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A Method for Measuring the Motility of Bacteria and for Comparing Random and Non-random Motility

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SUMMARY

The motility of *Escherichia coli* was measured in capillary tubes by determining the distribution of bacteria throughout the tube (the complete assay) or simply by locating the point of furthest advance of the bacteria—the frontier of the migration (the frontier assay). The diffusion of ^{14}C-glucose was similarly measured in capillary tubes by determining the distribution of radioactivity throughout the tube. The diffusion of glucose under the conditions used was correctly described by the known diffusion equation. The method gives a measure of the net forward velocity of the bacteria. Interpretations, advantages and disadvantages of these assays are given. The method also gives a measure of the degree of randomness of the motility. When chemotaxis is taking place, a very high proportion of the bacteria leave the origin and migrate as a band. When bands are not allowed to form, by omitting methionine, the motility of the bacteria qualitatively resembled a random process, such as the diffusion of glucose.

INTRODUCTION

A quantitative objective method for measuring the motility of bacteria should be a useful tool for exploring various questions of bacterial movement. Such a method is examined in this paper. The bacteria were introduced at one end of capillary tubes filled with a medium suitable for showing motility, and at various times a tube was marked off into compartments and each compartment sampled to determine how much progess the bacteria had made. In the complete assay, the number of viable bacteria in each compartment was measured by plating and counting colonies. This told how many bacteria had moved how far along the tube. Alternatively, in the frontier assay simply the presence or absence of bacteria in each compartment was determined by incubating the contents of each compartment in broth to see whether or not turbidity developed. This located the furthest point of advance of the bacteria—the frontier of the migration.

The method was used in the work reported here to contrast the motility of bacteria under conditions where a chemotactic band of bacteria formed and moved out into the tube with motility under conditions where such a band did not form. A comparison was made with a known random process—the diffusion of glucose.

METHODS

Description of assay method. A 10 cm. long melting point capillary tube (Kimax no. 34502, 0·8–1·2 mm. internal diameter, Owens-Illinois, Toledo, Ohio) was filled from end B with medium (described below) to 8 cm. from end B (see Fig. 1 for a diagram of the completed assay tube). At end B, agar was then added by plunging the tube into a Petri plate containing hardened agar (15 g. agar/l. distilled water, and containing no other components) until the column of liquid had been moved to 1 mm. from end A. This 1 mm. was then filled almost to overflowing with about 10^5 to 10^6 bacteria (prepared as described below) from a drawn-out Pasteur pipette (no. A–2882/B transfer pipette, Clay-Adams, Inc., New York, N.Y.). Next, about 5 mm. agar was added at end A as before; this resulted in pushing out some of the agar at end B. Finally, the tube was sealed at end A with a 2–3 mm. plug of modelling clay to keep the column of liquid from moving when fractions were removed later.

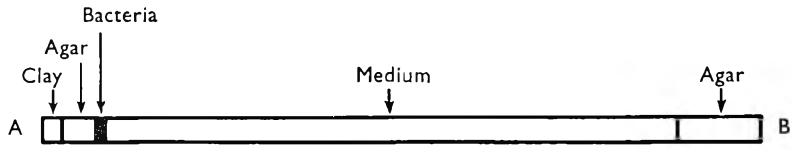

Fig. 1. Description of assay tube. For details see Methods.

The tubes were incubated in a horizontal position at 35°. Recovery of viable bacteria was complete up to 10 hr, but incubations longer than 10 hr sometimes resulted in some loss of viability. At the end of the incubation period, each tube was marked off with a pen into ten 8 mm. compartments beginning at the liquid/agar junction (the 'origin') near end A. The tube was then broken at the liquid/agar junction near end B and the contents of each compartment removed one at a time, beginning at end B with a finely drawn-out Pasteur pipette.

In the complete assay the number of viable bacteria in each compartment was measured by plating appropriate dilutions on EMB glucose agar, incubating overnight, and then counting colonies. In the frontier assay, the contents of each compartment were added to a tube containing EMB glucose broth and the tubes incubated at 35° for 28 hr. Then the presence or absence of turbidity in each tube was noted.

Organism used. The organism used in this work was a strain of *Escherichia coli* K 12 called B 275. It was prepared from Dr A. Garen's strain F3–W 1–6, a derivative of the Lederberg strain W1. Strain B275 is F^- threonine$^-$ leucine$^-$ methionine$^-$ lactose$^-$, phosphatase$^-$, lysogenic for λ, resistant to λ and T1, streptomycin-resistant and motile.

The organism was adapted to the growth medium described below by growing once each week at 35° with rotatory shaking in a New Brunswick Gyrotory shaker to an extinction at 590 mμ (E_{590}) of about 0·8 (about 6×10^8 bacteria/ml.). Longer shaking in this medium caused serious loss in viability. Without any shaking, the bacteria grew poorly in the growth medium and were poorly motile. Shaking speeds anywhere between 100 and 250 rev./min. (200 rev./min. is the speed usually used) resulted in equally motile cultures.

Bacteria previously adapted in this way were inoculated into the growth medium described below to give an initial extinction (E_{590}) value of about 0·04; inoculation with lower concentrations of bacteria caused a very long lag period in this medium. The bacteria were incubated at 35° with rotatory shaking to a final E_{590} of 0·4 (about 3×10^8 bacteria/ml.).

Then 2·5 ml. of the culture were centrifuged for 10 min. at 8000 rev./min. in the International Centrifuge (head no. 856). The pellet was then gently suspended in 5 ml. of a medium containing 1×10^{-2} M-potassium phosphate buffer (pH 7·0), 1×10^{-3} M-$MgSO_4$ and 1×10^{-4} M-EDTA. Again the suspension was centrifuged, the bacteria were resuspended in this medium and again centrifuged, and the supernatant fluid decanted. This final pellet was suspended without any addition of medium by gently shaking the tube, and this suspension served directly as the inoculum for the capillary tubes. More than 90% of these bacteria were very motile when observed under the microscope. After three additional washes the motility was still excellent, so this washing procedure was safe for preserving the motility.

Media. The inorganic portion of the growth medium contained (g./l.): 11·2, K_2HPO_4; 4·8, KH_2PO_4; 2·0, $(NH_4)_2SO_4$; 0·25, $MgSO_4.7H_2O$; 0·0005, $Fe_2(SO_4)_3$; 1 l. distilled water; this is the H medium of Kaiser & Hogness (1960). The organic portion of this growth medium contained the 20 amino acids commonly encountered in proteins at a final concentration of each amino acid 0·25 g./l.; final pH 7·0. The pure amino acids, desirable because they allowed a completely defined medium, could be fully replaced by vitamin-free casein hydrolysate.

The medium used for filling the capillary tubes contained 1×10^{-2} M-potassium phosphate buffer (pH 7·0), 1×10^{-4} M-EDTA, 1×10^{-3} M-$MgSO_4$, 1×10^{-3} M-$(NH_4)_2SO_4$, 1×10^{-2} M-L-serine; final pH 7·0. Only glass-distilled water was used. Serine was chosen as energy source because this strain of *Escherichia coli* can use serine either aerobically or anaerobically to supply energy for motility (Adler & Templeton, 1966). This allowed motility to persist everywhere in the tube, even where oxygen had been exhausted. Growth of the bacteria was inhibited in these experiments, since in no case were all three of the required amino acids, leucine, methionine and threonine, added.

EMB glucose broth contained (g./l.): 10, glucose (autoclaved separately); 10, Difco Bacto-Tryptone; 5, NaCl; 2, K_2HPO_4; 1, Difco yeast extract; 0·4, eosin; 0·065, methylene blue; 1 l. of distilled water. EMB glucose agar contained in addition 15 g. agar/l. medium. This is the recipe of Lederberg (1950) modified by Kaiser & Hogness (1960). The eosin and methylene blue in this case served to discourage the growth of contaminants. As a further precaution, the strain used whenever possible was streptomycin-resistant and in that case streptomycin (0·1 g./l.) was added to the EMB glucose broth and to the EMB glucose agar.

RESULTS

Before the results with bacteria are presented, the diffusion of glucose under conditions identical to those used for the study of bacterial motility will be described. The diffusion of a chemical is a random process that follows well-known laws for diffusion.

The diffusion of glucose

Instead of bacteria, ^{14}C-glucose was introduced at one end of capillary tubes filled with medium, and then the tubes were incubated at room temperature. All the details of the procedure were otherwise identical to those described under Methods.

The diffusion of glucose measured by the complete assay. Figure 2 shows for several time points a complete assay of the diffusion of glucose, i.e. a determination of the distribution of glucose throughout the tube. The diffusion equation which applies to the present experimental conditions, where a thin layer of diffusion substance is placed at one end of a column of liquid, is the one of Ljunggren & Lamm (1957):

$$\ln (c/c^{\circ}) = -1/2 \ln (\pi Dt) - (x^2/(4Dt)). \quad (1)$$

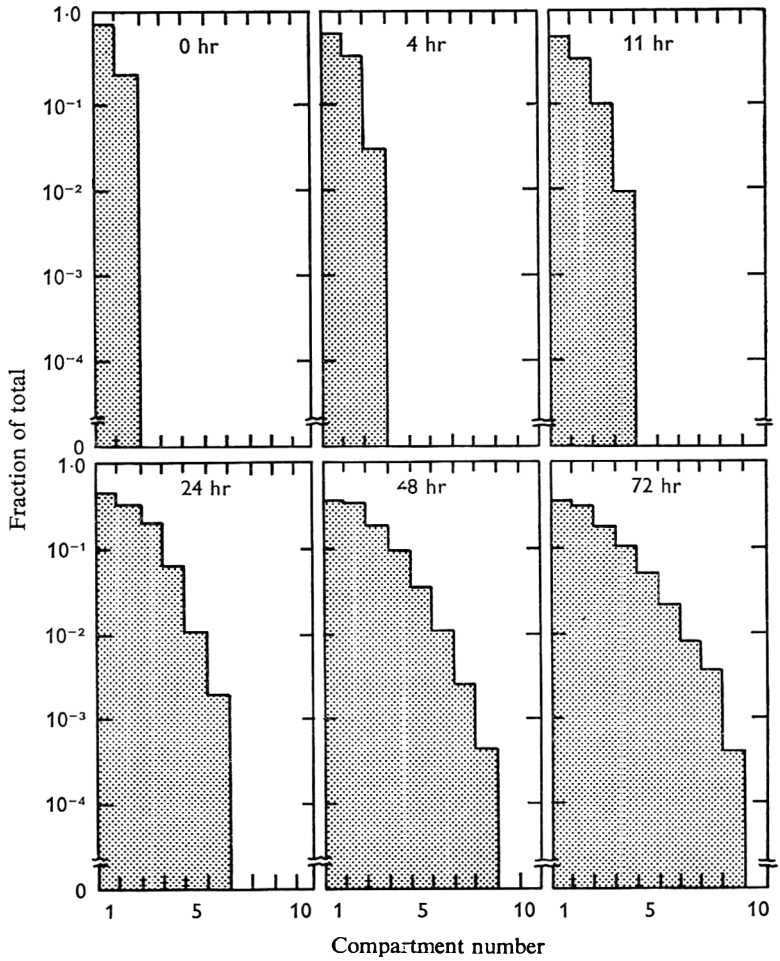

Fig. 2. The diffusion of glucose measured by the complete assay. Each capillary tube contained sterile 10^{-2} M-potassium phosphate (pH 7·0) and 10^{-4} M-EDTA. At one end 45,000 c.p.m. (0·034 μmoles) of sterile ^{14}C-glucose was introduced. Bacteria were not added and were not involved in this experiment. After incubation at room temperature for various times, the tubes were fractionated into ten 0·8 cm. compartments and the contents of each compartment were dried on a planchet and counted in a windowless gas-flow counter. See Methods for details. A value of zero means that no readily detectable radioactivity (less than 80 c.p.m.) was found in that compartment.

Here c° represents the initial concentration, c the concentration at any distance x from the origin at any time t, and D the diffusion coefficient. According to this equation, a plot of the logarithm of the fraction of the total radioactivity in each compartment ($\ln (c/c^\circ)$) against the distance moved from the origin squared (x^2) for any particular time should give a straight line whose slope is $-(1/4Dt)$. The data of Fig. 2, when replotted in this manner (Fig. 3), do indeed yield straight lines, and from the slopes of these lines D was calculated as ranging from 0·017 to 0·022 cm.²/hr. This value is in good agreement with 0·024 cm.²/hr., a published diffusion coefficient for glucose at 25° obtained by Gladden & Dole (1953) with much more sophisticated techniques. We may conclude that under the experimental conditions used here the above equation correctly describes a process of random movement such as the diffusion of glucose.

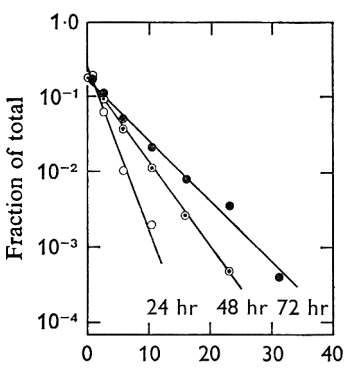

Fig. 3

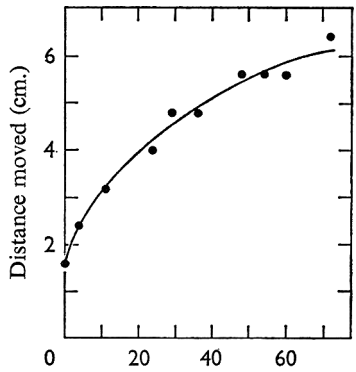

Fig. 4

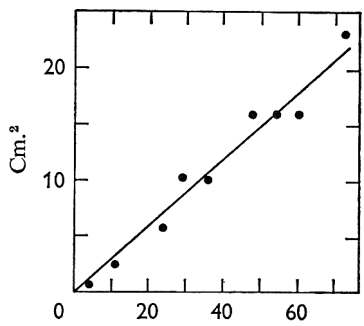

Fig. 5

Fig. 3. The data of Fig. 2 replotted. The abscissa plots the distance (cm.) moved away from the origin, squared. Since at zero time compartments 1 and 2 (the origin) were occupied, the length of two compartments (1·6 cm.) was subtracted in every case. This left only one point to plot for the 4 hr data and only two points for the 11 hr data; therefore no plots are presented for these time-points. In addition to the data shown for 24, 48 and 72 hr, complete sets of data for 29, 36, 54 and 60 hr were also collected. These are not reported in order to keep the figure readable, but in every case the points fell very well on straight lines. D calculated from the slope of each of these seven lines is 0·021, 0·020, 0·019, 0·022, 0·017, 0·019 and 0·018 cm.²/hr, respectively.

Fig. 4. The diffusion of glucose analysed by the frontier assay. The figure plots the farthest advance of at least 80 c.p.m. for each time-point. The data come from the complete assays described in Figs. 2 and 3.

Fig. 5. The data of Fig. 4 replotted. As in Fig. 4, except the ordinate represents cm.² instead of cm.; and 1·6 cm. has been subtracted in every case since at zero time compartments 1 and 2 were occupied.

To study the effect of glucose concentration on the diffusion coefficient, capillary tubes were inoculated with 0·034, 0·0043, or 0·00063 μmoles ^{14}C-glucose, incubated for 77 hr and then analysed. The diffusion coefficient was 0·020 cm.²/hr in all three cases. As expected, the value found for the diffusion coefficient does not depend on the concentration of glucose, at least within the range studied.

The diffusion of glucose analysed by the frontier assay. The results from a complete assay include information about the 'frontier' of the migration—the farthest advance of at least 80 c.p.m., a minimum number of readily detectable counts. This information

was collected from Fig. 2 and from the other complete assays mentioned in the legend of Fig. 3, and then replotted in Fig. 4. To apply equation (1) to the frontier assay, the equation can be rearranged to yield:

$$x^2 = -4Dt \ln (c/c^\circ) - 2Dt \ln (\pi Dt). \quad (2)$$

In the frontier assay, c is the minimum amount of radioactivity readily detectable (80 c.p.m.), c° is the amount of radioactivity added to each assay tube (45,000 c.p.m.) and ln c/c° is therefore constant. Under conditions used here, the second term, $2Dt \ln (\pi Dt)$, becomes negligible compared to the first term, $4Dt \ln c/c^\circ$, and therefore a plot of x^2 against t should give a straight line whose slope is $-4D \ln c/c^\circ$. The data of Fig. 4 were accordingly replotted (Fig. 5). A straight line was indeed obtained, and D can be calculated from the slope to be 0·015 cm.²/hr, in good agreement with the value obtained from the complete assay. This again supports the conclusion that equation (1) correctly describes a process of random movement such as the diffusion of glucose under the experimental conditions used here.

Motility under conditions where the bacteria do not migrate in a band

This section considers the motility of bacteria in the medium described under Methods. In this case, we shall see that the spreading of bacteria along the tube resembled a random process such as the diffusion of glucose but there were certain differences. A later section will concern itself with motility under conditions where the bacteria migrate in a sharp, easily visible, chemotactic band and do not at all behave like diffusing molecules.

Motility measured by the complete assay. Figure 6 shows complete assays of the movement of *Escherichia coli* at several times of incubation. This figure resembles Fig. 2 for the diffusion of glucose although the profile of the histogram is different. It is clear from Fig. 6 that initially all the bacteria were located in the first two compartments (the 'origin'); the remaining eight compartments were free of any viable bacteria. This shows that the sampling technique did not cause any objectionable amount of stirring.

To find how well equation (1) for diffusion (which is also the equation for random movement) describes the swimming of these bacteria, the data of Fig. 6 were replotted as ln c/c° against x^2, as was done for the diffusion of glucose. The resulting curves (Fig. 7) were not straight lines; in the case of the diffusion of glucose, straight lines were obtained (Fig. 3). The explanation for the shape of the curves in Fig. 7 is not known; several possibilities will be considered in the Discussion.

Just as the slope in Fig. 3 for the diffusion of glucose was used to calculate a diffusion coefficient, so the slope in Fig. 7 (which is $-(1/4Mt)$) was used to calculate a motility coefficient, M. Although the slope of the curves in Fig. 7 is not constant, the smallest slope (corresponding to the fastest motility) was used to calculate motility coefficients of 0·57, 0·48 and 0·24 cm.²/hr for the 1-, 2- and 5-hr samples, respectively. In twenty different determinations this value ranged from 0·08 to 0·57 cm.²/hr, with an average value of 0·25 cm.²/hr.

To study the effect of inoculum size on the motility coefficient, $2{\cdot}2 \times 10^6$, $1{\cdot}3 \times 10^6$, $7{\cdot}7 \times 10^5$, $2{\cdot}6 \times 10^4$, or $2{\cdot}3 \times 10^3$ bacteria were used in a 4-hr experiment. The motility coefficient was found to be 0·22, 0·49, 0·27, 0·19 and 0·18 cm.²/hr, respectively.

Motility measured by the frontier assay. The farthest point of advance of the bacteria

can be obtained fron the results of a complete assay (see data of Fig. 6), but this information is much easier to get by means of the frontier assay. A typical result from frontier assay of the movement of bacteria is shown in Fig. 8. The top curve shows the farthest point of advance (the frontier) at various times for this motile strain of

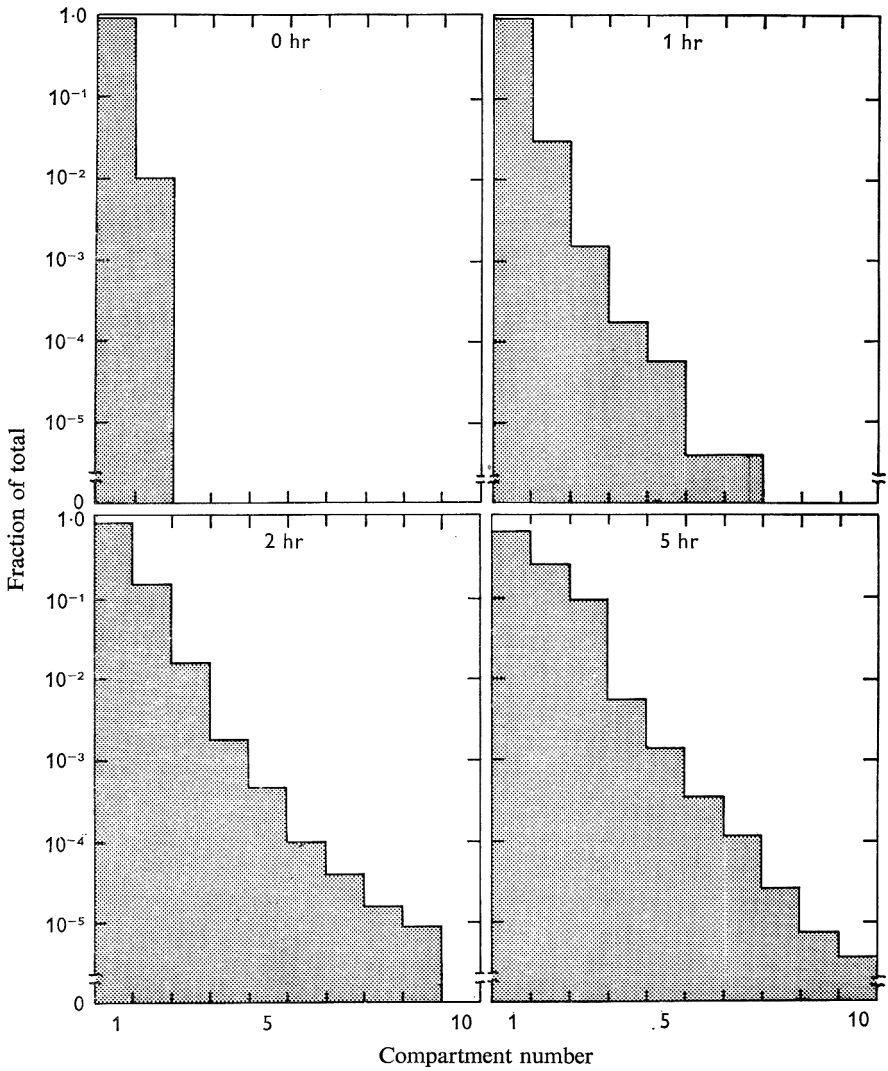

Fig. 6. The movement of bacteria under conditions where they did not migrate in a band, as measured by the complete assay. The capillary tubes contained the medium described under Methods. No methionine was added. After incubation at 37° for 0, 1, 2 and 5 hr, the tubes were fractionated into 0·8 cm. compartments and viable *Escherichia coli* in each compartment were measured by plating and counting colonies. The total recovery of viable bacteria was $5{\cdot}5 \times 10^5$, $2{\cdot}4 \times 10^5$, $3{\cdot}4 \times 10^5$ and $1{\cdot}1 \times 10^6$, at the respective times. These were not growth conditions since the required leucine, methionine and threonine were not added. See Methods for details. The figure includes the following data: at 0 hr, compartments 2–10 contained no bacteria; at 1 hr compartments 6 and 7 had 1 bacterium each, and compartments 8, 9 and 10 contained none; at 2 hr compartment 8 had 5 bacteria, 9 had 3, and 10 had none; at 5 hr compartment 9 had 8 bacteria and 10 had 4.

Escherichia coli K12. This curve resembles the comparable curve for the diffusion of glucose (Fig. 4). The bottom curve of Fig. 8 shows that *E. coli* B, a non-motile strain, remained at the origin (it did so even at 24 hr, not shown in figure). This shows again that the sampling technique was reliable, and also that Brownian movement, diffusion, or convection of bacteria did not play a great enough role to interfere with the assay for motility.

To determine whether the equation for random movement (equation 2) describes the movement of these furthest moving bacteria, the data in the top curve of Fig. 8 were replotted in the same manner as was done for the diffusion of glucose. In this case,

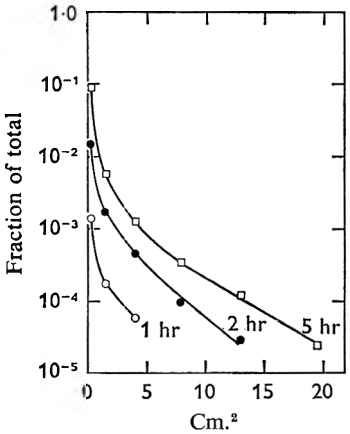

Fig. 7

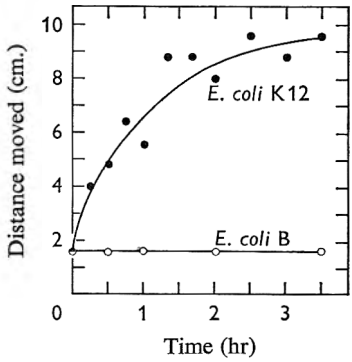

Fig. 8

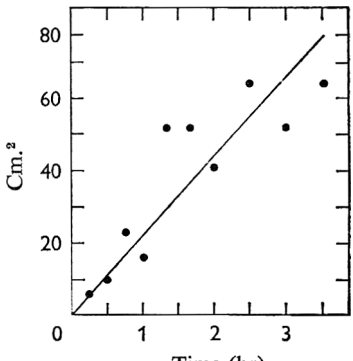

Fig. 9

Fig. 7. The data of Fig. 6 replotted. The abscissa plots the distance (cm.) moved away from the origin, squared. Since at zero time compartments 1 and 2 (the origin) were occupied, the length of two compartments (1·6 cm.) was subtracted in every case. Values smaller than 10 bacteria/compartment are not included here. (These values are enumerated in the legend of Fig. 6). Such very low values tend to give scattered points in this kind of figure, probably because they are statistically so unreliable.

Fig. 8. The movement of bacteria under conditions where they do not migrate in a band, as measured by the frontier assay. Exactly as in Fig. 6, except that the frontier assay was used and more time-points were included. The farthest advance of the bacteria (the frontier) is shown for *Escherichia coli* K12, strain B275, the motile strain used throughout this study, and for *E. coli* B, a non-motile strain. In this experiment the tubes were longer than the usual ones.

Fig. 9. The data of Fig. 8 for *Escherichia coli* K12 replotted. As in Fig. 8, except the ordinate represents cm.² instead of cm. Since at zero time compartments 1 and 2 (the origin) were occupied, the length of two compartments (1·6 cm.) was subtracted.

c in equation (2) is the minimum number of bacteria detectable (one bacterium), c° is the number of bacteria added to each assay tube (10^6 bacteria), and $\ln c/c^\circ$ is therefore a constant. The second term of equation (2) becomes negligible as before, and a plot of x^2 against t should give a straight line. Such a plot is shown in Fig. 9. Although the experimental points are somewhat scattered, a best-fitting straight line has been drawn. (In some experiments the points fell very much more closely on a straight line.) From the slope of this line (which is $-4M \ln c/c^\circ$), a motility coefficient, M, was calculated to be 0·40 cm.²/hr. In seven different experiments this value has ranged from 0·13 to 0·40 cm.²/hr., with an average of 0·26 cm.²/hr. This is in agreement with the motility coefficient determined above from the complete assays.

Motility under conditions where the bacteria migrate in a band

When the medium described under Methods was supplemented with methionine, a band of bacteria formed and travelled out into the tube. Such a band was clearly visible to the naked eye, and it has also been shown by photography, densitometry and microscopy (Adler, 1966*a*, *b*), as well as by assaying for viable bacteria along the tube, as will be seen. The band results from chemotaxis toward oxygen (Adler, 1966*a*, *b*), but the mechanism by which methionine promotes the formation of the band is unknown (see Discussion).

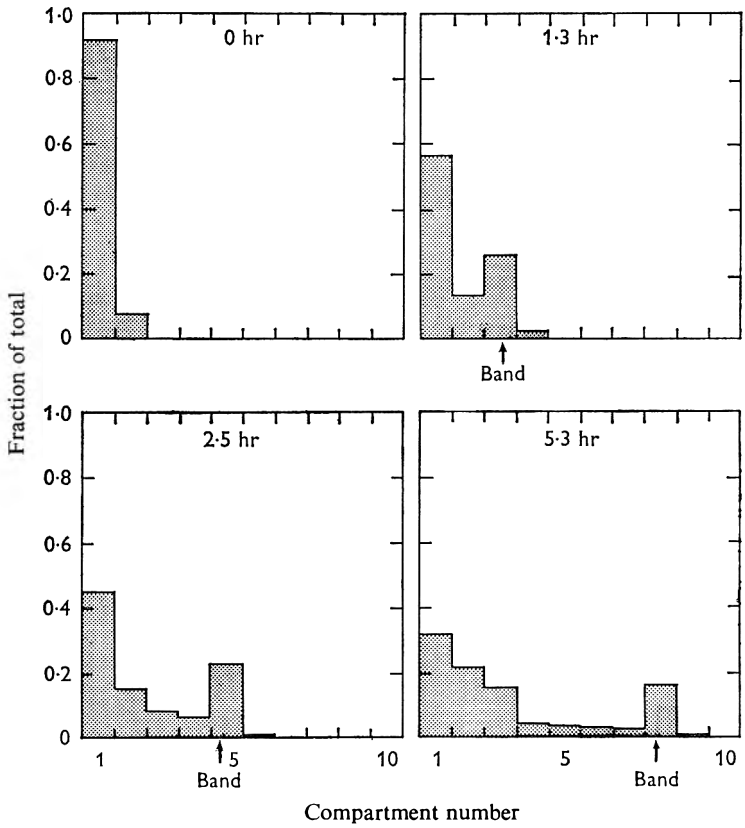

Fig. 10. The movement of bacteria under conditions where they migrate in a band (presence of methionine), as measured by the complete assay. The capillary tubes contained the medium described under Methods, $+3 \times 10^{-5}$ M-methionine. After incubation at 37° for 0, 1·3, 2·5 and 5·3 hr, the tubes were fractionated into 0·8 cm. compartments and the viable bacteria in each compartment measured. The total recovery of viable bacteria was $3{\cdot}0 \times 10^5$, $5{\cdot}8 \times 10^5$, $8{\cdot}2 \times 10^5$, and $9{\cdot}6 \times 10^5$, at the respective times. The conditions used here were not conducive to growth since the additionally required leucine and threonine were not added. The bands were visible to the naked eye, and their location is shown by the arrows. See Methods for details.

Motility measured by the complete assay. Figure 10 shows complete assays of the movement of *Escherichia coli* under conditions where a band of bacteria formed and moved out into the tube. The band appeared as a peak fraction. Figure 10 is strikingly different in this regard from the comparable Fig. 2 for the diffusion of glucose or

Fig. 6 for the motility of bacteria under conditions where they did not migrate in a band.

Motility measured by the frontier assay. Figure 11 shows the results of a frontier assay (top curve) of the movement of *Escherichia coli* under conditions where the bacteria migrated in a band, as well as a plot of the progress of the band itself (bottom curve). It may be seen that the band moved at a constant speed. The farthest point of advance of the bacteria, the frontier (top curve), was always some centimeters ahead of the band; the shape of this curve approximated a straight line in several experiments.

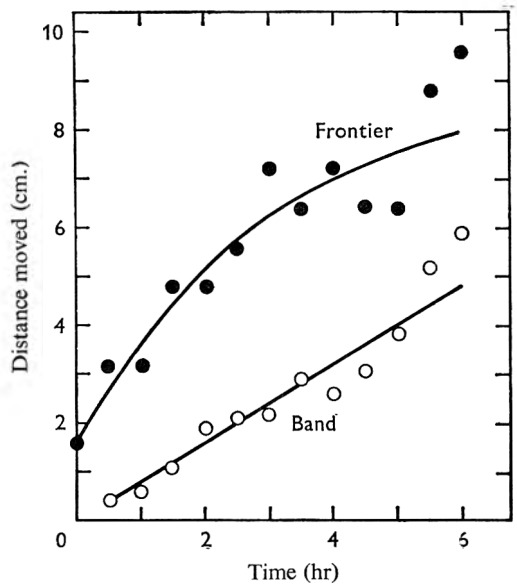

Fig. 11. The movement of bacteria under conditions where they migrate in a band (presence of methionine), as measured by the frontier assay. Exactly as in Fig. 10, except that the frontier assay was used and more time-points were included. The top curve shows the farthest advance of the bacteria (the frontier). The bottom curve shows the location of the visible band as measured with a ruler. In this experiment the tubes were longer than the usual ones.

DISCUSSION

Measurements of motility. The microscope allows a rapid and direct indication of motility. At a moment's glance one can estimate the velocity of the bacteria, the frequency of change of direction, the degree of co-ordination, or the fraction of a population that is motile. However, these observations are subjective and qualitative, and this tends to make such work with a microscope less credible and less easily reproduced by others.

In the work reported here the motility of bacteria was measured in capillary tubes by determining the distribution of bacteria throughout the tube (the complete assay) or simply by locating the point of farthest advance of the bacteria—the frontier of the migration (the frontier assay). These assays have the advantage of being objective and quantitative. They reduce the complicated motions of individual bacteria to a measurable number, the motility coefficient, and give a measure of the net forward velocity of the bacteria. They do not measure the actual velocity, because net forward movement includes numerous detours. Thus, a decrease in the net forward velocity might

mean either a decrease in the actual velocity or an increase in the frequency of changes of direction. A bacterium that has moved out into the tube 4 cm. in the first hour might have either an actual velocity of 4 cm./hr, if it was lucky in picking the right direction and in making no changes in direction or it might have a much higher actual velocity if it made very frequent changes in direction. The net forward velocity of the average bacterium, $\bar{v}$, equals $\sqrt{(2M)}$. For a motility coefficient, M, of 0·25 cm.²/hr, $\bar{v}$ is 0·7 cm./hr. By using a microscope and a clock, Ogiuti (1936) measured the actual velocity of *Escherichia coli* as 9 cm./hr, and we measured the actual velocity as about 10 cm./hr with the strain and conditions used in this report.

The frontier assay described here has the advantage that it is much simpler to carry out than the complete assay. Although the frontier assay curve (top of Fig. 8) is not a straight line, it is nevertheless useful because one can state the distance that the bacteria have moved forward in a certain time and one can compare this distance for that time under a variety of experimental conditions. The curve can usually be changed into a straight line, however, by plotting the square of the maximal distance moved against time, as shown in Fig. 9. The slope of this line is then a convenient measure of the motility of the bacteria. The reliability of the assay has been confirmed by frequent use of the microscope: whenever an assay indicated either lack of motility or excellent motility, it was always possible to verify this by microscopic examination. Several demonstrations that bacteria with various degrees of motility really give various results in the frontier assay are included in the following paper (Adler & Templeton, 1967).

On the other hand, the frontier assay has some disadvantages which may be mentioned. The outermost compartment that contains bacteria may have only a very few or even only one bacterium out of the million that were put into the tube. The result for any one time-point may then be determined by a very few individuals. This is the main cause for a very considerable amount of variability in this assay. Results which differ by one or even two compartments (0·8 or 1·6 cm.) are not regarded as significantly different. While bacteria are usually found in every compartment from the origin up to a certain point, occasionally at the end away from the origin there is a skip which must have been produced by an especially lucky or fast bacterium. A policy of not scoring these skips was adopted, in order not to put reliance on these statistically insignificant events. The results obtained by the frontier assay depend on the number of bacteria put into the tube, as expected, so that it is necessary to keep the inoculum size approximately constant. For example, when capillary tubes were inoculated with $1{\cdot}5 \times 10^6$, $2{\cdot}3 \times 10^5$, $2{\cdot}8 \times 10^4$ or $3{\cdot}0 \times 10^3$ bacteria, the movement in 1 hr advanced 5·6, 3·2, 2·4 and 0·8 cm., respectively. Similarly, the result would also be expected to depend on what fraction of the bacteria is motile. The shape of the frontier assay curve may also depend on whether or not chemotaxis is taking place, since bacteria will be held back in the chemotactic band.

Previous descriptions of quantitative assays for motility of bacteria should be mentioned, namely those of Clowes, Furness & Rowley (1955), Gabritschewsky (1900), Liachowetzky (1910–11), Ogiuti (1936) and Shoesmith (1960). Of these, the first three are similar in principle to the frontier assay in that they measured the furthest point of advance of the bacteria (in a column of agar, Clowes *et al.* 1955; or on filter paper, Gabritschewsky, 1900, Liachowetzky, 1910–11), while the last two methods involved measurements with the use of a microscope.

A comparison of random and non-random motility. When the bacteria do not migrate in a band, the motility qualitatively resembles the diffusion of glucose. The similarity is evident from a comparison of Figs. 2 and 6, 4 and 8, and 5 and 9. This similarity in kinetics indicates that the bacteria were swimming randomly when they did not form bands, just as the glucose was diffusing randomly. By random swimming we mean that any direction of swimming is equally likely at all times throughout the container.

However, Fig. 3 for the diffusion of glucose has straight lines while Fig. 7 for the swimming of bacteria has curved lines. Several possible explanations for the curved lines can be offered. (1) The bacteria are heterogeneous with regard to their motility i.e. some swim faster than others. Preparations stained for flagella did show a range of 0–8 flagella/bacterium. Simple genetic heterogeneity was eliminated, since descendants from one of the farthest-moving bacteria again yielded the same curves as shown in Fig. 7. (2) The conditions in the capillary tube are heterogeneous. The crowded condition near the origin leads to rapid exhaustion of oxygen and substrates and to extensive accumulation of waste products, and is therefore less favourable to motility than the relatively unpopulated medium further out. For example, it is known that swimming in presence of serine is slower anaerobically than it is aerobically (Adler & Templeton, 1967). (3) Crowding at the origin causes frequent collisions of bacteria and this decreases the net forward velocity near the origin.

Chemotaxis in bacteria has been known ever since the end of the nineteenth century when Engelmann, Pfeffer and others observed chemotaxis towards oxygen, minerals and organic nutrients microscopically (for a review see Weibull, 1960). In 1893 Beijerinck demonstrated macroscopically chemotaxis towards oxygen by showing that a variety of motile bacteria placed at the bottom of a test-tube filled with water formed a sharp easily visible band that ascended until it came to a stop near the meniscus. More recently, Sherris, Preston & Shoesmith (1957) and Baracchini & Sherris (1959), with capillary tubes instead of test-tubes, confirmed and extended these results.

The band of bacteria which formed in presence of methionine, described above, was due to chemotaxis towards oxygen. Measurements of oxygen and serine (Adler, 1966*a*, *b*) have shown that this band travelled along consuming all the oxygen to oxidize a part of the serine.

When chemotaxis was taking place (Fig. 10), the kinetics of movement did not resemble a random process like the diffusion of glucose (Fig. 2). The bacteria (at least those in the neighbourhood of the band) were not swimming randomly. By this we mean that the gradient of oxygen which the bacteria created in the vicinity of the band somehow influenced the bacteria to swim preferentially in the direction of increasing oxygen concentrations. Microscopic examination showed that the bacteria in the band swam in a short line and then stopped for an instant. The appearance was one of very rapid and highly jerky motion. This suggests that an 'avoiding reaction' (or 'shock reaction') was taking place (see Weibull, 1960): whenever a bacterium enters a region of lower oxygen concentration, it stops for an instant and then goes off in a new randomly chosen direction (or in some species it backs up). When it finds itself at a higher oxygen concentration, it does not change its direction. The net result is a non-random swimming which causes the organisms to vacate a region of low oxygen concentration and to accumulate where the concentration of oxygen is higher. (Actually, very high concentrations are also avoided.)

The mechanism by which methionine promotes the formation of the band remains

to be elucidated. Judged by microscopic examination, the motility is just as vigorous with or without methionine. However, in the absence of methionine the bacteria swam in long straight lines with only rare gradual changes in direction, instead of in a jerky manner. This suggests that the avoiding reaction was not taking place when methionine was absent. The strain of *Escherichia coli* used for these studies requires methionine for growth, but it also requires leucine and threonine for growth and these were not needed for the formation of bands. A prototrophic strain of *E. coli*, such as w3110, formed bands without the addition of any methionine.

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The Effect of Environmental Conditions on the Motility of *Escherichia coli*

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SUMMARY

A simple chemically defined medium for examining the motility of *Escherichia coli* K12 was designed. The essential components were: (1) a chelating agent to protect the motility against inhibition by traces of heavy metal ions; (2) a buffer to keep the pH value at the optimum between pH 6·0 and 7·5; (3) an energy source to stimulate the motility above that allowed by an endogenous energy source. Oxygen was required unless an energy source was provided which yielded energy anaerobically. A temperature optimum was determined.

A chemically defined growth medium capable of producing motile bacteria was devised. It was found that the presence of glucose or growth above 37° prevented synthesis of flagella.

INTRODUCTION

For many studies of bacterial motility, it would be desirable to work with chemically defined media and to know the effect of commonly encountered variables. The present work aimed to meet the following objectives. (*a*) To design for studying motility a medium which contained only known chemicals and which did not allow growth. (*b*) To determine the optimal conditions for motility in this medium, including the effects of pH value, temperature, ionic strength and the concentration of oxygen. (*c*) To find a chemically defined growth medium and suitable growth conditions for producing motile bacteria.

Escherichia coli was chosen because the vast knowledge of its biochemistry and genetics should be applicable to the study of numerous problems of bacterial motility. Many strains of *E. coli* are motile by virtue of having several flagella distributed around the cell.

To make the necessary measurements of motility, an assay described in the preceding paper by Adler & Dahl (1967) was used. By omitting methionine from the medium, it was possible to study motility in the absence of chemotaxis.

METHODS

The strain B275 of *Escherichia coli* was the same as that described by Adler & Dahl (1967); this strain is F^-, threonine$^-$, leucine$^-$, methionine$^-$, lactose$^-$, phosphatase$^-$, lysogenic for λ, resistant to λ and T1, streptomycin-resistant and motile. The bacteria

were grown in a growth medium containing the 20 amino acids commonly encountered in proteins, as described by Adler & Dahl (1967), except where otherwise mentioned.

The medium for washing the bacteria free of growth medium, for studying motility, and for filling the capillary tubes contained 1×10^{-2} M-potassium phosphate buffer (pH 7·0) and 1×10^{-4} M-EDTA. Various additions, such as an energy source, were made, as described in the text. Glass-distilled water was used for making solutions for this medium. Solutions of all organic components were sterilized by filtration. Since the leucine, methionine, and threonine required for growth were omitted, this motility medium did not sustain growth.

Capillary tubes were filled with this motility medium, inoculated at one end (the origin) with about 10^6 *Escherichia coli* organisms and then closed off at both ends with agar plugs and at one end with clay, according to the procedure of Adler & Dahl (1967). After incubation horizontally at 35°, the tubes were fractionated into ten compartments each 8 mm. long by breaking the end away from the origin at the liquid/agar junction and then withdrawing samples with a smaller capillary tube. Each sample was placed into EMB glucose streptomycin broth. The presence or absence of turbidity after 28 hr incubation told whether or not the compartment contained viable bacteria. Usually there was turbidity in every tube from the origin to a certain point, but sometimes at the end away from the origin there was a skip which must have been due to an especially lucky or fast bacterium. A convention of not scoring these skips was adopted. Non-motile bacteria remained at the origin even after many hours of incubation. This assay locates the point of furthest advance of the bacteria—the frontier of the migration. Results which differ by one or even two compartments (0·8 or 1·6 cm.) are not regarded as significantly different in this assay. The main reason for a considerable amount of variability is that a statistically small number of bacteria—even one—can determine the location of the frontier.

RESULTS

A motility medium and optimal conditions for motility

Peptone or other such complex media have usually been used in the study of motility because these media allow excellent movement. The present aim was to determine which components of peptone are essential for motility. Table 1 shows that peptone could be completely replaced by a mixture of the 20 amino acids normally found in proteins, or even by a single amino acid such as L-glutamine or (not shown in the table) nearly any other of the amino acids. Surprisingly, however, glucose did not support motility (Table 1).

The effect of a chelating agent. The explanation for the phenomenon that amino acids supported motility but glucose did not, is the following; motility is highly sensitive to inhibition by trace amounts of heavy metal ions and amino acids are good chelating agents for metal ions while glucose chelates very poorly at neutral pH (Greenberg, 1951; Martel & Calvin, 1952). The following experiments supported this explanation.

A chelating agent such as ethylenediaminetetraacetic acid (EDTA) added with glucose and buffer allowed excellent motility (Fig. 1). Maximum stimulation was obtained at 1×10^{-6} M-EDTA and above (Fig. 2). Inhibition of motility began to appear

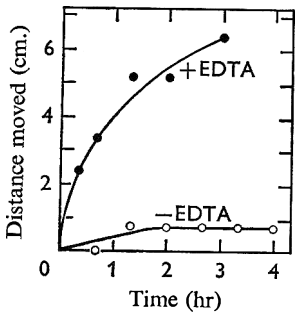

Fig. 1

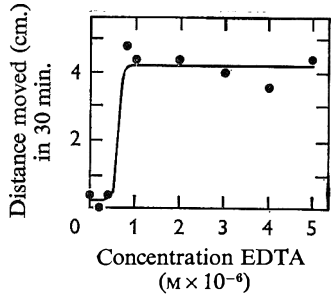

Fig. 2

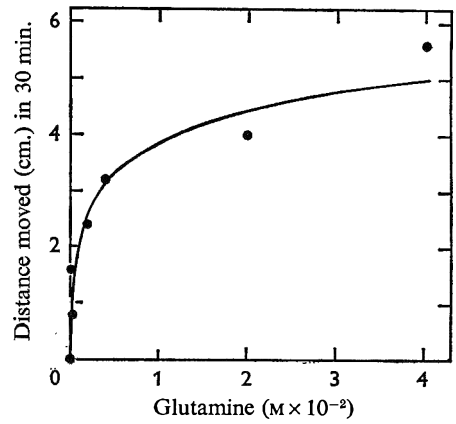

Fig. 3

Fig. 1. The effect of EDTA on motility of *Escherichia coli*. This and subsequent figures plot the point of furthest advance of the bacteria—the frontier of migration. A zero time blank of 1·6 cm. was subtracted from all values in every figure. The farthest movement physically possible was 6·4 cm. in the arrangement used in all the experiments reported in this paper. Phosphate buffer (pH 7·0; 1×10^{-2} M), glucose (1×10^{-2} M) and EDTA (1×10^{-4} M) were present. In this experiment the tube was fractionated into twenty 0·4 cm. compartments.

Fig. 2. The effect of EDTA concentration on motility of *Escherichia coli*. Phosphate buffer (pH 7·0; 10^{-2} M) and glucose (10^{-2} M) were present. In this experiment the tube was fractionated into twenty 0·4 cm. compartments.

Fig. 3. The effect of glutamine concentration on motility of *Escherichia coli*. Phosphate buffer (pH 7·0; 10^{-2} M) and glucose (10^{-2} M) were present; no EDTA.

Table 1. *Replacement of peptone by a simpler motility medium for Escherichia coli*

The components listed were added at a final concentration of 0·5 %. In each case 1×10^{-2} M-potassium phosphate (pH 7·0) was also present.

This and subsequent tables report the point of furthest advance of the bacteria—the frontier of the migration. For details see Methods. A blank of 1·6 cm. has been subtracted from all values in every table, since at zero time the first two compartments were occupied.

	Distance moved (cm.)	
	15 min.	60 min.
Peptone	1·6	4·0
Mixture of 20 amino acids	2·4	4·0
L-glutamine	2·4	4·8
L-glutamine + peptone	2·4	4·0
D-glucose	0	0

at 5×10^{-2} M-EDTA. The position of the curve of Fig. 2 would depend on the content of inhibitory metal ions in the particular supply of water and other reagents used.

Glutamine stimulated motility in the presence of glucose and buffer (Fig. 3), but the concentration required for maximum stimulation was much higher for glutamine (about 2×10^{-2} M) than for EDTA, presumably because glutamine is not as effective a chelating agent. In the presence of 2×10^{-2} M-glutamine, EDTA did not stimulate further. Besides glutamine, several other materials known to have chelating ability also stimulated motility; this list includes peptone, albumin, citrate, oxalate, iminodi-

acetic acid, mercaptoethanol, nearly all of the L-amino acids, D-serine, D-threonine and D-glutamine.

Bacteria which were highly motile completely lost their motility within 30 min. when they were washed free from medium and then placed into a medium lacking a chelating agent. The motility was fully restored at once by adding EDTA or amino acids, even 2 hr after movement had first stopped. This result showed that the inhibition of motility by metal ions was reversible.

A medium inhibitory of motility, such as glucose+phosphate buffer, that was first passed through a chelating resin (Dowex A 1, also called Chelex 100, which has iminodiacetic acid covalently bound) to remove metal ions, allowed excellent motility even in the absence of any added chelating agent (line 1 of Table 2). Such chelating-resin-treated medium served for measuring the effect of a large number of metal ions on motility. In this way copper ion, a common contaminant of water and reagents, was shown to be a potent inhibitor (Table 2); AgCl and $HgCl_2$ also inhibited at nearly as low concentrations.

Table 2. *The effect of cupric chloride on motility of Escherichia coli*

The motility medium, containing 10^{-2} M-glucose and 10^{-2} M-phosphate buffer (pH 7·0), was first passed through Dowex A 1 chelating resin to allow examination of motility in the absence of a chelating agent.

Concentration of $CuCl_2$ (M)	Distance moved in 4 hr (cm.)	No. of bacteria viable at 4 hr
0	6·4	$2·4 \times 10^8$
10^{-8}	3·2	$4·7 \times 10^8$
10^{-7}	1·6	$2·2 \times 10^8$
10^{-6}	0	$1·7 \times 10^8$
10^{-5}	0	0

At much higher concentrations, most metal ions inhibited motility. At 10^{-5} M inhibition appeared with $BaCl_2$, $CdCl_2$, $CoCl_2$ and $NiCl_2$; at 10^{-3} M inhibition by $CaCl_2$, $MgCl_2$, $MnCl_2$ and $ZnCl_2$ appeared. There was no inhibition by $PbCl_2$, $Na_2B_4O_7$ or Na_2MoO_4 at 10^{-4} M, and a stimulation at that concentration was produced by $Al_2(SO_4)_3$, $CrCl_3$, $Fe_2(SO_4)_3$ and $SnCl_4$.

Even though first treated with a chelating resin, NaCl, KCl, and NH_4Cl were somewhat inhibitory at 10^{-2} M, and more so at 10^{-1} M; the Cl^- or NO_3^- salts tended to be less inhibitory than SO_4^{2-} salts. Potassium phosphate buffer (pH 7·0) at 10^{-1} M partially inhibited, even though first treated with chelating resin. It is clear from this information that high ionic strengths inhibited motility and should be avoided when optimum motility is desired.

The effect of an energy source. Motility was observed in the absence of any added energy source, although the addition of a chelating agent such as EDTA was necessary to demonstrate it (lines 1 and 2 of Table 3). This motility must have been due to an endogenous energy source, since after three extra washes to remove contaminating growth medium (by alternate centrifugation and resuspension of the bacteria) there was still good motility (Table 3). For further evidence see the discussion about galactose in the next section. The addition of an energy source such as glucose or glutamine did, however, stimulate the rate of movement (Table 3).

The effect of oxygen. The following experiments show that glutamine could serve

as an energy source for motility only when oxygen was available. A suspension of motile *Escherichia coli* in L-glutamine + EDTA + phosphate buffer (pH 7·0) remained motile for days in a hanging drop on a slide, as judged by microscopic examination. However, when a coverslip, surrounded by stopcock grease to prevent evaporation, was placed on a drop, the motility came to a stop within 10 or 20 min., except near air bubbles. Removal of the coverslip, even 2 days after movement had stopped, resulted in immediate restoration of motility. This cessation of movement in presence of glutamine under a coverslip was fully prevented by including Chlorella algae with the bacteria and shining light on the suspension; the motility of the bacteria could be turned on or off at will by removing or inserting a green filter in the light path. The motility in the presence of Chlorella and light, which persisted for at least 3 days, presumably was due to a supply of oxygen generated by the photo-synthetic algae. The results confirm similar studies reported by Engelmann in 1881 (for a review see Weibull, 1960).

Table 3. *Effect of energy source on motility of Escherichia coli*

In the first experiment, the bacteria were washed free from growth medium twice as usual (Adler & Dahl, 1967). In the second experiment, the bacteria were washed an additional two times. When this was followed by still another wash, exactly the same data as in Expt. 2 were obtained. Phosphate buffer (pH 7·0, 10^{-2} M) and EDTA (10^{-4} M) were present. Glucose or glutamine was added to 10^{-2} M.

Expt. no.	Energy source added	Distance moved in 30 min. (cm.)
1	None	2·4
	None; EDTA omitted	0
	Glucose	5·6
	Glutamine	5·6
2	None	1·6
	Glutamine	4·8

With tryptone or a mixture of the 20 amino acids commonly found in proteins, *Escherichia coli* grown aerobically on a mixture of the 20 amino acids (Adler & Dahl 1967) remained weakly motile under a coverslip for at least a day, in contrast to its behaviour with glutamine. Each of the amino acids in this mixture was tested singly to determine which were responsible for this anaerobic movement. Only L-serine allowed movement to continue under a coverslip; this anaerobic motility on L-serine persisted for many hours, but it was not as vigorous as when the coverslip was removed. A direct demonstration that serine can be consumed both aerobically and anaerobically is presented elsewhere (Adler, 1966). *E. coli* contains L-serine dehydrase (Wood & Gunsalus, 1949; Pardee & Prestidge, 1955; Umbarger & Brown, 1957), an enzyme which can catalyse the anaerobic conversion of L-serine to pyruvate; the pyruvate can then be dismutated anaerobically to yield energy. As expected from this, pyruvate allowed good movement for many hours under a coverslip. Also glucose allowed such anaerobic movement for 2 days, although again the movement was more vigorous without the coverslip, presumably because the supply of energy is more abundant aerobically than anaerobically.

Escherichia coli grown anaerobically on a mixture of the 20 amino acids were motile anaerobically on L-threonine and L-tryptophan, as well as on L-serine. Anaerobic

conditions induce L-threonine dehydrase (Wood & Gunsalus, 1949; Umbarger & Brown, 1957) and apparently also tryptophanase.

On D-serine there was anaerobic movement for many hours, but only when the bacteria were first grown in the presence of D-serine; *Escherichia coli* is known to contain a D-serine dehydrase inducible by D-serine (Pardee & Prestidge, 1955).

Without any added energy source, extensively washed *Escherichia coli* were motile in a hanging drop containing EDTA + phosphate buffer, but the movement under a coverslip quickly stopped. This showed that the endogenous energy source required oxygen for its utilization. Even when the bacteria were grown on galactose, swimming of the washed bacteria stopped under anaerobic conditions. Since galactose allowed both aerobic and anaerobic motility, this is additional evidence that the endogenous energy source is not some residual energy source from the medium.

Salmonella abortusequi, strain NCTC 5727, grown aerobically on a mixture of 20 amino acids, remained motile under a coverslip for a day or more on tryptone or on a mixture of 20 amino acids. Each amino acid was tested singly and the support of motility was traced to L-serine and L-threonine; presumably these bacteria contain an L-serine dehydrase and L-threonine dehydrase. Sherris, Preston & Shoesmith (1957) reported that *Pseudomonas viscosa* required oxygen for movement except in the presence of arginine, and that this strain could metabolize arginine anaerobically to yield adenosine triphosphate. Different strains and species of bacteria may be able to swim anaerobically on different substrates, depending on the repertoire of enzymes available for yielding energy anaerobically. In fact, this test for anaerobic motility would seem to be a rapid, effective technique for learning about the anaerobic metabolism of motile bacteria.

The conclusion from this study is that motility requires oxygen if the particular substrate yields energy only aerobically, but motility does not require oxygen if pathways are present for obtaining energy from a substrate anaerobically. Even when a substrate can yield energy anaerobically, the aerobic motility on that substrate is more vigorous, presumably because the aerobic production of energy is more abundant.

The effect of pH value. The optimum pH value for motility was between pH 6 and 7·5 (Fig. 4). Cacodylate (not shown in Fig. 4) or tris could replace phosphate buffer, so there was no requirement for added phosphate in this system. The shape of the pH curve must be determined by the effect of pH value on energy generation and perhaps other processes, as well as on motility. Flagella are known to disintegrate into subunits at pH 3–4 (Weibull, 1948; Stocker & Campbell, 1959).

The effect of temperature. Figure 5 shows that the optimum temperature for motility was about 25–37°. This optimum applies only to the particular time of incubation (30 min.) used here. A detailed study of the effect of temperature on the motility of Salmonella by Ogiuti (1936) showed that the optimum temperature decreased as the incubation time increased.

A growth medium for producing motile Escherichia coli and the effect of growth conditions

Ordinarily peptone or other such complex media have been used to grow bacteria that are highly motile. The present aim was to find a medium that was chemically defined and still produced *Escherichia coli* capable of excellent movement in the motility medium. It was found that peptone could be replaced by inorganic salts and a mixture

of 20 amino acids. This growth medium (Adler & Dahl, 1967) was used in all of the experiments mentioned up to this point.

The effect of glucose in the growth medium. When glucose replaced the mixture of 20 amino acids in the growth medium (except that leucine, methionine and threonine, which are necessary for the growth of this strain of *Escherichia coli*, were added) surprisingly the migration of organisms grown in this way was very slow and did not proceed far in capillary tubes containing glucose + EDTA + phosphate buffer (pH 7·0) (bottom curve of Fig. 6). Even when a chelating agent (e.g. citrate) was present in the glucose-containing growth medium, and even though the pH value of the growth medium remained near pH 7·0, about 99% of these bacteria were not motile, as judged by microscopic examination. The explanation for this lack of motility of the vast majority of these bacteria grown in presence of glucose was that these bacteria lacked flagella. Plate 1 compares bacteria grown in the presence of the 20 amino acids (fig. 1) with bacteria grown in the same medium except that glucose (and a supplement of leucine, methionine, and threonine) replaced the amino acid mixture (fig. 2). Bacteria grown in the presence of the amino acid mixture had numerous flagella, while those grown in the presence of glucose for the most part had no flagella,

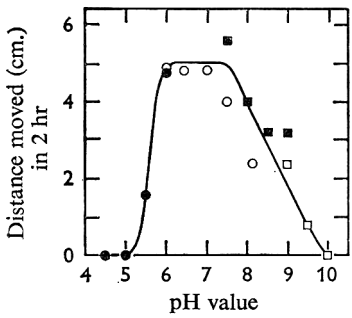

Fig. 4

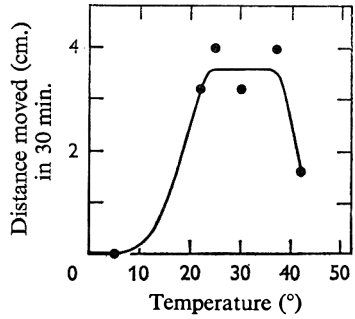

Fig. 5

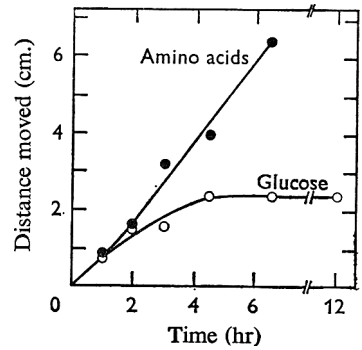

Fig. 6

Fig. 4. The effect of pH value on motility of *Escherichia coli*. ●—●, potassium acetate buffer; ○—○, potassium phosphate buffer; ■—■, tris hydrochloride buffer; □—□, β-alanine buffer. EDTA (10^{-4} M) was present. No energy source was added; in the absence of an added fermentable energy source, motility stopped when the oxygen was used up. The observed motility was therefore due to those bacteria which migrated from the crowded oxygen-deficient region at the origin. Complete loss of viability in 2 hr occurred when more extreme pH values (pH < 4·0 and > 10·5) than those reported in the figure were used.

