resistant to the drug. Therefore, if a polymutational theory were invoked to explain the non-existence of pre-formed mutants, it would have to be of a highly, and it may be felt improbably, complex form.

With brilliant green the behaviour is different, but here, too, pre-existent mutants do not seem to be detectable. The culture is remarkably viable, and small numbers of cells all survive even though they develop into full-sized colonies only after the lapse of many days. Very slow growth seems to occur until something, presumably the generation of acid, has occurred to antagonize the action of the dye, and then a burst of more rapid growth ensues. There is no sign in the untrained cultures of a few rapidly growing resistant forms: all the cells grow but much more slowly than in the absence of the dye. Once again, therefore, the view that the phenomena depend upon mutation can only be maintained with the help of elaborate auxiliary hypotheses.

REFERENCES


The rate of development of colonies of Bacterium lactis aerogenes on agar plates containing drugs

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When bacteria are plated on a solid medium containing a drug, only a fraction \(x_\infty\), often very small, of the cells ever form colonies, \(x_t\), the fraction which have done so at time \(t\), has been determined as a function of time, together with \(x_\infty\), for various trained and untrained strains of bacteria in presence of a series of drugs. Bacterium lactis aerogenes (Aerobacter aerogenes) has been studied in this way in presence of brilliant green, proflavine, chloramphenicol, streptomycin, terramycin, thymol, sodium azide and isonicotinyl hydrazine; and Bact. coli mutabile in presence of chloramphenicol, and propamidine.

In some of the examples the curves of \(x_t/x_\infty\) against time are clearly and obviously incompatible with the postulate that the resistant forms in the trained culture are pre-existent in the original. Separate experiments, moreover, have shown in these examples that a large concentration of non-resistant forms does not in fact delay the growth of forms already adapted.

In other examples the \(x_t/x_\infty\) against \(t\) curves are less sharply contrasted for the trained and untrained strains. Here, however, \(x_\infty\) for the trained strain may represent practically the whole population, while for the untrained strain it is \(10^{-6}\) or less.

The statistical result of comparing, in attempts to estimate numbers of mutants, the average of one population with the extreme tail of another is examined. It is concluded that the \(x_t/x_\infty\) relations, even in the second type of example, are as consistent with the occurrence of adaptations in competition with lethal actions on the plate as they are with the growth of mutants.

The resistance of strains re-tested after isolation from resistant colonies is highly variable.
INTRODUCTION

When a large number of bacterial cells are plated on a solid agar medium containing an inhibitory or toxic substance in a suitable concentration range, a small measurable proportion of them \( \alpha_\infty \) may eventually succeed in forming reasonably well-developed colonies. There are several possible interpretations of such behaviour, and these, it should be emphasized, may correspond not to conflicting theories so much as to a series of possible occurrences.

1. Pre-existing resistant mutants may grow on the plate, and \( \alpha_\infty \) may represent the fraction of these in the population under test.

2. There may be a competition between death of the cells and adaptation of the survivors to resist the toxic agent. \( \alpha_\infty \) here would represent the fraction of the population which, according to the often approximately exponential death-curve, lives long enough to overcome the lag caused by the drug.

3. The actual adaptability to the drug may vary statistically in the population according to a distribution, the extreme end of which might constitute the fully resistant mutants. Since structural factors and the dynamics of enzyme balance are both unquestionably of importance in determining the reactions of a cell, a whole range of behaviour would not be surprising.

In the present paper an answer is sought to the following question. If \( \alpha_t \) is the fraction of the cells plated on the drug plate which have formed colonies (of a standard size) by a time \( t \) from inoculation, what relation exists between the curves of \( \alpha_t/\alpha_\infty \) against \( t \) for an original strain and one previously ‘trained’ (by adaptation or selection) to full resistance? If the resistant mutants existed fully developed in the original culture \( (\alpha_\infty)_{\text{original}} \) would be smaller than \( (\alpha_\infty)_{\text{trained}} \), but for the two cases \( \alpha_t/\alpha_\infty \) would be the same function of \( t \) (unless the mutants were impeded from developing by other cells). If adaptation occurred, the relations would be different, and might vary with circumstances. In particular, if there were a competition between adaptive and lethal processes, some rather paradoxical results might follow, as will be explained.

Some observations on the degree of resistance on re-test of the cells in colonies formed on drug plates will also be recorded. This too is relevant to the question whether survival on a drug plate is merely a selection of the appropriate spontaneous mutants or whether it is influenced by many other factors. Evidence in support of the latter interpretation has already been published (Dean & Hinshelwood 1952a, b, 1953; Dean 1955).

MATERIALS

Strains

The strains of Bact. lactis aerogenes (Aerobacter aerogenes) and Bact. coli mutabile used in these experiments were obtained by plating stock strains and isolating single colonies. These colonies were transferred to ‘Lemo’ broth and incubated overnight. Inocula from the broth cultures were then transferred to a glucose-ammonium sulphate medium and the resulting strains were fully adapted to this
Development of colonies of Bacterium lactis aerogenes

medium, before being used in the drug experiments, by at least 20 serial subcultures. By this time the growth rate has reached its optimum value.

