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A Microphotometric Method for the Estimation of Penicillinase in Single Bacteria

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SUMMARY

A microphotometric technique is described by which the penicillinase content of a single bacterium in a stable microdrop is measured. The microdrop contains a buffer, benzylpenicillin and bromocresol purple. As the penicillinase hydrolyses the penicillin to penicilloic acid, the concentration of the cationic (purple) form of bromocresol purple decreases. This decrease is measured by a specially designed single-beam microphotometer, and, from the change in light transmission and the volume of the drop, the total penicillinase content of the drop is calculated. The microphotometer and the calibration of the assay with soluble penicillinase preparations are described.

INTRODUCTION

The specific enzyme activity of a bacterial culture is often of value in describing the state of the bacteria under experimental conditions, but the variation in enzyme content between the individual bacteria in the population has not been directly investigated before. The estimation of an enzyme in a single organism, in which only a few enzyme molecules may be present, requires novel assay techniques. A microphotometric technique has been developed for the assay of such quantities of penicillinase, and this technique may also be of general application to other enzyme assays. The basis of the technique is the observation of the enzymic reaction in a stable microdrop, which can contain either a bacterium whose penicillinase is to be estimated or a known concentration of soluble penicillinase for calibration purposes. Penicillinase catalyses the hydrolysis of penicillin to penicilloic acid, which has a secondary amino group and a carboxyl group that were not present in the original penicillin. At neutral pH values, the amino group is unionized but the new carboxyl group ionizes, and a proton is released. The solution thus tends to become acidic as the reaction proceeds, and in the assay system this proton release decreases the concentration of the ionized form and increases the concentration of the unionized form of a pH indicator, bromocresol purple, present in the assay mixture. The decrease in concentration of the purple (ionized form) is accompanied by an increasing light transmission through the solution, which is most marked at 5900 Å, the peak absorption of the purple form of the indicator. The optical measurements which enable the rate of the enzymic reaction in the microdrop to be calculated are made with the aid of a specially designed microphotometer, by which the transmission of the drop at a selected wavelength can be measured and recorded continuously. Since the observations are made of the transmission through a spherical

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drop with an uncertain light path instead of the more usual parallel-sided container, the interpretation of the measurements is more complicated than in normal photometry, and a modified equation has been derived from Beer's law in which the concentration of the absorbing substance is introduced, but from which the light path and the extinction coefficient have been eliminated. Under the assay conditions used, this concentration can be assumed with only small errors.

The special microphotometer designed for the micro-assay is a single-beam instrument by which the light transmitted through a circular area about 1 μ diameter in the centre of the drop under observation is detected by an 11-stage 'venetian blind' photomultiplier, and the output signal amplified and recorded on a potentiometric recorder. The instrument is designed to give high sensitivity together with stability over considerable periods of time.

The calibration of the method has shown the response to be linear over a considerable range of enzyme concentration, and the sensitivity is high enough to detect penicillinase activity corresponding to less than 50 molecules of *Bacillus cereus* penicillinase (Kogut, Pollock & Tridgell, 1956).

METHODS

Reaction mixture. The usual reaction mixture used for the estimation of penicillinase contains 2·0 ml. 4 % (w/v) bromocresol purple (G. T. Gurr Ltd.; adjusted to pH 6·15 with sodium hydroxide), 1·0 ml. 0·2 M-K_2HPO_4, 0·4 ml. benzylpenicillin solution (containing 2×10^5 i.u./ml. saline) and 0·2 ml. saline. This mixture is at about pH 7·4. To make a drop preparation, a small quantity (0·02 ml.) of the test solution is mixed with 0·18 ml. of the reaction mixture. The test solution containing the penicillinase should have no strong buffering capacity.

Preparation of drops. Water-repellent tubes (treated with a solution of dimethyldichlorosilane: 'Repelcote', Hopkin and Williams Ltd.) were used for mixing the reaction mixture and the test solution. After vigorous mechanical mixing, the mixture was pipetted into a prepared water-repellent capillary tube which had been drawn out to a fine point on an automatic 'electrode' pulling machine (Alexander & Nastuk, 1953). From the capillary tube, the mixture was sprayed into a drop of silicone oil (Midland Silicones Ltd., MS 702) on a water-repellent coverslip. By the use of a shield, drops were prevented from falling on to the surface of the coverslip itself. A second water-repellent coverslip was placed on top of the oil, and the oil and the drops trapped in it spread out between the two coverslips. The drops were just lighter or heavier than the oil, depending on the reaction mixture used, and came to rest touching one of the coverslips, but not wetting it, and retaining their spherical shape. Once the oil ceased flowing under the coverslip, the drops were stationary and stable for very long periods (i.e. days).

The microphotometer: the optical system. A restricted beam of light was used to illuminate the drop under observation on the stage of the microscope, and the light transmitted through the central area of the drop was measured with a photomultiplier. A schematic diagram is shown in Fig. 1. A Watson 'Bactil-60' microscope was used with a $\times 40$ air objective and a second matching objective in place of the normal substage condenser. The light source *A*, collector lens *B*, filter *C* and field aperture *D* were mounted on an optical bench. The light source was a 6 V.

30 W. bulb 20 in. from the substage condenser; the collector lens was $2\frac{1}{4}$ in. from the bulb, so that the filament was focused at the substage condenser, giving Köhler illumination. The interference filter (max. transmission 5900 Å, 74 % transmission, band width 80 Å; Barr and Stroud Ltd.) was mounted in a swinging holder between the collector and the field aperture. The field aperture, 10 in. from the substage condenser, consisted of a demountable circular plate in a fixed holder, the aperture (0·4 mm. diam.) being drilled centrally in this plate. The mirror (*F*) deflecting the light to the substage condenser was surface-silvered, to avoid multiple images of

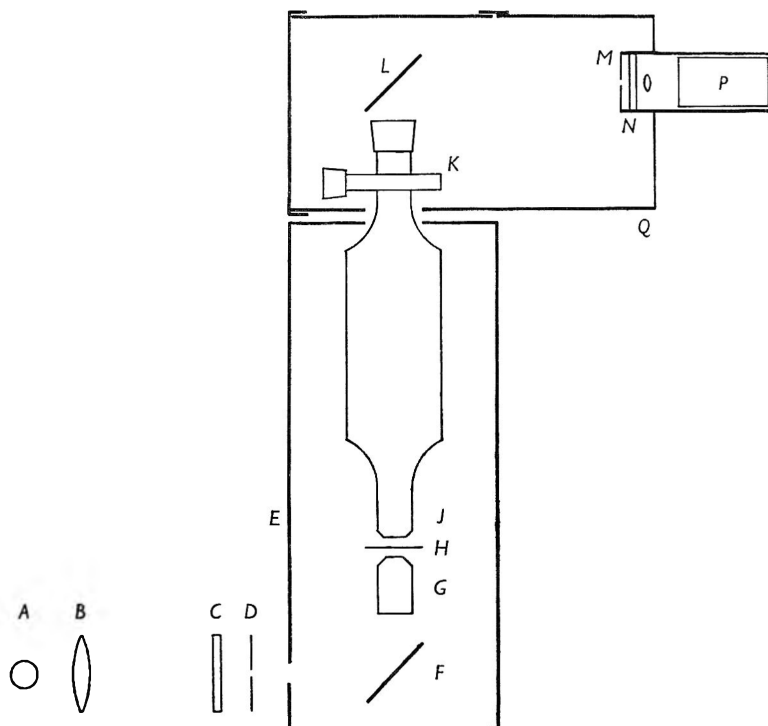

Fig. 1. Schematic diagram of microphotometer. *A*, lamp; *B*, condenser; *C*, filter; *D*, field aperture; *E*, thermal enclosure; *F*, mirror; *G*, *J*, ×40 objectives; *H*, drop preparation; *K*, micrometer eyepiece; *L*, mirror; *M*, screen with central aperture; *N*, camera shutter; *P*, photomultiplier; *Q*, light-tight enclosure.

the field aperture. The substage condenser (*G*) was identical with the objective (*J*), a 4 mm. (×40) air objective, N.A. 0·70. It was adjusted for each drop observed so that the image of the field aperture was in the same plane as the drop. A Baker ×10 micrometer eyepiece (*K*) was used, over which could be fitted a surface-silvered mirror (*L*) deflecting the light through 90°. About 10 in. away the image of the drop was focused on a white screen (*M*), and moved till it lay centrally about a 1 mm. diam. hole, through which the light passed to the photomultiplier (*P*). A camera shutter (*N*) and a small lens to diverge the light beam over the face of the photomultiplier were mounted behind the screen in a light-tight enclosure (*Q*).

The microphotometer: electronic system. A single-beam instrument was chosen for overall simplicity, and adequate stability of the light source was obtained by using

a 6 V. 30 W. bulb (F/25; G.E.C. Ltd.) fed from a 6 V. 7 A. transistor-stabilized d.c. power supply having an output impedance less than 0·01 Ω and a stabilization factor for variations of input voltage greater than 5×10^3. The photomultiplier was an EMI type 6097 with an overall gain of the order of 10^7. The EHT supply, which had a stabilization factor > 500 for variations of input voltage, provided 1250 V. with a positive earth, and was applied between the cathode and last dynode of the photomultiplier, giving about 125 V. across each dynode, the dynodes being joined by 330 kΩ resistors. The last dynode was earthed and the collector held at +108 V. with respect to it by the stabilizer valve V_2 (Fig. 2). The voltage developed across the collector load R_L by the current through the photomultiplier was applied to a balanced cathode follower amplifier (Fig. 2). A photomultiplier current of 0·1 μA. when R_L = 1 MΩ provided an input to the amplifier of 100 mV., and this was arranged to give full-scale deflexion in the output. A balanced system was therefore

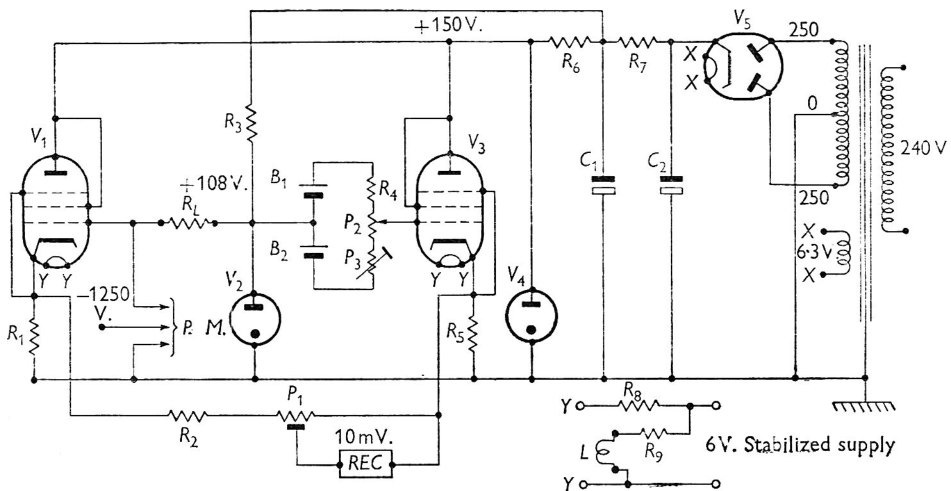

Fig. 2. Circuit of balanced cathode follower amplifier. V_1, V_3, ME1400; V_2, M8224; V_4, M8223; V_5, 6X4; R_1, R_5, 220 kΩ, 1 %; R_2, 2·7 kΩ; R_3, 18 kΩ; R_4, 68 kΩ; R_6, 12 kΩ; R_7, 1 kΩ; R_8, 5·5 Ω; R_9, 0·5 Ω, 120 W.; R_L, 1 MΩ, 500, 250, 150, 100, 50, 33 or 10 kΩ; P_1, 500 Ω potentiometer; P_2, 10 kΩ potentiometer; P_3, 100 kΩ potentiometer; C_1, C_2, 32 μF.; B_1, B_2, RM–12R 1·35 V. batteries; *P.M.*, EMI 6097 photomultiplier; *L*, 6 V. 30 W. F/25 lamp; *REC*, 10 mV. servorecorder. Eight position selector switch for R_L omitted from diagram.

necessary to ensure that variations in the reference voltage were cancelled out. The cathode follower also provided a low output impedance from an input circuit of high impedance, allowing a conventional 10 mV. servorecorder to be used.

A pair of matched ME 1400 electrometer valves were used operating at 50 V. V_a (anode voltage), 0·5 mA. I_a (anode current) and 4·5 V. V_f (heater voltage), fed from the low voltage stabilized supply. Under these conditions grid currents were of the order of 10^{-12} A. A series resistor in the output circuit dropped the 100 mV. to the 10 mV. required for the recorder and limited the output current to approximately 30 μA., which was small compared to the mean current through each valve (500 μA). The potentiometer P_2 enabled the amplifier to be adjusted to give zero output on the recorder when the photomultiplier collector was disconnected. The dark current

from the photomultiplier was approximately 10^{-3} μA. at room temperature. To obtain a wide sensitivity range, eight collector loads covering a range of 100:1 could be selected.

The microphotometer: mechanical system. The apparatus was mounted on an integral platform supported by four antivibration mountings. The microscope was fixed to a plate which could be moved over three levelled studs, and locked to the platform. The optical bench was joined to a crossbar which was located at both ends against stops on the board. The microscope was lined up optically with source and aperture on the optical bench and then locked in position.

All the mechanical movement of the microscope stage was dismantled because it gave rise to slight movement of the stage after a drop had been centred. The moving slides were lubricated with a molybdenum disulphide suspension in grease, which enabled the stage to be manipulated satisfactorily, and precise centring of a drop was readily achieved.

A thermal jacket made of sheets of expanded polystyrene was built round the microscope, with a hole to allow the light to reach the instrument, and a removable side to allow adjustment of the stage and other controls. The thermal enclosure contained a heating coil and tank through which water at 30° was continuously circulated. The temperature in the enclosure was measured near the stage, and with the side of the box closed the temperature rose rapidly to between 22 and 25°, depending on the ambient temperature. The eyepiece was enclosed in a light-tight tube leading to the photomultiplier; the end of this tube could be removed when direct observation either of the screen or through the eyepiece was required.

Operation of the microphotometer. The electrical circuits were switched on about 30–60 min. before the photometer was required. When an enzyme assay was made, the drop preparation was made as described. It was then placed on the stage of the microscope, and the mirror on the eyepiece removed. The condenser was lowered, the field aperture removed and a neutral filter swung into the illuminating beam. The drops were then inspected carefully to find one which contained only a single organism. The diameter of the selected drop (typically 7–10 μ) was measured by means of the micrometer eyepiece. The field aperture was replaced, and the condenser racked up till the image of the field aperture appeared in the plane of focus of the drop. The mirror was then mounted on the eyepiece, and the field aperture again removed. An orange filter was introduced in place of the neutral filter, and adjustment of the fine focusing produced an image of the microscopic field on the screen M (Fig. 1). The microscope stage was then moved manually until the image of the selected drop was near, but not over, the aperture in the middle of the screen. The field aperture was replaced, and its image centred over the aperture in the screen M by means of the condenser centring screws. The light-tight cover between the microscope and the photomultiplier was closed, and the orange filter replaced by the interference filter. The camera shutter behind the screen M was opened, and the total light recorded on a low-sensitivity range of the photometer. After a short period to record this, the shutter was closed, the field aperture removed and the orange filter re-introduced in place of the interference filter. The end cap was taken off the tube between the microscope and the photomultiplier, and the microscope stage adjusted to bring the image of the drop centrally round the aperture in the middle of the screen M. The cap was replaced, the field aperture re-inserted and the

interference filter replaced in the light path. The shutter was opened, and the light passing through the drop produced a signal which was recorded by the servorecorder. The collector load was chosen to give a reading about 25 % of full scale on the recorder, which was then allowed to run for about 30–45 min., that is, until a stable temperature was reached inside the thermal jacket E, and a steady recording of the light signal obtained, from which the slope could be measured. The dark current was checked at intervals by interrupting the light path. It was also possible to check the response and stability of the system by introducing a fixed load across the input to the cathode follower, giving a steady signal.

Calculation of enzyme activity in a drop

Under the conditions of assay used, the rate of destruction of penicillin by the enzyme should be proportional to the amount of enzyme present, and for the initial period of the reaction the amount of penicillin hydrolysed changes a proportional amount of purple (cationic) bromocresol purple into the yellow form. Thus, if E = total enzyme in drop, c = concentration of purple bromocresol purple, V = volume of the drop and A is a constant:

$$E = AV\frac{dc}{dt}. \tag{1}$$

Now, Beer's law states that

$$I = I_0 e^{-\alpha cd}, \tag{2}$$

where I = transmitted light, I_0 = incident light, α = extinction coefficient, c = the concentration of the absorbing material, and d = the light path. If I and c are the only variables, differentiation of (2) with respect to time gives

$$\frac{dI}{dt} = -\alpha d I_0 e^{-\alpha cd}\frac{dc}{dt}$$

$$= -\alpha d I \frac{dc}{dt}. \tag{3}$$

While the extinction coefficient and the light path may be considered to be known quantities, both of them could be open to question under the present circumstances because of: (1) possible changes in extinction coefficient under the high concentration conditions of the indicator, or changes caused by interaction with cell material; (2) the variable light path through the drop, which could be as long as the diameter of the drop, but which could be considerably shorter for light rays not passing through the exact centre of the drop. These two factors were therefore eliminated from the calculation in the following way. Taking logs of equation (2), and rearranging the terms

$$\log_e \frac{I_0}{I} = \alpha cd,$$

or

$$\frac{1}{c}\log_e \frac{I_0}{I} = \alpha d, \tag{4}$$

I and I_0 are known, and c can be assumed to be the initial concentration (C_0) of the purple form of bromocresol purple, if the readings are made within about 1 hr of

the start of the reaction (for the basal enzyme), by which time about 15 % of the purple bromocresol purple has been converted (on average) into the yellow form. The final equation used for the calculation of the total enzyme present in the drop was obtained from equations (1), (3) and (4):

$$E = -AV\frac{C_0}{I}\frac{1}{\log_e I_0/I}\frac{dI}{dt}. \tag{5}$$

The slope of the recording (dI/dt) was measured about 30 min. after the reaction started, but the recording was allowed to run on so that any gradual change in drop position which produced a gradual change in transmission would be more readily noticed. The temperature of the thermal enclosure was also noted, and the reaction rates were adjusted to the equivalent ones under the standard assay temperature of 30°.

RESULTS

Stability and calibration of microphotometer

Since the microphotometer was a single-beam machine, it was necessary to obtain a high degree of stability in the light source and the electronic equipment. The lamp (and valve heater) supply from the d.c. power supply and the EHT supply were held within close limits, and the stability of the whole system was tested by recording

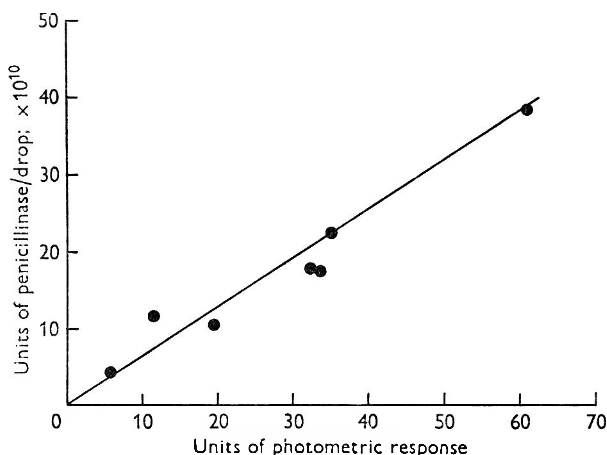

Fig. 3. Calibration curve of photometer. The relation between the soluble penicillinase content and the photometric response of mierodrops.

the output from the photomultiplier illuminated through the optical system without a drop in the field. The recording showed no change over 3–4 hr, and this was accepted as a measure of the stability of the entire system, both optical and electrical.

The recording of the photomultiplier output signal was used to show that the components of the assay system were stable under the conditions of illumination used. Drop preparations containing only bromocresol purple indicator were rapidly bleached on illumination with white light, but the monochromatic light (wavelength 5900 Å) obtained from the interference filter caused no detectable change in light transmission for a period of 4 hr. Similar results were obtained when penicillin at

the normal concentration was added to drops. Drops prepared from the bromocresol purple solution plus washed organisms showed a very slow change in transmission comparable to the least change produced by uninduced *Bacillus licheniformis* strain 749 organisms in the presence of the complete assay mixture. The contribution to the change in light transmission by the residual internal metabolism of one organism could be ignored under these circumstances, and no large error was introduced by attributing the entire change observed to penicillinase action.

Calibration. The microphotometer was calibrated by recording the transmission of drops of assay mixture in which had been mixed known concentrations of the soluble penicillinase released by *Bacillus licheniformis* organisms into the culture medium. Cell-bound penicillinase appeared to be readily accessible to the substrate, since the release of cell-bound penicillinase was not accompanied by an increase in the total enzyme activity measurable; hence the calibration with soluble penicillinase could be validly applied to the results obtained for cell-bound enzyme. The results of the calibration experiments are shown in Fig. 3. A linear relation between the amount of penicillinase present in the drop and the photometric response was obtained.

DISCUSSION

The technique and the microphotometer. The development of the technique for the assay of small amounts of enzymes in microdrops has enabled investigations of the variation within a bacterial population to be undertaken (Collins, 1964). This technique uses basically the photometric method common to many ordinary enzyme assays. In the microdrop the progress of the reaction caused by the enzyme is observed at a selected wavelength, and the continuous recording of the reaction allows the calculation of the rate of enzymic reaction per unit volume of the drop. The volume of the drop is estimated from its visible diameter, assuming it to be spherical. This assumption was justified, since a moving drop coming to rest against the coverslip underwent no visible change of appearance. The moving drop is practically spherical, and on touching the coverslip there will be a contact pressure equal to the difference in weight between the drop and the oil it displaces. This contact pressure, transmitted through the drop, causes the horizontal (visible) diameter of the drop to increase slightly and the vertical diameter to decrease slightly, but the changes in diameter were calculated theoretically to be of the order of 0·1 % of the diameter of the sphere, and were disregarded. The stability of drops in the preparations in oil, between water-repellent coverslips, enabled the method to be used successfully.

The light path through the drop was eliminated from the calculation of the rate of reaction, but the transmitted light through the drop has an added component of light diffracted round the edge of the drop. Trials with slides covered with droplets of mercury (evaporated on to the slide) in oil showed that this diffracted light could be as much as 20 % of the light transmitted through a bromocresol purple drop. However, in the drop preparations the intensity of the diffracted light is decreased from that in the model system, since the refractive index change at the oil–drop interface is much smaller. It is difficult to estimate the diffracted light for the drop preparations, but from the good calibration figures it is unlikely the contribution to the transmitted light could be more than 10 % of the light signal for an average

sized drop. The fact that the light path has been eliminated from the calculations means that the method is not restricted to spherical drops, but can be used to assay enzyme in any stable preparation whose volume is known.

The single-beam microphotometer has been successfully used in the measurements of light transmitted through drops, because of the high stability that has been achieved in the electronic and mechanical components. The manner in which this stability has been achieved has already been indicated in Methods. It may be added that the apparatus was operated in a laboratory containing the usual quota of centrifuges, etc.

The stability of the equipment was shown in the series of control experiments that were made with actual drop preparations. The use of soluble enzyme preparations enabled calibration of the response of the microphotometer over a wide range of enzyme concentrations; the results (Fig. 3) gave good agreement with the linear response expected. The errors involved in the calculation have been assessed roughly as $\pm 5\%$ for C_0, $\pm 2\frac{1}{2}\%$ for the diameter (equivalent to $\pm 7\frac{1}{2}\%$ for the volume V), $\pm 10\%$ for the total and transmitted light and the rate of change of light transmission. These give an estimated error of about $\pm 35\%$ in the calculated values of total enzyme in the drop. The calibration figures lie mostly within these limits, though a few larger deviations were also found.

The assay mixture. The assay mixture was so designed that, when all the penicillin had been destroyed, the pH value should be approximately 6·15, the pK value of bromocresol purple. The concentration of the purple form of the indicator was initially practically the same as the total concentration of the indicator itself, and the final concentration should be half this; in this way the concentration of the purple form needed for the calculations could be assumed without gross error to be that initially present, and the error should always be in the same sense. During the initial period of the enzymic reaction, there is competition between the purple form of the indicator and phosphate ions for the protons being released, and the change in concentration of the purple form of the indicator should bear a linear relation to the amount of penicillin destroyed. Thus the assumption of a linear relation between photometric units and the amount of enzyme present in the drop is reasonable.

Application to bacteria. The analysis of the distribution of penicillinase in bacterial populations as determined by using the technique described here is given in the next paper (Collins, 1964). Changes in most drops containing single organisms of *Bacillus licheniformis* were readily observed. It was noted on occasion that, after a drop preparation had been left overnight, drops containing organisms had changed colour, but drops without organisms remained purple.

The general method. The method should be applicable in principle to the assay of an enzyme which can be made to produce a measurable change in the light transmission of a microdrop. There are, however, other requirements for the successful operation of the method, which have not been stressed so far. It is particularly important that the enzyme substrate and product should be insoluble in oil so that they do not leave the microdrop during the observations. The indicator or chromogen used must also be insoluble in oil and stable to the lighting conditions used. In the case of penicillinase, these requirements have been easily met, and the high molecular activity of the penicillinase used has enabled the activities of 50 molecules

of it to be detected. It may not be possible to reach such sensitivity with other enzymes. Since relatively few enzyme molecules are present in a microdrop, it may be necessary to guard against enzyme inactivation at the oil/water interface, by the addition of carrier protein. In the case of penicillinase this was not necessary.

The viability of the organisms after they had been sprayed into the microdrops for penicillinase assays was not tested. To retrieve such an organism would require very careful micromanipulation, and was not attempted because the high concentration of penicillin in the assay mixture made survival rather unlikely. The reverse operation of inserting an organism into a drop may be simpler and more useful in practice.

We wish to thank Dr M. R. Pollock, F.R.S., for his interest and encouragement during the development of this technique, Mr J. Smiles, O.B.E., and Mr M. R. Young for valuable advice, and Mr W. Perrin for building the amplifier.

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The Distribution and Formation of Penicillinase in a Bacterial Population of *Bacillus licheniformis*

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SUMMARY

The distributions of penicillinase in populations of uninduced, induced and constitutive cultures of *Bacillus licheniformis* strains 749 and 749/c each showed a large positive skew. Analysis of the enzyme distribution in the uninduced population as a Poisson distribution showed that penicillinase molecules were produced not as independent random events, but in clusters containing 1100 molecules. There were 0–4 (average 0·85) clusters/organism. The fundamental random event resulting in the formation of a cluster of penicillinase molecules may be the formation of one enzyme-forming site capable of producing 1100 such molecules. Analysis of the enzyme distribution in the constitutive population showed that in this case penicillinase was produced in groups of 55,000 molecules, or fifty of the clusters of penicillinase found in the organisms of the uninduced population. This may be the number of enzyme-forming sites made by a constitutive penicillinase gene during the period between its formation and replication. Analysis of the enzyme distribution in the induced population showed that the organisms did not respond equally after induction, but all organisms probably increased their rates of penicillinase synthesis. Enzyme partition can be highly asymmetric when an organism divides; the enzyme molecules do not appear to be able to diffuse freely within the cell cytoplasm, but may be associated with some structurally rigid material in the organisms.

