The Effect of Mycophenolic Acid on the Growth of *Staphylococcus aureus* in Heart Broth

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During an investigation of the production of antibiotics by fungi, Wilkins & Harris (1943) found that the culture liquid from *Penicillium brevi-compactum* Dierckx. 3568 inhibited the growth of *Staph. aureus*. The substance responsible for the inhibition was isolated in this laboratory and found to be mycophenolic acid (Florey, Gilliver, Jennings & Sanders, 1945). The molecular constitution of this substance had been studied by Clutterbuck & Raistrick (1933), who advanced the following structure for normycophenolic acid:

In mycophenolic acid itself one of the phenolic groups is methylated.

Florey *et al.* (1945) studied the biological properties of this antibiotic and noticed that when its activity against staphylococci was determined by the serial dilution method the titre varied greatly with the number of organisms used for the inoculum. To study this in more detail a quantitative investigation has been made of the effect of the antibiotic on the growth of staphylococci. It was soon apparent that the effect is a complicated one, involving a rapid adaptation of the staphylococci to growth in the presence of mycophenolic acid.

EXPERIMENTAL

Conditions of growth. *Staph. aureus* (H. strain, National Collection of Type Cultures (N.C.T.C.) no. 6571) was grown in aerated heart-extract broth at pH 7. The medium (20 ml.) was contained in pyrex glass tubes. The tubes were attached, by a ground-glass joint, to a hollow glass head-piece having an air inlet tube, an outlet and an opening with a ground-glass cap for taking samples (Fig. 1). The tubes and head-pieces were sterilized separately and fitted together aseptically, without grease, after sterile medium and the inoculum had been added to the tubes. The tubes were placed in a water-bath at 37° and a slow stream of sterile air, saturated with water-vapour at the same temperature, was blown through the medium. Samples were taken with long Pasteur pipettes.

Except in special circumstances, mentioned later, the staphylococci grew in the form of perfectly discrete cells.

Total counts. The total number of organisms was counted in a Neubauer haemocytometer, 0.1 mm. deep, with a grating of sixteen sets of sixteen squares. A drop of formalin and a trace of methylene blue were added to the sample,

Fig. 1. *a*, manifold delivering sterile air; *b*, tap; *c*, cotton-wool plugs; *d*, opening for taking samples; *e*, air inlet; *f*, air outlet; *g*, rubber ring for supporting tube in rack *h*; *i*, ground-glass joints.

which was then diluted, if necessary, until it contained about 20×10^6 organisms/ml. The cells were allowed to settle for about 15 min. in the haemocytometer before being counted. The majority of counts was made on samples

containing more than 10×10^6 organisms/ml., a concentration at which a turbidity in the medium was just perceptible; no attempt was made to count samples having less than 4.0×10^6 /ml., corresponding to about one cell/small haemocytometer square or 256 in the whole grating.

If counts are made in N small haemocytometer squares giving a total Nn cells, the mean number of cells/square is n and the standard deviation of the distribution is $\sqrt{n}$. The standard deviation of the mean of N counts (standard error) $= \sqrt{(n/N)}$. Thus, if the whole haemocytometer grating is used to count a sample containing 4.0×10^6 cells/ml. the standard error can certainly not be less than 6.2%. Counting in a haemocytometer 0.1 mm. deep presented no difficulty with staphylococci, which were easily seen and settled readily, but it is not suggested that the method would necessarily be satisfactory for counts of other species of bacteria.

Viable counts. The number of viable organisms was counted by the roll-tube method of Wilson (1922) or by spreading two drops of a suitably diluted culture, with the aid of a glass spreader, on the surface of three nutrient-agar plates. Dilutions were made in sterile tap water with calibrated dropping pipettes.

For counting samples containing relatively small numbers of organisms in the presence of the antibiotic the plate method was preferred. In these cases the roll-tube method gave rather lower values, possibly because it involved a smaller dilution of the antibiotic and also brought the latter into contact with the cells for a short time at 45° .

Inoculum. A 16 hr. culture in Lemco broth was made from a culture of *Staph. aureus* kept on an agar slope in the ice-chest. One loopfull of this culture was added to 5 ml. of heart broth in a 25 ml. Erlenmeyer flask and the flask shaken at 37° for 15–16 hr. The resulting culture contained about 3000×10^6 organisms/ml., at least 90% of which were viable. It was suitably diluted in sterile tap water and about five drops of the bacterial suspension, delivered by a calibrated dropping pipette, were used to inoculate 20 ml. of the medium in the tubes.

GROWTH CURVES

In all the growth curves $\log_{10} n_t$ or $\log_{10} n_v$ was plotted against t , where n_t was the total number of organisms/ml., n_v the number of viable organisms/ml., and t the time in minutes.