Glucose-ammonium sulphate medium

This was prepared by mixing sterile solutions of glucose (50 g/l.), phosphate buffer (Na₃H₂PO₄·12H₂O 16 g/l., KH₂PO₄ 2·96 g/l., pH, 7·1), ammonium sulphate (5 g/l.), and magnesium sulphate (1 g/l.) in the amounts 10, 10, 5 and 1 ml. respectively. Ferrous sulphate sufficient to give 0·2 mg/l. in the final medium was included in the magnesium sulphate solution.

Glucose-ammonium sulphate medium containing drugs

Small volumes of the drugs in solution in glass-distilled water were added to glucose-ammonium sulphate medium.

Drug-agar

An agar medium containing Na₃H₂PO₄·12H₂O, 8·0 g; KH₂PO₄, 1·5 g; (NH₄)₂SO₄, 0·6 g; MgSO₄·7H₂O, 0·05 g; agar, 15·0 g; glass-distilled water, 800 ml., was prepared. Its pH was 7·1. This medium was sterilized, and to aliquot parts was added the requisite amount of a sterile solution of the drug. The drug-agar was distributed in tubes in 8 ml. quantities. Before the plating, 1 ml. sterile solution of glucose was added to each tube of molten agar. When streptomycin or terramycin was being tested the above procedure was modified in that the drug solution was added to the tubes of molten agar after they had been cooled to 45°C in a thermostat.

Experimental methods

‘Training’ to drugs in liquid media

This was achieved by serial subculture in the glucose-ammonium sulphate medium containing amounts of drug which were gradually increased until the desired concentration had been reached. The bacteria were then given 10 to 20 passages at this drug strength. With Bact. lactis aerogenes the ‘training’ was carried out at 40°C and with Bact. coli mutabile at 37°C.

Plating techniques

The bacterial culture was centrifuged and resuspended in phosphate buffer. 1 ml. volumes of suitable dilutions of this suspension were added to tubes of molten drug-agar which had been kept at 45°C in a thermostat. After mixing, the inoculated agar was then poured into a Petri dish. When the agar had set, a second layer of drug-agar (without bacteria) was poured on top. In the platings with trained strains of bacteria the inoculum consisted of 50 to 200 cells so that all would have the opportunity of forming colonies.
Retesting of colonies from drug plates

The colonies were transferred to ‘Lemco’ broth, incubated overnight and 1 loopful of the broth culture was inoculated into 26 ml of glucose-ammonium sulphate medium. After incubation the cultures derived from these colonies were plated on drug-agar in the normal manner.

RESULTS

The time of appearance of colonies on drug plates

The results obtained when trained and untrained strains of *Bact. lactis aerogenes* were plated on agar containing brilliant green, proflavine sulphate, chloramphenicol, streptomycin sulphate and terramycin (oxytetracycline hydrochloride) are given in figures 1 and 4 to 7. Results with sodium azide and with *isonicotinyl hydrazide* will also be described. The behaviour of *Bact. coli mutabile* on chloramphenicol-agar and on propamidine-agar is shown in figures 2 and 3. It will be convenient to deal with these results in groups.

![Graph 1](image)

**Figure 1.** *Bact. lactis aerogenes* and brilliant green, 40 mg/l. *Un*, untrained strain: $x_\infty = 3.6 \times 10^{-5}$. *Tr*, trained strain: $x_\infty = 1.0$. *T*, total, *L*, large.

In general, the trained strains have $x_\infty = 1$, that is 100% colony formation. The untrained strains mostly gave values of $10^{-5}$ to $10^{-7}$ at the drug concentrations used.

The number of colonies gradually increased, and at a certain time reached a size which, according to a pre-determined convention, was designated ‘large’. Curves are given showing the increase with time both in the total number of visible colonies (marked $T$) and in the number of ‘large’ colonies (marked $L$).

The most marked differences in time between trained and untrained strains were observed with *Bact. lactis aerogenes* and brilliant green (figure 1), with *Bact. coli mutabile* and chloramphenicol (figure 2), and with *Bact. coli mutabile* and propamidine (figure 3). In the first of these examples there was a difference of
Development of colonies of Bacterium lactis aerogenes

5 days in the time for formation of 100% of total or large colonies. For 50% the corresponding times were 2.5 days for the totals and 2 days for the large (figure 1). With Bact. coli mutabile and chloramphenicol the time differences were 7 days for 100%, and 4 to 5 days for 50% of both total and large colonies (figure 2).

**Figure 2.** Bact. coli mutabile and chloramphenicol, 10 mg/l. Un, untrained strain: $a_\infty = 1 \cdot 10^{-6}$. Tr, trained strain: $a_\infty = 1 \cdot 0$. T, total; L, large.

**Figure 3.** Bact. coli mutabile and propamidine, 70 mg/l. Un, untrained strain: $a_\infty = 1 \cdot 6 \times 10^{-4}$. Tr, trained strain: $a_\infty = 1 \cdot 0$. T, total; L, large.

With Bact. coli mutabile and propamidine differences of about 3 days throughout are shown in figure 3.

When an untrained culture of Bact. lactis aerogenes was plated on proflavineagar (253 mg/l.) a very small fraction (2 $\times$ 10$^{-6}$) of the cells survived to form colonies and the number of these colonies gradually increased and reached a maximum after 4 days of incubation (figure 4). At the same time the colonies gradually
increased in size, and on the fifth day were ‘large’. With the trained strain, however, practically all the cells plated had formed colonies by the second day, and on the third day of incubation these colonies were ‘large’.