INTRODUCTION

The formation of a particular enzyme by a bacterial cell depends in the first place upon the necessary genetic information being present in the cell. Other factors then determine the amount of the enzyme actually produced. These factors will include the presence or absence of any agent (such as an inducer or repressor) which specifically affects the formation of the particular enzyme, and local environmental factors which affect the availability of the intermediates necessary for the formation of protein molecules, or which affect the stability of any of the compounds involved. Since these factors may vary from cell to cell in a single culture, the behaviour of genetically identical cells need not necessarily be the same, though the possible variation cannot be easily predicted. From the nature of the variation in enzyme content in individual bacteria certain deductions may be made about the nature and significance of the various factors which control specific enzyme synthesis and which could not be obtained from observations of large bacterial populations.

The behaviour within a bacterial population has been studied by indirect methods in the case of β-galactosidase in *Escherichia coli*. Benzer (1953) showed that

gratuitous induction of this enzyme proceeded in an apparently homogeneous fashion throughout the bacterial population, but non-gratuitous induction gave a heterogeneous response. Novick & Weiner (1957) showed that gratuitous induction at low inducer concentrations gave a heterogeneous response; in this system, however, the induction of β-galactosidase is complicated by the presence in the normal strains of *E. coli* studied of a galactoside-permease which concentrates galactosides (the inducers) inside the cell, and which is itself inducible. The homogeneous and heterogeneous formation of β-galaetosidase in *E. coli* appears to reflect the distribution of the permease within the population (Cohn & Horibata, 1959*a*, *b*). The indirect methods used in the study of this system do not reveal other details of the enzyme distribution among individuals of the population.

The microspectrophotometric method for the assay of penicillinase described in the paper by Collins, Mason & Perkins (1964) enables the amount of this enzyme in a single bacterium to be estimated directly with sufficient sensitivity to detect less than 50 molecules of penicillinase, assuming (as appears reasonable from preliminary findings with purified penicillinase from *Bacillus licheniformis* strain 749/c; Dr M. R. Pollock, private communication) the molecular activity of the penicillinase studied to be similar to that of the penicillinase of *Bacillus cereus* strain 569 (Kogut, Pollock & Tridgell, 1956).

The distribution of the penicillinase has now been studied in populations of *Bacillus licheniformis* strain 749 (Kushner, 1960) in which it is an inducible enzyme, and in a mutant strain derived from this in which penicillinase is a constitutive enzyme. Three populations have been studied to examine the distribution of penicillinase amongst organisms (*a*) uninduced, (*b*) after growth for one generation in the presence of an inducer, (*c*) of the mutant constitutive strain.

METHODS

Organisms. Bacillus licheniformis strain 749 (Kushner, 1960) and a mutant strain constitutive for penicillinase (*B. licheniformis* strain 749/c) were used in the experiments. The mutant strain was isolated by Dr M. R. Pollock from *B. licheniformis* strain 749 spores by a method based on that of Novick (1963). Both strains were maintained as spore suspensions in distilled water.

Growth media, etc. For the production of spores, 'S' broth (Pollock & Perret, 1951) cultures were shaken at 35° for 10 days. The spores were washed three times with distilled water and then resuspended in distilled water at a concentration of 3×10^8 viable spores/ml. Viable counts were made on diluted spore suspensions which were poured in 3 ml. molten 3 % agar-Hedley Wright broth (Wright, 1933) on to the same solid medium, and incubated for 18 hr at 35°.

For liquid cultures casein hydrolysate medium (Kogut *et al.* 1956) was used without citrate, and with the addition of 0·1 % (v/v) 'salts' solution (Collins & Kornberg, 1960) which contains a variety of metal ions. This was called CHS medium. Cultures (20 ml. CHS medium in a 250 ml. flask) were inoculated with 3×10^6 spores/ml., and shaken on a reciprocating shaker (86 cyc./min.; 1 in. throw) at 35°. After 12 hr the culture contained the equivalent of 0·25 mg. dry wt. bacteria/ml., and was still growing exponentially. Fresh warm medium (20 ml. CHS medium in a 250 ml. flask) was inoculated from this culture to a concentration of 0·07 mg. dry

wt. bacteria/ml. and transferred to another part of the reciprocating shaker where the throw was 3·5 in. Growth was measured turbidimetrically. The culture was allowed to grow to a density equivalent to 0·2 mg. dry wt./ml. before harvesting. For the investigation of cell-bound enzyme, the organisms were harvested by filtering the culture through a cellulose acetate membrane ('Oxoid'). The organisms on the filter were washed with a small volume of cold saline to remove traces of soluble enzyme, and were resuspended in saline to a concentration of 10^{10} organisms/ml. (equivalent to about 10 mg. dry wt./ml.). Kept at 0°, the organisms held the bound enzyme firmly, losing about 1 % per hr into the saline. (At room temperature about 40 % was lost in 1 hr.)

Determination of the mean residence time of penicillinase molecules in organisms. In an exponentially growing culture, the formation of free penicillinase lags behind the total penicillinase production because of the period during which the enzyme stays associated with the bacilli. The mean residence time is equivalent to the time that would be required for the rate of release, increasing exponentially with the same growth constant as the culture, to equal the present rate of formation. This may be expressed as

$$\frac{dF}{dt}2^{r/t} = \frac{dE}{dt}, \tag{1}$$

where dF/dt = rate of formation of free penicillinase, dE/dt = rate of formation of total penicillinase, r = mean residence time, t = doubling time of culture, and hence r/t = mean residence time expressed in doubling times of the culture. In order to measure the ratio of these rates of formation, steady-state growth experiments were performed in a 'turbidostat' so that observations could be made over prolonged periods of exponential growth. In the steady state, penicillinase was being synthesized by the bacteria in the culture vessel as fast as it was being washed out with the medium, i.e. at a rate proportional to (*a*) the total concentration of penicillinase present, and (*b*) the flow rate through the vessel. Similarly, free penicillinase was also being formed as fast as it was washed out, i.e. at a rate proportional to (*a*) the concentration of free penicillinase in the medium, and (*b*) the flow rate through the vessel. The rates of formation of total and free penicillinase could thus be compared as the ratio of the total and free enzyme concentrations in the steady state, and hence the mean residence time could be calculated. In the steady state,

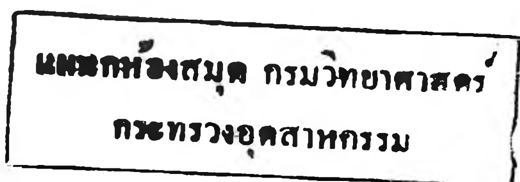
$$\frac{dE}{dt} = E\frac{dv}{dt} \quad \text{and} \quad \frac{dF}{dt} = F\frac{dv}{dt},$$

where dE/dt = the rate of formation of penicillinase, dF/dt = the rate of release of penicillinase into the medium, E = total penicillinase concentration, F = free penicillinase concentration and dv/dt = flow rate through the vessel.

Hence
$$\frac{1}{E}\frac{dE}{dt} = \frac{1}{F}\frac{dF}{dt}$$

and from equation (1)
$$2^{r/t} = E/F. \tag{2}$$

Steady-state growth experiments. Exponentially growing cultures of *Bacillus licheniformis* strains 749 and 749/c were maintained in steady multiplication by



pumping fresh CHS medium into aerated cultures, and pumping out the culture at a balancing rate. The pumping rates were adjusted manually to maintain a constant optical density and a constant culture volume. After growth for 4–5 hr the penicillinase activity of the whole culture and that bound to the bacteria were measured. Several samples were taken in each experiment and each experiment was repeated several times. In this manner the steady-state distribution of penicillinase between the bacteria and the medium was measured for the basal and for the induced enzyme in *B. licheniformis* strain 749, and for the constitutive enzyme in *B. licheniformis* strain 749/c. In the case of the induced enzyme, the culture was grown in the presence of 1 or 5 μg. cephalosporin C (Glaxo Laboratories Ltd.)/ml., with the cephalosporin added to the growing culture in the flask and also pumped in at this concentration in fresh medium.

Induction experiments. For routine induction experiments in bulk cultures in shaken flasks, cultures containing the equivalent of 0·1 mg. dry wt. bacteria/ml. were induced with cephalosporin C at 5 μg./ml.; this concentration produced no change in the growth rate. The organisms were harvested and washed when the culture contained the equivalent of 0·2 mg. dry wt. bacteria/ml.

Large-scale penicillinase assays. The iodometric assay of Perret (1954) was used for the assays of penicillinase in whole cultures, suspensions of washed organisms or in cell-free culture media.

Penicillinase microassay. The basis of the penicillinase microassay was the observation of the rate of the enzymic reaction in a microdrop containing a single organism (Collins, 1962). The method of preparing the stable microdrops, the basic assay mixture and the microphotometer have been described in the previous paper (Collins *et al.* 1964). The reaction mixture for the estimation of the basal enzyme contained: 2·0 ml. 4 % (w/v) bromocresol purple (G. T. Gurr Ltd.; adjusted to pH 6·15 with sodium hydroxide); 1·0 ml. 0·2 M-K_2HPO_4, 0·4 ml. benzylpenicillin solution (containing 2×10^5 i.u./ml. dissolved in saline); 0·2 ml. saline. The final pH value of the mixture was about 7·4. For each experiment 0·18 ml. of this reaction mixture was mixed with 0·02 ml. washed bacterial suspension, and drops were prepared by spraying the mixture through a fine capillary tube into a drop of silicone oil on a water-repellent coverslip. This was covered with a second water-repellent coverslip. The selection of a drop of suitable diameter (7–10 μ) containing a single organism was made by eye, using white light illumination and with the condenser of the microscope racked down somewhat from the normal position. Drops could then be searched carefully for organisms, and when necessary a purple filter could be used to decrease the contrast between the purple drop and the surrounding area. The sequence of operations after the drop was selected is described in the previous paper (Collins *et al.* 1964). The calculation of the total amount of enzyme in a drop was made from the recorded output from the photometer, which gives the total incident light, the transmitted light and the rate of change of transmitted light and from the diameter of the drop (Collins *et al.* 1964).

The reaction mixture was modified when organisms containing large amounts of penicillinase were tested; in order to obtain satisfactory records from the microphotometer it was necessary to slow the reaction, while maintaining proportionality between rate and amount of enzyme. This was achieved by adding 6-(2,6-dimethoxybenzamido)-penicillanic acid ('Methicillin'; Beecham Research Laboratories Ltd.),

which is a poor substrate for the enzyme and behaves as a competitive inhibitor when mixed with benzylpenicillin, to the benzylpenicillin solution to 4 mg./ml. This mixed substrate solution was used instead of the benzylpenicillin solution in the reaction mixture. The K_m for *Bacillus licheniformis* strain 749 penicillinase, measured by the micro-iodometric method of Novick (1962), was found to be $6{\cdot}7 \times 10^{-5}$ M for benzylpenicillin, and the K_i for methicillin was found to be $1{\cdot}9 \times 10^{-6}$ M. This value of the K_m is about 36 % of that found by Manson, Pollock & Tridgell (1954); the method used by these authors has been found to give higher values than other methods (Dr M. R. Pollock, private communication). With the mixed substrate solution in the reaction mixture, the observed enzymic activity was about 5 % of the activity with benzylpenicillin alone. The maximum velocity of hydrolysis of methicillin was about 0·6 % of that of benzylpenicillin, and the hydrolysis of the methicillin in the mixed substrate solution by penicillinase was negligible as compared with that of benzylpenicillin under the experimental conditions. The precise degree of inhibition was confirmed for each reaction mixture by testing a known amount of soluble penicillinase with the mixed substrate solution diluted tenfold with 0·1 M-K phosphate buffer (pH 7·2). The tenfold dilution brought the final concentrations of the penicillins to those found in the reaction mixture after the suspension of organisms had been added. Penicillinase solutions of known concentration were used to calibrate the response of the mixed substrate reaction mixture in the microassay; the calculated enzyme activity was multiplied by the factor determined in the bulk mixed substrate assay to correct for the inhibition of the enzyme reaction.

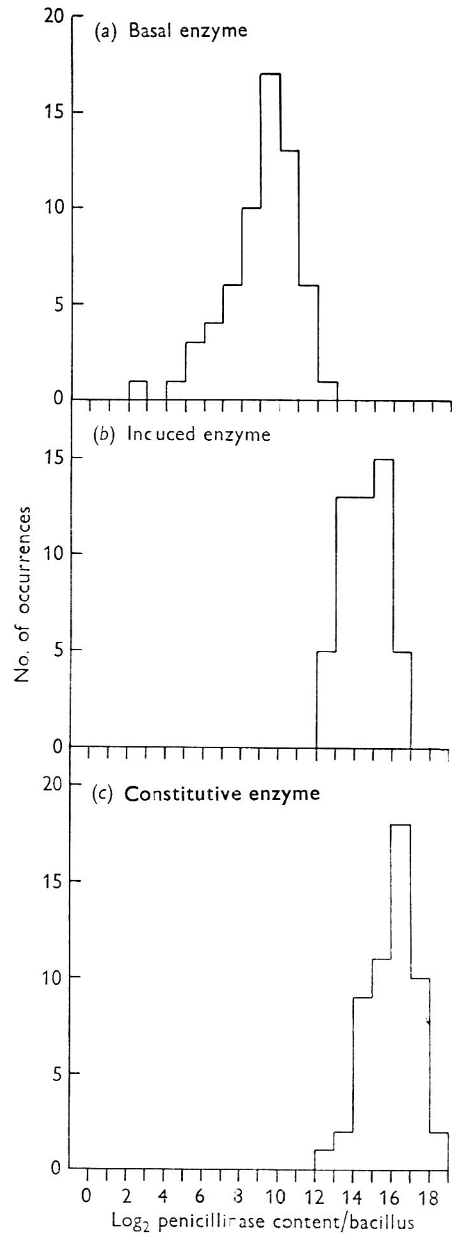
RESULTS

The penicillinase from Bacillus licheniformis strain 749

The amount of penicillinase in an individual organism is most simply expressed as the number of molecules of enzyme; one molecule of penicillinase must represent the fundamental unit of enzyme activity, and the use of these units aids the interpretation of results in terms of possible biochemical mechanisms. The molecular activity of the purified enzyme from *Bacillus licheniformis* strain 749/c (Dr M. R. Pollock, private communication) is close to that of crystalline penicillinase from *B. cereus* strain 569 (Kogut *et al.* 1956). One molecule of this penicillinase is equivalent to about $1{\cdot}5 \times 10^{-11}$ units of penicillinase activity, the unit being defined as that amount of enzyme which hydrolyses 1 μmole benzylpenicillin/hr at pH 7·0 and 30° in the presence of enzyme-saturating concentrations of benzylpenicillin. The experimental results given here are expressed as molecules of enzyme/single organism, by using this conversion factor.

Mean residence time of penicillinase molecules in the bacilli

The mean residence time was determined for the penicillinase molecules present in the bacilli in uninduced, induced and constitutive populations. In all three populations 68 % of the total enzyme was found attached to the bacilli, and 32 % was found free in the medium in the steady state. The mean residence time was therefore calculated (by equation (2)) to be 1·6 doubling times for the enzyme in these populations. In the steady-state growth experiments, the differential rate of formation of penicillinase with respect to bacterial growth is proportional to the specific



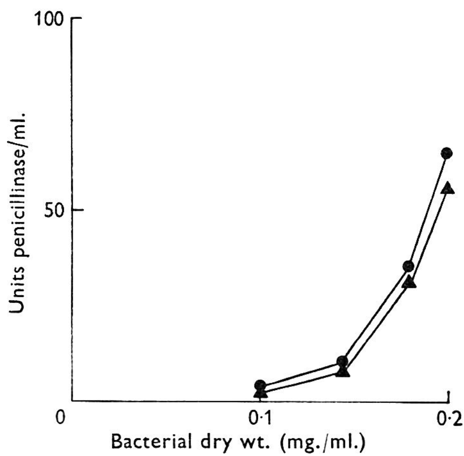


Fig. 1

Fig. 2

Fig. 1. Course of penicillinase formation by *Bacillus licheniformis* strain 749 after induction with 5 μg. cephalosporin C/ml. The cephalosporin C was added to the culture when it contained the equivalent of 0·1 mg. dry wt. bacilli/ml.

Fig. 2. Distributions of penicillinase in (*a*) uninduced, (*b*) induced populations of *Bacillus licheniformis* strain 749, and in (*c*) constitutive population of *B. licheniformis* strain 749/C; expressed as the logarithm to the base 2 of the number of penicillinase molecules/bacillus.

activity in the culture, given by total penicillinase present divided by quantity of bacteria. Total penicillinase is 1·47 times the cell-bound enzyme, from the result given above; hence it is possible to calculate enzyme synthesis and loss from the bacteria for one doubling time as follows. If initially in the steady state a bacteria contain b units of penicillinase, there will be a total of $1{\cdot}47b$ units of penicillinase in the culture. One doubling time later, there will be $2a$ bacteria, a total of $2{\cdot}94b$ units of penicillinase, $2b$ of which will be cell-bound, since the specific activity of the bacteria remains constant. The amount of penicillinase synthesized will be $1{\cdot}47b$ units; hence, if no enzyme had been released from the bacteria, they would contain $b+1{\cdot}47b = 2{\cdot}47b$ units. Thus $0{\cdot}47b$ units have been released during this period. The course of induction (Fig. 1) shows that the penicillinase formed during one doubling time after induction was predominantly cell-bound, though the free penicillinase (the difference between the curves of the total and the cell-bound enzyme) began to increase towards the end of the experiment. This suggested that the mechanism of release of penicillinase, which functions in the three populations examined in a similar manner, operated principally on the older penicillinase present, and that release might be achieved only after a period of maturation of the enzyme after formation. A simple assumption that might be made was that the enzyme was released sequentially from the bacilli 1·6 doubling times after formation.

The comparative distribution of penicillinase in the uninduced, induced and constitutive populations

The distributions of penicillinase in the three populations are shown in Fig. 2. The microassays were performed on organisms from exponentially growing cultures of *Bacillus licheniformis* strain 749 for the uninduced population (63 assays) and the induced population (51 assays), and on organisms from exponentially growing cultures of *B. licheniformis* strain 749/c for the constitutive population (53 assays). The penicillinase content is expressed as the logarithm to the base 2 of the number of molecules present in the organism so that the wide range of results can be accommodated in a single figure. The three populations are clearly distinct, with mean values in the ratios 1:30:90, which compare well with the values found in suspensions of the bacilli. The induced and constitutive populations contain few organisms with less than 8192 (2^{13}) molecules of penicillinase, the upper limit reached in the uninduced population. The range of enzyme contents was large in all three populations, being about 2000-fold in the uninduced population, 32-fold in the induced population and 128-fold in the constitutive population. The induced population does not appear to be a mixture of the uninduced and constitutive populations.

Analysis of the penicillinase distribution in the uninduced population

The distribution of penicillinase in the uninduced population is shown on an arithmetic scale in Fig. 3. The distribution has a large positive skew; the range extends from almost 0 to 4000 penicillinase molecules/bacillus; the mean value is 940. This value compares closely with the mean penicillinase content/bacillus (1010 molecules) measured in the suspensions of washed organisms used in the

microassay. Several of the bacilli produceed no change in the light transmission through their assay drops during the period of observation (up to 2 hr in one case). It is not possible to be certain that no penicillinase was present in these organisms, but an upper limit may be set to the penicillinase content as the amount that would have produced the least detectable change over the period of observation. This upper limit for two bacilli was 30 molecules, less than 3 % of the mean value. The highest content measured was four times the mean value. Because of the large range in enzyme contents found, it was necessary to confirm that individual spores of

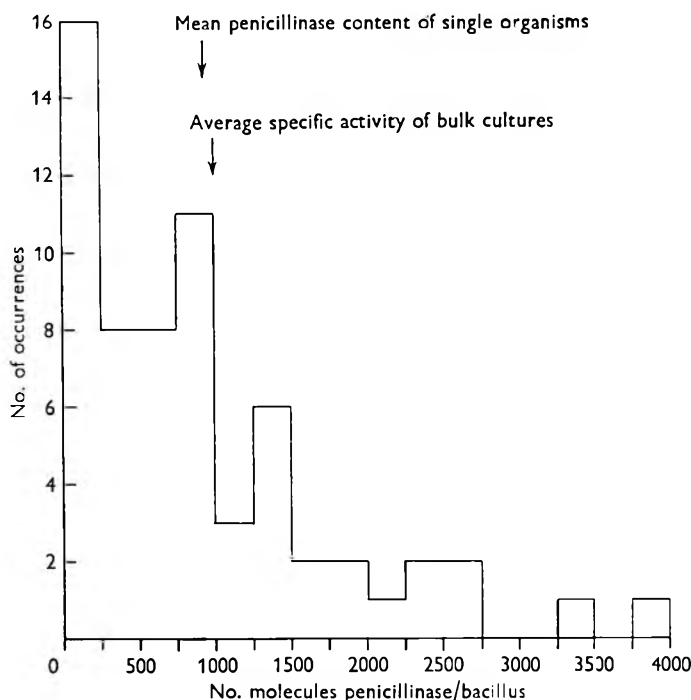


Fig. 3. The distribution of penicillinase in an exponentially growing uninduced population of *Bacillus licheniformis* strain 749.

Bacillus licheniformis strain 749 did not give rise to populations of bacilli with widely differing penicillinase contents. In control experiments, therefore, the mean specific penicillinase activity of bacilli in cultures grown from single colony isolates of the main spore stock was measured using the Perret assay technique with about 10^9 washed organisms. The variation found in a number of these isolates (shown in Fig. 4) was similar to the day-to-day variations in specific penicillinase activity measured in the same way in the suspensions of washed bacilli used for the microassay. Since the cultures used to provide these suspensions were grown from large inocula of spores, it was concluded that the spores were genetically homogeneous with respect to the production of penicillinase, the variations arising perhaps from slight changes in the environmental conditions from day to day. The large range of penicillinase contents of single bacilli found could not be explained therefore on the grounds of either genetic or environmental differences between the organisms, but

must reflect the process of penicillinase production in individual organisms, which, taken in the total population, gives a constant ratio of penicillinase to bacterial dry weight. It was therefore reasonable to treat the sixty-three observations of single organisms as a single distribution for further analysis. This distribution is a property of an exponentially growing culture which should be stable on further increase of the population; i.e. not only is the specific enzyme activity/bacillus constant, but the proportions of the population with any given enzyme content must also remain constant (see Collins & Richmond, 1962). It is possible to visualize from the logarithmic distribution different patterns of enzyme synthesis and release from the original bacilli which will achieve this stable distribution again after one doubling of the organisms. All the bacilli could be synthesizing some enzyme during the doubling time, since bacilli with very little enzyme content could always have arisen from an asymmetric partition of the enzyme when the parent organisms divided. With any pattern of release and partition, synthesis must have occurred in at least 17 out of the population of 63 bacilli, i.e. 27 % of the population, during the doubling period, so that penicillinase synthesis by an uninduced organism is not a rare event, although the uninduced bacteria produce only about 1 % as much penicillinase as the constitutive bacteria.

Table 1. *Comparison of the distribution of basal penicillinase (expressed as groups of* 1100 *molecules) and the theoretical Poisson distribution*

Groups of enzyme molecules/bacillus	No. of occurrences in distribution	
	Basal enzyme	Poisson distribution
0	26	27
1	26	23
2	9	9
3	1	3
4	1	1
Total	63	63

The average rate of penicillinase synthesis during one doubling period is 1·47 times the average content, that is, 1380 molecules. The highest content measured was 3900 molecules; if one doubling time later two bacteria have this content (to maintain the distribution constant in proportion) a minimum synthesis of 3900 molecules must occur in the bacterium already containing this amount, assuming no enzyme to be lost. Therefore, penicillinase synthesis must occur in a small proportion of the population at least at this rate, which is 2·8 times the average rate. There is therefore evidence in the distribution of a range of rates of penicillinase synthesis in the bacilli.

It is also possible from the distribution to deduce whether or not penicillinase molecules are synthesized as independent random events. If the molecules are synthesized as independent events, the distribution of enzyme in the population should be a Poisson distribution when the content is expressed as the number of molecules present. If there is any association in penicillinase production, i.e. a tendency for enzyme molecules to be produced in groups, the distribution will be a Poisson distribution only when expressed in terms of the total activity of such a group of penicillinase molecules. The penicillinase distribution in the uninduced

population can be expressed as a Poisson distribution (whose mean is equal to the variance) only when the penicillinase content is expressed as multiples of 1100 molecules. The Poisson distribution arises when the occurrence of an event in an individual in a large population does not affect the probability that a similar event will occur again in that individual. Thus the penicillinase distribution found might arise if the penicillinase gene were functioning as a source of information for the enzyme infrequently and randomly, but, each time it functioned, 1100 molecules of penicillinase were synthesized. The group of 1100 penicillinase molecules would then represent the output of either a single enzyme-forming site, or of a number of such sites resulting from the copies of the penicillinase gene made during its period of activity. The distribution (Table 1) shows that the bacteria contain from 0 to 4 of these groups of 1100 penicillinase molecules, the average content being equal to 0·85 group. The average rate of synthesis is then equal to 1·25 groups of 1100 penicillinase molecules per doubling time.

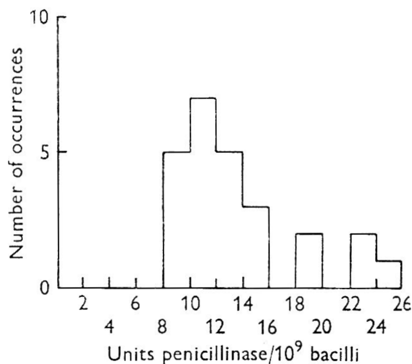


Fig. 4

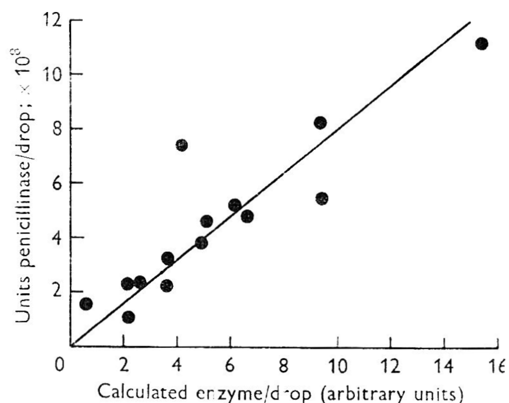


Fig. 5

Fig. 4. Variation in the cell-bound penicillinase activity from different uninduced cultures of *Bacillus licheniformis* strain 749.

Fig. 5. Calibration of the assay mixture containing benzylpenicillin + 6-(2,6-dimethoxy-benzamido)-penicillanic acid, by using soluble penicillinase. This mixture was used to assay the penicillinase content of induced and constitutive bacilli.

The penicillinase distribution in the population after induction

This penicillinase distribution was measured using the modified assay mixture containing methicillin to inhibit the enzymic reaction to a convenient rate. The calibration of the modified assay mixture was made by using soluble penicillinase from *Bacillus licheniformis* strains 749 and 749/c. A linear relation between the amount of enzyme in a microdrop and the photometric units calculated from the transmission of the microdrop was obtained over a wide range of enzyme concentration (Fig. 5); the errors were similar to those found in the calibration of the micro-assay when using benzylpenicillin alone with much lower concentrations of the soluble penicillinase (Collins *et al.* 1964).