If there is a sudden transition from the lag phase to the logarithmic phase the common growth curve of a bacterial culture is given by

$$n = n_0 e^{k(t-L)} \text{ or } \log_{10} n = \log_{10} n_0 + \frac{k}{2.303} (t-L),$$

where n_0 is the original number of organisms, n the number at time t , and L the length of the lag phase; k is a growth constant. The lag is defined here in accordance with this equation; it was measured by extrapolating the straight line representing the change of $\log_{10} n$ with t in the logarithmic phase until $n = n_0$ and reading the corresponding value of t .

The mean generation times (m.g.t.'s), or the times taken for dividing cells to double in numbers,

were calculated from the slopes of the curves during the phase of logarithmic growth:

$$\text{m.g.t.} = \frac{2.303 \log_{10} 2}{k}, \text{ where } k = 2.303 \frac{d \log_{10} n}{dt}.$$

Normal growth

Growth in the batch of heart broth used for the first series of experiments is shown by curves 1, 2 and 3 in Fig. 2. The lag was 40–50 min. and did not vary significantly with the size of the inoculum. The m.g.t. was 28.2–28.7 min. The logarithmic phase of growth came to an end abruptly as the number of organisms approached 1000×10^6 , which was defined as the stationary population (n_s): the rate of increase in numbers beyond this was relatively very slow. The pH of the medium, as measured by indicators, did not change by more than 0.5 unit before the stationary population was reached.

Growth in a $1:10^4$ dilution of mycophenolic acid

The growth curves in the presence of mycophenolic acid at a dilution of $1:10^4$ are shown by curves 4–8 in Fig. 2. It is convenient to consider the lag phase and logarithmic growth phases separately.

(1) The lag phase

The length of the lag phase, as estimated by extrapolation of the total counts during logarithmic growth, increased greatly as the size of the inoculum was decreased. That this extrapolation was justified was shown by making viable counts during the lag phase itself, the results of which are shown in Fig. 3. There is reasonably good agreement between the length of the lag determined by the two methods (Table 1). The viable counts also indicated that, at this concentration of the antibiotic, the estimated lag was due to non-division only and not partly to the killing of a significant number of cells before multiplication began.

Table 1. Variation of length of the lag period with inoculum size

(*Staph. aureus* in heart broth.)

Inoculum (n_0) no. of organisms (millions)	Length of lag by extrap- olation of total counts (min.)	Inoculum (n_0) no. of organisms (millions)	Length of lag by viable counts (min.)
9.0	40	—	—
0.56	280	0.96	240
0.14	380	—	—
0.014	640	0.013	700
0.010	920	—	—

(2) Phase of logarithmic multiplication

Adaptation to growth in mycophenolic acid. In the presence of the antibiotic the m.g.t. was increased.

Fig. 2. Effect of inoculum size on growth. Curves 1, 2 and 3, growth in normal heart broth. Curves 4-8, growth in heart broth containing mycophenolic acid at a dilution of $1:10^4$. Curves V and VIII, subculture from 5 and 8 in the presence of mycophenolic acid ($1:10^4$).

Fig. 3. Effect of inoculum size on the lag period.
○—○ Viable counts. ● Total count.

The curves (Fig. 2) representing the rates of multiplication present three points of interest: (1) The rate of division when total counts were first possible ($n \approx 10 \times 10^6$) increased as the inoculum used was made smaller. (2) Except with the

Table 2. Variation of the rate of division with inoculum size in the two parts of the logarithmic growth phase

(*Staph. aureus* in heart broth containing mycophenolic acid ($1:10^4$)).

Inoculum (n_0) no. of organisms (millions)	m.g.t.* first phase $\log_{10} n = 7.2-7.7$ (min.)	m.g.t.* second phase $\log_{10} n = 8.2-8.7$ (min.)
9.0	137	83
0.56	80	70
0.14	63	58
0.014	41	41
0.010	39	39

* m.g.t. = mean generation time.

smallest inocula the growth curves were clearly composite, the rate of division increasing fairly suddenly after n had reached approximately 100×10^6 . Growth in the separate parts of the curves was nearly logarithmic. (3) The size of the stationary population was not changed significantly by the antibiotic. The data relating to (1) and (2) are summarized in Table 2.

The changes in the m.g.t.'s during logarithmic growth showed that the staphylococci were rapidly becoming adapted to multiplication in the presence of mycophenolic acid. The effect of this adaptation was clear from a study of the entirely different growth curves of subcultures.

Growth and stability of the adapted staphylococci. Curves V and VIII (Fig. 2) represent the growth of inocula taken from the cultures recorded in curves 5 and 8 (Fig. 2) a few hours after these had reached their stationary concentrations. In both cases the lag was short (about 90 min.) and the m.g.t. low (39 and 35 min.).

After subculturing culture VIII twice more in heart broth containing a $1:10^4$ dilution of mycophenolic acid there was little, if any, increase in adaptation. The lag was then 80 min. ($n_0 = 1.0 \times 10^6$) and the m.g.t. 36 min.