This variable difference of up to 2 days in the time for the appearance of colonies on proflavine-agar when trained and untrained strains of *Bact. lactis aerogenes* are plated is intermediate in the range of behaviour observed with the other drugs. For example, on thymol-agar there was no apparent difference in the time relationships with trained and untrained strains, although with the latter very few of the cells in the inoculum (about $10^{-7}$) succeeded in forming colonies at all (table 2).

![Graph showing time (days) vs. $10^6n$ for trained (Tr) and untrained (Un) strains](image)

**Figure 4.** *Bact. lactis aerogenes* and proflavine sulphate, 253 mg/l. *Un*, untrained strain: $\alpha_\infty = 2 \times 10^{-6}$. *Tr*, trained strain: $\alpha_\infty = 1.0$. *T*, total; *L*, large.

At low concentrations of streptomycin (between 1 and 20 µg/ml) the pattern of events was similar to that on thymol-agar, whilst at higher concentrations (30 to 100 µg/ml) the time differences were comparable with those observed with proflavine. For example, out of nine experiments carried out in the concentration range 1 to 20 µg/ml. there were six in which the thymol type of behaviour was observed, two experiments in which there was a difference of 1 day, and one in which there was a difference of 2 days in the time of incubation necessary for the appearance of the total number of colonies. Of the six experiments carried out at concentrations of streptomycin between 30 and 100 µg/ml., however, there were three in which the difference in time was 1 day, two in which it was 2 days and one in which it was 3 days. In figure 5 two examples of this latter type of behaviour are given, whilst all the experiments with streptomycin are summarized in table 1.

On chloramphenicol-agar (20 mg/l.) 94% of the cells in the inoculum from a trained strain of *Bact. lactis aerogenes* had formed colonies within 1 day, but none of the colonies was large in size. By the second day the proportion had reached practically 100%, and all the colonies were large. The percentage of total colonies formed with the untrained strain was 79 on the first day, 89 on the second and
Table 1. The resistance of Bact. lactis aerogenes on streptomycin-agar

<table>
<thead>
<tr>
<th>concentration of drug (µg/ml)</th>
<th>culture no.</th>
<th>fraction surviving</th>
</tr>
</thead>
<tbody>
<tr>
<td>1·0</td>
<td>1</td>
<td>$9 \times 10^{-7}$</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>$4 \times 10^{-8}$</td>
</tr>
<tr>
<td>2·0</td>
<td>1</td>
<td>$10^{-8}$</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>$1\cdot4 \times 10^{-8}$</td>
</tr>
<tr>
<td>4·0</td>
<td>2</td>
<td>$3 \times 10^{-8}$</td>
</tr>
<tr>
<td>5·0</td>
<td>1</td>
<td>$2 \times 10^{-8}$</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>$3 \times 10^{-8}$</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>$10^{-8}$</td>
</tr>
<tr>
<td>10·0</td>
<td>1</td>
<td>$10^{-8}$</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>$1\cdot5 \times 10^{-8}$</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>$6 \times 10^{-9}$</td>
</tr>
<tr>
<td>20·0</td>
<td>3</td>
<td>$10^{-8}$</td>
</tr>
<tr>
<td>30·0</td>
<td>3</td>
<td>$10^{-8}$</td>
</tr>
<tr>
<td>40·0</td>
<td>3</td>
<td>$10^{-8}$</td>
</tr>
<tr>
<td>50·0</td>
<td>3</td>
<td>$3 \times 10^{-9}$</td>
</tr>
<tr>
<td>60·0</td>
<td>3</td>
<td>$5 \times 10^{-9}$</td>
</tr>
<tr>
<td>70·0</td>
<td>3</td>
<td>$5 \times 10^{-9}$</td>
</tr>
<tr>
<td>100·0</td>
<td>3</td>
<td>$3 \times 10^{-9}$</td>
</tr>
</tbody>
</table>

Notes. (a) In the 16 tests carried out with concentrations of streptomycin between 1 and 60 µg/ml the total number of colonies had appeared and were large in size by the second day of incubation in 9 tests; 3 days were necessary in 4 tests and 4 days in the other 3 tests. The time relations for the experiments at 70 and 100 µg/ml are given in figure 5.

(b) Colonies picked from the drug plates were found to be fully resistant in re-test at the same concentration and also at $10^3$ µg/ml. In the experiments with 2, 3 and 5 µg/ml plates were obtained, at very high inoculum sizes (about $10^3$ cells) which contained a large number of late-appearing very small colonies. These small colonies were only slightly more resistant than the parent strain when re-tested.

Figure 5. Bact. lactis aerogenes and streptomycin sulphate. Left-hand set: 70 µg/ml. Un, untrained strain: $x_\infty = 5 \times 10^{-5}$. Tr, trained strain: $x_\infty = 1\cdot0$. Right-hand set: 100 µg/ml. Un, untrained strain: $x_\infty = 2\cdot7 \times 10^{-6}$. Tr, trained strain: $x_\infty = 1\cdot0$. T, total; L, Large.
third days, and then it gradually increased to reach 100 by the fifth day of incubation. There was thus little apparent difference between the behaviour of the trained cells and that of 89% of that minute fraction (4.4 \times 10^{-6}) of the untrained cells which succeeded in forming colonies at all. When large colonies are con-

**Figure 6.** *Bact. lactis aerogenes* and chloramphenicol, 20 mg/l. *Un*, untrained strain: \( \alpha_\infty = 4 \times 10^{-6} \). *Tr*, trained strain: \( \alpha_\infty = 1.0 \). *T*, total; *L*, large.

sidered the pattern of events is seen to be similar (figure 6) except that only 90% of the colonies from the untrained strain became large within the time limits of the experiment.