The distribution of the cell-bound penicillinase produced by *Bacillus licheniformis* strain 749 was measured in cultures which had grown for 1 doubling time after induction with cephalosporin C (5 μg./ml). After 1 doubling time most of the

penicillinase synthesized in response to the inducer was still bound to the cells (Fig. 1), while most of the original bacteria had divided only once. Thus the distribution should reflect primarily the response of the original bacteria to the inducer before the processes of enzyme release and partition at division dominate the picture. The distribution is plotted logarithmically in Fig. 2, and arithmetically in Fig. 6. There is a positive skew in the latter, the range being from 4000 to 90,000 molecules of penicillinase/bacillus. The mean content was 30,400 molecules, compared to average specific activity of 25,500 molecules/bacillus found in washed suspensions from bulk cultures.

The penicillinase distribution in this population represents an intermediate stage between the distribution on the uninduced population and the distribution after prolonged induction. Hence the pattern will not be stable as the population continues growing. It can be seen in Fig. 2 that the distributions of the uninduced and induced populations just overlap, but it is clearly unlikely that any of the bacilli induced with cephalosporin C have failed to respond by increasing the rate of synthesis of penicillinase. In fact, a bacterium which would have synthesized 1380 molecules of penicillinase, the average amount in one doubling time without induction, has synthesized about 60,000 molecules of penicillinase (the average content of two of the induced bacteria) in this period after induction. As the penicillinase content in the induced population reaches 89,500 molecules, some bacilli have synthesized penicillinase considerably faster than the average rate. A lower limit for the rate of synthesis is similarly twice the lowest content, or 9910 molecules, though this may be less than the smallest response in the population if enzyme partition at cell division were uneven.

The distribution of penicillinase in the constitutive population

The mixed substrate assay system was used to examine the penicillinase distribution in the constitutive strain of *Bacillus licheniformis*, 749/C. The distribution is shown logarithmically in Fig. 2, and arithmetically in Fig. 7, where it has a positive skew. The distribution ranges from 4500 to 360,000 molecules/bacillus. The mean content was 84,500 molecules, about 90 times the mean content of uninduced bacilli. As a control, twenty-four single-colony isolates of the constitutive strain were grown under the usual conditions and the cell-bound penicillinase measured. The specific activities of washed suspensions showed only a 2:1 range, confirming the homogeneity of the strain with respect to its penicillinase production.

The constitutive population should have a penicillinase distribution which is stable, i.e. unchanging with growth of the culture. It may be analysed in a similar manner to that of the uninduced population. The most economical arrangement of penicillinase release and synthesis which retains the shape of the distribution after growth of the population for one doubling time requires at least 18 (34%) of the bacilli to synthesize at least 2048 molecules of penicillinase. In order to maintain a constant proportion of the population with a penicillinase content of 360,000 molecules synthesis should also occur at least at the rate equal to 360,000 molecules/doubling time in a small proportion of the population. The mean rate of synthesis of penicillinase is 1·47 times the average content, or 124,200 molecules per doubling time. The higher rate is 2·9 times the average rate, similar to the value found for the same ratio in the induced population. The analysis of the penicillinase distribution

in the constitutive population as a Poisson distribution suggests that penicillinase molecules are synthesized in large groups of about 55,000 molecules, the bacilli containing from 0 to 7 of these groups (average 1·53). These large groups of penicillinase molecules are the equivalent of 50 of the groups of penicillinase molecules found in the penicillinase distribution of the uninduced population. If the formation of a constitutive mutant involves a change in the DNA which affects the rate of synthesis but not the properties of the messenger-RNA molecule (Jacob & Monod, 1961), the large group of penicillinase molecules may represent the product of the messenger-RNA molecules produced by one constitutive penicillinase gene between its formation and replication.

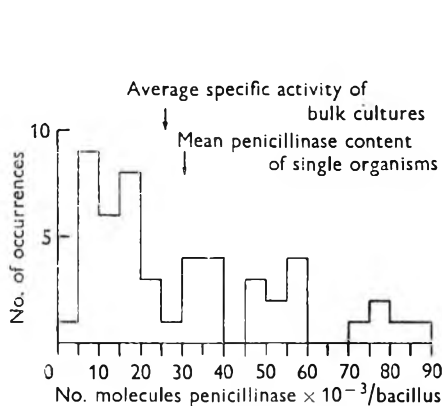


Fig. 6

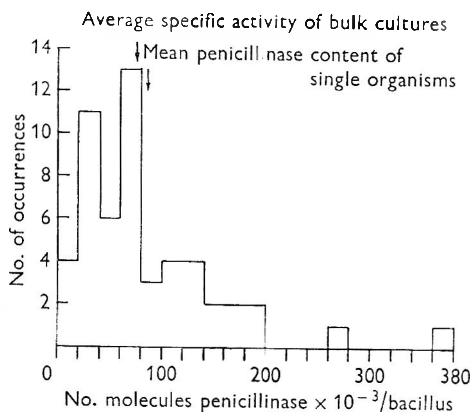


Fig. 7

Fig. 6. The distribution of penicillinase in an exponentially growing population of *Bacillus licheniformis* strain 749. One doubling time after induction with 5 μg. cephalosporin c/ml.

Fig. 7. The distribution of penicillinase in an exponentially growing population of *Bacillus licheniformis* strain 749/c.

If, moreover, the penicillinase gene in this constitutive mutant has lost a specific controlling substance (a genetic repressor) (Jacob & Monod, 1961), then every such gene should produce messengers and at least one large group of 55,000 penicillinase molecules should be found in a bacillus when it divides. The presence of bacilli (12 out of 53, i.e. 23 %) containing half this amount suggests that penicillinase partition is often uneven; indeed, the lowest content observed was only 8 % of one large group of 55,000 penicillinase molecules, or one-sixth of a half-share. The division of bacilli of the two strains of *Bacillus licheniformis*, 749 and 749/c, was observed to give two daughter bacilli of nearly equal size by microscopic examination of growing cultures. Highly asymmetric partition of penicillinase might arise if the enzyme were not freely diffusible within the cell cytoplasm, but distributed non-randomly in association with some structural elements within the bacillus. That this is true is suggested by the findings of Dr J. O. Lampen (personal communication) about the location of the cell-bound penicillinase in this organism and of Kushner & Pollock (1961) about the cell-bound penicillinase in *B. licheniformis* strain 6346, which they concluded was largely, if not entirely, bound to insoluble cell structures probably associated with the cell envelope. If penicillinase molecules were bound,

for example, into the cell membrane, both the unequal partition of the enzyme at division and the lag before release of the penicillinase from the bacilli could be explained. Thus extension of the cell membrane during growth might be associated with transfer of bound protein molecules within the permeability barrier of the bacillus to the outside, from which they could be released in a soluble form into the medium.

DISCUSSION

The penicillinase system of *Bacillus licheniformis* strains 749 and 749/c was chosen for the study of enzyme distribution within exponentially growing populations of bacilli because it was possible to obtain populations with a wide range of specific cell-bound penicillinase activities, and in different phases of expression of the same gene (i.e. in the uninduced and induced populations). Further, the sensitivity of the microphotometric method (Collins *et al.* 1964) was sufficient to measure the penicillinase distribution in the population of uninduced bacilli in detail, but could be decreased (by use of the competitive inhibitor methicillin) so that the higher penicillinase contents found in the bacilli of the induced and constitutive populations could be measured by the same technique.

The cell-bound penicillinase has been shown to consist mainly of the more recently synthesized enzyme molecules which have a mean residence time on the bacilli of 1·6 doubling times. This time was the same in the uninduced, induced and constitutive populations, which had average penicillinase contents in the ratio 1:30:90. The mechanism of enzyme release appeared to be the same in these three cases, and it might be related to the pattern of events which occurs during the bacterial division cycle, particularly since the cell-bound penicillinase was largely associated with insoluble cell structures (Dr J. O. Lampen; personal communication). Since the more recently synthesized penicillinase was attached to the bacilli, the enzyme distribution reflected mainly the recent activity of the penicillinase genes in the population. In individual bacilli, the penicillinase content is determined by the amounts of enzyme inherited, synthesized and lost since the bacillus was formed. While specific figures cannot be given for the behaviour of any individual bacillus, it was possible to deduce information about the rates and variation in rates of synthesis of penicillinase from the distributions of uninduced and constitutive populations, because these distributions were stable patterns unchanging with further growth. In both these populations there must be a range of rates of penicillinase formation at least up to three times the average rate. However, these populations differ in a significant manner.

In the uninduced population forming penicillinase at about 1 % of the rate reached in the constitutive population, it could be shown that penicillinase synthesis was not a rare event, so that the penicillinase genes are capable of functioning at rates less than the constitutive rate during one doubling time. The penicillinase is distributed within the bacterial population as if it were distributed into bacilli at random in groups of 1100 molecules. If the penicillinase gene can be expressed only in 'quanta' of 1100 penicillinase molecules, the synthetic ability of one copy of the penicillinase gene may in fact be about that number of molecules of penicillinase. It is in fact likely that this is the case, since, if the 1100 molecules were the product of more than one copy of the gene, the number of copies formed by the

gene should vary in a random fashion, and the distribution would be of the double Poisson type. Fitting a double Poisson distribution to the observed data scarcely alters the number of penicillinase molecules in the group, so that either the original suggestion is correct, or when the gene functions a predetermined number of copies are always made—clearly a more complex explanation.

There are other features of the penicillinase system in *Bacillus licheniformis* strain 749 that are consistent with the idea that the enzyme-forming site has a considerable lifetime, and might make a large number of penicillinase molecules. The lag (about 60 min.) that occurs upon induction before a constant differential rate of penicillinase synthesis is attained, and the continued production of penicillinase in the absence of any free inducer for up to 2 hr, might both reflect the presence of long-lived enzyme-forming sites. Thus, in uninduced bacilli, the formation of penicillinase appears to follow the production of one copy of the penicillinase gene, this copy being produced as a random event about once per gene per doubling time (the exact frequency depending upon the average number of penicillinase genes/bacillus). With such a low rate of functioning of the penicillinase gene, it is possible that the gene might be copied into messenger-RNA molecules not entirely at random during its lifetime, but perhaps with greater probability soon after it had been formed, if control were normally exercised by a specific repressor molecule. Thus a new penicillinase gene may be copied as soon as it is completed, before a repressor molecule is attached.

In the constitutive population, penicillinase appeared distributed in the bacilli in large groups of 55,000 molecules—50 times the number produced by one copy of the gene. These large groups (0–7 per bacillus) may be the product of one constitutive gene, during its lifetime. The number of large groups should then be related to the number of functional penicillinase genes present in the bacillus. If the constitutive penicillinase gene is of the type proposed by Jacob & Monod (1961), functioning in the absence of a normal repressor, every bacillus should contain at least half a large group of penicillinase molecules. The large number of exceptions suggest uneven partition of penicillinase at division. The cell-bound enzyme is known to be associated with insoluble (probably membranous) particles, which, if part of the cell envelope, would maintain their gross spatial organization as the cells grew and the membrane extended. To account for the non-random distribution on these structures, a free cytoplasmic stage of the penicillinase molecule is rendered unlikely, unless specific receptor sites are also non-randomly spread in the envelope. The high penicillinase content of the constitutive strain makes this receptor hypothesis difficult to maintain, since the penicillinase molecules could cover a substantial portion of the cell envelope as a monomolecular layer. An alternative hypothesis is that penicillinase molecules are synthesized at or near the cell envelope, the new molecules being absorbed into the envelope immediately after production. If the enzyme-forming sites were also immobilized in a similar manner, the enzyme-forming sites produced by one penicillinase gene might all be held in a small region near the membrane of the cell, and the group of penicillinase molecules produced might have as a result a spatial as well as a statistical association.

In the induced population, the distribution of penicillinase shows that the original bacilli treated with cephalosporin C have all responded to the stimulus and produced more penicillinase than bacilli in the uninduced population, though the range of

response is not defined well. The average synthesis (60,000 molecules) was half the average rate in a bacillus of the constitutive strain.

The induction process (Fig. 1) accelerates gradually, and the slow start may be caused in a number of ways, e.g. by delay in the inducer reaching its operative site, delay in the repressed gene becoming functional after the inducer has acted, or delay while the new penicillinase-forming sites begin to operate. It is hoped to investigate further the mechanism of control of the penicillinase gene in this organism under other physiological conditions.

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Composition of Cell Walls of Ageing *Pseudomonas aeruginosa* and *Salmonella bethesda*

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SUMMARY

Anaerobic cultures of *Pseudomonas aeruginosa* died rapidly in the absence of nitrate and death was normally followed by extensive autolysis. A mutant which did not undergo extensive autolysis was isolated. Anaerobic cultures of *Salmonella bethesda* did not die or lyse even after prolonged incubation. The protein and lipid content of the parent *P. aeruginosa* cell walls altered during ageing in contrast to *S. bethesda* walls which did not alter greatly as the organisms aged. The amino acid and amino sugar content of the three strains was determined. The diaminopimelic acid, glycine, alanine, glutamic acid, glucosamine, muramic acid and glucose content of the parent cell walls decreased by 50% as the organisms aged. The mutant strain of *P. aeruginosa* and *S. bethesda* walls showed no such change in ageing. Chemical changes similar to ageing could be produced in the cell walls of *P. aeruginosa* by incubation with an 'autolysin' obtained from old cultures of the parent strain.

INTRODUCTION

Pseudomonas aeruginosa is a strict aerobe which can grow under anaerobic conditions when nitrate is added to the medium to act as an alternative to oxygen as hydrogen acceptor. Under these conditions rapid growth occurs until the exhaustion of the nitrate supply after which all growth ceases and the culture rapidly passes into the decline phase. Such cultures afford an opportunity to examine some of the changes which occur when non-proliferating organisms are held in an otherwise non-toxic environment.

Rapid death and extensive autolytic changes have previously been observed in anaerobically incubated cultures of *Bacillus subtilis* (Nomura & Hosoda, 1956). Kaufmann & Bauer (1958) noted rapid lysis of anaerobically incubated cultures of *B. subtilis* and isolated an autolytic enzyme from the medium. They suggested that this 'autolysin' was released in an active form by the organisms when the normal cellular respiration was inhibited. Lysis of several species of sporing bacilli has been examined in some detail (Strange, 1959) and a bacterial lysozyme has been shown to be involved in some instances (Richmond, 1959). The cell walls of Gram-negative bacilli are more complex in nature than those of the Gram-positive bacteria (Salton, 1958, 1960) and in general, lysozyme treatment of Gram-negative bacteria does not result in cell lysis except under special conditions (Warren, Gray & Bartell, 1955; Repaske, 1958). A number of Gram-negative bacteria undergo autolysis on ageing, but for the most part little information is available on the mode of action of the enzymes involved. The similarities between

the rapid death and autolysis observed in the anaerobic cultures of *Pseudomonas aeruginosa* and that reported for the corresponding anaerobically incubated Bacillus cultures suggested that a similar mechanism may be responsible for both phenomena. An 'autolysin' has been found in the culture medium of old *P. aeruginosa* cultures and comparison of the composition of cell walls isolated from ageing organisms with that of young cell walls treated with the enzyme showed similar changes to occur in both.

METHODS

Organisms. Pseudomonas aeruginosa NCTC 6750 and *Salmonella bethesda* strain Md. 2 (kindly supplied by Dr N. Atkinson, University of Adelaide) were grown on nutrient agar slopes at 37° for 24 hr and stored at 4°. Subcultures were prepared from these slopes from time to time. A mutant strain of *P. aeruginosa*, which was not subject to extensive autolysis on ageing, was isolated from an old broth culture of NCTC 6750.

Medium. The growth medium contained the following nutrients: acid hydrolysate of casein (Oxoid Ltd., England), 5·0 g.; sodium nitrate, 5·0 g.; ammonium sulphate, 1·0 g.; magnesium sulphate, 0·1 g.; trace elements solution, 1·0 ml. (Meiklejohn, 1950); 0·067 M-2-amino-2-hydroxymethyl propane-1,3-diol (Tris) buffer (pH 7·0), 1 l. The medium was autoclaved at 115° for 15 min.

Cultural conditions. The medium was dispensed in 1 l. quantities in screw-capped serum bottles. Each bottle was inoculated with approximately 10^7 viable bacteria. The air was removed with a vacuum pump after the insertion of a sterile hypodermic needle through the rubber wad. A positive gas pressure was introduced by returning sterile pure nitrogen into the bottle and removing the needle. The positive pressure ensured that air did not leak into the cultures during the prolonged incubation period. All cultures were incubated at 37°.

Counting methods. Viable bacteria were counted by the method of Miles & Misra (1938). Total counts were made by the Williams (1952) method.

Quantitative analyses. Total-nitrogen, protein (Folin–Ciocalteau method and biuret method), total reducing sugar, total hexosamine and total phosphorus determinations were made by methods described by Kabat & Mayer (1961). Nitrate was estimated as previously described (Collins, 1956).

Individual sugars were identified chromatographically after hydrolysis of the walls with 2 N-sulphuric acid at 100° for 2 hr. The descending chromatograms were run in ethyl acetate + acetic acid + water (3 + 1 + 3 by vol.) for 18 hr at 20°. The papers were sprayed with aniline phthalate reagent (Cramer, 1954) and the sugar content of each spot estimated by the method of Baar (1954). Muramic acid was estimated by the method described by Strange & Kent (1959).

Amino acids were identified, after hydrolysis of cell walls with 6 N-HCl at 105° for 18 hr, by two-dimensional chromatography as described by Salton (1953). The intensities of the colours obtained with ninhydrin were compared with those for standard spots of known amino acids developed under identical conditions.

Lipids were estimated by the method described by Salton (1953).

Preparation of cell walls. Organisms were washed twice in saline and once in distilled water and suspended in cold distilled water at a concentration equivalent to 20 mg. dry wt./ml. The cells were then exposed for 15 min. to sonic vibration in

a Raytheon disintegrator with an output of 50 W. at 9 kc./sec., the suspension being kept at 0° throughout the experiment. The cell walls were washed by the method of Munoz, Ribi & Larsen (1959) and their purity checked by the examination of palladium shadowed preparations with a Philips model EM 100 electron microscope. The cell walls were suspended in distilled water and stored at −20° until required.

Concentration of autolysin from old cultures of Pseudomonas aeruginosa. Eight-day anaerobic cultures of *P. aeruginosa* were freed from whole cells by centrifugation at 8000 rev./min. for 30 min. The supernatant fluid was adjusted to pH 7·0 with N-acetic acid. Solid ammonium sulphate was slowly added in the cold to a final concentration of 3 M. The precipitate which formed was spun down, washed with 3 M-ammonium sulphate and re-dissolved in 0·067 M-Tris buffer (pH 7·0). The preparation was dialysed against 0·067 M-Tris buffer (pH 7·0). The precipitate in the dialysis sac was spun off and discarded. Nucleic acids were removed with protamine sulphate (Korkes, del Campillo & Ochoa, 1950). The autolysin was re-precipitated with 3 M-ammonium sulphate and dialysed against 0·067 M-Tris buffer (pH 7·0). After centrifugation the clear supernatant fluid was assayed for protein and diluted with buffer until it contained 5 mg. protein/ml. Three ml. of the enzyme preparation was added to the equivalent of 200 mg. dry wt. 24 hr *P. aeruginosa* NCTC 6750 cell walls suspended in 5 ml. of 0·01 M-Tris buffer (pH 8·0) and the volume made up to 10 ml. with Tris buffer (pH 8·0). The preparation was incubated at 45° for 20 hr and the cell walls were spun down at 20,000 ***g*** for 30 min. The walls were washed twice in cold 0·067 M-Tris buffer (pH 7·0) and stored in distilled water at −20° until required for assay.

RESULTS

The growth of Pseudomonas aeruginosa and Salmonella bethesda in anaerobic cultures

Both the parent and the mutant strain of *Pseudomonas aeruginosa* grew vigorously in the anaerobic cultures. A maximum viable population of $3–5 \times 10^9$ organisms/ml. was maintained until exhaustion of the nitrate supply (shown by an arrow in Fig. 1) whereupon a rapid and extensive decline in viable count was regularly observed (Fig. 1). Following the decrease in viable count the *P. aeruginosa* NCTC 6750 culture showed 40–50 % lysis on ageing, whereas the mutant showed very little lysis (5–10 %; see Fig. 1). The maximum viable population of *Salmonella bethesda* remained constant at $4–5 \times 10^8$ organisms/ml. throughout the experiment and the culture did not undergo any observable autolytic changes during ageing. The extensive lysis observed in the older cultures of *P. aeruginosa* NCTC 6750 suggested the presence in those cultures of an autolytic enzyme and so lysis experiments with young washed suspensions in the presence of cell-free culture filtrates were attempted. Preliminary experiments showed that some lytic activity was associated with the fluid of old cultures; to obtain significant results it was necessary to first concentrate the 'autolysin' by ammonium sulphate precipitation.

Incubation of young resting cell suspensions of *Pseudomonas aeruginosa* NCTC 6750 with the crude autolysin concentrate resulted in the lysis of 40–50 % of the organisms in 10–15 min. Total counts confirmed that after 60 min. only 20 % of the original organisms were still intact. Control suspensions which were incubated with heated autolysin (100° for 10 min.) showed little or no lysis during this time (Fig. 2). The addition of ethylenediaminetetraacetic acid (EDTA) to the test mixture greatly

increased the rate of lysis of the parent Pseudomonad and the mutant so that 60–70 % lysis was observed within 2–4 min. Control suspensions (containing EDTA only) decreased by 10 % over the 10 min. period. Ammonium sulphate precipitation of cell-free culture filtrates of aged mutant broth cultures yielded preparations with little or no lytic activity for parent or mutant Pseudomonads either in the presence or absence of EDTA. Lysis presumably resulted from the hydrolysis of the cell wall by the autolysin and attempts were, therefore, made to determine the possible substrates of the enzyme by making chemical analyses of the cell walls of the ageing bacteria.

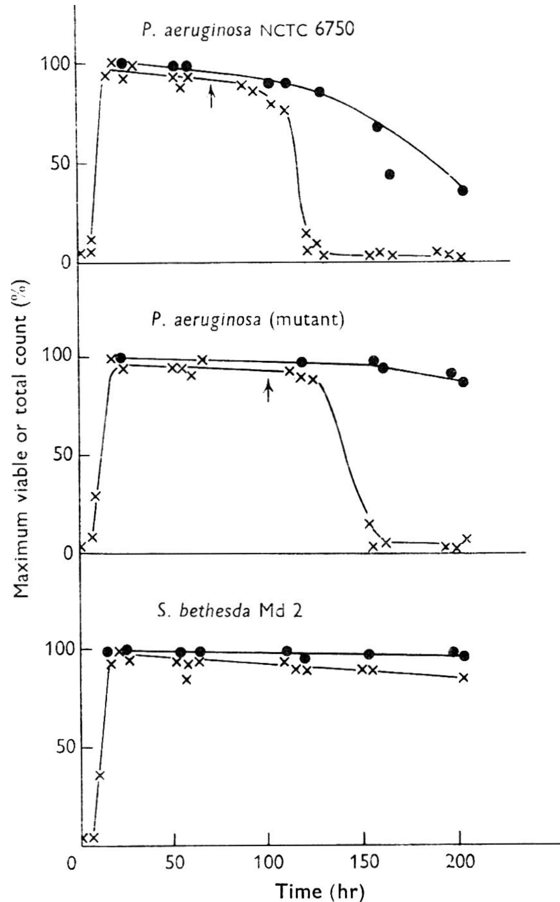

Fig. 1. Growth curves of *Pseudomonas aeruginosa* NCTC 6750, *P. aeruginosa* (mutant) and *Salmonella bethesda* in casein hydrolysate medium incubated anaerobically at 37°. The arrow indicates the time the nitrate supply became exhausted. ×—× = viable count; ●—● = total count. Nitrate was still present in the *S. bethesda* culture at 192 hr.

Composition of ageing bacterial cell walls

The details of the balance sheets for washed cell walls of the three organisms are set out in Tables 1 and 4.

Total nitrogen and protein. The total-N content of the walls of the three organisms accounted for 8–11 % of the dry weight and showed very little change with age.

The cell walls of young *Pseudomonas aeruginosa* NCTC 6750 cultures contained 50–60 mg. protein/100 mg. dry wt. wall and this rose to almost 70 mg. in old organisms. By contrast, the cell walls of the Pseudomonas mutant together with

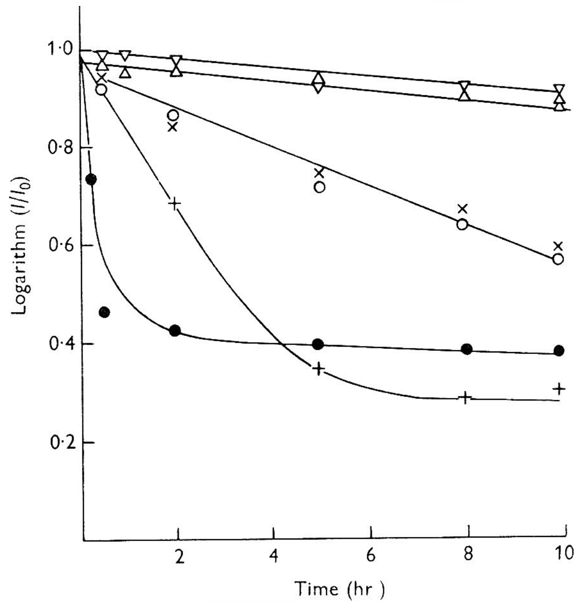

Fig. 2. Lysis of resting suspensions of *Pseudomonas aeruginosa* NCTC 6750 and *P. aeruginosa* (mutant) by crude autolysin in the presence of 0·01 M-Tris (pH 8·0) and 100 μg. ethylenediaminetetraacetic acid (EDTA)/ml. ●—●, *P. aeruginosa* NCTC 6750+autolysin +EDTA; ○—○, *P. aeruginosa* NCTC 6750+autolysin; +—+, *P. aeruginosa* mutant+ autolysin+EDTA; ×—×, *P aeruginosa* mutant+autolysin; △—△, *P. aeruginosa*+ EDTA; ▽—▽, *P. aeruginosa* control.

Table 1. *Composition of cell walls of ageing bacteria*

Age (hr)		Total-N	Protein*	Total reducing sugar	Total-P	Lipid Free	Lipid Total
		(mg./100 mg. dry wt. cell walls)					
24	*Pseudomonas aeruginosa* NCTC 6750	8·4	54	8·0	1·7	5·2	15
48		8·4	50	—	1·1	—	15
96		8·8	61	—	1·4	—	12
192		8·7	64	6·1	1·6	5·4	4
24	Enzyme treated†	—	63	6·0	—	7·6	10
24	*P. aeruginosa* (mutant)	10·1	70	12·1	1·6	6·3	16
96		10·5	72	—	1·4	—	14
120		10·0	69	—	1·0	—	11
192		10·9	70	13·5	1·0	5·8	13
48	*Salmonella bethesda*	8·0	77	18·5	1·7	8·7	12
96		9·0	63	—	1·7	6·0	11
192		9·0	63	15·4	1·6	7·2	10

* Average of Folin–Ciocalteau and biuret determinations.