After subculturing culture VIII twice through heart broth not containing the antibiotic there was no significant loss in adaptation, the lag being 80 min. ($n_0 = 1.0 \times 10^6$) and the m.g.t. 37 min.

Fig. 4. Adaptation to growth in presence of mycophenolic acid (1 in 10^4). Curve 1, primary growth curve. Curve 2, subculture from 1. Curve 3, growth in normal heart broth.

The course of adaptation to a $1:10^4$ dilution of mycophenolic acid when starting from a fairly large inoculum ($n_0 = 6.0 \times 10^6$) is indicated by the curves in Fig. 4. Curve 1 shows the primary growth and curve 2 the curve obtained with an inoculum taken from culture 1 a few hours after the latter had reached its stationary population. The adaptation consisted in a shortening of the time during which

the slower rate of growth occurred and in a transition to a faster rate of division than the corresponding rate in the primary culture. In the absence of the antibiotic the adapted staphylococci grew at the same rate as the normal organisms.

Effect of adding mycophenolic acid during logarithmic growth. In Fig. 5 curves 2 and 3 show the effect on growth of mycophenolic acid when added to give a dilution of $1:10^4$ at the beginning (curve 2) and in the middle of the logarithmic phase (curve 3). The rate of division decreased immediately, the m.g.t. being approximately doubled. After a short time this was followed by a further decrease to a very slow rate of growth before adaptation began.

Fig. 5. Addition of mycophenolic acid (to a dilution of $1:10^4$) at two stages of logarithmic growth.

Effect of culture-filtrates on growth in the presence of mycophenolic acid. The dependence of the length of the lag phase on the inoculum size in the presence of mycophenolic acid made it desirable to investigate the effect on growth of filtrates from young cultures.

(1) An inoculum of 1.0×10^6 cells/ml. was allowed to grow in normal heart broth to about 20×10^6 and the culture then centrifuged clear and sterilized by filtration through a Gradacol membrane. 5 ml. of heart broth were added to a tube containing 15 ml. of the filtrate and the mixture inoculated so that it contained 0.8×10^6 organisms/ml.; a similar inoculum was used for a control tube containing 20 ml. of heart broth; mycophenolic acid was added to both media to give a dilution of $1:10^4$. Viable counts were made at intervals on samples from the two tubes. In the normal heart broth the number of organisms remained practically constant for 500 min. In the filtrate a very slow rate of growth began but stopped after about one division. When the inocula grew up the organisms in the filtrate

reached a count of 100×10^6 , 30 min. before those in normal heart broth. The final effect of the filtrate was therefore rather small.

(2) An inoculum of 5.0×10^6 cells/ml. was allowed to grow to about 50×10^6 in a $1:10^4$ dilution of mycophenolic acid in medium, at which stage it had almost reached the point at which transition occurred from the slower to the faster rate of growth. The culture was centrifuged and filtered. Growth was then compared in the filtrate and in normal heart broth containing the same concentration of the antibiotic. (It had been shown that mycophenolic acid was not inactivated during growth of the staphylococci.) The growth curves are shown in Fig. 6. In normal heart broth (curve 1) there was

Fig. 6. Abolition of the lag phase by filtrate from a young culture. Curve 1, growth in heart broth containing mycophenolic acid ($1:10^4$). Curve 2, growth in filtrate from a young culture containing mycophenolic acid ($1:10^4$). ○—○ Viable counts. ●—● Total counts.

a lag period of 600 min.; in the filtrate (curve 2) division began immediately, with a m.g.t. of 125 min. which was maintained until transition occurred to a faster rate of growth. Culture (2) reached a count of 100×10^6 , 170 min. before culture (1). There was thus no doubt about the effect of the filtrate in abolishing the lag period.

Effect of concentration of mycophenolic acid. In Fig. 7 curves 1–5 represent growth from inocula of similar size ($n_0 = \text{approx. } 5.0 \times 10^6$) in the presence of concentrations of mycophenolic acid increasing from a dilution of $0.25:10^4$ to a dilution of $5.0:10^4$. With the apparent exception of curve 5 all the curves are composite, consisting of a relatively short lag, a period of slow division and a period of more

rapid division. In the case of the highest concentration (curve 5) the dotted line indicates that the long lag represented by the number of organisms counted does not give a true picture of the course of events. During this period morphological changes occurred, resulting in the adherence of some of the cells to the sides of the tube; the numbers counted in the supernatant liquid, which contained for the

Fig. 7. Effect of the concentration of mycophenolic acid on growth. Curve 1, 0.25 in 10^4 ; curve 2, 0.5 in 10^4 ; curve 3, 1.0 in 10^4 ; curve 4, 2.5 in 10^4 ; curve 5, 5.0 in 10^4 ; curve 6, control, growth in heart broth without mycophenolic acid.

most part discrete cells, therefore gave low values in terms of bacterial mass. When more rapid growth set in, this was normal in character and most of the adhering cells disappeared. The stationary population ($n_s = 550$) was lower than that reached in the presence of lower concentrations of the antibiotic ($n_s = 1100$).