Fig. 5. The effect of temperature on motility of *Escherichia coli*. Phosphate buffer (pH 7·0, 10^{-2} M), glutamine (10^{-2} M) and EDTA (10^{-4} M) were present. Since glutamine was not fermentable, motility stopped when the oxygen was used up. The observed motility was therefore due to those bacteria which migrated from the crowded oxygen-deficient region at the origin.

Fig. 6. The effect of amino acids on the motility of *Escherichia coli* grown on glucose. The cells were grown in the growth medium described in the preceding paper (Adler & Dahl, 1967), except that the mixture of 20 amino acids was replaced by glucose (5 g./l. medium) and a supplement of leucine, methionine and threonine (250 mg. each/l. medium). The bacteria were then washed free of this medium and placed into motility medium. In the bottom curve, the motility medium contained glucose (1×10^{-2} M), phosphate buffer (pH 7·0; 1×10^{-2} M) and EDTA (1×10^{-4} M). In the top curve, a mixture of the 20 amino acids normally found in proteins (each at about 1×10^{-3} M) replaced glucose in the motility medium.

sometimes one short flagellum, and very rarely (0·1–1%) a normal complement of flagella. This conclusion was confirmed by the following two independent tests.

Escherichia coli grown in the presence of the amino acid mixture agglutinated when anti-flagella serum was added while glucose-grown bacteria did not agglutinate. (The antiserum was prepared and kindly given to us by M. L. DePamphilis.) In addition, the bacteria grown on the amino acid mixture readily adsorbed χ, a phage that attacks only flagellated bacteria (Meynell, 1961), while the bacteria grown on glucose adsorbed phage χ at a just barely detectable rate (S. Z. Schade, unpublished).

Motile *Escherichia coli* organisms grown in medium containing the 20 amino acids were non-motile and lacked flagella beginning at about 6 divisions after they were transferred into a medium containing glucose instead of the amino acids (or even glucose in addition to the amino acids). Bacteria grown for as many as 20 divisions in the presence of glucose quickly regenerated flagella when placed into a medium free from glucose and containing the 20 amino acids. This restored motility, as shown in the top curve of Fig. 6. Omission of leucine, methionine and threonine from this mixture of amino acids prevented restoration of motility, presumably because this strain is unable to synthesize the protein of flagella without these amino acids which are required for growth. It is clear that glucose shut off the synthesis of flagella and that this effect was annulled by removal of the glucose. It was possible in this way to go back and forth between flagellate and largely non-flagellate phases.

Lactate behaved like glucose in stopping synthesis of flagella, but the following carbon sources when replacing the amino acid mixture in the growth medium produced normally motile bacteria with normal flagella: galactose, glycerol, succinate, α-ketoglutarate. The inhibitory effect of glucose on the synthesis of flagella was also shown, though to a very much lesser degree, with another strain of *Escherichia coli*, W3110; but the motility and flagellation of *Salmonella typhimurium*, strain TM2, was not affected by glucose in the growth medium, even after 25 divisions.

The effect of pH value of growth, temperature of growth and age of culture. Escherichia coli was adapted for at least eight divisions to grow at values ranging between pH 6·0 and 8·0 in the medium described under Methods. The bacteria grown at each pH value were normally motile and had a normal complement of flagella. Therefore, variation in the pH value of growth within the range pH 6·0–8·0 was not critical for studies of motility in this strain of *E. coli*.

The effect of temperature of growth was studied. *Escherichia coli* grown at 24° was more motile than when grown at 37°, as judged by the motility assay. Organisms grown at 40° or above were very weakly motile and had very few flagella, even though the growth rate was near normal. Such organisms were resistant to bacteriophage χ (S. Z. Schade, unpublished). It is clear that temperatures above 37° should be avoided to get good synthesis of flagella; even 37° is border-line. The inhibition of synthesis of flagella by elevated temperatures has been noted previously (Ogiuti, 1936, for *E. coli*; Meynell, 1961, and Quadling & Stocker, 1962, for Salmonella).

Escherichia coli organisms harvested at various stages of growth were tested in the motility assay. It was found that organisms in the exponential phase were the most motile; these were used for all the studies reported here. Organisms from lag phase or from late stationary phase cultures were distinctly inferior for motility.

DISCUSSION

The aim of developing a completely defined medium for the expression of motility led to the finding that heavy metal ions at very low concentrations inhibited the motility of *Escherichia coli* organisms, and that amino acids could stimulate motility by virtue of chelating these metals. Stocker & Campbell (1959) and Meynell (1961) noted that Salmonellas washed in saline lost their motility and that the motility was regained when a complex broth, albumin, or an amino acid was added. Apparently no other study of the requirements for a chemically defined motility medium has been reported before for bacteria. Tyler (1953) and Rothschild & Tyler (1954) showed that amino acids protected the motility and fertilizing ability of sea-urchin spermatozoa and that chelating agents could replace the amino acids. The mechanism by which heavy metal ions inhibit bacterial movement is not understood; it is not known whether metal ions inhibit the movement directly, or whether the effect on movement results indirectly from an inhibition of some other process—for example, production of adenosine triphosphate. The protective effect of chelating agents for the viability of bacteria has been generally recognized.

The results reported here show that it has been possible to replace a complex motility medium such as peptone with simply a chelating agent, an energy source and a buffer and still obtain excellent motility of *Escherichia coli*. By using a strain of bacteria which requires certain nutritional factors (in this particular case leucine, methionine, threonine) and not providing these factors in the motility medium, one can obtain motility without the complications of chemotaxis (Adler & Dahl, 1967) or growth.

The attempt to devise a chemically defined growth medium for producing motile bacteria led to the observation that glucose prevented the synthesis of flagella. The mechanism of this effect of glucose remains unknown. The glucose effect has been commonly observed for many enzymes (see review by Magasanik, 1961), and the effect is now extended to include a structure or organelle—the flagellum. Apparently there are no other published studies on the requirements of a chemically defined growth medium for producing bacteria with high motility, although it has been noted that a defined medium may lead to synthesis of fewer flagella (Kerridge, 1959).

For many helpful discussions we thank Drs H. Echols and H. M. Temin. Dr Temin called our attention to the work of Rothschild & Tyler (1954) and suggested the use of EDTA and the idea that amino acids protect bacterial motility by chelation.

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EXPLANATION OF PLATE

The effect of carbon source on the synthesis of flagella by *Escherichia coli*.

Fig. 1. *Escherichia coli* grown in the growth medium of Adler & Dahl (1967) which contains a mixture of 20 amino acids.

Fig. 2. The amino acid mixture of the growth medium was replaced by glucose (5 g./l.) and a supplement of leucine, methionine and threonine (each 250 mg./l.); in this picture several bacteria each with only one flagellum may be seen. The flagella were stained according to Leifson (1951).

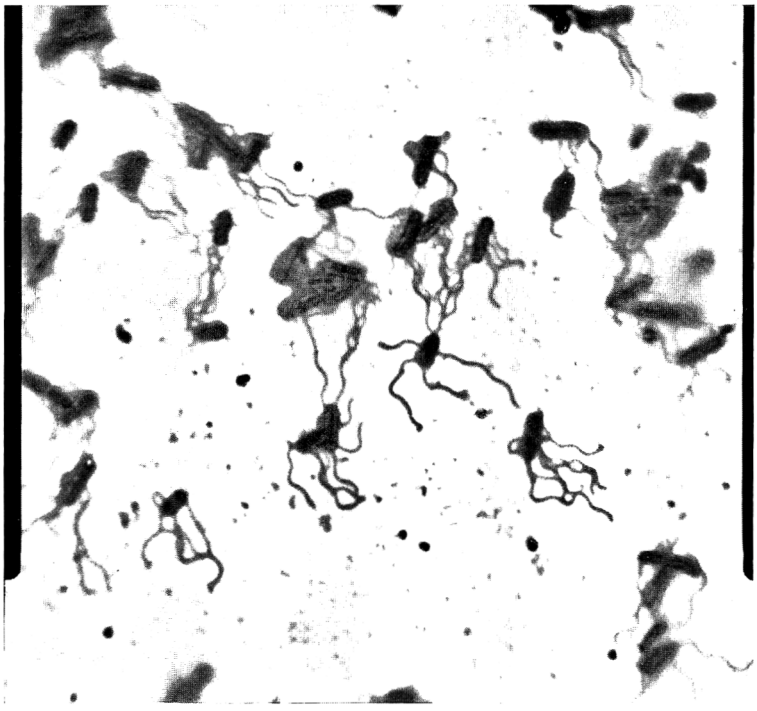

Fig. 1

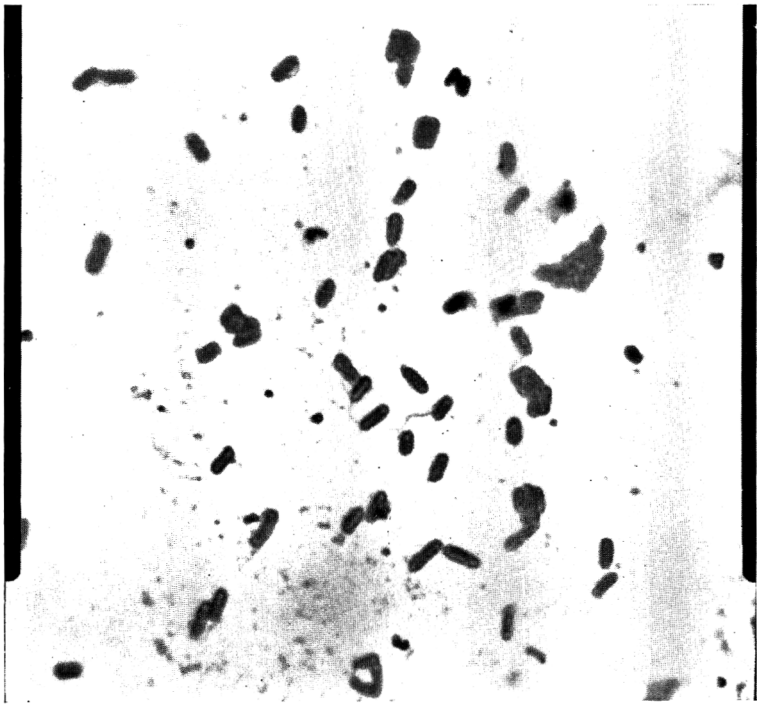

Fig. 2

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Variability in Mallomonas

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SUMMARY

This paper deals with variability within certain species of the genus *Mallomonas* as revealed under the electron microscope. New varieties and two new species are described (*M. annulata*; *M. pillula*), one of these having previously been considered a variety, partly through confusion. An attempt is made to consider different kinds of variability and to make a decision on what range can be tolerated within the limits of a species. The importance of examining the whole organism under the electron microscope as well as a few scales is now realized. It is desirable also to examine the flagellum since several species which have been described as belonging to the genus *Mallomonas* must be separated from it because they prove to have two flagella.

INTRODUCTION

In this paper I discuss different aspects of variability in the genus *Mallomonas* derived from working with wild material viewed with the light and electron microscopes. Some species have scales of a constant pattern, others show a series of easily separable varieties and one, *M. pillula*, has a series of forms all living together, which I refrain from classing as varieties from the material available. Some of these taxonomic units (e.g. *M. annulata*) are found in all sorts of habitats, but others seem to occur in only a few habitats which are of much the same kind. In addition there may be a range in the scales of a single organism. This is particularly noticeable in organisms of an elongated shape such as *M. annulata*. Also, while most scales of an individual may be perfectly formed, one may find a few scales that appear to be unfinished and these look very different; I have called them immature.

DESCRIPTION OF SPECIES

Mallomonas papillosa Harris & Bradley 1957 and 1960
(Figs. 1, 2, 4; Pl. 1, figs. 1, 2)

History. Harris & Bradley (1957, in part), pl. 4, fig. 9 (replica of scale and bristle tip and foot). The description in the text and text-figures H, K, L, M, which we called *Mallomonas papillosa*, is now recognized as *M. annulata*.

Emended diagnosis. Cellula ellipsoides ferme, setis ubique contecta tenuibus, brevibus, curvatis. Squamae parvae, prope aequatae per totam cellulam; squama ovalis cum cupola, scuto, margine. Scuti pars exterior papillis aequaliter contecta, pars interior levis. Margo distalis costas exhibet obliquas. Cupola saepissime paucas papillas ferens in uno latere, permulata foramina minuta in altero. Setae paucis dentibus aequalibus instructae. Cystum leve obovatum, parte latiore saepissime anteriore.

Dimensiones. Cellula 12–18 μ, squamae 3–4 × prope 2 μ, cystum 10–11 μ latitudine.

Emended English diagnosis. Cell broadly ellipsoidal, covered all over with delicate short curved bristles. Scales small, nearly uniform all over cell. Scale oval showing dome, shield and flange. Shield with regularly arranged papillae on the outer surface, inner surface smooth. Distal margin showing oblique ribs. Dome usually showing a few papillae on one side and numerous fine perforations on the other. Bristles showing a few evenly marked serrations. Cyst smooth, obovate, broad end usually anterior.

Occurrence. The type locality of *Mallomonas papillosa* (Harris & Bradley, 1957, pl. 4, fig. 9) is Stan Lake (Nat. Grid, 480·0 E. 175·5 N.). The locality given in the text refers to the light-microscope drawings and description. Stan Lake is a slowly moving stream widened into an ornamental lake. It has now been greatly altered and *M. papillosa* has not been found there lately in quantity and the material described in this paper is from Benyons Lake (Nat. Grid, 464·0 E. 163·3 N.) where *M. papillosa* has been found in large numbers together with *M. annulata.*

Mallomonas papillosa var. *monilifer* var.nov. (Figs. 1, 2, 3; Pl. 2, figs. 3–5)

Latin diagnosis. Ab typo discrepat quod scutum ex parte solum papillis obtectum: sed in iunctura scuti et cupolae sunt papillae in uno ordine curvato: cupola autem sine papillis. Cystum ferme ellipsoides.

Est unus ordo curvatus (quasi monile) papillarum.

English diagnosis. Differs from the type in scale, having only a patch of papillae on shield and in addition a single curved row of papillae where the shield adjoins the dome. The dome has no papillae. Cyst broadly ellipsoidal.

Occurrence. The var. '*monilifer*' occurs in a small artificial acid pond by Farley Court (Nat. Grid, 475·3 E. 164·3 N.).

The name, 'monilifer', necklace-bearing, refers to the row of papillae.

Mallomonas papillosa var. *ellipsoida* var.nov. (Figs. 1–3; Pl. 3, fig. 6)

History. Mallomonas papillosa Harris & Bradley, 1960, (see Pl. 5, figs. 30, 31).

Latin diagnosis. Ab typo discrepat setis serratis validioribus: scutum squamae papillis paucioribus, superficie non ab omni parte contecta: cupola sine papillis: cystum ferme ellipticum.

English diagnosis. Differs from the type in having more strongly serrate bristles. Shield of scale with fewer papillae which do not cover the whole surface, dome without papillae. Cyst very broadly ellipsoidal.

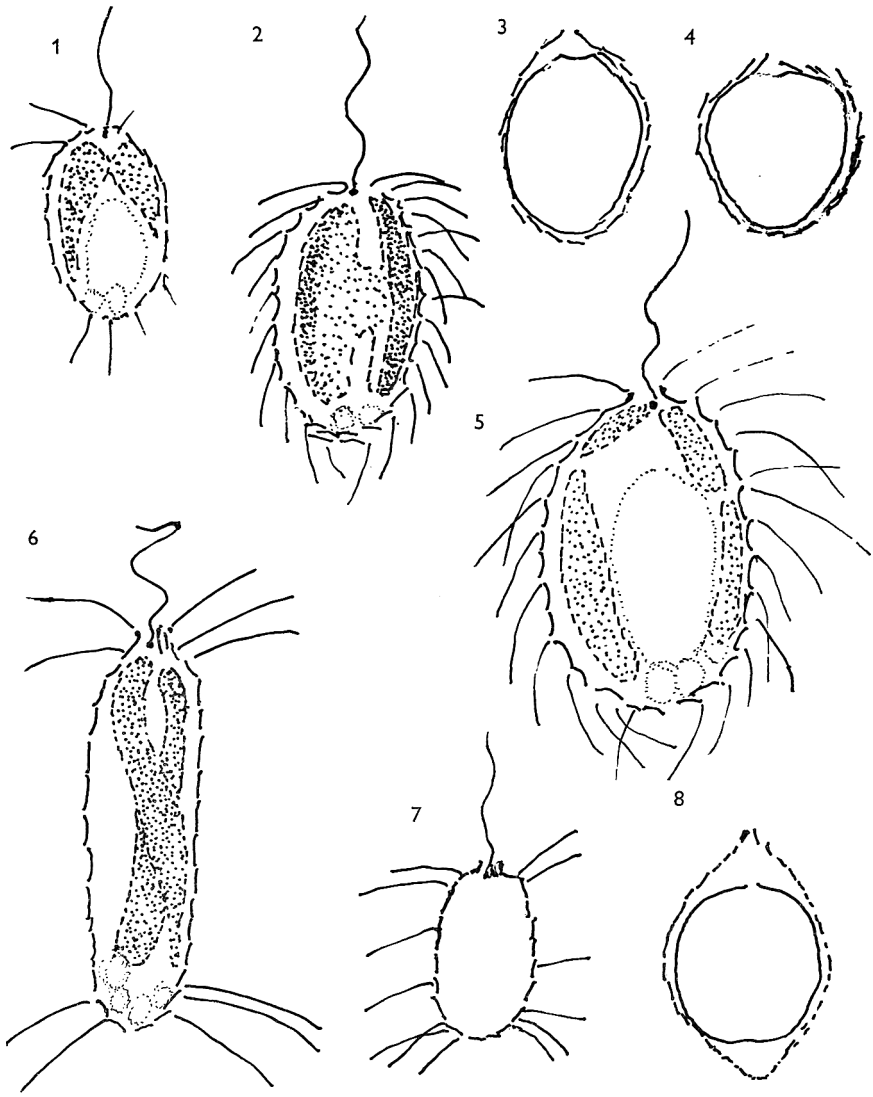
Occurrence. The variety *ellipsoida* was found in the eutrophic oxbow pool at Grazely (Nat. Grid, 470·0 E. 166·8 N.). Specimens with similar scales were found and their scales seen under the electron microscope in Spencer's Wood roadside pond, but their cyst was not seen.

Discussion. Similar scales have been seen by Takahashi (1959) in Japan. The small differences are a slightly broader scale and even deeper serrations on the bristles. *Mallomonas papillosa* is indistinguishable from its varieties under the light microscope. Another species which looks very similar was described as *M. radiata* Conrad var. *ovalis* by Lund (1942, see p. 64). In my opinion forms as small as these are only to be distinguished when we have electron micrographs of the scales and bristles.

Mallomonas papillosa may be rather like species and varieties of the '*striata*' group under the light microscope. The scales of the '*striata*' group are larger and more concave, and their pattern as seen with the electron microscope is completely different.

Mallomonas annulata sp.nov. (Figs. 6–8; Pl. 3, figs. 7–10; Pl. 4, fig. 11)

History. This species has been previously figured but its taxonomic position was not recognized. (Harris & Bradley, 1957, text-figs. H–M; 1960, pl. 5, figs. 32, 35). It was confused with *Mallomonas papillosa* and referred to as *M. papillosa* forma *annulata*. Bradley (1966) raised its status to that of a variety, *M. papillosa* var. *annulata*. Further



Figs. 1–8. 1, Young organism of *Mallomonas papillosa*. 2, Older organism of *M. papillosa*. 3, Cyst in mother cell of *M. papillosa* var. *monilifer*. 4, Cyst in mother cell of *M. papillosa*. 5, *M. striata* Asmund, form. 6, 7, *M. annulata* at different ages. 8, *M. annulata* cyst in mother cell. ×2000

study has demonstrated numerous differences which are sufficient to raise it to specific rank.

Latin diagnosis. Cellula adulta elongata extremis rotundatis: sed cellula nondum adulta brevis et elliptica. Pars anterior et pars posterior, excepta parte postrema, setas ferunt: sed pars media nuda vel setis paucis sparsis. Squamae parvae trium generum: (i) squamae corporis setiferae; (ii) squamae corporis non setiferae; (iii) squamae extremae partis setiferae quoque. Squamae corporis rhomboides; setiferae cupola et scuto et margine; non setiferae scuto et margine solum. Squamae extremae partis asymmetricae. Flagellum torque circumdatum squamis corporis erectis setiferis. Squamae omnes papillis simili modo ornatae in scuto: papillae nonnunquam in circulos iunctae parte distali. Cystum leve globosum.

Dimensiones. Cellula 17–28 × 6–10 μ, squamae 3–4 × prope 2 μ, cystum 10–12 μ latitudine.

English diagnosis. Mature cell elongated with rounded ends but immature cell short and ellipsoidal. Anterior and rear ends, except extreme rear, with bristles but middle region bare or with few scattered bristles. Scales small, of three kinds: body scales bearing bristles, body scales without bristles and small scales at extreme rear also without bristles. Body scales rhomboidal, those with bristles having dome, shield, and flange those without bristles having shield and flange only. Scales of extreme rear asymmetrical. Flagellum surrounded by a collar of erect body scales bearing bristles. All scales decorated on shield with similar pattern of papillae which may be joined in rings at distal end. Cyst smooth spherical.

Occurrence. Mallomonas annulata occurs in many kinds of pools—acid, eutrophic and intermediate, in the open and woods. The type locality, Stan Lake (Nat. Grid, 480·05 E. 175·5 N.), is a slow-moving stream artificially widened. After *M. akrokomos* it is the commonest species near Reading and has been noticed in nearly all months of the year. Bradley (1966) has found it in Scotland and Asmund (private letter) in Denmark.

Comparison. Mallomonas papillosa and *M. annulata* are difficult to distinguish in their early stages and their small scales are very similar as seen with the light microscope, but when mature they are easy to distinguish and with the electron microscope their scales have little resemblance. *Mallomonas annulata* has been found in the localities of all varieties of *M. papillosa* in most months of the year. In 1957 and 1960 when Harris & Bradley examined mallomonas scales they were in the habit of using isolated scales for the electron microscope and described *M. papillosa*, *M. papillosa* var. *serrata* and *M. annulata* all as *M. papillosa*, and gave light-microscope drawings of *M. annulata* (1957, pl. IV, fig. 9; 1960, pl. 5, figs. 32, 35). Further investigation has cleared up the confusion and differentiated the species.

Mallomonas striata Asmund its var. *serrata* Harris & Bradley (Pl. 5, fig. 12)

Asmund (1959) described *Mallomonas striata* and Harris & Bradley (1960, pl. 3, figs. 19, 20; pl. 4, fig. 29) described a variety *serrata* found in a flooded field. It differed from the type in having a smaller cell and a ring of triangular collar scales not bearing bristles, and its bristles on the other scales were serrate. A second variety also having small cells and serrate bristles but without collar scales was found in a small permanent pond on acid soil (Nat. Grid, 475.3 E. 164·3 N.). Electron micrographs of several whole organisms were examined; they are not illustrated here.

Mallomonas striata has scales of rather varied ornamentation. Asmund figured and

described several slightly different forms and I have noted other forms of scale in material from near Reading. I refrain from describing the forms as varieties from the material I have available.

Mallomonas cratis Harris & Bradley, 1960 (Pl. 5, fig. 14)

Mallomonas cratis is interesting in showing a constant appearance with the light microscope or with the electron microscope, a constant pattern on its scales and a constant form to its bristles, though occurring in very varied waters. The type locality was a brackish pool (I did not analyse the water but it tasted very salt). It has now been found near Reading (Nat. Grid 462·0 E., 163·3 N.) in a woodland lake (Benyons Lake) on slightly acid soil. *Mallomonas cratis* is not unique in this respect. *Mallomonopsis elliptica* var. *salina* Asmund & Hilliard (1965) which was first found in brackish water was also found in an inland fresh-water pool (Harris, 1966); the organisms scales and bristles from both waters look alike.

Mallomonas pillula sp.nov. (Pl. 5, fig. 13; Pl. 6, figs. 19–23)

Latin diagnosis. Cellula parva globosa saepe setis contecta sed nonnunquam setis perpaucis solum. Flagellum circumdatum circulo squamarum erectarum praelongarum asymmetricarum cupola lata; squamae saepe leves vel minus valide signatae in scuto quam squamae corporis. Squamae corporis rhomboides cupola parva vel nulla. Squamae setiferae scuto et margine et cupola. Cupola levis saepe parvis foraminibus. Scutum parvis papillis signatum iunctis fascia in globos sex septem continentes, nonnunquam papillam mediam includentes. Scutum mitram ferens et fascia circumdatum punctis obliquis obscuris signata. Pars extrema distalis extra fasciam unum ordinem ferens papillarum in margine et laciniam labro revoluto. Squamae setas singulas ferentes, raro duo vel tres, leves, curvatas, breves, attenuatas. Cystum ignotum.

Forma exannulata discrepat maiores papillas ferens in scuto non in fasciam iunctas et marginem ferens distalem signatum uno ordine altarum lacunarum.

Dimensiones. Cellula prope 10 μ diam., squamae prope $2 \times 1{\cdot}75$ μ.

English diagnosis. Cell small, spherical, often covered with bristles, sometimes having only a few. Flagellum surrounded by a ring of erect asymmetrically elongated scales with a broad dome, often smooth or less strongly marked on shield than are body scales. Body scales rhomboidal with small dome or no dome. Bristle bearing scales have shield, flange and dome. Dome smooth, often with some small perforations. Shield marked by small papillae joined by a band in groups of about six, sometimes enclosing a central papilla. Shield has a hood and is surrounded by a band which has oblique dark dots. Distal end, outside the band, has border with single row of papillae and flange with turned back edge. Bristles 1, rarely 2 or 3 per scale, smooth, curved, short, tapering. Cyst unknown.

Forma '*exannulata*' (Pl. 6, figs. 15–18) differs in having larger papillae not joined by band, on shield, and having a distal border decorated by a single row of deep pits.

Occurrence. Mallomonas pillula and its form were found in a woodland lake on slightly acid soil, Benyons Lake (Nat. Grid, 463·0 E. 163·3 N.), in spring.

The name '*exannulata*' refers to the absence of rings on the shield.

Discussion of Mallomonas pillula. Mallomonas pillula varies remarkably in the same water. One form has been described. Another form, illustrated in Pl. 6, fig. 21, has some large perforation on the basal plate while the basal plate in Pl. 6, fig. 23 has large

perforations all over it, though the basal plate is normally smooth. The degree of annular or papillose markings is also variable. We do not know the nature of this variability but it recalls the variability one meets in naturally breeding plants in which some individuals differ genetically from others.

Bradley (1964, pl. 2, fig. 17) showed, in a specimen from Iceland, a group of scales and bristles which agree with those of a typical Berkshire specimen, and these I identify with *Mallomonas pillula*. In Bradley's (1964) fig. 14 there is a figure from the same locality, differing only in showing numerous minute perforations on the basal plate and striations instead of dots on the band surrounding the shield. I think this too is a form of *M. pillula*. I express no opinion about Bradley's fig. 13, which is of the same size and shape as *M. pillula* but has markings different from any I have seen; it was from a different locality and might be yet another variant.

CONCLUSION

Different kinds of variation occur in the scales of species of the genus *Mallomonas*. In one Mallomonas species the scales differ in anterior, middle and posterior parts of the armour, and in another species there may be differences, presumably genetical, between all the scales of one individual and of another. Harris & Bradley (1960) showed a striking variation in scales due to their immaturity. All this variability is clearly important to the taxonomist. I feel that we shall only understand this variability deeply when it is studied in material grown in pure cultures. One subsidiary aspect of my conclusions is that I now realize that we should study whole fixed organisms, at any rate of the smaller species, with the electron microscope, as well isolated scales.

I wish to thank Professor R. W. Ditchburn, F.R.S., and Dr T. Evans for allowing me to use the electron microscope in the J. J. Thomson Physical Laboratory, Reading University, Professor P. Allen for allowing me occasional use of the electron microscope in the Geology Department, Reading University, and Mr F. Robertson, Reading University, for translating my diagnoses into Latin.

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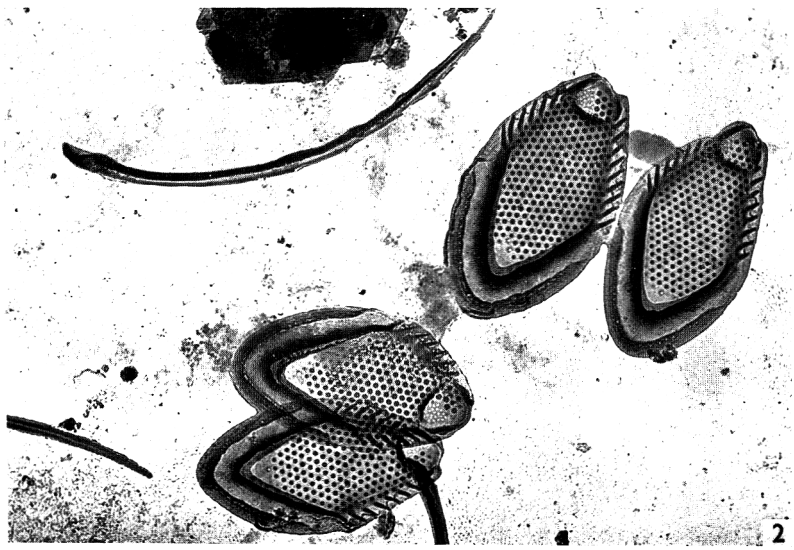
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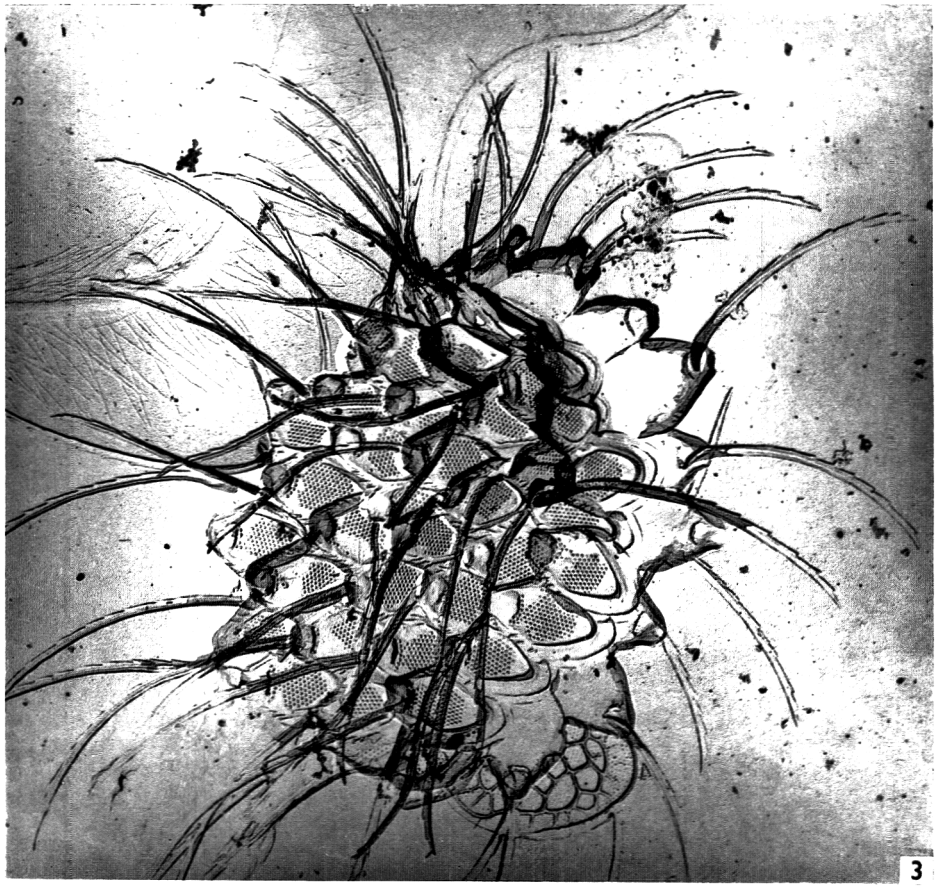
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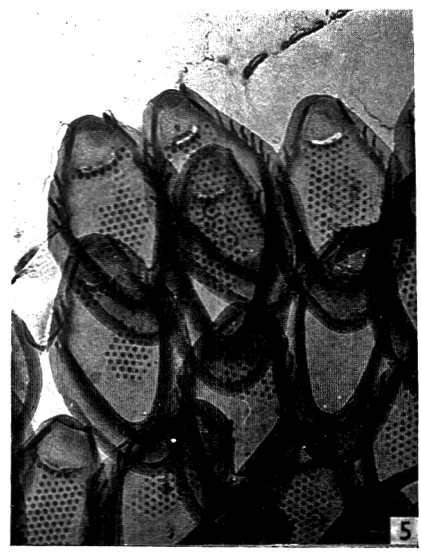
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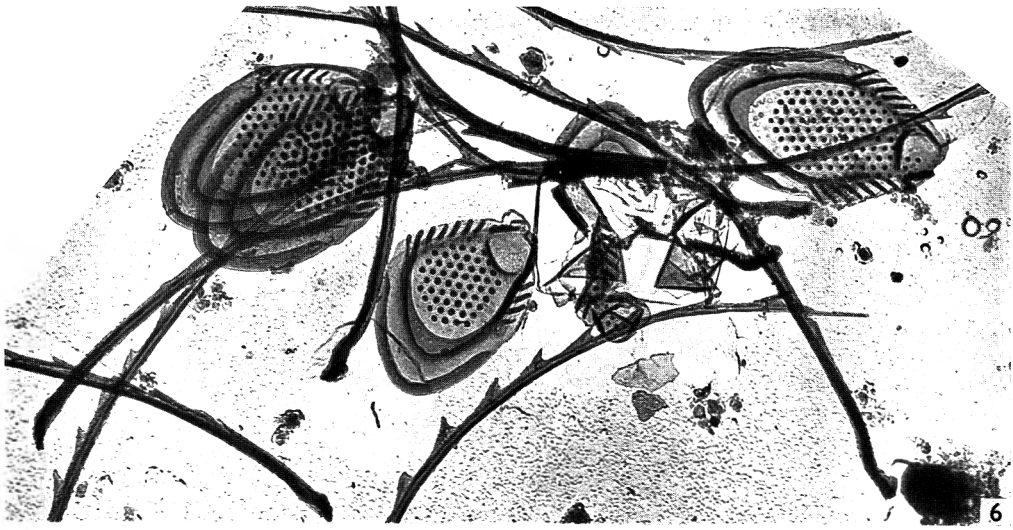
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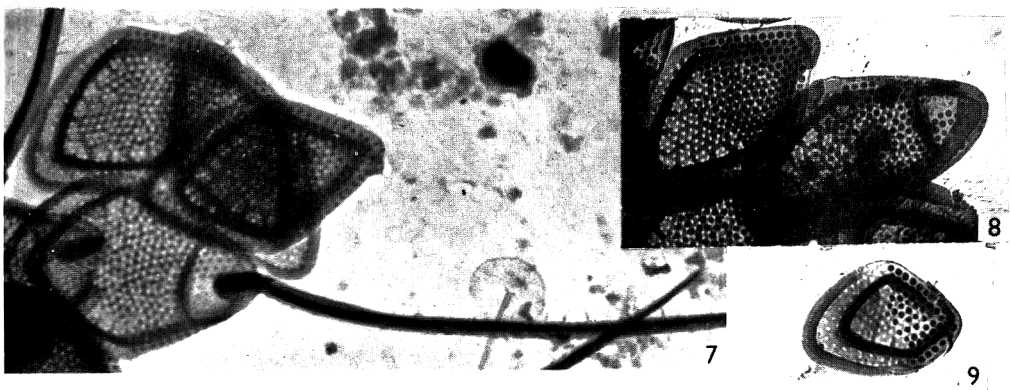
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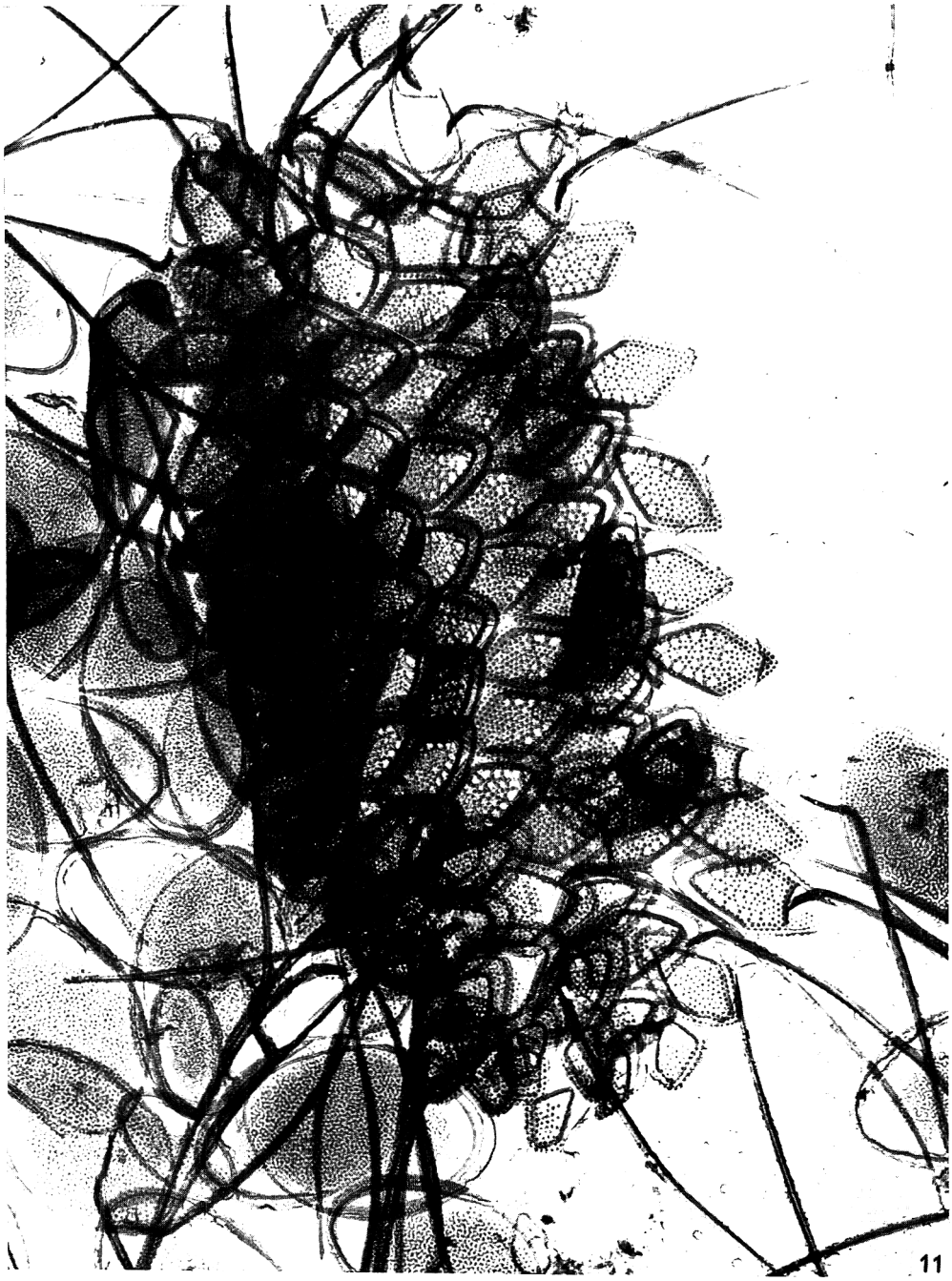

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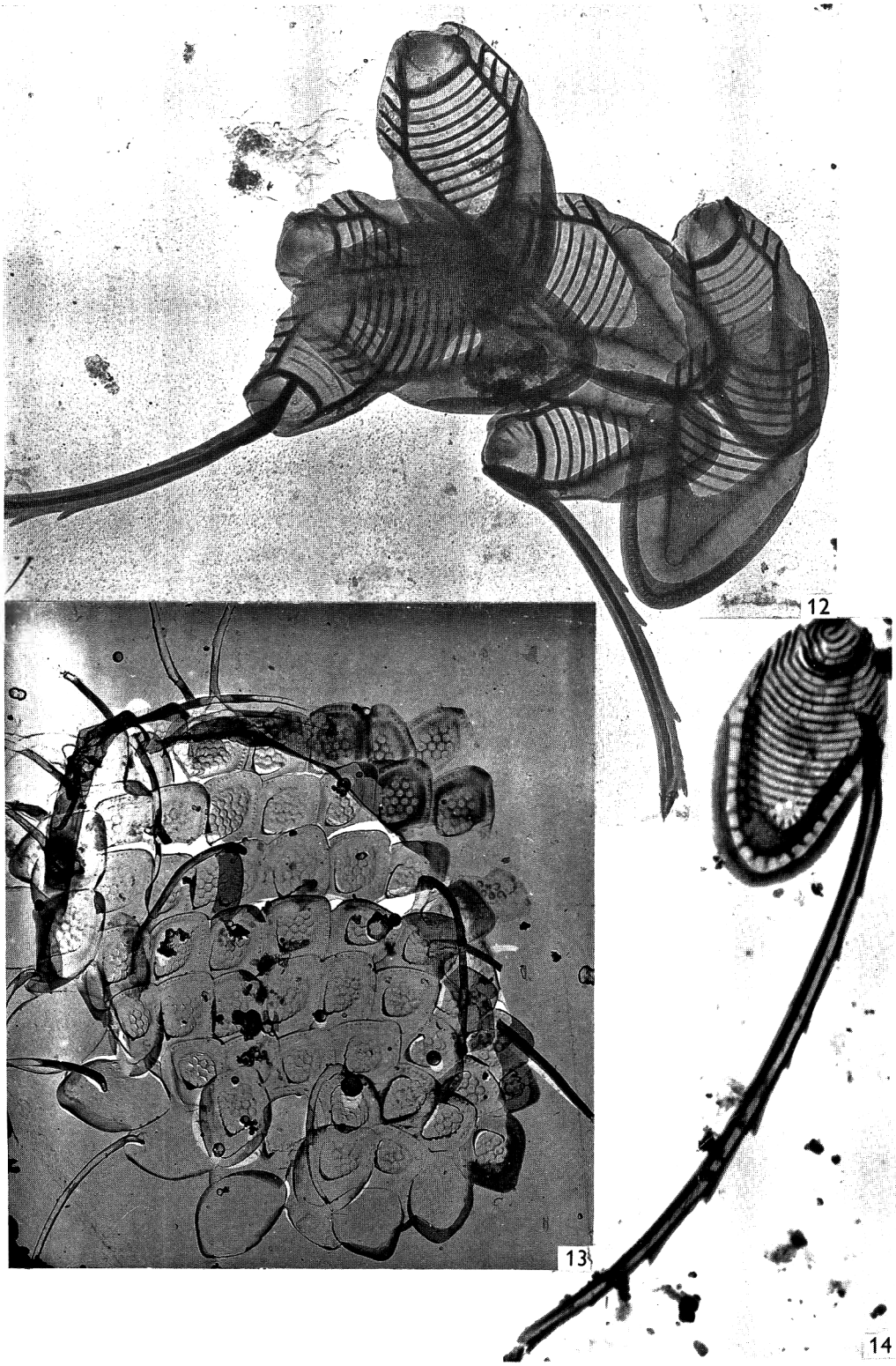
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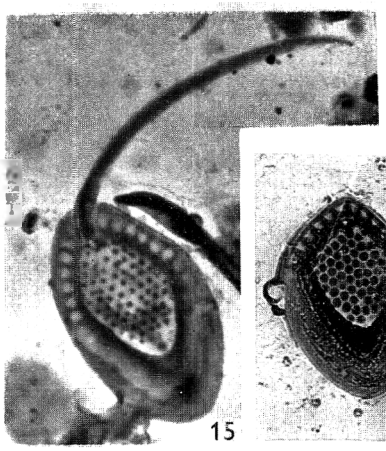
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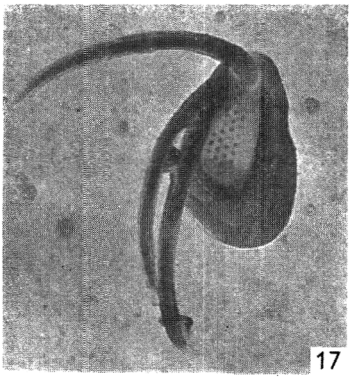
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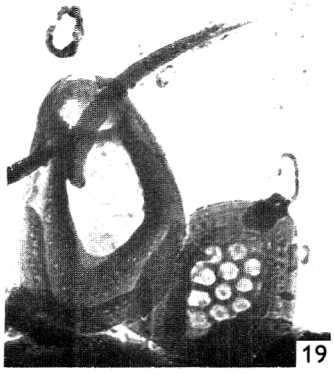
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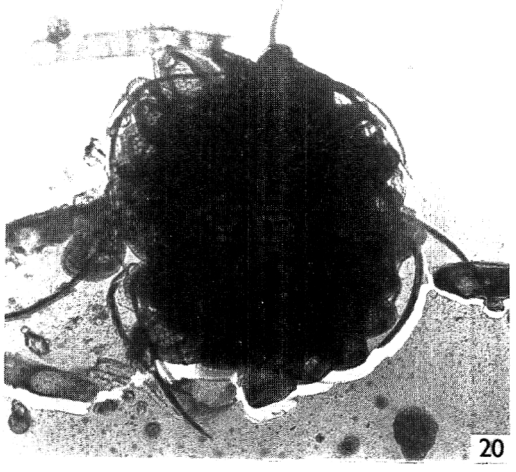
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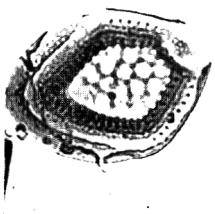
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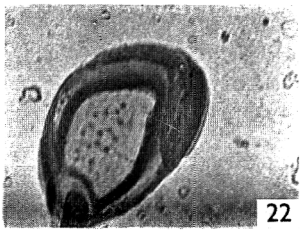
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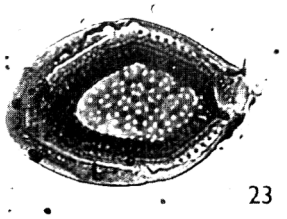
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EXPLANATION OF PLATES

PLATE 1

Mallomonas papillosa

Fig. 1. Whole organism, direct. × 5000.

Fig. 2. Scales and bristle, direct. × 1200.

PLATE 2

Mallomonas papillosa var. *monilifer*

Fig. 3. *Mallomonas papillosa* var. *monilifer*, whole of small cell replica; flagellum at top. × 5000.

Fig. 4. Scale and bristle, direct. × 12,000.

Fig. 5. Array of scales, direct. × 12,000.

PLATE 3

Fig. 6. *Mallomonas papillosa* var. *elliptica*, scales and bristles, direct. × 12,000.

Fig. 7. *Mallomonas annulata*, anterior scale with dome and two without, direct. × 12,000.

Fig. 8. *Mallomonas annulata*, rear scale with dome and one without, direct. × 12,000.

Fig. 9. *Mallomonas annulata*, extreme rear small scale, direct. × 12,000.

Fig. 10. *Mallomonas annulata*, whole small organism, direct. × 5000.

PLATE 4

Mallomonas annulata

Fig. 11. Whole organism also some scales of *Mallomonopsis elliptica.* var. *ovalis*, direct. × 5000.

PLATE 5

Fig. 12. *Mallomonas striata* form, direct. × 12,000.

Fig. 13. *Mallomonas pillula*, replica. × 6000.

Fig. 14. *Mallomonas cratis*, scale and bristle, direct. × 12,000.

PLATE 6

Mallomonas pillula

Figs. 15–18. *Mallomonas pillula* var. *exannulata*, all direct.

Fig. 15. Body scale with bristle. × 15,000.

Fig. 16. Body scale. × 15,000

Fig. 17. Collar scale and three bristles. × 15,000.

Fig. 18. Whole organism with flagellum. × 5000.

Figs. 19–23. *Mallomonas pillula*, all direct electron micrographs.

Fig. 19. Collar scale nearly smooth and body scale well marked, and bristle. × 15,000.

Fig. 20. Whole organism with faint flagellum, direct. × 5000.

Fig. 21. Body scale fairly well marked. × 15,000.

Fig. 22. Collar scale faintly marked. × 15,000.

Fig. 23. Body scale of a different form. × 15,000.

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The Influence of Dissolved Oxygen Concentration on the Respiration and Glucose Metabolism of *Klebsiella aerogenes* during Growth

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SUMMARY

The influence of dissolved oxygen concentration on the metabolism and respiration of growing *Klebsiella aerogenes* NCTC 8017 was studied by means of a continuous-flow culture technique. Different dissolved oxygen tensions (equivalent partial pressures) were obtained by varying the partial pressure of oxygen in the gas phase. The respiration rate (oxygen uptake rate per unit mass organism) was independent of dissolved oxygen tensions above 10–15 mm. Hg when the culture was said to be in the 'excess oxygen state'. With dissolved oxygen tensions below about 10 mm. Hg, the 'limited oxygen state' developed. In the transition to this state, complex oscillations in the dissolved oxygen tension occurred. The oscillations reflected alternate stimulation and inhibition of the respiration rate. A decrease in dissolved oxygen tension to below 5 mm. Hg immediately increased the respiration rate; an increase in dissolved oxygen tension from 5 to 10 mm. Hg immediately decreased the respiration rate. These effects formed part of the mechanism of the oscillations which could be sustained indefinitely. The oscillations in respiration rate occurred irrespective of change from pH 6·0 to 7·4 or whether growth was limited by glucose or ammonium supply. The pattern of oscillations in respiration rate varied with growth rate over the range 0·2–0·5 hr^{-1}. It is suggested that decreasing the oxygen tension to about 5 mm. Hg acted like an uncoupler of oxidative phosphorylation.

In the excess oxygen state with excess glucose and growth limited by ammonium supply, the glucose carbon was largely accounted for as CO_2, pyruvate and organisms, irrespective of the pH value. Pyruvate was not accumulated when growth was glucose-limited. In the limited oxygen state, the glucose carbon was largely accounted for as: organisms, CO_2, 2:3-butanediol, ethanol, acetic acid, formic acid, lactic acid. The proportions of these fermentation products varied with oxygen supply, pH value and whether growth was ammonium-limited or glucose-limited.

INTRODUCTION

Studies on the influence of oxygen on growth and metabolism have been severely limited by the lack of a convenient and reliable means for the measurement of dissolved oxygen concentration. But the recent development of oxygen electrodes has made measurement of dissolved oxygen concentration over long periods much more feasible. In the present work, oxygen electrodes were used in the type of continuous-flow culture referred to as a chemostat (Herbert, 1958). By this method it should be

possible to maintain a constant dissolved oxygen concentration with a constant rate of organism growth provided the oxygen supply rate is constant. The relation of the dissolved oxygen concentration to the oxygen uptake rate and oxygen supply rate is given below. The oxygen electrode responds to oxygen activity or oxygen tension rather than to oxygen concentration (Kinsey & Bottomley, 1963). The tension (T) and concentration (C) are related by the expression $T = fC$, where f is an activity coefficient. Consequently the electrode readings are expressed in terms of equivalent oxygen partial pressure or tension; convenient units are mm. Hg.

Most measurements of dissolved oxygen tension in cultures have been concerned with the effect of oxygen tension on respiration rate (e.g. Baumberger, 1939; Winzler, 1941; Longmuir, 1954; Chain & Gaulandi, 1954; Chance, 1957; Phillips & Johnson, 1961). The results of these and other workers generally indicate that respiration rate is independent of the dissolved oxygen tension until it reaches values in the range 0–15 mm. Hg. There is a considerable variation in the value of the 'critical O_2 tension'; that is, the value at which the respiration rate (q_{O_2}) begins to decrease; for the same organism, yeast, values from 0 mm. Hg (Baumberger, 1939) to 12 mm. Hg (Hixon & Gaden, 1950) have been reported. The influence of growth rate on q_{O_2} has not been taken into account; however, it needs to be, because, as the data of Herbert (1958) showed, the q_{O_2} of bacteria increases linearly with their growth rate.

The existence of qualitative differences between the metabolic response of growing (multiplying) and non-growing bacteria to oxygen tension may be deduced from the observations on the adaptive response of enzyme systems to changes in oxygen supply. Several workers (e.g. Chin, 1950; Ephrussi, Slonimski & Perrodin, 1950; Schaeffer, 1950) have shown the adaptive development of cytochromes in cells in response to access of oxygen. Moss (1952, 1956) investigated the relation between adaptive formation of cytochromes and dissolved oxygen tension in a growing culture of *Aerobacter aerogenes*. He used a continuous culture technique to maintain growth and an oxygen electrode to measure dissolved oxygen tension. He found that with decrease in the dissolved oxygen tension the amount of cytochrome a_2 in the bacteria increased and reached a maximum at an oxygen tension of about 0·2 mm. Hg. This quantitative control over the cytochrome content exerted by the environment is reminiscent of the quantitative control over the enzyme content of bacteria observed by Tempest & Herbert (1965), rather than 'all or none' induced synthesis. Facultative anaerobic bacteria provide a particularly interesting subject for the study of oxygen relationships because their metabolism undergoes a profound change with change from an aerobic to an anaerobic environment (Gray, Wimpenny & Mossman, 1966). The facultative anaerobe *Klebsiella* (*Aerobacter*) *aerogenes* was chosen for the present study because the effect of oxygen supply on the glucose metabolism of the growing organism had already been extensively studied (Pirt, 1957; Pirt & Callow, 1958*a*, 1959). These workers compared fully aerobic growth with oxygen-limited growth, but they had no means of measuring the dissolved tension. The present study relates the glucose metabolism of *Klebsiella aerogenes* NCIB 8017 to the dissolved oxygen tension and reveals some new aspects of the behaviour of the organism at low oxygen tensions.

METHODS

Relation between oxygen supply rate, oxygen uptake rate and dissolved oxygen tension. The parameters which affect the oxygen concentration of a submerged culture may be expressed as follows. Let C = concentration of dissolved oxygen; C_s = saturation concentration of dissolved oxygen; t = time; $(dC/dt)_s$ = oxygen solution rate, that is, the rate of oxygen transfer from the gas to the liquid phase; A = area of interface between gas and liquid.

For the oxygen solution rate or supply rate we can write (see Finn, 1954).

$$\left(\frac{dC}{dt}\right)_s = \Phi A(C_s - C), \tag{1}$$

where Φ = a constant which depends on the aeration conditions.

Let T_L and T_G (mm. Hg) be the oxygen tensions in the liquid and gas phases respectively.

$$\text{Then } T_L = fC \tag{2}$$

$$\text{and } T_G = fC_s, \tag{3}$$

where f is a constant for a given medium.

From (1), (2) and (3) we have,

$$\left(\frac{dC}{dt}\right)_s = K(T_G - T_L), \tag{4}$$

where $K = \Phi A/f$.

For a culture we can write,

$$\begin{aligned}\text{(net rate of increase in dissolved oxygen)} &= \text{(rate of oxygen supply)} \\ &\quad + \text{(rate of oxygen uptake)};\end{aligned} \tag{5}$$

that is,

$$\frac{dC}{dt} = K(T_G - T_L) - N, \tag{6}$$

where N = oxygen uptake rate. In the steady state of a chemostat culture $dC/dt = 0$, and then,

$$T_L = T_G - N/K. \tag{7}$$

It follows from (7) that as long as N, the oxygen uptake rate of the culture, is constant, with constant aeration conditions (i.e. constant K) the plot of T_L against T_G will be a straight line with a slope of unity, and the line will cut the T_G axis at a point equal to N/K.

In the experiments to be described here it was not possible to control independently the dissolved oxygen tension since the required apparatus (MacLennan & Pirt, 1966) was developed subsequently. Different oxygen tensions (T_L) were obtained by varying the oxygen tension in the gas phase (T_G). This was done by mixing nitrogen with the air supply. The total gas flow rate and the stirring rate were kept constant so that the term K in equation (7) should be constant. Under such conditions any departure from a linear relation between T_L and T_G must be ascribed to a change in the oxygen uptake rate, N.

Dissolved oxygen measurement. Although polarographic techniques have been used for many years for the measurement of dissolved oxygen concentration in cell suspensions, at the beginning of the present work there was no oxygen electrode which was proved suitable for use over long periods (up to several weeks) as required in continuous cultures. Consequently we investigated several techniques for the purpose. The first electrode used in this work was the E.I.L. Bishop Electrode (E.I.L. Richmond, Surrey) described by Bishop (1959). The Bishop electrode was initially used with a 'Lectrona' oxygen analyser kindly supplied by the British Oxygen Company Limited, Medical Department, London, S.W.1. The Lectrona (Bracken, Glover & Longmuir, 1960) differs from the normal system of measuring the diffusion current produced by the electrode in that the electrode is used as a capacitor which is given a charge, and the rate of discharge of the electrode by oxygen is then measured. This system was found to be less suitable than was the measurement of diffusion currents. The zero current was very high (equivalent to 10 mm. Hg of O_2) and the calibration drift over a few days was large. When used to measure diffusion currents the Bishop electrode had a very low zero reading (equivalent to < 1 mm. Hg pressure of O_2) but the calibration still drifted. However, this drift in calibration became slight after the electrode had been in use for 7 days, and after this period the electrode could be used with greater confidence but it was less sensitive. When the electrode was set up, left for a week, then calibrated and used in a fermentation lasting 2 weeks, the maximum relative error due to drift was estimated to be $\pm 5\%$.

In the latter part of the present work a scaled-down version of the Mackereth oxygen electrode (Mackereth, 1964) was obtained from E.I.L. Richmond, Surrey. This electrode was adapted for insertion into a culture vessel by replacing the Perspex cap by a Perspex rod. The Mackereth electrode is much more sensitive than the Bishop electrode and the calibration was found to be stable for over 1000 hr continuous use in air-saturated solutions. A straight-line response was obtained up to over 160 mm. Hg O_2 (air-saturated solution), and the lower limit of sensitivity was about 0·2 mm. Hg of O_2.

Oxygen electrodes were sterilized by ethylene oxide according to the method used by Callow & Pirt (1956) for pH electrodes.

Continuous culture apparatus. The continuous culture apparatus used was of the chemostat type, similar to that used by Pirt & Callow (1958*a*). The 2-l. culture vessel was equipped with automatic pH control.

The percentage oxygen in the gas supply to the culture was varied by mixing nitrogen from a cylinder ('White-spot' nitrogen supplied by British Oxygen Company, Wembley, Middlesex) and air from a pressure line. The gas flow rates were adjusted by means of Flostats (G. A. Platon Limited, Croydon, Surrey), which would compensate automatically for any variation in back pressure in the culture vessel. It was necessary that the composition of the gas-mixture supplied to the culture should be maintained very constant. A Manostat pressure regulator (John Watson and Smith Limited, Leeds) was used to ensure that the air pressure applied to the Flostat was constant. The gas mixture was passed through a 1-l. mixing bottle to smooth out any small sudden variations in gas composition. The gas flow rates were measured by Rotameter flow meters (Rotameter Manufacturing Company, Croydon, Surrey).