† 24 hr *P. aeruginosa* NCTC 6750 cell walls incubated at 45° for 20 hr in 0·01 M-Tris buffer (pH 8·0) with the enzyme preparation.

those of *Salmonella bethesda*, contained 65–70 mg. protein/100 mg. dry wt. wall irrespective of the age of the cells (Table 1).

Amino acids. The amino acid composition of the cell walls altered significantly as the cells aged (Table 2). Although the over-all recovery of amino acids increased with age, considerable decreases were observed in the amounts of diaminopimelic acid (DAP), alanine, glutamic acid and glycine present in the older cell walls of the parent strain. The amino acid composition of the cell walls of young mutant Pseudomonads closely resembled that of the parent but there was no corresponding decrease in the DAP, alanine, glutamic acid or glycine content as the organisms aged (Table 3). The amino acid composition of the *Salmonella bethesda* cell walls differed quantitatively but not qualitatively from that of the *Pseudomonas aeruginosa* preparations.

Table 2. *Amino acid composition of Pseudomonas aeruginosa* NCTC 6750 *cell walls*

	Age of cell walls (hr)				Enzyme treated*
	24	40	88	192	24
Amino acid	(mg./100 mg. dry wt. cell walls)				
Phenylalanine	6·0	7·8	9·0	9·5	6·3
Leucine and isoleucine	2·2	2·9	3·5	3·5	2·4
Valine	3·0	2·5	2·6	2·0	1·7
Tyrosine	3·3	2·6	3·4	2·8	2·4
Arginine	0·9	0·6	0·6	0·9	0·9
Lysine	1·1	0·8	0·9	1·1	0·8
Alanine	7·2	7·5	4·2	3·3	3·1
Glycine	11·0	11·4	6·3	6·6	5·6
Serine	5·0	—	4·0	3·4	4·1
Glutamic acid	6·6	5·7	4·7	3·2	4·6
Aspartic acid	14·4	11·6	9·6	11·4	10·9
Diaminopimelic acid	2·8	3·0	1·7	1·2	0·9

* As in Table 1.

Table 3. *Amino acid composition of Pseudomonas aeruginosa (mutant strain) and Salmonella bethesda cell walls*

	P. aeruginosa (mutant)			*S. bethesda*		
	Age of cell walls (hr)					
	24	96	192	48	88	192
Amino acid	(mg./100 mg. dry wt. cell walls)					
Phenylalanine	7·3	9·3	10·8	4·1	6·6	7·2
Leucine and isoleucine	2·8	3·2	4·0	1·6	2·5	2·7
Valine	3·9	6·8	4·7	2·0	2·3	2·6
Tyrosine	4·2	6·4	5·4	2·5	3·0	4·2
Arginine	1·3	1·9	1·7	0·4	0·3	0·7
Lysine	1·7	2·5	2·3	0·5	0·5	0·6
Alanine	5·1	6·2	7·3	4·3	3·5	3·5
Glycine	7·1	8·4	8·7	5·5	8·0	14·0
Serine	5·4	8·4	8·9	2·6	2·0	3·0
Glutamic acid	5·8	8·6	6·0	4·9	4·9	7·8
Aspartic acid	9·3	8·9	7·0	14·5	15·0	22·5
Diaminopimelic acid	3·2	5·5	5·1	2·1	1·9	2·9

Carbohydrate. The details of the total reducing sugar content of the three cell wall preparations are given in Table 4. *Pseudomonas aeruginosa* NCTC 6750 showed a decrease in total reducing sugar content with ageing whilst the cell walls of the mutant did not show any ageing effect. However, the cell walls of the mutant contained more reducing sugar residues than did the walls of the parent strain. The *Salmonella bethesda* cell walls contained 18·5 mg. reducing sugar/100 mg. wall and this decreased slightly with ageing (Table 4). The walls of all three organisms contained 1·5–2·0 mg. of amino sugar/100 mg. wall, of which about half was muramic acid (Table 4). The amino sugar content of *P. aeruginosa* NCTC 6750 cell walls decreased by 50 % as the organisms aged. Little change in the amino sugar content of the other two organisms was noted. Chromatographic identification of the individual cell wall sugars and amino sugars disclosed the presence of glucose, mannose, rhamnose, glucosamine and muramic acid (Table 4). The cell walls of *P. aeruginosa* NCTC 6750 contained approximately equimolar amounts of glucose and rhamnose. The glucose content of the walls decreased by 60 % as the organisms aged, whereas the rhamnose and the mannose content changed very little (Table 4). The glucose and mannose content of the cell walls of the mutant was almost double that of the parent and this offers an explanation for the higher reducing sugar content observed with the cell walls of the mutant. The sugar content of the *S. bethesda* cell wall was quantitatively quite distinct from both Pseudomonads and there was no detectable alteration in sugar content on ageing.

Table 4. *Carbohydrate and amino sugar composition of cell walls of ageing bacteria*

Age (hr)	Strain	Glucosamine	Muramic acid	Glucose	Mannose	Rhamnose
		(mg./100 mg. dry wt. cell walls)				
24	*Pseudomonas aeruginosa* NCTC 6750	0·7	0·8	2·9	0·3	2·2
64		—	—	2·7	0·3	2·0
88		—	—	1·8	0·3	2·0
192		0·2	0·6	1·1	0·3	1·8
24	Enzyme treated*	tr.	0·4	0·6	0·3	1·9
24	*P. aeruginosa* (mutant)	0·7	0·8	5·0	0·6	3·0
72		—	—	4·4	0·7	3·0
192		0·5	0·9	3·4	0·5	2·8
48	*Salmonella bethesda*	1·1	1·0	3·9	3·7	9·6
88		—	—	4·8	3·3	8·2
192		1·6†	—	4·1	3·0	8·1

* As in Table 1.
† Estimated only as total hexosamine.
tr. = trace.

Lipid and total phosphorus. The phosphorus content of the cell walls of the three strains varied from 1 to 2 mg./100 mg. wall and there was no change on ageing. The free lipid content varied between 5·2 and 8·7 mg./100 mg. wall and did not alter with ageing. However, the total lipid content of the parent pseudomonad walls decreased by almost 25 % (Table 1). The lipid content of the walls of the mutant and of *Salmonella bethesda* showed very little change on ageing. The total recovery

of wall material varied from 80 to 100 %. The high recoveries for the mutant walls were mainly due to the unexplained 15–20 mg./100 mg. wall increase in the protein content of these walls. The incomplete recoveries for *Pseudomonas aeruginosa* NCTC 6750 and *S. bethesda* suggest that there may be other undetected minor components in these walls.

Autolysis of washed Pseudomonas aeruginosa cell walls

Incubation of young *Pseudomonas aeruginosa* cell walls with the crude autolysin obtained from old cultures resulted in a change in opacity of 15–20 % in 18 hr. Chemical assays carried out on the hydrolysed cell walls disclosed changes in composition similar to those recorded above for aged cell-wall preparations. The protein content increased somewhat but the total reducing sugar was not apparently affected (Table 1). However, chromatographic examination of the cell-wall hydrolysates disclosed that the amino acids DAP, alanine, glycine and glutamic acid decreased by 50 % (Table 2). Similarly, the glucosamine and muramic acid content of the walls decreased sharply (Table 4). The total lipid content of the walls also decreased considerably (Table 1). The composition of a control cell wall suspension treated with boiled autolysin resembled, within experimental error, the initial 24 hr old preparation recorded in Table 1.

DISCUSSION

The observed lysis of the anaerobically incubated *Pseudomonas aeruginosa* NCTC 6750 was due to the presence of an extracellular autolysin. Cultures of the mutant strain and *Salmonella bethesda* did not produce detectable amounts of autolysin in ageing cultures, although the mutant was still susceptible to lysis. Thus the mutation appeared to involve the loss of ability to produce an extracellular autolysin, rather than the formation of a lysis-resistant cell wall. The mutant cells were still strictly aerobic and were subject to a rapid and extensive degree of killing when incubated under anaerobic conditions. The absence of subsequent lysis of these cells suggests that the lethal and lytic processes observed in cultures of *P. aeruginosa* NCTC 6750 are distinct and not directly inter-related.

The gross chemical composition of the cell walls of the three strains of bacteria underwent a number of changes as the cultures passed through their growth phases. The most interesting alterations occurred in the amino acid and amino sugar content of the ageing cell walls of the parent strain. The over-all amino acid content of the walls increased slightly as the cells aged. This was to be expected from the observed increases in total protein. The coincidence of the sharp decline in the DAP, alanine, glycine and glutamic acid content of the walls of the parent strain with the onset of autolysis suggested a causal relationship between the two phenomena. Significantly, the cell walls of neither the mutant strain nor *Salmonella bethesda* showed any comparable decrease in these amino acids. In view of the known importance of these amino acids in the cell wall mucopeptides of other Gram-negative bacteria (Salton, 1958, 1960; Brown, 1958) it seems reasonable to presume that the autolysin present in the cultures of the parent strain removed part of the rigid mucopeptide layer from the *Pseudomonas aeruginosa* cell walls so that cell lysis resulted. The removal of at least 50 % of the muramic acid and glucosamine from the aged

P. aeruginosa NCTC 6750 cell walls was taken as confirmation that the entire mucopeptide moiety was affected. However, the Pseudomonas autolysin was not a simple bacterial lysozyme since the crude autolysin preparation was unable to lyse *Micrococcus lysodeikticus* suspensions.

The removal of complex lipid from the cell walls of the parent strain during ageing suggested the presence of an esterase in the autolysin. Sierra (1957) demonstrated the presence of several esterases in *Pseudomonas aeruginosa* cells and noted a correlation between their presence in the culture medium and the subsequent induction of cellular lysis. In the present study, both the whole organisms and the autolysin concentrate were found to have lecithinase activity when tested against serum lecithin. The loss of lipid from the ageing cell walls of the parent but not the mutant organism suggested that lysis of the cell was initiated only after some lipid complex in the wall had been removed. This was confirmed by the increased autolysis of *P. aeruginosa* following treatment of the cells with acetone or Teepol. Increased autolysis following treatment of cells with lipid solvents has previously been observed by Warren *et al.* (1955) and others. Presumably the removal of a lipid moiety exposed the mucopeptide layer which could then be attacked by other enzymes present in the autolysin. Thus it seems likely that the *P. aeruginosa* autolysin consists of a mixture of at least two enzymes and is distinct from the autolysins produced by the Gram-positive bacteria. Further purification of the autolysins of this and other Gram-negative bacteria would, therefore, be useful and should reveal whether or not the autolysin of *P. aeruginosa* is typical of the enzymes produced by other Gram-negative micro-organisms.

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Accumulation of Glucose and Galactose by *Streptococcus faecalis*

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SUMMARY

Glucose and galactose are accumulated against a concentration difference by *Streptococcus faecalis* strain 10cl which has been treated with iodoacetate to eliminate glycolysis. The rate and extent of accumulation are greater for glucose than for galactose but both processes show saturation kinetics, stereospecificity, and sensitivity to metabolic inhibitors. Accumulated sugar does not exchange with glucose or galactose in the external medium. Loss of internal sugar is a relatively slow first-order reaction. Both accumulation and loss are temperature dependent. Preincubation of the organisms in arginine increases the rate and extent of accumulation and decreases the rate of loss. A model is proposed in which hexose accumulation results from an energy-dependent adsorption to specific, thermolabile receptors.

INTRODUCTION

Experiments designed for the study of carbohydrate transport in bacteria have frequently made use of two alternative conditions in order to dissociate the entry of the sugar from its subsequent phosphorylation and metabolic breakdown: (1) an analogue of the sugar is chosen which is able to penetrate the cell but which is not a substrate for the available glycolytic enzymes (Cohen & Monod, 1957); or (2) a mutant is selected which can accumulate the natural sugar but which is unable to metabolize it (Horecker, Thomas & Monod, 1960). During investigations in this laboratory on the metabolism of carbohydrates by *Streptococcus faecalis* strain 10cl, it was noted that free glucose and galactose were accumulated by cocci in which glycolysis had been eliminated by treatment with iodoacetate (Wilkins & O'Kane, 1961). This observation suggested the possibility of studying hexose transport independently of both substrate analogues and mutants. The work presented here is concerned with characterizing the accumulation of glucose (a metabolizable sugar) and galactose (which is not metabolized by glucose-grown cocci) in terms of entry and exit rates, stereospecificity, energy requirements, and sensitivity to metabolic inhibitors.

METHODS

Preparation of suspensions of cocci. The organism, *Streptococcus faecalis* strain 10cl, was grown at 37° in a medium containing 0·1 % glucose, 0·3 % K_2HPO_4, 1·0 %

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yeast extract, and 1·0 % tryptone. The cocci were harvested in the late logarithmic growth phase, washed in and resuspended in 2-amino-2-hydroxymethylpropane-1,3-diol (tris)+maleate buffer (pH 7·0; 0·03M) so that a 1 ml. volume of reaction system would contain the equivalent of 1·6 ± 0·2 mg. dry-weight cocci. This quantity represents a compromise between an amount of cocci small enough to permit rapid collection on a membrane filter and large enough to accumulate measurable amounts of radioactivity from low concentrations of ^{14}C-labelled substrate. The equivalent dry weight of cocci was determined turbidimetrically in an Evelyn Photoelectric Colorimeter by using a suitable calibration curve.

Apparatus. In most experiments the cocci were removed from a reaction mixture containing 10^{-3}M-iodoacetate and a radio-active hexose by means of a closed-system membrane filter that could be immersed in a water bath for the control of temperature (Fig. 1). Cocci, substrate and other reagents could be placed in the reaction tube compartments (shown inverted in Fig. 1) before the two sections of the filter unit were clamped together. Subsequent additions were made by syringe through Port 1 (Fig. 1, (3)). At the end of the reaction time, the mixture was tipped on to a Millipore HA filter (25 mm. diam.) by rotating the entire unit. Suction was applied at the filtrate tube (Port 2, Fig. 1, (7)) and the soluble reaction constituents passed through the filter within 20 sec. One ml. of tris+maleate buffer was injected through Port 1 immediately after tipping to wash down the drop of reaction mixture that hangs from the reaction tube. The cocci were washed with two additional 1 ml. portions of buffer and the membrane, with adhering cocci, was promptly removed. Since individual determinations entailed several manipulations extending over a considerable period of time, the apparatus was arranged so that four independent filter units could be used simultaneously.

When, as in exit rate experiments, preloaded cocci were to be resuspended above the filter membrane, the suction at Port 2 was turned off, a slight suction was applied at Port 3 (Fig. 1, (2)) and the appropriate solution was added through Port 1. The resuspending solution did not leak through the filter under these conditions but could be quickly removed by reversing the direction of suction.

The substrate solutions were prepared from weighed quantities of glucose-^{14}C (uniformly labelled) from Nuclear-Chicago Corp. and galactose-1-^{14}C obtained from the National Bureau of Standards. The radioactivity was measured with a Nuclear Instrument Corporation Geiger counter (model 153). The glucose ^{14}C had 18,000 c.p.m. per μmole and the galactose-1-^{14}C had 48,000 c.p.m. per μmole. Sufficient counts were recorded to yield a probable error of less than 2 %.

The radioactivity remaining with the cocci after they had dried on the filter membrane was usually interpreted as the μmoles sugar accumulated/g. dry wt. of cocci. The internal substrate concentration was estimated by dividing the amount of sugar accumulated by the volume of intracellular water. The intercellular water content of the packed cell mass determined by the inulin method of Conway & Downey (1950) was very close to the theoretical value of 26 % for close-packed spheres. The water content of the cocci was assumed to be 80 % (Luria, 1960), and the percentage of the packed cell mass representing intracellular water presumably available to dissolve hexose was therefore taken to be 0·74 (correction for intercellular water) × 0·80 (intracellular water) × the packed cell volume.

Chemical analyses. Reducing sugars were determined by Nelson's (1944) modi-

fication of the Somogyi method. Lactic acid was measured by the method of Barker (1957). Ammonia production from arginine was measured with Nessler's reagent prepared by the method of Vanselow (1946). Chromatography of free sugars was carried out by the method of Jermyn & Isherwood (1949) using ethyl acetate + pyridine + water (2 + 1 + 2, by vol.) as the developing solvent and aniline oxalate as the spray.

Kinetics of sugar transport. Hexose accumulation in *Streptococcus faecalis* can be considered in terms of the equations presented by Cohen & Monod (1957) for permease mediated sugar transport in *Escherichia coli.* In this model, net accumulation is a balance between an entry reaction which follows saturation kinetics and an independent first-order exit reaction. The change in the internal substrate concentration, S_i, may be represented by

$$\frac{dS_i}{dt} = y\left(\frac{S_e}{S_e+K}\right) - c(S_i), \tag{1}$$

where S_e is the external concentration, K is the external concentration at which half-maximum accumulation occurs, y is the maximum transport capacity of the system, and c is the exit rate constant.

At equilibrium, S_i is constant. Substituting $dS_i/dt = 0$, and $y/c = Y$ in equation (1) and taking reciprocals gives

$$\frac{1}{S_i} = \frac{1}{Y} + \frac{K}{Y}\left(\frac{1}{S_e}\right), \tag{2}$$

from which the constants may be determined by the conventional Lineweaver–Burk plot of $1/S_i$ vs $1/S_e$.

RESULTS

Inhibition of glycolysis by iodoacetate

Preliminary experiments with concentrated coccal suspensions (1 g. wet wt./ml.) showed that iodoacetate-poisoned suspensions (exposed to either glucose or galactose) accumulated reducing activity that could be extracted from the cocci by 5 min. exposure to 100° after treatment of a 1 ml. sample with 1 ml. acetone, allowing to stand a few min., and adding 1 ml. of water. Chromatography of samples of the extracts showed either free glucose or free galactose in approximately the amount expected (indicating that phosphorylation and subsequent metabolic steps had been eliminated by treatment with iodoacetate). To facilitate experimentation, the coccal concentration was decreased, and the sugar uptake followed by radioactivity alone.

Glucose metabolism was not stopped immediately upon the addition of 10^{-3}M-iodoacetate. Manometric experiments showed that oxygen consumption and acid production did occur in reaction systems similar to those used in the accumulation studies described below unless the iodoacetate was added 15 min. before the addition of glucose. The cocci were therefore routinely incubated with iodoacetate for 20 min. before the sugar was added. Under these conditions, chemical analyses showed no loss of glucose from the medium and no production of lactic acid. The procedure used for the determination of glucose was not sensitive enough to detect the amount removed from the medium by the accumulation process but it could be

expected to reveal, over an extended reaction time, a very slow metabolic utilization of glucose. Although galactose was not fermented by glucose-grown cocci, iodoacetate was included in the experiments with galactose.

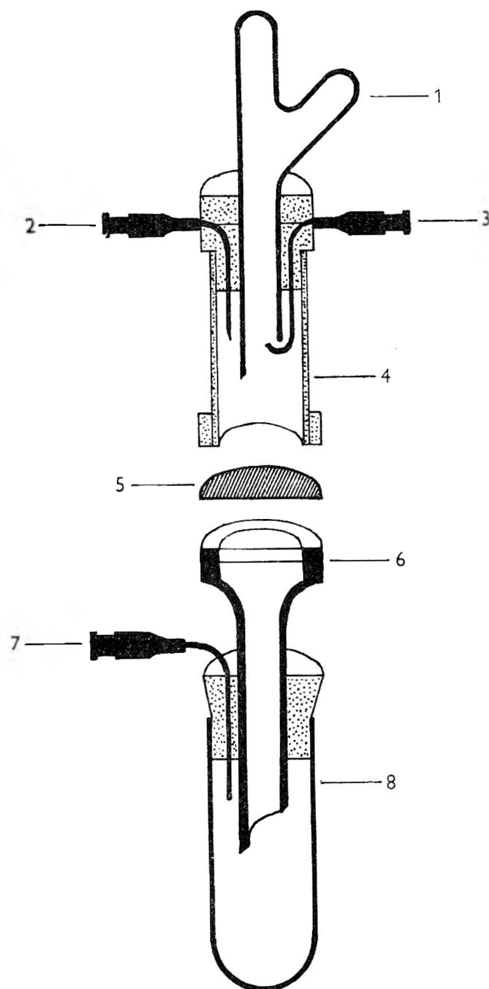


Fig. 1

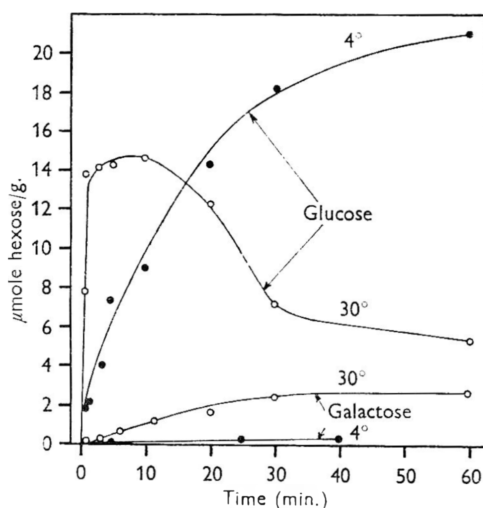


Fig. 2

Fig. 1. Closed system membrane filter. (1) Glass reaction tube; (2) Port 3 (no. 20 hypodermic needle) suction line coupling; (3) Port 1, inlet for solutions; (4) Acrylite cylinder; (5) Millipore HA filter membrane (25 mm. diam.) (6) Millipore filter base; (7) Port 2, suction line coupling; (8) filtrate tube. The cylinder is clamped to the base after the compartments of the reaction tube (shown inverted) have been filled. For operational procedures, see text.

Fig. 2. Time course of glucose and galactose accumulation. Each reaction tube contained (1·0 ml.): cocci, equiv. 1·6 mg. dry weight; tris+maleate buffer (pH 7·0), 13 µmole; hexose-^{14}C, 0·1 µmole; iodoacetate, 1·0 µmole added 20 min. before the addition of the sugar. At the end of the indicated accumulation times, the cocci were collected, washed (in three 1 ml. portions of buffer) and allowed to dry on the filter membrane. The specific activities of the hexoses were: glucose 18,000 c.p.m./µM, galactose 48,000 c.p.m./µM.

Accumulation of glucose and galactose

The time course of glucose and galactose accumulation, at 4° and 30°, from external concentrations of 10^{-4} M is shown in Fig. 2. The initial rate of glucose uptake was too rapid for accurate measurement, even at low temperatures, and thus the Q_{10} for glucose entry could not be determined. The time and extent of maximum glucose accumulation tended to vary with the coccal preparation despite efforts to keep growth conditions and preparatory manipulations constant. At 30° the cocci accumulated 90 % of the maximum amount in less than 1 min.; a gradual loss of most, but not all, of the internal glucose followed. At 37° maximum glucose values were considerably lower than those at 30° possibly because, as Winters, Delluva, Deyrup & Davies (1962) postulated for sulphate accumulation in kidney mitochondria, uptake and subsequent loss at 37° may occur within a very short time interval. The internal glucose value at 20° was higher than at 30°, and it remained relatively stable. At temperatures below 20°, accumulation values were still higher and no loss of previously accumulated glucose was observed.

Table 1. *Hexose accumulation by Streptococcus faecalis against a concentration difference*

The experimental conditions were those given in the legend to Fig. 3. The internal concentration, S_i, was estimated as described in Methods. The external concentration, S_e, at the steady state equals the amount of sugar originally added less the amount accumulated.

S_e at start (Molar × 10^5)	S_i/S_e at the steady state	
	Glucose	Galactose
100	4·2	3·0
70	6·2	4·3
50	8·6	6·4
30	13	9·1
10	38	14
5	69	14
1	90	—

The initial rates of galactose uptake were readily measured (Fig. 2) and were reproducible within narrow limits. Unlike glucose uptake, the accumulation of galactose was negligible at 4°. Between 20° and 37° the Q_{10} value was 2·2. Net loss of internal galactose did not occur for at least 3 hr, but, when the starting external concentrations were the same, the maximum value for galactose was less than the final value for glucose.

Figure 3 shows the effect of external concentration on maximum accumulation values of glucose and galactose at 30°. The apparent Michaelis constants, calculated from the data in Fig. 3 by the method indicated in equation (2), are $3{\cdot}1 \times 10^{-5}$ M for glucose, and $3{\cdot}4 \times 10^{-4}$ M for galactose. The extent of accumulation against concentration differences may be determined by converting the accumulation values in Fig. 3 to internal molar concentrations (Table 1). Both sugars were unquestionably transported 'uphill' and the ratio of internal to external substrate concentration (S_i/S_e) increased with a decrease in S_e. Higher ratios were obtained for glucose than for galactose, but S_i/S_e did not reach the extremely high values found for sugar transport in *Escherichia coli* (Cohen & Monod, 1957; Horecker *et al.* 1960).

Since uphill accumulation depends in part on the amount of energy available, the presence of an energy-yielding substrate in the reaction system would be expected to increase S_i/S_e. Arginine fermentation by *Streptococcus faecalis*, which has been shown to produce ATP (Oginsky, 1955), was found not to be inhibited by iodoacetate. Pre-incubation of the cocci in 10^{-2}M-arginine for one hour before addition of glucose or galactose increased the accumulation of both sugars (Table 2) and eliminated the loss of internal glucose which normally follows uptake at temperatures above 20° (Table 3). However, under these conditions, S_i/S_e did not increase with a decrease in S_e (Table 2) even though accumulation times were extended from 10 to 60 min. for glucose, and from 30 to 120 min. for galactose.

Table 2. *Effect of arginine on accumulation of hexose by Streptococcus faecalis against a concentration difference*

Arginine (10^{-2}M) was added 60 min. before the addition of the sugar. The other reaction components were the same as those given in the legend to Fig. 2. Accumulation times: glucose, 30 min.; galactose, 120 min. Temperature, 30°. S_i = internal concentration: S_e = external concentration

S_e at start (Molar × 10^5)	S_i/S_e at the steady state	
	Glucose	Galactose
20	165	18
10	180	19
5	120	21
2	190	17

Table 3. *Effect of arginine on glucose accumulation by Streptococcus faecalis*

The reaction components were the same as listed in the legend to Fig. 2 plus 10 μmoles arginine added 60 min. before the addition of glucose-^{14}C. Accumulation time, 45 min.

Temperature	μmole glucose/g. equiv. dry wt. cells	
	Without arginine	With arginine
20°	10	25
35°	4	28

The exit process

In the experiment illustrated by Fig. 4 glucose was accumulated without pre-incubation with arginine, and the cocci were washed and resuspended above the filter membrane in tris + maleate buffer. The initial exit rates, shown by the tangents in Fig. 4, were proportional to the initial internal concentrations. The kinetics of glucose exit do not correspond to a simple process, but if slopes for a first-order reaction constant of 0·1 min.$^{-1}$ are drawn they are approximately tangent to the initial exit rates (Fig. 4). It seems probable from inspection of Fig. 4 that part of the internal glucose is retained by the cocci indefinitely. When appropriate values for the retained glucose are subtracted, curves 1 and 2 quite closely follow first-order exponentials from which an average rate constant of 0·2 min.$^{-1}$ can be calculated. However, a consistent relationship between the amount of internal sugar

that is retained by the cocci and that which diffuses out was not found. The backward projection technique (Van Liew, 1962) applied to semilog plots of curves 1 and 2 did not indicate that more than one exponential process was involved.