The rate of division in the first phase decreased, but by no means linearly, with the increasing concentration of the antibiotic. The rate of division in the second phase decreased to a value, about 0.4 , as great as that of a normal culture, at which it remained practically constant during a further fivefold increase in concentration of antibiotic. This is shown graphically in Fig. 8. In the presence of a $5.0:10^4$ dilution of mycophenolic acid the numbers of viable organisms also were counted at intervals during the first 700 min.; there was good agreement between the viable and total counts.

To see whether the highest concentration of antibiotic was approaching one at which it would exert a bactericidal action, viable counts were made at intervals for 420 min. on samples from a tube inoculated with 10×10^6 organisms/ml. and containing a $1:10^3$ dilution of mycophenolic acid. There was a very slow increase in the number of cells which, at the end of this time, reached 20×10^6 . A corresponding change occurred in the total num-

ber of organisms present. Thus, under these conditions, 0.1 % mycophenolic acid exerted no detectable lethal effect on the cells.

Fig. 8. Variation of the ratio of the growth constants with various concentrations of mycophenolic acid to the growth constant in normal heart broth.

Morphological changes during growth in the presence of mycophenolic acid

The first series of measurements on growth in the presence of a $1:10^4$ dilution of mycophenolic acid (Fig. 2) was made using a single batch of heart broth, which was kept sterile in 100 ml. bottles at room temperature. After 2 months, although the lag, m.g.t. and n_s of the staphylococci grown in this medium were not measurably different from their original values, morphological changes, consisting in the formation of clumps of cells which had a tendency to adhere to the walls of the vessels, occurred when growth took place in the presence of a $1:10^4$ dilution of mycophenolic acid. As these changes continued to occur when the staphylococci were taken from different agar slopes, while growth was normal in a fresh sample of heart broth in the

same concentration of mycophenolic acid, it was concluded that alterations which had taken place in the medium on standing were responsible for the abnormal mode of growth. This medium is subsequently referred to as 'old heart broth' (O.H.B.).

The physical properties of the clumps of cells made it impossible to investigate quantitatively their numbers and sizes, but the following facts were noted: (1) Clumping only occurred after growth had begun. At the end of the lag period about one division took place in an apparently normal manner before any morphological changes appeared. The degree of clumping then increased to a maximum and subsequently decreased as the growth cycle proceeded. (2) The morphological changes were more pronounced when growth occurred in filtrate, to which mycophenolic acid had been added, from young cultures in O.H.B. When small inocula were used the filtrate greatly shortened the time which elapsed before clumping began, a result which might be expected if the filtrate abolished the lag period. (3) After two subcultures in O.H.B. containing mycophenolic acid the staphylococci had become trained to grow in this medium without any morphological changes taking place. The growth curve was then very similar to that of the staphylococci which had become adapted to mycophenolic acid by growth in a sample of heart broth in which clumping did not occur at all.

RESULTS

The main facts which emerge from a study of the growth curves are: (1) When *Staph. aureus* (H. strain, N.C.T.C. no. 6571) is grown in normal heart broth the short lag phase is not altered appreciably by changes in the inoculum size. (2) Mycophenolic acid lengthens the lag phase and the mean generation time; at a dilution of $1:10^4$ it has little effect on the size of the stationary population. (3) As the inoculum size is made smaller the effect of mycophenolic acid on the lag phase is greatly increased. The reverse effect is found on the m.g.t.; within certain limits the smaller the inoculum the greater the rate of division when logarithmic multiplication has set in. (4) Filtrate from a young culture grown in the presence of mycophenolic acid abolishes the long lag phase of small inocula. Growth is also stimulated by filtrate from a young normal culture. (5) During multiplication in the presence of the antibiotic a slow rate of division may be superseded by a faster one, resulting in composite growth curves. (6) The growth rates in both parts of the growth curve are decreased, but not linearly, by increasing the concentration of the antibiotic; the faster growth rate reaches a limiting value below which it does not fall while the concentration of antibiotic is increased fivefold. (7) During

growth a rapid adaptation of the staphylococci occurs which may be almost complete in one culture cycle. The adapted organisms are characterized by a short lag and by a high rate of division in the presence of the antibiotic. Reversal of the adaptation, if it occurs, is comparatively very slow. (8) Minor alterations in the medium can result in gross morphological changes during growth in the presence of mycophenolic acid, but the staphylococci are rapidly adapted to grow without these changes occurring. The changes are accentuated by filtrate from a young culture grown in the same medium in the absence of the antibiotic.

