Fig. 3. At pH 6.8 and 4.0, growth did not occur in filtrate + proflavine. At pH 6.0 and 5.5, growth occurred at 12–14 % of the normal rate. Leaving aside for a moment the fact that growth did not occur at pH 4 in the undiluted medium, these results confirm that the interaction of filtrate and proflavine, which decreased the inhibitory action of the drug, occurred only when the filtrate was acid and was abolished when the acidity was neutralized. The absence of this effect at pH 4 may be due either to the pH value being too low, or to the fact that no fresh medium was added.

It was observed that there was a coarse red precipitate in this flask at the end of the experiment.

Experiment 7. The possibility that the culture filtrate contained a proflavine-inactivating enzyme was investigated in this experiment. As part of Expt. 6, some of the diluted filtrate of pH 5.5 was boiled for 10 min. The proflavine

Fig. 2

Fig. 3

Fig. 2. Effect of bacterial filtrate from a sensitive culture in glucose ammonia medium on the inhibitory action of proflavine on *Bacterium lactis aerogenes*. The relative amounts of growth were measured by turbidity. 1, Filtrate 40 ml. + fresh medium 10 ml., to which proflavine was added gradually then inoculated with washed logarithmic phase organisms; 2, proflavine added gradually from 210 min. after inoculation; 3, all proflavine added at 210 min. after inoculation; 4, no proflavine added. - - - - = gradual proflavine addition; = 66 µg. proflavine/ml. present; — = no proflavine added. Time from addition of organisms in (1) and from 210 min. after inoculation in others.

Fig. 3. Effect of varying pH of bacterial filtrate on the inhibitory action of proflavine on growth of *Bacterium lactis aerogenes* in glucose ammonia medium. 1, Filtrate diluted by fresh medium 4:1, pH 6.8; 2, filtrate diluted by fresh medium 4:1, pH 6.0; 3, filtrate diluted by fresh medium 4:1, pH 5.5; 4, filtrate undiluted, pH 4.0. Gradual proflavine additions to final concentration of 61 µg./ml., then inoculated with washed organisms 210 min. after inoculation. Time was measured from addition of organisms to proflavine + filtrate; amount of growth was measured by turbidity.

was added at intervals in the usual way. After 2 hr., with proflavine at 61 µg./ml., an inoculum of packed *Bacterium lactis aerogenes* was added and incubation continued for 90 min. Growth occurred in the boiled and unboiled filtrate at the same rate of 14% normal. Although it is possible that a heat-resistant enzyme was present in culture filtrates, the more likely explanation is that the effect was not due to an enzyme.

Experiment 8. We attempted to prove that the phenomenon of growth in increasing concentrations of proflavine was due to the antagonistic action of a

low pH value on the inhibition by proflavine. If this were so, two consequences follow. First, if the pH were kept neutral during the gradual addition of proflavine, no growth should occur. Secondly, if the pH of the culture were lowered at the beginning of the experiment, then the addition of all the proflavine at once should not stop growth; it would do so in the usual Baskett experiment because insufficient acid had at this time been produced. In a

Fig. 4

Fig. 5

Fig. 4. Effect of maintaining neutral pH in culture during proflavine additions, and of acidifying the culture with large single addition of proflavine made on growth of *Bacterium lactis aerogenes* in glucose ammonia medium. 1, pH maintained neutral throughout incubation, proflavine added gradually; 2, culture acidified to pH 4.9, then all proflavine added at once; 3, proflavine added gradually; 4, no proflavine added. ---- = gradual proflavine additions; = 65 μ g. proflavine/ml. present; — = no proflavine added. Time was measured from beginning of proflavine additions; growth was measured by turbidity.

Fig. 5. Effect of acidifying medium before gradual proflavine additions on growth of *Bacterium lactis aerogenes* in glucose ammonia medium. 1, Medium acidified to pH 5.5, proflavine added gradually, then inoculated with washed packed organisms; 2, gradual proflavine additions to culture in logarithmic phase. ---- = Gradual proflavine additions; = 65 μ g. proflavine/ml. present. Time was measured from inoculation in flask 1, and from beginning of proflavine additions in flask 2; growth was measured by turbidity.

series of preliminary experiments, we measured with a pH meter the changes in pH of a growing culture with and without gradual additions of proflavine. As well as this we measured the effect on growth of the addition of NaOH at intervals in amounts needed to restore the pH value to 7.2.

Experiment 8. A flask was set up in the usual way, for the gradual addition of proflavine to a culture of *Bacterium lactis aerogenes* in the logarithmic phase. Before the beginning of the addition, and throughout the addition, the pH was kept constant at 7.0–7.2 by the addition of NaOH. No growth occurred, and there was a decrease in turbidity (Fig. 4).

The pH of a growing culture was adjusted to 4.9 just before the addition of proflavine in one amount to a concentration of 65 $\mu\text{g./ml.}$; incubation was then continued. Growth occurred, the rate increasing during incubation from 10 to 30 % normal and the pH declining further to 4.2. In other flasks, whose pH values were not adjusted, the pH decreased during incubation, with or without proflavine to about 4.5 roughly in proportion to bacterial density. The results (Fig. 4) therefore confirmed the prediction made on the basis that the growth of the culture during addition of proflavine was due to the antagonism of the inhibitory action of proflavine by an acid medium.