Since most permeation systems are readily reversible, it was expected that the rate of exit of glucose-^{14}C would increase when the cocci were resuspended in a solution containing non-radioactive glucose rather than in buffer alone. No significant change in exit rate was found, however, when the cocci were resuspended in concentrations of glucose-^{12}C as high as 0·1 M.

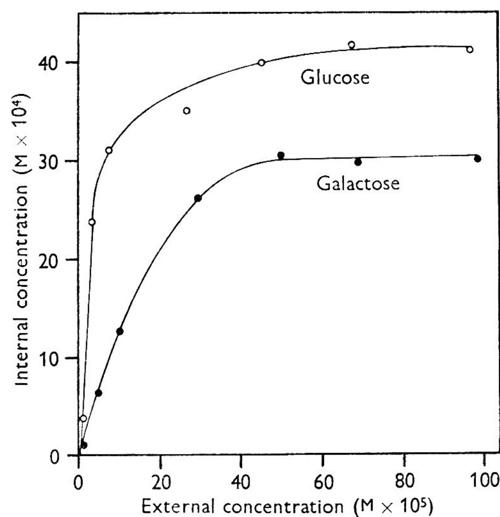


Fig. 3

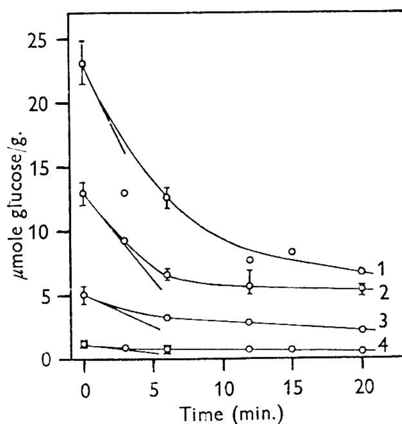


Fig. 4

Fig. 3. Effect of external concentration on the steady-state values of accumulated glucose and galactose. The reaction tubes contained cocci and iodoacetate as described in the legend to Fig. 2. Temperature, 30°. Ordinates: internal sugar estimated by the procedure given in Methods. Abscissa: steady-state external sugar concentrations; the amount of sugar accumulated was subtracted from the amount originally added to the medium. Accumulation times: glucose, 10 min.; galactose, 30 min.

Fig. 4. The effect of internal concentration on the time course of glucose exit. The cocci were incubated for 5 min., in the absence of arginine, in reaction systems similar to that described in the legend to Fig. 2 but containing the following amounts of glucose-^{14}C: curve 1, 0·5 μmole; curve 2, 0·1 μmole; curve 3, 0·05 μmole; curve 4, 0·01 μmole. The cocci were washed and resuspended above the filter membrane in tris + maleate buffer (pH 7·0, 0·03 M) for the time intervals indicated. Temperature, 30°.

Table 4. *Exit of glucose and galactose from Streptococcus faecalis pre-incubated with arginine*

The cocci were loaded with sugar after 60 min. pre-incubation in 10^{-2} M-arginine. First-order exit rate constants were calculated from the initial slopes of exit time course curves. Temperature 30°.

Internal sugar	Sugar (10^{-2} M) in resuspending medium	Exit rate constant
Glucose-^{14}C	None	0·04
	Glucose-^{12}C	0·03
Galactose-^{14}C	None	0·02
	Glucose-^{12}C	0·02

Table 4 shows that pre-incubation of the cocci in arginine before they were loaded with sugar decreased the glucose exit rate about fourfold. This effect was less marked for galactose since galactose exit from cocci that had not been incubated with arginine was slower than glucose exit. The failure of internal hexose to exchange with external sugar is also shown in Table 4.

The exit rate constant, *c*, of the permease model (Horecker *et al.* 1960) is calculated from accumulation time course data expressed as

$$2{\cdot}303 \log \frac{S_\infty - S_i}{S_\infty} = c(t), \tag{3}$$

where S_∞ is the equilibrium value of the internal concentration, S_i, and t is the accumulation time. Appropriate values of S_i could not be measured for glucose accumulation by *Streptococcus faecalis* but, for galactose, the data in Fig. 2 yield $c = 0{\cdot}04$ min.$^{-1}$, which is within the same order of magnitude as values obtained from a direct measurement of exit rate.

Stereospecificity

Table 5 shows that the uptake of glucose and galactose was inhibited by the presence of other hexoses. Lactose, sucrose and inulin (used as a control) did not appreciably decrease hexose accumulation. Abrams (1960) showed that oligosaccharides are able to penetrate protoplasts of *Streptococcus faecalis* but our results indicate that they do not compete for the hexose accumulation mechanism.

Table 5. *Inhibition of accumulation by stereoisomers*

The competing sugars were added at the time of the addition of the substrate. Accumulation time: glucose, 2 min.; galactose, 30 min. Temperature, 20°. Other conditions were the same as given in the legend to Fig. 2.

Substrate (10^{-4} M)	Competing sugar (10^{-3} M)	μmole substrate accumulated/g. equiv. dry wt. of cocci	Inhibition (%)
Glucose	None	14·1	0
	Galactose	13·0	7
	Fructose	5·0	64
	Mannose	6·3	55
Galactose	None	2·5	0
	Glucose	0·2	92
	Fructose	1·6	36
	Mannose	0·4	34

Sensitivity to metabolic inhibitors

Inhibition of accumulation by *p*-chloromercuribenzoate (PCMB) and uranylacetate is shown in Table 6. PCMB did not inhibit uptake unless it was added to the cocci some minutes before the addition of the sugar. Uranylacetate was immediately effective, which suggests that it acts in *Streptococcus faecalis*, as it does in yeast (Rothstein, 1954), by blocking uptake at the cell surface. Inhibition of sugar transport by PCMB has been observed with several cell types (Le Fevre, 1954; Randle, 1960; Kepes, 1960) and is considered to be evidence for the participation in

transport of an enzyme-like protein. The accumulation mechanism is also sensitive to iodoacetate, since an increase in concentration beyond the limit that ensured the blocking of glycolysis resulted in lower values for internal sugar.

Table 6. *Inhibition of accumulation by uranylacetate and p-chloromercuribenzoate (PCMB)*

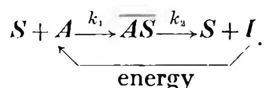
The reaction system was the same as given in the legend to Fig. 2. PCMB (10^{-4} M, final concentration) was added to the cell suspension 30 min. before the addition of the substrate; uranylacetate (10^{-3} M, final concentration) was added at the same time as the substrate. Accumulation time: glucose, 2 min.; galactose, 30 min. Temperature, 18°.

Substrate (10^{-4} M)	Inhibitor	μmole substrate accumulated/g. of equiv. dry wt. cocci	Inhibition (%)
Glucose	None	18·0	0
	Uranylacetate	0·8	96
	PCMB	3·1	83
Galactose	None	2·5	0
	Uranylacetate	0·1	96
	PCMB	0·1	96

DISCUSSION

The accumulation of glucose and galactose by *Streptococcus faecalis* (in the absence of glycolysis) has two principal characteristics: (1) an energy-dependent mechanism that permits uptake against a concentration difference and which shows saturation kinetics, stereospecificity and sensitivity to metabolic inhibitors; (2) a relatively slow exit that can be inhibited by incubating the cocci with arginine but which is independent of the external substrate concentration. These features agree in several respects with the permease theory for sugar transport (Cohen & Monod, 1957; Horecker *et al.* 1960) but the lack of exchange between internal and external substrate is an important difference. Since the permease theory depends to a considerable extent on experiments involving substrate displacement reactions (Cohen & Monod, 1957; Horecker *et al.* 1960; Kepes, 1960), its applicability to sugar accumulation in *S. faecalis* seems questionable.

An alternative hypothesis was suggested by Dr V. P. Cirillo (personal communication) in which accumulation results from intracellular adsorption to specific receptors. According to this view, free internal sugar is in equilibrium with the external sugar at all times, irrespective of the mechanism of transport; but accumulation against a concentration difference depends on two irreversible processes as shown in the following scheme:



The substrate, S, combines with an activated receptor, A, to form a thermolabile complex, $\overline{AS}$, which can dissociate to form free S and an inactivated receptor, I. The regeneration of A from I requires metabolic energy. In the absence of a sufficient number of activated sites, desorbed S will diffuse out of the cell. Since

the cell membrane is freely permeable to hexoses, the substrate retained by washed cocci is entirely in the bound form. Thus the change in the internal concentration can be expressed as

$$\frac{d\overline{AS}}{dt} = k_1(S)(A) - k_2(\overline{AS}). \tag{4}$$

The total number of receptors, A_t, may be defined as $A_t = A + \overline{AS} + I$. Therefore

$$\frac{d\overline{AS}}{dt} = k_1(S)(A_t - \overline{AS} - I) - k_2(\overline{AS}). \tag{5}$$

In the steady state, $d\overline{AS}/dt = 0$. Thus

$$S(A_t - I) - \overline{AS}\left(S + \frac{k_2}{k_1}\right) = 0. \tag{6}$$

If we define $k_2/k_1 = K$,

$$\overline{AS} = (A_t - I)\frac{S}{S+K}. \tag{7}$$

Taking reciprocals yields an expression that is formally equivalent to equation (2),

$$\frac{1}{\overline{AS}} = \frac{1}{(A_t - I)} + \frac{K}{(A_t - I)}\left(\frac{1}{S}\right). \tag{8}$$

When energy resources are sufficiently high, the concentration of I will be negligible and $(A_t - I)$ will be a constant equal to the maximum number of receptor sites. The constant, K, which represents the ratio of the rate of breakdown to the rate of formation of the complex, can then be evaluated from a Lineweaver-Burk plot of equation (8).

This scheme offers an explanation for most of the data on hexose accumulation by *Streptococcus faecalis*. It predicts that the energy supplied by arginine metabolism would have the observed effect of increasing the accumulation rate and decreasing the exit rate. It would also account for the prevention of the loss of internal glucose after an initial accumulation period at temperatures above 20°. In this respect the adsorption scheme is similar to the model proposed by Winters *et al.* (1962) for kidney mitochondria in which uptake and subsequent loss of sulphate is a function of thermolabile binding sites that can be partially stabilized by oxidative phosphorylation. The adsorption model, like the permease model, does not explain why the ratio of internal to external sugar remains constant over a tenfold range in external concentration after pre-incubation with arginine. It also does not account for the observation that part of the accumulated sugar is retained by the cocci indefinitely. One might postulate, however, that two types of adsorption complexes are formed, one of which does not dissociate under the conditions studied.

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The Preparation of ^{14}C–Labelled Gibberellic Acid

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SUMMARY

Gibberella fujikuroi was allowed to grow on unlabelled medium until gibberellic acid (GA) was being produced, and samples of mycelium were then transferred to a substrate containing ^{14}C-acetate. Pure ^{14}C-GA was isolated from the medium. The specific activity of the ^{14}C-GA was increased by transferring mycelium to a second labelled substrate 6 hr after the first transfer. The specific activity was not increased when unlabelled glucose was present in the transfer medium, or when anaerobic conditions were maintained after transfer. Most of the ^{14}C-GA was produced during the 48 hr following transfer. A large proportion of the ^{14}C was lost as $^{14}CO_2$, and most of this was produced during the 40 hr after transfer. After this period little GA was produced, but the evolution of unlabelled CO_2 continued.

INTRODUCTION

The work described below was done in order to devise a satisfactory technique for preparing gibberellic acid (GA) labelled with ^{14}C, for use in physiological experiments with higher plants. Several publications describe the isolation of ^{14}C-GA from cultures of *Gibberella fujikuroi* containing labelled substances (Watanabe, 1957; Watanabe & Scully, 1957*a*; Zweig, DeVay & Cosens, 1958; Zweig & DeVay, 1959; Zweig, Yamaguchi & Mason, 1961; Birch, Richards & Smith, 1958; Birch *et al.* 1959; Redemann & Meuli, 1959). Much of this published work was concerned with possible paths of synthesis of GA within the fungus, but Watanabe & Scully (1957*b*, *c*) and Zweig *et al.* (1961) used some of the ^{14}C-GA produced by the fungus in experiments with higher plants. In these cases the fungus was grown from the time of inoculation on a medium containing labelled substrate, a method which is without doubt the most satisfactory when labelled GA of very high specific activity is required. However, much of the labelled carbon is inevitably lost with such a technique, since the amount of GA produced is high only after the fungus has been growing for a considerable time (Borrow *et al.* 1955). Further, the concentration of carbon should be high initially, as the best conditions for GA production are those in which nitrogen and not carbon becomes the limiting factor for growth (Brian, Radley, Curtis & Elson, 1955). A more practicable means of producing ^{14}C-GA is to allow the fungus to grow on an unlabelled substrate until GA is being produced, and then to transfer mycelium to a labelled substrate. A transfer technique of this kind was apparently first tried by Zweig *et al.* (1958), and a similar technique was devised for the following work.

^{14}C-acetate was chosen as a carbon source. Zweig & DeVay (1959) used several

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carbon sources, and found that acetate was relatively efficient for ^{14}C-GA production. Birch *et al.* (1958) found that alternate carbon atoms of the gibbane ring system of GA (Brian, Grove & MacMillan, 1960) became labelled when ^{14}C-1-acetate was used as a precursor.

METHODS

Organism. Gibberella fujikuroi (Saw.) Wr.; conidial state *Fusarium moniliforme* (Sheld.) emend. Snyder & Hansen was used. A culture of the organism, Strain 917, was kindly supplied by Professor P. W. Brian.

Chemicals. Sodium ^{14}C-1-acetate, specific activity 7·23 mc./mM. (88·1 µc./mg.), was obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England. Unlabelled GA was obtained from L. Light & Co. Ltd. (Slough).

Medium and growth of Gibberella fujikuroi

Medium. The following medium was used for the growth of the fungus before transfer of mycelium to samples of labelled substrate (%, w/v): glucose 10; KH_2PO_4 0·5; $MgSO_4.7H_2O$ 0·2; NH_4NO_3 0·24. The substances were dissolved in distilled water, and to every litre of culture solution 2 ml. of the following minor element concentrate were added (g.): $FeSO_4.7H_2O$ 0·59; $CuSO_4.5H_2O$ 0·075; $ZnSO_4.7H_2O$ 0·5; $MnSO_4.7H_2O$ 0·059; $(NH_4)_2MoO_4$ 0·079, dissolved in 500 ml. distilled water, with a few drops of HCl added to clear the solution. (The medium is essentially the same as one recommended by Professor P. W. Brian.) A standard volume (100 ml.) of culture solution was used for the preparation of samples of mycelium. Except where otherwise stated, the labelled medium was identical in composition with that described above, except that acetate replaced glucose as the only carbon source. Medium and apparatus were autoclaved at 120° for 5 min.

Culture apparatus. The cultures were aerated. The air was first passed through concentrated H_2SO_4, and then through sterile water to remove acid fumes and to humidify the air stream. In experiments where CO_2 was collected, the air was passed through a soda-lime tower before entering the H_2SO_4, and after escaping from the culture vessel was allowed to bubble through 0·2 N-NaOH held in Pettenkofer tubes of 20 ml. capacity. Different types of culture vessels have been used, but the most satisfactory was a round-base tube, 22 cm. long and 3 cm. diameter, held vertically, and provided with a ground joint and glass cap. The air passed through a narrow tube sealed into the cap, and bubbled into the culture from a simple orifice near the base of the vessel. Another tube sealed into the cap provided the outlet for the air stream. In a preliminary experiment air was passed into the culture from a sintered-glass bubbler, but the fungus grew on the sinter and eventually cut off the air supply. The culture vessels and the flask of sterile water were kept in a water bath at 25°.

Inoculation and growth. Subcultures were maintained on nutrient agar slopes. At the time of inoculation of the main culture about 1 ml. sterile water was added to one of the subcultures, and hyphae dislodged from the surface into the water with a sterile loop. The suspension of hyphae was poured into the main culture vessel. In one experiment samples (1 or 2 ml.) of culture liquor were withdrawn from time to time after inoculation, and any GA present was extracted and assayed as described below. The fungus was found to be producing GA at a rate of about 8 mg./l.

culture/day, from the 9th to the 14th days (Fig. 1). On subsequent occasions cultivation was discontinued some 14 days from the time of inoculation, so that samples of mycelium, known to be producing GA actively, could be prepared for transfer to samples of labelled medium. In each case a sample of the culture was withdrawn and the presence of GA confirmed before the transfer was made.

Preparation of samples of mycelium for transfer to labelled media. The unlabelled culture was filtered on a Büchner funnel through a 7 cm. diam. Whatman No. 50 filter paper which had previously been subdivided with pencil lines into a convenient number of segments. After filtration the paper and mycelial mat were turned over and cut along the pencil lines with sterile scissors. Each sample of mycelium obtained in this way was used to inoculate a quantity of labelled medium comparable in volume to that from which it was derived. For example, half of the mat was used to inoculate 50 ml. fresh medium, a quarter, 25 ml., and so on. Segments not used immediately were stored at 6° in sterile tubes for use later. A segment stored in this way for 29 weeks liberated about 140 μg. GA in 72 hr after being transferred to 10 ml. of medium, but in the experiments reported here much shorter times of storage were used. These operations were carried out in a sterile room.

Measurement of respiration and estimation of specific activity of $^{14}CO_2$

A study was made of the rate of liberation of $^{14}CO_2$ from cultures to obtain some measure of the rate of utilization of the labelled substrate. CO_2 trapped in the NaOH was precipitated as $BaCO_3$ by shaking with excess 0·05 M-$BaCl_2$ solution. The precipitate was removed by centrifugation, shaken with distilled water, and again centrifuged. The mass of $BaCO_3$ precipitate (and from this the amount of CO_2 collected) was determined by titrating the excess alkali contained in measured samples of the bulked supernatant liquid against HCl of known strength (about 0·01 N), with phenolphthalein as indicator. A sample of the $BaCO_3$ precipitate was suspended in water and transferred to a 20 mm. diameter disc of Whatman No. 42 filter paper by using the apparatus described by Calvin *et al.* (1949). Ethanol and then chloroform were passed through the sample and the dry disc of $BaCO_3$, supported by filter paper, was stored in a CO_2 free atmosphere ready for counting with a thin-window Geiger-Müller tube; counts were taken at infinite thickness. The product of the mass of $BaCO_3$ in the whole precipitate and the counts/min. of the samples was used to obtain an estimate of the radioactivity of the whole precipitate, and hence of the evolved CO_2. This product is referred to as the 'relative counts'. It is an important property of this estimate that it is independent of any contamination of the air stream by extraneous unlabelled CO_2.

The extraction and assay of gibberellic acid

The methods used for extracting GA were largely based upon those described by Curtis & Cross (1954), Borrow *et al.* (1955) and Brian *et al.* (1955). Small volumes of culture (less than 10 ml.) were filtered, adjusted to pH 2·8–3·0 with dilute HCl, and extracted three times by shaking with equal volumes of ethyl acetate. The lower phase was discarded, and the bulked upper ethyl acetate-rich phase evaporated *in vacuo* at not more than 30° to leave a solid residue. The crude extract was then either taken up in 30% (v/v) ethanol in water and assayed for GA in the manner described below, or transferred in ethyl acetate or acetone to chromatography

paper. Larger volumes of culture (10 to 100 ml.) were filtered, adjusted to pH 2·8–3·0 with dilute HCl, and shaken with activated carbon in the ratio of 2 g. carbon to 100 ml. culture. The carbon was removed on a Büchner funnel and shaken with acetone. The acetone + carbon mixture was filtered, and the dry carbon shaken with a small volume of distilled water and again shaken with acetone; this was repeated a third time. The bulked extract was evaporated *in vacuo* to an aqueous residue, which was adjusted to pH 2·8–3·0 and extracted three times with an equal volume of ethyl acetate. The whole extraction procedure was repeated three times, and the bulked ethyl acetate extract was evaporated to a small volume *in vacuo* and transferred to chromatography paper.

Chromatography. This was done by the descending technique, the solvent being the upper phase of a mixture of 3 vol. *n*-butanol + 1 vol. aqueous 1·5 N-NH_4OH, unless stated otherwise. Spots of GA were detected either by their blue-green fluorescence under ultraviolet (u.v.) radiation after treatment with a mixture of ethanol + sulphuric acid (Curtis & Cross, 1954; Radley, 1956), or, along with other organic substances, by dipping in 0·4 % $KMnO_4$ and washing in tap water (Bird & Pugh, 1958). On other occasions spots or bands of GA were eluted in 'elution tubes' (Canny, 1960). In earlier work acetone was used as a solvent for elution, but it was later found that a mixture of acetone and water was more effective. The acetone was evaporated away, and the aqueous residue extracted with ethyl acetate. Radioactive spots were detected by standard autoradiographic technique, or by scanning with an automatic scanning device.

Assay by the use of dwarf pea seedlings. This test was used as a routine bioassay for gibberellin. The method was essentially that described by McComb & Carr (1958) except that seedlings of the dwarf pea variety 'Meteor' were raised in a greenhouse from seed sown in John Innes' No. 2 compost (without fertilizer) instead of perlite.

Assay using Callitriche rosettes. Callitriche rosettes elongate strongly after treatment with GA (McComb, 1959), and unknown amounts of GA in solution may be assayed through the response of this plant. Rosettes of Callitriche were cut from their parent shoots and placed with their upper surfaces downwards into 0·2 ml. of aqueous solution to be assayed, ten replicates/treatment. After at least 12 hr the rosettes were transferred to tap water and allowed to grow for 3 days under continuous illumination. The shoots were measured, and an estimate of the amount of GA present in the experimental solution made by reference to the response of shoots treated with known amounts of GA. The test does have some advantage over the pea assay in that no initial measurement need be taken, and the time of assay is relatively short. However, the response of the rosettes is quite variable, the test is not useful over a wide range of concentrations, and plant material can be difficult to maintain. For these reasons the pea assay is to be preferred as a routine method.

RESULTS

The effects of glucose and anaerobic conditions on the production of labelled gibberellic acid

The possibility was investigated that the amount of ^{14}C incorporated into GA would be increased if unlabelled glucose were provided at the same time as labelled acetate (so as to act as an alternative carbon source), or if the fungus were grown

under anaerobic conditions from the time of transfer to labelled medium. Three test tubes, A, B, and C, were used as culture vessels. A 10 ml. sample of medium containing 10 μc. labelled acetate/ml. was added to each tube. Glucose (0·1 g.) was added to tube B. Each tube was inoculated with a similar sample of mycelium taken from a culture of the fungus known to be actively producing GA. Tubes A and B were aerated, but pure nitrogen instead of air was passed into tube C. After 43 hr a 1 ml. sample was withdrawn from each vessel; the experiment was terminated at 88 hr after inoculation, when further 1 ml. samples were withdrawn. All the samples were extracted and chromatographed on Whatman No. 1 paper. Autoradiographs of the chromatograms revealed apparently identical results for the two sets of samples withdrawn at 43 and 88 hr. In all cases spots could be seen at the same R_F as known GA, but it was clear that the samples from tube A had much more labelled carbon at this R_F than those from the other tubes (Pl. 1, fig. 1). The chromatograms were then dipped in ethanolic sulphuric acid and examined under u.v. radiation, when the greenish fluorescence characteristic of GA could be seen in all cases, at the same R_F as known GA.

The GA present in tube A was extracted with activated carbon and purified by chromatography. Assay with Callitriche revealed that the culture contained about 30 μg. of GA. Samples of the ^{14}C-GA were chromatographed in six different solvent systems, including that used above, selected from the work of Radley (1956), Takahashi *et al.* (1956), Van Overbeek, Racusen, Togami & Hughes (1957) and Phinney, West, Ritzel & Neely (1957). In each case only one spot of labelled compound was detected by autoradiography, coincident in R_F with a spot of known GA.

The GA in tubes B and C was extracted with ethyl acetate, and assayed by using dwarf pea seedlings. B was estimated to contain 34 and C 26 μg of GA. Clearly the most satisfactory method of the three for producing ^{14}C-GA was that in which labelled acetate was provided as the only carbon source in aerated culture. CO_2 was collected during the experiment, and it was found that most of the $^{14}CO_2$ produced by the fungus was evolved during the first 24 hr of cultivation. It seemed possible that most of the ^{14}C-GA might also be produced during that time. It was important that the ^{14}C-GA obtained should have a specific activity high enough to be of use in physiological experiments. A simple test was made to see whether ^{14}C could be detected in Callitriche plants after they had been treated with the ^{14}C-GA isolated from the culture of tube A. A number of rosettes were each treated with about 2 μg. labelled GA in the same way as in the assay test, and were then washed and allowed to grow in tap water. The plants were dissected, pressed as herbarium specimens at different times after treatment and autoradiographs taken of the dried specimens. Radioactivity was detected (Pl. 1, fig. 2) and it was concluded that ^{14}C-GA of this specific activity could be used in physiological work.

The rate of formation of ¹⁴C-gibberellic acid

An experiment was made to determine the rate of production of ^{14}C-GA, and to see whether this was related to the production of $^{14}CO_2$. One sample of 10 ml. of culture was used, containing ^{14}C-acetate, 5 μc./ml. and this was inoculated as described. The apparent respiration rate remained fairly constant, some variation being introduced because the culture vessel was opened from time to time to with-

draw samples (Table 1). On the other hand, the rate of evolution of $^{14}CO_2$ was far from constant (Fig. 2).

Samples of medium were withdrawn from time to time, at first of 1 ml. but later 0·5 ml. These were extracted with ethyl acetate and chromatographed. Autoradiographs revealed spots at the same R_F as known GA, and these were counted with a Geiger tube. Counts were adjusted to the same sample size and plotted against the time of sampling (Fig. 3*a*). When the experimental data were plotted against the

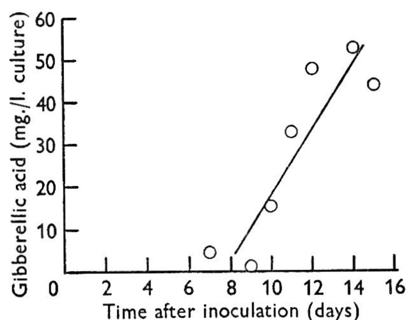

Fig. 1

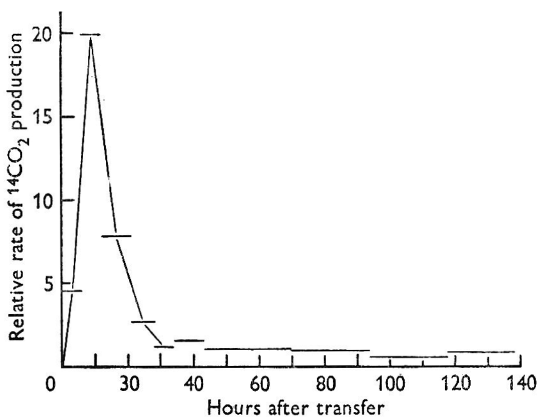

Fig. 2

Fig. 1. Estimates of the amount of gibberellic acid present in a culture at different times after inoculation.