The delay in the formation of colonies by *Bact. lactis aerogenes* on terramycin
plates was similar in some respects to that observed with high concentrations of streptomycin in that there was a difference of 3 days between trained and untrained strains. At the lowest concentration of streptomycin tested, however, when the fraction surviving was about $10^{-7}$, there was no difference between the times for trained and untrained strains. With terramycin, on the other hand, 5 days of incubation were necessary before all the surviving cells of the untrained strain had formed colonies. This time was required when the fraction surviving was $2.4 \times 10^{-6}$ (10 units per ml.) and also when it was as high as 1 in 3 (1 unit per ml.) (figure 7).

On azide-agar (40 mg/l.) the fraction of the inoculum of an untrained strain of *Bact. lactis aerogenes* which survived to form colonies was $1.2 \times 10^{-5}$ on the second day of incubation and $1.4 \times 10^{-5}$ on the third day. There was no increase in the number after the third day. Of these colonies none was large on the second day of incubation and 38% were large by the third day. The 62% which were small on the third day remained small even after 7 days of incubation.

In contrast, when a small inoculum of a trained strain was plated at 40 mg/l. all the cells formed colonies, and these were large by the second day of incubation. This strain had had 37 subcultures in liquid medium containing 88 mg/l. of azide. Training was carried out at this higher concentration since tests during the training procedure had shown that, although growth took place readily in liquid media containing 40 mg/l. azide, the trained strain was not fully resistant when plated at this concentration. Indeed after 21 subcultures in liquid media containing azide at 88 mg/l. only 9 cells from an inoculum of 98 cells formed colonies at all when plated at 40 mg/l. It is of interest to note that azide appears to be more active in solid media than in liquid media, unlike many drugs for which the reverse holds.

With isonicotinyl hydrazine when an untrained strain of *Bact. lactis aerogenes* was plated, 6 colonies were obtained from an inoculum of $4.8 \times 10^3$ cells; they first appeared on the third day of incubation and were large by the fourth day. With a strain which had been given 61 subcultures at 1190 mg/l. and plated on agar containing this concentration of drug only 65 colonies were obtained from an inoculum of $2 \times 10^3$ cells and no colonies when the inoculum was 100 cells. These colonies were large on the second day of incubation.

The failure, in spite of repeated opportunities for favourable selection, of resistant colony-forming types to show more than a modest increase is to be noted.

**Effect of an excess of sensitive cells on the growth of resistant cells**

That delay in the appearance of colonies from untrained cells was not due to the inhibition of resistant cells by the presence of large numbers of sensitive cells was shown by experiments in which a large number of untrained cells (about $10^9$) were mixed with about 100 resistant cells and the mixture plated on drug-agar. The resistant cells formed colonies in the same time as when plated alone, in the following examples tested: *Bact. lactis aerogenes* with proflavine, chloramphenicol, brilliant green, streptomycin and terramycin, and *Bact. coli mutabile* with chloramphenicol.
The resistance of colonies from drug plates when re-tested

Cultures derived from colonies of *Bact. lactis aerogenes* on streptomycin plates were all found to be fully resistant on re-test at the concentration at which they had originally been plated and also at $10^3 \mu g/ml$. This represents complete resistance to as much as 1000 times the concentration to which the cells had been

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc. (mg/l.)</th>
<th>Fraction surviving</th>
<th>No. of colonies tested</th>
<th>Fraction surviving</th>
<th>Resistance on re-test</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brilliant green</td>
<td>32</td>
<td>$8 \times 10^{-7}$</td>
<td>4</td>
<td></td>
<td>$4 \times 10^{-7}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$9 \times 10^{-7}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$1.3 \times 10^{-6}$</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$8 \times 10^{-3}$</td>
<td></td>
</tr>
<tr>
<td>two colonies from test b were re-tested in e and f and two from d in g and h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$&lt;3 \times 10^{-7}$</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>$6 \times 10^{-8}$</td>
<td>1</td>
<td></td>
<td>$0.9 \times 10^{-3}$</td>
<td>in test f the colonies appeared over the first 4 days and in tests g and h they had all appeared within 2 days</td>
<td></td>
</tr>
<tr>
<td>two colonies which appeared on the 3rd day and two which appeared on the 4th day were re-tested in b, c, d and e</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>41</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$5 \times 10^{-5}$</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>$6 \times 10^{-8}$</td>
<td>4</td>
<td></td>
<td>$0.07$</td>
<td>in tests b, c and e the colonies appeared on the 4th day and in test d on the 3rd and 4th days</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>$8 \times 10^{-8}$</td>
<td>5</td>
<td></td>
<td></td>
<td>$&lt;10^{-5}$</td>
<td></td>
</tr>
<tr>
<td>sodium azide</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$2 \times 10^{-4}$</td>
<td></td>
</tr>
<tr>
<td>terramycin</td>
<td>10</td>
<td>$2 \times 10^{-6}$</td>
<td>5</td>
<td></td>
<td>$3 \times 10^{-4}$</td>
<td></td>
</tr>
<tr>
<td>(*) ug/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$10^{-4}$</td>
<td></td>
</tr>
</tbody>
</table>

In primary test colonies appeared on the 2nd day and increased in number up to the 5th day. In the re-tests they appeared on the 2nd day and increased in number and size up to the 6th day.
exposed (table 1). Moreover, the delay in the appearance of colonies, which was in evidence in some of the primary platings, was not observed even at concentrations of $10^8$ µg/ml. in the re-tests.