DISCUSSION

The inoculum size in the serial dilution method of estimating antibacterial activity

Within certain limits the variation of dilution test readings with inoculum size does not in itself imply that the size of the inoculum has any effect on either the lag or the rate of division. It is obvious that, provided two inocula differ sufficiently in size, one can be fully grown before any growth is visible from the other, so that on reading the tubes *within a given time* there would be a clear-cut difference in turbidity.

Suppose n_{01} is the large and n_{02} the small inoculum. When n_{01} has just grown to its stationary population n_s , n_{02} has grown to a value n_2 , where

$$\log n_s - \log n_{01} = \log n_2 - \log n_{02} = k(t - L),$$

or

$$n_{01}/n_{02} = n_s/n_2.$$

The condition that at any time there should be full growth in one tube and no visible growth in the other is that $n_2 < 10 \times 10^6$ or $n_{01}/n_{02} > n_s/10 \times 10^6$. If n_s is 1000×10^6 this condition is satisfied when $n_{01}/n_{02} > 100$.

With a hundredfold dilution of the inoculum this state of affairs holds only momentarily. With a thousandfold dilution, however, it is maintained during a time T sufficient for n_{02} to increase tenfold. Therefore $T = \text{m.g.t.}/0.3$. If a given concentration of an antibacterial substance increased the m.g.t. from 30 min. to, say, 100 min., $T = 5.5$ hr. If n_{01} and n_{02} were 1000×10^3 and 1×10^3 respectively the larger inoculum would reach its stationary population in 1000 min. and the smaller inoculum its visible population in 1333 min. Suppose, as has been shown in some cases (e.g. Dagley & Hinshelwood, 1938), that the growth constant k varied linearly with the concentration of the antibacterial substance, i.e. $k = k_0 - dC$, where C is the concentration and d is a constant. Then it is easily calculated that, if the concentration of the antibacterial substance were halved, the smaller inoculum would reach its stationary population in 920 min. If the concentration of antibacterial substance were doubled, k would

appear as a negative quantity, and there would be no growth at all. Thus, in a twofold dilution test carried out under these conditions, and read between 16.5 and 22 hr., complete inhibition of growth would be shown in one extra tube in the serial dilutions in the case of the smaller inoculum. If the effect of the inoculum size were considerably greater than this it would be expected either that growth depended in some specific manner on the size of the inoculum or that the rate of growth was altered relatively little by changes in the concentration of the antibacterial substance. Both these factors were operative in the case of mycophenolic acid. Inspection of curve 7, Fig. 2, and curve 5, Fig. 7, shows that, between 15 and 17 hr. after inoculation, there was no visible growth from the smaller inoculum while an inoculum 360 times as large had reached its stationary population in a fivefold greater concentration of mycophenolic acid. The determining factors here were the considerable lengthening of the lag phase as the inoculum size decreased and the adaptation of the cells to grow rapidly even in relatively high concentrations of mycophenolic acid.

Adaptation and natural selection

In the vast literature on bacterial variation widely different suggestions have been made to account for the changes which can occur within the same bacterial species (cf. Hadley, 1937). Apart from the detailed mechanism of such variations, however, a difference in properties between a given culture and its parent must have arisen in one or both of two ways: (1) by natural selection of cells from a heterogeneous population; (2) through changes induced in the cells themselves.

Some 'adaptive' enzymes not directly concerned with growth appear to be formed in the absence of cell division (Stephenson & Yudkin, 1936; Knox & Pollock, 1944) which would preclude their emergence through a natural selection. Hinshelwood (1944) has stated a number of arguments in favour of the view that, at least in those cases in which the variation consists in the adaptation of bacteria to grow in new media or in the presence of drugs, the essential role is played by a modification of the enzyme systems of the cells. It is thought that the behaviour of *Staph. aureus* to mycophenolic acid is to be interpreted most satisfactorily from this point of view.

It is clearly possible to obtain a rough approximation to any one of the composite curves in Fig. 2 by superimposing curves calculated for given inocula of two sets of organisms growing independently with appropriate lags and m.g.t.'s. The slope of such a curve at any moment is given by

$$dn/dt = k_1 n_1/n + k_2 n_2/n,$$

where n is the total number of organisms and n_1 and n_2 the numbers of cells of the separate substrains, whose growth constants are k_1 and k_2 . Plotting $\ln n$ against t never gives a really straight line, but in general, as the faster-growing strain overtakes the slower-growing one, the slope of the curve may change from a value not far from k_1 to a value not far from k_2 during three or four divisions. To obtain a reasonable approximation to all the curves in Fig. 2, however, on the assumption that these represent only a selection of strains of different sensitivity, it would appear necessary to assume the presence of several substrains whose lags varied in a complicated manner with the inoculum size. Moreover, suppose curve 8 resulted from the least sensitive substrain multiplying while the rest of the organisms remained in the lag phase. From curves 5 and 8 this substrain would be assigned a short lag of about 80 min. It would therefore have apparently grown up from an initial value of 0.12 cells/tube. The chance that a given tube would not contain one cell of this substrain would be $e^{-0.12}$ or 9 to 1; while if it did contain one cell the experimental and synthetic curves would no longer fit. It would not seem plausible to assume that the adaptation depended solely on natural selection.