Fig. 2. The evolution of $^{14}CO_2$ from a culture of *Gibberella fujikuroi* containing ^{14}C-acetate. Samples were collected during the times indicated by the horizontal lines. The relative count of each sample was divided by the sample time $\times 10^4$, to give an estimate of the relative rate of $^{14}CO_2$ production.

Table 1. *Estimates of the rate of production of CO_2 at different times, following the transfer of mycelium from a culture of Gibberella fujikuroi containing glucose to one containing acetate as the only carbon source*

Time from inoculation (hr.)	mg. CO_2 evolved/hr/ml. culture	Time from inoculation (hr)	mg. CO_2 evolved/hr/ml. culture
0– 6	0·12	34– 43	0·16
6–12	0·10	43– 70	0·08
12–21	0·10	70– 94	0·11
21–28	0·13	94–118	0·08
28–34	0·12	118–138	0·14

logarithm of the time of sampling, they were found to approximate to a straight line with a correlation coefficient of 0·97. The curve shown in Fig. 3*a* was calculated from the regression line, as was a curve showing the rate of labelled GA formation (Fig. 3*b*). It can be seen that most of the GA was produced during the first 48 hr, and the amount produced after about 90 hr was negligible. The spots of labelled GA were eluted, and the eluates assayed with dwarf-pea seedlings. From the assay data and the counts/min. recorded from the chromatograms, estimates were made of the relative specific activities of the various samples (Table 2). The results,

though variable, suggest that an increase in the specific activity of the GA took place with time. It seemed possible that higher specific activity might be achieved if the fungus were placed in a labelled medium for some hours, and then transferred to a second batch of labelled medium.

The effect of transferring mycelium to a second batch of labelled substrate

Mycelium derived from 50 ml. of unlabelled culture was used to inoculate 50 ml. of culture containing 0·5 mc. labelled acetate (medium A). Six hr later the mycelium was transferred to another 50 ml. of culture containing 0·5 mc. labelled acetate

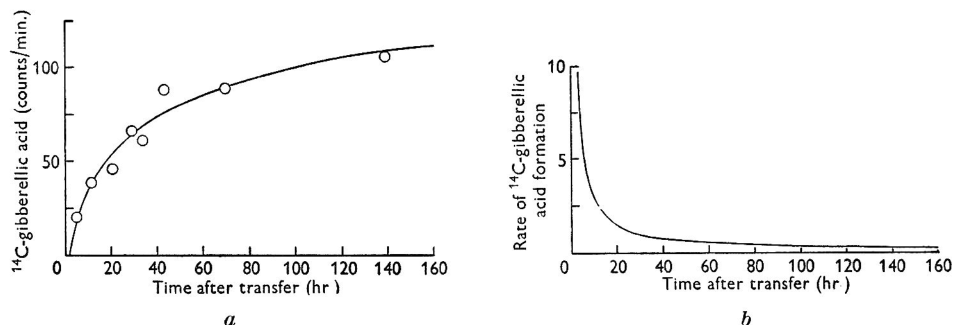

Fig. 3(*a*). Relative amounts of ^{14}C-gibberellic acid present in a culture of *Gibberella fujikuroi* containing ^{14}C-acetate. The estimates are based on counts/min. recorded from chromatograms at the R_F of known gibberellic acid.

Fig. 3(*b*). An estimate of the rate of production of ^{14}C-gibberellic acid, based on the data shown in Fig. 3(*a*). The rate has been calculated as the increase in number of counts/min. with each hour of incubation.

Table 2. *Estimates of the amount and relative specific activity ^{14}C-gibberellic acid present in a culture of Gibberella fujikuroi at different times after inoculation*

Time after inoculation (hr)	Amount of gibberellic acid (μg./ml. culture)	Relative specific activity*
6	62	0·3
12	39	1·0
21	49	1·0
28	50	1·3
34	108	0·6
43·2	60	1·5
69·7	70	1·3
138	25	4·3

$$* \frac{\text{Counts/min. at } R_F \text{ of gibberellic acid}}{\text{Mass of gibberellic acid on chromatogram}}.$$

(medium B). A 2 ml. sample was withdrawn from medium A, which was then inoculated with a further sample of hyphae, derived from 50 ml. of culture. The relative counts of $^{14}CO_2$ evolved from the three cultures are shown in Fig. 4. As comparatively little was evolved from medium A after the second inoculation, it is assumed that most of the ^{14}C-acetate was taken up by the fungus during the first inoculation of that medium.

The incubation was discontinued 120 hr after the first inoculation of medium A, and 2 ml. samples were withdrawn from the culture media. Thus, three samples were available for study: A, withdrawn from medium A when mycelium was transferred to medium B; B, withdrawn from medium B at the end of the experiment; and A^1, withdrawn from medium A at the end of the experiment. Ethyl acetate

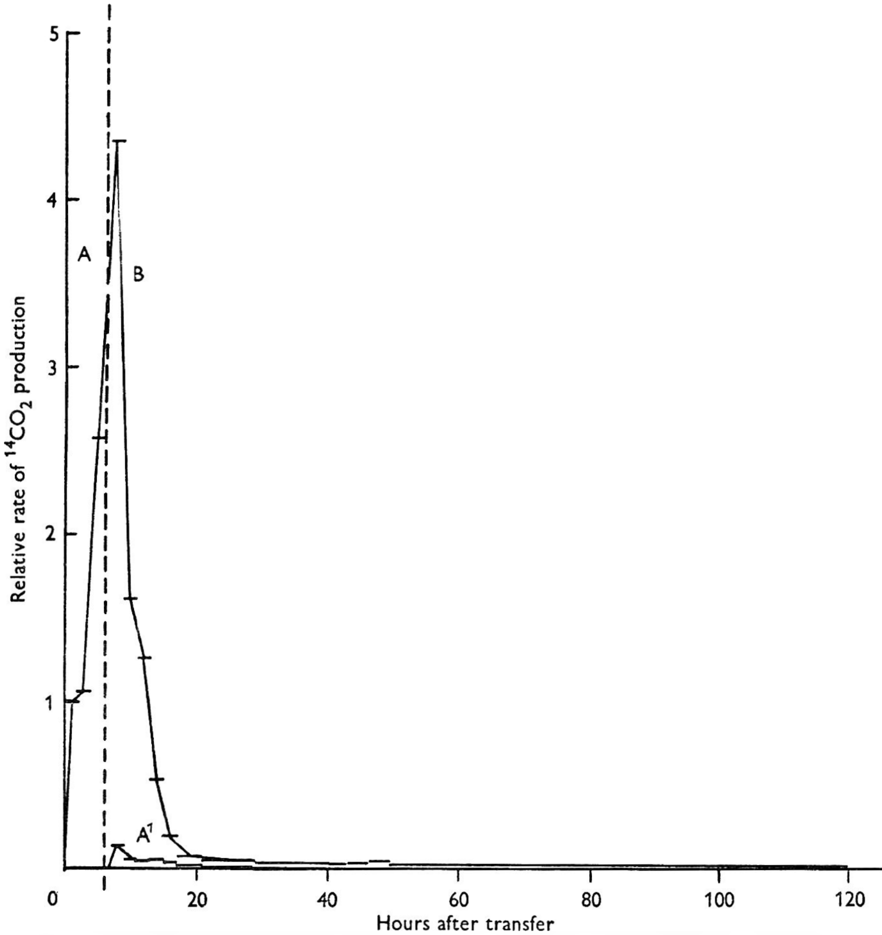

Fig. 4. The evolution of $^{14}CO_2$ from cultures of *Gibberella fujikuroi* containing ^{14}C-acetate. Samples were collected during the times indicated by the horizontal lines. The relative counts of each sample was divided by the sample time $\times 10^7$. At the time indicated by the broken line, the mycelium in one medium, (A), was transferred to a second medium, (B). Medium A was then inoculated with a further sample of mycelium, and the $^{14}CO_2$ evolved from the culture is represented by the line, (A^1), which begins at the time of this inoculation.

extracts of the samples were chromatographed, and the chromatograms scanned. Radioactivity was estimated by measuring the areas under the traces at the R_F of known GA. The GA spots were eluted and assayed in the same batch of assay plants; the results are given in Table 3. It is clear that sample B contained the most GA, and also GA of the highest specific activity. The lower specific activity of the GA

in sample A_1 is attributed to the introduction of unlabelled GA (or GA precursors) at the time of the first inoculation. A further decrease in specific activity accompanied the introduction of more mycelium into medium A (sample A_2). In addition, there was less radioactivity in A_2 than in A_1, suggesting that some of the ^{14}C-GA present in A had disappeared. It is possible that during the period without an external carbon source (e.g. glucose, acetate) some of the GA present in the medium was broken down by the fungus. Samples of the assayed sample B were counted on 5 mm. squares of filter paper, and the count rate compared with that of a known amount of ^{14}C-acetate under the same counting conditions. The specific activity of the labelled GA was determined as about 4 μc./mg. The ^{14}C-GA present in medium B was extracted with activated carbon, and has been used in experiments with higher plants.

Table 3. *Estimates of the amount and relative specific activity of ^{14}C-GA present in chromatograms of extracts from cultures of Gibberella fujikuroi*

Sample (2 ml.)	Mass gibberellic acid in sample (μg.)	Radioactivity. Area under trace drawn by scanning machine (mm.^{2})	Relative specific activity. (Area divided by mass)
A_1	4·9	328	67
B	14·1	1149	82
A_2	7·3	192	26

DISCUSSION

The ^{14}C-gibberellic acid obtained by the method described has a specific activity high enough to allow studies to be made on the distribution and breakdown of GA in higher plants, but further increase in specific activity would be advantageous. This might be achieved by simply supplying more labelled acetate, and also by supplying acetate of higher specific activity, labelled in both the methyl and carboxyl groups. The rates of production of $^{14}CO_2$ and ^{14}C-GA had fallen to a relatively low value within 40 hr after the transfer of mycelium to the labelled substrate; this can be attributed to exhaustion of the supply of labelled carbon in the medium. After this time respiration continued, the unlabelled CO_2 being derived from carbon reserves which had accumulated in the mycelium before transfer (Borrow *et al.* 1961). Since the specific activity of the GA did not fall with time (Table 2), it is assumed that GA was not produced from these unlabelled reserves. This is important as far as the production of ^{14}C-GA is concerned, for any further diminution in specific activity would be undesirable. It seems that carbon derived from reserves within the mycelium passed into the pathways of 'endogenous respiration' (Cochrane, 1958) rather than into those of GA synthesis.

I wish to record my debt to Dr M. J. Canny for advice and encouragement during the course of these experiments. Thanks are also due to Professor P. W. Brian, F.R.S. for the gift of a culture of *Gibberella fujikuroi*, and for information about the growth of the organism. This work was carried out during the tenure of an Overseas Scholarship awarded by the Royal Commission for the Exhibition of 1851, and forms part of the requirements for the Degree of Doctor of Philosophy at the University of Cambridge.

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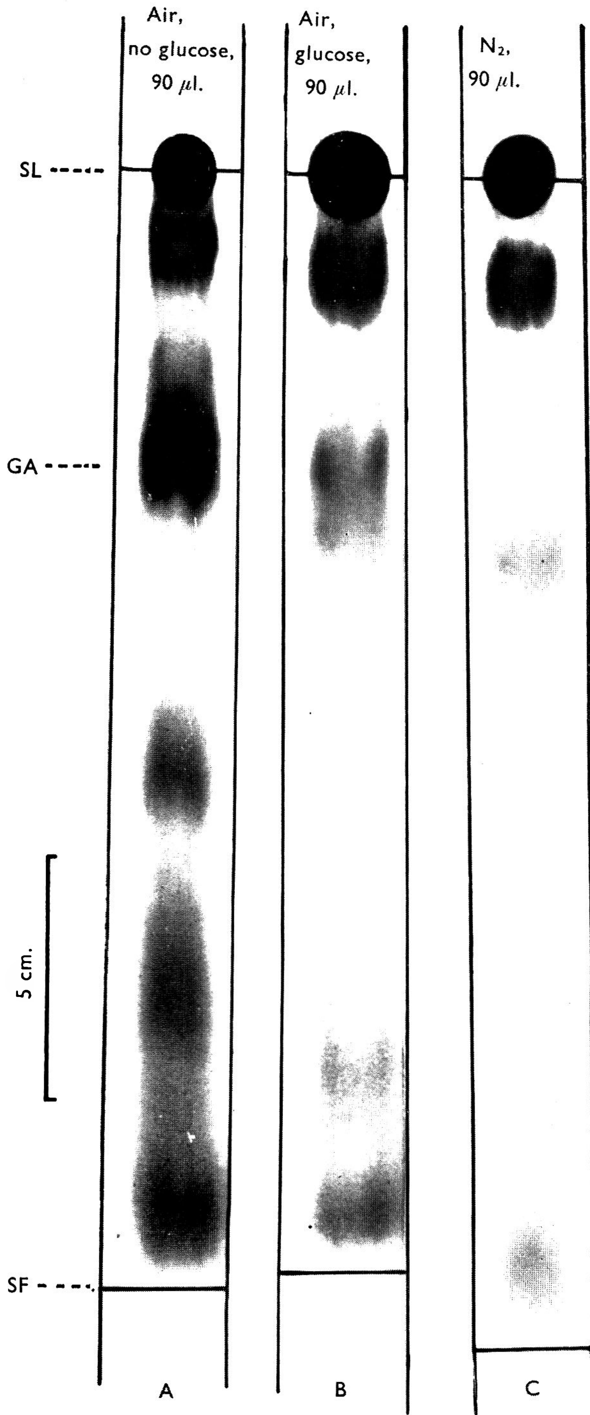

Fig. 1

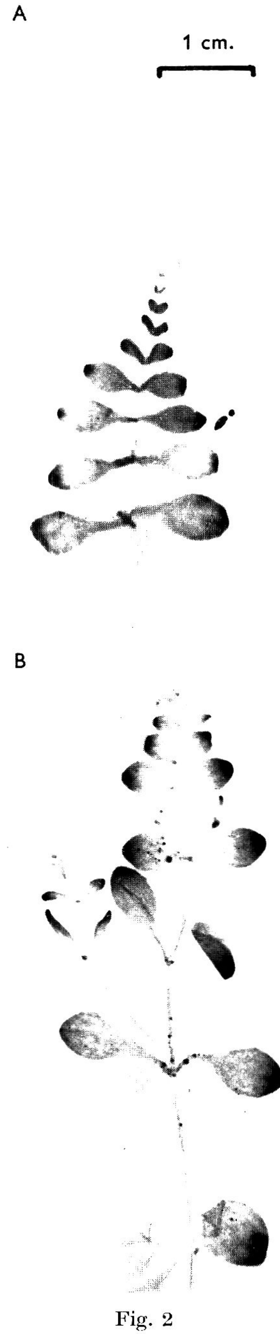

Fig. 2

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EXPLANATION OF PLATE

Fig. 1. Autoradiographs from chromatograms of extracts taken from cultures of *Gibberella fujikuroi* containing ^{14}C-acetate 43 hr after inoculation. A: Aerated culture containing ^{14}C-acetate as sole carbon source. B: Aerated culture containing unlabelled glucose and ^{14}C-acetate. C: Culture containing ^{14}C-acetate as sole carbon source. Nitrogen instead of air was bubbled through the medium. SL: Start line. GA: R_F of known gibberellic acid. SF: Solvent front. Exposure time 7 days.

Fig. 2. Autoradiographs from *Callitriche* plants treated with ^{14}C-gibberellic acid. Rosettes were each treated for 12 hr with about 2 μg. of ^{14}C-gibberellic acid, and transferred to tap water. The autoradiographs shown above are from plants harvested (A) 24 hr, (B) 72 hr after transfer. Radioactivity at the leaf tips is in the region of the hydathodes. Exposure time 35 days.

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Effect of Infection with Phage Lambda on the Synthesis of Protein, RNA and DNA in *Escherichia coli*

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SUMMARY

Delay in deoxyribonucleic acid (DNA) synthesis after use of ultraviolet radiation to induce *Escherichia coli* lysogenic for phage λ was due to the irradiation procedure; the same delay was found in non-lysogenic bacteria exposed to the same dose of radiation. After infection with phage λ_c or λ_v in 0·02 M-$MgSO_4$, DNA synthesis began without delay when complete medium was added. During vegetative development of phage in induced bacteria, 81–89 % of the DNA synthesized was accounted for in the phage progeny, indicating that host DNA synthesis is much diminished and may be completely inhibited. Mature phage particles arose soon after the appearance of serum blocking power (SBP) and endolysin activity. Induced bacteria synthesized up to 15 times more SBP than was incorporated into complete phage particles: the excess SBP was not sedimented at 40,000 **g** (unlike phage particles), but about half was sedimented at 90,000 **g**, the rest remaining in the supernatant fluid. Whilst protein and RNA synthesis in infected or induced bacteria was initially similar to that in the control, there was a marked decrease of synthesis in the second half of the latent period.

INTRODUCTION

The extent to which host-cell metabolism is affected by the vegetative development of bacteriophage has been studied almost exclusively in *Escherichia coli* infected with the T series of coliphages. Thus in bacteria infected with phage T_2 or T_4, there are profound effects on the net synthesis of ribo- and deoxyribo-nucleic acids (Cohen, 1948), and early in the latent period several new enzymes must be produced before the synthesis of specific phage components can take place (see Flaks, Lichtenstein & Cohen, 1959; Bessman, 1960). Analogous studies of the events during the latent period in cells infected with temperate bacteriophage (i.e. bacteriophage which can exist in the lysogenic state) have until recently received comparatively little attention and most of the data concerned *Bacillus megaterium* (Siminovitch & Rapkine, 1952). When *B. megaterium* lysogenic for phage 1 was induced by ultraviolet (u.v.) radiation, there was little change in the rate of synthesis of ribonucleic acid (RNA) and protein. On the other hand, no net synthesis of deoxyribonucleic acid (DNA) was detected during the first part of the latent period. Since after infection of *E. coli* with phage T_2 there is also a lag period before DNA synthesis is resumed (Cohen, 1948), it was of interest to know whether the lag observed in the induced organisms was a direct effect of the onset of phage development, or only a secondary and indirect effect brought about by the irradiation

procedure. The exposure of the non-lysogenic parent *B. megaterium* to the same dose of u.v. radiation was in fact found to produce no lag in the synthesis of DNA (Siminovitch & Rapkine, 1952). However, with *E. coli* it has long been known that exposure to u.v. radiation causes inhibition of DNA synthesis, and the period of this inhibition is directly related to the dose of radiation (Kelner, 1953). In the present work, a study has been made of the effect of vegetative phage development on host-cell metabolism first in *E. coli* lysogenic for wild-type phage λ after induction by u.v. radiation, and secondly in *E. coli* after infection with phage mutants λ_c and λ_v. The latter are mutants of phage λ which have lost the ability to lysogenize *E. coli*; phage λ_c can infect and develop in bacteria which are not lysogenic for phage λ, whilst phage λ_v (virulent inducer mutant) will infect and develop in *E. coli*, whether or not the bacteria are lysogenic for phage λ (Jacob & Wollman, 1954). Recently Séchaud (1960) showed that when starved *E. coli* organisms were infected with phage λ_c in the presence of $MgSO_4$ and then transferred to complete medium, there was a delay of about 8 min. before net DNA synthesis was resumed. Most, if not all, of this period appeared to be required for events concerned with the injection of the phage DNA into the host cell.

METHODS

Organisms. Escherichia coli K12 strains K112 and C600, and the phages λ_{22} (wild-type) and the two mutants λ_{gc} and λ_{gv} (large plaque-forming mutants) have been described by Jacob & Wollman (1954, 1956*b*) and Fry (1959).

Growth media and assay methods. In general the media and plating procedures were based on those of Adams (1950). Peptone broth (PB) contained (g./l.) 10, Oxoid peptone; 1, Lab. Lemco; at pH 7. Tryptone broth (TB) contained (g./l.) 20, Oxoid tryptone; at pH 7. Phage stocks were stored and diluted in medium PB. For plating, medium PB was supplemented with 1 % NaCl and agar (Parke-Davis) as required (1% for the bottom layer of plates for phage and viable counts; 0·7 % for the small tubes in which samples were mixed before plating). *Escherichia coli* strain C600 was used as indicator organism in the phage assays.

Preparation of phage stocks. Stocks of phage λ_{22}, prepared by the induction of *Escherichia coli* (λ_{22}) and concentrated by centrifugation (see Fry, 1959), were stable for several months at 4° and contained about 1×10^{12} active particles/ml. Small stocks of phage λ_{gv} and λ_{gc} were prepared from plates by the confluent lysis technique and these were used to infect cultures (1 l.) of *E. coli* C600 in the log phase of growth (5×10^8 bacteria/ml.) at a multiplicity of 5 λ phage/cell. Cultures were incubated until lysis was complete, and then any whole bacteria and cell debris removed. The phage particles were collected by centrifugation (as for phage λ_{22}) and the pellet suspended in a small volume of medium PB.

Ultraviolet irradiation. An Hanovia Chromatolite low-pressure Hg lamp (Hanovia Ltd., Slough, England) without the filter was placed 75 cm. from the sample (100 ml.), which was rocked gently to and fro in an enamel dish (32 × 28 cm.). Irradiations were done in a dark room and in these conditions a dose of 60 sec. was just sufficient for the optimal induction (i.e. 90 % or more) of *Escherichia coli* K112 (λ_{22}): this dose allowed 30 % survival of non-lysogenic coli K112 (measured as ability to form colonies) and 45 % survival of phage λ_{22} (measured as ability to form plaques).

Preparation of samples and the determination of protein, RNA and DNA. Samples of cultures were taken at the required times and 1 ml. used for the determination of growth (in terms of turbidity), whilst 6 ml. were rapidly mixed with 0·6 ml. 50 % trichloroacetic acid (TCA). After standing overnight at 4° the precipitates were collected by centrifugation and washed once with 5 ml. 5 % TCA. Each precipitate was then suspended in 2 ml. 5 % TCA and heated at 90° for 30 min. in a water bath; double aluminium foil caps on the tubes prevented evaporation. After centrifugation the supernatant fluids were carefully removed and kept for the assay of RNA and DNA. Residues were dissolved in N-NaOH and 0·5 ml. samples were used for the assay of protein by the method of Lowry, Rosebrough, Farr & Randall (1951). Each sample received 5 ml. of the reagent prepared from 50 ml. 2 % Na_2CO_3 and 1 ml. 0·5 % $CuSO_4$ in 1 % NaK tartrate, followed after 10 min. by 0·5 ml. Folin–Ciocalteu reagent in N-HCl (British Drug Houses Ltd). After standing for 1 hr at 37°, the absorption of the blue colour was determined at 500 mμ (blue photo cell). A standard protein solution was prepared from bovine plasma albumin fraction V (Armour Pharmaceutical Co. Ltd., Eastbourne) in N-NaOH. For the determination of RNA, a suitable sample of the hot TCA extract in a total volume of 1·5 ml. 5 % TCA was mixed with 1·5 ml. of 0·5 % $FeCl_3$ in concentrated HCl and 0·15 ml. 10 % orcinol in absolute ethanol, and then heated at 100° for 30 min. (Schneider, 1945). After cooling, the absorption of the green colour was read at 660 mμ (red photocell). D-Ribose (L. Light, Colnbrook, Bucks.) served as the standard, and results are expressed in terms of μg. apparent ribose. DNA was determined by the method of Burton (1956). Samples (1 ml.) of the hot TCA extract were each mixed with 0·2 ml. of 3 N-$HClO_4$ and 1·2 ml. of Burton's acetaldehyde-Dische reagent. After overnight incubation at 37°, the blue colour was read at 600 mμ (blue photocell). 2-Deoxy-D-ribose (L. Light, Colnbrook, Bucks.) served as the standard and results are expressed in terms of μg. apparent deoxyribose. (The word 'apparent' is used because only the ribose and deoxyribose bound to purine nucleotides reacts in these procedures).

Determination of DNA content of phage λ_{22}. The phage particles were collected as a pellet by centrifugation for 1 hr at 40,000 *g*, the supernatant fluid removed and the pellet extracted overnight with 2 % tryptone solution. Insoluble material was removed (8000 *g* for 15 min.) and then the phage suspension centrifuged at 105,000 *g* for 1 hr. After removing the supernatant fluid as completely as possible, the pellets were extracted overnight with 0·05 M phosphate buffer (pH 7) containing 0·01 M-$MgSO_4$ and insoluble material removed (8000 *g* for 15 min.). The supernatant fluid was assayed for phage particles and 1 and 2 ml. samples used for the determination of DNA. The purified phage preparation contained $3{\cdot}8 \times 10^{11}$ particles/ml. (from mean of four plates; standard deviation $\pm 0{\cdot}2 \times 10^{11}$) and 8·14 μg. apparent deoxyribose/ml. (from mean of 3 determinations; standard deviation ±0·41), giving $2{\cdot}14 \times 10^{-11}$ μg. apparent deoxyribose/particle of phage λ_{22}.

Assay of serum blocking power (SBP). The methods used were based on those of DeMars (1955) and Jacob & Wollman (1956). Samples of the cultures were stored in ice until they were placed in the ultrasonic generator (2 min. exposure in a 1 kW. Mullard generator). The disintegrated suspensions were stored at 0° until required (usually overnight). Samples (0·2 ml.) containing the SBP material to be assayed were mixed with 0·8 ml. of $1{\cdot}25 \times 10^{-4}$ dilution of phage antiserum (neutralization

value, K, = 200) and incubated for 6 hr at 37°. The test phage (λ_{gv}) was then added (0·2 ml. containing about 2×10^7 particles) and the mixture incubated for a further 3 hr. before plating appropriate dilutions with the indicator organism, strain K112 (λ_{22}). Standard curves were prepared by using stock phage suspensions.

Assay of endolysin. The method used was based on that of Jacob & Fuerst (1958). The lytic activity of the suspensions of bacteria disrupted by ultrasonic treatment was tested against bacterial suspensions prepared as follows. Cells of *Escherichia coli* K112 were harvested from growing cultures (5×10^8 organisms/ml.), washed once with distilled water and resuspended in 0·1 M ethylenediaminetetra-acetate (EDTA) at pH 8·3. After 5 min. at 37°, the bacteria were collected by centrifugation, washed and suspended in distilled water. Such bacterial suspensions (optical density, E, = 1·0) could be kept at 0° for 48 hr. Endolysin activity was determined by adding the sample (1·5 ml.) to 1·5 ml. of the washed bacterial suspension and the optical density read at intervals at 610 mμ (blue photocell). When endolysin is present, the optical density at first decreases linearly for a period, and then less rapidly to a plateau value, after which there is little or no further change with time. The rate of change in optical density (ΔE/min.) over the initial linear portion of the graph was taken as a measure of the amount of endolysin in the sample.

Determination of viable counts. The sample was diluted in 0·9 % NaCl solution and then plated by the soft agar layer technique on to PB agar plates.

Measurement of turbidity. Measurements of optical density, E, were made at 610 mμ (blue photocell).

Spectrophotometry. All measurements were made in a Unicam spectrophotometer Model SP 600 with 1 cm. glass cells.