Culture medium. The medium contained (amounts in g./l.): $MgSO_4.7H_2O$, 0·20; $ZnSO_4.7H_2O$, 0·001; $MnSO_4.4H_2O$, 0·001; $FeSO_4.7H_2O$, 0·005; $CuSO_4.5H_2O$,

0·001; $CaCl_2.6H_2O$, 0·005; EDTA, 0·25; KH_2PO_4, 6·0; $NaH_2PO_4.2H_2O$, 1·07. In experiments where growth was ammonium (nitrogen)–limited, ammonium sulphate, 0·6 g./l. was added. When glucose was the growth-limiting substance, ammonium sulphate, 2·5 g./l., was added to the medium. Glucose was fed to the culture separately in the amounts stated below. The reservoir glucose solution contained 200 g./l. for the experiments in which growth was nitrogen-limited, and 15 g./l. for experiments in which growth was glucose-limited. The salts solution was adjusted to pH 6·3 with NaOH when the final value was to be pH 6·0; for experiments at pH 7·4 the salts solution was adjusted to pH 7·0. The final pH was achieved in the culture vessel by automatic addition of either 2 N-NaOH or 2 N-H_2SO_4. The trace metal salts were dissolved separately in the EDTA solution with constant addition of NaOH to keep the pH value about 7·0. The salt solutions, the ammonium sulphate and the glucose were each autoclaved separately at 121° for 20 min.

Foam control. The apparatus included means for the automatic addition of antifoam at regular intervals (Pirt & Callow, 1958*b*). The antifoam used was polyglycol P200 (Dow Chemicals, R. W. Greef and Co. Ltd., London). Foaming occurred occasionally and with the appearance of foam the oxygen tension in the culture decreased rapidly. The addition of antifoam to the culture was equally undesirable as it was noted that at pH 6·0 the addition of antifoam caused an increase in oxygen tension of about 10 mm. Hg, even when there was no foam present. When antifoam was added to a culture at an alkaline pH (7.4), however, there was a sudden decrease in oxygen tension (about 5 mm. Hg) followed by a slow increase back to the original reading. The results reported here were obtained at periods when the culture was not foaming and antifoam had not been added.

Inoculum. The organism used was *Klebsiella aerogenes* NCIB 8017, obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen. A fresh ampoule of freeze-dried culture was used to inoculate a shake-flask containing 20 ml. medium. When maximum growth was obtained in the shake-flask it was used as an inoculum for the continuous culture.

Other cultural conditions. All experiments were done at $30 \pm 0{\cdot}5°$ The pH value was controlled to $\pm 0{\cdot}05$ pH units. On changing the conditions of culture, at least 4 volumes were allowed to flow through the culture vessel before samples were taken. At least two samples were taken for each steady state, one or more volumes being allowed to pass through the culture vessel between samples. Aeration was by the vortex method with a stirring rate of 1050 rev./min. unless otherwise stated. The stirrer was a vaned disc of 65 mm. diameter; vane height, 13 mm. above and 4 mm. below disc.

Treatment of samples. Samples were taken into a boiling tube standing in iced water to effect rapid cooling. The opacity of the sample was measured immediately. The organisms were removed by centrifugation and the supernatant fluid stored at 4° with a trace of mercuric chloride (one drop of a saturated solution/20 ml.) added as a preservative.

Analytical methods. The methods of determining dry weight of organism, glucose, butanediol, acetoin, ethanol, lactic acid, formic acid, and volatile acid, were as described by Pirt (1957) and Pirt & Callow (1958*a*). Acetic acid was determined by subtracting the formic acid present from the total volatile acid. Pyruvic acid was found to interfere with the lactic acid determination, but a correction was made for this, 1 mg. pyruvic acid giving the same colour reaction as 0·06 mg. lactic acid. When large

amounts of pyruvic acid were present in the sample a correction was made to the volatile acid; about 75% of the pyruvate distilled over in the first 200 ml. collected. Pyruvic acid was determined by the colorimetric method of Friedemann & Haugen (1943). The method of Koepsell & Sharpe (1951) for differentiating between pyruvic and α-ketoglutaric acids was used on selected samples. No α-ketoglutarate was found except in samples taken under anaerobic conditions at pH 7·4, where small amounts were detected. Ammonia was determined by the method of Conway (1957).

The opacity of the culture was not used as an estimate of the dry weight of organisms in it, as the relationship between dry weight and opacity may vary with the conditions of growth. However, the opacity reading provided a useful check as to whether the culture was in a steady state. The cell-carbon content was taken as 50·2% of the organism dry weight (Pirt, 1957).

Oxygen and carbon dioxide in the gas phase were determined with the Orsat apparatus as described by Pirt & Callow (1958*a*). Analyses were always made on duplicate samples. There was an unavoidable error in the readings because no allowance could be made for the difference in water-vapour content between the gas fed to the culture and the effluent gas. However, as both were measured under the same conditions, the relative error from this source cannot have exceeded 3% and was probably less than 1·5%. Towards the end of this work a paramagnetic oxygen analyser (Paramagnetic Oxygen Analyser Mark II, Servomex Controls Limited, Crowborough, Sussex) became available and was used in preference to the Orsat apparatus because of the greater speed and accuracy of the former; with it the oxygen content of the gas phase could be measured within ±0·06%. The partial pressure of oxygen in the gas phase over the culture was taken as equal to that in the effluent gas. The gas pressure in the vessel was less than 0·5 mm. Hg above atmospheric pressure.

RESULTS

The fate of glucose at different oxygen tensions

Glucose in excess. To study the effect of dissolved oxygen tension on a culture with glucose in excess the culture was grown at a growth rate of 0·20 hr^{-1} with nitrogen as the growth-limiting factor, and with excess glucose. The amount of glucose utilized was independent of that supplied provided that the supply was over 10 mg./ml. The culture was at pH 5·0. The fate of the glucose carbon at different oxygen tensions is given in Fig. 1*a*, *b*. The culture showed three different states according to different ranges of dissolved oxygen tension. The 'excess oxygen state', which occurred at dissolved oxygen tensions above about 15 mm. Hg, had the following characteristics. The rate of oxygen uptake by the culture was constant and independent of the oxygen tension. The dissolved oxygen tension fell linearly with the oxygen partial pressure in the gas phase in accordance with equation 7. About 95% of the carbon in the glucose utilized was accounted for in organisms, CO_2 and pyruvic acid, with the remainder as 2:3 butanediol and ethanol. It appeared that the fermentation end-products (butanediol, ethanol) could not be completely eliminated by excess oxygen conditions with glucose in excess. The respiratory quotient (R.Q.) with excess oxygen and excess glucose was 0·8. This low value is accounted for by the formation of pyruvate.

The second state, called the 'transition state' of the culture, was entered when the dissolved oxygen tension decreased below 15 mm. Hg; in this range the oxygen tension

fluctuated between the values of < 1 and 13 mm. Hg (Fig. 2). Although the oxygen supply was maintained constant for over 48 hr (about 13 generations) the fluctuations in the dissolved oxygen tension showed no sign of diminishing. With the medium used and specific growth rate 0·2 hr^{-1} the fluctuations in dissolved oxygen tension were apparently permanent. Decrease of the gas partial pressure of oxygen still further eliminated the fluctuations in the dissolved oxygen tension and the dissolved oxygen reading became indistinguishable from the zero reading (i.e. < 1 mm. Hg of O_2). Thus the transition state was characterized by the occurrence of oscillations in the dissolved oxygen tension.

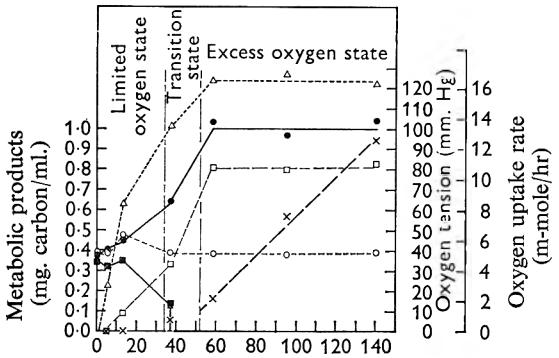

Fig. 1*a*

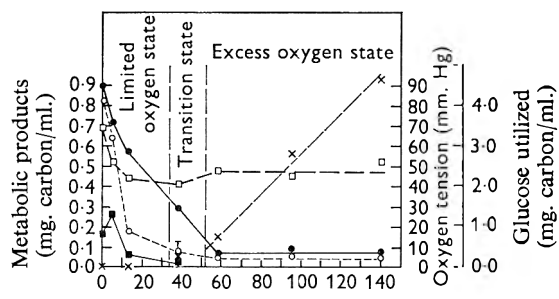

Fig. 1*b*

Fig. 1. *a,b*, *Klebsiella aerogenes* NCIB 8017. The fate of glucose at different oxygen tensions with nitrogen-limited growth and excess glucose, at pH 6·0. Dilution rate: 0·20 hr^{-1}.
(*a*) ×– –×, dissolved oxygen tension; △– –△, oxygen uptake rate. Products: ●—●, organism dry weight; ○– –○, CO_2; □– –□, pyruvic acid; ■—■ volatile acid (as acetic). I, denotes the amplitude of oscillations in O_2 tension.

(*b*) ×– –×, dissolved oxygen tension; □– –□, glucose utilized. Products: ●—●, 2:3 butanediol; ○– –○, ethanol; ■—■, lactate. I, denotes the amplitude of oscillations in O_2 tension.

When the oxygen supply was decreased below that which gave rise to the transition state, the third or 'limited oxygen state' was reached; here the dissolved oxygen was stable and indistinguishable from zero. Also in the limited oxygen state, the dry weight of organism and pyruvic acid production decreased with decrease in the oxygen supply, and the production of fermentation products butanediol, acetic acid, ethanol and lactate increased. It can be seen from Fig. 1*b* that butanediol production started to increase at a higher oxygen supply rate than did ethanol production. Acetoin production was very low, accounting for only 0·01 mg. carbon/ml. The decrease in the dry weight organism at the lower oxygen tensions may be attributed to a decrease in stored polysaccharide rather than in cell protein since it was shown that growth was still nitrogen-limited and therefore probably the bacteria were still synthesizing the same amount of protein.

A similar pattern of results in response to changes in oxygen supply was obtained at growth rates of 0·10, 0·40 and 0·50 hr^{-1}, but at the higher growth rates of 0·40 and 0·50 hr^{-1} the amounts of the products formed per ml. were much decreased. At a

growth rate of 0·10 hr^{-1} no unsteady transition state was found. However, it may be that, at such a low growth rate, the unsteady state extends over a much narrower range of oxygen supplies and so was missed in these experiments. At growth rates of 0·40 and 0·50 hr^{-1} the type of fluctuations found for the dissolved oxygen tension in the transition state (Fig. 3) differed from that found at 0·20 hr^{-1}. A more regular pattern was found at the higher growth rates and the amplitude of the oxygen tension oscillations was smaller.

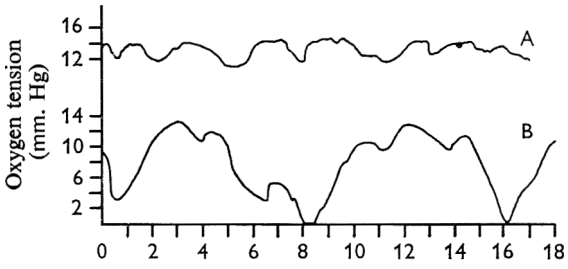

Fig. 2

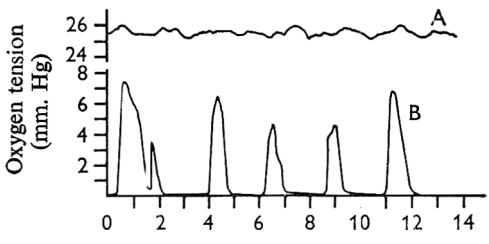

Fig. 3

Fig. 2. *Klebsiella aerogenes* NCIB 8017. Tracings of culture oxygen tension obtained with nitrogen-limited growth and excess of glucose at pH 6·0. Dilution rate 0·20 hr^{-1}. A, tracing obtained in the excess oxygen state. B, tracing obtained in the transition to the limited-oxygen state.

Fig. 3. *Klebsiella aerogenes* NCIB 8017. Tracings of culture oxygen tension obtained with nitrogen-limited growth and excess glucose at pH 6·0; dilution rate, 0·40 hr^{-1}. A, tracing obtained in the excess oxygen state. B, tracing obtained in the transition to the limited-oxygen state.

Effect of an alkaline pH value. The metabolism of *Klebsiella aerogenes* may alter considerably with changes in pH value of the culture (Mickelson & Werkman, 1938; Pirt & Callow, 1958*a*), and so a culture was grown with excess glucose, controlled at pH 7·4, to investigate the effect of dissolved oxygen at an alkaline pH value.

Again the metabolism fell into three states. In this case the transition state extended over a narrower range of dissolved oxygen tension. With a growth rate of 0·18 hr^{-1} the dissolved oxygen tension remained below 1 mm. Hg, but once in every 24 hr increased and fluctuated between 1·0 and 3·3 mm. Hg about once per hour for 6 hr and then decreased again to the steady value.

From Fig. 4 it can be seen that the pattern of metabolic end-products in the culture at pH 7·4 differed considerably from that obtained at pH 6·0. In the excess-oxygen state pyruvate production was considerably less than that obtained at pH 6·0 and substantial amounts of acetic acid were formed. The transition from excess oxygen to limited oxygen was marked by a large increase in acetic acid production and a steep decrease in organism dry weight. The production of butanediol, ethanol, lactate and formate also increased immediately the oxygen supply became limiting. The organism dry weight reached a minimum before the culture was fully anaerobic, and pyruvate production did not cease completely under anaerobic conditions at pH 7·4 as was the case at pH 6·0. Maximum butanediol and lactate production was reached, not under anaerobic conditions, but in the limited oxygen state. Acetoin was only detected in samples showing maximum amounts of diol, and then only accounted for 0·01 mg.

carbon/ml. Very large amounts of volatile acids were formed at pH 7·4 under oxygen-limited conditions. The volatile acid was mostly acetic acid with the higher oxygen supply, but with decreased oxygen supply formic acid production increased until it accounted for about half as much glucose carbon as the acetic acid, i.e. the number of molecules of formic acid and acetic acid produced were equal.

Glucose-limited growth. To investigate how much the phenomena observed were a result of glucose being in excess, cultures were made with glucose as the growth-limiting substance, and with an excess of ammonium sulphate as N source. The glucose feed was adjusted to give a supply of about 2·5 mg./ml., the culture controlled at pH 6·0 and the growth-rate kept at 0·16 hr^{-1}.

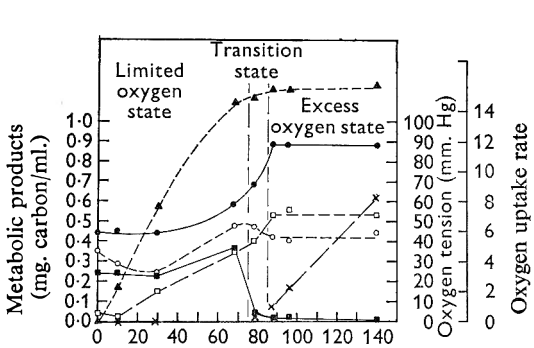

Fig. 4*a*

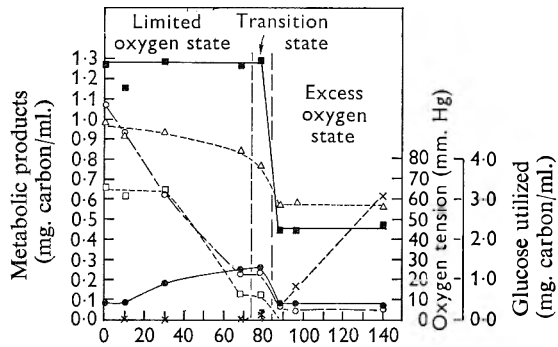

Fig. 4*b*

Fig. 4. *a*, *b*. *Klebsiella aerogenes* NCIB 8017. The fate of glucose at different oxygen tensions with nitrogen-limited growth and excess glucose at pH 7·4; dilution rate 0·18 hr^{-1}.

(*a*) ×– – –×, dissolved oxygen tension; ▲– – –▲, oxygen uptake rate. Products: ●—●, organism dry weight; ○– – –○, CO_2; □– – –□, pyruvic acid.

(*b*) ×– – –×, dissolved oxygen tension; △– – –△, glucose utilized. Products: ●—●, 2:3 butanediol; ○– – –○, ethanol; ■— ■, acetic acid; □– – –□, formic acid. I, denotes the amplitude of oscillations in O_2 tension.

The influence of oxygen tension on the fate of glucose is shown in Fig. 5. Again the excess oxygen, the transition and the limited oxygen states were apparent. In the excess oxygen state 95–100% of the glucose carbon was recovered as organisms and CO_2, and pyruvic acid did not accumulate. In keeping with the complete oxidation of glucose, the R.Q. was 1·02. In the limited oxygen state the organism dry weight and the CO_2 production decreased with the decreased oxygen supply and the glucose-carbon appeared instead in butanediol, ethanol and volatile acid. Acetoin was only detected when no oxygen was supplied to the culture; it then accounted for 0·07 mg. carbon/ml.

The transition state was characterized by continuous oscillations in the dissolved oxygen tension. As with excess glucose the oscillations occurred at oxygen tensions below about 15 mm. Hg (Fig. 6). The dropwise addition of glucose solution caused the oxygen tension to oscillate over a range of about 1 mm. Hg. These small oscillations were imposed on the major oscillations. Since the respiration rate was glucose-limited it increases and decreases slightly in rhythm with the drops of glucose solution. The pattern of oscillations in oxygen tension in the transition state with glucose-limited growth was much more irregular than was the case with excess glucose.

In Fig. 5 it is apparent that the respiration rate (q_{O_2}, expressed as m-mole O_2 consumed/g. organism dry wt/hr) increased by about 33% in the limited oxygen state. This may be taken to represent a true increase in the oxygen uptake rate per bacterium because, with glucose-limited growth, polysaccharide is not stored in the organism (Holme, 1957) and so the increase apparent in q_{O_2} cannot be explained by a decrease in the mass of individual bacteria.

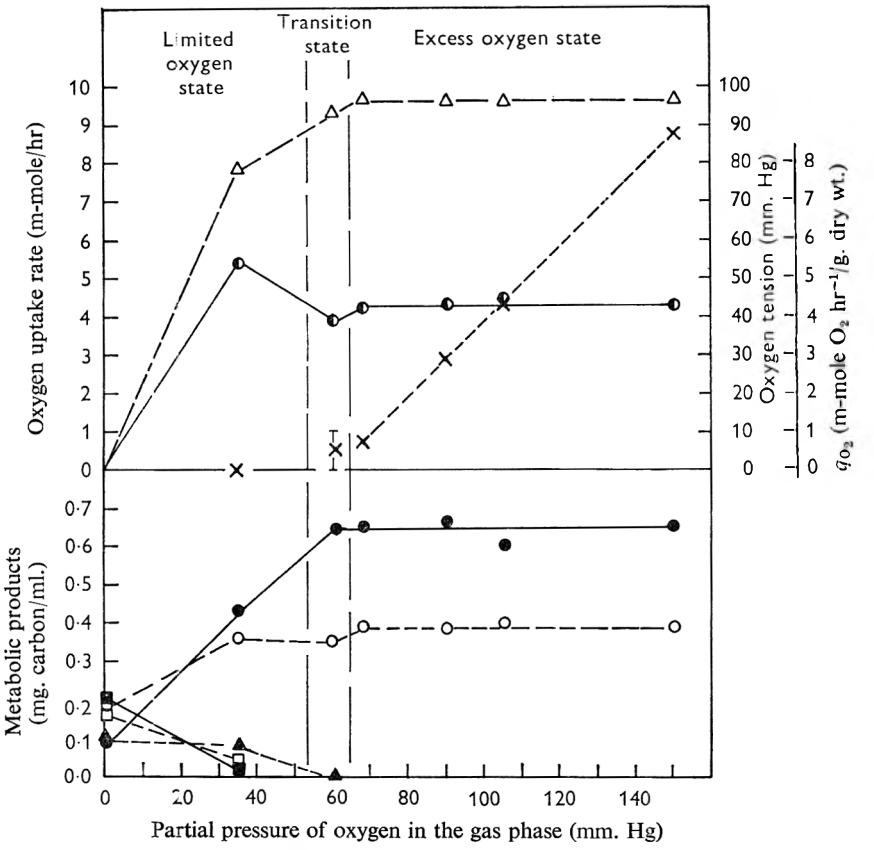

Fig. 5. *Klebsiella aerogenes* NCIB 8017. The fate of glucose at different oxygen tensions with glucose-limited growth at pH 6·0, dilution rate 0·16 hr^{-1}. Glucose concentration in entering medium 2·6 mg./ml. ×---×, dissolved oxygen tension: △---△, oxygen uptake rate; ◐—◐, respiration rate (q_{O_2}). Products: ●—●, organism dry weight; ○---○, CO_2; ■—■, 2:3 butanediol; □---□, ethanol; ▲---▲, volatile acid. I, denotes the amplitude of oscillations in O_2 tension. Glucose utilization was over 97% of that supplied, under all conditions.

The transition state

The possibility that the fluctuations in oxygen tension obtained during the transition phase were not caused by the growing organisms but were an artifact due, for example, to small variations in oxygen supply, was investigated as follows. The aeration conditions were simulated by the oxidation of a sulphite solution (Cooper, Fermstrom & Miller, 1944) in the culture vessel. The sulphite solution (0·17 M) containing $CuSO_4$ (0·0005 M) was fed into the culture vessel at a constant rate to give a constant oxygen uptake rate in the solution. The sulphite feed-rate was adjusted to decrease the dis-

solved oxygen tension to about 5 mm. Hg, that is, well within the transition state range of a culture. With the same stirring rate, gas flow rate and gas phase partial pressure of oxygen which gave oscillations in the culture it was not found possible to induce oscillations in the dissolved oxygen tension in the sulphite solution. The oxygen tension in the sulphite solution varied less than $\pm 0{\cdot}75$ mm. Hg. Since the oscillations in the dissolved oxygen tension in the transition state of a culture were up to ten times as great, they cannot be attributed to variation in the oxygen supply rate.

The meaning of changes in dissolved oxygen tension during the transition state. Although the patterns of fluctuations obtained during the transition phase varied with different conditions of growth, there were certain basic similarities between the patterns. The lowest dissolved oxygen tension reached in each case was close to zero. This was followed by a steep increase in the oxygen tension reading, which then remained high for a period before decreasing again. However, the maximum oxygen tension reached during the fluctuations, and the frequency and length of the peaks and troughs, varied considerably. From the similarities, and the fact that the fluctuations were only obtained during the transition from aerobic to anaerobic metabolism,

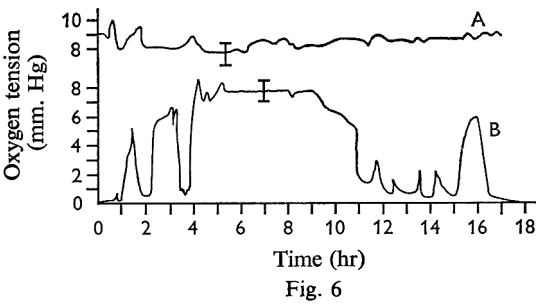

Fig. 6

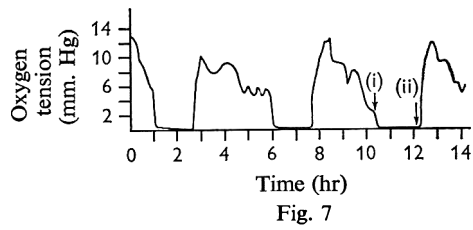

Fig. 7

Fig. 6. *Klebsiella aerogenes* NCIB 8017. Tracings of culture oxygen-tension reading, obtained with glucose-limited growth at pH 6·0; dilution-rate 0·16 hr^{-1}. A, tracing obtained in the excess oxygen state. B, tracing obtained in the transition to the limited oxygen state. $\mathbb{I}$, indicates the amplitude of oscillations (of frequency 45/hr) superimposed on these tracings, which were caused by the drop-wise addition of glucose.

Fig. 7. *Klebsiella aerogenes* NCIB 8017. Tracing of culture oxygen-tension readings obtained with nitrogen-limited growth and glucose in excess at pH 6·0, dilution-rate, 0·20 hr^{-1}. Points (i) and (ii) are referred to in Table 2.

it would seem that, in spite of the differences in the actual form of the fluctuations, the states of flux obtained were all homologous.

The phenomenon of an unsteady state between aerobic and anaerobic metabolism was further investigated in the following way. A transition state was obtained in a culture with a growth rate of 0·20 hr^{-1} at pH 6·0 with glucose in excess. The oxygen tension in the culture was followed by using a Mackereth electrode. Figure 7 shows the tracing obtained. Another Mackereth electrode was used to follow the changes in oxygen partial pressure in the effluent gas. This electrode was inserted into a rubber tube (25 mm. diameter) joined to the effluent gas pipe-line from the culture, so that the effluent gas flowed out around the membrane. There was no temperature-compensating device with this system, but it was found, by using a temperature recorder, that there were no sudden changes of room temperature and a steady recording could be obtained.

The oxygen uptake rate in this experiment was measured by means of the paramagnetic oxygen analyser.

It was thus found that every increase in oxygen tension recorded in the culture corresponded to an increase in oxygen partial pressure in the effluent gas, and every sudden decrease in oxygen tension recorded in the culture corresponded to a sudden decrease in oxygen partial pressure in the effluent gas. An increase in oxygen partial pressure in the effluent gas accompanied by an increase in the dissolved oxygen tension must reflect a decrease in the oxygen uptake rate of the culture. A decrease in oxygen partial pressure in the gas phase accompanied by a decrease in the dissolved oxygen tension must be the result of an increase in the oxygen uptake rate of the culture.

Table 1. *Klebsiella aerogenes* NCIB *8017. Comparison between oxygen uptake rates in different aerobic states*

Growth rate 0·20 hr^{-1}, pH 6·0, glucose in excess.

Culture state	Oxygen in gas supply (%)	Dissolved oxygen tension (mm. Hg)	Oxygen uptake rate (m-mole/hr)	Number of samples
Excess oxygen	17·0–20·9	27·0–70·0	16·5 ± 0·34	5
Transition (at high oxygen tension in cycle)	16·2	11·0–13·0	15·15 ± 0·26	2
Transition (at low oxygen tension in cycle)	16·2	< 0·2	17·65 ± 0·21	2

Table 2. *Klebsiella aerogenes* NCIB *8017. Comparison between the amounts of growth and* CO_2 *production in the excess oxygen state and in the transition to the limited oxygen state*

Excess glucose present, pH 6·0, dilution rate 0·2 hr^{-1}.

Culture state	Organism dry weight carbon (mg/ml.)	CO_2-carbon (mg./ml.)
Excess oxygen	1·09 ± 0·06	0·39 ± 0·02
Transition at high dissolved oxygen tension (about 5 mm. Hg, point i in Fig. 7)	1·15 1·11	0·37 0·39
Transition at low dissolved oxygen tension (< 0·2 mm. Hg; point ii in Fig. 7)	0·89 0·83	0·50 0·54

Table 1 shows the results of measurements of oxygen uptake rates taken at various points in the transition state. It can be seen that the oxygen uptake rate was significantly higher when the oxygen tension had decreased to the minimum, than when the oxygen tension was at the maximum of the cycle. Also it can be seen from Table 1, that when the culture was in the transition state and the oxygen tension was at the minimum, the oxygen uptake rate was significantly higher than that obtained with excess oxygen. Since the organism dry weight fell about 20% in the transition state (Table 2) and the amount of nitrogen taken up by the organisms was the same or slightly less, the increased oxygen uptake cannot be attributed to increase in cell mass or cell nitrogen.

Further evidence of an increase in the respiration rate when the oxygen tension of the culture decreased to the minimum in the transition state is shown in Fig. 8. With dissolved oxygen tensions above about 12 mm. Hg the relation between the oxygen tensions in the liquid and the gas phases was linear, in accordance with equation 7. However, some of the values of the dissolved oxygen tension during the transition phase lay below the extrapolated straight line. Since the aeration conditions were constant (K in equation 7 was constant) the increased slope of the line must indicate an increase in the oxygen uptake rate (N).

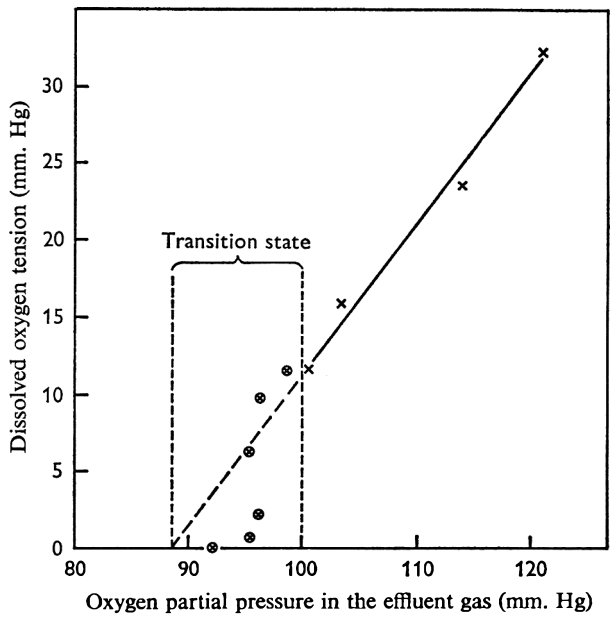

Fig. 8. Dissolved oxygen tension as a function of the partial pressure of oxygen in the gas phase. ×, oxygen tension readings during the excess oxygen state. ⊗, oxygen readings during the transition to the limited oxygen state.

The fate of glucose in the transition phase when the oxygen tension was high and when at the minimum (points i and ii in Fig. 7) was determined and compared with that in the excess oxygen state. The pyruvate production was the same, and there were slight increases in the amounts of butanediol, ethanol and volatile acid in the transition state. The only marked differences were in organism dry weight and CO_2 production; these are shown in Table 2. In the transition state when the oxygen tension was minimal, the organism dry weight decreased and CO_2 production increased greatly, although the glucose utilization did not change. Thus in the transition phase increased respiration rate was accompanied by an increased CO_2 production rate and a decrease in the yield of organism/g. glucose utilized.

Considering the large change in CO_2 production during the transition phase, it seemed possible that some of the changes occurring might be caused by changes in CO_2 partial pressure. To test this the partial pressure of CO_2 in the gas phase was increased by feeding 5% CO_2 into the gas supply and adjusting the nitrogen flow to keep the oxygen content the same. The increase in CO_2 partial pressure (about threefold) had no detectable effect on the fluctuations in oxygen tension.

Response to induced changes in oxygen tension in the transition state. Experiments were made to find whether the changes in oxygen uptake rate in the transition phase were a direct response to changes in the dissolved oxygen tension. Changes in dissolved oxygen tension were brought about by altering the percentage of oxygen in the gas supply until the desired oxygen tension was reached, when the oxygen supply was returned to its original value. The experiments were repeated with alteration of the oxygen tension by changing the rate of flow of gas through the culture, to check that the organisms were not responding to nitrogen partial pressure. The results were the same in each case.

When the dissolved oxygen tension reading was in the 'high' part of the cycle (i.e. the oxygen uptake rate was low) it was found that decreasing the dissolved oxygen tension to 6 mm. Hg had no effect on the oxygen uptake rate. When the oxygen feed was returned to its previous value, the dissolved oxygen tension increased again. However, when the dissolved oxygen tension was decreased to 4 mm. Hg, it did not increase again on returning the oxygen supply to its previous value, but continued to decrease until it reached the zero reading. The oxygen uptake rate had increased. The oxygen tension then remained low for about 1·5 hr before showing a sudden spontaneous increase to about 13 mm. Hg, like that found in the cycle of fluctuations shown in Fig. 7. This response was obtained at any point on the 'high' part of the cycle of oxygen tension fluctuations, whether the oxygen tension had just increased from zero or whether it had remained high for more than 2 hr. It was noticed, however, that the longer the culture was maintained in the fluctuating state the lower was the threshold oxygen tension at which the respiration rate was stimulated. Initially the threshold was about 4·0 mm. Hg of O_2, but after 24 hr it was about 2·0 mm. Hg. The same trend can be seen in Fig. 7, where in the first fluctuation, the sudden decrease in oxygen tension occurred when the reading had reached about 4·0 mm. Hg, but in the third cycle the threshold was closer to 2·0 mm. Hg.

When the dissolved oxygen tension was in the 'low' part of the cycle of fluctuations, that is, the oxygen uptake rate was stimulated, increasing the dissolved oxygen tension to 5 mm. Hg had no effect on the oxygen uptake rate. When the oxygen supply was returned to its previous value the oxygen tension decreased again. When the dissolved oxygen tension was increased to 10 mm. Hg, however, the oxygen tension did not decrease again on returning the oxygen supply to its previous value, but continued to increase; it increased to 17 mm. Hg and remained at this value, that is, the culture entered the 'high' part of the cycle. Hence the respiration rate was partially inhibited by an increase in the dissolved oxygen tension to 10 mm. Hg.

The spontaneous increase in oxygen tension during the 'low' part of the cycle might be attributed to a continued decrease in the actual oxygen tension which the electrode was not sensitive enough to show; then, when the oxygen tension reached a certain value below the sensitivity of the instrument (about 0·2 mm. Hg) the organism responded by decreasing the respiration rate. To test this possibility when the culture was in the 'low' part of the cycle (i.e. while the respiration was in the stimulated state and the oxygen tension reading was minimal) the oxygen supply to the culture was decreased temporarily. This was done by stopping the air supply to the culture and feeding pure nitrogen for a few minutes. The oxygen supply was then returned to its previous value. There was no apparent decrease in the oxygen demand of the culture; the oxygen tension remained low after the partial pressure of oxygen in the effluent

gas was restored to its previous value. This procedure was repeated at various points on the 'low' part of the cycle, always with no response from the culture. Therefore, it was concluded that the inhibition of oxygen uptake rate which occurred spontaneously in the cycle was not an immediate response to a decrease in dissolved oxygen tension to a very low critical value, below the sensitivity of the electrode.

Interpretation of the fluctuations in oxygen tension. From the observations in the above experiments, the fluctuations shown in Fig. 7 can be interpreted as follows. When the dissolved oxygen tension decreased below a certain threshold value (about 5 mm. Hg) the culture responded by increasing the respiration rate so that there was a sudden fall in dissolved oxygen tension. The increased oxygen uptake rate was maintained for about 1·5 hr and was accompanied by a decrease in organism production and an increase in CO_2 production. When the dissolved oxygen tension had been at the minimum for about 1·5 hr there was a sudden reversion to the lower respiration rate, causing a rapid increase in dissolved oxygen tension. It is postulated that this reversion to the lower respiration rate was caused by the build-up of an inhibitor. The inhibitor quickly disappeared once the oxygen tension increased, because under these conditions decreasing the dissolved oxygen tension below the threshold value of about 5 mm. Hg caused the culture to take up oxygen at the higher rate again. When the dissolved oxygen tension increased to about 13 mm. Hg the oxygen uptake rate was at first a little lower than that obtained under fully aerobic conditions, because the organism dry weight decreased while the oxygen tension was low. The slow decrease in dissolved oxygen tension which followed was probably caused by the increase in organism dry weight so that the oxygen uptake rate increased slowly.

Although this interpretation was deduced from a regular pattern of fluctuations such as was obtained in Fig. 7 the same principles can account for the more irregular types of fluctuations obtained in the transition phase. The fact that the pattern of the fluctuations varied from one culture to another, even when the conditions of growth were apparently similar (as in Fig. 2 and 7), suggests that the past history of the culture has an influence on its behaviour in the transition phase. In the case illustrated in Fig. 7, the culture had not been kept growing in the excess oxygen phase as long as in the experiment depicted in Fig. 2.

DISCUSSION

In response to changes in dissolved oxygen tension the continuous culture of *Klebsiella aerogenes* NCIB 8017 exhibited three states. In the excess oxygen state obtained at oxygen tensions above 10–15 mm. Hg the end-products of glucose metabolism and the respiration rate were independent of dissolved oxygen tension. In the transition state the oxygen uptake rate of the culture varied although the aeration conditions were constant. The varying oxygen uptake rate caused the dissolved oxygen tension to oscillate within the range $< 0{\cdot}2$–15 mm. Hg, the range of the fluctuations seeming to depend upon the conditions and the history of the culture. In the limited-oxygen state the dissolved oxygen tension decreased to < 1 mm. Hg and the oxygen uptake rate of the culture was dependent on the oxygen supply rate. Pirt (1957) characterized three phases in the metabolism of *Aerobacter cloacae*: a fully aerobic, a partially aerobic and an anaerobic phase. The present observation of an unsteady state in the transition between a fully aerobic and a partially aerobic metabolism is new.

In the excess oxygen state when the growth of *Klebsiella aerogenes* was glucose-limited, all the glucose carbon could be accounted for as organisms and carbon dioxide, and the R.Q. was 1. However, when growth was nitrogen-limited and glucose was supplied in excess, a large part of the glucose utilized was converted into pyruvate and into material (probably glycogen) stored in the organisms; in this state the R.Q. was 0·8. It is interesting to note that small amounts of fermentation products (butanediol, ethanol) were found in the excess oxygen state when glucose was in excess, although, since the amounts were small, it might be argued that in spite of the immediate cooling of samples the products could have been formed after the sample had been removed from the culture, when the oxygen supply was cut off. In the limited oxygen state, fermentative pathways began replacing the aerobic oxidation of glucose as the major energy-generating mechanism.

The production of formic and acetic acids in equimolar amounts under anaerobic conditions at pH 7·4 would seem to confirm suggestions by other workers (Wood, 1961; Hadjipetrou, Gerrits, Teulings & Stouthamer, 1964) that the thioclastic cleavage of pyruvate occurs in Aerobacter. The breakdown of formic acid to H_2 and CO_2 is suppressed at an alkaline pH as reported by Blackwood, Neish & Ledingham (1956). Wood (1961) suggested that the ethanol formed by Aerobacter is produced from the acetyl phosphate or acetyl CoA which is produced by the cleavage of pyruvate. If this were so, then the number of molecules of formic acid produced at an alkaline pH by *Klebsiella aerogenes* should equal the number of molecules of acetic acid + ethanol formed. It can be seen from Fig 4*b* that a large amount of ethanol was formed under anaerobic conditions at pH 7·4, although formic and acetic acids were formed in equimolar amounts. Thus it would seem that ethanol was not produced via the thioclastic cleavage of pyruvate under these conditions.

The experiments reported here are concerned with the change from growth in presence of excess oxygen to growth with limited oxygen. The reverse change, from steady-state anaerobic to aerobic growth, was not studied because it was found that when the culture was kept anaerobic for about 10 or more generations the change to aerobic growth took several days (20 or more generations) to reach a steady state; this suggests that complex adaptive changes or selection of variants were involved.

The concept of 'critical oxygen tension' as the tension below which the oxygen uptake rate of an organism decreases with a decrease in oxygen tension, has been widely quoted by workers since its introduction by Gerard & Falk (1931). However, from the work of Moss (1956) and White (1963) there are indications that growing organisms may not respond in such a straightforward manner at low oxygen tensions. This has been confirmed in the present work, where it has been shown that the response of *Klebsiella aerogenes* to decreased oxygen tension may involve an increase in respiration rate rather than a decrease. A modified definition of 'critical' oxygen tension to meet the new situation would be: the oxygen tension above which the respiration rate of an organism is independent of changes in dissolved oxygen tension; below this tension the oxygen uptake rate of the organism may increase or decrease in response to a decrease in oxygen tension, according to cultural conditions.

From the interpretation of the fluctuations in oxygen tension obtained in the transition phase, it would seem that a growing culture of *Klebsiella aerogenes* may show two types of aerobic respiration: (i) at oxygen tensions above the 'critical' value the oxygen uptake rate of *K. aerogenes* is constant and independent of oxygen tension; (ii) when the

oxygen tension is decreased below the 'critical' value the organism at first takes up oxygen at a higher rate and converts more glucose to CO_2 and less to organism.

The actual value of the critical oxygen tension in the cultures investigated here varied with cultural conditions from 2 to about 10 mm. Hg. Induction of respiratory enzymes at low oxygen tensions may be involved. However, in the case studied here the response to a decrease in oxygen tension below the 'critical' value was so very rapid (less than 5 min.) that it seems doubtful whether the simple induction of enzymes could explain this. Similarly, when the oxygen tension was increased above the 'critical' value the culture immediately reverted to the lower oxygen uptake rate.

It may be thought that there was some connexion between the oscillations in respiration rate and the generation time of the organism possibly involving synchronization of cell division. This would be ruled out, firstly because, even when the oscillations in oxygen tension were fairly regular the period of the oscillations was always much greater than the generation time of the organisms; and secondly because the rise and fall in the respiration rate occurring with dissolved oxygen concentration approximately in the range 0–10 mm. Hg could be produced at will, irrespective of the previous history of the culture.

Many workers have shown that it is the rate of transfer of electrons to the terminal oxidase which limits the oxygen uptake rate under fully aerobic conditions (Winzler, 1941; Chance, 1952; Longmuir, 1957), so that changes in the terminal oxidase system alone would not be expected to increase the respiration rate. If the electron transport chain were the rate-limiting factor for fully aerobic respiration, then an increase in respiration rate at low oxygen tensions might be brought about by the functioning of an alternative pathway for electron transport.

In recent years there have been reports indicating that ADP and ATP concentrations are important in the control of glucose utilization (Barker, Khan & Solomos, 1964; Maitra, Estabrooke & Chance, 1963) and of electron transport systems (Chance, 1961). It is tempting to propose that the stimulation of respiration in *Klebsiella aerogenes* by a low oxygen tension is caused by a decreased ATP yield which accompanies either a change to an alternative electron transport pathway or the uncoupling of oxidative phosphorylation. Uncoupling of phosphorylation similarly can cause more rapid electron transfer and oxygen uptake (Racker, 1965).

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Kinetic Aspects of the Growth of *Klebsiella aerogenes* with Some Benzenoid Carbon Sources

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SUMMARY

The aerobic growth of *Klebsiella aerogenes* was studied in chemically defined media consisting of mineral salts (ammonium as N source) and various benzenoid compounds singly as sole source of carbon + energy. A strain adapted to a defined glucose mineral salts (ammonium as N source) medium grew readily in concentrations of benzaldehyde, benzoate, *p*-hydroxybenzoate or phenylacetate of the order 10^{-3} M to 10^{-2} M. The initial growth rate in benzoate, *p*-hydroxybenzoate or phenylacetate increased and then decreased with increasing concentration of benzenoid compound, according to a relationship which is interpreted by analogy with enzyme inhibition by excess substrate. The kinetics of growth inhibition by the above compounds in glucose salts medium were similar to those in the simplest case of inhibition of an enzyme or heterogeneous catalyst. During repeated subculture in benzoate, *p*-hydroxybenzoate or phenylacetate as sole carbon source, the bacteria showed various adaptive responses which include an immediate decrease in the lag and an increase in the growth rate. As the concentration of benzoate to which the bacteria had become adapted to utilize as sole carbon + energy source was increased, the molar growth yield and the growth rate changed in a related manner and the inhibitory action of benzoate in glucose salts medium decreased.

INTRODUCTION

Klebsiella aerogenes can utilize a variety of carbon + energy ($C+E$) sources and in doing so often shows adaptive responses (see Hinshelwood, 1946; Baskett & Hinshelwood, 1950) which can be interpreted on the basis of kinetic principles (Hinshelwood, 1946; Dean & Hinshelwood, 1963, 1964). The purpose of the work to be described was to investigate certain kinetic aspects of the growth of *K. aerogenes* in chemically defined media containing a benzenoid compound as sole $C+E$ source; ammonium was the N source.

METHODS

Organism. The organism used was *Klebsiella aerogenes* NCTC 418 (*Aerobacter aerogenes*) originally obtained from Professor Sir Cyril Hinshelwood's laboratory.

Apparatus. Each culture was contained in a sterile Pyrex 150 mm. boiling tube of 25 mm. diam. sealed with a sterile cotton-wool plug and maintained at 40·0° in a water bath. Through the cotton-wool plug passed a sterile 1 ml. graduated pipette for withdrawing samples of culture and a sterile Pasteur pipette through which filtered

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air was passed for aeration and agitation. All glassware was thoroughly washed before use.

Media. Each culture developed in 26 ml. medium which was prepared by adding to the culture tube 16 ml. of a sterile solution of salts and 10 ml. of a sterile solution of the $C+E$ source. These solutions were prepared from glass-distilled water and 'AnalaR' grade reagents or recrystallized laboratory reagents. The salts solution consisted of: Na_2HPO_4, 3·91 g./l.; KH_2PO_4, 1·83 g./l.; $(NH_4)_2SO_4$, 1·54 g./l.; $MgSO_4.7H_2O$, 61·7 mg./l.; $FeSO_4.7H_2O$, 0·16 mg./l.; pH 7·12. The solution of the $C+E$ source was either a glucose solution (50 g./l.) or a solution of a benzenoid compound or a solution of a benzenoid compound + glucose (50 g./l. unless otherwise stated). Solutions of carboxylic acids were neutralized to pH 7·12 with sodium hydroxide. In naming the medium after the predominant species, the carboxylate anion (> 99·7 mole %), there is no intention of implying that the anion is the biologically active species. Indeed, the un-ionized molecule of a weak acid is often more active than the corresponding anion in inhibiting the functions of bacteria (Cruess & Richert, 1929; Goshorn & Degering, 1938) and of yeasts, fungi, protozoa and other organisms (for a discussion, see Albert, 1965). Since the media were buffered to the same pH value in all experiments, the concentrations of the active species in a particular medium were constant fractions of the total molar concentration throughout those phases of growth during which measurements were made. Therefore, no matter what the active species were, the conclusions drawn from the experiments on the inhibitory action of benzoate, *p*-hydroxybenzoate and phenylacetate are not invalidated.

Growth was normally terminated by accumulation of toxic waste products, change in pH value and shortage of oxygen. When it was considered desirable that the lag on subsequent subculture should be as short as possible, exhaustion of glucose was arranged to limit growth. The medium then contained only 0·8 ml. of the glucose solution (50 g./l.) instead of 10 ml. and is referred to as the limited-glucose medium.

Media containing benzenoid substrates were inoculated with a sufficient volume of bacterial suspension to give a defined initial bacterial concentration in the whole medium (see later). The inoculum was either taken directly from a limited-glucose culture or else consisted of a suspension in phosphate buffer of bacteria which had been taken from the same culture and washed twice by centrifugation with phosphate buffer; this buffer (pH 7·12) contained Na_2HPO_4 6·34 g./l. and KH_2PO_4 2·96 g./l.

The organism used in these experiments had previously been grown only in meat extract media and in the glucose medium, to which it was fully adapted by repeated subculture. Subculture of bacteria into fresh medium was made daily unless otherwise stated. One subculture corresponded to about 7 generations.

Measurement of bacterial concentration. The bacterial concentration (M) was determined turbidimetrically by means of a Hilger 'Spekker' photoelectric absorptiometer calibrated in terms of the total number of bacteria in a sample taken from a culture in glucose medium just after the end of the logarithmic phase. The bacteria were counted in a Helber counting chamber under a microscope. The M value of a bacterial suspension is thus expressed as the equivalent number of standard-sized living and dead bacteria/10^{-6} ml. which would give the same turbidity as the suspension under consideration. The dry weight of bacteria in 1 ml. of a suspension of unit bacterial concentration ($M = 1·0$) was 0·48 μg.

All cultures were initiated with a standard inoculum ($M = 3·0$). At various times

samples were removed from each culture and immediately placed in the 0·5 ml. microcell of the absorptiometer for the determination of M. From the plot of log M against the time after inoculation, the lag and the mean generation time of the culture were calculated. Lags less than about 40 min. were considered to be negligible.

RESULTS

Benzenoid compounds as sole carbon+energy sources

The original glucose-adapted strain was tested for its ability to grow in media containing various benzenoid compounds as sole carbon+energy ($C+E$) source. For this, media containing a given $C+E$ source at concentrations from 0·03 to 4 g./l. were inoculated with sufficient unwashed exhausted limited-glucose culture to give the standard inoculum. This was repeated from 2 to 4 times for each benzenoid $C+E$ source. Growth occurred with benzaldehyde, benzoate, *p*-hydroxybenzoate and phenylacetate but not with *o*-hydroxybenzoate, cinnamate, mandelate, benzilate, acetophenone, phenol or resorcinol. Catechol and quinol were also tested but the results were indefinite because these compounds were rapidly oxidized by the air used for aerating the cultures.

There exists the possibility that the compounds which did not support growth under the conditions of the previous experiment might begin to do so after glucose had been used up from a medium containing glucose+benzenoid compound. As a test for this, bacteria adapted to glucose were subcultured in media containing glucose +benzenoid compound and over 8 subcultures, the glucose concentration was decreased stepwise from 1·54 g./l. to zero, while the concentration of the benzenoid compound was simultaneously increased stepwise. The maximum bacterial concentration (M_s) in the stationary phase of each culture was determined. After growth in the presence of cinnamate, mandelate, benzilate, acetophenone, resorcinol or *o*-hydroxybenzoate, the values of M_s were never significantly greater than the corresponding values in glucose alone, which confirms that these benzenoid compounds did not support growth. Phenol at 0·077 g./l. or greater completely inhibited growth under the conditions described.

Initial growth in benzoate, p-hydroxybenzoate and phenylacetate media

Initial growth in media containing benzoate, *p*-hydroxybenzoate or phenylacetate as sole $C+E$ source was slower than in the glucose medium and was preceded by a lag. In benzoate medium the lag always exceeded 6 hr but was very variable. Indeed, with benzoate at $1{\cdot}99\times10^{-2}$ M or greater growth often did not occur, whereas with $1{\cdot}58\times10^{-2}$ M or less growth always occurred after lags which ranged from 6·4 hr to 187 hr in parallel tests and which appeared to bear little relation to the concentration.

With *p*-hydroxybenzoate and in phenylacetate the initial lag was less variable than with benzoate and tended to increase with concentration. The initial lags with phenylacetate, washed and unwashed inocula are shown in Fig. 1; those with *p*-hydroxybenzoate were between 1 and 4 hr in the range $1{\cdot}39\times10^{-4}$ M to $5{\cdot}57\times10^{-2}$ M, and 15–30 hr in the range from $8{\cdot}35\times10^{-2}$ M to $1{\cdot}11\times10^{-1}$ M; no growth occurred in $1{\cdot}39\times10^{-1}$ M or greater. The data just given for *p*-hydroxybenzoate refer to unwashed inocula. In general, in all media washing the bacteria of the inocula led to longer lags.

The mean generation time (T) during initial growth in a given concentration of benzoate was variable but less so than the lag. T for a given concentration of *p*-hydroxybenzoate or phenylacetate was somewhat less variable than for benzoate. For each $C+E$ source and at each concentration the average value of T over several experiments is plotted against concentration of $C+E$ source in Fig. 2. The results show essentially the same pattern for different $C+E$ sources. As the concentration was increased from small values, T decreased to a minimum and then increased again as the concentration of $C+E$ source was further increased. This increase in T indicated

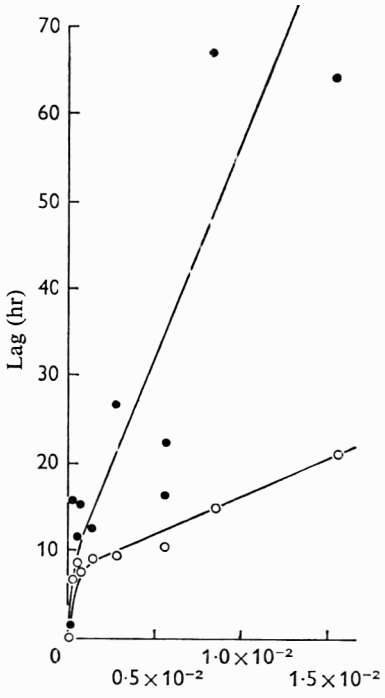

Fig. 1

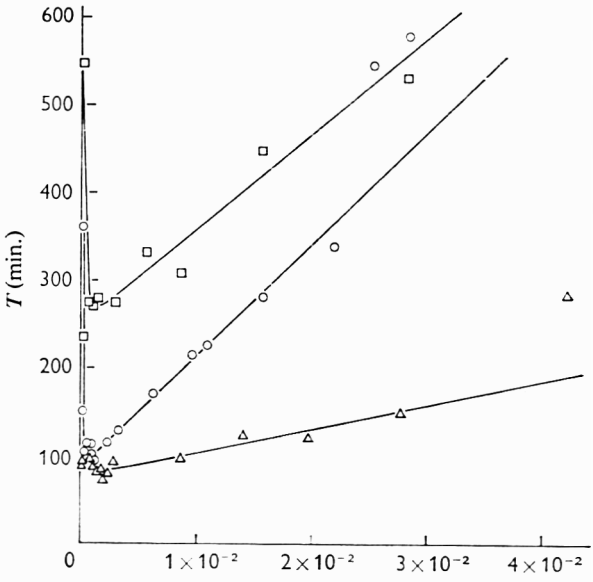

Fig. 2

Fig. 1. Lags preceding initial growth of unwashed (○) and washed (●) *Klebsiella aerogenes* from the original glucose-adapted strain in a chemically defined medium containing various concentrations of phenylacetate as sole $C+E$ source. (In $2{\cdot}83\times10^{-2}$ M-phenylacetate, lag = 135 hr, ∞ in separate experiments.)

Fig. 2. Relationships between T of the original glucose-adapted strain of *Klebsiella aerogenes* and the molar concentration (c) of the following benzenoid compounds, provided as sole $C+E$ source: ○, benzoate; △, *p*-hydroxybenzoate; □, phenylacetate. (In $5{\cdot}57\times10^{-2}$ M-*p*-hydroxybenzoate, T = 470 min.; in $8{\cdot}35\times10^{-2}$ M-*p*-hydroxybenzoate, T = 630 min.; in $1{\cdot}11\times10^{-1}$ M-*p*-hydroxybenzoate, T = 1280 min.)

that the $C+E$ source was then acting as an inhibitor of growth. In moderate concentrations of each $C+E$ source T was an approximately linear function of the concentration, thus, $T = a+bc$, where a and b are constants for a given $C+E$ source and where c is the molar concentration of the $C+E$ source (Fig. 2). In higher concentrations of benzoate or *p*-hydroxybenzoate (exceeding about 2·5 to $3{\cdot}0\times10^{-2}$ M) T increased more and more rapidly with c than is suggested by the linear relationship.

Serial subculture in benzoate as sole carbon+energy source

The original glucose-adapted strain was subcultivated repeatedly in media containing certain chosen concentrations of benzoate as sole $C+E$ source. After the first subculture in benzoate the lag was never more than 100 min. and during repeated subculture T tended to decrease to an approximately constant minimum value. Whenever the organism was grown in glucose after being subcultured in benzoate, the lag was never more than 60 min. and T remained at its optimum value of 29–33 min.

The original glucose-adapted strain readily became adapted to $9{\cdot}45 \times 10^{-4}$ M and $3{\cdot}15 \times 10^{-3}$ M-benzoate but with $9{\cdot}45 \times 10^{-3}$ M and above it did not grow after 2 or 3 successive subcultures. When trained to $3{\cdot}15 \times 10^{-3}$ M-benzoate it readily became adapted to $9{\cdot}45 \times 10^{-3}$M, and when trained to $9{\cdot}45 \times 10^{-3}$M it readily became adapted to $2{\cdot}20 \times 10^{-2}$M.

When T had become minimal during subcultivation in the chosen concentration of benzoate, adaptation to that concentration was judged to have occurred. Growth curves were then plotted for growth in a number of different concentrations of benzoate, and in the glucose medium as a control. The variation of T with the concentration of benzoate as sole $C+E$ source was of a similar pattern for the various strains trained to benzoate to that for the untrained strain, with the exception that the linear relationship at moderate concentrations of benzoate broke down more and more as the training concentration increased, showing that the adaptive response was rather complex. For example, in the presence of moderate concentrations of benzoate ($0–3 \times 10^{-2}$ M), a linear relationship was again found with strains which had been previously trained to benzoate $9{\cdot}45 \times 10^{-4}$ M and $3{\cdot}15 \times 10^{-3}$ M. With strains trained to higher concentrations of benzoate, however, the relation between T and c was more complex (Table 1).

Inhibitory effect of certain benzenoid compounds on growth in glucose medium

The inhibitory actions of *p*-hydroxybenzoate, phenylacetate and benzoate on growth were studied in isolation by plotting growth curves of the organism during incubation in the standard glucose medium to which known amounts of benzenoid compound were added before inoculation.

At low concentrations of *p*-hydroxybenzoate or phenylacetate the lag was negligible (40 min.), but at higher concentrations the lag increased more and more rapidly with increasing concentration (e.g. Fig. 3). Similar variations in the lag with the concentration of other inhibitors have been found (e.g. Hinshelwood, 1946). On the other hand, the lag preceding growth in the glucose medium + benzoate at a range of concentrations ($0–5{\cdot}85 \times 10^{-2}$ M) remained negligible.

In glucose medium containing moderate concentrations of benzoate, *p*-hydroxybenzoate or phenylacetate T increased with the concentration of the benzenoid compound according to the general linear relationship, $T = a+bc$, described previously (see Figs. 3, 4; Table 1). The relationship again broke down when the concentration of the inhibitor was high, i.e. when the benzoate exceeded 3×10^{-2} M and the *p*-hydroxybenzoate $8{\cdot}5 \times 10^{-2}$ M.

The inhibitory action of benzoate on growth in the glucose medium of several strains trained to grow in various concentrations of benzoate as sole $C+E$ source was also investigated. The lag preceding growth in the presence or absence of benzoate was

negligible and the variation in T was very similar in form to that for the untrained strain (Fig. 4). The gradient of the line, i.e. the constant b, for a given strain is a measure of the inhibitory action of benzoate on the growth of that strain in glucose and b tended to decrease as the training concentration of benzoate increased (Table 1). Training to benzoate as sole $C+E$ source therefore tended to decrease the inhibitory action of benzoate on growth in the glucose medium.

Table 1. *Values of the constants a, b and b/a in the relationship $T = a+bc$ for certain strains of Klebsiella aerogenes, where T (min.) is the mean generation time of the organism growing in media containing moderate concentrations (c mole/l.) of various single benzenoid compounds*

Basal medium: salts+ammonium+carbon source (pH 7·12). Incubation temperature: 40°.