Two kinds of change in the enzyme systems of bacterial cells might be responsible for an adaptation to growth in the presence of an antibiotic. First, enzymes concerned with normal or alternative growth mechanisms might increase in quantity/cell. Secondly, an enzyme might be produced which inactivated the antibiotic itself. A change of the latter type was not concerned in the adaptation to mycophenolic acid. Filtrates from cultures which had reached their stationary populations from small inocula in the presence of the antibiotic showed no decrease in antibacterial activity when tested by the ring method (Heatley, 1944).

The lag phase

A decrease in the length of the lag phase with an increase in the inoculum size means that the individual cell is influenced through the medium by its neighbours; it clearly suggests that a substance diffusing from the cells is concerned with the ending of the lag.

It would also be possible, if less likely, that an inhibitor diffused from the medium to the cells where it was destroyed. The two cases would be sharply differentiated by attempts to concentrate the diffusible substance.

There are many recorded instances of growth depending on the size of the inoculum. Thus Mueller (1939) found that *C. diphtheriae* grew well when seeded thickly on a solid medium, while when it was thinly sown growth either did not occur at all or else only after a prolonged lag. Later Cohen,

Snyder & Mueller (1941) obtained evidence that the poor growth from a light inoculum might be due in part to absence of oleic acid. Hoffmann & Rahn (1944) found that crystal violet increased the lag phase of *Esch. coli* and *Streptococcus lactis* and that this increase was greater the smaller the size of the inoculum. Lodge & Hinshelwood (1943) found that when *Bact. lactis aerogenes* was grown in an artificial medium with ammonium sulphate as the nitrogen source, the lag was greatly affected by the age of the inoculum. With very young cultures the lag was considerable, but as the age increased it rapidly fell to a minimum and finally again increased. The first or 'early lag' increased as the inoculum size was made smaller; it was abolished by filtrate from an actively growing culture which was approaching the age at which it showed the minimum lag, proving that a diffusible lag-removing substance was present in the medium. Lag was thought to end when this substance reached a threshold concentration in the cells. Later it was found that 'early lag' was increased by the presence of proflavine, methylene blue and sulphonamides and that this effect of these substances was antagonized by sterile filtrate (Davies & Hinshelwood, 1943; Davies, Pryce & Hinshelwood, 1944). It was therefore concluded that, *inter alia*, these inhibitory substances hindered the synthesis or utilization of the diffusible substance which abolished the lag.

The part played by the inoculum size under the conditions used in the present work is similar but not identical. Thus, the lengthening of the lag phase appears only in the presence of mycophenolic acid and not with small inocula in normal cultures. If the reasonable assumption be made that the first effect of the antibiotic is to damage but not destroy the normal growth mechanism this might be explained in several ways: (1) A diffusible growth-intermediate might be supplied in great excess by the normal cells so that even after considerable dilution it would still be present in optimum amounts. If the antibiotic interfered with the production of the intermediate this might no longer be true. (2) The antibiotic might change the permeability of the cell membranes, allowing the diffusion of a substance normally retained in the organisms. (3) The antibiotic might affect the rate of utilization of a diffusible intermediate. Suppose, as a very simple case, that the consecutive reactions

occur in the cells and that substance B is diffusible. If, in the normal cell, the velocity constant k_2 is much greater than k_1 the concentration of B will remain low and very little may be lost by diffusion during the conversion to C ; the over-all reaction rate will be governed by k_1 . If now k_2 be decreased

by the action of an antibiotic, B will accumulate and for a time after inoculation the rate of formation of C in the cells may be greatly dependent on the inoculum size. An explanation of a similar kind would apply if the antibiotic altered the dissociation constant of a coenzyme and its protein carrier.

The consequences of these hypotheses are different; hypothesis (1) implies that the diffusible substance would be present in larger amounts in filtrate from a young normal culture than in filtrate from a culture grown in the presence of the antibiotic, while (2) and (3) imply the opposite. Further work is necessary to estimate the relative quantities of the diffusible substance in the two filtrates. Although the gross effect of the normal filtrate was much the smaller of the two it may have been complicated by morphological changes (see below). At present it is reasonable to conclude that the primary action of mycophenolic acid on the staphylococci consists partly in hindering the utilization or synthesis of a diffusible growth-intermediate produced in the cells. With large inocula the substance reaches a sufficient concentration in the cells to allow slow multiplication to begin very soon, but with small inocula this concentration is not attained and the first slow growth is replaced by a lag period.