Experimental procedure. Large-scale cultures were grown in conical flasks (150 ml. medium TB/1 l. flask) shaken in a water bath at 37°. Cultures were normally harvested when they reached 5×10^8 bacteria/ml. When the bacteria were to be u.v.-irradiated, they were collected by centrifugation and resuspended at an optical density of about 0·6 in 0·9 % NaCl solution containing 0·1 vol. of medium PB or TB (i.e. about 6×10^8 bacteria/ml.). The suspension was divided into two parts; one was irradiated and the other kept as the control. Within about 2 min. of irradiation, 100 ml. of each suspension were placed in a 1 l. flask, 20 ml. 10 % tryptone at 65° added (to bring the culture to 37° quickly), and then shaken in a water bath at 37°.

For infection with phage λ_{gc} or λ_{gv} in the growth medium, cultures were grown to 5×10^8 bacteria/ml., $MgSO_4$ added to a final concentration of 0·02 M, and then infected with a high-titre phage stock (multiplicity 10 particles/bacterium). For infection in the absence of growth medium, the culture was centrifuged and re-suspended in 0·02 M-$MgSO_4$; a small volume of high-titre phage suspension was then added, and after 15 min. at 37° the bacteria were collected by centrifugation and resuspended in warm 2 % tryptone medium.

All percentages of solutes are w/v and all incubations were done at 37° unless otherwise stated.

RESULTS

Effect of induction of Escherichia coli lysogenic for wild-type phage λ_{22}

When the induced bacteria were incubated in complete medium, the turbidity of the culture increased for about 70 min. (phase of residual growth) and then lysis began (Fig. 1). Although for the first few minutes the rates of protein and RNA synthesis were the same in the induced and control (unirradiated) bacteria, the rates in the induced culture eventually became slower than in the control culture (Fig. 2).

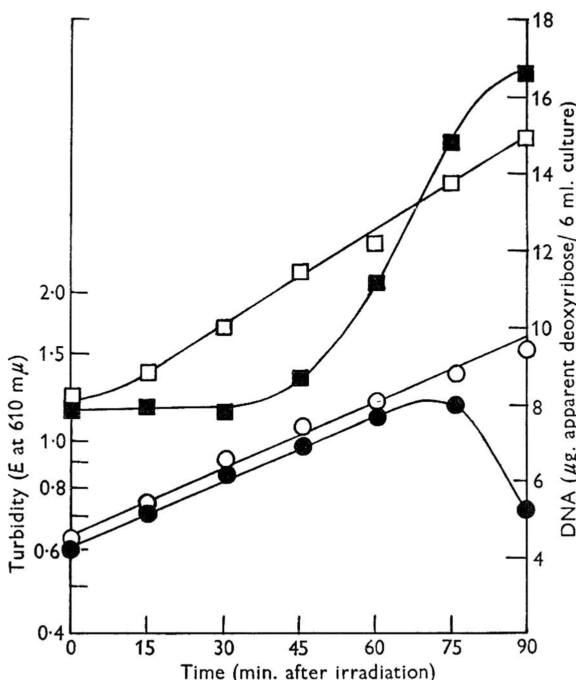

Fig. 1. Growth of *Escherichia coli* K 112 (λ) and the synthesis of DNA after the induction of phage development by ultraviolet radiation. Experimental procedure described in Methods (p. 416). Samples were taken for the determination of turbidity (○, ●) and DNA (□, ■): closed symbols for irradiated culture and open symbols for control (unirradiated). Initially $5{\cdot}6 \times 10^8$ bacteria/ml. and 96 % were induced.

In the induced bacteria, the net synthesis of DNA was completely inhibited during the first 30 min. of the latent period (Figs. 1, 2). After this lag, synthesis was resumed and eventually became faster than in the unirradiated bacteria (Fig. 1). Similar experiments were done with the non-lysogenic parent strain. By comparing Figs. 2 and 3 it can be seen that the changes in DNA, RNA and protein synthesis which occurred in the induced bacteria were similar to the changes produced in the non-lysogenic bacteria by the same dose of radiation as used in the induction experiments. It was therefore concluded that the lag in DNA synthesis and the falling off in the synthesis of RNA and protein observed in the induced organisms were due to the effects of the irradiation on the general metabolic processes of the bacteria and were not a reflexion of events concerned with phage development.

Comparison of amount of DNA synthesized with DNA present in phage progeny

An attempt was made to determine how much of the DNA made after the induction of *Escherichia coli* K 112 (λ) was phage DNA. The base composition of DNA from phage and host are the same (Lwoff, 1953; Kaiser & Hogness, 1960), so there is no direct way of following the synthesis of phage DNA as distinct from host DNA. However, the phage yield can be determined, and, when the DNA content of a phage λ

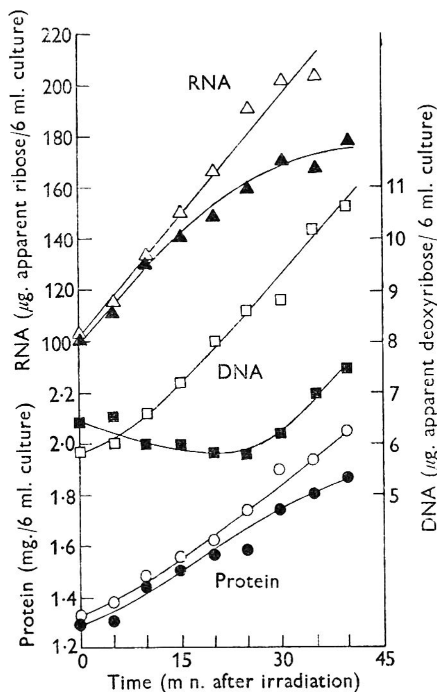

Fig. 2

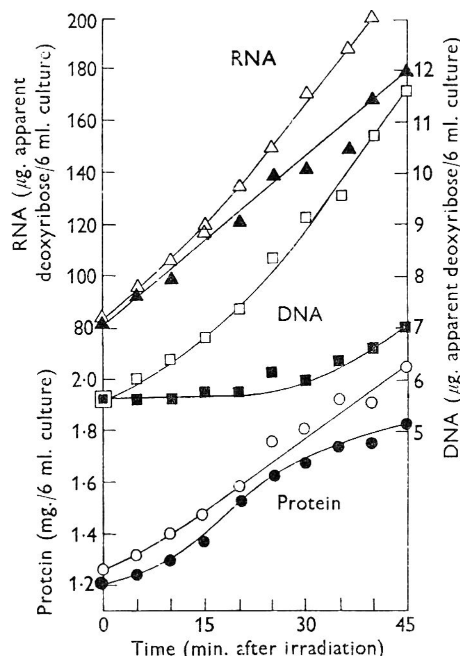

Fig. 3

Fig. 2. The synthesis of protein (○, ●), RNA (△, ▲) and DNA (□, ■) in *Escherichia coli* K 112 (λ) during the first half of the latent period after induction of phage development by ultraviolet radiation (closed symbols for irradiated culture and open symbols for control culture). Experimental procedures as for Fig. 1. Initially $3{\cdot}8 \times 10^8$ bacteria/ml. and 89 % were induced.

Fig. 3. The synthesis of protein (○, ●), RNA (△, ▲) and DNA (□, ■) in non-lysogenic *Escherichia coli* K 112 after exposure to ultraviolet radiation (closed symbols for irradiated culture and open symbols for control culture). Experimental procedure and dose of ultraviolet radiation as for Fig. 2. Initially $3{\cdot}9 \times 10^8$ bacteria/ml. and 77 % were killed.

particle is known (see Methods, p. 415), the amount of DNA present in the phage progeny can be compared with the total amount of DNA synthesized in the culture. Experiments with induced bacteria (Table 1) showed that the DNA estimated to be present in the phage yield accounted for an appreciable part (between 81 and 89 %) of the DNA synthesized after irradiation. Hence the synthesis of host DNA was at least very much decreased during the vegetative development of phage λ; perhaps no synthesis took place at all and the excess DNA was phage DNA which had not

been incorporated into complete phage particles (see similar experiments with phage T_2 by Hershey, 1953, and Hershey & Burgi, 1956). One of the main difficulties in this type of experiment is to measure the phage yield. If this is done by assay of the bulk culture when lysis is complete then some of the phage particles will have been lost by adsorption to cell debris and unlysed bacteria and thus the estimate of the phage yield will be inaccurate. If to avoid this the practice of Ellis & Delbruck (1939) is followed and a sample of the culture is diluted several thousand times before lysis begins, the assumption has to be made that the phage yield/bacterium in the diluted culture is the same as in the bulk culture from which samples for the estimation of DNA are obtained. The figures in Table 1, Expt. 1, show how great the difference was between phage yield as determined by direct assay of the bulk culture and the value obtained from the diluted culture.

Table 1. *Synthesis of DNA and the production of phage particles after the induction of Escherichia coli* K112 (λ) *by ultraviolet radiation*

Bacteria were harvested from cultures in the log phase of growth, resuspended, irradiated and, after the addition of warm medium, incubated at 37° (details as in Methods, p. 416). After 40 min., a sample was diluted 6×10^5 times in tryptone broth (at 37°) and also incubated with shaking. A sample of the diluted culture was immediately plated to determine the number of induced bacteria. After 120 min. a sample of the diluted culture was incubated for a further 30 min. in TB+0·02 M-KCN (Doerman, 1952; Weigle & Delbruck, 1951), and then assayed for phage particles. Figure in parentheses shows phage particles found in sample of bulk culture taken at 120 min. and incubated in TB+0·02 M-KCN for 30 min. The phage particles present at the time of irradiation were determined after incubating a sample of the unirradiated bacteria in broth + 0·02 M-KCN + a drop of chloroform for 30 min. In Expt. 1, at time 0 min. $5{\cdot}9 \times 10^8$ bacteria/ml., and 93 % were induced: in Expt. 2, $8{\cdot}6 \times 10^8$ bacteria/ml. and 93% were induced: in Expt. 3, $6{\cdot}7 \times 10^8$ bacteria/ml., and 90 % were induced. All results are expressed per 6 ml. of culture. DNA content of phage $2{\cdot}14 \times 10^{-11}$ μg. apparent deoxyribose/particle (Methods, p. 415).

	Total apparent deoxyribose (μg./6 ml. culture). At 0 min. (A)	At 120 min. (B)	DNA synthesized (μg. apparent deoxyribose) (B−A)	Phage (particles/6 ml.) At 0 min.	Phage At 120 min.	Apparent deoxyribose in phage (μg.) (C)	% of DNA synthesized in phage (C × 100)/(B−A)
(1)	8·26	28·58	20·32	$5{\cdot}4 \times 10^4$	$7{\cdot}7 \times 10^{11}$ ($2{\cdot}5 \times 10^{11}$)	16·47	81
(2)	11·52	42·40	30·88	$3{\cdot}8 \times 10^6$	$1{\cdot}2 \times 10^{12}$	25·62	83
(3)	9·54	24·50	14·96	—	$6{\cdot}2 \times 10^{11}$	13·26	89

Effect of infection of Escherichia coli K112 *with phage* λ_{gc}

Infection with phage λ_{gc} or λ_{gr} can be done in a medium which does not support growth, e.g. in 0·02 M-$MgSO_4$ alone, or in the growth medium itself supplemented with 0·02 M-$MgSO_4$. The former conditions enable the majority (90 %) of the bacteria to be infected, and, when they are subsequently transferred to the growth medium, phage development will begin in all the bacteria at the same time. This procedure is preferred since, when organisms are infected in the growth medium, unless infection of the population as a whole is very rapid the events which occur in the infected bacteria will tend to be blurred by those in non-infected bacteria. After exposure to phage λ_{gc} in 0·02 M-$MgSO_4$, 95 % of the bacteria were infected, and there

was little residual growth when they were placed in broth (though the non-infected bacteria grew without lag). One reflexion of this was the small amount of protein synthesis (Fig. 4*a*). The synthesis of DNA occurred without delay in both infected and non-infected bacteria, but the rate of synthesis in infected bacteria was somewhat more rapid during the latter part of the latent period (Fig. 4*b*). In the experiment reported, no net synthesis of RNA was detected, although the amount of RNA in the control bacteria increased steadily (Fig. 4*b*). Lysis of the infected bacteria began at about 35 min.

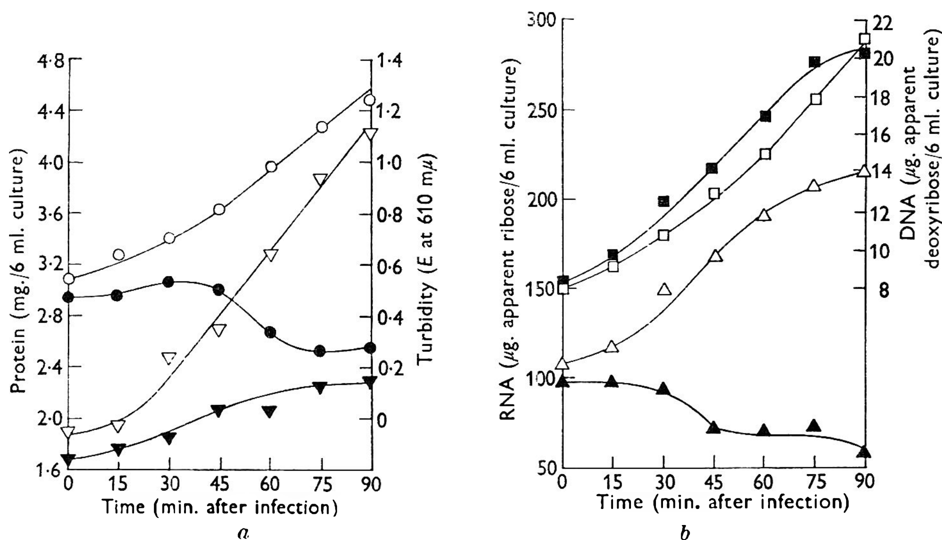

Fig. 4. Growth of *Escherichia coli* K112 and the synthesis of protein, RNA and DNA after infection with phage λ_{gc} in the presence of 0·02 M-$MgSO_4$ alone. A log-phase culture was harvested, suspended in 0·02 M-$MgSO_4$ ($5·7 \times 10^8$ bacteria/ml.) and divided into two parts. One part received a small volume of phage λ_{gc} ($8·5 \times 10^9$ particles/ml.) in 0·02 M-$MgSO_4$: the other part was mixed with $MgSO_4$ alone. After 15 min. at 37°, the cells were harvested and resuspended in warm TB medium, and samples taken at intervals for the determination of (*a*) turbidity (○, ●) and protein (▽, ▼) and (*b*) RNA (△, ▲) and DNA (□, ■). Closed symbols for infected culture and open symbols for control culture.

When bacteria in the log phase of growth were infected in the growth medium with phage λ_{gc} there was an appreciable amount of residual growth before lysis began after about 30 min (Fig. 5*a*). Total protein synthesis in the infected bacteria was less than in the control bacteria (Fig. 5*a*), and again there was no lag in the synthesis of DNA, the rate of synthesis of which during the latent period was greater than in the uninfected organisms (Fig. 5*b*). The synthesis of RNA in the infected bacteria was much reduced (Fig. 5*b*).

Effect of infection of Escherichia coli K*112 with phage* λ_{gv}

As in the experiments with phage λ_{gc}, infection was brought about in 0·02 M-$MgSO_4$ or in growth medium. When the former procedure was used, the rate of growth of the infected bacteria was less than that of the control bacteria, which grew without lag when resuspended in the tryptone medium (Fig. 6). Phage λ_{gv}, like phage λ_{gc}, markedly decreased the synthesis of protein and RNA in the infected

bacteria. There was no delay in the synthesis of DNA and, even though lysis of the culture began at 30 min., the amount of DNA present continued to increase for about 30 min. at a faster rate than in the control culture, implying that the rate of synthesis in bacteria still unlysed was extremely high (Fig. 6). When infection in the growth medium was studied, and DNA estimated at 5 min. intervals, no lag in the synthesis of DNA was observed and the rate of synthesis was always greater than in the uninfected bacteria.

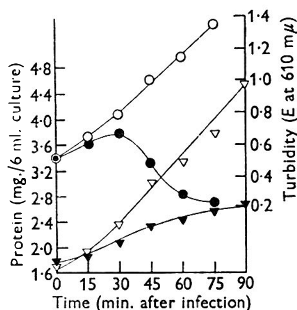

Fig. 5*a*

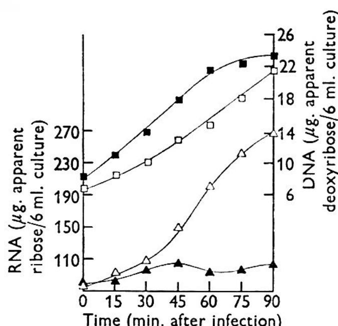

Fig. 5*b*

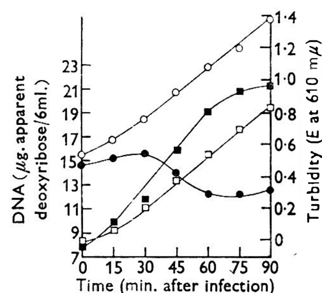

Fig. 6

Fig. 5. Growth of *Escherichia coli* K112 and the synthesis of protein RNA and DNA after infection of a growing culture with phage λ_{gc}. A log-phase culture ($5\cdot7 \times 10^8$ bacteria/ml.) in TB was divided into two parts. One part received phage λ_{gc} plus $MgSO_4$ (final concn. 0·02 M and $8\cdot0 \times 10^9$ phage/ml.) and the other $MgSO_4$ alone. After 15 min., 98 % of the cells were infected. Samples were taken for the estimation of (*a*) protein (▽, ▼) and turbidity (○, ●): and (*b*) RNA (△, ▲) and DNA (□, ■). Closed symbols for infected culture and open symbols for control culture.

Fig. 6. Growth (○, ●) of *Escherichia coli* K112 and the synthesis of DNA (□, ■) after infection with phage λ_{gv} in the presence of 0·02 M-$MgSO_4$ alone. Experimental procedure as for Fig. 4. Initially, $5\cdot0 \times 10^8$ bacteria and $1\cdot0 \times 10^{10}$ phage/ml. in 0·02 M-$MgSO_4$: 95 % of the bacteria were infected. Closed symbols for infected culture and open symbols for control culture.

Synthesis of phage-specific proteins

During the vegetative development of phage new proteins may be formed in the host organism. Some of these are enzymes concerned with the synthesis of specific phage components (e.g. the new DNA polymerase in *Escherichia coli* infected with phage T_2; Aposhian & Kornberg, 1962), whilst others will be the phage components themselves (e.g. head protein, tail fibres). Specific methods are not yet available for identifying and assaying many of these phage proteins, but two may be studied, namely serum blocking power and endolysin. Serum blocking power (SBP) is the name given to antigenic material in the tail of a phage particle which combines with antibody in phage antiserum and as a result of this combination the phage can no longer adsorb to sensitive bacteria. Endolysin is the name given to a lytic enzyme, similar to lysozyme, which appears in bacteria during the vegetative development of phage and which is regarded as being the same as the lytic enzyme in the tails of mature phage particles.

The time course of the appearance of SBP and endolysin was determined after the induction of λ phage development in *Escherichia coli* K112 (λ_{22}). Since only small amounts of these materials were to be expected in the early part of the latent period, 100 ml. samples of the induced culture were quickly cooled in an ice–salt

mixture. The bacteria were then collected by centrifugation at 0°, suspended in 10 ml. of medium PB, and ultrasonically treated. The same disintegrated suspensions were tested for SBP, endolysin activity and plaque-forming units. The assay for plaque-forming units underestimates the number of mature phage particles present in the bacteria at the time of sampling, since ultrasonic treatment has a lethal effect on some of the phage particles (ultrasonic treatment causes a decrease in the titre of a phage suspension). The experimental procedure was standardized as far as possible, and it was assumed that the proportion of phage which survived would be of the same order in each sample, and that the measured phage titres would reflect changes in the number of mature phage particles in the bacteria.

Table 2. *Time course of the appearance of SBP, endolysin and phage particles during onset of the induced vegetative development of phage λ*

An exponential culture of *Escherichia coli* K112 (λ) was harvested, resuspended, induced and reincubated in the normal way (Methods, p. 416). At time 0 min., 4.5×10^8 bacteria/ml.: 92% were induced. Samples (100 ml.) were taken at intervals and quickly cooled. The bacteria were collected by centrifugation, suspended in 10 ml. PB and then subjected to 5 min. ultra sound. Each disintegrated sample was assayed for SBP, endolysin activity and plaque-forming units. Other samples were taken at intervals to determine turbidity and hence follow residual growth and lysis of induced culture. Temperature during endolysin assay was 22–23°.

Time after induction (min.)	SBP (phage equivalents/ml.)	Endolysin activity (ΔE/min.)	Plaque-forming units (per ml.)
0	6.3×10^7	0.001	—
15	6.0×10^7	—	6.5×10^5
20	6.0×10^7	0.001	4.9×10^5
25	5.9×10^7	0.03	2.4×10^5
30	6.6×10^7	0.05	1.2×10^6
35	7.6×10^7	0.20	3.8×10^6
40	7.7×10^7	0.40	3.9×10^6

A small degree of spontaneous induction occurs naturally in a culture of *Escherichia coli* (λ), and it is to be expected that the sample taken immediately after u.v.-irradiation would show small amounts of SBP, endolysin and mature phage particles (Table 2). In the experiment reported, endolysin activity began to increase 25 min. after induction, whilst SBP and mature phage showed an increase 30 min. after induction. Since the method for the detection of endolysin is more sensitive than that for SBP, it is not possible to conclude unequivocally that endolysin formation precedes the production of SBP. However, it does seem that the first appearance of these proteins is followed within 5 min. by the formation of the first mature phage particles. In this experiment, lysis began between 52 and 55 min. after incubation of the induced cultures began. In previous experiments (see Fig. 1), lysis normally began at about 60–65 min. The difference was probably due to the time taken to set up the larger amount of culture required in the present experiment. In later experiments the total amount of SBP in the final lysate of an induced culture (105 min. after induction) was as much as 15 times the number of plaque-forming units; that is to say the SBP antigen appears to be produced in great excess as compared with the number of mature phage particles which are eventually assembled. When the phage particles were removed from such a lysate by centrifuga-

tion at 40,000 *g* for 90 min., a large part of the excess SPB remained in the supernatant fluid (Table 3). However, after this first centrifugation, the recovery of phage particles and SPB was only about 50 % of that initially present. In spite of all precautions, and the use of media which contained 0·001 % gelatin and working at 4°, this result was consistently obtained in five experiments. Evidently high-speed centrifugation caused inactivation of some of the SBP and phage particles. Part of the losses of phage particles were probably due to aggregation of particles in the pellet and failure to disperse them completely when resuspended in fresh medium. Kaiser & Hogness (1960) encountered difficulties in the quantitative recovery of λ phage from crude lysates. After the removal of the majority of the phage particles, the amount of SBP in the supernatant fluid was about 18 times greater than the number of phage particles detected by plaque counts. About a quarter of this SBP remained in the supernatant fluid after centrifugation at 96,000 *g* for 90 min., whilst about half was found in the pellet. The SBP remaining in the supernatant fluid probably represents molecules of antigenic phage material which was not incorporated into organized structures at the time of lysis of the host bacteria, whilst the SBP in the pellet is probably composed of phage tails and/or fibres; compare the experiments of Franklin (1961) with lysates from *E. coli* B infected with phage T_2.

Table 3. *Distribution of serum blocking power and phage particles after differential centrifugation of lysates from induced Escherichia coli* K112 (λ)

Escherichia coli K112 (λ) was grown, harvested, induced (200 ml. containing $1·9 \times 10^8$ bacteria/ml.) and reincubated with nutrient medium in the usual way (Methods, p. 416) until lysis was complete (105 min.). After removal of cell debris etc. by 5000 *g* for 20 min., the lysate was centrifuged in the Model L Spinco centrifuge. Pellets were suspended in the same volume of TB as the original supernatants and 'insoluble' material removed at 5000 *g* for 15 min.

	Phage (particles/ml.)	SBP (phage equivalents/ml.)
Lysate	$3·01 \times 10^{10}$	$1·5 \times 10^{11}$
Supernatant after 40,000 *g* for 90 min.	$2·34 \times 10^{9}$	$4·8 \times 10^{10}$
Suspension of 40,000 *g* pellet	$1·55 \times 10^{10}$	$1·60 \times 10^{10}$
Supernatant after 96,000 *g* for 90 min.	$6·23 \times 10^{7}$	$1·1 \times 10^{10}$
Suspension of 96,000 *g* pellet	$7·91 \times 10^{8}$	$2·25 \times 10^{10}$

DISCUSSION

In the experiments reported here, no lag was observed in the synthesis of DNA following infection of *Escherichia coli* with phage λ_{gc} or λ_{gv}. Moreover, the lag in DNA synthesis in induced bacteria was the same as in bacteria of the non-lysogenic parent strain which had received the same dose of u.v. radiation. This implies that the length of this lag was due to an effect of the irradiation on a process common to lysogenic and non-lysogenic bacteria. These results therefore contrast with those obtained with *Bacillus megaterium* where, although there was a delay in DNA synthesis after induction of the lysogenic strain, there was no delay after u.v.

irradiation of the non-lysogenic parent (Siminovitch & Rapkine, 1952). Séchaud (1960) has studied the effect of infection of *E. coli* with phage λ_c. In her experiments the synthesis of DNA in the bacteria was temporarily inhibited by starvation (incubation in 0·01 M-$MgSO_4$) or by starvation and subsequent u.v. irradiation. When such bacteria were returned to a growth medium, there was a lag before DNA synthesis was resumed. This lag was reduced to about 8 min. when the bacteria were infected with phage λ_c before their return to complete medium. The majority of the adsorbed phage did not inject their DNA into the bacteria until the latter were placed in the growth medium. Injection of the phage DNA took about 4 min. and another 4 min. elapsed before DNA synthesis was resumed. Injection of the phage DNA only when the host organism is in a growth medium appears to be a phenomenon associated with starved bacteria and has not been observed with freshly harvested bacteria. It would therefore seem that the lag in DNA synthesis observed by Séchaud was due to changes brought about by the treatment of the bacteria before infection with phage. We have avoided using starved bacteria, because the primary effects of starvation on cellular metabolism are still unknown and also because starvation by incubation in the absence of sources of nitrogen, carbon and energy profoundly alters the size and composition of the ribonucleoprotein particles of a bacterial cell (Bowen, Dagley & Sykes, 1959), especially when the starvation medium contains 0·01 M-$MgSO_4$ (Dr J. Sykes, personal communication). Such particles play an important role in the synthesis of protein and perhaps RNA (see McQuillen, 1961). Starvation procedures may also alter the size and integrity of host DNA molecules, and thus diminish or inhibit DNA and RNA synthesis.