		Benzenoid compound as sole carbon+energy source (nutrient and inhibitor)			Benzenoid compound in the glucose medium (as an inhibitor only)		
Strain	Benzenoid compound	a (min.)	b (min. l. mole^{-1})	b/a	a (min.)	b (min. l. mole^{-1})	b/a
Original glucose-adapted	*p*-Hydroxy-benzoate	75	2680	35·7	29	197	6·8
Original glucose-adapted	Phenyl-acetate	251	10800	43·0	29	576	19·9
Original glucose-adapted	Benzoate	88	12700	144	29	3100	107
B1 trained* to $9·45\times10^{-4}$ M-benzoate	Benzoate	64	9670	151	31	3580	115
B2 trained to $3·15\times10^{-3}$ M-benzoate	Benzoate	60	6600	110		2860	95·3
B3 trained to $9·45\times10^{-3}$ M-benzoate	Benzoate	55	Two linear portions $b = 1190$ up to $1·63\times10^{-2}$ M $b = 8920$ up to $3·75\times10^{-2}$ M		30·5	2850	93·5
B4 trained to $2·20\times10^{-2}$ M-benzoate	Benzoate	79	J-shaped curve above curve of B3 and cutting curves of B2 and B1		31·5	2260	71·8

* History of strains B1–B4 of *Klebsiella aerogenes* trained to utilize benzoate as sole carbon+energy source:

Strain B1, 30–40 subcultures in $9·45\times10^{-4}$ M.
Strain B2, 40–50 subcultures in $3·15\times10^{-3}$ M.
Strain B3, 38 subcultures in $3·15\times10^{-3}$ M followed by 70–100 subcultures in $9·45\times10^{-3}$ M.
Strain B4, 38 subcultures in $3·15\times10^{-3}$ M followed by 84 subcultures in $9·45\times10^{-3}$ M followed by 40–50 subcultures in $2·20\times10^{-2}$ M.

To compare the degre of inhibition (as measured by b) caused by a given benzenoid compound on the value of T for growth with itself as sole $C+E$ source and for growth with glucose, the value of T at zero inhibition (as measured by a) must be taken into account. The quantity b/a fulfils this requirement and can be considered to be the

initial percentage increase in T brought about by an increase in the concentration of benzenoid compound from 0 to 10^{-2} M. Table 1 shows that b/a for growth in benzoate, *p*-hydroxybenzoate or phenylacetate was greater when each compound was sole $C+E$ source than when each was present singly in the glucose medium. Adaptation to low concentrations of benzoate ($9{\cdot}45\times10^{-4}$ M and $3{\cdot}15\times10^{-3}$ M) as sole $C+E$ source did not alter this pattern. Since strains adapted to higher concentrations of benzoate ($9{\cdot}45\times10^{-3}$ M and $2{\cdot}20\times10^{-2}$ M) gave more complex relationships between T and benzoate concentration provided as sole $C+E$ source, b had more than one value over moderate concentrations and so proper comparisons of b/a cannot be made.

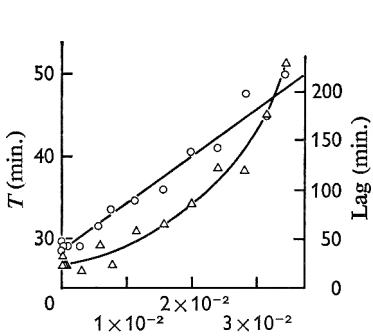

Fig. 3

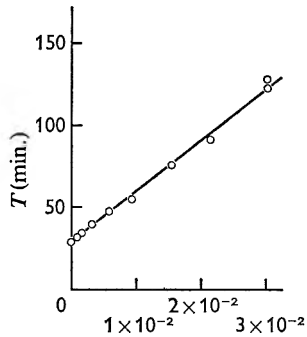

Fig. 4

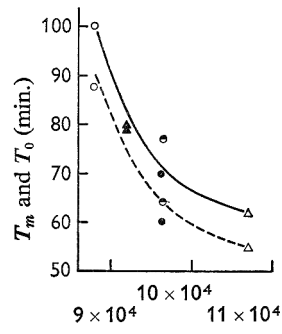

Fig. 5

Fig. 3. Lag (△) and T (○) for the growth of the original glucose-adapted strain of *Klebsiella aerogenes* in glucose medium containing various concentrations of phenylacetate.

Fig. 4. T for the growth of the original glucose-adapted strain of *Klebsiella aerogenes* in glucose medium containing various concentrations of benzoate. Lag negligible (< 40 min.). (In $4{\cdot}47\times10^{-2}$ M-benzoate, lag = 0, T = 292 min.; in $5{\cdot}85\times10^{-2}$ M-benzoate, lag = 0, T = 1090 min.; in $7{\cdot}18\times10^{-2}$ M-benzoate, no growth.)

Fig. 5. Relationship between the mean generation time, measured by T_0 (broken curve) and T_m (continuous curve), and the molar growth yield, measured by $\Delta M_s/\Delta c$, for the following strains of *Klebsiella aerogenes* growing in benzoate as sole $C+E$ source. Final training concentration of benzoate (for detailed histories of the strains see Table 1): ○, untrained; ◓, $9{\cdot}45\times10^{-4}$ M; ●, $3{\cdot}15\times10^{-3}$ M; △, $9{\cdot}45\times10^{-3}$ M; ▲, $2{\cdot}20\times10^{-2}$ M.

Maximum bacterial concentration (M_s) after exhaustion of a benzenoid carbon+energy source

When exhaustion of the $C+E$ source limited growth, plots of M_s against the initial molar concentration, c, of $C+E$ source were linear. At zero substrate concentration the lines intercepted the ordinate at small positive values of M_s and there was usually perceptible growth in media to which no $C+E$ source had been added (Bauchop & Elsden, 1960). This 'impurity' growth accounted for the intercept.

The gradient of the plot of M_s against c ($\Delta M_s/\Delta c$) is termed the molar growth yield (Monod, 1942; Herbert, 1958; Bauchop & Elsden, 1960), and is a measure of the efficiency with which the particular $C+E$ source is converted to bacterial substance. On general grounds, faster growth might be expected to result in a higher molar growth yield. In fact an inverse correlation between T and ($\Delta M_s/\Delta c$) was found for strains of *Klebsiella aerogenes* trained to grow in various concentrations of benzoate as sole $C+E$ source (Fig. 5). The values of T chosen for this plot must be those at which the concentration of $C+E$ source is neither so great as to slow down growth by

inhibition, nor so small as to limit the growth rate by starvation. The following two attempts were made to fulfil these requirements. The values of T corresponding to the upper (continuous) curve of Fig. 5 are the minimum values (T_m) interpolated from the plots of T against c and those corresponding to the lower (broken) curve are the hypothetical values (T_0) extrapolated to zero concentration from the inhibitory portions of the plots of T against c. T_0 is thus equal to the constant a in Table 1. The interpolated quantity T_m can be determined with greater accuracy than the extrapolated quantity T_0 but T_0 is theoretically preferable because the inhibition is then strictly zero.

On account of the complexities of adaptation, the inverse correlation between T and the molar growth yield for strains in various stages of training to benzoate, when it is acting both as an inhibitor and as a nutrient, could hardly be as simple as the relationship found when a given strain is grown under closely similar conditions within a restricted range (Pirt, 1965). Indeed, Table 2 shows that there was no correlation between T_m or T_0 and the molar growth yield for the original glucose-adapted strain growing with various benzenoid compounds as sole $C+E$ source, no doubt because the conditions of growth were so widely different. The molar growth yield for glucose was, however, higher than that for the benzenoid $C+E$ sources.

Table 2. *Molar growth yield (as measured by $\Delta M_s/\Delta c$) and T of the original glucose-adapted strain of Klebsiella aerogenes growing with single benzenoid carbon+energy ($C+E$) sources*

Basal medium: salts+ammonium+carbon source (pH 7·12). Incubation temperature: 40°.

Carbon source	$\Delta M_s/\Delta c$	T_m	T_0
Glucose	$14{\cdot}2\times10^4$	30	30
p-Hydroxybenzoate	$6{\cdot}83\times10^4$	80	75
Phenylacetate	$10{\cdot}6\times10^4$	286	251
Benzoate	$8{\cdot}80\times10^4$	100	88

M_s = maximum bacterial concentration in the stationary phase.
c = molar concentration of $C+E$ source.
T_m = minimum value of the mean generation time.
T_0 = value of the mean generation time extrapolated to zero concentration of $C+E$ source.

Serial subculture in p-hydroxybenzoate and phenylacetate

A glucose-adapted strain which had not been in contact with any other chemically defined medium was repeatedly subcultivated in a medium containing a suitable fixed concentration of the appropriate benzenoid compound as sole $C+E$ source, and the lag and T during growth in both the benzenoid and glucose media were determined at various stages of training. During repeated subculture in the *p*-hydroxybenzoate medium (Table 3) the decrease in T to about 45 min. and the decrease in the lag after the first subculture indicated that adaptation to the $C+E$ source was taking place. Table 3 also shows that adaptation to *p*-hydroxybenzoate disturbed growth in glucose medium. Thus partial adaptation increased the value of T in glucose, whereas on prolonged adaptation T was normal but the lag increased.

During subcultivation in medium containing phenylacetate as sole $C+E$ source (Table 4) the sudden decrease in the lag from 700 min. to zero indicated that appreciable adaptation had taken place during the previous subculture. During repeated

subculture in phenylacetate medium T increased at first and then decreased later. When subculture was made daily (series A) the final value of T was about one half the initial value, but with subculture every second day T did not decrease below the value obtained at the first subculture (series B).

Table 4 shows that during subcultivation in phenylacetate medium the small or zero lag on return to glucose medium was maintained but the value of T in glucose medium was increased, although in series A it showed some tendency to decrease as the value of T in phenylacetate medium decreased. Subculture in medium containing phenylacetate as sole $C+E$ source was therefore incompatible with optimum growth in glucose medium.

Table 3. *Growth of Klebsiella aerogenes during serial subculture with* $2{\cdot}78 \times 10^{-2}$ M-*p-hydroxybenzoate as sole carbon+energy source*

Basal medium: salts+ammonium+*p*-hydroxybenzoic acid (pH 7·12). Incubation temperature: 40°.

No. of previous daily subcultures in *p*-hydroxybenzoate before test	Growth in *p*-hydroxybenzoate		Growth on return to glucose	
	Lag (min.)	*T* (min.)	Lag (min.)	*T* (min.)
0	100	82	2	33
1	51	50	60	36
3	35	47	87	58
10	0	76	0	78/40*
15	18	54	0	76/37*
28	57	44	89	56/35*
31	24	44	193	33
43	45	46	175	33

* Sudden decrease in T during growth.

Table 4. *Growth of Klebsiella aerogenes during serial subculture in* $2{\cdot}83 \times 10^{-2}$ M-*phenylacetate as sole carbon+energy source*

Basal medium: salts+ammonium+phenylacetic acid (pH 7·12). Incubation temperature: 40°.

No. of previous subcultures in phenylacetate before test	Growth in phenylacetate		Growth on return to glucose	
	Lag (min.)	*T* (min.)	Lag (min.)	*T* (min.)
	Series A. Subcultured each day			
0	645	166	2	33
3	0	208	0	51
8	0	104	0	97
12	0	132	0	86
22	0	135/88*	0	45
25	0	83	30	38
	Series B. Subcultured every 2 days			
0	708	151	60	32
1	0	150	—	—
7	0	159	0	63
16	0	213	—	—
18	0	235	44	47
35	0	150	0	52
40	0	159	0	62

* Sudden decrease in T during growth.

DISCUSSION

When *Klebsiella aerogenes* NCTC418 grew in medium containing glucose and benzoate, *p*-hydroxybenzoate or phenylacetate or when the organism utilized one of these aromatic compounds as sole $C+E$ source, then the following equation was found to relate the mean generation time (T) to the molar concentration (c) of the aromatic compound:

$$T = a+bc, \tag{1}$$

where a is the value of T at zero concentration of inhibitor, b is a constant independent of c, and c can have values which lie within the limit specified. The specific growth rate, v ($= \ln 2/T$), therefore obeys the following relationship:

$$v = d/(1+ec), \tag{2}$$

where d ($= \ln 2/a$) is the value of v at zero concentration of inhibitor and e ($= b/a$) is a constant independent of c. Equation (2) is also the expression for the rate of reactions catalysed by enzymes or by heterogeneous catalysts in the presence of an inhibitor whose concentration or partial pressure in the bulk phase is represented by c, where d and e are constants with the same interpretation as above (see Hinshelwood, 1940; Dixon & Webb, 1958; Ashmore, 1963). Some apparently more complex expressions for enzyme inhibition reduce to equation (2) or can be made to do so by making suitable approximations.

For the initial growth in benzoate, *p*-hydroxybenzoate or phenylacetate the plot of T ($= \ln 2/v$) against the molar concentration of the $C+E$ source (Fig. 2) closely resembles the plot of the reciprocal of the rate of an enzyme-catalysed reaction against the molar concentration of the substrate which simultaneously acts as an inhibitor (see the graphical representation of the data of Murray (1930) for the hydrolysis of ethyl butyrate by sheep liver carboxylesterase, and of the data of Dixon, Massey and Webb for the oxidation of leucine by methylene blue with snake venom L-amino acid oxidase in Dixon & Webb (1958)). Figure 2 and the plots by Dixon & Webb (1958) are similar both in form and in the order of magnitude of the molar concentration of the substrate corresponding to the minima.

The above similarities could arise from analogies between an enzyme-catalysed reaction proceeding *in vitro* and an actively growing bacterial culture. Both processes take place in solution containing various chemical species, among which are either the substrate(s) of the enzyme or the analogous nutrients for the bacteria. An enzyme reaction can be written as follows:

$$\text{reactants} + \text{enzyme} \rightarrow \text{enzyme} + \text{products},$$

whereas bacterial growth can be represented thus:

$$\text{nutrients} + \text{bacterium} \rightarrow \text{new bacterium} + (\text{metabolic products} + \text{more new bacteria}).$$

Both are slowed down by inhibitors which can act on one or more elementary physical or chemical processes or steps within the enzyme reaction or within the growing cell. The kinetics of inhibition are therefore essentially the same in these two cases and the inhibitor can be a nutrient for the bacteria, a substrate for the enzyme or a foreign substance.

The simplicity of the observed kinetics of inhibition of bacterial growth summarized by equations (1) or (2) is readily explicable if benzoate, *p*-hydroxybenzoate and phenylacetate are each acting as an inhibitor of a cellular process or reaction whose rate determines the rate of multiplication of the bacteria. The inhibited process might be not only an enzyme-catalysed metabolic reaction but also membrane transport catalysed by a permease, and this need not necessarily be the same for different benzenoid inhibitors.

As the concentration of the inhibitory compound is increased beyond a certain value, the lag rapidly increases and the mean generation time usually increases more steeply with concentration than is predicted by the linear relationship obeyed at lower concentrations. These changes are observed no matter whether the $C+E$ source is glucose or solely the benzenoid compound itself and suggest that other processes or reactions now begin to be inhibited also and determine the rate of growth.

The increase of lag that results from washing the bacteria of the inoculum may be due to the removal of diffusible metabolites needed for the biochemical reactions involved in cell division (Hinshelwood, 1946; Dagley, Dawes & Morrison, 1950).

Since the lag preceding growth of the original glucose-adapted strain in glucose media containing a wide range of concentrations of benzoate (0–$5{\cdot}85 \times 10^{-2}$ M) remained negligible, the appreciable lag preceding initial growth in even the lowest concentrations of benzoate as sole $C+E$ source cannot be a consequence of the inhibitory nature of benzoate, and would appear to be a result of the temporary inability of the cells to metabolize the compound.

Repeated subculture in either benzoate, *p*-hydroxybenzoate or phenylacetate as sole $C+E$ source calls forth the usual pattern of adaptive responses which are observed for aliphatic carbon sources (e.g. Hinshelwood, 1946; Baskett & Hinshelwood, 1950). Thus, the lag decreases to a small or negligible value after the first subculture and the mean generation time eventually decreases to a steady minimum value during repeated subculture in a constant concentration of benzoate or *p*-hydroxybenzoate. Adaptation to benzoate as sole $C+E$ source is at every stage fully compatible with optimum growth in glucose, whereas adaptation to *p*-hydroxybenzoate disturbs growth in glucose. During repeated subcultivation in phenylacetate growth in glucose was impaired.

Comparison of the values of b/a in Table 1 shows that benzoate, *p*-hydroxybenzoate and phenylacetate were stronger inhibitors when they were supporting growth than when glucose was. Furthermore, adaptation to moderate concentrations of benzoate as sole $C+E$ source did not change this pattern, and therefore the relatively greater sensitivity of benzoate metabolism cannot be ascribed to its incomplete development but was probably a consequence of differences inherent in the metabolism of glucose and benzoate.

The molar growth yield for glucose was greater than that for the benzenoid compounds. This cannot be entirely ascribed to the fact that the strain of *Klebsiella aerogenes* used was already fully adapted to utilize glucose as sole $C+E$ source, because, after the organism had become adapted to benzoate as sole $C+E$ source, the molar growth yield increased by not more than 21% and was still much smaller than it was for growth in glucose. The metabolism of glucose must therefore be intrinsically more efficient than that for the benzenoid compounds.

As the constant concentration of benzoate which the organism had been trained to

utilize was increased, the following adaptive responses occurred: (1) the inhibitory action of benzoate on growth in the glucose medium tended to decrease (Table 1), showing that the glucose metabolism of the organism had become less sensitive to inhibition by benzoate; (2) the molar growth yield in benzoate increased somewhat and the uninhibited value of the mean generation time decreased provided that the concentration used for training did not exceed about 10^{-2} M (Fig. 5). Although training to $2{\cdot}20 \times 10^{-2}$ M-benzoate did not affect growth in glucose and further decreased the inhibitory action of benzoate in glucose media, it damaged the metabolism of benzoate as sole $C+E$ source. This is shown by the decrease in the molar growth yield and the increase in the uninhibited value of the mean generation time (Fig. 5) and by the upward sweep of the J-shaped curve connecting the mean generation time and the concentration of benzoate provided as sole $C+E$ source (Table 1).

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Path of Glucose Breakdown and Cell Yields of a Facultative Anaerobe, *Actinomyces naeslundii*

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SUMMARY

Actinomyces naeslundii fermented glucose primarily by the Embden–Meyerhof pathway, as based on ^{14}C-glucose fermentation data and enzyme studies. Enzymes of the oxidative pentose phosphate cycle were also present, but functioned only to a minor extent. Growth on glucose was increased 2- to 4-fold in the presence of substrate amounts of CO_2 or O_2. This increase was attributed to the additional energy (ATP) made available from the breakdown of pyruvate to acetyl coenzyme A. In the absence of CO_2 or O_2, pyruvate was reduced to lactate. The weight of organism produced/mole ATP (Y_{ATP}) was 15–18 g. units under anaerobic conditions with CO_2, dependent on the growth medium, and 20 under aerobic growth conditions.

INTRODUCTION

Actinomyces naeslundii is a pathogenic organism which ferments sugars and requires substrate amounts of CO_2 for maximal growth. Pine & Howell (1956) and Buchanan & Pine (1963) investigated the glucose fermentation of *A. naeslundii* and found that fermentation products depended on the conditions of culture. Without added CO_2 (gaseous or bicarbonate), growth was limited and glucose was fermented to lactate

$$\text{glucose} \rightarrow 2 \text{ lactate}. \qquad (1)$$

When substrate amounts of CO_2 were present, lactate formation was decreased, and, for each mole of CO_2 fixed, an equimolar amount of acetate, formate, and succinate was produced (Pine & Howell, 1956):

$$\text{glucose} + CO_2 \rightarrow \text{formate} + \text{acetate} + \text{succinate}. \qquad (2)$$

The ratio of products with added CO_2 was not constant, and dependent upon the specific fermentation a combination of equations (1) and (2) was observed. Based on the average results of three fermentations (Buchanan & Pine, 1963, table 1, no. 3, 5, and 7), a general equation was derived:

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$$3{\cdot}5 \text{ glucose} + 3\ CO_2 \rightarrow 3 \text{ formate} + 3 \text{ acetate} + 3 \text{ succinate} + 1 \text{ lactate.} \quad (3)$$

CO_2 was fixed into the carboxyl groups of succinate (Pine, 1956, 1960). Presumably CO_2 functioned in the formation of oxaloacetate and malate, both of which acted as electron acceptors and permitted the formation of acetate and formate (Buchanan & Pine, 1963) from pyruvate. CO_2 could be partially replaced by either malate or fumarate, with a simultaneous increase in the formation of acetate and formate. Recently, Buchanan & Pine (1965) found that CO_2 was also required for the biosynthesis of aspartate for which the organisms lacked a permease.

When *Actinomyces naeslundii* grew aerobically, O_2 served as an electron (hydrogen) acceptor and CO_2 was required only to initiate growth (Pine & Howell, 1956). Buchanan & Pine (1963) found that glucose was degraded quantitatively to acetate and CO_2 in accordance with the equation :

$$\text{glucose} + O_2 \rightarrow 2 \text{ acetate} + 2\ CO_2 + 2\ H_2O. \quad (4)$$

As based on the above equations, glucose breakdown by *A. naeslundii* might occur by several pathways, and there was until now no evidence concerning which pathway was involved. The present paper presents evidence that *A. naeslundii* ferments glucose principally by way of the Embden–Meyerhof pathway, although the possible operation of other pathways to a minor degree is indicated from isotope data. Previous conclusions about the functions of CO_2 and O_2 in *A. naeslundii* were confirmed in the present work, and CO_2 and O_2 are related to the energy which is available for the growth of this organism.

METHODS

Actinomyces naeslundii (*A. bovis*, ATCC 10049), described previously (Buchanan & Pine, 1963), and *Actinomyces propionicus* (Buchanan & Pine, 1962) were grown at 37° in the casitone medium of Pine & Watson (1959) or in the casein hydrolysate medium of Pine & Howell (1956). Fermentation products were analysed as described previously (Buchanan & Pine, 1963). Lactate and succinate were isolated by Dowex-1 column chromatography (Busch, Hurlbert & Potter, 1952) as modified by Buchanan & Pine (1963). Purity was established by co-chromatography on paper of a sample of the isolated radioactive acids with authentic lactate or succinate. Chromatograms were developed descendingly by using ethanol + water + concentrated ammonium hydroxide (80 + 15 + 5, by vol.) as the solvent (Cheftel, Munier & Macheboeuf, 1953). Acids were located as yellow spots against a blue background by spraying the chromatograms with bromcresol green (0·01% (w/v) in 95% (v/v) ethanol in water neutralized to pH12 with 0·1 N-NaOH) and by radioautography. In all cases, yellow spots coincided exactly with the darkened areas of the X-ray film. Acetate and formate were isolated by steam distillation and were identified by their Duclaux constants.

For radioactivity determinations, compounds were oxidized to CO_2 and counted as $BaCO_3$. Oxidations were done in a combustion chamber equipped with a train of three collection tubes, one of which served as a $KMnO_4$ scrubber (5% (w/v) $KMnO_4$ in 0·3 N-H_2SO_4), leading from the chamber to two tubes containing a saturated solution of $Ba(OH)_2$. Helium was used to flush the system continuously. The $BaCO_3$ was separated by centrifugation in stoppered tubes, washed three times with boiling water, and suspended in 95% (v/v) ethanol in water. Suitable samples were pipetted into lightly greased planchets and dried under heat-lamps.

Radioactivity was measured with a continuous flow counter equipped with a 'micromil' window. 'Q-gas' (98·4% (v/v) He + 1·6% (v/v) butane) was used as the ionizable gas. Samples were corrected for background and self-absorption by reference to a standard curve obtained with known amounts of $BaCO_3$.

Succinate, lactate and acetate were wet-ashed by the method of Van Slyke, Plazin & Weisiger (1951), and the specific activity of the $BaCO_3$ (c.p.m./μmole $BaCO_3$) was multiplied by the number of carbon atoms in the molecule to give the average total specific activity. Formate was converted to CO_2 in the presence of acetate by oxidation with mercuric sulphate by the method of Friedemann (1938) as modified by Rabinowitz & Barker (1956). The residual acetate was collected by steam distillation and its purity verified by its Duclaux constants. Acetate was decarboxylated with the Schmidt reaction (Phares, 1951). After decarboxylation, the solution was made alkaline with KOH, and the methylamine was isolated by distilling into diluted HCl; methylamine was oxidized to CO_2 with alkaline permanganate (Katz, Abraham & Chaikoff, 1955). Lactate was decarboxylated with chromic acid (Calvin *et al.* 1949); the acetate formed was isolated and degraded as described above. Succinate was decarboxylated by the method of Phares & Long (1955). The specific activity of the methylene carbons of succinate was determined by subtracting the specific activity of the carboxyl carbons from the total specific activity obtained by wet-ashing. The validity of this method for determining the activity of the methylene carbons was established by decarboxylating a sample of synthetic succinate 2,3-^{14}C. No radio-activity was released with decarboxylation and when the residual ethylenediamine was isolated on paper chromatograms (Buchanan, 1962), its specific activity was in excellent agreement with the specific activity of the original succinate.

For enzyme studies, the organism was grown in the Casitone medium containing 25 μmoles $NaHCO_3$/ml., under Na_2CO_3 + pyrogallol seals. A volumetric flask of either 1 or 2 l. capacity filled to the base of the neck was inoculated with 10 ml. of an actively growing culture and incubated for 3–4 days. Organisms were harvested by centrifugation, washed three times with 0·01 M-potassium phosphate buffer (pH 7·0) containing 0·01% (w/v) neutralized cysteine HCl. The packed cells were resuspended in this buffer to give a 50% (v/v) suspension.

Like corynebacteria and lactobacilli (Gunsalus, 1955) the actinomyces organisms were difficult to break. Treatment with lysozyme, grinding with glass beads, sonic oscillation with a 12 kc. apparatus (50 W.), and treatment in a French pressure cell were ineffective in releasing soluble protein. The only procedures found to break the organisms were grinding with alumina or subjection to a 20 kc. (75 W.) sonic oscillator (Heat Systems Co., Great Neck, New York).

For alumina-broken preparations, 8–10 g. alumina was added per g. wet wt. organism, and the suspension was ground at 4° in a Sorvall Omnimixer (Ivan Sorvall, Norwalk, Connecticut) for 10 min. in 30 sec. bursts at 1 min. intervals to prevent heating. For sonic preparations, suspensions of organism were subjected to sonic treatment at 4° for 10 min. in 30 sec. bursts at 1 min. intervals to prevent heating. Treated suspensions were centrifuged for 15 min. at 23,500 *g* at 4° to remove debris and unbroken organisms. The precipitate was discarded; the supernatant fluid was dialysed for 4 hr against 0·01 M-potassium phosphate buffer (pH 7·0) containing 0·01% (w/v) neutralized cysteine HCl. When the oxidation or reduction of nicotinamide nucleotides (NAD) was to be measured, the dialysed extract was centrifuged for

60 min. at 100,000 *g* in a preparative ultracentrifuge. The supernatant fraction, which contained the soluble enzymes, was saved; the black precipitate, which contained a very active $NADPH_2$ oxidase system, was discarded.

Enzymes were assayed at room temperature (25° ± 2°). Oxidation and reduction of NAD were measured by the change in extinction at 340 mμ with a Beckman DU spectrophotometer. Fructosediphosphate aldolase (Enzyme Commission, subsequently referred to as E.C., no. 4.1.2.13) was assayed according to Sibley & Lehninger (1949) as modified by Bard & Gunsalus (1950), glucosephosphate isomerase (E.C. 5.3.1.9) according to Slein (1955), phosphate acetyltransferase (E.C. 2.3.1.8) according to Stadtman (1955) based on the reaction of acetyl phosphate with hydroxylamine (Lipmann & Tuttle, 1945), isocitrate dehydrogenase (E.C. 1.1.1.42) and malate dehydrogenase (E.C. 1.1.1.37) according to Ochoa (1955*a*, *b*), aconitate hydratase (E.C. 4.2.1.3) according to Anfinsen (1955), fumarate hydratase (E.C. 4.2.1.2) according to Massey (1955), and glutamate dehydrogenase (E.C. 1.4.1.3) according to Strecker (1955). Protein was estimated by the phenol method of Sutherland, Cori, Haynes & Olsen (1949) with bovine serum albumin as a standard. For each enzyme, the initial rate was directly proportional to the protein concentration. The rates varied little in different preparations.

The chemicals used were of reagent grade and were obtained from commercial sources.

RESULTS

One of the most useful techniques in elucidating metabolic pathways in microorganisms is the use of ^{14}C-labelled substrates (Wood, 1961). For heterotrophic organisms, an organic substrate labelled in a specific position is supplied to growing cultures and the fermentation products isolated and degraded to determine the positions of the label. For saccharolytic organisms such as *Lactobacillus casei* and *Streptococcus faecalis* (Gibbs, Dumrose, Bennet & Bubeck, 1950), glucose 1-^{14}C and glucose 6-^{14}C have been used most frequently. In the Embden–Meyerhof pathway, 1-^{14}C and 6-^{14}C glucose are both converted to fermentation products, such as lactate, which show identical labelling patterns. When glucose is fermented by other mechanisms (for example, the oxidative pentose phosphate pathway) the lactate formed in glucose 1-^{14}C and glucose 6-^{14}C fermentations shows quite different labelling patterns.

Table 1 shows the results of the fermentation of glucose 1-^{14}C and glucose 6-^{14}C by *Actinomyces naeslundii* grown anaerobically in substrate amounts (25 μm./ml.) of CO_2 added to the medium as bicarbonate. The ratio of the fermentation products was essentially as is shown in equation (3). The radioactivity recovered in the formate, CO_2 and carboxyl groups of lactate and succinate was low. This eliminated the oxidative pentose phosphate cycle, Entner–Doudoroff, and hexose monophosphate pathways (see review by Wood, 1961) as major mechanisms for glucose dissimilation. Based on radioactivity recovered in the formate, CO_2 and carboxyl groups of acetate, formate and succinate, not more than 6% of the total glucose fermented was channelled through any one of these pathways.

In the Embden–Meyerhof pathway, the glucose 1-carbon and the glucose 6-carbon atoms are precursors of the methyl-carbon atoms of lactate, of acetate, and of a methylene carbon of succinate (assuming succinate synthesis from a C_3 unit + CO_2) Chemically the two methylene carbons of succinate are indistinguishable, and therefore

the total activity in both carbons must be considered. In general, the degradation data in Table 1 fit these requirements for formation by the Embden–Meyerhof pathway. The specific activities of acetate, lactate and succinate were approximately 50% of the original ^{14}C-glucose molecules. For unknown reasons, the specific activity of acetate isolated from the glucose 6-^{14}C fermentation was low and was only 33% of the original ^{14}C-glucose. Of the total radioactivity in acetate and lactate, at least two-thirds resided in the methyl carbon atoms. Of the succinate, at least 90% of the total activity resided in the two methylene carbons.

Table 1. *Fermentation of glucose 1-^{14}C and glucose 6-^{14}C by Actinomyces naeslundii*

Organisms were grown anaerobically in 50 ml. Casitone medium (0·5%, w/v) containing either glucose 1-^{14}C or glucose 6-^{14}C with 25 μmoles $NaHCO_3$/ml. under Na_2CO_3 + pyrogallol seals. The following specific activities and total c.p.m., respectively, were used: glucose 1-^{14}C 1012 c.p.m./μmole and $12{\cdot}75 \times 10^5$ c.p.m.; glucose 6-^{14}C, 601 c.p.m./μmole and $7{\cdot}25 \times 10^5$ c.p.m. Assimilated glucose was determined by counting samples of washed organisms at infinite thinness. Other experimental details are given in the text.

	μmoles/100 μmoles glucose fermented		Specific activity of ^{14}C-products in % of the original ^{14}C-glucose	
	Glucose 1-^{14}C	Glucose 6-^{14}C	Glucose 1-^{14}C	Glucose 6-^{14}C
CO_2 remaining	—	—	5·0	0·2
CO_2 fixed	52·5	54·9	—	—
CH_3—COOH	47·1	49·7	45·2	33·6
CH_3—	—	—	40·0	21·4
—COOH	—	—	7·0	9·3
HCOOH	48·2	52·9	5·8	0·8
CH_3—CHOH—COOH	21·4	23·6	52·7	48·0
CH_3—	—	—	37·8	37·8
—CHOH—	—	—	6·6	3·8
—COOH	—	—	4·0	6·5
HOOC—CH_2—CH_2COOH	54·9	54·5	53·1	47·5
HOOC—	—	—	2·0	0·4
—CH_2—	—	—	23·6	23·5
Redox index	0·98	0·98	—	—
Glucose assimilated	14·0	10·2	—	—
Carbon recovery (%)	77·9	76·7	—	—
^{14}Carbon recovery (%)	78·0	79·4	—	—

Pertinent to demonstrating a particular metabolic pathway in an organism is the demonstration of its constituent enzymes. Aldolase is considered indicative of the Embden–Meyerhof pathway, and so far it has not been shown to function in other saccharolytic pathways (Buyze, van den Hamer & De Haan, 1957). Table 2 shows that aldolase was present in cell-free extracts of *Actinomyces naeslundii* and that its capacity was relatively high (cf. McDonald, Cheldelin & King, 1960). Extracts of a related organism, *Actinomyces propionicus* (Buchanan & Pine, 1962), also showed aldolase activity, with a rate about two-thirds that observed in *A. naeslundii*, thus indicating the Embden–Meyerhof pathway is possibly widespread in this group of organisms. Glucosephosphate isomerase, an enzyme of the Embden–Meyerhof pathway, and phosphate acetyltransferase, which often occurs in anaerobic bacteria (Stadtman, 1955), were also present in *A. naeslundii* (Table 2).

In a previous study, *Actinomyces naeslundii* was grown anaerobically in $^{14}CO_2$ and

glutamate and aspartate were isolated and degraded (Buchanan & Pine, 1965). The labelling pattern observed in glutamate was consistent with its biosynthesis from α-ketoglutarate, formed by the tricarboxylic acid cycle. This conclusion is supported by results of the present investigation which show that isocitric dehydrogenase, aconitase, and NADP-linked glutamic dehydrogenase were present in relatively high amounts in *A. naeslundii* (Table 2).

Table 2. *Demonstration of certain enzymes in cell-free extracts of Actinomyces naeslundii*

	μmoles substrate used/mg. protein/hr
Fructosediphosphate aldolase	1·6
Glucosephosphate isomerase	0·5
Phosphate acetyltransferase	18·0
Aconitate hydratase	1·7
Isocitrate dehydrogenase	8·5
Glutamate dehydrogenase (NADP linked)	7·5
Malate dehydrogenase	59·0
Fumarate hydratase	3·8
Glucose-6-phosphate dehydrogenase	0·5
Phosphogluconate dehydrogenase	2·6
Other enzymes of oxidative pentose phosphate pathway	0·1*

* This value does not represent the activity of any single enzyme, but instead is the over-all rate of conversion of ribose 5-phosphate to glucose 6-phosphate.

The mechanism by which CO_2 was incorporated into the carboxyl carbons of succinate by *Actinomyces naeslundii* was not determined unequivocally. However, a low but significant exchange reaction between $^{14}CO_2$ and the β-carboxyl carbon of oxaloacetate was demonstrated (Buchanan, 1962), and this provided evidence that the primary carboxylation reaction in *A. naeslundii*, leading to succinate, is the phosphopyruvate carboxylase (E.C. 4.1.1.32) reaction originally studied by Utter & Kurahashi (1954). Oxaloacetate would then be reduced to succinate by a reversal of the malic dehydrogenase, fumarase and succinic dehydrogenase (E.C. 1.3.99.1) reactions. The present demonstration (Table 2) of fumarase and malic dehydrogenase activities in *A. naeslundii* extracts supports this conclusion.

Glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) and phosphogluconate dehydrogenase (E.C. 1.1.1.44) were demonstrated in extracts of *Actinomyces naeslundii* (Table 2). As based on the reduction of NADP with ribose 5-phosphate as substrate (Table 2), the other enzymes of the pentose cycle which convert ribose 5-phosphate to glucose 6-phosphate were also present. These data therefore indicate that *A. naeslundii* has a complete oxidative pentose phosphate cycle but, as pointed out above, its operation as a major mechanism of glucose breakdown in *A. naeslundii* was excluded on the basis of the glucose 1-^{14}C fermentation data (Table 1). These data indicated that, at most, 6% if the total glucose fermented could go by this pentose pathway. Similar results were obtained in independent glucose 1-^{14}C fermentations carried out under aerobic conditions (Buchanan, 1962), thus eliminating the possibility of its function as a major pathway in aerobic growth.

Because of its relation to *Actinomyces naeslundii*, *A. propionicus* was tested for certain enzymes found in *A. naeslundii*. Other than the aldolase mentioned above, 6-

phosphogluconic dehydrogenase and isocitric dehydrogenase were found in extracts of *A. propionicus*, at essentially the same concentration as in *A. naeslundii* (Buchanan, 1962).

With the recognition that glycolysis was the major pathway used for the fermentation of glucose by *Actinomyces naeslundii*, and that the fermentation products depended on the availability and nature of the added electron acceptors, we tried to correlate the relative yields of organism to the energy theoretically released in a particular type of

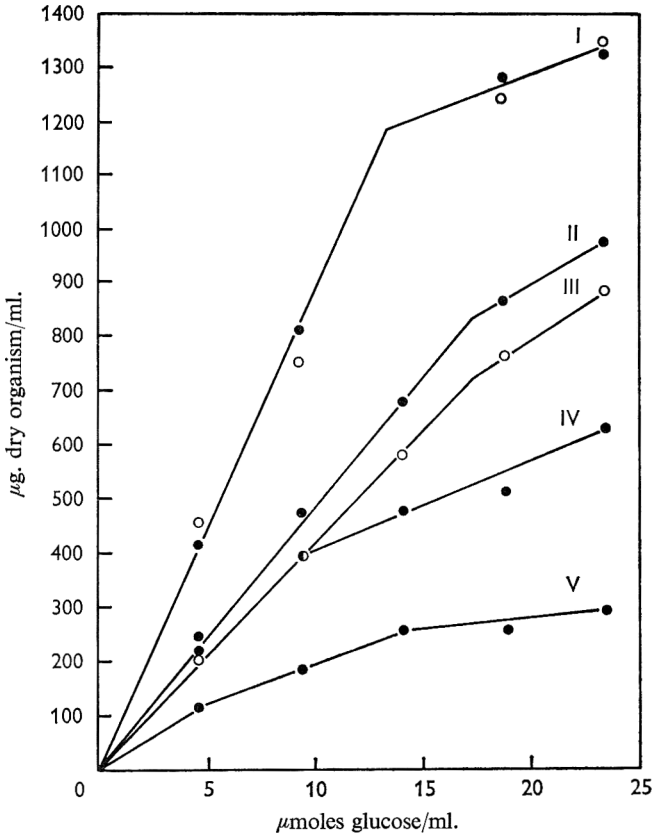

Fig. 1. Dependence of the yields of *Actinomyces naeslundii* on the conditions of culture. I, Aerobic growth in Casitone medium with CO_2= ●; without CO_2 = ○. II, Anaerobic growth in Casitone medium with CO_2. III, Anaerobic growth in casein hydrolysate medium with CO_2. IV, Anaerobic growth in casein hydrolysate medium minus CO_2 plus malate. V, Anaerobic growth in casein hydrolysate medium minus CO_2. Cells were grown on a rotary shaker in test-tubes containing 5 ml. Casitone medium under $KH_2PO_4 + Na_2CO_3$ seals to supply gaseous CO_2 (I); in side-arm fermentation tubes (Pine, 1956) containing 50 ml. Casitone medium with 25 μmoles $NaHCO_3$/ml. under Na_2CO_3 + pyrogallol seals (II); in 50 ml. volumetric flasks containing 48 ml. casein hydrolysate medium with 25 μmoles $NaHCO_3$/ml. under either Na_2CO_3 + pyrogallol seals (III) or without $NaHCO_3$ under NaOH + pyrogallol seals (IV, V). Sodium malate was added to IV to 0·5 % (w/v). Above values represent the average growth observed in duplicate vessels. Aerobic tubes were inoculated with 1 drop of a homogenized 72 hr culture diluted with water to an extinction of 0·5; anaerobic vessels were inoculated with the equivalent of 1 mg. dry wt. organisms. Growth was measured by the extinction at 600 mμ in a Spectronic-20 colorimeter. Extinction was related to dry weight by reference to a standard curve. The abscissa represents the initial concentration of glucose in the medium. All cultures were incubated 96 hr; maximum growth was usually attained in 48 hr; the ordinate represents the maximum value attained for each culture.

fermentation. The yields of organism obtained under various anaerobic or aerobic conditions of growth are given in Fig. 1. Except for anaerobic growth without CO_2, the extent of growth in all cases was proportional to the initial glucose concentration, at least for the first two points. As observed previously (Pine & Howell, 1956; Buchanan & Pine, 1963), growth was better in the presence of CO_2 or air, presumably owing in part to the additional energy released in pyruvate breakdown. In anaerobic growth without CO_2, growth was not proportional to the initial glucose concentration, although it was approximately linear up to 9·4 μmoles glucose utilized/ml. The non-linearity in the growth response was, in part, due to lack of aspartic acid, which could not by synthesized *de novo* without CO_2 and which could not be assimilated when added to the culture medium because of the absence of a permease (Buchanan & Pine, 1965).

Table 3. *Yields of Actinomyces naeslundii under various conditions of growth*

Curve of Fig. 1	Medium and substrate	Conditions	Glucose concentration (μmole/ml.)	Y*	Equation no. (see text)	Net ATP/mole glucose†	Y_{ATP}‡
I	Casitone, glucose	Air or air + CO_2	4·7	87·2	4	4	21·8
			9·4	86·7	4	4	21·7
II	Casitone, glucose	Anaerobic + CO_2	4·7	48·9	3	2·8	17·5
			9·4	51·0	3	2·8	18·2
			14·1	48·2	3	2·8	17·2
III	Casein hydrolysate, glucose	Anaerobic + CO_2	4·7	42·5	3	2·8	15·2
			9·4	41·5	3	2·8	14·8
			14·1	41·1	3	2·8	14·7
IV	Casein hydrolysate, glucose + malate	Anaerobic, no CO_2	9·4	40·4	5	4	10·1
V	Casein hydrolysate, glucose	Aneraobic, no CO_2	4·7	24·7	1	2	12·3

* μg. dry wt. organism/μmole glucose fermented (Bauchop & Elsden, 1960).

† Based on the fermentation equations shown in the text, in which the formation of 1 mole of acetate represents a net gain of 2 moles of ATP and 1 mole of lactate or succinate/mole ATP.

‡ μg. dry wt. organisms/μmole ATP (Bauchop & Elsden, 1960).

Anaerobic growth in the absence of CO_2 but with malate (curve IV, Fig. 1) showed that malate partially replaced CO_2 by functioning as a source of aspartate and as an electron acceptor to become succinate, pyruvate being converted to acetate and formate. Analysis of fermentations done in absence of CO_2 but with added malate showed that *Actinomyces naeslundii* fermented glucose (at initial values not exceeding 10 μmoles/ml.) in accordance with the equation:

$$\text{glucose} + 2 \text{ malate} \rightarrow 2 \text{ formate} + 2 \text{ acetate} + 2 \text{ succinate}. \quad (5)$$

At this low concentration of glucose, malate effectively substituted for CO_2 with a yield of organism 80% of that observed for anaerobic growth with added CO_2. At higher glucose concentrations, growth was no longer proportional to the glucose concentration and large amounts of lactate were formed. Analysis of the products showed the fermentation occurred according to the equation:

$$2 \text{ glucose} + 2 \text{ malate} \rightarrow 2 \text{ formate} + 2 \text{ acetate} + 2 \text{ lactate} + 2 \text{ succinate}. \quad (6)$$

Table 3 shows the yields of *Actinomyces naeslundii* calculated from data presented in Fig. 1, where growth was proportional to the added glucose. Growth is expressed in

the Y and Y_{ATP} units as defined by Bauchop & Elsden (1960); the net moles ATP formed/mole glucose, as calculated from the appropriate fermentation equation given above, is shown also. For these calculations, we assumed that one net ATP is released in the formation of one mole of lactate or succinate; 2 ATP are formed in the production of acetate (one additional ATP being due to the breakdown of pyruvate to acetyl coenzyme A + CO_2 in air, or to acetyl coenzyme A + formate anaerobically). Bauchop & Elsden (1960) defined the Y value as the g. dry wt. organism formed/mole glucose fermented; the value found for most organisms is 22 (Senez, 1962). The Y values observed with *A. naeslundii* grown anaerobically with substrate concentrations of CO_2 were 41·1–51·0, depending on the growth medium. These values are at least twice those reported for most other organisms. However, Gunsalus & Shuster (1961) reported a Y value of 37·5 of *Propionibacterium pentosaceum*, an organism which in many ways is closely related to the actinomyces species (Stanier & Van Niel, 1941; Buchanan & Pine, 1962). As a result of the high Y values observed with *A. naeslundii*, the Y_{ATP} values (g. dry wt. organism formed/theoretical mole ATP; Bauchop & Elsden, 1960) are correspondingly 1·5–2 times higher than the Y_{ATP} value of 10·5 observed for most organisms (Senez, 1962). Similar but somewhat higher values were obtained for aerobically grown *A. naeslundii* (Table 3). However, when the fermentation was homolactic (curve V, Fig. 1) or when malate was substituted for CO_2 (curve IV, fig. 1), the Y_{ATP} values were 10·1 and 12·3, respectively. If the Y_{ATP} value of 10·5 be accepted as a universal constant (Senez, 1962), the data in Table 3 imply that *A. naeslundii* obtains additional energy in its glucose fermentation in the presence of CO_2, perhaps at some point in the formation of succinate. However, this suggestion is complicated by the observation that the aspartate necessary for growth must be synhesized from a C_4-dicarboxylic acid (Buchanan & Pine, 1965).

DISCUSSION

This communication shows that *Actinomyces naeslundii* ferments glucose primarily by the Embden–Meyerhof pathway. Enzymes of the oxidative pentose phosphate cycle are present, but isotopic data show that this is a minor pathway and does not account for more than 6% of the glucose fermented. The operation of phosphoketolase, although we have no direct evidence for its presence, could explain in part the low activity of ^{14}C in the methyl carbons of acetate when glucose 6-^{14}C was the substrate. It is possible that the pentose pathway is essential for providing certain intermediates, such as pentoses, which are used for growth and that it does not function in energy production. Such a role would restrict the function of the pentose pathway in *A. naeslundii* to the interconversion of sugars needed for biosynthesis.

The dependence of the fermentation products on the conditions of growth shows that the pyruvate (or phosphoenolpyruvate) formed from glucose can take alternative routes. In the absence of CO_2 or O_2, pyruvate is reduced to lactate. When substrate amounts of CO_2 are present, lactate formation is relatively minor and CO_2 is used in the synthesis of oxaloacetate and malate which function as electron acceptors to form succinate. CO_2 is believed to be fixed into phosphoenolpyruvate to yield oxaloacetate, which is reduced to succinate by a reversal of the malic dehydrogenase, fumarase and succinic dehydrogenase reactions. The reduction of oxaloacetate to succinate requires four electrons, and four electrons are released in the conversion of a glucose molecule

to two molecules of phosphoenolpyruvate. Therefore, when CO_2 is present, a second molecule of phosphoenolpyruvate is free and is converted to acetate and formate, by way of pyruvate. This over-all process establishes a redox index of unity in the heterolactic fermentation as shown in equation (3). In aerobic growth, oxygen serves as an electron acceptor and glucose is oxidized to acetate, CO_2 and H_2O; the mechanism by which the electrons react with oxygen has not been determined. The over-all mechanisms of glucose fermentation for *Actinomyces naeslundii* are depicted in Fig. 2, as determined from this and preceding reports (Buchanan & Pine, 1963, 1965).

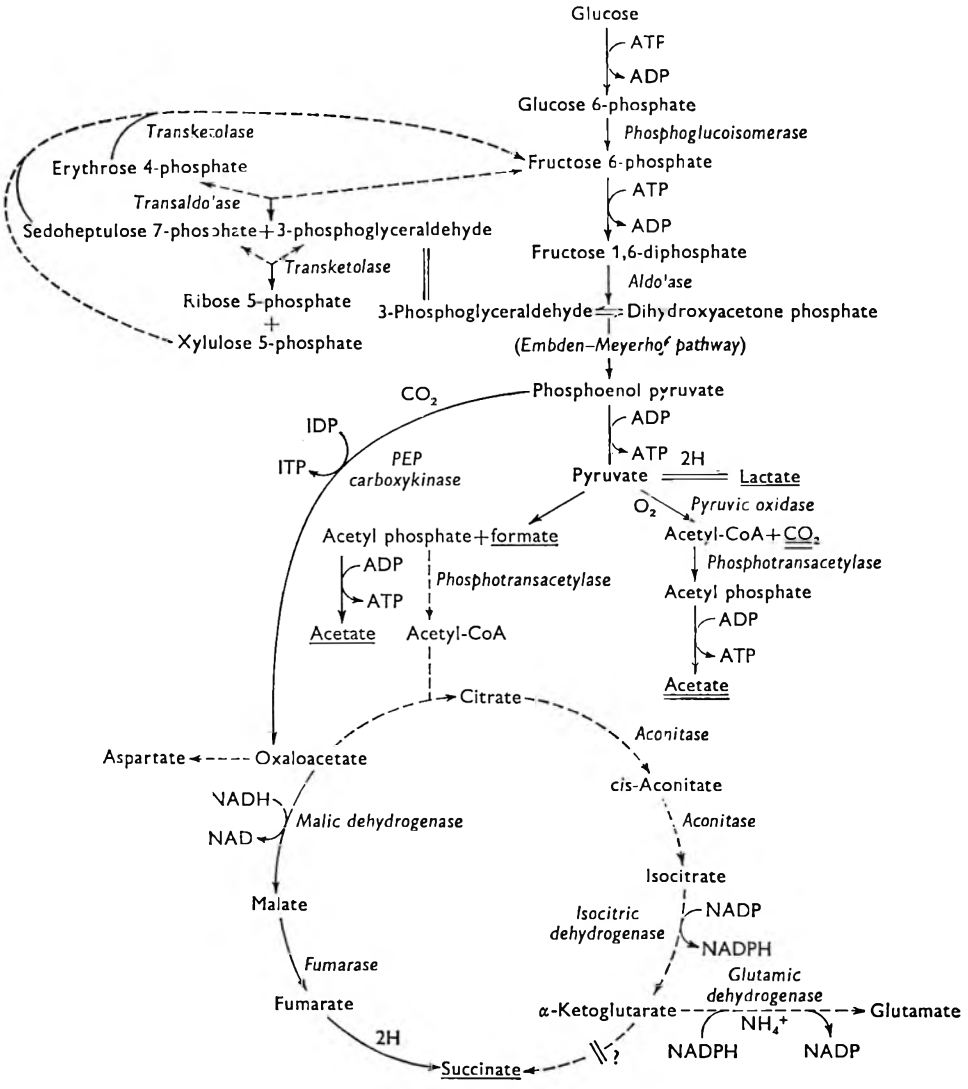

Fig. 2. Proposed mechanism of glucose breakdown by *Actinomyces naeslundii*. Major anaerobic fermentation products are singly underlined; major aerobic fermentation products are doubly underlined. Reactions believed to be involved principally in energy production are shown with solid arrows; reactions believed to be involved principally in biosynthesis are shown with broken arrows. The enzymes found in extracts are shown.

The breakdown of pyruvate to acetyl coenzyme A or acetylphosphate releases additional energy for growth. Under conditions promoting pyruvate breakdown, the yields of *Actinomyces naeslundii*/glucose molecule are higher than for most other organisms. Whether or not this is peculiar to the actinomyces (and the propionibacteria; Gunsalus & Shuster, 1961) is an interesting point that may be decided only by further experiments.

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Note added in Press

Recent cell wall analyses of strain ATCC 10049 showed the major amino acids to be glutamic acid, alanine, lysine, and glycine. The major sugars were galactose and mannose. Large quantities of hexosamine were also present. On the basis of cell wall analysis this strain is more correctly classified as *Actinomyces israelii* than *A. naeslundii*.

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The Cell Wall of *Escherichia coli*: Early Effects of Penicillin Treatment and Deprivation of Diaminopimelic Acid

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SUMMARY

The morphological effects on bacterial walls of the early stages of inhibition of mucopolymer synthesis have been investigated in *Escherichia coli*. Inhibition was achieved in strain B by penicillin treatment and in auxotrophic strains M203 and M173–25 by deprivation of diaminopimelic acid (DAP). Only growing organisms were affected; the walls of organisms in stationary phase remained unchanged. The first effects of treatment with penicillin appear in 20 min., whereas changes in DAP-deprived auxotrophs become visible only after 40–60 min. The soft layers of the wall formed bag-like protrusions and, at the same time, wide gaps or holes developed in the proteinaceous portion of the rigid layer. Widening of these gaps was paralleled by a weakening of the rigid layer, resulting eventually in bursting of the cell, or in protective media of high osmotic strength, in the formation of spheroplasts. The initial appearance and the subsequent widening of gaps in the rigid layer, observed almost exclusively in organisms multiplying logarithmically, are taken to indicate the presence of a mechanically weak mucopolymer formed under conditions that prevent crosslinking. The discontinuities, interpreted as sites of mucopolymer synthesis, were randomly distributed over the bacterium. Their appearance at the poles of the bacteria seemed frequently to be suppressed by media of high osmotic pressure, in which plasmolysis was first seen in the polar regions. The observations suggest that morphogenesis of the protein layer is dependent on the secondary structure of the mucopolymer in newly synthesized wall areas.

INTRODUCTION

The wall of Gram-negative bacteria consists of a multilayered structure (Glauert, 1962; Murray, 1962; Salton, 1964) which can be altered in its morphological properties by chemical or enzymic treatment as well as by growth inhibition (Liebermeister & Kellenberger, 1956; Hofschneider, 1960; Murray, Steed & Elson, 1965). In agreement with these reports are results of our own studies on the wall structure of *Escherichia coli* obtained with frozen-state microtomy. A general model of the organization of the cell wall has been proposed (Bayer & Anderson, 1965) as follows. The innermost portion of the wall consists of a rigid mucopolymer which serves many functions: it provides the wall with the necessary strength to maintain the shape of the cell against the osmotic pressure of the cytoplasm (Mitchell & Moyle, 1957); it is needed for priming its own synthesis (Lark & Lark, 1961; McQuillen, 1960); it supports the soft outer lipid layers of the wall which are 'pasted' to it by non-covalent bonds (Weidel & Pelzer, 1964).

In an investigation of the wall structure of *Escherichia coli* (Bayer & Anderson, 1965) it was shown that the rigid layer of the walls contained scattered gaps in an otherwise fairly uniform rigid layer. It was suggested that these gaps might represent the sites of growth, where autolytic enzymes had opened the wall structure to allow for synthesis of new wall elements. To achieve the introduction of a new building block (Park & Strominger, 1957), the rigid structure has to be opened without endangering the mechanical stability of the whole wall. It can be assumed that such a loosening in the 'centres of growth' (Toennies & Shockman, 1958) is caused by the action of several mucopeptide hydrolases (Pelzer, 1963).

After a new building block has been placed into the older rigid structure, the 'weak point' is closed up, and the cell regains its former rigidity in that area. Any interference with the restoration will leave the rigid structure incomplete at the sites of synthesis. To achieve such an inhibition of wall synthesis and to observe parallel early alterations of the wall structure, we used penicillin, which is considered (Wise & Park, 1965; Tipper & Strominger, 1965) to block specifically the production of a rigid mucipolymer in the last steps of polymerization, the cross-linkage of the glycopeptides which is indispensable for the required mechanical stability of the polymer (Martin 1964). All previous steps in the synthesis of the wall-glycopeptides have been shown to be insensitive to penicillin (Anderson, Matsuhashi, Haskin & Strominger, 1965) The production of larger amounts of defective mucopolymer will eventually lead to rupture of the whole wall of a growing bacterium. Because penicillin interferes with the synthesis of the wall, it affects growing bacteria only and remains ineffective against bacteria in the stationary phase. Interference with the cross-linkage of the mucopolymer can also be achieved in another way: by deprivation of diaminopimelic acid (DAP), an essential constituent of the mucopolymer in *Escherichia coli* (Weidel & Primosigh, 1957) which is involved in the cross-linkage of neighbouring peptide side chains of the glucosaminopeptide (Wise & Park, 1965; Tipper & Strominger, 1965). Certain mutants of *E. coli* (e.g. M203 or M173/25) are unable to synthesize DAP and require for growth an external supply of this amino acid. When such an auxotrophic mutant is not given DAP, an un-crosslinked 'weak' mucopolymer is produced leading eventually to bursting of the bacterium or formation of a spheroplast.

In the present report we describe the early effects of penicillin treatment and DAP-deprivation on the structure of the wall of *Escherichia coli*. The dependence of the effects of penicillin on the state of growth and on the osmotic conditions of the cultures will be shown.

METHODS

Bacteria. Escherichia coli B, wild type, was obtained from Dr E. W. Six (State University of Iowa) and has been cultivated in our laboratories for several years. In 10 ml. amounts of L medium (see below), with aeration by bubbling of 150 ml. air/min. or by shaking, the generation time at 37° in the log phase was 30 min.

The auxotrophic mutant *Escherichia coli* M203, derived from *E. coli* W-ATCC 9637 by the penicillin method (Davis, 1949; Lederberg, & Zinder, 1948) was kindly provided by Dr C. Gilvarg (Princeton University). This strain requires for growth to be given diaminopimelic acid. When grown without aeration in 10 ml. minimal medium (Davis & Mingioli, 1950) +diaminopimelic acid+lysine, its generation time was 100 min.; aeration by shaking and with yeast extract added to the same medium

decreased the generation time to 30 min. Cultures of *E. coli* M203 in medium containing yeast extract+lysine, but without DAP, showed about a twofold increase in optical counts for the first 30 min., then a decreasing count for the remaining time of the experiment.

Another DAP-requiring mutant of *Escherichia coli* M 173–25, was kindly provided by Dr B. D. Davis (Harvard Medical School, Boston), and was grown as described for mutant M203.

Culture media. L medium consisted of (%, w/v): 1, Bacto-tryptone (Difco Laboratories, Detroit, Mich.); 0·5, yeast extract (Difco); 0·5, NaCl; 0·1, glucose; adjusted to pH 7·0 with N-NaOH.

For growth of mutant M203, 20 μg. α-ϵ-DL-diaminopimelic acid (DAP) (Cyclo Chemical Co., Los Angeles, Calif.)+20 μg. of L-lysine (Calbiochem, Los Angeles, Calif.) were added/ml. minimal medium. To most of our cultures yeast extract (Difco), 500 μg./ml., was added.

For osmotic protection the S–P medium of Hirokawa (1962) was used with a slightly lower sucrose concentration (18 % instead of 20 %).

Procedures. The bacteria were counted in Neubauer chambers with a phase microscope. When the number of bacteria reached between 6×10^7/ml. and 2×10^8/ml. they were pelletted at 4000 rev./min. in a Servall centrifuge at 4° for 15 min., and the pellet stored at 1° in a moist atmosphere. A portion of each culture was grown for 6–8 hr longer, reaching optical counts of 5×10^9/ml. and more. The bacteria were then collected in pellets as described above.

The maximum loss of viability of logarithmically growing bacteria after pelleting and resuspending was about 15 %, as tested by colony-counting on L medium agar after incubation for 12 hr at 37°.