As judged by the values of $\alpha_\infty$ the colonies from the thymol plates were almost completely resistant on re-test, whilst those from brilliant green, azide and terramycin plates were slightly more resistant than the original untrained cultures when re-tested at the same drug concentration (table 2).

**Table 3. Resistance on re-test of colonies of Bact. lactis aerogenes from proflavine plates**

<table>
<thead>
<tr>
<th>expt.</th>
<th>drug concn. (mg/l.)</th>
<th>glucose concn. (g/l.)</th>
<th>buffer strength</th>
<th>primary plating</th>
<th>no. of colonies</th>
<th>resistance on re-test</th>
<th>$\alpha_\infty$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>320</td>
<td>40-0</td>
<td>normal</td>
<td>$2 \cdot 10^{-7}$</td>
<td>10</td>
<td>$0.6 \times 10^{-6}$*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>—</td>
<td>—</td>
<td>$1.8 \times 10^{-6}$</td>
<td>10</td>
<td>$4.2 \times 10^{-6}$*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>—</td>
<td>—</td>
<td>$1.3 \times 10^{-3}$</td>
<td>10</td>
<td>$0.9 \times 10^{-3}$*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>—</td>
<td>—</td>
<td>0-17</td>
<td>10</td>
<td>0.29*</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>204</td>
<td>4-2</td>
<td>normal</td>
<td>$1.4 \times 10^{-6}$</td>
<td>5</td>
<td>$a, 0.34$</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$b, 0.11$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$c, 0.07$</td>
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</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>$d, 0.47$</td>
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</tr>
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<td></td>
<td></td>
<td></td>
<td>$e, 0.03$</td>
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</tr>
<tr>
<td>3</td>
<td>204</td>
<td>40-0</td>
<td>normal</td>
<td>$6.1 \times 10^{-7}$</td>
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<td>$a, 4 \times 10^{-6}$</td>
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<td>$b, 1.9 \times 10^{-5}$</td>
<td></td>
</tr>
<tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>$c, 1.7 \times 10^{-6}$</td>
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<td></td>
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<td>$d, 4.5 \times 10^{-6}$</td>
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<td>$1.6 \times 10^{-7}$</td>
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<td>$a, 0.05$</td>
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<td>1-3 times</td>
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<td>$d, 0.08$</td>
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* Mean value.

In some of the experiments the colonies appeared more quickly in the re-tests but this was not always true even with the same drug. For example, with brilliant green in one experiment the colonies appeared more quickly and in another no more so than in the original test.

The behaviour of Bact. lactis aerogenes on chloramphenicol-agar has been considered elsewhere (Dean & Hinchelwood 1953). Of fifteen colonies picked from a 100 mg/l. plate, two were found to be completely resistant and three others slightly more resistant than the parent culture when re-tested at 100 mg/l. The remaining ten colonies, while no more resistant than the original cultures to 100 mg/l., were all fully resistant to 50 mg/l. At 50 mg/l. the survival of the parent culture was $0.5 \times 10^{-6}$.

With proflavine (table 3) the behaviour on re-test can be varied from almost complete resistance to the same resistance as in the original test, either by altering the amount of glucose on the plate or modifying the buffering capacity of the agar medium.
DISCUSSION

The difference in the time of appearance of colonies on drug plates when trained and untrained bacteria are plated varies from drug to drug in an interesting way.

There can be little doubt, in view of the large differences in time for trained and untrained strains, that the behaviour of Bact. lactis aerogenes on brilliant green plates and that of Bact. coli mutabile on chloramphenicol-agar and propamidine involves a gradual adaptation to the drug. Seeing that large excesses of sensitive cells demonstrably do not inhibit resistant cells, figures 1 to 3 seem to show quite clearly that the small numbers which grow from the untrained cultures are not pre-existent examples of the types which grow from the resistant cultures.

The experiments with brilliant green in table 2 are similar to those reported earlier for chloramphenicol in that colonies picked from plates varied in their resistance on re-test. In the first experiment given, one of the colonies was more resistant on re-test, while the other three were not. Moreover, when colonies from this high-survival plate were re-plated at the same drug concentration they were fully resistant, whilst colonies from one of the low-survival plates were no more resistant than the parent strain. It could be argued that the colonies on this former plate arose from resistant mutants which, during their growth, antagonized the drug in their immediate neighbourhood and so allowed non-mutants to grow and give rise to mixed colonies. Experiment has shown, however, that when a few trained cells are plated on brilliant green-agar in the presence of about $10^9$ untrained cells, mixed colonies do not result to any extent, the colonies formed from these trained cells being fully resistant on re-test. An analogous result has also been observed with the other drugs used in the present experiments, and incidentally answers the objection that a low survival on re-test might be due to reverse mutation during the procedure of re-testing. Further, in another experiment (41 mg/l.), where there was a higher degree of resistance on re-test than in the primary test, four colonies from the second plating were found to be no more resistant when tested again on brilliant green-agar.