Experiment 9. Fresh glucose ammonia medium was adjusted to pH 5.5. Proflavine was added gradually to 65 $\mu\text{g./ml.}$ This acid proflavine medium was then inoculated with packed *Bacterium lactis aerogenes* from a 3.5 hr. culture and incubated with aeration for 90 min. The experiment was controlled by a flask submitted to the usual Baskett procedure, that is, a flask in which proflavine was gradually added to a growing culture.

The medium acidified to pH 5.5 supported growth in presence of 65 $\mu\text{g.}$ proflavine/ml.; the growth rate was 27 % normal (Fig. 5). *Bacterium lactis aerogenes* was thus able to grow in fresh glucose ammonia medium in the presence of high concentrations of proflavine, provided the pH was low enough. This low pH value is that which is reached in experiments like those of Baskett in which continuous growth was achieved by gradual additions of proflavine which eventually reached the same final high concentration.

DISCUSSION

Our experiments confirm that *Bacterium lactis aerogenes* is able to grow in high proflavine concentration while this drug is added gradually to a culture growing in glucose ammonia medium. The explanation, however, is not, as Baskett (1952) suggested, that the organisms adapt themselves to the drug, but that during growth in presence and absence of proflavine the pH value of the culture drops from neutral to about 4.5–5.0. Low pH values antagonize the inhibitory action of proflavine (Peacocke & Hinshelwood, 1948; Albert *et al.* 1945).

Let us now consider Baskett's experiment in the light of our findings. Baskett started adding proflavine gradually to a culture in logarithmic phase (already turbid). In this the pH value was already sufficiently low to enable the organisms to tolerate 5 $\mu\text{g.}$ proflavine/ml. Growth therefore continued, the pH value was decreased again and this in turn enabled the organisms to

tolerate 10 μg . proflavine/ml. Further growth led to further decrease in pH value, and to increasing tolerance to proflavine. When Baskett added all the proflavine at once, no growth occurred because at the beginning of the logarithmic phase the pH value was not sufficiently small to enable this large amount of proflavine to be tolerated.

The ability to grow in high proflavine concentration is not inheritable. Baskett found that when he subcultured a small proportion of the 'adapted' cells into proflavine medium they did not grow. But when he subcultured a high proportion (dilution about 1/3), growth continued at the same high drug concentration. This is understandable when we remember that with this inoculum he lowered the pH value in the fresh proflavine medium. Here also the increased number of mutants, and the slight increase in resistance that we found, might play a role.

We have proved in several ways that there is no phenotypic adaptation in the Baskett phenomenon. The sterile filtrate of a 5 hr. culture of sensitive organisms, which were never in contact with proflavine, supported growth of *Bacterium lactis aerogenes* in the presence of the same high proflavine concentration and at a similar rate as in the usual Baskett type of experiment. This is the most important evidence against adaptation because in this filtrate there were no products of 'adapted' cells. The main factor which antagonizes the proflavine action in this filtrate is its low pH value. This was supported by two further observations. When the pH value was maintained at neutral during the usual Baskett experiment, no growth occurred, and when the pH value was decreased to that achieved at the end of Baskett's experiment and the whole amount of proflavine was added at once, growth occurred at a similar rate to that in the Baskett type of experiment. Finally, the low pH value itself is sufficient to explain Baskett's results, because growth occurred when fresh medium was acidified to pH 4.9, proflavine added and then sensitive organisms inoculated into it.

Although the decrease in pH value is sufficient to explain the Baskett phenomenon, it seems likely that other factors contribute to the actual rate of growth achieved in the presence of proflavine. These include: selection of mutants of high resistance; slight increase in resistance in most of the organisms; the products of metabolism (other than H ions) of a growing culture which might supply metabolic intermediates (Albert, 1951).

There still remains to mention the different rates of growth in our experiments. The growth rates in the Baskett type of experiment varied from 5 to 21 % of normal at 65 μg . proflavine/ml., and were somehow dependent on the bacterial density at which the proflavine additions started. In our experiment with filtrates supporting growth, the growth rates varied with different pH values of filtrates and dilution by fresh medium, the maximum at 65 μg . proflavine/ml. being 14–23 % of normal. In a culture acidified to pH 4.9 and with all proflavine added at once, the rate was 10 %, increasing to 30 %; in the acidified medium it was 27 %. We consider these are in reasonable agreement with the values found by Baskett.

The probable reason why *Escherichia coli* growing in broth did not grow in

the Baskett type of experiment is that the pH value did not decrease, owing to the absence of glucose. We conclude that the experiments of Baskett, which appeared to give the strongest support to Hinshelwood's theory, do not in fact support it. The fact that, in producing proflavine resistance, phenotypic adaptation does not occur rapidly and to a high degree does not, however, exclude the possibility that it may occur more slowly or to a lower degree. This possibility is investigated in the following paper.

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