When suspensions of *Escherichia coli* B and *E. coli* M203 were to be treated with penicillin, they were first pelleted, and the pellets then resuspended in L medium containing 20 g. sucrose and 0·2 g. anhydrous $MgSO_4$ per 100 ml. of total fluid. Sucrose and $MgSO_4$ provide the osmotic stabilization of spheroplasts (Hirokawa, 1962). To stationary phase cultures appropriate amounts of solid sucrose and anhydrous $MgSO_4$ were added.

Potassium penicillin G (Abbott Laboratories, Chicago, Ill.) was added to a final concentration of 10^3 units/ml. After 20–30 min. the bacterial suspensions were rapidly cooled and pelleted at 6000 rev./min. for 20 min. at 4°.

To exclude the plasmolyzing effects of the sucrose, some of the suspensions were treated with penicillin in media of the same composition, but without sucrose, When the optical counts of the bacteria in cultures of the mutants *E. coli* M203 and M 173–25 had reached 6×10^7/ml. the bacteria were pelleted as described above. The pellets were resuspended in minimal medium containing yeast extract, lysine and sucrose, but lacking DAP. After incubation at 35° for 60–120 min. and gentle shaking, the cultures were pelleted at 6000 rev./min. and the pellet stored at 1°.

To study the walls of dead bacteria, cultures of logarithmically growing *Escherichia coli* B were heat-treated at 67° for 10 min. in L medium and rapidly cooled in water at 5 to 7°. Only 1 out of 2×10^4 bacteria remained viable when plated on L medium agar and incubated at 37°. The pellets were obtained as described above.

Our technique for microtomy in the frozen state (Bayer & Anderson, 1965) began with quick freezing of small portions of the unfixed bacterial pellets in liquid nitrogen.

The frozen bacteria were opened by the knife of an ultramicrotome at −30°, and the cytoplasm allowed to escape during a subsequent brief washing with distilled water at room temperature. The remaining cell walls were then stained negatively in silicotungstate, pH 6·5. When treated with 0·5% (w/v) sodium dodecyl sulphate (SDS) on the electron microscope grid, the lipid layers of the cell walls dissolved. A period of 5 min. at 20° was sufficient to remove the lipids in *Escherichia coli* B walls, whereas walls of *E. coli* M203 and M173–25 needed treatment for 12 hr at 45°. After a subsequent washing in distilled water for 20 sec., the wall residues were negatively stained and immediately dried inside the microscope. Electron micrographs were taken with a Siemens Elmiskop I equipped with double condenser at magnifications of ×20,000 and 40,000. Light microscope observations and photomicrographs were made with a Zeiss GFL microscope, equipped with a phase-contrast oil-immersion objective (N.A. 1·25) and condenser and Zeiss electronic microflash.

RESULTS

Phase-contrast microscopy

The size and shape of *Escherichia coli* organisms depend on the growth phase. Logarithmically growing bacteria are rod-shaped and measure 1·5–2 μ in length and 0·5–1 μ in width, with an axial ratio of 2 or more (Pl. 1, fig. 1). Stationary phase bacteria are smaller and measure slightly more than 1 μ in length with an axial ratio of about 1·5. In both types of cultures, however, one finds occasionally very long rod-shaped bacteria.

The effects of sucrose concentrations of 18–20% (w/v) on the protoplasmic contents of *Escherichia coli* B can easily be observed in the light microscope: plasmolysis becomes visible immediately after exposing the cultures to the sucrose. Usually the protoplast seems to be retracted from one or both poles of the cell (Pl. 1, figs. 2, 3); occasionally also other portions of the protoplasm are involved. Transfer of such bacteria to a medium of normal osmotic pressure quickly reverses the plasmolysis. Continued exposure to 18% (w/v) sucrose results in gradual reversal of the plasmolysis; after incubation for 10–15 min., most of the protoplasts seem to occupy again the entire space inside the walls. The multiplication (generation) time seems to be prolonged during the reversal in sucrose and returns to its former value after about 20–30 min. This effect might be caused by the sucrose or by the disturbance of growth during pelleting and resuspension.

The effects of penicillin become visible after 20 min., while DAP-deprival did not produce any noticeable effects until growth had proceeded for 45–60 min. In both instances the changes consisted of a swelling of the bacteria, followed by the appearance of hernia-like extrusions of the wall; some of these extrusions measured up to 1 μ in length (Pl. 1, figs. 4–6). In later stages during either treatment the extruding cytoplasmic contents form large spherical bodies, frequently with portions of the walls still attached to them (Lederberg, 1956; Liebermeister & Kellenberger, 1956). Bacteria without sucrose protection seem either to burst very suddenly or to leak at many small areas of their wall simultaneously, thus frequently obscuring observation with the light microscope of the site of burst. Finally, 2 or 3 hr. later, the bacteria became spheroplasts in the penicillin-treated series. The later stages differed somewhat among the strains.

In DAP-deprived cultures of *Escherichia coli* M203 many bacteria became rather long (5 μ and more) and seemed to form extrusions less frequently; instead, they were often blown up to bottle- and spindle-shaped forms (Pl. 4, fig. 12). The phenomenon could usually be observed in the central portions of the bacterium, less frequently at the poles or at zones where the cytoplasm had retracted from the wall. After *E. coli* M173–25 was deprived of DAP for 60–90 min., most of the swollen bacteria had gradually rounded up and formed spherical spheroplasts without bursting. Only occasionally were longer forms (3–4 μ) found, some of them with asymmetrical deformations. Formations of spheroplasts remained incomplete in DAP-deprived cultures of *E. coli* M203, even after prolonged incubation (4 hr.); furthermore, long forms were observed with irregularities in their form of growth; branching, Y-shaped forms were frequently found.

Electron microscopy

Negative staining of whole bacteria in the electron microscope confirmed the light microscope results about the size and shape of *Escherichia coli*. The surfaces of bacteria in logarithmic and in stationary phases looked similar.

The application of frozen-state microtomy (Bayer & Anderson, 1965) yielded no apparent difference between *Escherichia coli* B and *E. coli* M203 except that the latter seemed to be more vulnerable to mechanical injury. The structures rendered visible by this technique represent mainly the two outer layers with their channels and small protrusions (Pl. 1, fig. 7). The particulate protein of the rigid layer became visible after removal of the lipoprotein and lipopolysaccharide from the walls with sodium dodecyl sulphate (Pl. 2, figs. 8, 9), while the mucopolymer seemed to remain invisible in negatively stained preparations. The rigid layer contained gaps and holes (100–200 Å) in the otherwise uniform distribution of its proteinaceous cover (Pl. 2, fig. 9). The number of discontinuities depended on the state of growth: almost all bacteria in the logarithmic phase showed numerous gaps (Pl. 2, fig. 9), whereas in stationary-phase bacteria gaps and holes were very rare. Untreated stationary-phase bacteria resembled closely the example in Pl. 2, fig. 8 in this respect. The distribution of the gaps seemed to be random.

Heat-killed bacteria exhibited gaps to the same extent and with the same distribution as did unheated controls; this seems to exclude the possibility that the gaps were formed by the action of autolytic enzymes during preparation (Weidel, Frank & Leutgeb, 1963).

The proteinaceous cover is composed of particles which were better defined in cell walls of stationary bacteria than in the walls of logarithmically growing bacteria (compare Pl. 2, figs. 8, 9). In both phases of a culture the smallest ball-shaped protein units in the rigid layer of the cell walls measured from 50 to 70 Å in diameter and frequently contained a less compact or possibly hollow centre, filled with the staining material (Pl. 2, fig. 8, arrows). This property was not affected by the penicillin treatment.

The effects of penicillin become visible only in logarithmically growing bacteria; no effect was observed on the walls of stationary-phase bacteria. In agreement with the light microscope observations, the first effects on the walls showed up after the bacteria had been exposed to penicillin for 15–20 min. They consisted of bag-shaped extrusions of the walls, occasionally rather large in size and frequently including portions of cytoplasmic membrane. In other organisms the wall was buckled out at

numerous small areas (Pl. 3, fig. 10); the visible wall structure seemed still to be continuous in these locations. Increasing time of penicillin treatment (to about 30 min.) caused drastic changes in the walls of most of the bacteria: the channels disappeared, the contour of the walls became lobulate and the surface appeared to be made of large round or oval patches. The rigidity of the walls decreased and the bacteria assumed a round or oval shape. In the final stages, and probably as a result of mechanical injury, the walls disintegrated into scattered circular discs of different sizes. The cytoplasmic membrane was visible inside the walls as a lighter grey structure with a continuous outline (Pl. 1, fig. 7; Pl. 3, fig. 10; Pl. 4, fig. 13). The outer zone was slightly brighter than the other portions and measured about 70 Å in width in penicillin-treated bacteria as well as in the control organisms. This value is in agreement with the thickness of cytoplasmic membranes in general.

When the lipid layers of bacteria treated with penicillin were removed, a considerable widening of the dark gaps and holes in the remaining rigid layer was observed (Pl. 3, fig. 11). The total number of gaps did not seem to increase as compared with untreated bacteria under similar growth conditions. In general, the widening of the discontinuities was randomly distributed over the entire wall residue. Sometimes, however, the poles of the walls appeared to be excluded or seemed less affected by this phenomenon than were the other portions of the wall (Pl. 5, fig. 15), an effect most commonly observed in bacteria growing in media which contained 18–20% sucrose. Penicillin treatment without sucrose protection caused a much more even distribution of the wall alterations, including those in the polar regions. Finally the widening discontinuities formed a network separating the 'older' portions of the rigid layer from one another (Pl. 5, fig. 15). Among the rigid residues occasionally circular or lobulate pieces with a central hole of 200–700 Å diameter were found (Pl. 3, fig. 11, enclosed in squares). The outside dimensions of these pieces varied from 800 to 1500 Å and fragments were found scattered all over the supporting film. They were originally covered by the lipid material of the wall, and provided the disc-shaped elements of the distintegrating wall with their rigid 'skeleton'. However, not all of the discs of disintegrating walls are necessarily equipped with a piece of rigid mucopolymer, since lipopolysaccharides extracted from walls spontaneously form sheets (Weidel, Frank & Martin, 1960) and discs (Bladen & Mergenhagen, 1964).

The alterations in the walls of the auxotrophic mutants *Escherichia coli* M203 and M173–25 after DAP-deprivation consisted of a large increase in the amount of soft lipid material which protruded from the walls in cloud-like patterns (Pl. 4, fig. 13). These clouds emerged from small localized areas on the wall. As in the penicillin-treated bacteria, DAP-deprivation of the auxotrophic mutants caused in the rigid layer a considerable increase of 'darker' areas (discontinuities) at the expense of the 'old' mucopolymer. As compared with the effects of penicillin, however, the following differences were observed: (1) the rigid layer of mutant M203 disintegrated into more numerous, smaller patches; (2) the gaps in the walls of DAP-deprived mutant M203 seemed not to be widened by stretching of the cell wall by osmotic forces: the gaps were no wider in the extended regions of spindle-shaped bacteria than in the less deformed or normal sized regions. An explanation may be sought in the slight 'leakage' of the M203 mutant (Dr C. Gilvarg, personal communication) which might provide for a low repair rate, a rate low enough to be initially overcome by osmotic forces, but eventually rapid enough to prevent bursting or even expansion to complete sphero-

plasts. DAP-deprived mutant M173–25 (which is not leaky) frequently showed a rigid layer which had a strong resemblance to the rigid layer of bacteria treated with penicillin.

DISCUSSION

Immunological studies of the pattern of synthesis of the surface antigens in Gram-negative bacteria have yielded support for the assumption of scattered growth zones in the outer layers of the wall (May, 1963; Cole, 1964; Beachey & Cole, 1966). However, firm conclusions about the growth of the rigid layer cannot be drawn from immunological reactions alone; the mucopolymer is apparently not exposed on the outside of the cell. Furthermore, it is not known how firm are the bonds between the soft lipid outer coat and the rigid layer (Rogers, 1965), thus raising the problem of lateral mobility of the antigenic soft material on the surface.

The relation between the lipid layers and the rigid mucopolymer seems to be altered by interference with the synthesis of the mucopolymer, especially after DAP-deprival of the auxotrophic mutants of *Escherichia coli*. The hernia-like extrusions visible in the early stages of penicillin treatment can be explained sufficiently by a relative increase of the osmotic pressure inside the wall when the bacteria are in an environment of lower osmotic pressure. In favour of this view is the fact that we occasionally found the cytoplasmic membrane driven into such an outpocketing. In DAP-deprived bacteria, however, a different mechanism has to be found to interpret the formation of the large amounts of soft material which protrudes from the wall with no deformation of the cytoplasmic membrane. One may tentatively assume that the rate of synthesis of the mucopolymer is decreased relative to the synthesis of wall lipids. Such a disproportionate growth would result in the accumulation of excess 'soft' material outside the bacterium.

The synthesis of the mucopolymer in *Escherichia coli* has been followed by using radioactive DAP (van Tubergen & Setlov, 1961). Their results indicated that the rigid layer contained many, more or less randomly scattered, synthesizing 'centres'; the label of the wall of the parent bacterium had been 'randomly' inherited in at least 200 fragments to the daughters.

If one assumes that the rigid layer has been opened at the gaps we observed in log-phase bacteria to allow for new synthesis, this synthesis would indeed seem to occur at many randomly scattered places. That autolytic processes produce a similar morphological pattern on the rigid layer was shown by Weidel *et al.* (1963) in autolytically damaged bacteria where wide zones free from the particulate proteinaceous layer appeared on the wall residues. The function of the proteinaceous particles which cover the mucopolymer is unknown. They are best defined and most evenly distributed in the walls of bacteria in stationary-phase cultures. On the other hand, they seem to disappear completely as morphological entities in certain L-forms of *Proteus mirabilis*. Martin (1964) therefore suggested that this protein in its particulate form might have an important function in the morphogenesis of the rigid mucopolymer. Our results suggest a more subtle interaction; for after penicillin treatment, when cross-linkage is prevented, the newly synthesized portions of the mucopolymer layer lack a layer of proteinaceous particles, indicating that the deposition of the protein as particles might depend on the conformation of the mucoprotein.

Murray *et al.* (1965) found that bacterial walls (*Escherichia coli* and some other

Gram-negative bacteria) lost about 20–35 Å of their thickness after penicillin-induced spheroplast formation; an even greater loss could be accounted for by the disappearance of the particulate protein. Taking the presence of proteinaceous particles to indicate an undisturbed rigid layer, we interpret the gaps normally found in this layer as sites where the mucopolymer had been temporarily opened to allow the introduction of new building blocks into the growing wall. The wider gaps in penicillin-treated bacteria as well as in the DAP-deprived mutants might represent locations where cross-linkage was not sufficiently achieved during an appreciable fraction of the growth period.

The distribution of the gaps in the wall of a penicillin-treated *Escherichia coli* organism seemed in most cases to be random, especially in the cylindrical part of the wall. In some of the bacteria, however, portions of the walls, predominantly the poles, were less affected or even completely free from lesions. Widened gaps were then found in the central portions of the cell wall, where the wall finally burst, a sequence of events which was followed frequently with the phase-contrast microscope. Also the formation of spindle-shaped cells after DAP-deprivation of the auxotrophic mutants can be explained by an initial disproportionate weakening of the central or cylindrical portions of the walls, the zones which contain the sites where the new cell boundary will be formed.

Assuming that the presence of the cell membrane is necessary for the action of hydrolysing and polymerizing enzymes, one can tentatively attribute uneven distribution of the widened gaps to uneven plasmolytic retraction of cell membrane from cell wall, e.g. in sucrose solutions. The action of enzymes would then be decreased or even interrupted at those areas of the walls where the cytoplasm had been retracted. When the plasmolysing effect of sucrose was not operative, a more even distribution of the lesions was achieved, including at the poles of the cell walls. It is of interest that the surface antigens at the poles of *Escherichia coli* seem to be less involved in synthesis than do other portions of the wall (Beachey & Cole, 1966). Under normal growth conditions, a less firm contact of wall and membrane at the cell poles might be involved in this phenomenon; plasmolysis is first detectable there. However, there might well be other factors involved in the exclusion of certain areas of the wall from such phenomena as formation of widened gaps after inhibition of mucopolymer synthesis. The possibility has to be considered of localized signals for the division of the cell wall from the bacterial DNA via the cell membrane. This might include an increase in synthesis and transport of mucolytic enzymes to the sites of intensified 'modelling' of wall material during division (Rogers, 1965).

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EXPLANATION OF PLATES

PLATE 1

Figs. 1–6. Phase-contrast micrographs, magnification × 3300. The bar equals 3 μ.

Fig. 1. *Escherichia coli* B from a culture growing logarithmically in L medium.

Figs. 2, 3. *E. coli* B, shortly after transfer to L medium containing 18 % (w/v) sucrose. The cytoplasm is retracted from the wall mainly at the poles of the bacteria (compare Pl. 5, fig. 15).

Figs. 4–6. *E. coli* B after 20 min. penicillin treatment in L medium. Some of the bacteria have developed extrusions of the wall.

Fig. 7. Electron micrograph showing channel-like structures in the wall of *E. coli* M 203 grown in the presence of diaminopimelic acid. × 100,000.

PLATE 2

Rigid layer of *Escherichia coli* B, magnification × 100,000.
In all the electron micrographs except Pl. 5, the bar represents 0·2 μ.

Fig. 8. Stationary phase, after treatment with penicillin in L medium with 18 % (w/v) sucrose for 20 min. The arrows point to ball-shaped particles with 'hollow' centres.

Fig. 9. Logarithmic phase, without penicillin or sucrose.

PLATE 3

Escherichia coli B after penicillin treatment of a culture growing logarithmically in L medium without sucrose, magnification × 100,000.

Fig. 10. Cell wall with numerous extrusions of the non-rigid portions of the wall.

Fig. 11. Rigid layer of such a wall showing wide gaps and holes in the proteinaceous cover of the mucopolymer. Two of the zones are enclosed in squares where wall components are separated into circular or lobular elements containing a central hole.

PLATE 4

Escherichia coli auxotrophic mutant M 203

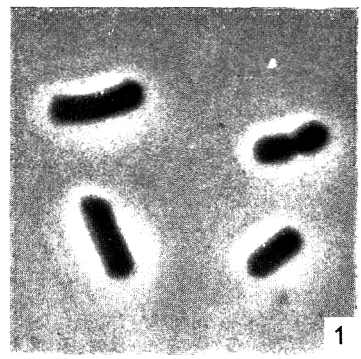
Fig. 12. After DAP-deprivation for 3 hr.; phase contrast; × 3300.

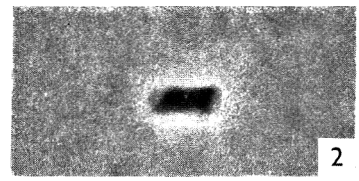
Fig. 13. Cell wall of an organism from the same culture; electron micrograph; × 100,000.

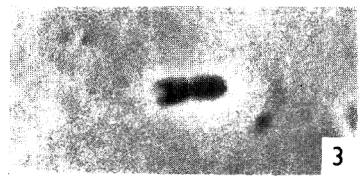
Fig. 14. Rigid layer of the wall of another organism from the same culture, revealed by extraction with sodium dodecyl sulphate; × 100,000.

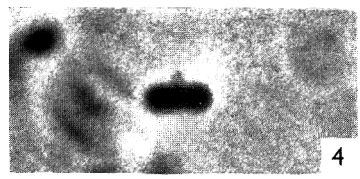
PLATE 5

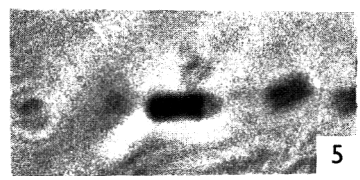
Fig. 15. Wall of *Escherichia coli* B, logarithmic phase, after penicillin treatment for 20 min. in L medium containing 18 % (w/v) sucrose; × 48,000. The bar represents 0·5 μ. Gaps and holes are suppressed in the polar regions (compare Pl. 1, figs. 2, 3).

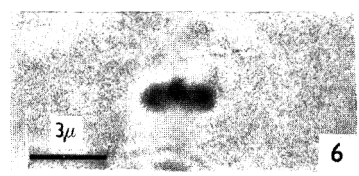












M. E. BAYER

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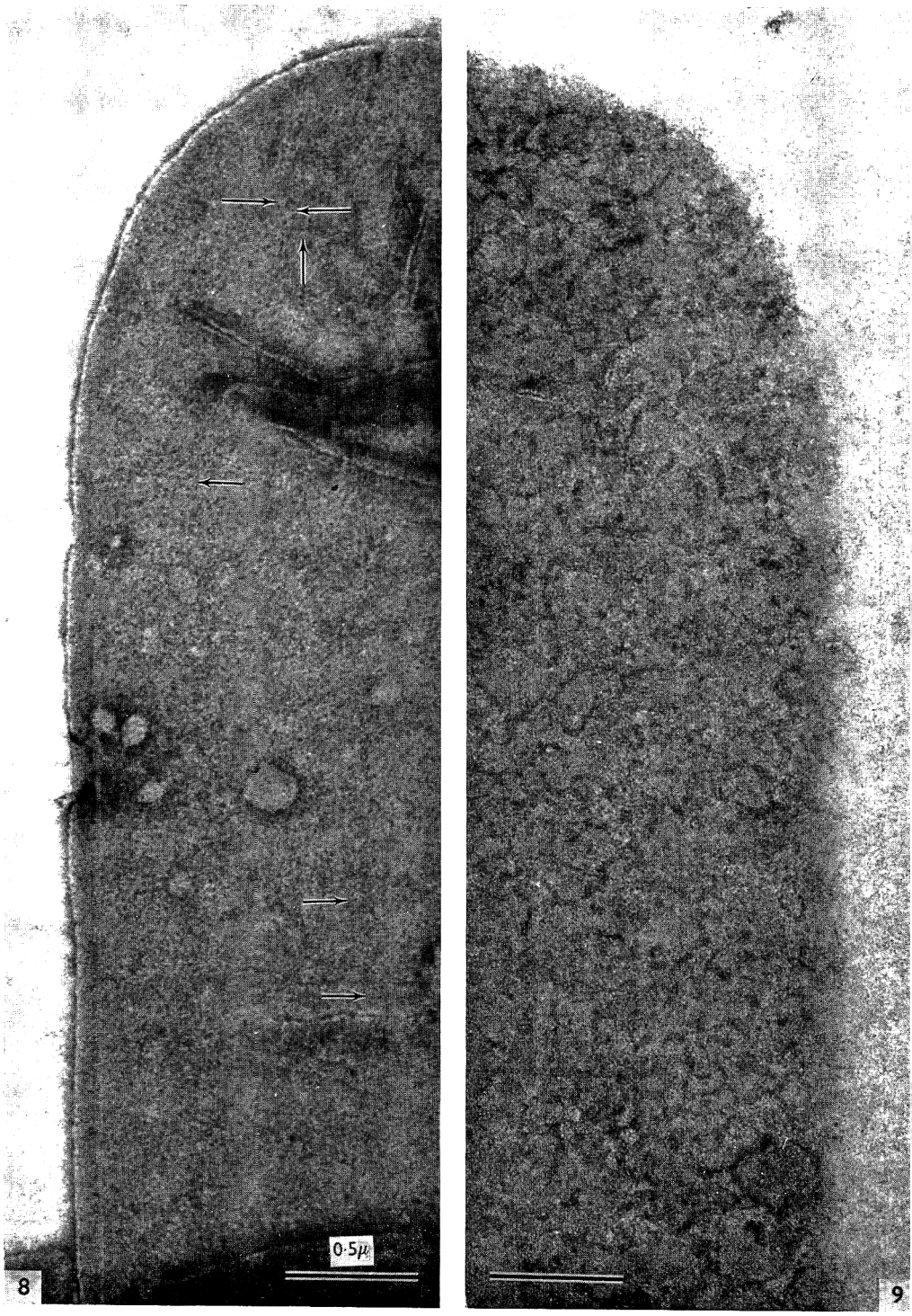

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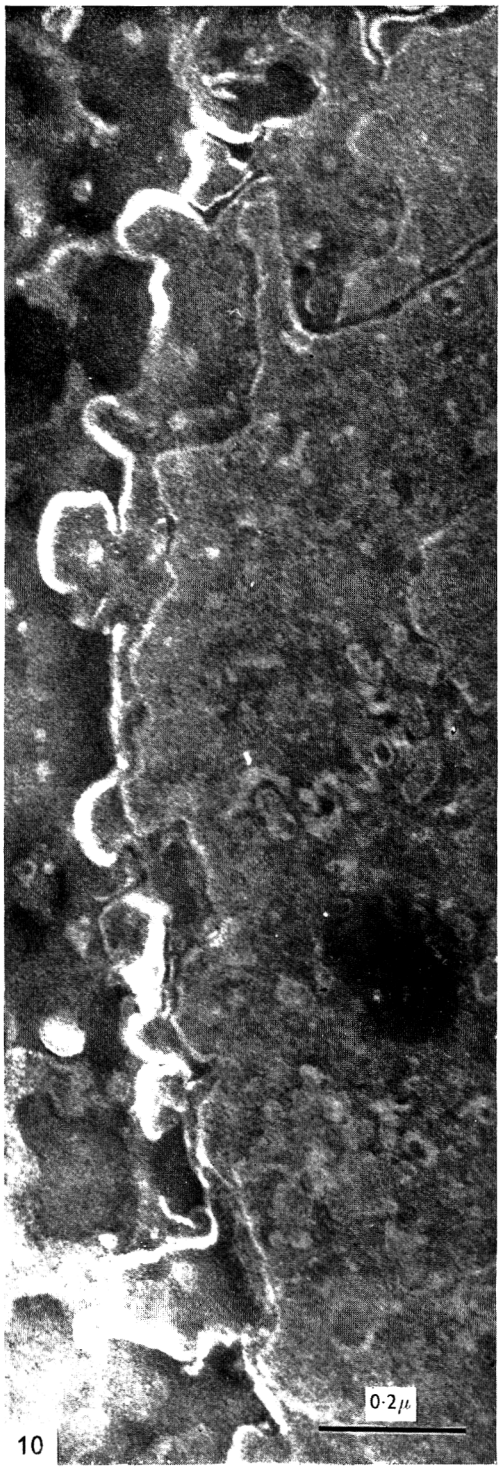

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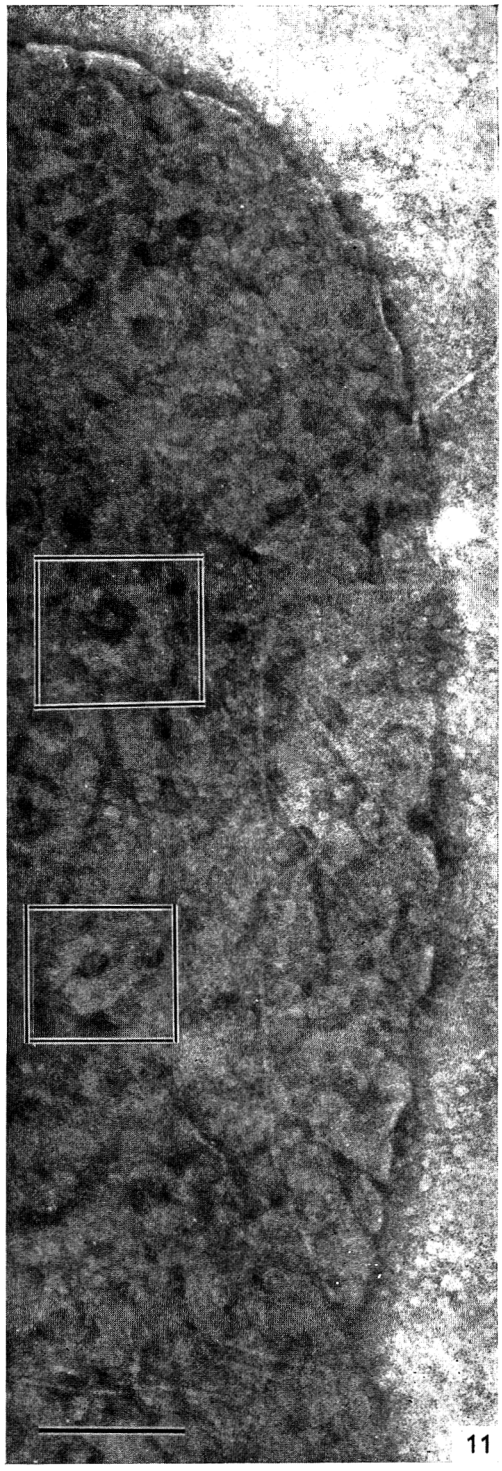

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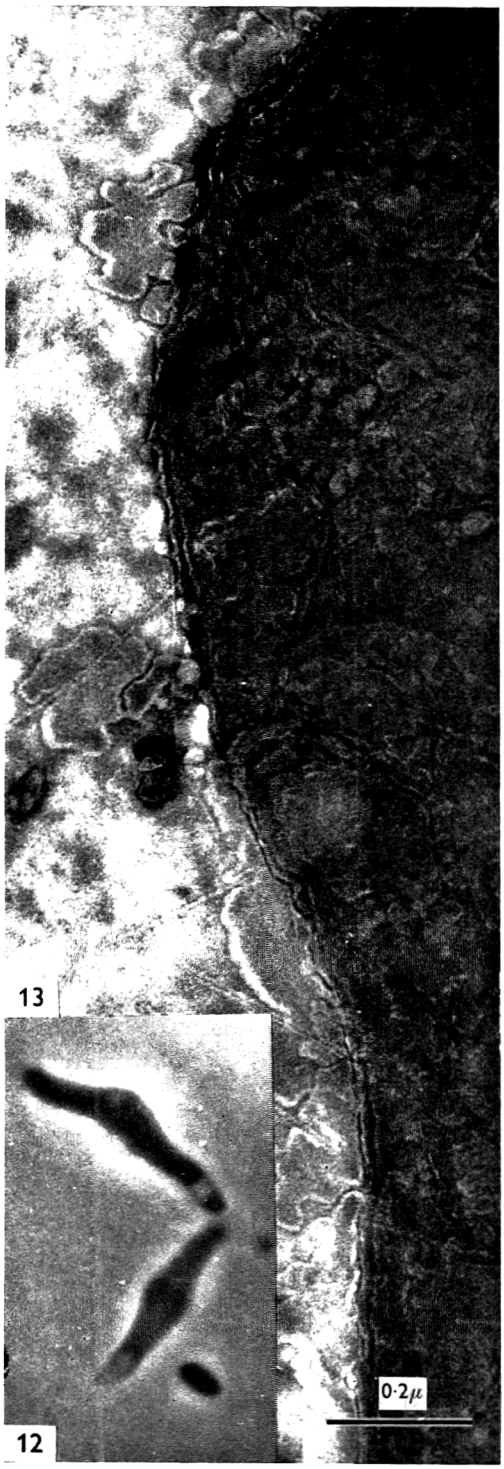

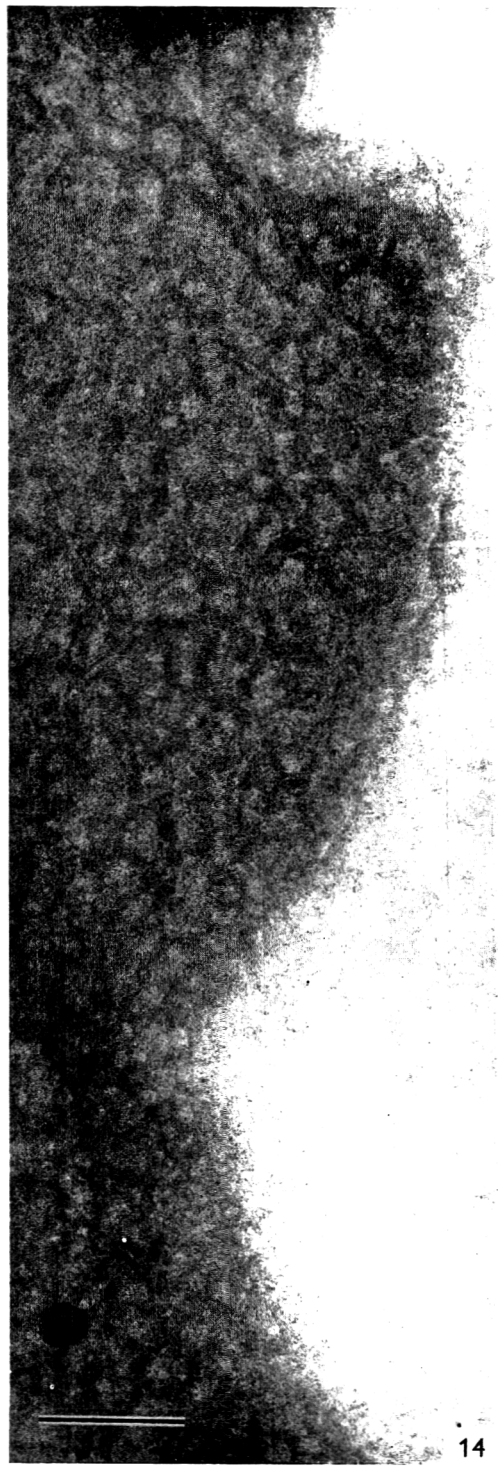

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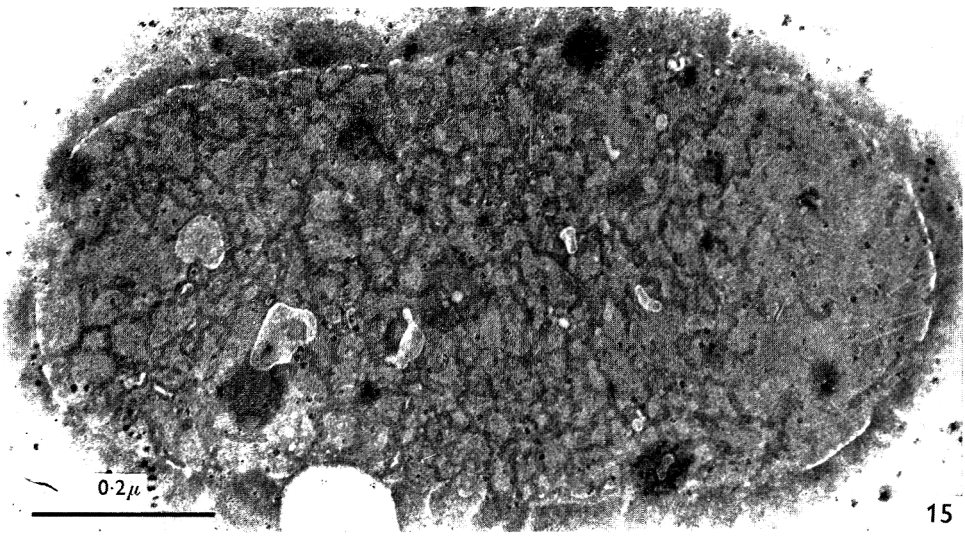
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Serum Mediated Killing of Three Group D Salmonellas

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SUMMARY

Salmonella enteritidis, *S. pullorum* and *S. gallinarum* were found to be sensitive to complement-mediated killing by fresh normal human serum but far less so to sera of the mouse, rat or rabbit. *S. pullorum* but not *S. gallinarum* was sensitive to the microbicidal activity of adult fowl serum. *S. enteritidis* was slightly sensitive to the latter serum. Day-old-chick serum was unable to kill either *S. gallinarum* or *S. pullorum* even when undiluted serum was used. When human serum was absorbed at 4° by dense suspensions of dead organisms it was found that all antimicrobial activity against the three organisms was absorbed only when the homologous organism was used as the absorbing strain. This suggests that minor somatic antigenic differences occur between the three related organisms. *S. pullorum* was extremely sensitive to the action of monospecific rabbit antisera. However, serum concentrations which were highly effective against *S. pullorum* were less effective for *S. enteritidis*. Even highly microbicidal serum concentrations were unable to kill 100% of the *S. enteritidis* within the time period of the test. The relevance of these findings to the known differences in the virulence of these three salmonellas for the mouse and the chicken is discussed.

INTRODUCTION

The high degree of resistance of some animals to bacteria which may be highly pathogenic for a closely related host species presents a fascinating problem. The factors involved in this so-called 'natural' immunity are known to be complex and variable, depending upon the nature of the parasite and host. For instance, a definite correlation has been shown between the presence of β-lysins in the blood and resistance to *Bacillus anthracis* and to infections by some other Gram-positive bacteria (*Topley & Wilson's Principles*, 1964). However, the β-lysins do not appear to be active against Gram-negative bacteria. In general, resistance to infection by the latter organisms shows no simple correlation with serum factors.

The microbicidal activity of fresh serum taken from apparently normal animals has been known for many years (Mackie & Finkelstein, 1928; Gordon & Carter, 1932), but it is only in recent years that the dual requirement for specific antibody and complement has been clearly established (Skarness & Watson, 1957). Adler (1953) showed that natural antibody present in the serum of uninfected animals combined specifically with surface antigens of susceptible bacteria. These antibodies were probably produced in response to cross-reacting bacterial antigens present in the normal enteric flora of most animals. Recently, Michael, Whitby & Landy (1962) demonstrated microbicidal antibody specific for *Escherichia coli* and *Salmonella typhi* in normal mice. They

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showed that serum from newborn mice did not contain antibacterial antibody but rapidly acquired it within a week of birth. Fisher & Manning (1958) reported that normal human serum contained antibodies against *E. coli*, Proteus species and *Staphylococcus aureus*, but not against *S. typhimurium*, *Klebsiella pneumoniae* or *Streptococcus pyogenes*. These workers suggested that there may be a significant correlation between the resistance of the host to infection by some bacteria and the presence of specific antibody in the circulating blood. Although a number of such correlations have been reported in the past, no general pattern has emerged, and recently Muschel, Chamberlin & Osawa (1958) re-emphasized that resistance to a particular disease cannot be explained simply in terms of an *in vitro* phenomenon. Nevertheless, there exist instances in which a definite correlation has been shown between the degree of microbicidal activity in the serum and resistance to a particular pathogen. In these circumstances serum-mediated killing may be a contributory factor to host resistance.

In the present study, considerable differences in the serum sensitivity of three closely related salmonella strains were observed. In view of the known differences in virulence of the three strains for mice and chickens, a further investigation was made of their sensitivity to serum-mediated killing.

METHODS

Organisms. The strains of organisms used were described elsewhere (Collins, Mackaness & Blarden, 1966). They were grown on Oxoid blood base agar slopes enriched with 1·0% (w/v) Casamino acids and 0·1% yeast extract (Oxo Ltd., London). Fresh subcultures were made at 3-month intervals. After four transfers the culture was discarded and a fresh freeze-dried culture was opened.

Sera. Fresh human group AB serum was obtained from the Red Cross Blood Bank, Adelaide, dispensed in 5 ml. ampoules and stored at −20° until required. Before use the serum was thawed and absorbed at 4° for 60 min. with 2 mg. dry wt. of the appropriate strain of bacteria (previously killed by heating to 56° for 30 min.) per ml. of serum. The bacteria were removed in a Serval refrigerated centrifuge (model RC2) and the absorption procedure was repeated. Absorbed serum was used immediately as an added complement source where indicated in the text. Haemolytic complement titrations, carried out before and after absorption (Mackie & McCartney, 1962), showed a decrease in titre of no more than one tube after the double absorption. Fresh fowl and chicken sera was obtained by heart puncture from certified *Salmonella pullorum*-free stock (Government Poultry Farm, Parafield). They were immediately used and were not enriched with absorbed human complement-containing serum. Mice were bled by the method described by Rosenberg & Tachibana (1962). Monospecific H and O rabbit immune sera were prepared as recommended by Kauffmann (1954). These sera were heated at 56° for 30 min. and stored at −20° until required. Absorbed human serum (1/10) was used as a source of complement in all bactericidal tests involving rabbit immune sera.

Bactericidal tests. Test sera were diluted in Davis & Mingioli (1950) basal medium without the addition of glucose. Where required, an equal volume of absorbed human serum was added to each sample (final dilution 1/10). Heated human and test sera (56° for 30 min.) controls were always included. After equilibration at 37° the serum dilutions were inoculated with 1000–2000 logarithmic-phase bacteria. Samples were

removed after thorough mixing and colony counts made at 0, 20, 40 and 60 min. The end titre was taken as the highest dilution of serum which (in the presence of excess complement) resulted in 50% killing within 1 hr.

RESULTS

Microbicidal activity of fresh normal serum for Salmonella enteritidis, S. pullorum and S. gallinarum

The microbicidal titres of fresh serum from five animal species showed considerable variation when tested against the three strains of Salmonella (Table 1). Whereas the rabbit and rat sera showed little or no activity against *Salmonella enteritidis*, human serum was active against all three strains. The absence of activity in rabbit serum was due to a lack of specific antibody, since fresh serum from rabbits immunized with a heat-killed vaccine of *S. enteritidis* was highly bactericidal for this organism. Fresh mouse serum was not microbicidal for *S. enteritidis* even when absorbed human complement was added. *Salmonella gallinarum* was killed by 1/4 dilution of mouse serum, but variable results were obtained when the test was repeated at a 1/5 dilution. *Salmonella pullorum* was sensitive to a 1/5 to 1/10 dilution of mouse serum.

Table 1. *Antimicrobial titres against three salmonellas of fresh normal sera obtained from animal species*

	Inverse of end titre*		
Normal sera	*S. enteritidis*	*S. gallinarum*	*S. pullorum*
Human	20	20	40
Rabbit	0	0	5
Rat	2	10	0
Mouse	0	4	5–10
Chicken			
1-day	5	0	0
7-day	5	0	5
30-day	10	0	20
90-day	8	0	16

* No extraneous source of complement was used in these tests.

Fresh adult fowl serum was microbicidal for both *Salmonella enteritidis* and *S pullorum* but not for *S. gallinarum*. This contrasted sharply with the titres obtained for day-old chicken serum (Table 1) which did not kill either *S. pullorum* or *S. gallinarum* even when undiluted serum was tested. The progressive change in the microbicidal titre of a serum pool taken from 20 maturing chickens is recorded in Table 1. Killing curves for the three Salmonella strains tested against 1-day chick and 3-month fowl sera are recorded in Figs. 1–6. The adult fowl serum was bactericidal for both *S. enteritidis* and *S. pullorum* at a dilution of 1/10, but as many as 20% of the *S. enteritidis* still survived after 60 min. This could be compared with less than 1% survival of *S. pullorum* in this time. The 10–20% survival rate for *S. enteritidis* occurred even when undiluted serum was used. Addition of fresh serum to the tube after 60 min. had little further effect on the viability of the survivors. After culture in broth, the survivors yielded a population of bacteria which gave a killing curve almost

identical to the original. Thus, the serum treatment had not selected a stable population inherently more resistant to serum-mediated killing. The significance of this finding will be discussed later.

Bactericidal activity of absorbed rabbit sera for the three strains

The observed variation in the rate and extent of killing of the three Salmonella strains by normal serum justified a further examination of the bactericidal reaction with absorbed rabbit serum specific for somatic antigens 0–9 or 0–12. Absorbed human serum was added as a complement source throughout. Preliminary experiments

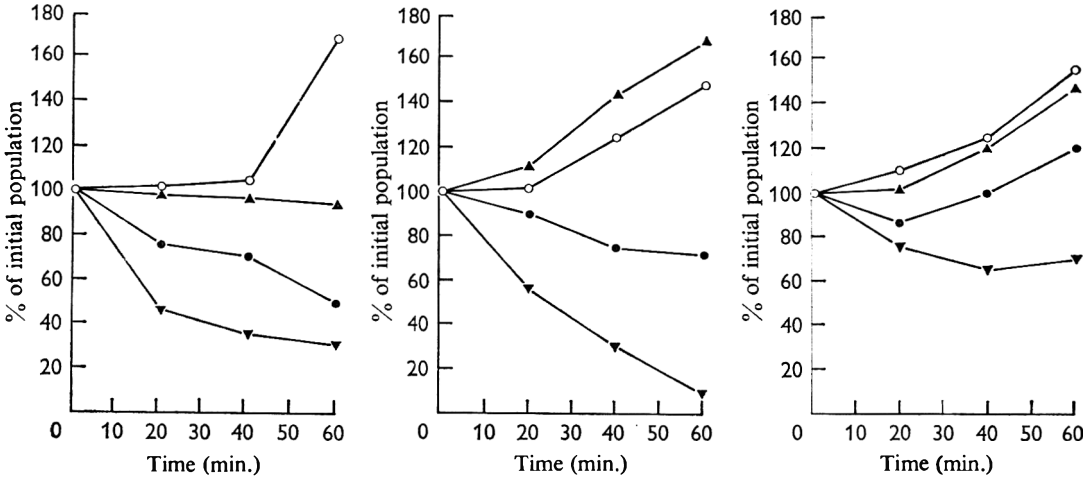

Fig. 1

Fig. 2

Fig. 3

Fig. 1. Killing curves for *Salmonella enteritidis* in decreasing concentrations of fresh 1-day chicken serum. The control contained a 1/5 dilution of serum heated to 56° for 30 min. Absorbed human serum (1/10) was added as a source of complement. ▼—▼, 1/2 serum; ●—●, 1/5 serum; ▲—▲, 1/10 serum ○—○, control.

Fig. 2. Killing curves for *Salmonella pullorum* in decreasing concentrations of fresh 1-day chicken serum. Absorbed human serum (1/10) added as a source of complement. Symbols as for Fig. 1.

Fig. 3. Killing curves for *Salmonella gallinarum* in decreasing concentrations of fresh 1-day chicken serum. Absorbed human serum (1/10) added as a source of complement. Symbols as for Fig. 1.

Table 2. *The microbicidal activity of human serum pre-absorbed at 4° by increasing amounts of three Salmonellas*

Absorbing suspension		Inverse of microbicidal titre*		
		S. enteritidis	*S. gallinarum*	*S. pullorum*
Nil		20	20	40
S. enteritidis	(2 mg./ml.)	2	5	10
	(4 mg./ml.)	0	5	5
	(10 mg./ml.)	0	0	0–2
S. gallinarum	(4 mg./ml.)	5	0	10
S. pullorum	(4 mg./ml.)	10	20	0

* No extraneous source of complement was used in these tests.

showed that absorption of the human serum with dense suspensions of *Salmonella enteritidis* rapidly removed all activity for *S. enteritidis*, but that at least five times as many bacteria were necessary to remove all activity against *S. gallinarum* and *S. pullorum* (Table 2). Similar results were observed when *S. gallinarum* or *S. pullorum* were used for the cross-absorptions. Absorption of human serum in these tests was therefore always made with killed suspensions of the organism to be used in the final test.

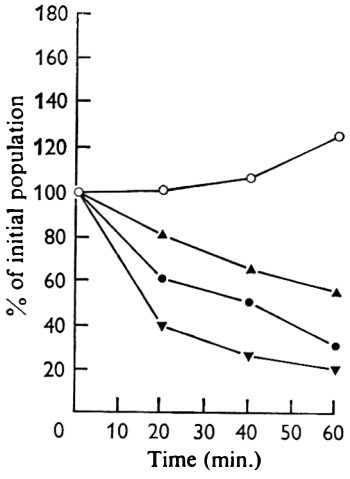

Fig. 4

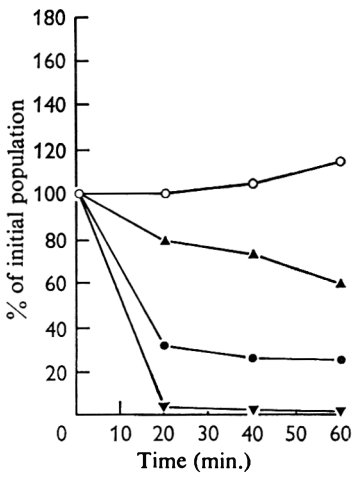

Fig. 5

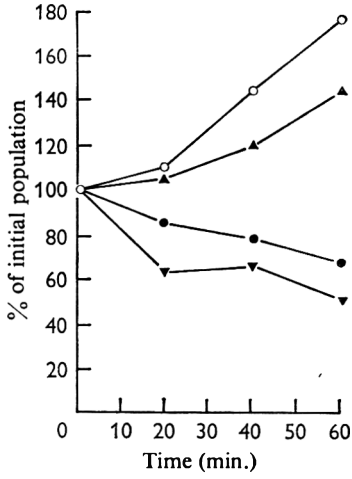

Fig. 6

Fig. 4. Killing curves for *Salmonella enteritidis* in decreasing concentrations of fresh adult fowl serum. No extraneous complement source added. ▼—▼, 1/5 serum; ●—●, 1/10 serum; ▲—▲, 1/20 serum; ○—○, control.

Fig. 5. Killing curves for *Salmonella pullorum* in decreasing concentrations of fresh adult fowl serum. No extraneous complement source added. ▼—▼, 1/10 serum; ●—●, 1/20 serum; ▲—▲, 1/40 serum; ○—○, control.

Fig. 6. Killing curves for *Salmonella gallinarum* in decreasing concentrations of fresh adult fowl serum. No extraneous complement source added. Symbols as for Fig. 5.

Table 3. *Microbicidal activity of monospecific rabbit antisera*

	Inverse of end titre*		
Serum	*S. enteritidis*	*S. gallinarum*	*S. pullorum*
Unabsorbed immune serum	10^4	10^5	10^6
Specific 'H' (g.)	10†	–	–
Anti-0-1	50	–	–
Anti-0-9	10^3	10^5	10^5
Anti-0-12	5×10^2	10^4	10^5

* Specifically absorbed human serum (1/10) was added as complement source throughout.
† This activity was probably due to traces of incompletely absorbed 'O' antibody.
– = not done.

The microbicidal titres of the monospecific rabbit serum are recorded in Table 3. *Salmonella gallinarum* and *S. pullorum* were 10 to 100 times more sensitive to monospecific antibody than was *S. enteritidis*. Essentially similar results were obtained with absorbed serum obtained from fowls vaccinated with heat-killed *S. pullorum*.

DISCUSSION

For the most part, early work failed to show any consistent correlation between the resistance of an animal species to infection and the microbicidal activity of the serum to the infecting organism (Skarnes & Watson, 1957). This is hardly surprising in view of the complex interactions which occur between host and parasite during the evolution of an infection. The failure of both rabbit and rat serum to kill *Salmonella enteritidis* even though both animals are totally resistant to infection by this organism exemplify this lack of correlation. However, microbicidal activity of sera from mice and developing chickens shows a closer correlation with the natural susceptibility of these animals to infection with the three test strains of Salmonella. Thus, the serum of the 1-day chick, which is susceptible to infection by both *S. pullorum* and *S. gallinarum*, was unable to kill either strain of organism *in vivo* when diluted more than 1 in 2, even in the presence of excess complement. An extraneous source of complement was thought essential for testing the 1-day chick sera. This was suggested by the finding of Dr K. Karthigasu (personal communication) that chick embryos were unable to kill even an avirulent variant of *S. gallinarum*. It was shown that bactericidal activity of the developing chick serum correlated well with its haemolytic complement value. Shortly after hatching the haemolytic complement activity of the chicken serum increased and serum-mediated killing could then be detected.

Adult fowls were susceptible to *Salmonella gallinarum* infections but not to *S. pullorum*; and the increased microbicidal activity of the adult birds' sera for *S. pullorum*, but not for the antigenically similar *S. gallinarum*, was therefore very interesting. Since cell walls of both organisms contain approximately equal amounts of lipopolysaccharide possessing antigen 0–9 and 0–12 specificity, the bactericidal activity of normal sera for these strains must depend on antibodies specific for antigens other than those listed in the Kauffmann–White scheme. The observed differences in the absorptive abilities of suspensions of the three Salmonella strains for the microbicidal antibody present in normal human serum (Table 2) lends further weight to the argument that minor cell-wall antigens may be important factors in determining the susceptibility of Gram-negative bacteria to complement-mediated killing.

Salmonella pullorum appeared to be exquisitely sensitive to serum killing, whereas *S. enteritidis* was relatively resistant. Furthermore, almost 100% of the *S. pullorum* cells were killed, in 20 min. or less, by concentrations of antibody which could kill only 80% of the *S. enteritidis* in 60 min. Specific anti-9 antibody was still bactericidal for *S. pullorum* and *S. gallinarum* at a dilution of 1/100,000, whereas a 1/1000 dilution was needed to kill *S. enteritidis*.

Rough strains of Gram-negative bacteria have been reported in general to be more susceptible to the microbicidal action of normal serum than are the antigenically smooth parent strains (Muschel *et al.* 1958). Suspensions of the *Salmonella pullorum* strain used in this study were therefore tested for smoothness. By the slide-agglutination test, the suspension was stable in the presence of 0·1% acriflavine (Mackie & McCartney, 1962) and only slight auto-agglutination was observed when the organisms were incubated at 56° overnight. The extreme sensitivity of *S. pullorum* to serum-mediated killing could not, therefore, be explained in terms of S–R variation.

One further possible explanation for the observed differences in sensitivity might be that the antigenic groups on *Salmonella pullorum* cell walls are optimally situated for

complement-mediated killing, whereas in *S. enteritidis* (and to a lesser extent in *S. gallinarum*) fewer such sites are available. However, this could not explain the observed differences in the final percentage kill of the three test organisms. Nevertheless, the correlation between the increasing degree of serum activity in developing fowls and the corresponding increase in resistance to infection may be causally related. Investigation of the levels of specific microbicins in the sera of other animal species which show similar striking differences in their susceptibility to infection by antigenically related Salmonella may reveal similar correlations to that found in the present study.

I wish to thank Mr R. Walsh for his excellent technical assistance during this study. I am indebted to the Red Cross Blood Bank, Adelaide, for generously supplying the human serum used.

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The Growth and Metabolic Versatility of the Gram-negative Bacterium NCIB 8250 ('Vibrio 01')

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SUMMARY

The nutritional requirements of the Gram-negative Bacterium NCIB 8250 (formerly referred to as 'Vibrio 01') have been determined. Growth was tested on almost 450 compounds, of which over 100 served as carbon + energy sources and almost 50 as nitrogen sources. The effects of various factors such as pH value, temperature and aeration on the growth rate and cell yield of organism have also been measured.

INTRODUCTION

Until recently the Gram-negative bacterium now in the National Collection of Industrial Bacteria (NCIB, Torry Research Station, Aberdeen, Scotland) as *Achromobacter* sp., NCIB 8250, was generally referred to as 'Vibrio 01', but Sebald & Véron (1963) and Fewson (1967) have shown that it belongs to the Acinetobacter–Moraxella group of bacteria. This organism has been used in experiments on the metabolism of aromatic compounds (e.g. Cain, 1961; Ali, Callely & Hayes, 1962; Chapman & Dagley, 1962; Griffiths, Rodriques, Davies & Evans, 1964; Callely & Jones, 1965), monocarboxylic and dicarboxylic acids (Dagley & Patel, 1955; Dagley, 1956; Callely, Dagley & Hodgson, 1958) and liquid paraffin (Linday & Donald, 1961). There appears, however, to have been no detailed study of its nutritional requirements or metabolic versatility. This paper records experiments on the growth and nutrition of the Bacterium NCIB 8250. The results obtained in testing growth on a large number of compounds have served to outline the metabolic capabilities of the organism and have indicated which catabolic pathways could be used in experiments on metabolic control.

METHODS

Organism. Stock cultures of Bacterium NCIB 8250 were maintained in Oxoid cooked-meat medium stored at 4°. Subcultures were made into Oxoid nutrient broth at intervals of approximately 2 months and were also kept at 4°. Inocula used in the growth experiments were produced by a further subculture into nutrient broth and incubated for 17–24 hr at 30° immediately before use.

Measurement of growth rates. Cultures were grown under conditions of vigorous aeration in an apparatus similar to that described by Schlegel, Kaltwasser & Gottschalk (1961). Medium (800 ml.) was contained in 1 l. flasks fitted with side-arms to facilitate sampling. Magnetic stirring drive assemblies, constructed by Mr N. Harvey in this Department, were arranged to accommodate 5 or 10 flasks in water baths equipped

with Circotherm II a constant temperature units (Shandon Scientific Co. Ltd., London). Aeration was effected by means of 45 mm. magnetic stirring bars encased in polypropylene. In all experiments the vortex produced by stirring extended to the bottom of the flask and was there disturbed by the stirring bar so that bubbles were introduced into the body of the medium (Schlegel *et al.* 1961). This arrangement allowed a large number of cultures to be grown in the laboratory under constant and identical conditions of aeration, agitation and temperature. Growth was followed by taking 4 ml. samples at 10 to 20 min. intervals and measuring the extinction at 350 mμ in a Spectronic 20 colorimeter.

When testing the effect of various concentrations of nutrients, the inoculum was grown in a chemically defined medium deficient in the appropriate compound. In some cases, e.g. potassium or iron, several subcultures through the defined deficient media were necessary to show any response to the nutrient under test. In all cases the concentration of only one ion was varied at a time and the accompanying ions were added in other forms.

Screening carbon and nitrogen sources. The basal media were as follows. (i) For testing carbon+energy sources: 4 g. KH_2PO_4+2 g. $(NH_4)_2SO_4$ dissolved in 1 l. glass-distilled water and adjusted to pH 7·0. (ii) For testing nitrogen sources: 4 g. KH_2PO_4 +5·4 g. sodium succinate.$6H_2O$ dissolved in 1 l. distilled water and adjusted to pH 7·0. Carbon or nitrogen sources at 10 mM, adjusted to pH 7·0, were sterilized by filtration through Millipore filters (GSWP 047 00, 0·22 μ). Suitable volumes of the sterile solutions and sterile water to give final concentrations of 0·1, 0·5 and 5 mM in a total volume of 50 ml. were then added to 25 ml. amounts of autoclaved basal medium in 250 ml. Erlenmeyer flasks plugged with cotton wool. Heat-stable compounds were frequently sterilized by autoclaving at 109° rather than by filtration. Water-insoluble volatile compounds (e.g. benzene, pentane) were placed in test tubes held upright in the growth flasks by the cotton-wool plugs, and thus could distil over into the half-strength basal medium as carbon sources. Other insoluble compounds were added to the half-strength basal medium either before or after autoclaving, depending on their heat stability. These substances, such as stearic acid, were therefore tested as saturated solutions and not at known concentrations. Then were added 1·0 ml. sterile 2% (w/v) $MgSO_4.7H_2O$ and, as inoculum, 0·1 ml. of a 24 hr nutrient broth culture. Growth was followed for 5 days at 30° on a rotary shaker (L. H. Engineering Co., Bells Hill, Stoke Poges, Bucks; Mk V) moving at about 180 oscillations/min. The growth response to different carbon or nitrogen sources was compared with that obtained in the presence of succinate as carbon source and ammonium as nitrogen source. Controls of the basal media alone were also included. Visual estimation of the growth responses to different substrates was made by comparison with standard flasks containing 0·1, 0·3, 1 and 3 mg. wet wt. bacteria/ml. This method gave results which were as satisfactory as turbidity measurements with an absorptiometer or nephelometer: indeed the final results of response to carbon and nitrogen sources reported in this paper are given as 'growth' or 'no growth'. In doubtful cases, such as those where the substrate itself produced turbidity, growth was checked by the colony count technique of Miles & Misra (Mackie & McCartney, 1953).

Materials. Dr W. H. Holms of this Department kindly synthesized the *N-o*-carboxyphenyl-D-glucosylamine (Bayne & Holms, 1952). All other compounds were from commercial sources.