In apparently sharp contrast with the examples in figures 1 to 3, Bact. lactis aerogenes on plates containing thymol, or streptomycin at less than 30 $\mu$g/ml. showed little difference in the time relations for trained and untrained strains. With higher concentrations of streptomycin (30 to 100 $\mu$g/ml.) there was a delay in some experiments of one day, in others of two days and in another of three days with the untrained strain.

On chloramphenicol-agar the pattern of events with Bact. lactis aerogenes was rather complex in that 89% of the cells of the untrained strain which formed colonies at all did so within the same time limits as those of the trained strain. There was a variable difference of up to 2 days with Bact. lactis aerogenes on proflavine-agar. On terramycin-agar, however, 5 days of incubation were required both at a concentration of drug where $a_\infty$ was about $10^{-7}$ and also when the concentration was low enough to allow of a survival of about 1 in 3.

It might be argued that some of these results represent merely a selection of the small fractions of pre-existent mutants, particularly since with streptomycin
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the fraction of the inoculum which survived did not vary to any appreciable extent as the drug concentration was increased.

Further evidence which might be cited for mutations is the fact that the survivors from all the streptomycin experiments were fully resistant, not only to the concentration of drug on the plates from which they had been isolated, but also to the highest concentration at which they were tested (10^9 μg/ml). An alternative explanation in terms of an adaptive mechanism has, however, been suggested for this particular kind of behaviour (Dean & Hinshelwood 1953), and further investigation is necessary before a decision can be reached. Some adaptation is in any case necessary to concentrations of streptomycin in the range 30 to 100 μg/ml since the trained cells form colonies on the plates more rapidly than the untrained cells.

The time relationships observed with Bact. lactis aerogenes and cloramphenicol (20 mg/l.) might apparently be interpreted by the assumption that the 89% of the survivors from the untrained strain which formed colonies in the normal time were fully adapted pre-existing mutants. The other 11% needed considerable adaptation, since the percentage of total colonies was 89 on the second and third days, 93 on the fourth day and 100 on the fifth day.

Before such a view of these examples is adopted, however, some more detailed statistical considerations are necessary. These will reveal this kind of test for mutants in a somewhat unexpected light, and indicate that while the behaviour shown in figures 1 to 3 is inconsistent with pre-existent resistant forms, that shown by some of the other figures is by no means inconsistent with gradual adaptation (see next section).

The resistance on re-test of colonies of Bact. lactis aerogenes picked from cloramphenicol plates (100 mg/l.) is not incompatible with an adaptive hypothesis. Of fifteen colonies, two were found to be fully resistant to 100 mg/l., three were slightly more resistant than the parent strain, and the remaining ten, while no more resistant to 100 mg/l., were more than 10^6 times as resistant to 50 mg/l. as the parent strain. Some difficulties encountered in the explanation of these results on a mutation basis are discussed elsewhere (Dean 1955).

The experiments with terramycin are interesting since not only would a delay of 3 days in the formation of colonies, but also a mutation rate of about 1 in 3 have to be explained if survival were assumed to be merely a selection of pre-existing resistant cells. Moreover, colonies from terramycin plates were not fully resistant on re-test.

The results of the experiments on the resistance of colonies grown on proflavine (table 3) can be explained in terms of the inactivation of proflavine by the acids produced from glucose by the growing cells. On plates with normal buffering capacity a high concentration of glucose would result in the production of sufficient acid to break the buffer in the immediate neighbourhood of the colony and so antagonize the drug, thus giving less stimulus to unstably adapted cells to retain their resistance or for their progeny to adapt so fully. Reduction of the glucose concentration or increase of the buffering capacity of the medium, as expected, counteracts this antagonism.
Some statistical considerations about survival on drug plates

In drugs such as proflavine or thymol the death-rate is high. An adaptive process like that which seems clearly to be occurring with those cultures whose behaviour is represented in figures 1 to 3 could only lead to colony formation in cells which are favoured by chance with an exceptionally long survival time. That the incidence of death is often not far from random is shown by the nature of the survival-time curve itself.

In any culture which is subject to lethal influences there is a wide distribution of actual survival times, and in any which undergoes an adaptive process attended with a lag there must be a statistical scatter in the delays preceding the growth of individual cells. Adaptation in a dying culture depends upon the interplay of these two statistical factors.

The following approximate theoretical treatment, although based upon equations which are only roughly true in the quantitative sense, brings to light a result which will almost certainly remain valid even if the detailed forms of the statistical laws assumed is considerably varied.

Let the most probable delay before a cell begins to develop into a colony be \( L \). If the actual delay is \( t \), then this represents a deviation of \( \theta \), where

\[
\theta = t - L.
\]

We assume as a rough approximation that the deviations are Gaussian, so that the probability of a deviation between \( \theta \) and \( \theta + d\theta \) is

\[
\frac{h}{\sqrt{\pi}} e^{-h^2\theta^2} d\theta
\]

or if \( x = h\theta = h(t - L) \), the probability is

\[
\frac{1}{\sqrt{\pi}} e^{-x^2} dx.
\]

We shall consider chiefly large negative values of \( x \), that is, exceptionally quickly developing cells.