The phase of logarithmic growth and the mechanism of adaptation

The curves representing the multiplication of the staphylococci from large inocula in the presence of mycophenolic acid show qualitatively some striking similarities to the curves obtained by Davies & Hinshelwood (1943) and by Lodge & Hinshelwood (1944) while investigating the adaptation of *Bact. lactis aerogenes* to growth in the presence of sulphonamides and to certain changes of medium. In all these cases there is a transition point at which a faster rate of growth replaces a slower one and the adaptation consists, partly at least, in a shortening of the period which elapses before the transition point is reached. Moreover, in the first two cases the m.g.t.'s fell with increasing concentration of the antibacterial substance, not to zero, but to limiting values. The composite curves obtained with *Bact. lactis aerogenes* were interpreted as showing the operation of alternative modes of growth, one less efficient but readily mobilized, the other more efficient, when in action, but only mobilized after a lag phase. This would appear to be the most satisfactory way of interpreting the composite curves obtained in the present investigation.

The effect of the concentration of mycophenolic acid on the growth constants could be expressed formally by an equation of the type

$$k = k_0 - \frac{\alpha C}{1 + \beta C},$$

where C is the concentration and α and β are constants. $\frac{\alpha C}{1 + \beta C}$ is the Langmuir expression for the variation with concentration of the amount of substance adsorbed on a surface. In these terms the constant $\left(1 - \frac{\alpha}{\beta k_0}\right)$ would be a measure of the proportion of the 'growth patterns' in the cells untouched by the antibiotic.

If the primary curves obtained with large inocula in the presence of mycophenolic acid are compared with those of the adapted organism it is evident that one result of adaptation is a greatly increased rate of growth and another is the decrease in the time interval before the increased rate begins. If the increased rate of division is itself considered as an adaptation, a comparison of curves from inocula of different sizes indicates that this is a more important function of the time that the organisms have been in contact with the antibiotic than of the number of divisions that have occurred in its presence. Once logarithmic growth begins from a small inoculum, after a lag period, it does not proceed through well-defined phases with successively faster growth rates connected by transition points, but attains fairly rapidly its maximum rate. This lends support to the view that alternative modes of growth are involved in the adaptation and that this is not merely an expansion of the original mechanism. It could be assumed that during the long lag period, in which the normal mechanism is unable to function, intermediates are being formed which finally set the alternative mechanism in action. Such a picture of the adaptation is, however, oversimplified. There are not only two rates of growth but a whole series, depending on the length of the lag which precedes the onset of logarithmic multiplication. It appears as though a number of intermediate states were stable for a time in the cell, in which both mechanisms were contributing to growth in varying proportions.

The adaptation to mycophenolic acid was not easily reversible. In the literature of bacterial variation there are many instances recorded in which an adapted or variant organism is very stable and many others in which it reverts easily. It has often been assumed that entirely different processes are involved in the two cases, but the differences are generally of degree rather than of kind and can hardly serve in themselves as a satisfactory basis for classification. If an adaptation consists in the development of an alternative mode of growth there is no reason to expect that it should be easily reversible, provided the new mechanism competes in efficiency with the old, under normal conditions. The staphylococci adapted to mycophenolic acid grew in the presence of the antibiotic with a facility approaching that of normal growth and in its

absence at a rate indistinguishable from the normal. It is therefore not surprising that reversion did not rapidly occur.

The way in which the enzyme balance in bacterial cells may change is at present largely a matter of speculation. Yudkin (1938) considered enzymes to be in equilibrium with precursors, the combination of an enzyme with a substrate or inhibitor leading to the formation of more enzyme from the precursor. Davies & Hinshelwood (1943) have suggested that bacterial protein is built up from suitable fragments by a process similar to crystallization. The type of reaction they envisage is formulated:

When the cells are undergoing steady multiplication the increase in mass of a given enzyme must follow the same law as cell growth, i.e. $E = E_0 \cdot e^{kt}$, where k is the growth constant. The physico-chemical implications of disturbing simple systems of this sort at specific points by the action of antibacterial substances have been worked out in some detail (Davies *et al.* 1944; Lodge & Hinshelwood, 1944). It has been shown that in such cases adaptation may occur automatically and rapidly, either by expansion of one enzyme system in relation to another or by readjustment of the balance in a single system. Under optimum conditions adaptation is almost complete when the new bacterial mass outweighs the old and if certain simple conditions are fulfilled it is not easily reversible. There does not appear to be anything in the behaviour of *Staph. aureus* to mycophenolic acid which is incompatible with this attractive theory. Processes which are more complicated but fundamentally of this nature may well play an important part in the adaptation of bacteria to the presence of antibacterial substances and to changes of medium, though it is not likely that they can account for the whole phenomenon of bacterial variation.