RESULTS

Composition of medium and conditions for growth

The composition of the basal medium was examined by varying the concentration of each nutrient in turn. Extinction measurements gave an adequate estimate of cell mass since even under conditions of nitrogen or phosphate limitation the specific extinction was found to be almost the same as that of the bacteria grown in complete medium ($E_{350} = 1$ was equivalent to 0·28 mg. dry wt. organism/ml.). The effects of different concentrations of phosphate, ammonium, magnesium and sulphate are shown in Fig. 1. The results from this experiment were used to formulate the basal media used subsequently.

Nitrate, nitrite or ammonium were used as sources of inorganic nitrogen. Hydroxylamine gave growth at low concentrations but hydrazine was not utilized nor was there any evidence of nitrogen fixation. The phosphate in the medium could be replaced by pyrophosphate, ribose-5-phosphate, fructose-1,6-diphosphate or glucose-6-phosphate. The usual source of sulphur was sulphate but bacterium NCIB 8250 could also utilize sulphite, thiosulphate, cysteine, cystine, methionine, glutathione or thiourea. Neither biotin nor 2-mercaptobenzoate was able to supply the sulphur requirements.

A significant dependence of bacterial yield on potassium was shown only after five successive subcultures, with 1 % (v/v) inocula, through K^+-deficient media. Neither sodium, caesium nor rubidium replaced potassium although rubidium had a small but reproducible sparing effect on the potassium requirement (Table 1). No requirement for sodium, caesium, rubidium or chloride in the growth of bacterium NCIB 8250 was observed. A decreased yield in the absence of added iron was found only after about seven subcultures (1 %, v/v, inocula) through deficient media. Iron (5 μM as $FeCl_3$) was added to routine media only when preliminary experiments had shown that the inoculum was such that there would be an increased yield. No increase in either the growth rate or yield was found when yeast extract (1 mg./l.) or a mixture or biotin (2·5 μg./l.), pyridoxine (0·5 mg./l.). nicotinamide (0·4 mg./l.) and thiamine (0·4 mg./l.) was added to a simple acetate + salts medium. No effect of the addition of any vitamins was observed after 130 subcultures (1 %, v/v, inocula) through chemically defined media.

Bacterium NCIB 8250 grows only under aerobic conditions. The degree of aeration under the conditions used was adequate to give maximum growth rates with acetate as carbon source (mean generation time, 45 min.). There was no increase in growth rate or yield when more air or oxygen was passed through the medium. No growth occurred under anaerobic conditions, even with 0·1 % (w/v) potassium nitrate present to act as a possible alternative electron acceptor.

The influence of temperature on the growth rate is shown in Fig. 2. The optimal temperature was from 29° to 32°; most subsequent experiments were made at 30°. The Arrhenius plot (Fig. 2, inset) gave a straight line up to 29° and the apparent activation energy was calculated to be 14,700 cal. $mole^{-1}$.

The effect of the initial pH value on the mean generation time of bacterium NCIB 8250 is illustrated in Fig. 3. The response to the pH value was independent of the carbon and nitrogen sources tested; the optimum was pH 7.

The variations of growth rate and yield with changing concentrations of the carbon + energy sources are illustrated in Fig. 4. Increasing amounts of acetate, succinate

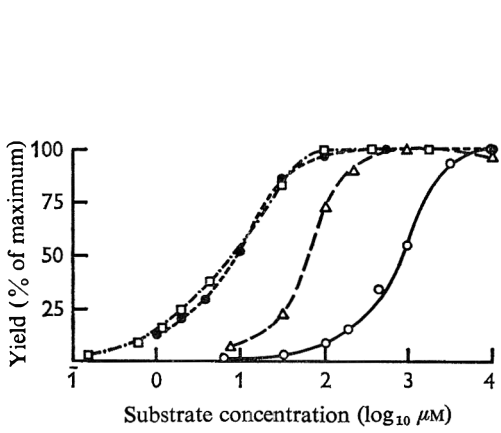

Fig. 1

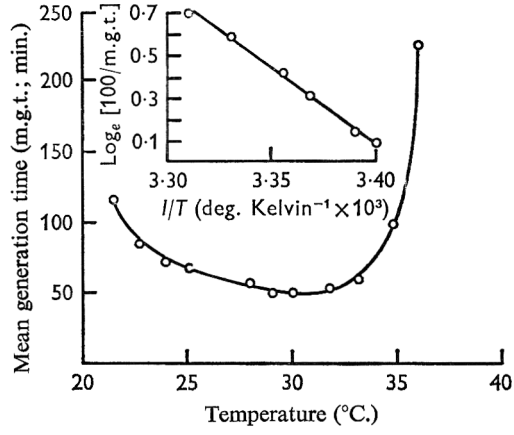

Fig. 2

Fig. 1. Effect of concentration of nutrients on final yield of Bacterium NCIB 8250. The carbon + energy source was 5 mM-succinate which gave a maximum yield (E_{350}) of about 1·0. ○, NH_4^+; △, PO_4^{3-}; ●, SO_4^{2-}; □, Mg^{2+}.

Fig. 2. Effect of temperature on growth rate of Bacterium NCIB 8250. The carbon + energy source was 5 mM-succinate. Inset: Arrhenius plot of growth rate.

Table 1. *Effect of potassium on growth of Bacterium NCIB 8250*

The basal medium contained 2·6 g. $(NH_4)_2SO_4$ + 1·0 ml. glacial acetic acid + 2·0 ml. 98 % (v/v) phosphoric acid + 0·4 g. $MgSO_4.7H_2O$ in 1 l. distilled water adjusted to pH 7·0. The inoculum was prepared by making 4 successive subcultures (1 %, v/v, inocula) in this medium in order to show a significant dependence on K^+. Erlenmeyer flasks (250 ml.) containing basal medium (50 ml.) + appropriate amounts of K_2SO_4, Na_2SO_4, Cs_2SO_4 or Rb_2SO_4 were inoculated with 0·1 ml. of the K^+-deficient culture and shaken at 30° for 48 hr. before measuring the extinction with a Spectronic 20 colorimeter.

Concentration of K^+ and other ions (μM) in medium	Final yield (E_{350})
0	0·29
10 K^+	0·50
100 K^+	0·73
100 Na^+	0·31
100 Cs^+	0·29
100 Rb^+	0·36
10 K^+ + 100 Na^+	0·50
10 K^+ + 100 Cs^+	0·52
10 K^+ + 100 Rb^+	0·57
100 K^+ + 100 Rb^+	0·72

and benzoate gave proportionate increases in yield. The results approximate to molar growth yields, although more sophisticated techniques would be required for a more accurate determination of this parameter. It is of interest that the yield from benzoate (E_{350} = 0·30/mM) was almost exactly equal to the sum of the yields from succinate (E_{350} = 0·19/mM) and acetate (E_{350} = 0·12/mM): benzoate is oxidized by conversion to succinate and acetyl-CoA (Ornston & Stanier, 1964). The change in mean generation time with concentration of substrate is shown in the upper half of Fig. 4. The effects of substrate concentration on growth rate would be better determined in a continuous

flow apparatus, but the primary purpose of the present experiments was to provide data for the growth of batch cultures for enzyme studies. Succinate and acetate tended to provide a constant maximum growth rate (mean generation time 45 min.); these two compounds gave the fastest rate of growth of Bacterium NCIB 8250 in chemically defined medium of any tested. Higher benzoate concentrations depressed the growth rate; this effect was noted with several substrates, e.g. 2-hydroxybenzyl alcohol, phenol and 2-aminobenzaldehyde.

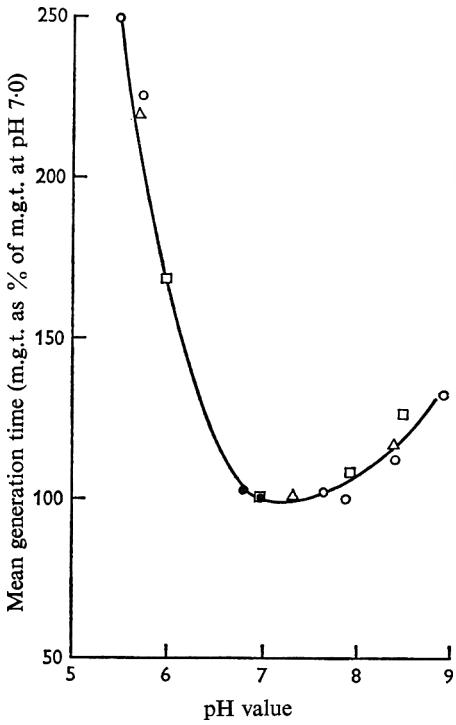

Fig. 3

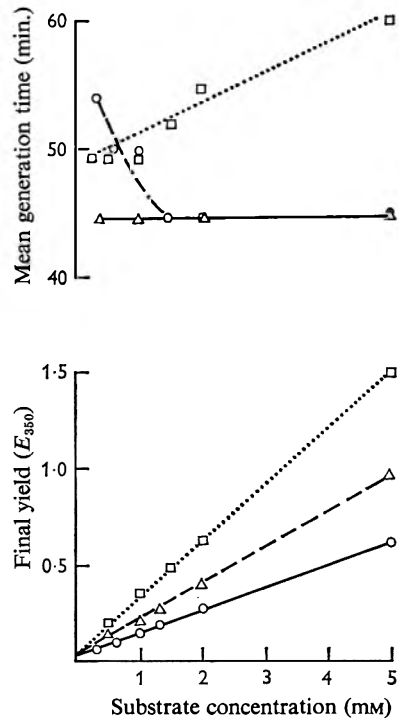

Fig. 4

Fig. 3. Effect of initial pH value on growth rate of Bacterium NCIB 8250. The pH values of the media were measured during growth but in no case did a change occur before the last generation when some cultures, initially at relatively low or high pH, shifted towards neutrality by up to 0·5 pH unit. Carbon and nitrogen sources were: ○, acetate + glutamate; △, mandelate + glutamate; ●, acetate + ammonium; □, acetate + nitrate.

Fig. 4. Effect of substrate concentration on growth rate and final yield of Bacterium NCIB 8250. ○, acetate; △, succinate; □, benzoate.

Screening carbon and nitrogen sources

The metabolic versatility of Bacterium NCIB 8250 was tested by screening a large number of compounds as carbon and nitrogen sources for growth. The yield of organism was recorded at three concentrations of substrate as indicated in Methods. Some compounds, e.g. 2-hydroxybenzoate, gave growth at 0·1 and 0·5 mM but not at 5 mM: toxic effects of this type were not uncommon. In contrast to this, a few compounds such as D,L-tropate showed growth only at the higher concentrations; this may have reflected the inefficiency of these compounds as inducers. With still other compounds,

such as vanillin (mean generation time about 200 min.), growth was extremely slow even at the optimum concentration. Most compounds which supported growth gave approximately the same yield of bacteria as when an equal amount of carbon or nitrogen was provided in the form of succinate or ammonium respectively. In a few cases, e.g. with some amino acids as nitrogen sources, the amount of growth was not as great as that given by the same molar concentration of ammonium or glutamic acid. When compounds did not support growth under the ordinary conditions they were occasionally further tested by growing the inoculum in nutrient broth or glutamate + salts medium + the compound under test, in an attempt to pre-induce enzymes necessary for their utilization. In no case, however, did this method show growth where it had not already occurred in the routine test system. Despite complications such as toxicity, slow growth rates and low yields, it was possible to divide the compounds tested into those which supported growth and those which did not, and thus to delineate the metabolic versatility of Bacterium NCIB 8250.

Table 2. *Sugars and related compounds which did not serve as carbon sources for growth of Bacterium* NCIB *8250*

D-isomers were used unless otherwise stated. The basal medium contained KH_2PO_4 + $MgSO_4$ + ammonium as nitrogen source. A nutrient broth culture was used as inoculum (0·2 %, v/v) and flasks were shaken at 30° for 5 days.

N-Acetylglucosamine	6-Phosphogluconate	Mannitol
L-Arabinose	Glucosamine	Mucate
Ascorbate	Glucose	Raffinose
Cellobiose	Glucose-6-phosphate	L-Rhamnose
2-Deoxyribose	Glucuronate	Ribose
Dihydroxyacetone	D,L-Glyceraldehyde	Ribose-5-phosphate
penta-Erythritol	Glycerol	Sorbitol
Fructose	α-Glycerophosphate	Starch
Fructose-1,6-diphosphate	*meso*Inositol	Sucrose
Galactose	Lactose	Xylose
Gluconate	Maltose	

Bacterium NCIB 8250 showed no growth with any of the sugars or related compounds which were tested (Table 2). Experiments in which the organism was grown on benzoate, succinate or glutamate in the presence of glucose or ribose showed no increase in yield over controls in the absence of sugars. The pattern of utilization of amino acids and related compounds is shown in Table 3. There was a much greater lag in the utilization of L-serine than of D- or D,L-serine, but this effect was not noted with the isomers of alanine or glutamate. Those amino acids which did not serve as carbon sources were further tested in the presence of nitrate rather than ammonium as nitrogen source, but in no case was growth obtained. Table 4 records the growth on normal alkanes, normal alcohols and monocarboxylic and dicarboxylic acids. The failure to grow on alkanes containing less than eight carbon atoms may have been due to their toxicity but they did not depress growth in nutrient broth. All the normal alcohols except methanol gave growth, as did isobutanol but not isopropanol or *sec*-butanol (Table 5). Monocarboxylic and dicarboxylic straight-chain acids, with the exception of formate, oxalate and malonate, supported growth. Growth also occurred with a number of other aliphatic and related compounds (Table 5) including several members

of common metabolic sequences. Most purines, pyrimidines and their derivatives served as nitrogen but not carbon sources (Table 6). Adenosine supported an extremely small amount of growth; confirmation of this result must await further work. A large

Table 3. *Amino acids and related compounds as carbon or nitrogen sources for growth of Bacterium* NCIB 8250

The basal medium contained KH_2PO_4 + $MgSO_4$ + either succinate as carbon source or ammonium as nitrogen source. A nutrient broth culture was used as inoculum (0·2 %, v/v) and flasks were shaken at 30° for 5 days.

	As carbon source	As nitrogen source		As carbon source	As nitrogen source
D-, L- or D,L-Alanine	+	+	δ-Hydroxy-D,L-lysine	−	Not tested
β-Alanine	+	+	L-Hydroxy proline	+	+
D,L-2-Amino-*n*-butyrate	−	+	L-Leucine	−	+
4-Amino-*n*-butyrate	−	−	D,L-Isoleucine	−	+
2-Aminoisobutyrate	−	−	D,L-Norleucine	−	−
L-Arginine	−	−	L- or D,L-Lysine	−	−
L-Asparagine	+	+	L- or D,L-Methionine	−	+
L- or D,L-Aspartate	+	+	D,L.-Ornithine	−	−
L- or D,L-Citrulline	−	+	L- or D,L-Phenylalanine	−	+
Creatine	−	−	*N*-Phenylglycine	−	−
L- or D,L-Cysteine	−	+	Phenyl-D,L-serine	−	+
L-Cystine	−	+	L-Proline	+	+
D,L-α-ε-Diaminopimelate	−	−	D-, L- or D,L-Serine	+	+
D-, L- or D,L-Glutamate	+	+	D,L-Homoserine	−	+
L-Glutamine	+	+	L- or D,L-Threonine	−	−
Glycine	−	+	L-Tryptophan	−	+
Glycylglycine	−	+	L-Tyrosine	+	+
Glycylglycylglycine	−	+	L- or D,L-Valine	−	+
L- or D,L-Histidine	+	+	D,L-Isovaline	−	−

Table 4. *Normal alkanes, normal alcohols, monocarboxylic and dicarboxylic acids as carbon sources for growth of Bacterium* NCIB 8250

The basal medium contained KH_2PO_4 + $MgSO_4$ + ammonium as nitrogen source. A nutrient broth culture was used as inoculum (0·2 %, v/v) and flasks were shaken at 30° for 5 days.

No. of C-atoms	$CH_3(CH_2)_nCH_3$		$CH_3(CH_2)_nCH_2OH$		$CH_3(CH_2)_nCOOH$		$HOOC(CH_2)_n$ COOH	
1	.	.	Methanol	−	Formate	−	.	−
2	.	.	Ethanol	+	Acetate	+	Oxalate	−
3	.	.	Propanol	+	Propionate	+	Malonate	−
4	.	.	Butanol	+	Butyrate	+	Succinate	+
5	Pentane	−	Pentanol	+	Valerate	+	Glutarate	+
6	Hexane	−	Hexanol	+	Caproate	+	Adipate	+
7	Heptane	−	Heptanol	+	Heptoate	+	Pimelate	+
8	Octane	+	Octanol	+	Caprylate	+	Suberate	+
9	Nonane	+	.	.	.	.	Azelate	+
10	Decane	+	Decanol	+	Caprate	+	Sebacate	+
11	Undecane	+	Undecanol	+	Undecylate	+	.	.
12	Dodecane	+	Dodecanol	+	Laurate	+	.	.
13	Tridecane	+	Tridecanol	+	Tridecylate	+	.	.
14	Tetradecane	+	Tetradecanol	+	Myristate	+	.	.
15	Pentadecane	+	Pentadecanol	+	.	.	.	.
16	Hexadecane	+	.	.	Palmitate	+	.	.
18	Octadecane	+	.	.	Stearate	+	.	.
22	.	.	.	.	Behenate	−	.	.

number of aromatic compounds was tested (Table 7) but only relatively few supported growth. The non-aromatic cyclic compounds which served as nitrogen or carbon sources are given in Table 8.

Table 5. *Some aliphatic and related compounds as carbon or nitrogen sources for growth of Bacterium* NCIB *8250*

The basal medium contained KH_2PO_4 + $MgSO_4$. A nutrient broth culture was used as inoculum (0·2 %, v/v) and flasks were shaken at 30° for 5 days.

(i) *The organism grew on the following carbon sources (ammonium as nitrogen source)*:

Acetoin	Diacetyl	Oxalacetate
Isoamylacetate	Fumarate	2-Oxoadipate
Butane-2,3-diol	3-Hydroxybutyrate	2-Oxoglutarate
Isobutanol	Lactate	Pyruvate
Citrate	Malate	

(ii) *The following compounds were used as nitrogen sources (succinate as carbon source) but not as carbon sources (ammonium as nitrogen source)*:

Cyanate Propionamide

(iii) *No growth on the following compounds as carbon sources nor, where possible, as nitrogen sources*:

Acetamide	Ethyl acetate	3-Methylvalerate
Acetone	Ethylene glycol	2-Oxobutyrate
Aconitate	3-Ethyl-3-heptanol	Pentane-1,5-diol
Acrylamide	4-Ethyl-3-hexanol	Propane-1,2-diol
Acrylate	3-Ethyl-3-pentanol	Propane-1,3-diol
Adipamide	D,L-Glycerate	Isopropanol
Butane-1,3-diol	Glycollate	Propiolate
Butane-1,4-diol	Glyoxylate	Sarcosine
sec-Butanol	Hexane-1,6-diol	Spermidine
n-Butylamine	Hydroxypyruvate	Spermine
Isobutylamine	Itaconate	Tartrate
sec-Butylamine	Linoleate	Taurine
tert-Butylamine	Linolenate	Thiocyanate
Cadaverine	Maleate	Tris (hydroxymethyl) aminomethane
Cyanide	2-Methoxyethanol	
Ethanolamine	Methylamine	Isovalerate

Table 6. *Purines, pyrimidines and derivatives as nitrogen sources for growth of Bacterium* NCIB *8250*

The basal medium contained KH_2PO_4 + $MgSO_4$ + succinate as carbon source. A nutrient broth culture was used as inoculum (0·2 %, v/v) and flasks were shaken at 30° for 5 days. None of the compounds served as carbon source when ammonium was the nitrogen source.

	Nitrogen source		Nitrogen source
Adenine	+	Inosine	+
Adenosine	−	Orotate	−
Allantoate	+	Thymidine	−
Allantoin	+	Thymine	+
Barbiturate	−	Uracil	+
Cytidine	−	Urate	+
Cytosine	−	Urea	+
Guanine	+	Uridine	−
Guanosine	+	Xanthine	+
Hypoxanthine	+	Xanthosine	+

DISCUSSION

The examination of the metabolic versatility of Bacterium NCIB 8250 involved testing about 450 substances of which over 100 served as sole carbon + energy sources and about 50 as sole nitrogen sources. It is probable of course that wider screening or the use of other basal media and inocula would uncover additional compounds which can support growth. Also some compounds may have failed to give growth because they do not serve as inducers for the appropriate enzymes and furthermore Bacterium NCIB 8250 might be impermeable to certain compounds and unable to form permeases for their transport into the bacterium. A number of types of growth response was noted in testing these compounds but the results have been recorded as 'growth' or 'no growth' since it was felt that many of the variations in response were trivial in that they depended on the size and type of inoculum or concentration of nutrient. There is no doubt that some compounds which did not support growth are partially metabolized by Bacterium NCIB 8250. An example of this is the oxidation of 3-hydroxymandelate to 3-hydroxybenzoate by Bacterium NCIB 8250 adapted to grow on mandelate (Kennedy & Fewson, 1966). This type of incomplete oxidation might have considerable ecological significance in the succession of microbial populations in soil (Alexander, 1964).

The experiments described in this paper confirm that Bacterium NCIB 8250 is a suitable organism in which to examine the control of catabolic enzyme systems. Growth occurs on a number of diverse compounds and the failure to utilize carbohydrates may simplify studies on catabolite repression. The survey of metabolic versatility has revealed several oxidative sequences which are inducible and amenable to experiments on the mechanism of control. These include the pathways for the degradation of purines, pyrimidines and their ribosides; indole and indolyl-3-acetate; alkanes, alcohols, and acids; butane-2,3-diol, acetoin and diacetyl; several amino acids; quinate and shikimate; the mandelate family. The pathway of oxidation of mandelate and related compounds in Bacterium NCIB 8250 has already been elucidated (Kennedy & Fewson, 1966). Work has also been done on other systems such as the oxidation of straight-chain acids (e.g. Callely *et al.* 1958), homogentisate (Chapman & Dagley, 1962) and protocatechuate (Cain, 1961).

The experiments on screening of substrates, in addition to indicating which catabolic systems can be induced in Bacterium NCIB 8250, have shown several features of the metabolism of this organism which merit further work. The basis of the inability to grow on carbohydrates should be of considerable interest, especially since members of the Acinetobacter–Moraxella group show a wide variation in their saccharolytic properties (Steel & Cowan, 1964; Henderson, 1965). It is noteworthy that whereas some *N*-glycosides such as *N*-*o*-carboxyphenyl-D-glucosylamine and the purine ribosides supported growth (presumably at the expense of the non-sugar components) thymidine, uridine and the *O*-glycoside salicin did not give growth even though in these cases also the non-glycosidic moieties were utilized (Tables 6, 7). Another problem is raised by the observation that phenylpyruvate but not phenylalanine served as carbon source (Tables 3, 7). This is surprising since phenylpyruvate can be shown to accumulate in the medium when Bacterium NCIB 8250 grows with phenylalanine as nitrogen source (unpublished results). Parallel results were obtained with tryptophan. The explanation would seem to lie in the specificity of enzyme induction and repression.

Table 7. *Aromatic compounds as carbon or nitrogen sources for growth of Bacterium* NCIB *8250*

The basal medium contained $KH_2PO_4 + MgSO_4$. A nutrient broth culture was used as inoculum (0·2 %, v/v) and flasks were shaken at 30° for 5 days.

(i) *Compounds used as carbon sources (ammonium as nitrogen source)*:

Acetylsalicylate	Ethylphenylacetate	L- or D,L-Mandelate
Benzaldehyde	Homogentisate	Phenol
Benzyl alcohol	2-Hydroxybenzaldehyde	Phenylacetaldehyde
Benzoate	4-Hydroxybenzaldehyde	Phenylacetate
Benzoylformate	2-Hydroxybenzoate	Phenylpyruvate
Catechol	4-Hydroxybenzoate	D,L-Tropate
3,4-Dihydroxybenzaldehyde	2-Hydroxybenzyl alcohol	Vanillate
3,4-Dihydrozybenzoate	4-Hydroxybenzyl alcohol	Vanillin
3,4-Dihydroxymandelate	4-Hydroxymandelate	Vanillyl alcohol
Ethylbenzoate	4-Hydroxyphenylpyruvate	Vanillylmandelate

(ii) *Compounds used as carbon and nitrogen sources*:

2-Aminobenzaldehyde	*N-o*-Carboxyphenyl-D-glucosylamine
Anthranilate	4-Hydroxy-3-methoxybenzylamine

(iii) *Compounds not used as carbon sources (ammonium as nitrogen source)*:

2-Acetamidobenzoate	Ethyl-*p*-aminobenzoate	2-Nitrophenol
2-Aminophenol	Eugenol	3-Nitrophenol
3-Aminophenol	Isoeugenol	4-Nitrophenol
4-Aminophenol	Ferulate	3-Nitrophthalate
Amygdalin	Gentisate	4-Nitrophthalate
Anisaldehyde	Guaicol	3-Nitrosalicylate
Anisyl alcohol	3-Hydroxybenzaldehyde	5-Nitrosalicylate
Anisate	3-Hydroxybenzoate	2-Nitrosophenol
Atrolactate	3-Hydroxybenzyl alcohol	Orcin
Atropine	3-Hydroxymandelate	Phenoxyacetate
Benzene	3-Hydroxy-4-methoxy-benzaldehyde	Phenylacetamide
p-Benzoquinone	3-Hydroxy-4-methoxy-mandelate	Phenyl-*n*-butyrate
Benzyl malonate	4-Hydroxy-3-methoxy-Phenylacetate	2-Phenylethanol
Caffeate	2-Hydroxyphenylacetate	D,L-1-Phenylethanol
2-Carboxybenzaldehyde	3-Hydroxyphenylacetate	3-Phenylpropanol
homo-Catechol	4-Hydroxyphenylacetate	3-Phenylpropionate
Chloramphenicol	4-Hydroxyisophthalate	Phenylsuccinate
Chlorogenate	Mandelamide	Phloretate
Cinnamaldehyde	D-Mandelate	Phloroglucinol
Cinnamate	2-Methoxybenzaldehyde	Isophthalaldehyde
Cinnamyl alcohol	3-Methoxybenzaldehyde	Phthalate
o-, *m*-, and *p*-Coumarate	2-Methoxybenzoate	Isophthalate
o-, *m*-, and *p*-Cresol	3-Methoxybenzoate	Terephthalate
2,4-Dihydroxybenzaldehyde	3-Methoxybenzyl alcohol	Piperonylate
2,5-Dihydroxybenzaldehyde	4-Methoxyphenol	Pyrogallol
2,3-Dihydroxybenzoate	2-Methoxyphenylacetate	Resorcinol
2,4-Dihydroxybenzoate	Methylbenzoate	Salicin
2,6-Dihydroxybenzoate	Methyl-2-hydroxybenzoate	Sinapate
3,5-Dihydroxybenzoate	Methyl-4-hydroxybenzoate	Sinapinate
3,4-Dihydroxyphenylacetate	Methylsalicylate	Sulphanilamide
3,4-Dihydroxyphenylalanine	1-Napthol	Syringate
2,4-Dimethoxybenzaldehyde	2-Naphthol	Toluene
3,4-Dimethoxybenzyl alcohol	2-Nitroaniline	*o*-, *m*-, and *p*-Toluate
3,5-Dinitrobenzoate	3-Nitroaniline	3,4,5-Trihydroxybenzoate
2,4-Dinitrophenol	4-Nitroaniline	*o*-Vanillin
Diphenate	3-Nitrobenzoate	Isovanillin

Table 7 (*cont.*)

(iv) *Compounds not used as either carbon or nitrogen sources*:

2-Aminobenzoate	Aniline	2-Nitrobenzaldehyde
4-Aminobenzoate	Benzamide	3-Nitrobenzaldehyde
3-Amino-4-hydroxybenzoate	Benzylamine	4-Nitrobenzaldehyde
D,L-α-Aminophenylacetate	2-Carboxyphenylglycine	2-Nitrobenzoate
4-Aminophenylacetate	Hippurate	4-Nitrobenzoate
4-Aminosalicylate	Mandelonitrile	Phenylhydrazine

Table 8. *Heterocyclic, alicyclic and related compounds as carbon or nitrogen sources for Bacterium* NCIB *8250*

The basal medium contained $KH_2PO_4 + MgSO_4$. A nutrient broth culture was used as inoculum (0·2 %, v/v) and flasks were shaken at 30° for 5 days.

(i) *Compounds used as carbon sources (ammonium as nitrogen source)*:

Quinate Shikimate

(ii) *Compounds used as carbon and nitrogen sources*:

Indole D,L-Kynurenine
Indolyl-3-acetate

(iii) *Compound used as nitrogen source but not as carbon source*:

Xanthurenate

(iv) *Compounds not used as carbon sources or, here possible, as nitrogen sources*:

Adamantanecarboxylate	2-Hydroxycyclohexanone
Adrenaline	Indolyl-3-acetamide
Noradrenaline	Indolyl-3-butyrate
Aneurine	Indolyl-3-propionate
Biotin	Indolyl-3-pyruvate
Camphor	Isatin
2,6-Dipicolinate	Kojate
Folate	Nicotinate
2-Furoate	Pyridoxine
Cyclohexane	Skatole
Cyclohexanecarboxylate	Urocanate
Cyclohexanol	

The pathways of degradation of some of the purines and pyrimidines are also in doubt (Table 6). Adenine appears to be oxidized via xanthine and uric acid to urea since all these compounds served as nitrogen but not carbon sources. In the metabolism of uracil, however, it would seem that the dihydrouracil pathway (White, Handler & Smith, 1964) did not function since the product would be β-alanine which unlike uracil was a carbon and a nitrogen source. The barbiturate pathway (Schulman, 1961) did not seem to operate since bacterium NCIB 8250 did not grow on barbiturate.

The central role of the Krebs cycle in the metabolism of bacterium NCIB 8250 has been indicated by previous workers (Dagley & Rodgers, 1953; Dagley & Patel, 1955; Callely *et al.* 1958). It may also be presumed that the organism possesses a glyoxylate cycle since it grows on acetate (Table 4), and possesses isocitratase and malate synthetase (Callely *et al.* 1958). The failure to grow on glycollate or glyoxylate (Table 5) might be caused by the lack of a suitable anaplerotic sequence (Kornberg, 1956), but neither glycerate nor glycerate with glyoxylate, glycollate or glycine supported growth. Another possible cyclic system of Bacterium NCIB 8250 is the butane-2,3-diol cycle (Juni & Heym, 1956) for the dissimilation of acetoin, diacetyl and butane-2,3-diol.

All these compounds supported growth (Table 5); an inducible specific enzyme which oxidises butane-2,3-diol to acetoin is at present under investigation.

The range of compounds which can support the growth of Bacterium NCIB 8250 is considerable, and perhaps, as suggested in a preliminary and unpublished communication by Stanier & Palleroni, the utilization of some of the compounds tested in this work may serve as a basis for classification of the Acinetobacter–Moraxella group. The range of versatility also helps to explain the apparent ubiquity of this group in nature (Henderson, 1965), especially since many of the compounds metabolized, such as vanillin, are derivatives of common organic polymers found in soil.

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Computer Analysis of *Acinetobacter lwoffii* (*Moraxella lwoffii*) and *Acinetobacter anitratus* (*Moraxella glucidolytica*) Strains

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SUMMARY

Adansonian analysis of 31 *Acinetobacter lwoffii* and 8 *A. anitratus* strains is described. The mean S-value was 81·9 and 87·3 % for groups *A. lwoffii* and *A. anitratus*, respectively. The intergroup value was 71·9 %. The computer distinguished well the *Alcaligenes faecalis* strains from the former two species. According to the computer analysis, *Bordetella bronchiseptica* is closely related to *A. faecalis*. *Pseudomonas aeruginosa* and *Aeromonas liquefaciens* link with the former groups at a much lower level.

INTRODUCTION

The systematics of Gram-negative non-fermenting bacteria has been fairly uncertain. Although numerous data have been available in the literature on the taxonomy of the genera *Pseudomonas*, *Alcaligenes*, *Moraxella*, *Achromobacter* and *Flavobacterium*, many questions have been left unanswered. Much dispute has arisen about the systematic position and nomenclature of the Gram-negative coccobacillary micro-organisms classified by various authors in the genera *Moraxella*, *Mima*, *Achromobacter*, *Acinetobacter* and *Cytophaga*, or designated '*Bacterium anitratum*'. The available biochemical, serological, and morphological data are too scanty to permit reliable classification. A new basis for the systematics of these genera is offered by Adansonian analysis, applied first to bacteria by Sneath (1957). During recent years many valuable results have been obtained in bacterial systematics by computer analysis. As to the Gram-negative non-fermenting bacteria, most reports have been published on the computer analysis of *Pseudomonas* species (Colwell & Liston, 1961; Rhodes, 1961). Information has been relatively scanty about other non-fermenting bacteria. Thornley (1961) succeeded in separating '*Bacterium anitratum*', *Mima* and *Alcaligenes faecalis* strains into distinct groups. In extensive studies involving numerous bacterial species, Beers, Fisher, Megraw & Lockhart (1962), and Focht & Lockhart (1965) found that on numerical analysis, the biochemically inactive non-fermenting bacteria appeared to form a distinct group and to link with other Gram-negative bacteria only at a low level.

The present work was done to examine the relations in 'polythetic' numerical analysis of bacterial strains isolated from clinical material and identified 'monothetically'. Mainly *Acinetobacter lwoffii* and *Acinetobacter anitratus* strains were studied, other Gram-negative non-fermenting bacteria (*Alcaligenes faecalis*, *Bordetella bronchiseptica*, *Pseudomonas aeruginosa*, one *Aeromonas liquefaciens* strain) being used for comparison.

METHODS

Organisms. Out of a total of 47 strains examined, 39 were isolated in our laboratory from clinical material (urine, blood, bile, sputum, pus). Identification was done mostly by means of the diagnostic tables of Cowan & Steel (1965). Four strains were kindly supplied by Dr S. T. Cowan (from the National Collection of Type Cultures, London, NCTC), and a further 4 strains from the collection of Statens Seruminstitut, Copenhagen, through the courtesy of Dr H. Lautrop. Our serial numbers, the designations and origins of the strains are listed below.

Nos. 1–28. *Acinetobacter lwoffii.* Our isolates.

No. 29. *A. lwoffii*, NCTC 5866.

Nos. 30, 31. *A. lwoffii*, Statents Seruminstitut. Original designation: '*Bacterium anitratum*' type B, A3 and A14.

Nos. 32–36. *A. anitratus.* Our isolates.

No. 37. *A. anitratus*, NCTC 7844.

Nos. 38, 39. *A. anitratus*, Statens Seruminstitut. Original designation: '*Bacterium anitratum*' type A, A1 and A4.

Nos. 40, 41. *Alcaligenes faecalis.* Our isolates.

No. 42. *A. faecalis*, NCTC 415.

No. 43. *Bordeteila bronchiseptica*, NCTC 8761.

Nos. 44–46. *Pseudomonas aeruginosa.* Our isolates.

No. 47. *Aeromonas liquefaciens.* Our isolate.

Cultivation of organisms and determination of characters. The strains were maintained on nutrient agar slopes. Before examination, the strains were streaked on nutrient agar plates. A single isolated colony was transferred to a nutrient agar slope and after incubation for 24 hr. the cultures were washed off with saline. This suspension was used as starting material. The incubation temperature used was always 25°. The tests applied and the characters found are shown in Table 1. During coding, the 79 characters listed in the table yielded 98 character states on the basis of secondary data. For biological and biochemical assay we used the procedures recommended by Cowan & Steel (1965).

Computer analysis. Programming and calculations were performed by the Computer Centre of the Hungarian Academy of Sciences (Budapest). The programmes were written by L. Ferenczy and computed on a Ural (USSR) type computer. The experimental data were coded and tabulated by two different methods and calculated in parallel. The following two coding systems were used:

Coding system 1. Data were tabulated with the designations +, −, or n.c. (not counted). On the basis of these data, and by appropriate programming, the computer gave the values of matching coefficient (affinity index) for each pair of strains (Sokal & Michener, 1958). Both the positive and negative properties were included in this analysis. Quantitative properties were coded by the additive method. Properties giving uniform responses with all strains were excluded from the analysis.

Coding system 2. Data were coded by the four-symbol (A, B, C, D) system introduced by Lockhart & Hartman (1963). The computer indicated similarity when two symbols were identical; in all other cases it indicated dissimilarity. In this sytem, too, 'negative' properties were also included in the evaluation under the appropriate symbol.

After the determination of the S-values the strains were grouped by the highest link-

sorting method. Highest link sorting was made with several starting pairs and served as the basis for forming groups. After clustering, mean intra-group and inter-group values were computed.

Table 1. *Properties of test organisms included in the numerical analysis*

Property no.	Property
1–3	Cell morphology
4–7	Colony morphology
8	Motility
9–11	Growth at 4°, 37°, 42°
12–14	Growth at pH 4·0, 5·0, 9·6
15–17	Growth in 3, 5, 10% NaCl
18–19	Growth in KCN and McConkey media
20–21	Resistance to 56°/30 min. and 60°/30 min.
22–24	Litmus milk acid, alkaline and reduced
25–27	Catalase, cytochrome oxidase, Kovács oxidase
28–32	Urea hydrolysis, nitrate reduction, nitrite reduction, H_2S production, indol production
33–35	Arginine and lysine decarboxylation, phenyl-alanin deamination
36–37	Gelatin and serum liquefaction
38–39	Glucose oxidation and fermentation
40–42	Gluconate oxidation, methyl red and Voges–Proskauer tests
43–52	Acid from galactose, maltose, arabinose, xylose, lactose, 10% lactose, mannitol, sorbitol, inositol, dulcitol
53–69	Utilization as sole carbon source: formate, benzoate, lactate, malonate, maleate, succinate, α-Ketoglutarate, citrate, acetate, glycine, alanine, cystine, aspartate, valine, lysine, arginine, lactose
70–79	Resistance to streptomycin, chloramphenicol, tetracycline, aureomycin, neomycin, erythromycin, oleandomycin, kanamycin, nitrofurantoin, sulphapyridine

Table 2. *Intra-group and inter-group S-values*

The upper values in each pair are from coding method 1;
The lower values from coding method 2.

Species					
Acinetobacter lwoffii	82·4	—	—	—	—
	81·9	—	—	—	—
A. anitratus	70·9	84·7	—	—	—
	71·9	87·3	—	—	—
Alcaligenes faecalis,	68·0	66·0	77·0	—	—
Bordetella bronchiseptica	69·5	67·1	78·1	—	—
Pseudomonas aeruginosa	55·1	67·8	67·5	82·0	—
	54·3	66·0	68·8	83·0	—
Aeromonas liquefaciens	60·0	59·5	62·0	60·5	100·0
	56·7	56·7	61·7	59·0	100·0
	A. lwoffii	*A. anitratus*	*A. faecalis*, *B. bronchiseptica*	*P. aeruginosa*	*A. liquefaciens*

RESULTS

In Table 2 are shown the average intra-group and inter-group S-values obtained by two methods of coding. The results obtained by two methods do not differ significantly. In every case, intra-group S-values were higher than 75%, representing, we believe, the species level, whereas the inter-group values were lower. Considering the mean values, linking at the highest level occurs between *Acinetobacter lwoffii* and *A. anitratus*; with *A. lwoffii* is linked the *Alcaligenes* group and with that the group *Pseudomonas aeruginosa*.

The dendrogram presented in Fig. 1 has been derived from highest link sorting based on the second coding system. The above groups are well separated by this method; links occur, at a higher level. The linking of *Acinetobacter anitratus* strains to each other ceases at the 90% level; that of *A. lwoffii* strains to each other at 84·7%. The highest link between the two groups is at 79·8%.

The three *Alcaligenes faecalis* strains and the *Bordetella bronchiseptica* strain formed a uniform group in all phases of analysis. They link with the *Acinetobacter lwoffii* at a

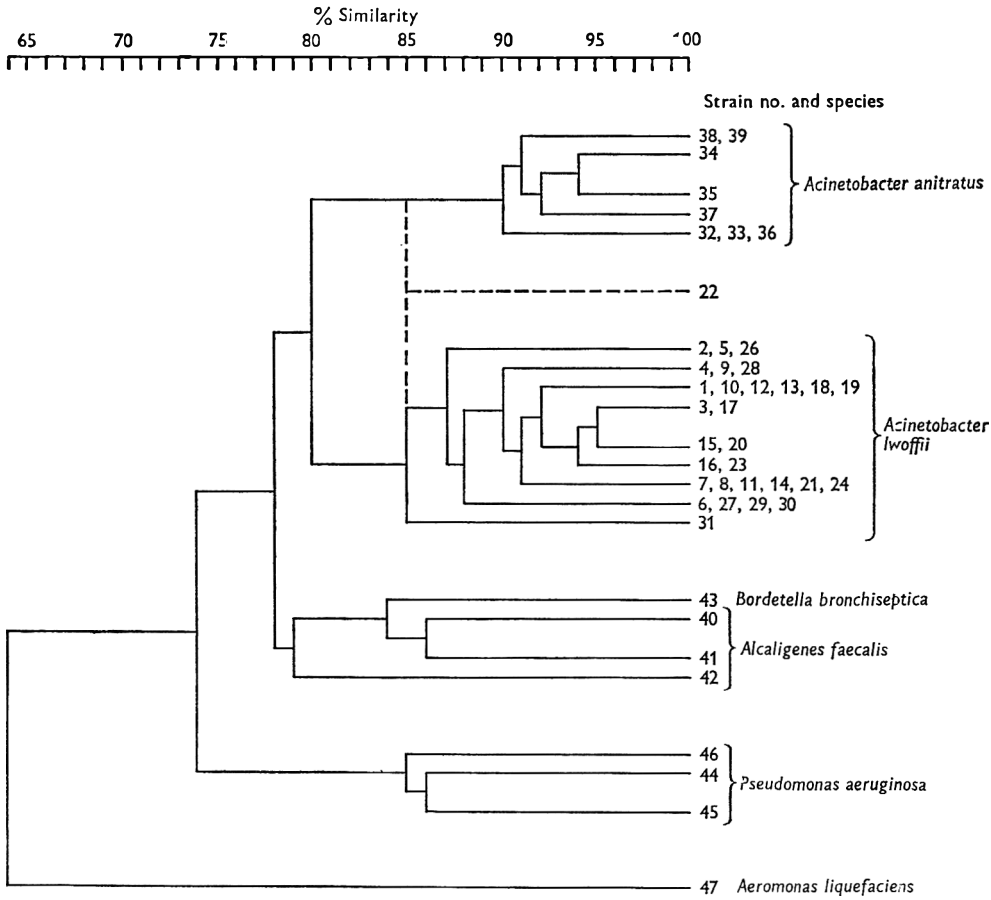

Fig. 1. Similarity relationships among the organisms as shown by coding method 2.

relatively high S-value (78·6%), but the much lower inter-group mean S-value shows them to be significantly different from *A. lwoffii*. The *B. bronchiseptica* strain was very similar to the two *A. faecalis* strains isolated in this laboratory, but the *A. faecalis* NCTH 415 strain showed lower S-values, in comparison with the other three.

Difficulties were encountered in the classification of one strain, serial no. 22, labelled as *Acinetobacter lwoffii*. This linked at nearly identically high levels with both the *A. lwoffii* and *A. anitratus* groups. Also, the average S-values between strain no. 22 and these two groups were nearly the same.

As expected, the *Pseudomonas aeruginosa* strains were well separated from the above-mentioned groups, and still lower S-values were obtained with *Aeromonas liquefaciens*.

DISCUSSION

The systematic position of Gram-negative non-fermenting bacteria has been discussed in numerous recent publications. A reliable classification of these organisms is rendered difficult by the low number of their biochemical reactions as well as by the uncertainty of serological tests. Their identifications is usually restricted to a few procedures (examination for flagella, mode of glucose decomposition, oxidase reaction, nitrate reduction, urease production, etc). The monothetic method of identification, however, involves always the risk of obtaining erroneous results. In the case of *Acinetobacter lwoffii* and *A. anitratus*, the polythetic analysis reported in the present paper has proved the validity of these two species. Nevertheless, no perfectly sharp borders could be drawn between the two species. This observation is in good accord with the data available on the antigenic structure of the same organisms. Common antigens have been demonstrated in the strains of *A. lwoffii* (Mima) and *A. anitratus* (Herellea) by Brodie & Henderson (1964) and Mitchell & Burrel (1964).

The relatively uniform features revealed by the analysis of *Alcaligenes faecalis* and *Bordetella bronchiseptica* support the suggestion of Steel & Cowan (1964) that *B. bronchiseptica* should be placed in the genus *Alcaligenes*. An attempt was made to compare a strain of *B. parapertussis* NCTC 5952 with the strains *Acinetobacter lwoffii*. The conditions for cultivation and the nutritional requirements of these strains were, however, so different as to render reliable comparison impossible. Thus we are not in a position either to agree or to disagree with the suggestion by Steel & Cowan (1964) to classify *B. parapertussis* in the genus *Acinetobacter*.

The results of Adansonian analysis depends on the methods of coding and computing. This is particularly true for Gram-negative non-fermenting bacteria characterized by a relatively poor range of biochemical activities. It is believed, however, that further progress requires not so much the application of new methods of computing, as the further extension and improvement of bacteriological methods.

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Response of Verticillium Species to Griseofulvin

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SUMMARY

Griseofulvin induced various morphological abnormalities of the hyphae of *Verticillium albo-atrum* and *V. dahliae*. The growth-response curves obtained were unusual, and were due to the behaviour of the antibiotic. Repeated subcultivation of mycelium on Dox medium agar containing constant concentrations of griseofulvin produced trained mycelium which was however unstable. True adaptation of *V. alboatrum* was obtained when faster growing sectors of mycelium were produced at the periphery of inhibited colonies. This adapted mycelium showed decreased morphological abnormality but still showed marked inhibition of growth. Chitin production, estimated as glucosamine, following growth in the presence of griseofulvin appeared to be stimulated in the walls of non-adapted mycelium, but remained at a relatively constant value in the walls of the adapted mycelium.

INTRODUCTION

The antibiotic griseofulvin was isolated by Oxford, Raistrick & Simonart (1939) as a metabolic product of *Penicillium griseofulvum* Dierckx. In 1946, Brian, Curtis & Hemming obtained by chloroform extraction of the culture medium or mycelium of *P. nigricans* a substance which they termed 'curling factor' because of the morphological abnormalities it induced on the germ tubes of *Botrytis allii*. Later, Grove & McGowan (1947) showed that griseofulvin and 'curling factor' were one and the same substance. Griseofulvin was also shown to cause morphological abnormalities of the hyphae of other fungi (Brian, 1949; Napier, Turner & Rhodes, 1956; Aytoun, 1956), although only fungi with cell walls based on chitin appeared to be affected. Fungi with cell walls based on cellulose, e.g. Oomycetes, were apparently unaffected.

The nature of the morphological responses of sensitive fungi to griseofulvin is still unresolved. Brian (1949, 1960) considered that they were caused by the antibiotic decreasing the rigidity of the cell wall in the region of the hyphal tip so that the internal pressures could no longer be supported by the weakened membranes; he also suggested that griseofulvin interfered with the biosynthesis of cell-wall chitin. Rhodes (1962) suggested that griseofulvin might become incorporated into the cell wall to form a modified chitin. El-Nakeeb & Lampen (1965), who used griseofulvin-^{3}H, reported that it was taken up by sensitive fungi and formed complexes with nucleic acids and proteins.

Verticillium species have been shown by Brian (1949) and Napier *et al.* (1956) to be sensitive to griseofulvin, and the latter workers found that *V. albo-atrum* and *V. dahliae* reacted differently in their sensitivity.

The present paper considers some responses to griseofulvin of *V. albo-atrum* and *V. dahliae* in culture.

METHODS

Organisms used. The isolates used were *Verticillium albo-atrum* (an unusually slow-growing strain when on Dox medium agar) isolated from potato, a hyaline variant of it (= *V. albo-atrum* var.) and *V. dahliae* isolated from Antirrhinum. Stock cultures were maintained on test-tube slopes of Dox medium agar stored at room temperature.

For agar media and liquid media, the inocula were mycelial discs, 3 mm. diameter, consisting of conidia, mycelium and resting bodies of the test fungus, cut from just inside the leading edge of 2-week cultures on Dox medium agar. Liquid cultures were incubated at 25° in the dark without shaking for 15 days.

Chemicals. All chemicals used were obtained from British Drug Houses Ltd. (B.D.H.) and were of A.R. grade where available. The griseofulvin used was kindly supplied by Dr A. Rhodes (Glaxo Ltd.) and by Professor P. W. Brian (University of Glasgow).

Before use all glassware was cleaned with chromic acid + sulphuric acid, thoroughly rinsed with tap water and then with distilled water.

Media. In all experiments Dox medium (containing 1·5% sucrose, 0·2% sodium nitrate, 0·05% magnesium sulphate, 0·05% potassium chloride, 0·1% potassium dihydrogen orthophosphate, 0·001% ferrous sulphate, 2% agar) was used. For liquid cultures 25 ml. medium was dispensed into 250 ml. Pyrex conical flasks. Agar media were poured to a depth of about 3 mm. into sterile Pyrex Petri dishes 9 cm. diameter.

Where griseofulvin was to be incorporated into media a dilution method of preparation was used, namely, a known weight of griseofulvin was shaken for 3 hr with a known volume of 50% (v/v) ethanol in water and a given volume of this solution (or suspension) removed and added to the growth medium to give the required concentration, in each case 1% (v/v) ethanol, in the medium. The remainder of the solution was diluted with a given volume of 50% (v/v) ethanol in water, shaken for 3 hr and a volume of this dilution removed and added to the growth medium. This process was repeated to give the range of griseofulvin concentrations required.

Recording of growth. Growth on Dox agar was recorded by measuring the diameters of colonies in two directions at right angles and taking the mean of six replicates. The mycelia from liquid cultures were collected on previously dried and weighed filter papers, washed thoroughly with distilled water and dried for 24 hr at 85°. The mean dry weight of ten replicate cultures was determined.

Test of the effect of griseofulvin on the germination of conidia. Glass microscope slides were placed inside 9 cm. diam. Petri dishes which were then sterilized at 300° for 6 hr. The slides were then covered with a thin layer of Dox medium agar containing griseofulvin and when cool dipped in a spore suspension of the test fungus prepared in sterile distilled water. The effect of the antibiotic on the germination and morphology of the germ tubes was observed after incubating the slides in the Petri dishes at 25° for 20 hr.

Spectrophotometric determination of griseofulvin. Griseofulvin in solution was determined by the method of Abbott & Grove (1959) at 326 mμ in a Unicam SP. 500 spectrophotometer with 1 cm. glass cells.

Training and adaptation to griseofulvin. Eight sets, each of 10 replicates, of Dox medium agars were prepared and to each set griseofulvin in 1% (v/v) ethanol was added at the following rates: 1, 10, 20, 40, 100, 250, 500, 1000 mg./l. The organisms

were grown on these media for 12 subcultures at 15 day intervals (total 180 days). The differences in the diameters of the colonies at the end of the first subculture from those at the end of the 12th subculture were taken as a measure of the degree of training. When a trained mycelium was stable, i.e. when the training was not lost after growth on griseofulvin-free media and when it was transmitted through the spore, then the trained mycelium was considered to be adapted mycelium.

Chitin estimation. Chitin was determined as glucosamine according to the method of Tracey (1955). Oven dried mycelium (30–40 mg.) was hydrolysed with sulphuric acid and the neutralized hydrolysate assayed for glucosamine by the modified colorimetric method of Elson & Morgan (1933) using an EEL photoelectric colorimeter at 520 mμ (green filter OGRI). A calibration curve was obtained with commercial glucosamine.

RESULTS

Spectrophotometric determination of the solubility of griseofulvin in Dox liquid medium

Various amounts of griseofulvin in 50% (v/v) ethanol in water were incorporated into Dox liquid media to final concentrations from 1 to 100 mg./l. + 1% (v/v) ethanol in each. The extinction of the solutions at 326 mμ was determined before and after filtration through 5/2 porcelain filters; the results are summarized in Table 1. After these readings the solutions (both filtered and unfiltered) were left for 24 hr and readings again taken; no differences in the values were found.

Table 1. *The extinction of Dox liquid medium containing various amounts of griseofulvin*

Medium	Extinction* ($E^{1\,cm}_{326\,m\mu}$) Filtered solutions	Unfiltered solutions
Dox + 1% (v/v) ethanol alone	0	0
Dox + 1% (v/v) ethanol + griseofulvin (mg./l.)		
1	7	14
10	125	130
20	241	245
25	320	323
30	409	421
35	450	470
40	509	574
45	455	520
50	448	470
60	360	387
90	285	305
100	108	210

* Each value is the average of five replicates.

The results show that the amount of griseofulvin in Dox liquid medium + 1% (v/v) ethanol increased to a maximum at 40 mg./l. As the amount added was increased beyond this level, instead of the media having a constant extinction equivalent to that at 40 mg./l. a gradual decline occurred so that at griseofulvin 100 mg./l. the medium had an extinction approximately the same as that given when griseofulvin 10 mg./l.

was added, the remaining 90% of the added griseofulvin presumably being in suspension. Since the inhibitory effect of griseofulvin is a function of the amount in solution these results offer an explanation for the unusual growth response of Verticillium to griseofulvin recorded below.

Effect of griseofulvin on the germination of conidia

Griseofulvin had no effect on the percentage germination of condia; it was 100% for *Verticillium albo-atrum* and *V. dahliae*. Depending on the concentration of the griseofulvin, various morphological effects on the hyphae were observed as follows: (1) With no griseofulvin, i.e. Dox medium agar + 1% (v/v) ethanol, the germ tubes of both species grew rapidly, were long, regularly branched and their tips appeared normal. (2) Griseofulvin 1 mg./l., no response. (3) Griseofulvin 10 mg./l., germ tubes of both species were waved, curled and their tips had often grown back on themselves; slight stunting of growth occurred. (4) Griseofulvin 20 mg./l., germ tubes of both species curled, waved and were torulose with spatulate tips; noticable inhibition of longitudinal extension occurred, the hyphae being shorter and thicker than those on control media. (5) Griseofulvin 40 mg./l., germ tubes of both species severely stunted, gnarled, torulose and extremely distorted (Pl. 1, fig. 1). (6) Griseofulvin 80 and 100 mg./l., similar effects to 10 mg./l., presumably because the same amount of griseofulvin was in solution.

Effect of griseofulvin on growth in Dox liquid media

The effect of various added amounts of griseofulvin on the growth of *Verticillium albo-atrum* and *V. dahliae* in Dox liquid medium is summarized in Table 2. With both isolates, increasing concentrations of griseofulvin progressively inhibited development, reaching a maximum effect at 20–40 mg./l. However, as the amount of added griseofulvin was increased beyond this value, a progressive apparent stimulation of growth took place until at 100–250 mg./l. the mycelial dry-weight values approximated to those at 10 mg./l.; from 250–1000 mg./l. a further gradual increase in the dry weight value occurred,

The average final pH values of the culture media after growth of the organisms are indicated in Table 2. There was the least drift to alkalinity at the point of maximum inhibition. Thick gelatinous mycelial mats pigmented with resting bodies were produced in the control cultures, but in the presence of griseofulvin in excess of 1 mg./l. the growth of both organisms was diffuse and appeared as small separate colonies. With *Verticillium dahliae* the griseofulvin cultures had a 'frog-spawn' like appearance, due to the microsclerotia of the small surface colonies being surrounded by hyaline mycelium. Griseofulvin had no effect apparently on the morphology of the resting mycelium of *V. albo-atrum* or on the microsclerotia of *V. dahliae*.

Microscopic examination of the mycelium of both isolates grown at the various griseofulvin additions showed morphological abnormalities. At griseofulvin 1 mg./l, slight waving of the hyphae occurred; at 10 mg./l. the hyphae were more intensely waved, curled and often contained large intercalary cells which resembled chlamydospores but lacked thickened walls (Pl. 1, fig. 2). At 20–40 mg./l. (the amount for maximum inhibition), the hyphae were gnarled, torulose and contained many chlamydospore-like cells. At greater than 20–40 mg./l. the morphological abnormalities became

gradually less severe until at 100–250 mg./l. and above, they were like those in mycelium grown at 10 mg./l.

This growth response to griseofulvin of these two Verticillium species was also found in media with the sucrose concentration raised to 5 %. When sucrose was omitted from the Dox liquid media containing griseofulvin, little or no growth occurred, indicating that the fungi were unable to utilize griseofulvin as a carbon source. In further tests where the griseofulvin-containing Dox liquid media were sterilized by filtration (i.e. griseofulvin not autoclaved), similar growth effects were recorded although no increase in the mycelial dry weight occurred with griseofulvin 250–1000 mg./l.

Table 2. *The effect of griseofulvin on the growth of Verticillium albo-atrum and V. dahliae after 15 days in Dox liquid medium*

	V. albo-atrum		*V. dahliae*	
Medium‡	Mycelial dry wt. (mg.*)	pH†	Mycelial dry wt. (mg.)*	pH†
Dox + 1 % (v/v) ethanol alone	48·5	7·4	119·5	8·4
Dox + 1 % (v/v) ethanol + griseofulvin (mg./l.)				
1	44·6	7·3	107·7	8·1
10	38·5	7·0	52·8	7·4
20	34·0	6·8	44·1	7·2
40	28·0	6·7	43·1	6·9
60	34·2	7·1	44·1	6·8
80	36·9	6·8	46·8	6·8
100	38·5	6·9	47·1	7·0
250	40·4	6·9	47·1	7·2
500	47·7	7·0	54·1	7·2
1000	48·9	6·9	67·1	7·2

* Average of ten replicates.
† The pH values given represent the average final pH values of the cultures.
‡ Initial pH of all media = 4·3

At the concentration of griseofulvin for maximum inhibition, griseofulvin particles were seen in the medium, and it was assumed that at this and all higher concentrations the medium was saturated with griseofulvin. By calculation it was apparent that the weight of griseofulvin particles contributing to the mycelial dry weight would only become significant at griseofulvin 250 mg./l. and above. It was concluded that the gradual increase in mycelial dry weight at griseofulvin > 250 mg./l. was due to griseofulvin particles. However, the mycelial dry-weight increase from griseofulvin 20–40 to 100–250 mg./l. was real and was presumably due to the reduction in the amount of griseofulvin going into solution as observed in the spectrophotometric determination recorded above.

Effect of griseofulvin on growth on Dox medium agar

The effects of various additions of griseofulvin on the radial expansion of colonies of *Verticillium albo-atrum*, *V. albo-atrum* var. and *V. dahliae* are summarized in Table 3. The response of all the organisms was essentially similar in that increased inhibition of radial expansion occurred to a maximum at griseofulvin 40–100 mg./l. followed by a slight apparent stimulation of expansion from 100 to 1000 mg./l.

At all griseofulvin additions greater than 1 mg./l. the superficial surfaces of the colonies of *V. albo-atrum* and *V. albo-atrum* var. were irregular in outline, gnarled, convoluted and creamy white in colour although sporulation in both and resting mycelium production in the parent type, *V. albo-atrum*, occurred. Inhibited colonies of *V. dahliae*, however, were identical in gross morphology with those on control media in producing abundantly sporulating white fluffy aerial mycelium and densely aggregated microsclerotia.