The cells are dying off according to a law which will be taken as roughly exponential, so that \( n_t \), the number of survivors at time \( t \), conforms to the equation

\[
\frac{dn_t}{dt} = -\lambda n_t + f(n_t, t),
\]

where \( \lambda \) is the death-rate constant, and \( f(n_t, t) \) is the rate at which some cells succeed in dividing and so escaping the lethal process. In the conditions considered very few do this, so that \( f(n_t, t) \) is a small fraction of \( \lambda n_t \). Thus the fraction surviving at time \( t \) is nearly \( e^{-\lambda t} \).

Of this, the number which make an early start and form colonies in the interval between \( t \) and \( t + dt \) is a fraction \( \frac{1}{\sqrt{\pi}} e^{-x^2} dx \), where \( dx \) is the deviation corresponding to \( dt \). Thus the fraction of the original population which starts to form colonies between \( t \) and \( t + dt \) is

\[
e^{-\lambda t} \frac{1}{\sqrt{\pi}} e^{-x^2} dx = \frac{1}{\sqrt{\pi}} e^{-\lambda(t+x/h)} e^{-x^2} dx
\]
since \( x = -h(L-t) \). The total number which do this from time 0 to time \( t \) is

\[
\frac{1}{\sqrt{\pi}} \int_{-hL}^{-h(L-t)} e^{-\lambda(x+\lambda/2h)^2} e^{-x^2} dx = \frac{e^{-\lambda L/4h^2}}{\sqrt{\pi}} \int_{-hL}^{-h(L-t)} e^{-(x+\lambda/2h)^2} e^{\lambda^2/4h^2} dx,
\]

where \(-h(L-t)\) and \(-hL\) are the limits of \( x \) corresponding to \( t = t \) and \( t = 0 \) respectively.

If \( y = x + \lambda/2h \) the last integral becomes, with the new limits of \( y \) corresponding to the old limits of \( x \),

\[
P = \frac{e^{-\lambda t} e^{\lambda^2/4h^2}}{\sqrt{\pi}} \int_{-hL + \lambda/2h}^{-h(L-t) + \lambda/2h} e^{-y^2} dy
\]

\[
= \frac{e^{-\lambda L} e^{\lambda^2/4h^2}}{\sqrt{\pi}} \left[ \int_0^{hL - \lambda/2h} e^{-y^2} dy - \int_0^{h(L-t) - \lambda/2h} e^{-y^2} dy \right]
\]

\[
= \frac{1}{2} e^{-\lambda L} e^{\lambda^2/4h^2} [\text{erf}(hL - \lambda/2h) - \text{erf}(hL - \lambda/2h - ht)].
\]

When \( \lambda = 0 \) this becomes

\[
P = \frac{1}{2} [\text{erf}(hL) - \text{erf}(hL - ht)].
\]

When \( t > L \) the limits are interchanged and we have

\[
P = \frac{1}{2} [\text{erf}(hL) + \text{erf}(ht - L)],
\]

and correspondingly for (1),

As \( t \) increases \((2')\) reaches a limit of unity. The final limit of (1) is

\[
e^{-\lambda L} e^{\lambda^2/4h^2} = \alpha_\infty,
\]

the fraction of the cells which eventually form colonies.

\( \alpha_t \), the fraction which has formed colonies at time \( t \), is not directly given by the above formulae, since in these \( t \) is the time at which the colony begins to form and not that at which it attains a standard size. Nevertheless, the difference between the two times may be taken as a more or less standard value, and the equations will apply to the observed times if the origin of the experimental curves is shifted. In any case \( t \) of the equations will give a generally correct model for the behaviour of \( t_{0th} \).

The most interesting consequence of these equations is that the existence of a finite death-rate shortens the value of the most probable delay for those cells which actually do grow to colonies. When \( \lambda = 0 \), equation (2) gives, as \( t \) increases, a limiting value of unity, which corresponds to \( \alpha_\infty = 1 \). When \( \lambda \) is not zero, \( \alpha_\infty \) is \( e^{-\lambda L} e^{\lambda^2/4h^2} \) which is less than unity and may be very small indeed. But \( \alpha_t/\alpha_\infty \), which is given by the quantity in the square brackets, rises from 0 to 1 as before, and the most probable value of the delay, that is, the time for \( \alpha_t/\alpha_\infty \) to reach the value 0.5, is now no longer \( L \) but \( L - \lambda/2h^2 \), as is clear by inspection.

The magnitude of \( h \) for a trained culture or for one growing in the absence of drug may be taken as about 1.8 (with the time unit in days), which corresponds to a spread of about one day between \( \alpha_t/\alpha_\infty = 0.1 \) and \( \alpha_t/\alpha_\infty = 0.9 \), as shown in curve I of figure 8.
With proflavine at the concentrations used, a culture would be sterilized in a few days, so that $\lambda$ is in the range 5 to 10 day$^{-1}$. Thus the reduction in the apparently most probable lag is of the order $7/(2 \times 1.8^2)$ if $h$ remains unchanged, or about one day.