Morphological changes

There are obvious resemblances between the occurrence of the morphological changes in the staphylococci and the formation of snake forms in cultures of *Bact. lactis aerogenes* (Hinshelwood & Lodge, 1944). The mean length of the abnormal cells of *Bact. lactis aerogenes* increased to a maximum at about the middle of the logarithmic growth phase and then decreased. Their formation was induced by changes in media or by the addition of certain antibacterial substances and was stimulated by the addition of culture filtrates; the abnormal forms disappeared on 'training'. These facts resemble so closely those relating to the clumped forms of staphylococci that it would seem likely that similar factors are operating in the two cases.

Wyckoff (1934) concluded from micro-motion

pictures of bacteria undergoing multiplication that growth and division depend on different factors which may vary independently. Hinshelwood & Lodge (1944) considered the phenomenon of snake-like cell formation in terms of two independent factors, one diffusible and responsible for elongation, one retained by the cells and responsible for division. Snake forms were thought to appear whenever, owing to changes in the medium or the action of antibacterial substances, the production of these factors was put out of balance. When this balance was restored reversion to normal growth took place. How far this simple hypothesis is capable of explaining the abnormal growth forms of the staphylococci it is inadvisable to decide without further information about the nature of the clumped cells; the physical properties of these clumps suggest that other factors may also be involved.

SUMMARY

1. When *Staph. aureus* (H. strain, N.C.T.C. no. 6571) was inoculated into heart broth containing mycophenolic acid the period of lag, preceding logarithmic growth, lengthened greatly as the size of the inoculum was made smaller. The long lag with a small inoculum was abolished when heart broth filtrates from young cultures were used as a growth medium. It is considered that a diffusible growth intermediate produced by the cells is concerned with the ending of lag and that the primary action of mycophenolic acid consists partly in hindering the synthesis or utilization of this substance.

2. The staphylococci were rapidly adapted to grow more easily in the presence of the antibiotic; this could be manifested during multiplication by a transition from a slower to a faster rate of division. The adaptation consisted in a shortening of the time which elapsed, after inoculation, before the rapid rate of growth set in; the adapted organism multiplied quickly after only a short lag even from a small inoculum. This adaptation was not easily reversible. The most likely mechanism of the adaptation is thought to consist in a modification of enzyme systems of the bacterial cells, resulting in the development of an alternative mode of growth.

3. The rates of division during logarithmic growth varied with the concentration of mycophenolic acid in a manner which suggested that a certain proportion of the growth mechanism was relatively insensitive to the antibiotic.

4. Minor alterations in the medium led to morphological changes during growth in the presence of the antibiotic, consisting in a failure of the dividing cells to separate in the normal manner. The organism was easily trained, by subculturing, to grow without these changes occurring.

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Metabolism of Acetoacetate in Animal Tissues. 1

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The utilization of acetoacetic acid in animal tissues is commonly ascribed to two types of metabolic processes: reduction to β -hydroxybutyric acid and oxidation to CO_2 and water. The reduction and its reversibility was first observed by Friedmann & Maase (1910) and Wakeman & Dakin (1910). The occurrence of an oxidative breakdown became established when Magnus-Levy and Knoop showed that acetoacetic acid is a normal intermediate in the decomposition of fatty acids. It was further demonstrated in conclusive experiments by Snapper & Grünbaum (1927). This pioneer work was later confirmed and amplified by many workers, especially when the tissue slice technique was applied (Jowett & Quastel, 1935; Quastel & Wheatley, 1935; Edson & Leloir, 1936). Reference to details of this work will be made later in this paper in connexion with our own findings.

As yet, relatively little is known about the intermediary steps in which acetoacetate disappears. Krebs & Johnson (1937) showed that the reduction can be brought about by dismutation with α -ketonic acids:

As for the mechanism of oxidation, Weil-Malherbe (1938) excluded α -hydroxyacetoacetate as an intermediate. Hoff-Jorgensen (1940) reported that erythro-1:2-dihydroxybutyrate is rapidly destroyed

after intravenous injection, whilst little or none of the threo-form is attacked under the same conditions. Weil-Malherbe (1938), in contrast, found that the threo-form increases the O_2 uptake of, and is removed by, kidney cortex, whilst the erythro-form was inactive. Kleinzeller (1943) found that crotonate, vinylacetate, γ -hydroxybutyrate and *dl*-2:3-dihydroxybutyrate are oxidizable in kidney cortex. The substances which are metabolized are possibly, but not necessarily, normal intermediates; further experiments will be required to decide whether they are normal metabolites.

Breusch (1943) and apparently independently Wieland & Rosenthal (1943) recently suggested that the $-\text{CH}_2\cdot\text{CO}_2\text{H}$ grouping of acetoacetate is transferred as a whole to oxaloacetate, resulting in the formation of citric acid:

These schemes attempt to explain the observation that under some conditions oxaloacetate and acetoacetate together form more citric acid than oxaloacetate alone.

In the present investigation an effort is made to examine as fully as possible the chemical changes which accompany the disappearance of acetoacetate from animal tissues. It is already known from previous work (Jowett & Quastel, 1935; Breusch, 1943) and was soon confirmed by our own experiments