Table 3. *The effect of griseofulvin on the growth of Verticillium species after 15 days on Dox medium agar*

Medium	*V. albo-atrum*	*V. albo-atrum* var.	*V. dahliae*
	Colony diameters (mm.)*		
Dox agar+1 % (v/v) ethanol alone	28·5	60·0	58·5
Dox agar+1 % (v/v) ethanol+griseofulvin (mg./l.)			
1	27·0	53·5	55·0
10	18·0	25·5	32·5
20	16·0	19·0	31·0
40	13·0	13·0	30·0
100	11·5	12·0	28·0
250	14·0	16·0	29·5
500	15·5	17·5	30·0
1000	17·0	18·0	31·0

* Average of six replicates.

The griseofulvin-containing cultures of all isolates, unlike the controls, exhibited marked aerial growth of hyphae, probably as a result of the suppression of lateral, prostrate growth by the antibiotic, so that the hyphae were raised well above the surface of the agar. An additional characteristic of the inhibited colonies was a considerable radial splitting of the agar.

Microscopic examination of the hyphae in contact with the agar showed the typical morphological abnormalities associated with griseofulvin whereas the aerial mycelium appeared normal.

Training to growth in presence of griseofulvin

Since the growths of *Verticillium albo-atrum* var. and *V. dahliae* at the 1st and 12th subcultures on Dox medium agar+griseofulvin (see Methods) were almost identical only the results for *V. albo-atrum* are given in Fig. 1. These results suggest that a slight training of both isolates occurred. Throughout the training period, the gross morphology of the colonies of both isolates did not change, although the extensive radial splitting of the agar, observed in earlier subcultures, was only slight at the end of the 12th subculture. Microscopic examination of mycelium at each griseofulvin concentration revealed the usual morphological abnormalities associated with the respective amount of griseofulvin added and no change in the severity of these abnormalities was ever observed.

To test the stability of such training, mycelium from each isolate which had been trained to a particular concentration of griseofulvin was transferred to normal Dox medium agar (i.e. no griseofulvin) for 75 days (five 15-day subcultures) and then placed back for 30 days (two subcultures) on Dox medium agar+added griseofulvin at the

concentrations to which it had originally been trained. Morphological abnormalities, usually associated with the presence of griseofulvin, were observed in the earlier stages of growth on normal Dox medium agar (1st subculture) but disappeared as the young advancing hyphae grew away from the griseofulvin contained in the inoculum. During the remaining four subcultures on Dox medium agar no morphological abnormalities of the mycelium of either isolate were apparent.

However, on Dox medium agar the diameters of colonies of both isolates were such that they formed growth response curves similar to those produced in the presence of griseofulvin, namely, mycelium trained to griseofulvin 20 mg./l. grew more slowly than mycelium trained to 10 mg./l.

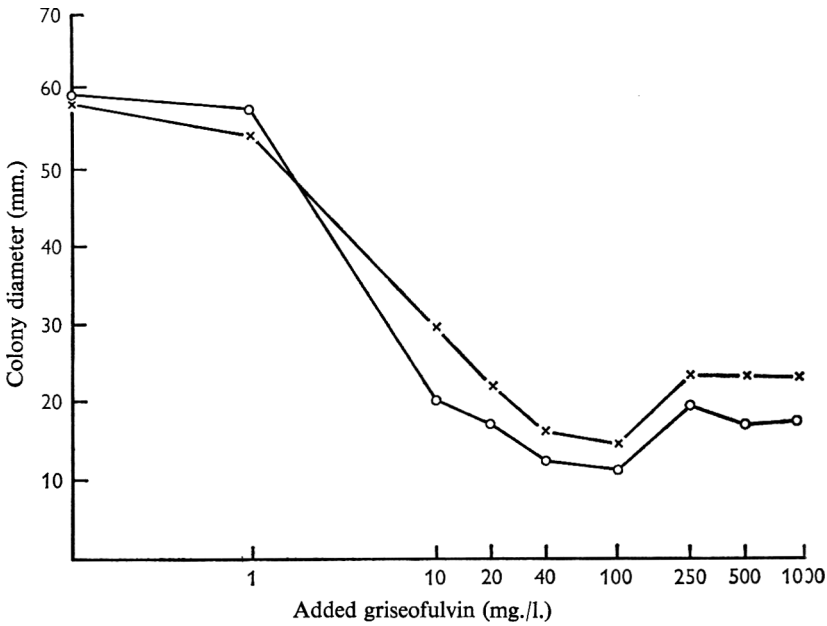

Fig. 1. Growth of *Verticillium albo-atrum* var. on Dox medium agar + 1 % (v/v) ethanol and various additions of griseofulvin. ———O———, after 15 days (i.e. first subculture). ———×———, after 15 days following twelve 15-day subculture periods.

On transferring previously trained mycelium back to Dox medium agar containing the training concentration of griseofulvin, the morphological abnormalities reappeared, and the diameters of the colonies after two subcultures resembled those of untrained rather than trained mycelium. It was concluded that the slight training of *Verticillium albo-atrum* var. and *V. dahliae* which developed after prolonged growth on griseofulvin-containing media was unstable and was lost on transfer to media without griseofulvin.

Adaptation to growth in presence of griseofulvin

During the training experiments, sectors of faster growing mycelium were occasionally produced at the circumference of colonies (Pl. 1, fig. 3) of *Verticillium albo-atrum* var. (but never of *V. dahliae*) which had grown in the presence of griseofulvin in excess of 1 mg./l. The mycelium of such sectors will be for convenience termed adapted. To

compare the growth of adapted and non-adapted mycelium both Dox medium agars and Dox liquid media with added griseofulvin were used.

Dox medium agar cultures. The results of attempts to determine the effects of various concentrations of griseofulvin on the radial expansion of colonies of adapted mycelium, of mycelium derived from spores of the adapted mycelium, and of the non-adapted mycelium, all of *Verticillium albo-atrum* var. are summarized in Table 4. In the presence of griseofulvin, colonies from spores and mycelium of the adapted isolate grew at the same rate, and faster than the non-adapted mycelium. In the absence of griseofulvin, however, the diameters of the colonies of the non-adapted mycelium were greater than those of the adapted mycelium. The adapted mycelium grew as smooth flat colonies with faint radiating furrows on a white fairly fluffy surface of slightly sporulating aerial mycelium. The mycelium on media with griseofulvin in excess of 1 mg./l. was curled and waved, and although the hyphae were not gnarled they still produced some intercalary swellings at the higher concentrations. The gross morphology and microscopic morphology of the non-adapted mycelium at the various griseofulvin concentrations was similar to that previously described.

Table 4. *The effect of griseofulvin on the growth of the adapted mycelium, mycelium derived from spores of this adapted mycelium and on non-adapted mycelium of Verticillium albo-atrum var., after 15 days.*

Medium	Adapted mycelium	Mycelium from spores of adapted mycelium	Non-adapted mycelium	Adapted mycelium	Non-adapted mycelium
	Colony diameter on Dox medium agar (mm.)			Mycelial dry weight (mg.)	
Dox agar + 1 % (v/v) ethanol alone	49·0	48·0	55·0	112·5	97·1
Dox agar + 1 % (v/v) ethanol + griseofulvin (mg./l.)					
1	48·0	48·0	50·5	91·4	78·3
10	33·0	30·5	18·0	45·2	36·5
20	28·0	27·0	14·5	37·0	24·2
40	24·0	23·5	11·0	37·5	21·1

Dox medium liquid cultures. The effects of griseofulvin on the growth of the adapted and non-adapted mycelium in Dox liquid media are summarized in Table 4. Both isolates produced approximately the same amount of mycelium on the control media without griseofulvin. In the presence of griseofulvin the growth of both isolates was progressively inhibited by increasing additions of griseofulvin to 20–40 mg./l., but the adapted mycelium grew better than did the non-adapted.

Both isolates developed as thick gelatinous mycelial mats on the media without griseofulvin but in the presence of griseofulvin the adapted isolate gave compact mycelial mats in contrast to the non-adapted isolate which formed small separate colonies showing no tendency to form mats.

Examination of mycelium grown in the presence of griseofulvin showed the following morphological abnormalities: with griseofulvin 10 mg./l. the mycelium of the non-

adapted isolate was curled, waved stunted and torulose, whereas mycelium of the adapted isolate was only slightly waved and extreme hyphal distortion was not seen. With griseofulvin 20 mg./l. the mycelium of the non-adapted isolate was extremely distorted (Pl. 1, fig. 4) but the mycelium of the adapted isolate was similar to the appearance shown at 10 mg./l. (Pl. 1, fig. 5). With griseofulvin 40 mg./l. extreme hyphal distortion and malformation appeared in the mycelium of the non-adapted isolate, whereas the mycelium of the adapted isolate, although showing curling and waving, never showed extreme distortion.

To test the stability of the adapted mycelium, the adapted and non-adapted isolates were grown for 75 days (five 15-day subcultures) on Dox medium agar alone. At each subculture, a sample of mycelium of each isolate was transferrred to griseofulvin-containing Dox medium agar. Since in the presence of griseofulvin the growth rate and morphology of the mycelium of the two isolates was similar to that described above, the conclusion may be drawn that no loss of adaptation occurred. Similarly, stock test tube cultures of the adapted isolate kept for 9 months on Dox medium agar still showed no loss of adaptation.

To test the effect of griseofulvin on chitin production in adapted and non-adapted strains of *Verticillium albo-atrum* var. mycelia of both strains were each separately grown in Dox liquid medium + 1 % (v/v) ethanol and in Dox liquid medium + 1 % (v/v) ethanol with added griseofulvin 10 and 40 mg./l. The mycelium from each treatment was harvested and separately analysed for chitin determined as glucosamine. Three separate analyses were done on each batch of mycelium. The results (Table 5) suggest that whereas the glucosamine content of the cell walls of the non-adapted mycelium increased with increasing griseofulvin concentration, the glucosamine content of the walls of the adapted isolate was almost unaffected by the presence of griseofulvin.

Table 5. *The effect of griseofulvin on chitin synthesis (as % glucosamine) in isolates of Verticillium albo-atrum var. adapted and non-adapted to griseofulvin*

	Medium in which mycelium was grown	Glucosamine-*N* in dry mycelium (%)
Non-adapted isolate	Dox liquid medium + 1 % (v/v) ethanol alone	0·089*
	Dox liquid medium + 1 % (v/v) ethanol + griseofulvin (mg./l.)	
	10	0·17
	40	0·25
Adapted isolate	Dox liquid medium + 1 % (v/v) ethanol alone	0·12
	Dox liquid medium + 1 % (v/v) ethanol + griseofulvin (mg./l.)	
	10	0·094
	40	0·16

* Each analysis is the average of three batches of mycelium each of dry weight 35–40 mg.

DISCUSSION

The observation that griseofulvin had an effect both on the morphology of the hyphae of the Verticillium species tested, and on their growth as measured by radial expansion of colonies and mycelial dry weight increases, is in agreement with the report of Brian (1949) that *Verticillium albo-atrum* was sensitive to griseofulvin 1 mg./l.

Higher concentrations of griseofulvin induced in *V. albo-atrum* and *V. dahliae* the formation of intercalary chlamydospore-like swellings apparently similar to those described by Brian (1960) in *Fusarium* species.

Although Napier *et al.* (1956) reported that *Verticillium albo-atrum* and *V. dahliae* differed in their response to various concentrations of griseofulvin, as judged by the severity of morphological abnormalities in the hyphae, in the present work both species reacted similarly in that the mycelia of both were much distorted at high concentrations. However, the gross appearance of the colonies of the two species was different on Dox medium agar containing griseofulvin: the surface of cultures of *V. albo-atrum* was gnarled and convoluted, whereas that of *V. dahliae* was more like the controls without griseofulvin, in that it had a smooth appearance.

The peculiar solubility characteristic of griseofulvin in which approximately the same amount went into solution at griseofulvin 100 mg./l. as at 10 mg./l. may to some extent explain the result obtained by Rhodes (1962) who reported that the uptake of griseofulvin by leaves decreased rapidly with concentration; 73% was retained at griseofulvin 20 mg./l. but only 1% at 5000 mg./l.

The loss of resistance to griseofulvin of trained mycelium of *Verticillium albo-atrum* and *V. dahliae* after a period of growth in the absence of griseofulvin was similar to that reported by Brian (1960) with *Botrytis allii*, by Bartlett (1959) and by Parry & Wood (1958, 1959*a*, *b*) with other fungi to other inhibitors. Using dermatophytic fungi, however, Aytoun, Campbell, Napier & Seiler (1960), and Rosenthal & Wise (1960) found that resistance to griseofulvin obtained in training experiments was stable and possibly resulted from the selection of mutants.

Brian (1960) kept colonies of *Botrytis allii* on a nutrient agar medium containing griseofulvin for several weeks, but never observed any tendency for colonies or sectors of colonies to overcome the inhibition. During the training experiments reported here sectors of faster growing mycelium were occasionally produced at the periphery of colonies of *Verticillium albo-atrum* var. (but never of *V. dahliae*) which had grown in the presence of griseofulvin > 1 mg./l. This mycelium was considered to be adapted since it was stable, and the adaptation was not lost after prolonged growth on griseofulvin-free media. The fact that the adaptation was transmitted through the spore suggests that it was based on mutation.

As a result of the abnormalities in the hyphae, the radial growth of fungal colonies on agar containing griseofulvin may be restricted (Brian, 1960). Adapted mycelium of *Verticillium albo-atrum* was not severely distorted even at high concentrations of griseofulvin (40 mg./l.) and grew faster in the presence of the griseofulvin than did non-adapted mycelium; the reverse was true in the absence of the griseofulvin. Similar morphological responses were observed in Dox liquid medium experiments and although the adapted mycelium appeared more normal than did the non-adapted, the decrease in development of both isolates was considerable, suggesting that inhibition of growth and the morphological effects of the griseofulvin are separate phenomena.

The chitin content (as % glucosamine) of the cell walls of the adapted and non-adapted isolates suggested that griseofulvin stimulated the production of excess chitin only in the non-adapted isolate. Bent & Moore (1966), however, found no differences in the composition of isolated walls of *Botrytis allii* after growth in the presence of griseofulvin, although overall increases in cell wall material occurred.

The diminution in the morphological effects shown by the adapted isolate may be a

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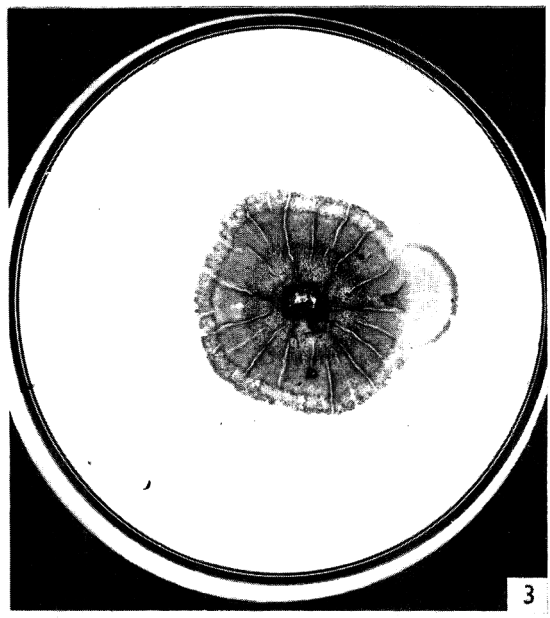

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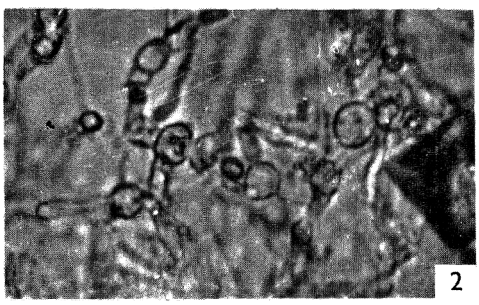

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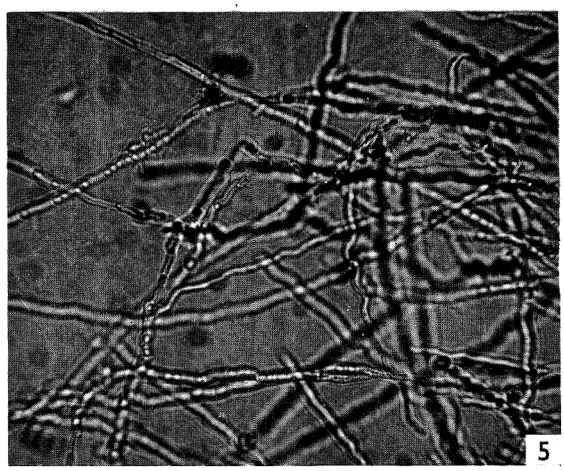

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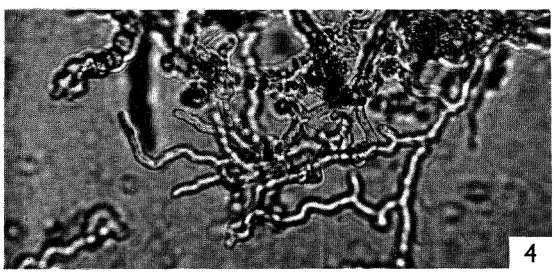

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reflexion of a mechanism which prevents griseofulvin from disturbing the morphogenetic system. It would appear unlikely that this is because the isolate breaks down the griseofulvin, for although its enzymic breakdown by various fungi has been reported (Abbott & Grove, 1959; Boothroyd, Napier & Somerfield, 1961), El-Nakeeb & Lampen (1965) found breakdown products only in cultures of griseofulvin sensitive fungi and suggested that uptake of the antibiotic was necessary for degradation to occur.

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EXPLANATION OF PLATE

Fig. 1. Germ tubes developing from a spore of *Verticillium albo-atrum* on Dox medium agar + 1 % (v/v) ethanol and added griseofulvin 40 mg./l. × 1200.

Fig. 2. Thin-walled chlamydospore-like cells of *Verticillium albo-atrum* in Dox liquid medium + 1 % (v/v) ethanol and added griseofulvin 20 mg./l. × 750.

Fig. 3. Development of a sector of adapted mycelium in a colony of *Verticilium albo-atrum* var. on Dox medium agar + 1 % (v/v) ethanol and added griseofulvin.

Fig. 4. Mycelium of *Verticillium albo-atrum* non-adapted to griseofulvin. × 300.

Fig. 5. Mycelium of *Verticillium albo-atrum* adapted to griseofulvin. × 300.

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The Activation of Spores of *Clostridium bifermentans*

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SUMMARY

The activation of spores of *Clostridium bifermentans* by various treatments was investigated. Mercaptoacetate did not activate the spores but inhibited the spontaneous activation which occurred in buffer alone. Sodium borohydride effected activation of spores by increasing the pH value above pH 10·0 rather than by its reducing action. The pH value of a suspension had a marked effect on activation; at 37° spores held at less than pH 3·0 or at pH 10·0 or more were activated within 1 hr. Continued incubation at an alkaline pH value led to a decrease in the specific requirements for germination, an effect not produced by acid pH values. Heat-activated spore suspensions became partially de-activated on storage unless they were continuously aerated. Spores activated at 37° and pH 2·0 or pH 7·4 readily became de-activated on storage, whereas spores activated at pH 10·5 remained activated. A specific requirement of heat-activated spores for sodium ions for germination was also found.

INTRODUCTION

The transition of dormant bacterial spores to fully active vegetative forms can be divided into three distinct phases: activation, germination, outgrowth. Activation of spores or the breaking of dormancy is commonly achieved by heating spores in aqueous suspension, a process first described by Evans & Curran (1943). It was reported, however, that reducing agents or exposure to acid pH values activated spores of an aerobic organism, *Bacillus cereus* (Keynan, Evenchik, Halvorson & Hastings, 1964). The effect of these agents as activators of spores of anaerobic bacteria has apparently not been investigated, although it has been reported that spores of *Clostridium roseum* require reducing conditions for germination (Hitzman, Halvorson & Ukita, 1957). In a previous publication (Gibbs, 1964), some of the factors which affect the germination of heat-activated spores of *C. bifermentans* were described. The present paper describes experiments on the effect of reducing agents and pH value as activating agents for spores of this organism and also investigations of the inorganic ion requirement for germination.

METHODS

The technique for the production of suspensions of spores of *Clostridium bifermentans* (CN 1617, Wellcome Research Laboratories' Culture Collection), activation by heat and estimation of germination were as previously described (Gibbs, 1964). A decrease in extinction of 55–60 % was found to correlate closely with phase-darkening of 95–100 % of the spores and, for speed and convenience, the decrease in extinction was used as a measure of germination. Since spores which were not activated did not

germinate within 1 hr at 37° on the addition of germinants, the degree of germination (% decrease in extinction) was taken as a direct measure of the degree of activation. All work with spore suspensions was done in 0·1 M-[Na/K] phosphate buffer (pH 7·4) unless otherwise stated.

Treatment of spores with reducing agents. For studies on the effect of mercaptoacetic acid on spores, the free acid was titrated to pH 7·4 with 10 N-NaOH, and added to spores suspended in phosphate buffer. Sodium borohydride and lithium aluminium hydride (L. Light and Co., Colnbrook, England) were added as solids (0·5–1·0 mg./ml.) to spores suspended in phosphate buffer (O.D. 680 mμ, *ca.*1·5; Hilger 810 Biochem. Absorptiometer, 15 mm. diam. tube). Treated spores were washed twice with phosphate buffer before testing for germination.

Treatment of spores with acid or alkali. Spores were suspended in phosphate buffer, titrated to the required pH value with NaOH or HCl, and incubated at 37°. Samples were removed and the spores washed twice with phosphate buffer and tested for germination.

De-activation of spores. After activation by heating or exposure to acid or alkali, spores were washed with phosphate buffer and stored as suspensions in this buffer at 4° and at room temperature (16–20°) in screw-capped bottles three-quarters full, tightly screwed down to minimize aeration. A heat-activation spore suspension was also stored at room temperature with a current of sterile air bubbled through it. Two samples were removed at intervals from the stored suspensions, one was heat-shocked (at 85° for 10 min.) and both were tested for germination.

Extraction of phosphate buffer with diphenyl thiosemicarbazone (dithizone). Phosphate buffer (200 ml.) was extracted ten times with 10 ml. portions of dithizone dissolved in chloroform (5 mg./100 ml.) Dithizone remaining in the buffer was extracted with chloroform and excess chloroform removed by warming and aerating.

RESULTS

Effect of reducing agents on spores of Clostridium bifermentans

Incubation of spores of *Clostridium bifermentans* at 16–20° in the presence of 0·01–0·5% mercaptoacetate for 2–3 days did not activate the spores to any greater degree than did incubation in buffer alone. However, after incubation for 7 days spores suspended in buffer alone had become markedly activated, whereas in the presence of mercaptoacetate little or no activation had occurred (Fig. 1 A, B). At all times heating was fully effective in activating the washed spores (Fig. 1, A′, B′).

Incubation of spores for 6 hr at 16–20° in the presence of the reducing agent sodium borohydride achieved full activation of the spores, but lithium aluminium hydride was without effect. However, it was found that the addition of sodium borohydride caused an increase of pH value to 9–10·5, depending on the amount added. It seemed possible, in the light of the results of Keynan *et al.* (1964), that either the pH value attained or the reducing conditions were reponsible for the activation observed. The effect of pH value was therefore investigated.

Effect of pH value of the suspension on the activation of spores

Since it appeared possible that alkaline pH values could activate spores of *Clostridium bifermentans* and it had been reported that acid pH values activated spores of

Bacillus cereus (Keynan *et al.* 1964), the effect of a range of pH values was examined; the results are summarized in Fig. 2. Marked activation occurred at pH 10·0 and above, an effect not noted by Keynan *et al.* (1964), and also at pH 2·0; but in the range pH 3·0–9·0 the activation was much less marked. It was shown that the high concentrations of Na^+ or Cl^- ions, added when adjusting the pH values, were not responsible for the activation observed. It appeared therefore that the activating effect of $NaBH_4$ could be entirely accounted for by the alkaline pH value attained on addition of this compound.

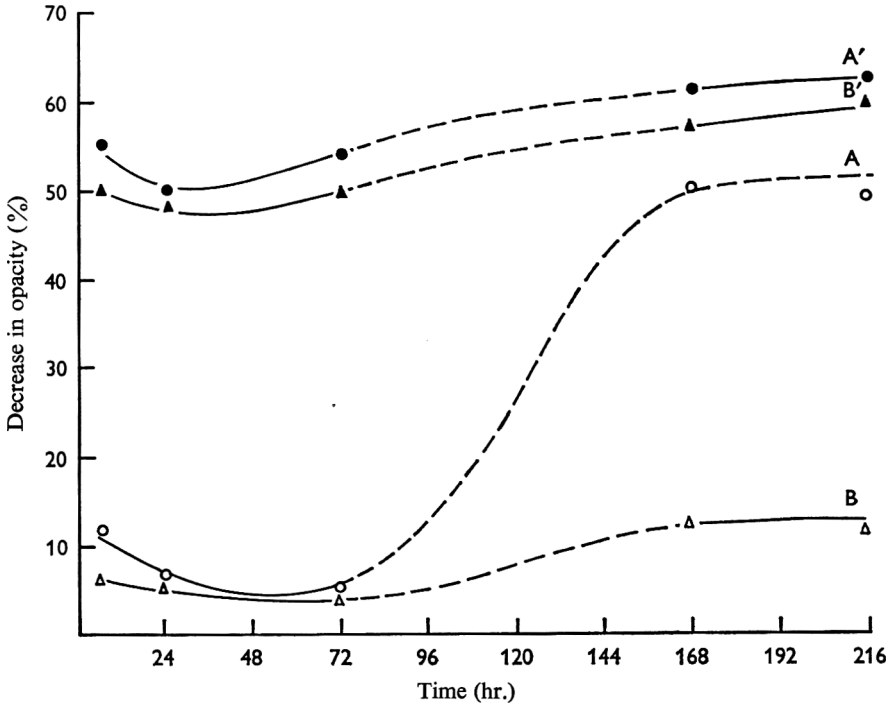

Fig. 1. Effect of mercaptoacetate on spores of *Clostridium bifermentans* during storage at 16–20°. Spores were washed twice before testing for germination. A, spores suspended in 0·1 M-phosphate (pH 7·4); B, spores suspended in 0·1 M-phosphate (pH 7·4)+0·01 % mercaptoacetate; A′ and B′, as A and B but the spores were heat shocked (85° for 10 min.) before testing for germination.

Samples removed at intervals from a spore suspension incubated at pH 10·5 and 37° showed an increasing degree of activation during incubation, rising to complete activation after 30 min.; the rate of activation appeared to be almost linear up to 20 min. (Fig. 3). Little activation occurred in spore suspensions incubated at pH 7·4 and 37° for periods of up to 1 hr., although continued incubation of these spores led to 60–70 % activation in 2 hr.

Annulment of activation ('de-activation') on storage

Although activated spores of aerobic bacteria have been reported to undergo de-activation on storage (Curran & Evans, 1947; Desrosier & Heiligman, 1956; Keynan *et al.* 1964), there appear to be no reports of the phenomenon in activated

spores of anaerobic bacteria. It was therefore decided to investigate whether spores of *Clostridium bifermentans* previously activated by the treatments outlined above, would undergo de-activation on storage.

Spores which were activated by incubation at 37° and pH 7·4 for 2 hr. when stored at 16–20° or at 4° in tightly capped bottles (conditions of minimal aeration) became fully de-activated in 6–7 days. Heating at 85° for 10 min. fully re-activated these spore suspensions at all times during storage.

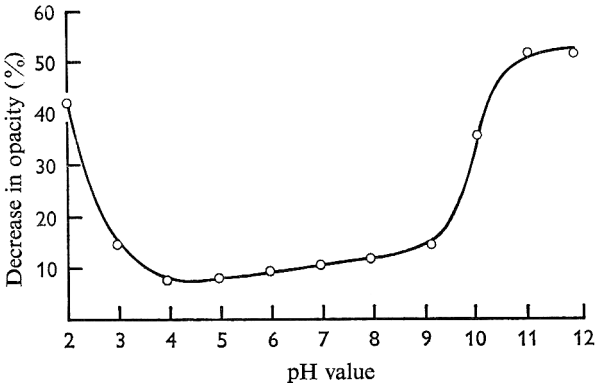

Fig. 2

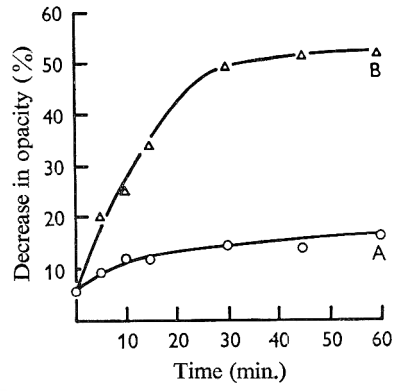

Fig. 3

Fig. 2. Activation of spores of *Clostridium bifermentans* by treatment at various pH values. Spores were suspended in 0·1 M-phosphate titrated to various pH values with NaOH or HCl, incubated for 2 hr. at 37°, washed twice with 0·1 M-phosphate (pH 7·4) and tested for germination.

Fig. 3. Activation of spores of *Clostridium bifermentans* at pH 10·5, 37°. A, spores suspended in 0·1 M-phosphate (pH 7·4), 37°, B, spores suspended in 0·1 M–phosphate (pH 10·5), 37°. Samples were removed at intervals, washed twice with 0·1 M-phosphate (pH 7·4) and tested for germination.

Spores heat-activated at pH 7·4 and stored under the same conditions, showed a rather slower rate of de-activation, a loss of 50% of activation being observed over a period of 10–14 days. Re-activation of these spores by heating at 85° for 10 min. did not occur. When heat-activated spores were stored with continuous aeration at 16–20° and pH 7·4, no de-activation was observed over a period of 10–14 days. A second heat treatment of these spores immediately before the addition of germinants, led to a much smaller degree of germination.

Spores activated at pH 2·0 showed a steady decrease in the degree of activation up to 10 days of storage, when approximately 30% of spores remained in the activated state. Heating of samples of these spore suspensions (85° for 10 min.) during the first 48 hr of storage showed a decrease in the degree of germination, but later heated samples showed an increased degree of germination on the addition of germinants as compared with unheated samples. It is possible that spores activated at pH 2·0 become sensitive to heat, but on de-activation regain their heat resistance, and also show a requirement for re-activation.

Germination of spores of Clostridium bifermentans: the minimum requirements

In a previous paper (Gibbs, 1964) it was reported that lactate, L-phenylalanine and L-α-alanine were all essential for the germination of heat-activated spores of *Clostridium bifermentans* suspended in phosphate buffer. During the present work it was found that germination of heat-activated spores occurred only when all three compounds were present simultaneously; addition and removal of these three compounds in all possible sequences did not lead to germination of spores. When similar experiments were made with spores activated at pH 10·5 and 37°, rapid and complete germination of the washed spores occurred in the presence of L-α-alanine+lactate or L-α-alanine+L-phenylalanine when the activation time was 2 hr or longer. All three compounds were required however for the germination of spores activated at pH 3·0 and 37° for 2 hr. Spores activated at pH 10·5 for 2 hr at 37° were therefore able to dispense with either L-phenylalanine or lactate, in the presence of L-α-alanine, for germination. The rates at which spores lost the requirement for lactate or L-phenylalanine during activation at pH 10·8 and 37° are shown in Table 1. It is seen that the requirement for L-phenylalanine was lost steadily during the activation period, but the requirement for lactate was not lost until after at least 60 min. exposure to pH 10·8 at 37°. The presence of L-α-alanine was essential for germination at all times.

Table 1. *Variations in the minimum requirements for germination*

Spore suspensions were incubated at 37° at pH 7·4 or pH 10·8. Samples were removed at intervals, the spores washed with phosphate buffer (pH 7·4) and tested for germination with L-α-alanine+lactate and with L-α-alanine+L-phenylalanine. Extinction values 680 mμ were measured over 60 min. and the decrease in extinction over 60 min. calculated (%), $\left(\frac{(E_0 - E_{60}}{E_0} \times 100\right)$.

Activation time at 37° (min.)	L-α-alanine+lactate		L-α-alanine+L-phenylalanine	
	pH 7·4	pH 10·8	pH 7·4	pH 10·8
	Decrease in extinction (E_{680}) over 60 min. (%)			
0	3·2	0	5	0
5	0	0	0	0
10	3·4	7·3	0	0
15	0	10·2	2·0	0
30	0	16·2	3·2	0
45	0	20·0	3·0	0
60	0	37	0	5
120	0	47	0	35

Inorganic ions required for germination. To investigate the requirements for inorganic ions for germination, spores were centrifuged from phosphate buffer suspension, washed five times with distilled water or with tris-HCl buffer, and resuspended in distilled water or tris-HCl. The suspensions were heat-activated (85° for 10 min.) and L-α-alanine, L-phenylalanine and lactate added. Germination of these suspensions was not observed during a period of 2 hr. When 0·1 M-phosphate buffer was added to these suspensions after 2 hr incubation, rapid and complete germination occurred. Further work showed that the degree of germination was proportional to the concentration of phosphate buffer, rising to a maximum at approximately 0·1 M. Heat treatment there-

fore was effective in producing activation of spores in distilled water or in tris-HCl buffer, but some component of the phosphate buffer appeared to be essential for the germination process. Trace metals have been shown to be active in promoting germination (Levinson & Sevag, 1953; Levinson & Hyatt, 1955). For this reason routine phosphate buffer was extracted with a solution of dithizone in chloroform. Spores suspended in dithizone-extracted buffer at first showed a decrease in the degree of germination, but this was traced to residual traces of chloroform in the buffer and when this effect was eliminated, the extracted buffer was found to be as effective as was non-extracted buffer in producing rapid and complete germination. The effect therefore appeared not to reside in the trace elements removed by dithizone but in the major ions present. The routine buffer was prepared from KH_2PO_4 titrated to pH 7·4 with NaOH. Heat-activated spores suspended in a buffer made by substituting KOH for NaOH did not germinate on addition of L-α-alanine + L-phenylalanine + lactate. From this it appeared that it was the sodium ions in the original buffer that were essential for germination. To test this, tris-HCl buffer was prepared in 0·1 M-NaCl. Heat-activated spores suspended in this buffer showed complete and rapid germination on the addition of the three specific germinants. However, when NaCl was replaced by KCl, germination did not occur. It would therefore seem that heat-activated spores of *Clostridium bifermentans* have an obligate requirement for sodium ions for germination.

DISCUSSION

It has long been known that many bacterial spores require some form of treatment before rapid germination in a nutrient medium will occur. This treatment has usually consisted of a short period of heating, so-called heat-shock, which was first described by Evans & Curran (1943) and has since been well-documented (Murrell, 1961). The term in use at present for this process is 'activation', describing an increase in metabolic activity of activated spores (Church & Halvorson, 1957) and also increased sensitivity to inimical reagents and heat (Halvorson & Church, 1957). Vinter (1960, 1961) showed that much of the inert nature of bacterial spores may be attributed to the presence of large numbers of cystine disulphide bonds present in the spore-coat protein, and that these are broken during germination. It was possible that reduction of these bonds would cause a modification of the structure of the spore-coat protein, thus activating spores either by increasing the permeability of the spore-coat to germination agents, or by exposure of the enzymes necessary for germination, or by a combination of these effects. The results obtained here with spores of *Clostridium bifermentans* treated with mercaptoacetate indicated that no activation occurred and indeed a marked inhibition of activation was observed. Such was not apparently the case with spores of *Bacillus cereus* since mercaptoacetate effected activation (Keynan *et al.* 1964). However, mercaptoacetate is not without some effect on spores of *C. bifermentans*, since it has been reported that spores so treated are lysed by hydrogen peroxide or lysozyme (Gould & Hitchins, 1963).

From our experiments on activation during incubation at various pH values, it would appear that activation occurs over a wide range of pH values, but that the rate of activation is greater at the extremes of the range. If activation is due to a reversible denaturation-like process of the spore-coat protein, as suggested by Keynan *et al.* (1964), then it is to be expected that the rate of activation would increase as extremes

of pH value or temperature are approached. However, it appears that activation of the spores of *Clostridium bifermentans* at pH 10·5 is more radical than activation at neutral or acid pH values or by heat-shock, since the germination requirements are then simplified and also de-activation does not occur on storage.

De-activation of heat-activated spores of *Clostridium bifermentans* has been shown to occur most readily in conditions of partial anaerobiosis and not in highly aerated conditions. This suggests that enzymic reactions are necessary for de-activation and not oxidation of sulphydryl groups since the organism is a strict anaerobe.

Many workers have noted a requirement for inorganic ions for spore germination (e.g. Levinson & Sevag, 1953; Rode & Foster, 1962*a*, *b*; Fleming & Ordal, 1964) but most of these results have been obtained with spores of species of the genus *Bacillus*. There has apparently been no other report of an obligate requirement for Na^+ ions for the germination of spores of an obligate anaerobe like *Clostridium bifermentans*.

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Studies on Tissue Specificity of Interferon

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SUMMARY

This study reports the investigation of tissue specificity of interferon induced with Chikungunya virus in lung and kidney cells obtained from 17-day chick embryos. Lung cells produced significantly more interferon than kidney cells under essentially the same conditions. Both lung and kidney interferon preparations repressed plaque formation on homologous and heterologous cells and lung cells were more sensitive to the protective effects of all the interferon preparations tested. No evidence for tissue specificity was obtained.

INTRODUCTION

Since the original observation that the cell which produces an interferon governs its specificity of action (Tyrrell, 1959), species specificity has become a well-documented property of interferon. However, no systematic study has been done to determine whether interferon made in different tissues obtained from the same animal exhibits tissue specificity. If tissue specificity is a property of interferon, this would be important in the understanding of interferon action, as well as in its use as a therapeutic agent. It would also imply that more than one cistron could be responsible for interferon synthesis. This study reports the investigation of tissue specificity of interferon induced in freshly dispensed lung and kidney cells obtained from 17-day chick embryos. Chick lung and kidney tissues differ in their embryonic origin. The kidney arises entirely from the mesodermal layer. The lung originates mainly from the endoderm, although the mesoderm makes some contribution to the connective elements and musculature of the lung.

METHODS

Cell cultures. Chick embryo fibroblast (CEF) cell cultures prepared from eviscerated and decapitated 10- to 11-day chick embryos were used for assay of virus and interferon (Lindenmann & Gifford, 1963*a*). Chick embryo lung and kidney cells, used for interferon production and assay, were prepared from 17-day chick embryos by a slight modification of the procedure used for whole embryos. The trypsin (Trypsin 1:250; Difco Laboratories, Detroit, Michigan, U.S.A.) used to prepare these cultures was increased to 0·1 % (w/v) for kidney cells, and 0·25 % (w/v) for lung cells. In culture, lung tissue was composed of 67 % fibroblast-like cells and 33 % epithelium-like cells; kidney tissue was composed of 80 % epithelium-like cells and 20 % fibroblast-like cells. Chick embryo fibroblast cell cultures were used at 44–48 hr; lung and kidney cells were used immediately after dispensing for interferon production, or at 27 hr

for interferon assays. The monolayers of all three cell types contained approximately 4×10^6 cells at the time of use.

Media. Growth medium consisted of Gey's BSS with 5% (v/v) calf serum, 0·1% (w/v) lactalbumin hydrolysate (enzymic; General Biochemicals, Chagrin Falls, Ohio, U.S.A.), 0·1% (w/v) Proteose peptone (Difco Laboratories), and 0·0025 M-tris or 0·06% (w/v) sodium bicarbonate. Maintenance medium was Gey's BSS with 0·11% (w/v) sodium bicarbonate, 0·1% (w/v) lactalbumin hydrolysate, 0·1% (w/v) yeast extract (Difco Laboratories) and 0·1% (w/v) Proteose peptone (Gifford, Mussett & Heller, 1964). Trypsin diluent consisted of Gey's BSS without magnesium and calcium. The medium used for the agar overlay consisted of maintenance medium with a final concentration of 5% (v/v) calf serum and 0·5% (w/v) 'Ion-agar' no. 2, omitting the yeast extract.

Virus strains. Vesicular stomatitis virus (VSV: Indiana strain) obtained from Dr S. Baron (National Institutes of Health, Bethesda, Maryland, U.S.A.) and vaccinia virus strain NY–914, were used for interferon assays. Stock preparations of VSV were derived by passage in primary cultures of chick embryo fibroblasts. Stock vaccinia virus was prepared by inoculating the chorioallantois of 11-day developing chick embryos. Infected membranes were removed and homogenized in maintenance medium 48 hr after inoculation. Chikungunya virus, used as the interferon inducer, was prepared by passage in the brains of new born mice. Brains were removed 44–48 hr after inoculation and homogenized in maintenance medium to make a 10% (w/v) suspension. All virus stocks were centrifuged to remove tissue debris and were stored in glass ampoules at $-70°$.

Interferon. Chick lung and kidney interferon was induced in kidney and lung cells by adding a 1/100 dilution of stock Chikungunya virus at the time the cells were dispensed. One hundred million (10^8) kidney and lung cells in 20 ml. growth medium were dispensed into 32 oz. bottles with a flat surface of 17×7 cm. Interferon-containing supernatant fluids were harvested 20 hr later. Chikungunya virus was inactivated by heating at 65° for 30 min. Interferon assays in which vaccinia virus was used as challenge virus followed the procedure of Lindenmann & Gifford (1963*b*). For interferon assays with VSV as challenge virus, cells were treated with the desired interferon dilutions for 6–7 hr before challenge with VSV. Virus was allowed to adsorb for 1 hr, unadsorbed virus was removed, and an agar-overlay added. In both systems, one p.d.d. 50 unit is the amount of interferon which depressed the plaque count by 50% (Lindenmann & Gifford, 1963*b*).

RESULTS

Production of interferon by kidney and lung cells

Low yields of interferon were detectable in the supernatant fluids of kidney and of lung cell cultures within 1 hr after infection with Chikungunya virus. Production continued linearly for about 20 hr. when a maximum titre was reached. Interferon was produced at a faster rate and reached a significantly higher titre in lung than in kidney cultures. Statistical analysis of differences between titres obtained at the point of maximum production in each system revealed that these differences were significant at the 1% level, or that they would be obtained by chance only 1 in 100 times (See Table 1). The method for interferon induction was identical in both systems, indicating

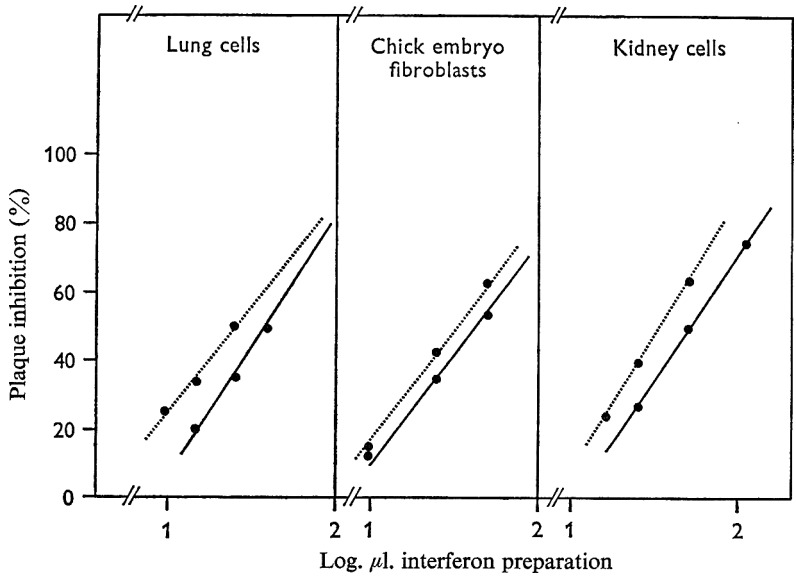

Fig. 1. Dose response curves of interferon prepared in kidney and lung cells on vesicular stomatitis virus plaque formation on lung, kidney and chick embryo fibroblast cultures. —, Kidney interferon; ....., lung interferon.

Table 1. *Comparison of lung and kidney interferon assayed on chick embryo fibroblast cultures**

Preparation no.	Activity of interferon preparations, p.d.d. 50 units/ml.	
	Lung interferon	Kidney interferon
1	345	116
	345	109
2	208	54
	169	61
3	200	96

* Vaccinia virus challenge, The difference between p.d.d. 50 units for lung and kidney interferon preparations was significant to the 1 % level.

that the greater yield of interferon from the lung system could probably be attributed to some property of the lung cells themselves. A difference in the capacity of cells to synthesize and release interferon was reported by Ho (1964).

Tissue specificity assays

Dose-response curves depicting the effect of kidney and lung interferon on VSV plaque formation on lung, kidney, and CEF monolayers are shown in Fig. 1. As expected from the titre of interferon on CEF cultures infected with vaccinia virus, interferon from lung cell cultures afforded significantly greater protection in all systems than did interferon obtained from kidney cells. Lung cultures were also more sensitive to interferon action than were kidney or CEF cultures. This greater sensitivity

was found even when interferon made in kidney cultures was used. p.d.d. 50 units were determined by interpolation, converted to units per ml. and are reported in Table 2.

Table 2. *Comparison of p.d.d. 50 units of interferon preparations produced and assayed in various systems**

p.d.d. 50 units/ml. of interferon

		Interferon assay system on		
Interferon producing system	Prep no.	Lung	Kidney	chick embryo fibroblasts
Lung	1	n.t.†	43	51
		n.t.	50	n.t.
	2	56	40	n.t.
	3	48	35	n.t.
Kidney	1	34	19	27
		n.t.	21	n.t.
	2	42	20	n.t.
	3	26	20	22
Chick embryo fibroblasts	1	51	37	n.t.

* Challenged with vesicular stomatitis virus
† n.t., not tested

DISCUSSION

The apparent lack of tissue specificity found in the present work should not be surprising since the two cell types are derived from the same animal. Since cellular DNA's exhibit species specificity rather than organ specificity, it is quite reasonable to postulate induction of the same interferon from kidney and lung cells, assuming that identical genetic information is possessed by each kind of cell. Statistical analysis performed on slopes of dose-response curves for both interferons revealed no significant deviation from parallelism. These results further indicate that the same interferon is induced in both systems.

The differences between the lung and kidney cultures might be explained by the differences in the proportions of fibroblast and epithelium-like cells in each culture. It is possible that the fibroblast cells were protected by less interferon and produced more interferon than epithelium-like cells. Thus, it is possible that some genetic information, presumably coded in all cells, cannot be as efficiently utilized by all cells.

Variation in sensitivity to interferon in different cells from the same embryo should be further considered. The sensitivity of cultures made from 10 to 11-day embryos (CEF) was intermediate to that found for the lung and kidney cultures. Procedures for the establishment of primary cell cultures vary in different laboratories (e.g. age of embryo, medium composition, trypsinization procedures) and it is possible that these differences will result in a variation in the ability of different cell types to survive and to become established. Furthermore, cells of various types may have a selective advantage under the cultural conditions imposed and populations may consequently change with time. It is possible that the sigmoid nature of the dose-response curve (Lindenmann & Gifford, 1963*b*) may be due, in part, to the presence of a cell type which is relatively insensitive to the action of interferon.

The findings presented in the present paper also have implications for the possible therapeutic use of interferon, since different kinds of cells from the same animal may show variability in their response to interferon. Consideration should therefore be given not only to the interferon sensitivity of a given virus, but also to the interferon sensitivity of the cell type in which the virus replicates. The sensitivity of a virus in an *in vitro* assay may not indicate the efficacy of interferon treatment *in vivo*, since the cell types in which the virus replicates in these two instances may differ. Also, the sensitivity to interferon exhibited by cells in culture may differ from the sensitivity of the same cell type in tissue.

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Antibiotic Inhibition and Binding Studies with a Group A Streptococcal L-Form

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SUMMARY

The minimal inhibitory concentrations of various antibiotics for a strain of *Streptococcus pyogenes* and a stable L-form of it were determined. These results were evaluated with those of other investigators similarly performed with *S. faecalis* and derived protoplasts. Differences in reaction to puromycin, bacitracin and ristocetin were apparent only between the wall-less derivatives. The polyene antibiotics nystatin and filipin and two antimicrobial agents, isatin 3-thiosemicarbazone and 1-methyl-isatin 3-thiosemicarbazone, were ineffective or only slightly inhibitory on growth of *S. pyogenes* and its L-form. Labelled penicillin studies with this group A streptococcus and its L-form indicated that the binding sites for penicillin were probably distinct from those concerned with streptococcal wall formation.

INTRODUCTION

Some information is available about the sensitivity of bacteria and their derived L-forms or protoplasts to various antibiotics (Ward, Madoff & Dienes, 1958; Shockman & Lampen, 1962; Williams, 1963; Molander *et al.* 1964; Kagan, Zolla, Busser & Liepnieks, 1964). The mode of action of such antimicrobial agents examined thus far has ranged from inhibition or disruption of a specific anatomical component (e.g. cell wall, membrane) to cessation of an essential metabolic process, e.g. protein synthesis. Although several antibiotics are known inhibitors of bacterial cell-wall biosynthesis, only penicillin and D-cycloserine (Krawitt & Ward, 1963) and bacitracin (Rotta, Karakawa & Krause, 1965) have been found capable of inducing 'L-phase variant' formation. The present work confirms the earlier results of others with certain group A streptococci (Ward *et al.* 1958; Rotta *et al.* 1965), staphylococci (Williams, 1963; Krawitt & Ward, 1963; Kagan *et al.* 1964; Molander *et al.* 1964) and their respective L-forms and expands upon these comparisons. The affinity for penicillin of the L-form and its parent group A streptococcus, *S. pyogenes*, with regard to the inability of the L-form to synthesize the rigid cell wall is discussed.

METHODS

The *Streptococcus pyogenes* and its derived stable L-form were the same as those used earlier (Panos, 1965). The L-form was grown in the absence of penicillin, otherwise the liquid medium used for each was as described previously (Panos & Barkulis 1959). For the present work, medium (100 ml.) in 250 ml. Erlenmeyer flasks equipped

with side-arm tubes were inoculated with 5 and 10 ml. overnight cultures, respectively, of the coccus or L-form. Each organism was grown at 35–36° until extinction values of 0·200 and 0·280–0·300 were attained by the L-form and parent streptococcus respectively, (approximately the middle of logarithmic growth), before addition of antibiotic. Changes in turbidity were followed at intervals with a Coleman model 14 spectrophotometer at 650 mμ. Uninoculated media served as blanks. Turbidity increase (growth) is due to an increase in viable numbers for this streptococcal L-form (Panos, 1965). Only logarithmic streptococcal and L-form cultures whose growth rates (doublings/hr) were close to 1·1 and 0·7, respectively, were used. Each antibiotic was assayed at least four different times with each organism. Testing of possible salt effects was done by comparable studies with the coccus grown in L-form medium (containing NaCl 3 %, w/v). Unless specified otherwise, all antibiotics were dissolved in sterile water just before use. Most of these antimicrobial agents were obtained from Dr G. Shockman (Temple University, School of Medicine) and had been used in similar earlier studies (Shockman & Lampen, 1962). Originally, they had been obtained from the sources indicated on request for the purest material available: actinomycin D (free base, 99 % pure) and novobiocin (sodium salt, lot no. L571789–2–4) and bacitracin (zinc salt, lot no. L474789–0–17) from Merck Sharp and Dohme Research Laboratories, Rahway, N.J.; cycloserine (free base, potency 1000 μg./mg.) and vancomycin (free base, potency 966 μg./ml.) from Eli Lilly Co., Indianapolis, Indiana; chloramphenicol (free base, 98·6 % pure) and penicillin G (potassium salt) from Parke Davis and Co., Ann Arbor, Michigan; polymixin B (sulphate, potency 7000 U./mg.) from Burroughs Wellcome and Co., Tuckahoe, N.Y.; puromycin (free base, lot no. 7–5402) from Lederle Laboratories, Pearl River, N.Y.; and ristocetin (free base, potency 1000 μg./ml.) from Abbott Laboratories, Chicago, Illinois. Filipin (free base, potency 1000 μg./mg.; Upjohn Co., Kalamazoo, Michigan) and nystatin (free base, potency > 4000 U./mg.; Squibb Institute for Medical Research, New Brunswick, N.J.) were also assayed. Samples of isatin 3-thiosemicarbazone and 1-methylisatin 3-thiosemicarbazone were a gift from Dr D. J. Bauer, Wellcome Laboratories of Tropical Medicine, London, England. The concentrations given (Table 1) refer to the antibiotics as received. The criteria for growth inhibition and method of expression are similar to those used by Shockman & Lampen (1962), as noted in the legend of Table 1.

For the determination of penicillin binding, a freshly prepared solution (0·6 mg./ml.) of tritium-labelled benzyl-T(G) penicillin (Radiochemical Centre, Amersham, Buckinghamshire, England), specific activity of 128 mc./m-mole, was added (20 μl.) to coccal and L-form cultures upon reaching an extinction of 0·200. Identical amounts of non-labelled crystalline penicillin-G were added to duplicate flasks as controls. Growth inhibition of only the parent coccus was observed after 1 hr, at which time 200 mg. non-labelled penicillin-G was added to all flasks. After an additional 15 min., 20 μl. solution of tritium-labelled penicillin-G was added to those flasks containing only non-labelled penicillin; i.e. non-labelled + labelled penicillin (Table 2). All organisms were harvested after 15 min. by centrifugation in a Serval centrifuge (4C) at 14,500 *g* for 10 min. Each pellet was washed twice with 10 ml. distilled water or 3 % (w/v) sodium chloride solution for the coccus and L-form, respectively. Organisms were extracted with 10 ml. 0·4 M-perchloric acid, centrifuged down and the pellets washed twice more as before. Distilled water (0·35 ml.) was added to each pellet and samples

collected by suction on tared cellulose acetate filter discs (Millipore Corp., Mass.) of pore size 0·45 μ, dried with an infrared lamp, and weighed. Filter discs were dried to constant weight before and after collection of samples and all weighings were done on an analytical microbalance. Radioactivity was determined with a Tricarb liquid scintillation spectrometer after placing each disc in 10 ml. of a counting solution [15.2 g. 2,5-diphenyloxazole (PPO) + 380 mg. 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) in 3·8 l. toluene]. A specific activity for labelled penicillin was similarly obtained by depositing known quantities on discs, drying and counting as done for the samples. From the specific activity of the penicillin and the dry weight of organisms used, the amount of penicillin bound per g. of organism was calculated.

Table 1. *Antibiotic inhibition of growth of Streptococcus pyogenes and its L-form*

	Minimal inhibitory concentration (μg./ml.) medium*		
	Streptococcus	L-form	Streptococcus in L-form medium
Actinomycin D	0·2	0·05	n.d.
Bacitracin	5	> 300†	5
Chloramphenicol	3	3	3
Cycloserine	240	> 300	n.d.
Novobiocin	2	30	75
Penicillin G	0·06	> 300	n.d.
Polymixin B	240	300	n.d.
Puromycin	30	2·5–25‡	n.d.
Ristocetin	1	> 300	2–2·5
Vancomycin	1·5–2	> 300	2

* Concentration which decreased growth at least 50 % of that in control cultures.
† Indicates highest concentration tested without significant inhibition of growth.
‡ Growth inhibition (50 %) or lysis (see Text).
n.d. = not done.

RESULTS

Table 1 tabulates the minimal inhibitory concentrations of those antibiotics found capable of producing at least 50 % inhibition of growth. Figure 1 illustrates a typical example of L-form growth, inhibition (polymixin B) and lysis (puromycin). As is apparent bacitracin, cycloserine, penicillin-G, ristocetin and vancomycin were ineffective in inhibiting L-form growth; this was expected since their primary site of action is purported to be inhibition of cell wall synthesis. Puromycin proved unusual in that above a minimal growth inhibitory concentration of 2·5 μg./ml. medium, lysis of this L-form usually occurred (Fig. 1); the lysis was confirmed by phase microscopy. The time of initiation of lysis, however, varied from 35 to 110 min. after addition of puromycin. A similar but lesser lytic effect was also noted with novobiocin. However, under the conditions of assay, novobiocin was apparently affected by the sodium chloride content (3 %, w/v) of the L-form medium (Table 1). Polymixin B, known to denature microbial membranes, was inhibitory for the parent coccus and for its L-form only at high concentrations. Two polyene antibiotics, nystatin and filipin (215 and 150 μg./ml. medium, respectively), solubilized with the aid of tolerable amounts of redistilled dimethylsulfoxide (DMS 3–4 %, v/v), before addition to media, were either ineffective or only slightly inhibitory of the growth of the L-form and the

parent coccus. Nystatin was without effect upon either organism. Filipin inhibited the growth only of the L-form, and that by 20%, as compared with cultures containing DMS as controls. Two other antimicrobial agents, isatin 3-thiosemicarbazone and 1-methyl-isatin-3-thiosemicarbazone (effective in the prophylaxis of smallpox) were examined. A DMS solution of isatin 3-thiosemicarbazone was added (80 μg./ml.) to the media with some effect, namely 14 and 25% growth inhibition for the coccus

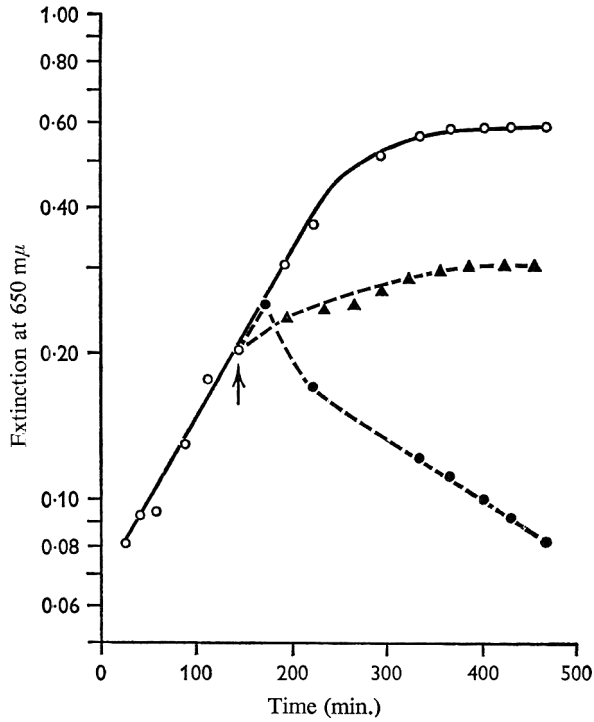

Fig. 1. Growth, inhibition and lysis of a stable L-form from *Streptococcus pyogenes*. ○—○, no addition; ▲—▲, polymixin B, 300 μg./ml. culture; ●—●, puromycin, 15 μg /ml. culture. Antibiotics added at point indicated by arrow.

Table 2. *Tritium-labelled benzyl-T(G) penicillin binding by Streptococcus pyogenes and its stable L-form*

Average of six determinations; L-form cultured in absence of penicillin.