With $\lambda = 7$ day$^{-1}$, $L = 2.5$ days, and $h = 1.8$, the value of $\alpha_\infty$ is about $10^{-6}$, which is comparable with that observed in many of the experiments described.

In such an experiment $L$ might have been increased by the presence of a drug to about a day more than the normal value in the absence of the drug, and yet

\[ L - \lambda/2h^2, \]

which gives the apparent value for the survivors, would be nearly the same as that observed with a resistant culture or a culture in a drug-free medium. This result is illustrated by a comparison in figure 8 of curves I, II and III.

The value of $h$ itself, however, will be different for the trained and the untrained cultures. The more scattered the distribution of lags, the smaller the value of $h$. Curves V and VI in figure 8 show the effect of a change in $h$ from 1.8 to 0.9. The difference in the forms of curve I, or any of the curves with $h = 1.8$, and VI ($\lambda = 3.5$, $L = 5$, $h = 0.9$) are in fact comparable with the differences between the
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$\alpha_U/\alpha_{\infty}$ against time curves for trained and untrained strains shown in figures 4 and 5. Thus it is reasonable to suppose that $k$ is in fact smaller for the untrained strains. When this is so, the effect previously described is much enhanced. In figure 8, curve VI, compared with curve I, shows how a true value of $L$ of 5 days in an untrained strain with $\lambda = 3.5$ and $k = 0.9$ may be replaced by an apparent value of the most probable delay of not much more than half this.

The comparison of curves VI and I is also interesting in that it shows how at first the untrained strain may appear to keep pace with or even outstrip the trained strain, but may fall back later. Figure 8 is of course illustrative only, but the relation of curves VI and I is suggestively like that shown in figures 4 and 5.

It will be useful to translate the above conclusions into non-mathematical terms. All properties of populations, and this applies particularly to physiological properties which vary in a highly complex way during the growth cycle of each individual cell, show a statistical distribution about a most probable value. Large deviations are rare and very large ones very rare, but up to a point have an assignable probability. In a plate test where all the cells are expected to grow, the number which can be used in the test is, for practical reasons, limited to about $10^2$, so that the chance of inclusion of the more highly deviating examples of the population is small. In experiments, on the drug resistance of untrained cultures, in order to obtain a comparable count of survivors, something like a million times as many cells may have to be plated as in the corresponding experiment with a trained strain (or in an experiment without drug). Thus the colony-forming cells represent the extreme end of a statistical distribution. Now if the population as a whole is rapidly dying (which happens with many drugs), the only survivors are those with exceptionally short lags, the majority of the cells never having a chance to contribute to a complete picture of the distribution of growth times. Thus the $\alpha_U/\alpha_{\infty}$ against time curve of a trained strain is representative of the average population while that of an untrained culture refers only to the exceptional tail of a very large population indeed. Yet in the latter case the distribution is finally recalculated by scaling up $\alpha_{\infty}$, which may be $10^{-6}$ or less, to unity for making comparisons. Thus from the nature of the test the surviving cells of the drug-sensitive population might even appear to develop into colonies faster than those of the corresponding resistant population. For a proper comparison something like a million times as many of the latter as are actually tested would, if practicable, be used, and the fastest developing few compared with the corresponding number of the sensitive cells.

Since this is not practicable, the experiments may give results simulating the growth of mutants, for reasons which are purely statistical.

Conclusion

The results in some of the examples are clearly and obviously incompatible with the view that the cells forming colonies on the drug plates are pre-existent and adapted mutants. A considerable degree of adaptation occurs on the plate. In other examples the time-number relations are not obviously inconsistent with the pre-existence of at least some of the adapted forms. But these relations are equally
Viability of adrenocortical tissue transplanted after freezing and thawing

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[Plate 20]

The average survival time after bilateral adrenalectomy of rats of the N.I.M.R. hooded strain, maintained on a low salt diet and not having visible accessory tissue, was about 6 days. This survival time was usually too short to permit the establishment of an effective graft, but by feeding NaCl it could be prolonged sufficiently to enable fresh cortical tissue to form grafts which were functional in that the animals survived subsequent transference to low salt diet for 2 weeks.

Exposure of the cortical tissue to low temperatures by the methods previously employed for ovarian tissue (Parkes & Smith 1953) greatly decreased the probability that it would form an effective graft, but a number of successful experiments were carried out, especially when a buffered Ringer–Locke medium containing glycerol was substituted for the glycerol-saline previously used. In all, more than a hundred functional grafts were obtained from adrenocortical tissue which had been exposed to a temperature of \(-79\) °C for up to 24 h.

Adrenocortical cells, therefore, like many others, are not necessarily destroyed by exposure to low temperatures (\(-79\) °C), though it seems that optimal conditions are not provided by the technique evolved for gonadal endocrine cells.

INTRODUCTION

The observation that glycerol protected fowl spermatozoa against the otherwise fatal effects of freezing and thawing (Polge, Smith & Parkes 1949), and permitted their long-term preservation at low temperatures, has been applied to a variety of other cells and also to endocrine and other tissues. Thus, rat ovarian tissue, soaked in 15% glycerol-saline, frozen slowly to \(-79\) °C and stored at \(-190\) °C readily