	Penicillin bound (μg./g. organism)	Organism dry weight* (mg.)
	Labelled + non-labelled†	
Streptococcus	0·81 ± 0·15	0·26 ± 0·12
L-Form	0·84 ± 0·23	0·42 ± 0·12
	Non-labelled + labelled†	
Streptococcus	0·11 ± 0·04	0·57 ± 0·33
L-form	0·15 ± 0·07	0·82 ± 0·28

* Average weight of organisms counted.
† Radioactive penicillin added followed by non-radioactive penicillin or vice versa.

and L-form, respectively; 1-methyl-isatin 3-thiosemicarbazone (23 μg./ml. medium) was only slightly inhibitory for the parent coccus (9 %) and its L-form (14 %) as compared with DMS-containing controls. Assay of higher concentrations of these agents, and filipin, was not possible because of their insolubility in aqueous media.

Table 2 tabulates the penicillin-binding capacity of *Streptococcus pyogenes* and its L-form grown in the absence of penicillin for two years, No apparent difference was observed in the affinity of each organism for labelled penicillin on a cell-weight basis.

DISCUSSION

These studies were closely patterned after those of Shockman & Lampen (1962) who found that, with the exception of penicillin and cycloserine, several other antibacterial agents were equally effective in inhibiting the growth of *Streptococcus faecalis* and protoplasts obtained from it, and that ristocetin and bacitracin were inhibitory at relatively small concentrations against both of these organisms. By comparison, the L-form of *S. pyogenes* displayed greater than 300- and 60-fold resistance, respectively, towards these two antibiotics as compared with the parent coccus (Table 1). Surprisingly, puromycin, an inhibitor of protein synthesis, caused lysis of this L-form. A similar response had not been observed with *S. faecalis* protoplasts (Dr G. D. Shockman, personal communication).

Bacitracin may either affect cell-wall synthesis (Salton,1960) or cause lysis of the cytoplasmic membrane (Hancock & Fitz-James, 1964; Snoke & Cornell, 1965). The data presented in Table 1 illustrates that the effect of bacitracin was an inhibition of wall formation in *Streptococcus pyogenes*. This is in agreement with similar findings of others with various group A streptococci (Levinson & Frank, 1955; Ward *et al.* 1958; Rotta *et al.* 1965), staphylococci (Krawitt & Ward, 1963; Williams, 1963; Kagan *et al.* 1964; Molander *et al.* 1964) and their respective L-forms. However, bacitracin apparently affected the membranes of *S. faecalis* protoplasts since the protoplast and the parent coccus were both inhibited by equal concentrations (5 μg./ml. medium) of this antibiotic (Shockman & Lampen, 1962). This is in agreement with similar findings reported for *Bacillus megaterium* and derived protoplasts (Hancock & Fitz-James, 1964).

A compilation of the results from two such serologically distinct streptococci as *Streptococcus faecalis* and *S. pyogenes* shows that they respond similarly to equal concentrations of certain antibiotics (puromycin, bacitracin, ristocetin). However, the fact that their wall-less derivatives required marked quantitative differences to obtain comparable responses, tempts the speculation of a membrane difference between protoplasts and L-forms after conversion from their respective parental streptococci. This is in accord with our findings that major fatty acid (Panos *et al.* 1966) and lipid (Cohen & Panos, 1966) differences and enzymic alterations (Panos & Cohen, 1966) also were shown in membranes from an L-form and from protoplasts derived from *S. pyogenes*. James, Hill & Maxted (1965) found other biochemical differences between protoplast membranes and L-form envelopes derived from *S. pyogenes* no. 416. Thus, it would appear that membrane differences may exist in biological derivatives from taxonomically diverse as well as within related streptococci.

To our knowledge, the present penicillin binding investigations represent the first

comparative study of a bacterium and its stable L-form. They illustrate that (*a*) the amount of penicillin bound by *Streptococcus pyogenes* is in close agreement with that found by others for Gram-positive bacteria; (*b*) there is no apparent difference in the binding capacity of *S. pyogenes* and its L-form for penicillin despite loss of wall synthesis in the latter; (*c*) that the binding mechanism for penicillin in these two organisms is probably distinct from that concerned with wall formation.

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An Electron Microscope Study of Dinoflagellate Flagella

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SUMMARY

The two flagella of the dinoflagellates examined possess the normal axoneme structure with 9+2 filaments, the nine outer doublets forming triplets in the basal region. Unlike other flagella which possess one basal disc, the dinoflagellates have two basal discs and two diaphragms in the transition region. The two flagella differ from each other in their external morphology and extra-axonemal structures. The longitudinal flagellum contains much packing material in its proximal two-thirds, whereas the distal third contains only the axoneme. This flagellum bears short fine hairs. The transverse flagellum differs from any other type which has been described. It has a helical ribbon-like form due to the presence of a striated strand, which is shorter than the axoneme, and an expanded sheath. It also bears long fine hairs in a unilateral array. The distal end of this flagellum is attached to the cell in the region of the flagellar bases. The structure of the two flagella appears to be compatible with their respective forms of movement.

INTRODUCTION

Dinoflagellates have long been known to possess two flagella which differ from one another in appearance and position (Fig. 1). One, the longitudinal flagellum, beats in a posterior plane in organisms belonging to the order Peridiniales and anteriorly in the order Prorocentrales. The other, the transverse flagellum, beats around the girdle in the Peridiniales and in an anterior position in the Prorocentrales. The structure of the transverse flagellum has been the subject of much speculation and it was at one stage thought to consist of a ring of cilia. Kofoid & Swezy (1921) however found that 'the longitudinal flagellum was thread-like and more rigid than the transverse flagellum which was frequently ribbon-like in form consisting of a deeply staining thread or stout fibril bordered on one side by a comparatively wide fin-like sheet of transparent protoplasm or membrane, somewhat greater in length than itself and thrown into ripples or folds of wider amplitude than the fibril'. These were very accurate observations considering that they were made with an ordinary light microscope. Later, Deflandre (1934) used a negative staining technique and described the transverse flagellum of *Glenodinium uliginosum* as having a single row of hairs. Thus he called it a 'stichonématé' flagellum. He also noted that this flagellum had a distinct outline on one side but not on the other and that the longitudinal flagellum was cylindrical with a long pointed tip.

Dragesco (1952) was the first to look at dinoflagellate flagella with the electron microscope. He reported that the two flagella of *Oxyrrhis marina* appeared to be identical, and were surrounded by a flattened sheath containing two marginal fibres. Pitelka & Schooley (1955) looked at the flagella of a *Gyrodinium* sp. They found the

longitudinal flagellum to be surrounded by 'apparently tubular sheath with no visible structure' which was continuous to the tapered end of the flagellum where it surrounded a single thick fibre from the axoneme. The transverse flagellum consisted of a dense axoneme and a sheath of low electron density which was expanded unilaterally, being about two or three times as wide as the axoneme. A unilateral array of fine mastigonemes (hairs) was found on one side of the flagellum. Pitelka & Schooley did not mention the presence of an accessory fibril although this is visible in their micrograph (Pitelka & Schooley, 1955, pl. 23*a*). This structure we have recently termed the striated strand (Leadbeater & Dodge, 1966).

Although the internal structure of many cilia and flagella is now well known, the dinoflagellates have been neglected in this respect. This is surprising since these organisms have obviously a unique flagellar system. The present paper shows that the dinoflagellates have the usual basic flagellar structure but that this is modified in several ways some of which are probably related to the peculiar mode of movement of the organisms.

METHODS

A wide range of dinoflagellates was examined during the present work but most of the observations were made on three species: *Woloszynskia micra*, *Gymnodinium vitiligo* and *Gymnodinium veneficum*. All organisms were maintained as uni-algal cultures under standard conditions described previously (Dodge, 1963). The cultures originated from the collection of Dr M. Parke at the Laboratory of the Marine Biological Association, Plymouth.

For electron microscopy, whole organisms were fixed with osmic acid vapour or by adding a few drops of 1 % (w/v) osmic acid solution to a small volume (about 5 ml.) of culture. Preparations were shadowed at 20° with a mixture of gold + palladium. For sectioned material two fixation methods were used: (*a*) 1 % (w/v) osmic acid in acetate veronal buffered balanced salt solution (Gibbs, 1962) at pH 8·2; (*b*) 6 % (v/v) glutaraldehyde in phosphate buffer and balanced salt solution at pH 8·0, followed after washing, by 1 % (w/v) osmic acid in the same medium. Dehydration was carried out in an ethanol/water series and after transfer to propylene oxide small pellets of organisms were embedded in Araldite. Sections were cut on a Porter Blum M.T.1 ultramicrotome and after staining in uranyl acetate in 50 % (v/v) ethanol/water followed by lead citrate (Reynolds, 1963), they were examined in a Zeiss EM 9 electron microscope.

RESULTS

External morphology from shadowed organisms

The difference in the appearance of the two flagella is clearly seen (Pl. 1, fig. 1; Fig. 1). When dried (for shadowing) the longitudinal flagellum becomes more or less straight and rigid. The basal two-thirds of the flagellum is wide and contains other material in addition to the axoneme (the 9 + 2 filaments). The distal third, or less, is narrow, with the sheath fitting firmly around the axoneme, it terminates in a rounded point into which extend some of the axoneme filaments, probably the central two. Fine hairs (about 0·5μ long) clothe the entire flagellum, probably in bilateral arrangement. The hairs are particularly abundant on the distal portion (Pl. 1, fig. 3).

The transverse flagellum is about twice as long as the longitudinal flagellum. When fixed it is quite distinct since it retains a series of waves (Pl. 1, figs. 1, 4) which are often

remarkably even in height and length. The axoneme is surrounded by a thin expanded sheath which also surrounds an accessory fibre, the striated strand (Pl. 1, figs. 1, 4). This strand describes a shorter path than the axoneme and is partly responsible for maintaining the expanded ribbon-like form of the sheath (Fig. 5). On one side of the flagellum is borne a single row of long fine hairs about 2 μ long (Pl. 1, figs. 2, 4). They appear to arise in groups of two or three from short stiff usually curved bases. The hairs appear to be attached to the sheath since they remain with the sheath when this is detached from the axoneme. The distal region of the transverse flagellum (Pl. 1, fig. 2) is tightly covered by its sheath, the final micron of its length is devoid of hairs and the flagellum ends in a smooth rounded tip. The striated strand appears to terminate at the point where the hairs cease.

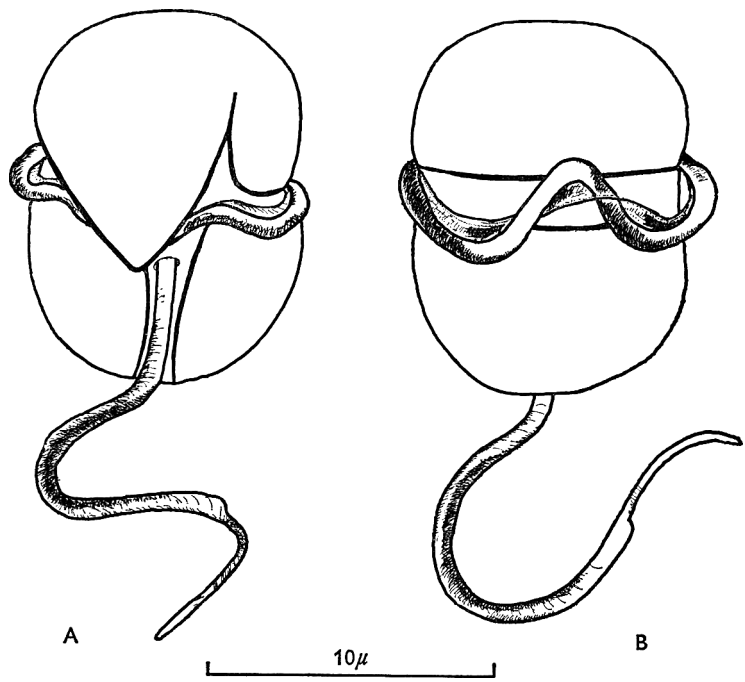

Fig. 1. Diagram of Gymnodinium to show the position of the longitudinal and transverse flagella. A, Ventral view of the organism; B, dorsal view of the organism.

The distal end of the transverse flagellum in some species of the Peridiniales is lightly attached to the organism in the region of the flagellar bases but readily becomes detached on fixation. As detached flagella show an entire distal end (Pl. 1, fig. 2) it would seem likely that there is little structural contact with the organism but that the tip reposes in a special infolding of the theca. Some evidence for this has been obtained from sectioned organisms.

Internal structure of the flagella

For convenience of description the flagella can be divided into three regions: the part outside the organism, the external morphology of which has already been described; the transition region where the flagellum enters the organism; the basal region

(kinetosome) which anchors the flagellum in the organism. The transition and basal regions are identical in the two flagella and so in the description of those regions no distinction is made between the two flagella.

The longitudinal flagellum outside the organism. In transverse section (Pl. 2, fig. 6) this part of the flagellum consists of the axoneme, occupying rather less than half the volume surrounded by the flagellar sheath, and a large amount of packing material. The axoneme is of the usual structure (Pl. 2, fig. 6; Fig. 2A) with nine outer pairs of

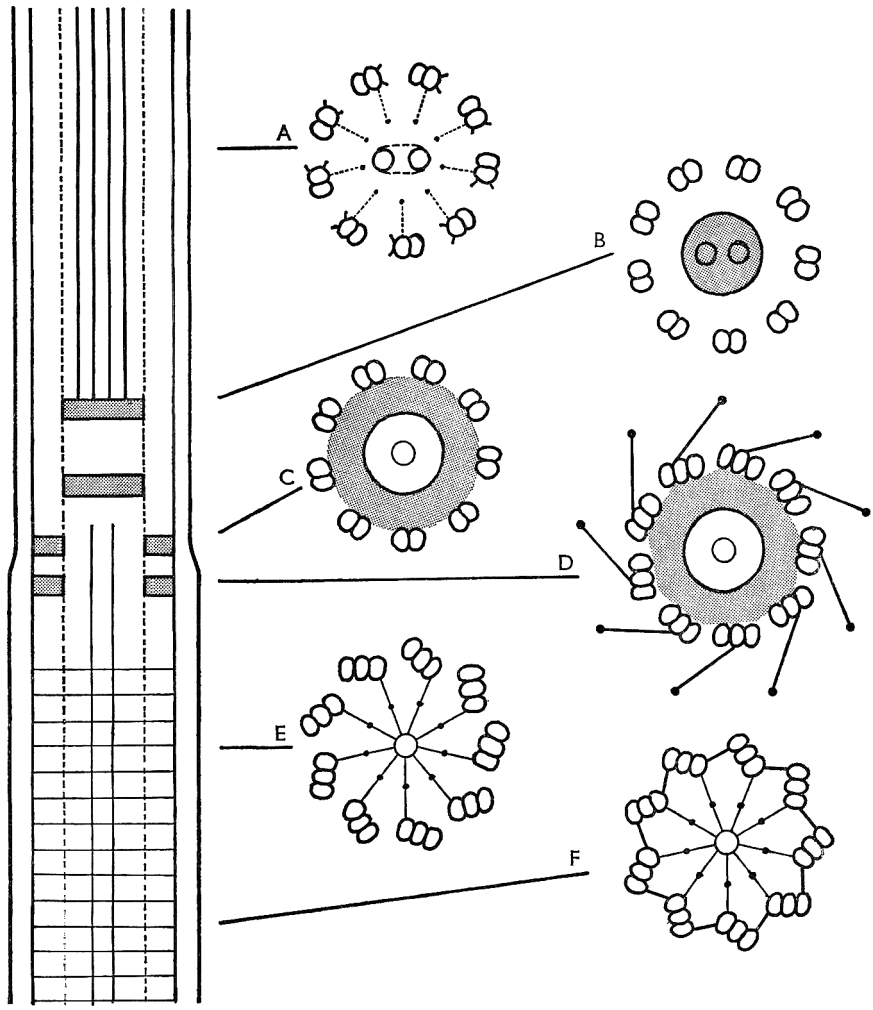

Fig. 2. Diagrammatic reconstruction of a flagellum and basal body. A–F show transverse sections at levels indicated on the median longitudinal section. The root fibres are omitted from the diagram.

tubular filaments and two inner filaments joined by delicate membranes. Between each peripheral doublet and the inner filaments is a secondary fibre. The secondary fibres appear to be connected by fine fibrils to the doublets (Fig. 2A). The centre two filaments are inclined at an angle of about 45° to a line drawn through the broad axis of the flagellum (Pl. 2, figs. 5, 6).

In longitudinal section (Pl. 3, fig. 16) the packing material is composed of regularly organized longitudinal rows approx. 360 Å apart. This material is absent from the narrow distal tip of the flagellum.

The transverse flagellum outside the organism. The axoneme is exactly as described for the longitudinal flagellum and is of the standard 9 + 2 type (Pl. 2, fig. 5). The unique feature of this flagellum is the striated strand which runs from just above the base of the flagellum (the exact point has not been determined) to near the distal end of the flagellum. In length it is shorter than the axoneme. In section the striated strand consists of a bundle of fibrils, or possibly tubules, generally situated at the opposite side of the flagellar sheath to the axoneme. In longitudinal section (Pl. 3, figs. 13–15; Fig. 3) it exhibits a complex transverse banding. The mean overall periodicity is 660 Å with a basic unit of banding about 110 Å long. Four light bands (440 Å) are followed by two dark bands (220 Å) in regular sequence. However, there is considerable variation in the measurements obtained, ranging from 400 to 800 Å for the overall periodicity. This suggests that the striated strand may alter in length. Regularly arranged packing material separates the striated strand from the axoneme and fills the remaining space within the flagellar sheath (Pl. 3, fig. 15). It is similar in structure to that described for the longitudinal flagellum.

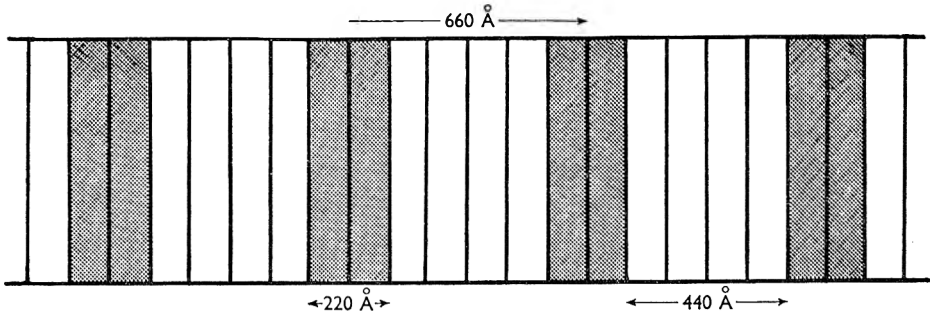

Fig. 3. Diagram showing the detailed pattern of cross-banding of the striated strand.

The transition region where the flagellum enters the organism. The outer extremity of the transition region is marked by the basal disc at which the two central filaments of the axoneme terminate (Pl. 3, fig. 13; Fig. 2B). A single thin-walled central filament runs from below the second basal disc to the base of the flagellum. The second diaphragm marks the proximal end of the transition region. The basal disc has a diameter equivalent to that of the secondary fibres. A short distance below is a second basal disc (Pl. 3, fig. 13; Pl. 4, fig. 17; Fig. 2) which is similar in appearance to the first disc. Connected to the second disc and beneath it is the first of a pair of diaphragms which project into the lumen of the axoneme but are pierced in the centre by an aperture of the same diameter as the two basal discs (Pl. 2, fig. 8, Pl. 3, fig. 13; Fig. 2). The second diaphragm is a short distance beneath the first but is clearly distinguishable from it in transverse section since the outer doublets of the axoneme have there increased to triplets (Fig. 2D). The addition of one filament to each doublet occurs mid-way between the two diaphragms. Also at this level the flagellar pore invagination of the outer cell membranes join the membrane of the flagellar sheath; and associated with each of the newly formed triplets is a centrifugally radiating spoke or fibre terminating in a densely stained circular body (Pl. 2, fig. 9; Fig. 2D).

The basal region (*kinetosome*) *and roots anchoring the flagellum.* The beginning of the basal region is marked by the centrifugally arranged accessory 'spokes' or fibres attached to the triplets (Pl. 2, fig. 9). The structure of the basal region is almost uniform throughout its length and consists of a single central tubule and nine peripheral triplets. Fine connexions or septa join the triplets to the central filament (Pl. 2, fig. 11;

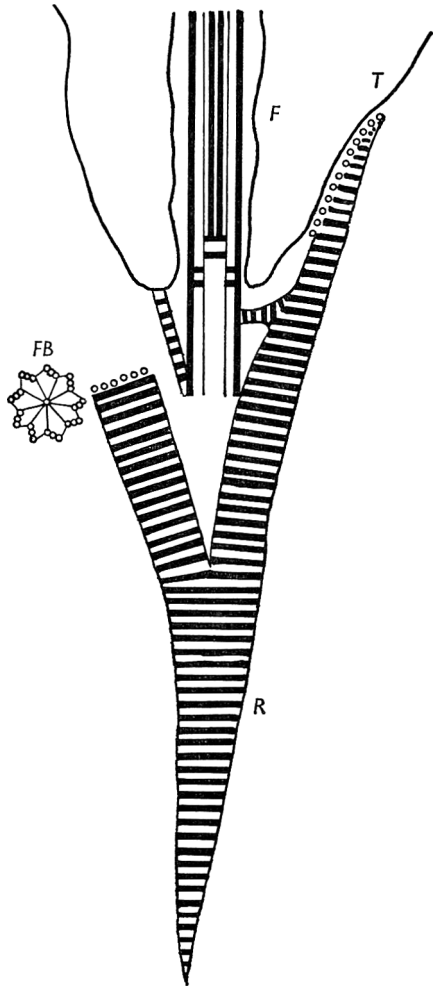

Fig. 4. Diagrammatic reconstruction of the root system associated with the flagellar bases (*FB*): flagellum, *F*; root, *R*; theca, *T*.

Fig. 2E). Towards the base of this region the angle which the triplets make to the radius to the centre of the flagellum decreases from about 85° to 50° (Pl. 2, figs. 9–12). At the base of this region the inner filament of each triplet is joined by a septum to the outermost filament of the next triplet in a clockwise direction (Pl. 2, fig. 12; Fig. 2F).

Both flagellar bases are joined by branches of a common striated root system (Pl. 4, fig. 19; Fig. 4). In transverse section the flagellar base is seen to be closely associated with a row of 15–20 fine filaments. These filaments join to form a single root which runs below the cell theca. In longitudinal section the root appears to be striated,

with a periodicity of about 360 Å. The filaments of the root appear to join the subthecal filament system (Leadbeater & Dodge, 1966). Smaller minor roots are also associated with the flagellar bases, one joining a triplet (Pl. 4, fig. 18) in the mid-basal region of the flagellum to another minor root which passes from the base of the flagellum to the subthecal region of the flagellar pore.

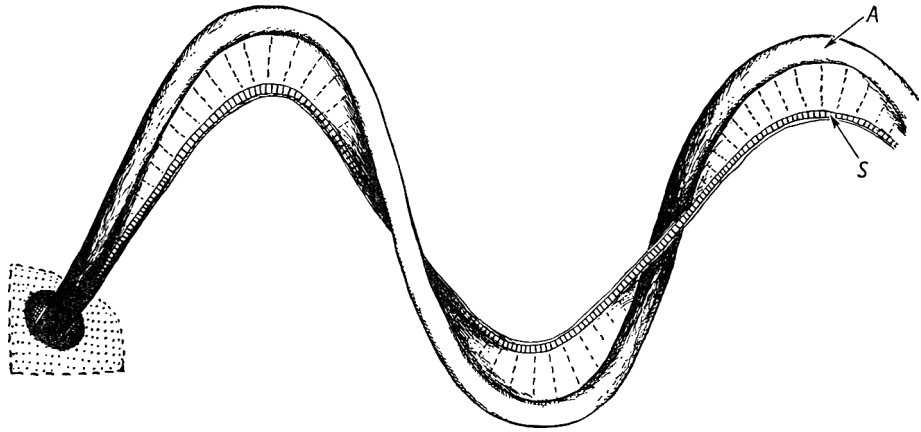

Fig. 5. Diagram of the transverse flagellum after its emergence from the flagellar pore, showing the suggested relationship between the axoneme (*A*), the striated strand (*S*) and the flagellar sheath. The fine hairs are not shown on this drawing.

DISCUSSION

The work reported in the present paper shows that the structure of the axoneme of dinoflagellate flagella conforms to the 9+2 (+secondary fibrils) pattern found in almost all cilia and flagella. In the dinoflagellates there are, however, several unique features in the construction of the flagella which are undoubtedly associated with the unusual movement, particularly that of the transverse flagellum.

The longitudinal flagellum is relatively simple in structure and externally has the shape described by Deflandre (1934) and Pitelka & Schooley (1955). The fine hairs, which we have observed for the first time, appear roughly similar in size to the deciduous hairs which are found in the Prasinophyceae (Manton, Rayns & Ettl, 1965). However, in dinoflagellates they do not appear to be easily detached and the flagella do not have a scaly covering like those of the Prasinophyceae. Internally the dinoflagellate longitudinal flagellum contains a large volume of packing material in the thick proximal region. This material is arranged in a definite manner (Pl. 3, fig. 16) and bears some resemblance to the 'paraxial rod' of trypanosome flagella (Vickerman, 1962).

The movement of the longitudinal flagellum of the large dinoflagellate Ceratium was studied in detail by Jahn, Harmon & Landman (1963) and by Brokaw & Wright (1963). They found it to show a base to apex planar wave which approximated to a sine wave. Our observations on living material (with phase microscopy) would suggest that this is normally the case in Gymnodinium, but as the wave is never retained after fixation we have been unable to make a detailed check of the form that this takes. It appears that sometimes the longitudinal flagellum is held straight, perhaps functioning like a rudder.

The transverse flagella examined possess several unique features. Externally, the

single array of long fine hairs appears rather similar to that of Euglena (Leedale, Meuse & Pringsheim, 1965) but internally, and in its movement, there is little similarity with the euglenoid flagellum. One distinctive dinoflagellate feature is the regularly banded striated strand which is probably constructed of fibrous protein. If this strand has elastic properties these will be of great importance in the structure of the flagellum which we describe below. The ribbon-like sperm tails of the toad; *Bufo arenarum* (Burgos & Fawcett, 1956) contain an accessory body (in addition to the axoneme), but unlike the striated strand, this body does not show banding and the tails move with a planar wave.

Observations of living organisms by high-speed photography (Jahn *et al.* 1963) suggest that the transverse flagellum moves in a base to apex circular or elliptical helical wave. From our sections it is clear that the flagellum is ribbon-like with the axoneme at one side of the hollow ribbon and the striated strand at the other (Pl. 2, fig. 5). Between these two structures is found packing material similar to that described from the longitudinal flagellum. From shadowed preparations it is clear that the striated strand, which normally runs in a more or less straight course, is much shorter than the axoneme. This latter is always thrown into a series of waves, even after fixation, and normally passes under and over the striated strand. These facts can be explained if the flagellum is visualized as having the form of a drawn out helix rather like a steep spiral staircase in which the axoneme forms the outer boundary of the spiral, and the striated strand the inner boundary. The packing material forms as it were the steps between the striated strand and the axoneme. This interpretation is illustrated in Fig. 5. From models which we have constructed it would appear that the tightness of the striated strand is to a considerable extent responsible for the helical form of the flagellum and therefore also for its unique helical movement. It is necessary, for this model to work, to have each end of the striated strand firmly attached to the axoneme. Our sections appear to show that it tapers off adjacent to the axoneme but no connexion has so far been observed.

Occasional micrographs have been obtained in which the striated strand and the axoneme do not appear to be intertwined but lie one over the other. In these cases it is possible that the relationship between the strand and axoneme was disturbed when the distal end of the flagellum became detached from the organism. Presumably if detachment takes place before fixation some rearrangement is likely to occur in a tensioned system like this. The model described above appears to be compatible with the observed movement of the flagellum. The broad spiral would appear to present a much greater surface area to the water than would a simple cylindrical structure. Whether this is also responsible for the forward movement of the organism, which Jahn *et al.* (1963) believe the transverse flagellum to provide, will only become clear after detailed hydrodynamic study.

The internal structure of the transition region differs somewhat from that described for certain other flagellates (e.g. Manton, 1963). Instead of the normal single basal disc two are found and, beneath these, two diaphragms. The pairs of discs and diaphragms may provide a useful taxonomic character for dinoflagellates. The size of the basal discs and the width of aperture of the diaphragms is of interest in that it corresponds with the diameter of the circle enclosed by the secondary fibrils of the axoneme. These fibrils appear to be continuous through the length of the flagellum, eventually joining the radial septae at the bottom of the flagellar base (Fig. 2).

As in Pseudotrichonympha (Gibbons & Grimstone, 1960) the peripheral doublets of the axoneme of the dinoflagellate flagella become triplets, but here this takes place not at the basal disc but between the two diaphragms. At this same point the outwardly radiating spokes make the structure appear like that of a centriole. The lower half of the basal region, the connexion between the triplets, their change in angle, and finally the central hub with radiating spokes, conform very closely with what has been found in other flagella. Similarly, the striated roots are like those of ciliates, but there appears to be no connexion with the nucleus of the type found in Prasinocladus (Parke & Manton, 1965). In fact the root in Gymnodinium does not penetrate very deeply into the organism but probably only joins the subthecal fibrillar system.

Acknowledgements are due to the S.R.C. for grants and to Dr M. Parke for supplying cultures.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Shadowed whole mount of *Gymnodinium vitiligo* showing longitudinal flagellum and transverse flagellum displaced above the organism. Shadowed gold + palladium. × 3500.

Fig. 2. Detached distal tip of transverse flagellum of *Gymnodinium vitiligo* showing unilateral array of long hairs with curved bases. The striated strand (S) terminates at the same level as the hairs. Shadowed gold + palladium. × 14,400.

Fig. 3. Distal portion of the longitudinal flagellum of *Woloszynskia micra* showing the firm sheath around the axoneme and the short fine hairs. One filament protrudes into the tip, the other filaments terminate short of this. Shadowed gold + palladium. × 17,500.

Fig. 4. Small portion of the transverse flagellum of *Gymnodinium veneficum* showing the undulating axoneme enclosed in an expanded sheath and bearing a unilateral array of long hairs. The striated strand runs more or less straight through the sheath. Shadowed gold+palladium. × 10,500.

PLATE 2

Transverse sections through the flagella of *Wolszynskia micra*.

Fig. 5. External portion of transverse flagellum. The expanded sheath contains the axoneme of nine outer doublets. two central filaments and the secondary fibres, and is expanded laterally to contain the fibrils of the striated strand (s). × 78,400.

Fig. 6. Proximal portion of longitudinal flagellum. The axoneme is surrounded by the sheath and a large amount of packing material. × 58,800.

Fig. 7. Distal transition region, the two central filaments are surrounded by the dense basal disc (*B*) of similar diameter to the secondary fibres. × 58,800.

Fig. 8. Proximal transition zone. A diaphragm (*D*) projects into the lumen of the axoneme from the periphery of the triplets. The diaphragm is penetrated by an aperture of similar diameter to the basal disc, and in the centre is a single filament. × 108,000.

Fig. 9. Distal basal region, the triplets are attached to centrifugally radiating fibres terminating in dense staining areas. The central filament is just visible. × 108,000.

Fig. 10. Mid-basal region, the triplets are orientated at approximately 30° to the circumference. × 71,000.

Fig. 11. Distal basal region, showing central filament. × 83,600.

Fig. 12. Proximal basal region. Triplets orientated at 35° to the circumference and joined to the central filament by fine fibres or septa. The inner filament of each triplet is joined to the outer filament of the adjacent triplet by a fine fibre (*F*). × 108,000.

PLATE 3

Longitudinal sections through the flagella of *Woloszynskia micra*.

Fig. 13. Transverse flagellum and basal body showing median and tangential longitudinal sections of axoneme and the striated strand. The two central filaments of the axoneme terminate in the first basal disc (*B*); immediately below this is the second basal disc and the two diaphragms (*D*). Attached to the base of the flagellum is part of the root extending to the subthecal region of the flagellar pore. The flagellum has recently divided and the duplicate base is seen above. × 37,500.

Fig. 14. External portion of the transverse flagellum showing fibrous connexions between the two central filaments of the axoneme and the complex cross banding of the striated strand (*S*). × 58,800.

Fig. 15. Transverse flagellum cut obliquely and showing the striated strand (*S*), some of the axoneme filaments and packing material. × 58,800.

FIG. 16. Longitudinal flagellum showing peripheral filaments of axoneme and regularly arranged packing material. × 84,800.

PLATE 4

Fig. 17. Longitudinal section of two flagellar bases of *Woloszynskia micra*. Showing the lower basal disc, the diaphragms and cross-striations in the basal region. (see Fig. 2). × 54,000.

Figs. 18, 19. Roof structure of *Woloszynskia micra*. Fig. 18. Transverse section of the basal region of two flagella showing the connection between one triplet and the root (*r*). × 71,000. Fig. 19. Longitudinal section of flagellar bases (*k*) with associated root structure (*r*). Below the lower base a narrow band of striated fibres joins the main root. × 63,000.

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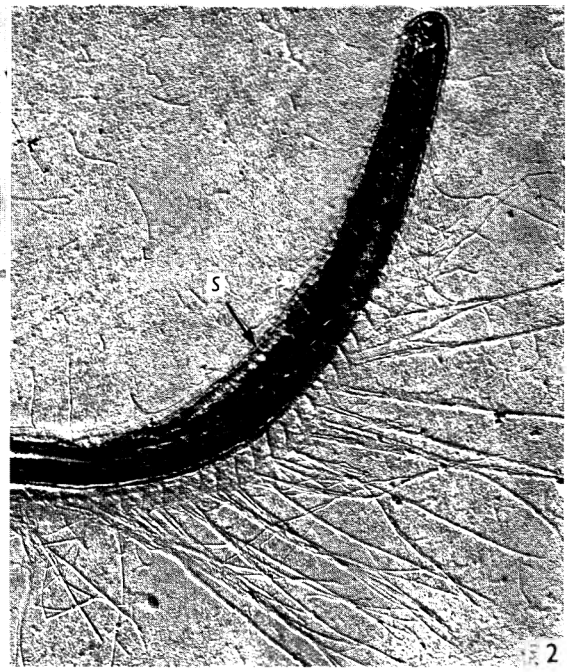

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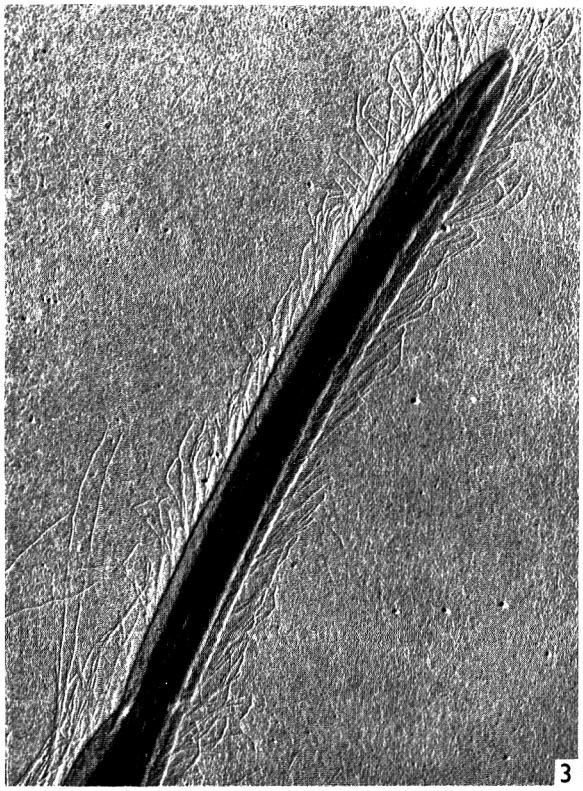

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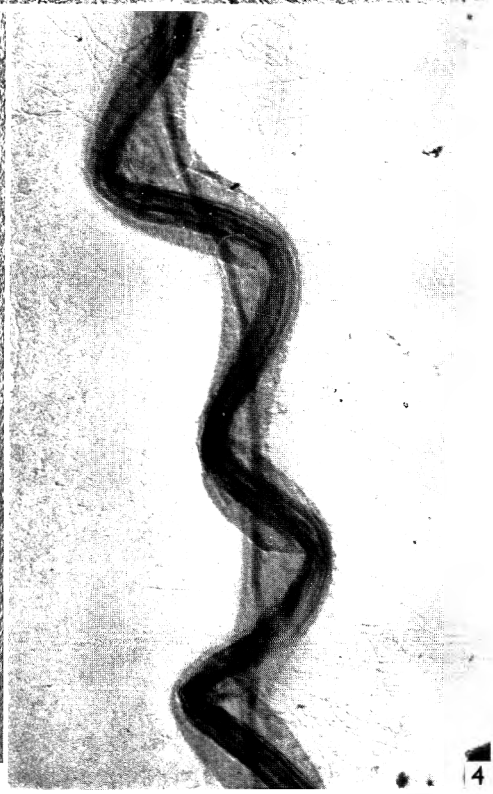

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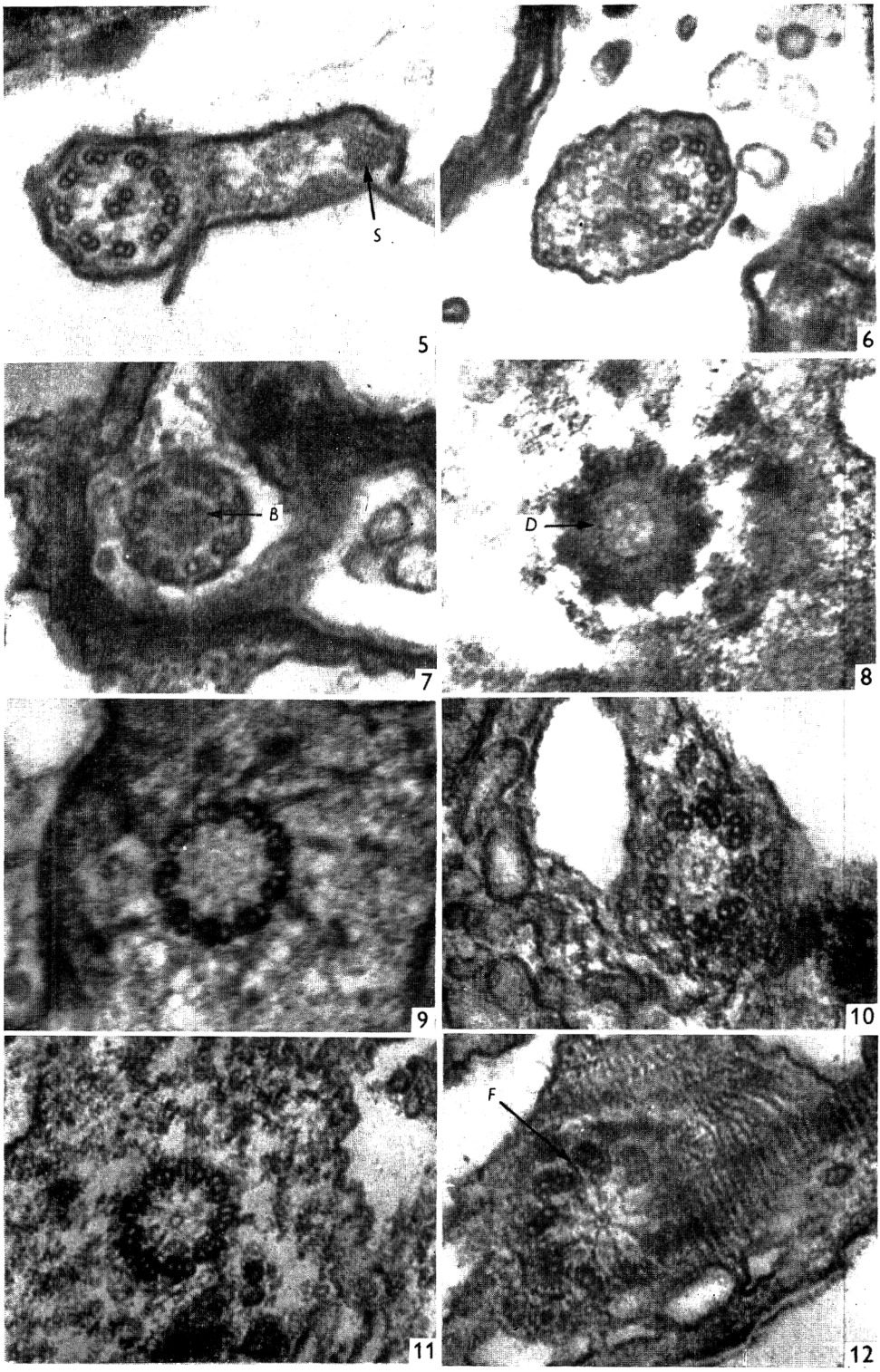
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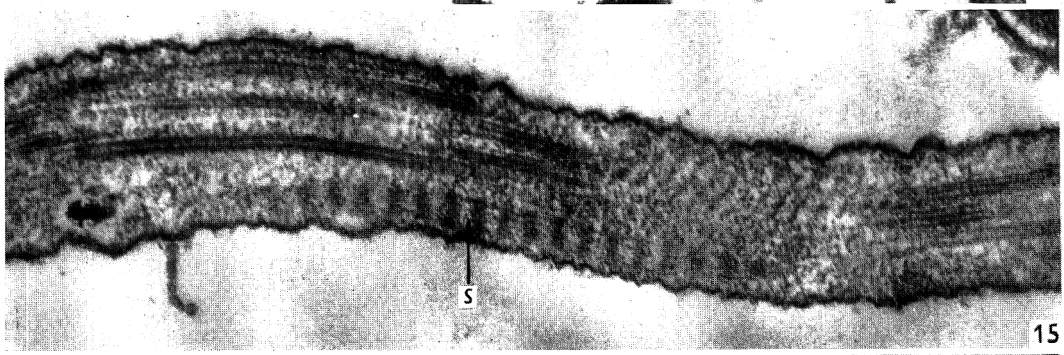
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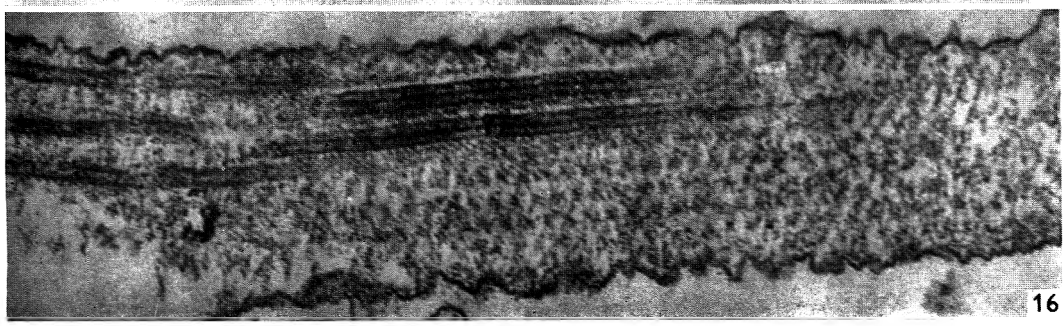
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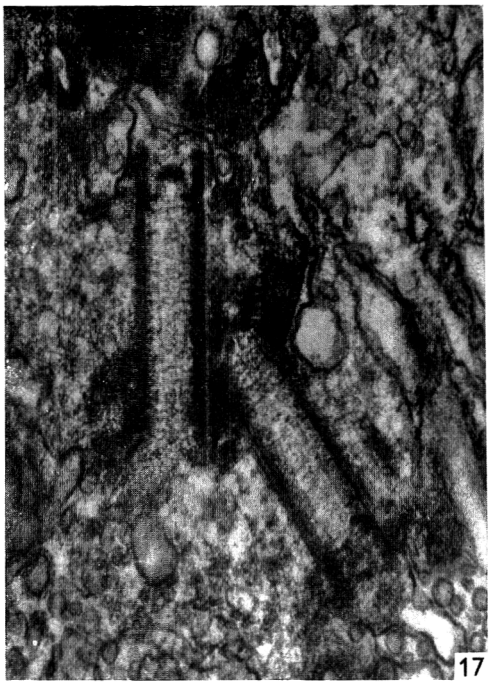

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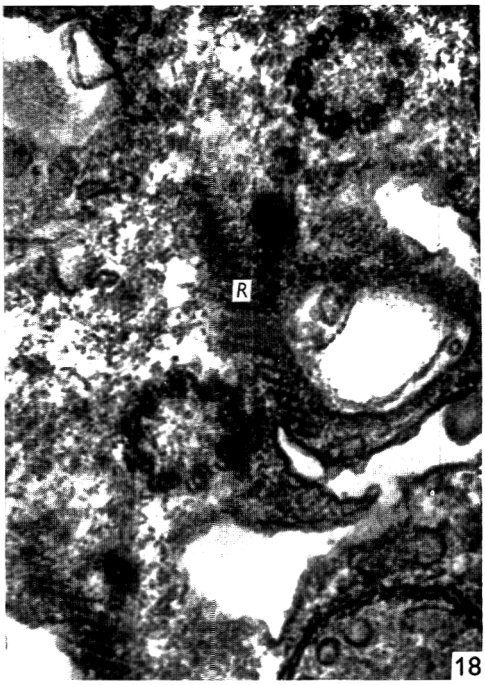

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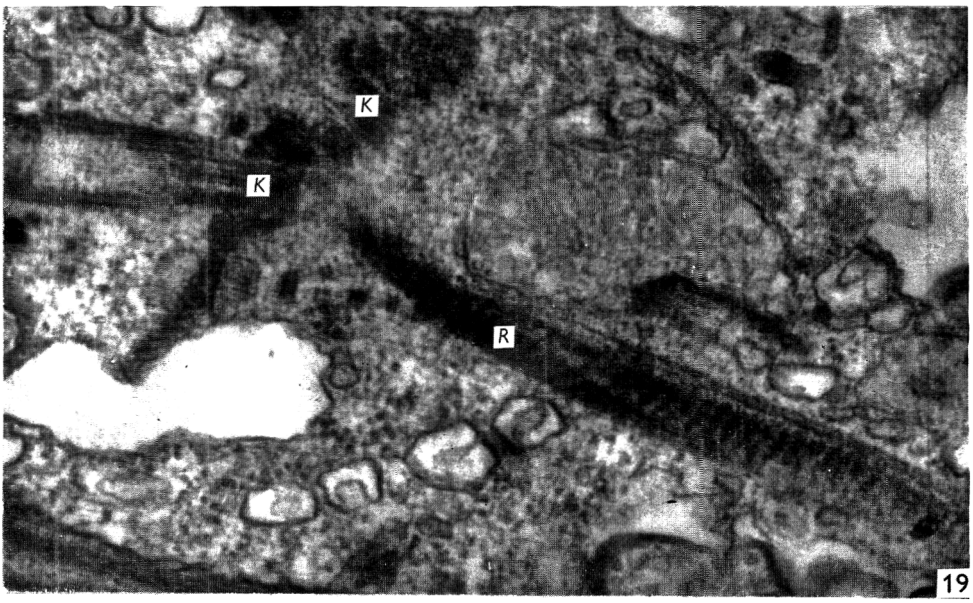

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A Technique for the Rapid Isolation of Single Bacterial Spores

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SUMMARY

Phase-bright bacterial spores are deposited singly and rapidly by means of a glass microloop at pre-marked sites on the surface of a cast block of agar gel. The block is then dissected and portions, each carrying a single spore, are transferred to fluid for heat-resistance or other tests. Under optimal conditions of culture 96–100% of these single spores germinated and gave visible growth.

INTRODUCTION

The technique was devised to enable heat-resistance tests to be made on single spores of *Bacillus subtilis* in batches of multiples of 24. One worker can select and isolate approximately 100 spores per hour and the isolates can be preserved at 3° without loss of phase-brightness or viability (i.e. capability of germination and production of visible growth), for at least 9 weeks. Thus large batches of spores can be prepared and the distribution of heat resistance amongst them estimated, knowing that each spore is phase-bright. It was important that the spores tested should be of approximately the same age because the age of spores is known to affect their response to germinants and recovery media (Magoon, 1926; Curran & Evans, 1947), to heat-shock treatment (Curran & Evans, 1947; Lawrence, 1957; Krishna Murty, 1957) and to heat resistance tests (Church, Halvorson & Bontempo, 1957; Rayman, 1957; Evans & Curran, 1960).

METHODS

The agar block. New Zealand agar (1·75%, w/v, in distilled water) was clarified with Hyflo-super-cel diatomaceous earth (Koch-Light Laboratories, Colnbrook, Buckinghamshire) to remove bacterial debris (Feinberg, 1956). Sterile blocks of the gel 2·0 mm. thick were cast between microscope slides. Each block, mounted on a sterile slide, was marked with 24 pairs of pits which were visible to the unaided eye (Fig. 1), being the imprints of the tips of 48 sterile steel needles mounted in an enclosed punch. The zone between each pair of pits was the site for the deposition of a single spore and was in register with the readings of the mechanical stage of the microscope, being 3·0 mm. distant from the adjacent sites in both directions. The agar blocks and their slides were protected from aerial contamination by enclosure in Petri dishes, each also containing a sterile duralumin humidifying trough 4 cm. × 2 cm. and 0·75 cm. deep (internal dimensions) holding glass wool saturated with distilled water, to minimize desiccation of the gel. During inoculation only, each block was protected from contamination of the sterile surface from droplets scattered from the inoculation loop, by a sterile metal cover, except for the extreme end remote from the pre-marked sites.

Preparation of spores. A single-spore strain of *Bacillus subtilis* CN788 (Wellcome Research Laboratories, Beckenham, Kent) designated CN788/1 was used. At 37°, 18 hr cultures on nutrient agar (Oxoid) produced mostly vegetative forms; a spore crop was obtained by inoculation from the nutrient agar culture on the surface of Tryptone soya broth (Oxoid) diluted 1/20 with distilled water and solidified with 2% (w/v) agar. After incubation for 18–24 hr at 37°, 95% of the organisms were as free spores. With *B. subtilis* strains NCTC 3610 and NCTC 6432 (National Collection of Type Cultures), the yield of free spores was much reduced. The spores were harvested in sterile Ringer's solution with 0·1% (w/v) gelatin and were not washed. Inoculation

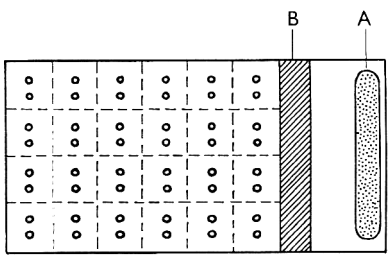

Fig. 1

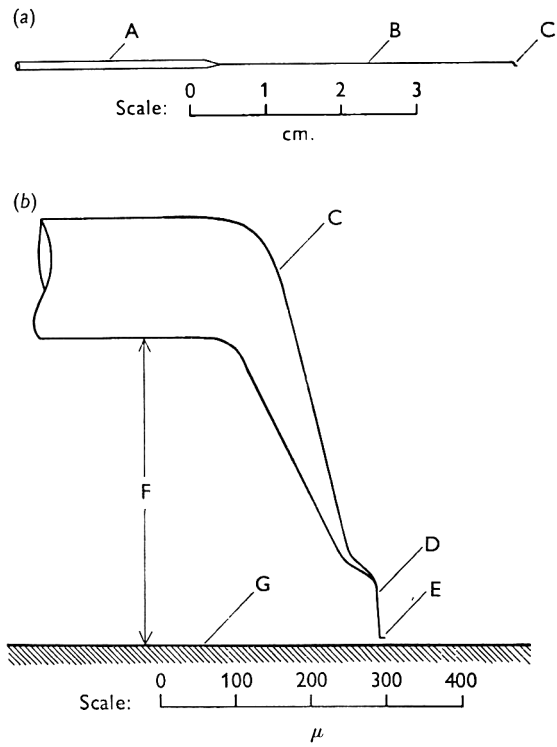

Fig. 2

Fig. 1. Plan of a completed agar block showing the site of inoculation A, the section B removed for sterility test and the 24 isolation sites, each carrying a single spore between the two locating pits. The broken lines indicate the knife cuts used to separate the isclation sites. Twice actual size.

Fig. 2. Elevation of a glass microloop: (*a*) showing the handle A, the shaft B and the tip C; (*b*) the tip C further enlarged, showing the filament D and the loop E in relation to the gel surface G. The distance F is the clearance between the shaft and the gel.

was made from a faintly turbid spore suspension with a wire loop 1 mm. in diameter along a strip A (Fig. 1) on the exposed upper surface of the gel. The fluid was rapidly absorbed, leaving the spores on the gel surface for selection and transfer.

The microloop. The soft glass microloop used consisted of a handle A (Fig. 2*a*) 2·5 cm. long and 1·0 mm. diameter, a shaft B 3·8 cm. long and 0·15 mm. diameter, with a shaped tip C (Fig. 2*a*, *b*) terminating in a minute oval loop made in a high-

power microforge. The latter, working at a magnification of $\times 400$, enabled the terminal glass filament D to be drawn out to 1 μ diameter and the loop E to be formed at its end, orientated to lie in the plane of the gel surface. For pure strain isolations, for which this method is very suitable, the loop can be as small as $3{\cdot}0\ \mu \times 1{\cdot}5\ \mu$ internal dimensions, its size being adjusted to limit its carrying power to a single spore. For multiple isolations of spores of the same strain, however, a larger loop $14{\cdot}0\ \mu \times 8{\cdot}0\ \mu$ is suitable and will carry 12 spores of *Bacillus subtilis*. The filament thickness and loop dimensions were determined by trial for a given strain. The loop must pick up or discharge the spores readily on the gel surface and when correctly formed and orientated can be used to make several thousand isolations if protected from damage.

The clearance F (approximately 400 μ) ensured that the shaft did not come into contact with the gel surface G. The dimensions of the tip allowed adequate working distance for the microloop between the gel and the protecting coverglass, which was held 1·0 mm. above the gel surface by a sterile Perspex cell enclosing the agar block and itself resting on the slide. The handle of the microloop was operated by the focusing movements of a second microscope (Johnstone, 1953), a conventional micromanipulator being unnecessary. The microloop was sterilized by immersion of the shaft and tip in chromic+sulphuric acid-cleaning solution, being carefully washed in sterile distilled water and then in 95% (v/v) ethanol in water before use.

Optical system. The $\times 40$ phase-contrast objective must have a working distance of at least 1·0 mm. below the coverglass and an N.A. of at least 0·70; the substage condenser must have a working distance of at least 3·5 mm. At $\times 750$ magnification, with a green filter (Wratten 58), the non-germinated spores appeared phase-bright, clearly defined and in sharp contrast to the phase-dark germinated spores and the vegetative forms. Catadioptric systems with long working distance can be used to simplify the manipulations, since the working distance below the coverglass can be greatly increased and condensation on the coverglass is then less troublesome, but only at the cost of loss in resolution and the necessary increase in illumination with the possibility of damage to the spores.

Manipulation of the spores. The margin of the inoculated strip nearest to the premarked sites was located and the microloop was centred to the optical axis whilst poised above the gel. On lowering the loop to encircle a selected spore and then raising it, the spore usually adhered firmly to the interior of the loop. Twelve selected spores were thus collected in the loop successively.

Whilst the loop was raised, the first pair of marking pits was located by operation of the mechanical stage controls and, from the reading of the scales and verniers of the stage, the positions of all the remaining sites were readily calculated. The charged loop was lowered to touch the gel surface gently *between* the two pits. At this point, the spores floated within the loop in the water exuded from the gel by the slight pressure of the glass. They were then readily discharged from the loop by vibration at the moment when the rising loop broke contact with the gel surface. This was effected by an electric bell mechanism mounted to impinge on the under side of the bench and controlled by a foot-switch and rheostat. A setting of the rheostat was found by trial which usually caused one spore to be left on the gel when the loop was raised during vibration. When more than one spore was ejected, these were clearly visible on the gel surface and any spores in excess were picked up with the loop, leaving an isolated spore on the first pre-marked site.

The second site was then brought into position, by using the readings of the stage, and thus twelve successive isolations were made. The loop was then re-charged with a further twelve spores from the inoculated area, for the completion of the block. Each block of 24 isolations can be completed in less than 10 min., re-sterilization of the loop being unnecessary for the same spore suspension.

Storage of the block. For *Bacillus subtilis* spores, it was necessary to store the isolates on the gel for at least 24 hr before transfer to fluid medium when the maximum viability was to be obtained with untreated spores. Under these conditions, the viability (i.e. the proportion of spores germinating and giving visible growth in optimal medium) was 96–100%, as compared with 83% when the blocks were dissected and transferred to medium immediately.

Dissection of the agar block. This was done under a stereoscopic microscope at × 5 magnification, with protection against aerial contamination by (*a*) a cabinet enclosing the stage with a plate-glass roof below the objectives; (*b*) a sterile transparent plastic hood covering the agar block and supporting slide, with an aperture to give access to the sharp stainless steel dissecting knives held in the hand.

The site of the inoculum A (Fig. 1) was first separated and discarded. A section of the gel B between the inoculum and the nearest isolation sites was then cut out and removed. This was cultured in Tryptone soya broth for 7 days at 37° and was proved to be sterile if the isolations were to be accepted as valid. The 24 isolation sites were then separated by knife cuts, by using the locating pits as guides, and were transferred in turn with a sterile platinum scoop to individual tubes of fluid for the required tests.

Accuracy of the method

(1) It was shown microscopically that, after dissection of a pre-marked agar block and transfer of 24 isolation sites with their isolated spores to a second slide, all the 24 spores were present on the gel surface and each was situated between its locating pits. Therefore exudation of water from the agar during cutting of the gel with a sharp knife did not displace the spores on the surface.

(2) In control series of isolates, transferred without heating or other treatment to an optimal medium, all 24 isolates frequently yielded the expected growth and must therefore each have been transferred with the portion of agar carrying the pre-marked site.

(3) Spread of growth from the inoculum did not occur on the sterile gel surface, as shown by the sterility of the section B (Fig. 1).

Effects of deviation from the preferred technique

The object of the method described was to prepare batches of single spores for heat-resistance tests, the interpretation of which is greatly simplified when the control (i.e. unheated) series gave a consistently high percentage viability. With spores of *Bacillus subtilis* CN 788/1, 96–100% of single spores germinated and gave visible growth in 24 hr at 37° in a carefully prepared Tryptone soya broth (Oxoid ingredients). Similar results were obtained with *B. subtilis* strains NCTC 3610 and NCTC 6432, but with slightly greater delay in the appearance of growth. This optimal result was decreased by: (*a*) the use of a medium which was inferior for the strain, especially when overheated during sterilization; (*b*) suspension or washing of the spores before isolation in distilled water or buffer solutions other than Ringer's solution containing 0·1%

gelatin; (*c*) exposure of the isolated spores to distilled water; (*d*) failure to store the isolates on the gel surface for at least 24 hr before dissection and transfer to medium.

The interrelation of these four factors is complex. Storage of the spores as isolates on the gel at 3° for 24 hr greatly decreased the loss in viability and the delayed germination which might arise from factors (*a*), (*b*) and (c), alone or together. Such storage should therefore be a standard technique for *B. subtilis* spores.

The method, without storage on the gel, is applicable to clostridial spores and has been used to obtain multiple single-spore strains of *Clostridium botulinum* type E, for which 82% of isolates were viable when cultured in meat broth.

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