Irrespective of whether the development of phage λ took place as a result of induction (Fig. 1) or after infection in $MgSO_4$ (Figs. 4*a*, 6) or in the growth medium (Fig. 5*b*), the synthesis of DNA occurred at a greater rate than in the control bacteria. When the amount of DNA estimated to be present in the phage progeny is compared with the net amount of DNA synthesized in the culture (Table 1), it appears that, during vegetative phage development, the synthesis of host DNA is at least very much decreased and perhaps completely inhibited. This is in agreement with the conclusions of Séchaud (1960) from experiments with phage λ_c. Since the bacterial DNA and phage DNA are of identical composition, chemical analysis cannot be used to determine whether only one type or both types of DNA are made during the latent period. If host-cell DNA synthesis is in fact markedly diminished or inhibited during the development of phage λ, a unique control mechanism would seem to be operating whereby the DNA polymerase is primed for the synthesis of phage DNA, but for some reason the bacterial DNA can no longer act as primer for the synthesis of more host DNA. In phage T_2-infected *Escherichia coli*, the phage initiates the production of enzymes which lead to the removal of one of the substrates (deoxycytidine triphosphate) required for making host DNA (Zimmerman & Kornberg, 1961). No such mechanism can operate in bacteria infected with phage λ since the phage and bacterial DNA are composed of the same four deoxyribonucleotides. It may be suggested that the onset of the vegetative development of phage λ causes disorganization or modification of the host DNA such that the latter can no longer act as a primer for the polymerase.

With regard to effects on the synthesis of RNA, the most notable result was seen

with bacteria which had been infected with phage λ_{gc} in the presence of $MgSO_4$: in such bacteria, net RNA synthesis was completely inhibited (Fig. 4*b*). Much less extreme effects were observed with induced bacteria (Fig. 2) and bacteria infected in the growth medium with phage λ_{gc} (Fig. 5*b*) or in $MgSO_4$ with phage λ_{gv}; in these experiments RNA synthesis was similar to that in the control bacteria for the first third of the latent period and only later became slower so that, at the commencement of lysis, total RNA was somewhat less than half that in the control cultures. The effect of phage development on net protein synthesis was similar to these effects on RNA synthesis, and, for example, became more noticeable in the latter half of the latent period in bacteria infected with phage λ_{gc} (Fig. 4*a*), where the rate fell to about 30–40 % of that in the control culture.

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Polarographic Evidence of the Production of Polythionates During the Bacterial Oxidation of Thiosulphate

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SUMMARY

A polarographic technique and Starkey's alkali method have been applied to the qualitative analysis of thiosulphate, trithionate and pentathionate and to the quantitative analysis of tetrathionate in filtrates of bacterial cultures grown in an inorganic medium containing sodium thiosulphate. Five strains of autotrophic thiobacilli and one strain of a heterotrophic bacterium produced polythionates. During thiosulphate oxidation by two autotrophic strains which resembled *Thiobacillus thioparus*, tetrathionate, small amounts of trithionate, pentathionate and abundant elemental sulphur were produced and the pH fell from *c.* 7·0 to *c.* 3·0. In cultures of one of the three autotrophic strains which resembled *T. thiocyanoxidans*, about half as much tetrathionate, with even smaller amounts of trithionate and pentathionate, was found; abundant sulphur was precipitated and the pH fell to *c.* 4·0. In cultures of the two other strains resembling *T. thiocyanoxidans*, small amounts of tetrathionate were detected only occasionally; trithionate and pentathionate were not found, sulphur was deposited and the pH fell to *c.* 4·5. Much tetrathionate, as well as trithionate and pentathionate, were all readily identified in cultures of the heterotrophic organism; the pH rose to *c.* 8·7 and no sulphur was formed.

INTRODUCTION

Since bacteria oxidizing thiosulphate were first described and isolated (Nathansohn, 1902; Beijerinck, 1904), the role of polythionates as possible intermediates has been debated. Over a period of many years several pathways for thiosulphate oxidation have been proposed, based on the results of tests for intermediates. Much of the work on thiobacilli has been reviewed by Baalsrud (1954), by Lees (1955), by Starkey (1956), by Vishniac & Santer (1957) and more recently by Peck (1962) and by Vishniac & Trudinger (1962). The intermediates and end-products which have been detected by various workers fall into the five main groups which are summarized in Table 1.

Numerous methods have been used to detect polythionates. Nathansohn (1902) made use of the solubility of the barium salts of thionates and of their subsequent oxidation by bromine. Starkey's quantitative determinations (1935*a*) depended on the reactions of trithionate, tetrathionate and pentathionate with sulphite, cyanate and mercuric chloride; he also developed a qualitative test, subsequently much used, in which polythionates are converted by treatment with alkali to sulphite and thiosulphate which are then titrated with iodine (1934*b*). Parker & Prisk (1953) used similar methods. Vishniac (1952) used mercurous nitrate as an

Table 1. *Summary of intermediates postulated or found during the bacterial oxidation of thiosulphate*

Intermediates	End-products	Organism	Reference
Group 1			
One or more polythionates	One or more of the following: sulphate, sulphuric acid, sulphur	Unnamed species (probably thioparus) isolated by Nathansohn (1902)	Nathansohn (1902)
		Thiobacillus thiooxidans isolated by Lipman, Waksman & Joffe (1921), Waksman & Joffe (1922) *T. concretivorus* isolated by Parker (1945) Thiobacillus X (thioparus or neapolitanus) isolated by Parker (1947)	Parker & Prisk (1953)
		T. thioparus Thiobacillus X	Santer, Margulies, Klinman & Kaback (1960)
		T. thioparus isolated by Parker (1947) (Thiobacillus X)	Trudinger (1959, 1961 *a*, *b*)
		T. thioparus, NCIB 8370 (Starkey's original non-motile strain) *T. thiocyanoxidans* isolated by Happold, Jones & Pratt (1958)	Pratt (1958)
		T. thioparus isolated by Vishniac (1952) *T. thioparus* isolated by Starkey (1935 *b*)	Vishniac (1952)
		T. thioparus, NCIB 8370 (Starkey's original non-motile strain) *T. denitrificans* isolated by Baalsrud & Baalsrud (1954) *T. thiocyanoxidans* isolated by Happold *et al.* (1958)	Jones & Happold (1961), Woolley, Jones & Happold (1962)
		T. novellus isolated by Starkey (1935 *b*) Thiobacillus X *T. thioparus*	Vishniac & Trudinger (1962)
Group 2			
Not identified	One or more of the following: sulphate, sulphuric acid, sulphur	*T. thioparus* isolated by Beijerinck	Beijerinck (1904)
		T. thiooxidans isolated by Lipman *et al.* (1921), Waksman (1922), Waksman & Joffe (1922)	Waksman & Starkey (1923)
		T. thiooxidans isolated by Lipman *et al.* (1921) *T. novellus* (culture A) isolated by Starkey (1935 *b*) *T. thioparus* (culture C) isolated by Starkey (1935 *b*)	Starkey (1934 *b*)
		T. novellus isolated by Starkey (1935 *b*) *T. thioparus* isolated by Starkey (1935 *b*)	Parker & Prisk (1953)
		T. thioparus isolated by Skarżyński & Szczepkowski (1959)	Skarżyński & Szczepkowski (1959)
Group 3			
Pyrosulphite, pyrosulphate	Sulphate, sulphuric acid	*T. thiocyanoxidans* isolated by Happold, Johnstone, Rogers & Youatt (1954)	Youatt (1954)
Group 4			
Sulphite, sulphide	Sulphate	*T. thioparus* ATCC 8158	Peck (1960), Peck & Fisher (1962)

Table 1 (*cont.*)

Intermediates	End-products	Organism	Reference
Group 5			
	One or more of the following: polythionates, alkali, sulphate, sulphur	*T. trautweinii* (culture B) isolated by Starkey (1935*b*) Culture T isolated by Trautwein (1921) Culture K isolated by Trautwein (1921) *Pseudomonas aeruginosa* *P. fluorescens* *Achromabacter stutzeri*	Starkey (1934*b*, 1935*a*)
		M-strains isolated by Parker (1947) Culture T isolated by Trautwein (1921) Culture K isolated by Trautwein (1921)	Parker & Prisk (1953)
		Culture T isolated by Trautwein (1921) Culture K isolated by Trautwein (1921)	Trautwein (1921) quoted by Starkey (1934*b*, 1935*b*)

indicator of thionate production and Pratt (1958) used a test depending on the reaction of polythionates with the Folin–Ciocalteu reagent. Other techniques employed include those of manometry, paper chromatography, paper electrophoresis and the use of radioactive substrate. In manometric investigations Vishniac (1952), Youatt (1954) and Jones & Happold (1961) used living cells, while Peck (1960) and Peck & Fisher (1962) used cell-free extracts. Paper chromatographic methods were used by Skarżyński & Szczepkowski (1959), Jones & Happold (1961), Woolley, Jones & Happold (1962) and Trudinger (1961*b*). Trudinger also used paper electrophoresis and both he and Skarżyński & Ostrowski (1958) used labelled thiosulphate as substrate.

During investigations into the bacterial oxidation of spent gas liquor, organisms oxidizing thiosulphate were isolated by the author. The pathway of thiosulphate oxidation was of interest because of the discrepancies already mentioned and in view of the fact that production of polythionates plays an important part in the classification of thiobacilli (*Bergey's Manual*, 1957). In the work described in this paper a polarographic technique not previously applied to the bacterial oxidation of thiosulphate by pure cultures has been used, supplemented by Starkey's alkali test. It is sensitive, relatively simple and quick; it facilitates the identification of individual polythionates and has both qualitative and quantitative applications.

METHODS

Organisms. Five strains of autotrophic thiobacilli were used. One strain (T. 1) was isolated from a mixed culture oxidizing thiosulphate, another (T. 2) from a mixed culture oxidizing thiocyanate. Two thiocyanate-oxidizing strains (T. 3 and T. 4) were isolated from mixed cultures kindly given to the author by the late Miss M. E. Adams of the National Chemical Laboratory, Teddington. A pure culture oxidizing thiosulphate (T. 5) was supplied by Mrs M. Townshend, formerly of the Houldsworth School of Applied Science, University of Leeds. A heterotrophic organism (C) was isolated as a contaminant of a thiosulphate medium. Morphological and biochemical characteristics indicated that T. 1 and T. 5 were strains of *Thiobacillus thioparus* and that T. 2, T. 3 and T. 4 were strains of *T. thiocyanoxidans*. Organism C was not classified. Strains were purified by several serial subcultures

of single colonies on thiosulphate agar and were tested frequently on nutrient agar for possible heterotrophic contamination. Stock cultures were maintained on thiosulphate agar by subculture at 14-day intervals; older cultures quickly became sterile. Throughout a 3-year period of regular subculture there was no evidence of the inter-species change reported by Johnstone, Townshend & White (1961), or of any variations in the cultural, morphological or biochemical characteristics of any of the strains.

Materials. Analytical grade chemicals were used for media and reagents. Samples of sodium trithionate, sodium tetrathionate and potassium pentathionate were prepared by Miss Mary Seaton (London Research Station, The Gas Council); they behaved characteristically in polarographic tests, although the pentathionate was contaminated with some tetrathionate (Fig. 3, 4 and 5). Membrane filters were obtained from Oxo Ltd.

Media. The liquid thiosulphate medium was similar to but not identical with Starkey's medium 2 (1934*a*). Its composition was: KH_2PO_4, 4·0 g.; $Na_2S_2O_3.5H_2O$, 10·0 g.; $MgSO_4.7H_2O$, 0·1 g.; $CaCl_2$, 0·1 g.; NH_4Cl, 0·1 g.; $FeCl_3.6H_2O$, 0·02 g.; $MnSO_4.4H_2O$, 0·02 g.; de-ionized water, 1 l. Its pH was adjusted to 7·2–7·4 with sodium hydroxide and it was solidified when necessary by the addition of 10 g. Oxoid ion agar No. 2. Both media were sterilized by autoclaving for 20 min. at a pressure of 10 lb./in.^{2}.

Nutrient agar in disposable plastic Petri dishes was obtained from Oxo Ltd.

Preparation of test cultures. Sterile 250 or 150 ml. conical flasks, containing approximately 100 or 30 ml. respectively of sterile liquid thiosulphate medium, were inoculated with a 2- to 7-day thiosulphate culture of the appropriate organism. The inoculated flasks were incubated at 30°, under static conditions or with shaking. After various incubation times, cultures were streaked on nutrient agar to test for contamination; some or all of the culture was then membrane filtered in order to remove organisms and precipitated sulphur. After its pH had been determined, the filtrate was analysed for polythionates. As preliminary work had shown that no decrease in thiosulphate concentration occurred before the sudden appearance of precipitated sulphur, the first analysis was usually delayed until sulphur had appeared. Except in cultures of the heterotroph (which did not produce sulphur), precipitation normally occurred 1–3 days after inoculation. Nutrient agar plates were examined carefully with a ×10 lens after incubation for 7–14 days. When an autotroph was used as test organism, growth on nutrient agar indicated heterotrophic contamination of the flask culture and analytical data from such cultures were ignored. The occasional growth of very tiny colonies was believed to have been made possible by the carry-over of enough medium with the inoculum to support minimal growth of the autotroph; nevertheless, such growth was regarded as contamination. When the heterotroph was used as test organism, typical growth on nutrient agar and the absence of other growth confirmed the purity of the culture.

Detection of polythionates in culture filtrates

Polarographic method

A conventional technique, employing a cell containing a dropping mercury electrode and a saturated calomel electrode, was used in this work; for details of

the principles and techniques of polarography the reader is referred to standard works by, for example, Kolthoff & Lingane (1952), Meites (1955), Milner (1957) and Brezina & Zuman (1958).

Cultures were prepared for analysis by mixing a known volume (1–10 ml.) of culture filtrate with 25 ml. of 2 N-diammonium hydrogen phosphate solution (as supporting electrolyte) in a 50 ml. volumetric flask; the mixture was made up to the mark with de-ionized water and well shaken. The cell was rinsed first with de-ionized water and then with some of the test solution. The rest of the test solution was poured into the cell and de-aerated by bubbling nitrogen through it for 10 min. Rinsing and de-aeration were facilitated by stirring the liquid in the cell magnetically. The time between mercury drops and the temperature of the cell contents were noted.

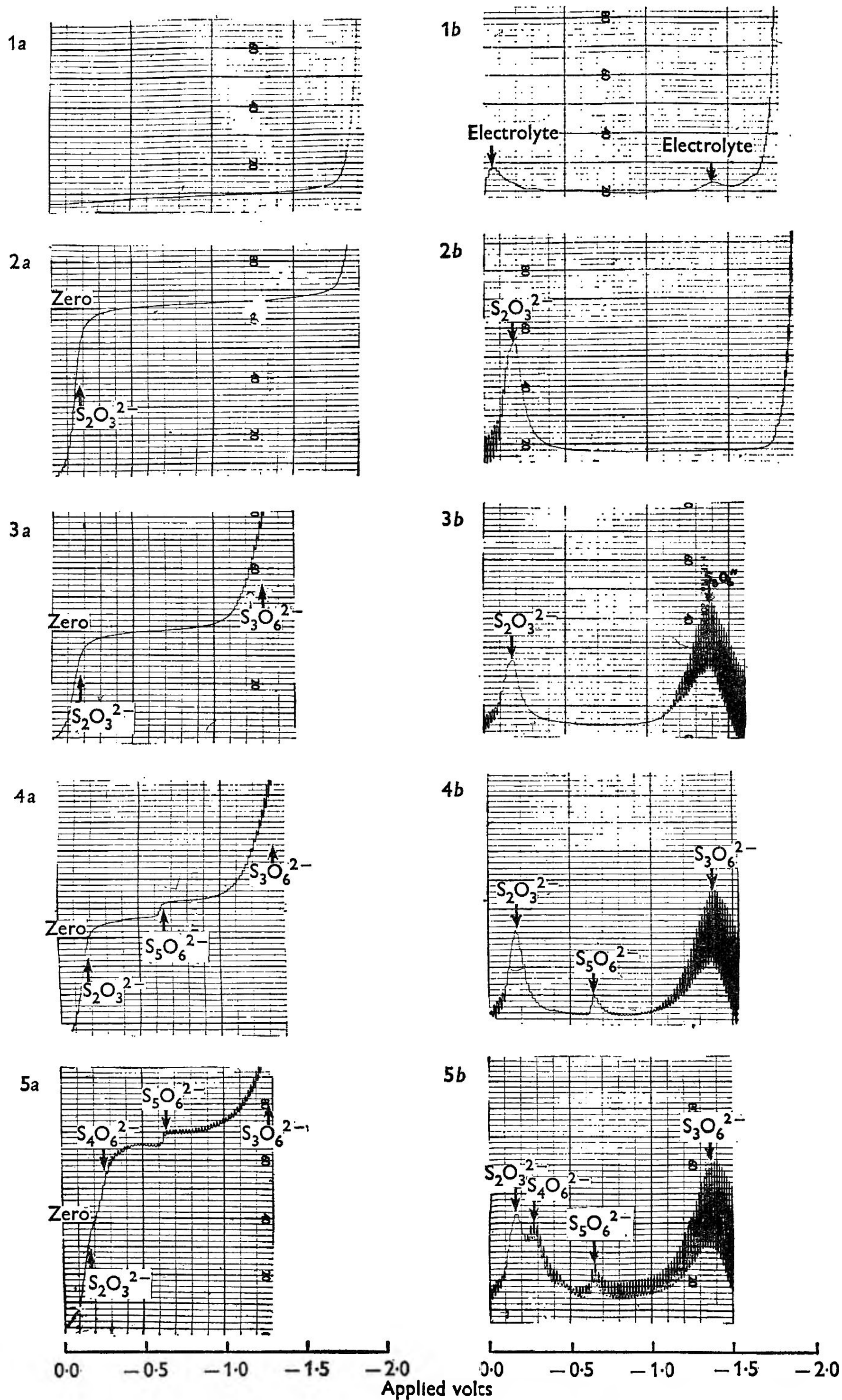
The same mercury electrode was used throughout the tests. The average time between drops was *c.* 4 sec. but values between 3·4 and 4·4 sec. were noted. At a drop time of 4 sec., 2 mg. of mercury dropped per second. The temperature of the cell contents was not controlled but varied only slightly during any one test; it was usually *c.* 20° but the range over which tests were conducted was 17–22°. For very accurate analysis, temperature and drop time should be controlled or corrections should be made for any variations.

An increasing negative voltage (over the range 0·0 to −1·5 or −2·0 V.) was automatically applied to the cell by means of a Tinsley type 15 recording polarograph and direct and derivative polarograms were obtained. For comparison polarograms of the supporting electrolyte, of the thiosulphate medium, and of sodium thiosulphate, tetrathionate and trithionate, and potassium pentathionate solutions were recorded. With diammonium hydrogen phosphate solution as supporting electrolyte, thiosulphate is reduced first, giving an anodic wave with a half-wave potential ($E_{\frac{1}{2}}$) of *c.* −0·18 V.; tetrathionate, pentathionate and trithionate are then reduced in that order, giving cathodic waves with $E_{\frac{1}{2}}$ values of *c.* −0·28, −0·67, and −1·32 V. respectively (Furness, 1950; Furness & Davies, 1952; unpublished work by Densham, Seaton & Noble, London Research Station, The Gas Council). The $E_{\frac{1}{2}}$ values vary slightly with concentration. When the concentration of thiosulphate greatly exceeds that of tetrathionate, the tetrathionate wave may be masked completely by the thiosulphate wave. Using solutions containing known amounts of sodium tetrathionate, a diffusion current of the order of 0·025 μA. was given by 1 p.p.m. tetrathionate ion. This value was used subsequently for the quantitative determination of tetrathionate in test solutions. The diffusion currents given by known amounts of thiosulphate, trithionate and pentathionate can be measured similarly.

At certain concentrations, maxima are obtained with tetrathionate (−0·5 V.) and trithionate (−1·62 V.). These maxima can be suppressed by the use of substances such as gelatin, but their infrequent appearance made such measures unnecessary. With diammonium hydrogen phosphate as electrolyte, small peaks with $E_{\frac{1}{2}}$ values of −0·05 and −1·4 V. are obtained at high sensitivities; these peaks are due either to the electrolyte itself or to trace impurities in it.

Starkey's alkali test (1934*b*)

A 5 ml. sample of culture filtrate was titrated with 0·01 N-iodine, using 1% (w/v) sodium starch glycollate as indicator. A second 5 ml. volume was boiled for 5 min.



Figs. 1–5. For legend see foot of p. 434.

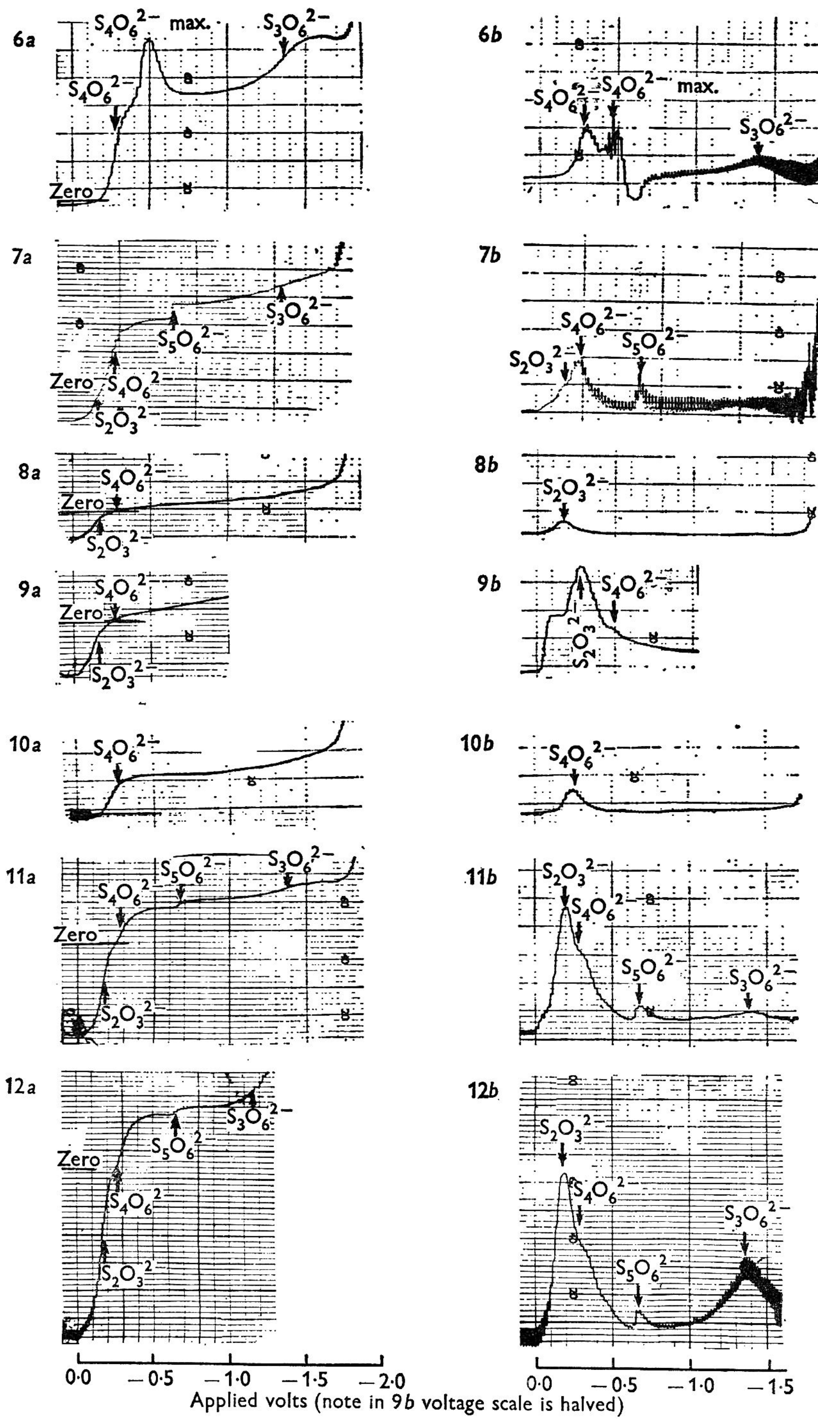


Fig. 6–12. For legend see foot of p. 434.

with 5 ml. of 10% (w/v) potassium hydroxide solution and a few ml. of de-ionized water. After cooling, the mixture was neutralized with 10 ml. of 10% (v/v) acetic acid and titrated with iodine as above. The iodine titre obtained before alkali treatment was subtracted from the titre obtained after treatment; the difference, if any, represented polythionate.

RESULTS

All the test strains produced tetrathionate and three of them produced trithionate and pentathionate as well. Very much greater concentrations of tetrathionate were detected in cultures of T. 1, T. 5 and organism C than in cultures of T. 2, T. 3, and T. 4. Acid was produced by all the strains except organism C, which formed alkali.

The results of the various analyses are shown in Table 2 and Figs. 6 to 12. Table 2 records the results for T. 2 only; the results for the other organisms were tabulated in a similar fashion, but only one table has been given as an example. Column (*a*) shows the differences between actual titration values before and after alkali treatment; these differences should be compared with the differences shown in column (*b*), which are calculated values based on the concentrations of tetrathionate found polarographically. The calculated differences were usually a little higher than the actual differences. This can be explained if, as was probable, hydrolysis of tetrathionate in the presence of alkali was not quite complete. Since tetrathionate hydrolyses theoretically to give 25% sulphite and 75% thiosulphate, and trithionate hydrolyses to give 66% sulphite and 33% thiosulphate (Starkey, 1934*b*), as would be expected the calculated differences were lower than the actual differences in the presence of high concentrations of trithionate (Fig. 12). The polarographic results (Figs. 6 to 12) should be compared with polarograms of the supporting electrolyte (diammonium hydrogen phosphate), of sterile thiosulphate medium and of thiosulphate medium to which trithionate, pentathionate and tetrathionate were added successively (Figs. 1 to 5).

In each of five experiments with T. 1 and six with T. 5 polythionates were detected (Figs. 6 and 11). The differences in iodine titre before and after alkali

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EXPLANATION OF FIGS. 1 to 12

Series *a*. Direct polarograms. The distance between adjacent vertical scale markings represents 0·2 μA. in Figs. 1, 2, 3, 4, 5, 7, 8 and 10, 0·4 μA. in Figs. 6, 11 and 12, and 0·1 μA. in Fig. 9.

Series *b*. Derivative polarograms. The distance between adjacent vertical scale markings represents 0·08 μA. in Figs. 2, 3, 4, 5, 7, 8, 10 and 11, 0·008 μA. in Figs. 1 and 9, 0·2 μA. in Fig. 6 and 0·1 μA in Fig. 12.

Fig. 1. Diammonium hydrogen phosphate solution only; this solution is the supporting electrolyte in Figs. 2 to 12.

Fig. 2. Sterile sodium thiosulphate medium.

Figs. 3, 4 and 5. Thiosulphate medium to which sodium trithionate, potassium pentathionate and sodium tetrathionate were added successively.

Fig. 6. Filtrate from a 3-day thiosulphate culture of T. 1.

Fig. 7. Filtrate from a 5-day thiosulphate culture of T. 2.

Figs. 8 and -9. Filtrate from a 5-day thiosulphate culture of T. 3.

Fig. 10. Filtrate from a 4-day thiosulphate culture of T. 4.

Fig. 11. Filtrate from a 4-day thiosulphate culture of T. 5.

Fig. 12. Filtrate from a 5-day thiosulphate culture of organism C.

treatment indicated several hundred p.p.m. polythionate in the culture filtrates. Polarographic analyses confirmed the presence of tetrathionate, trithionate and pentathionate. There was always more tetrathionate (up to 1690 p.p.m. for T. 1 and up to 1840 p.p.m. for T. 5) than trithionate and pentathionate, although the concentrations of these were not determined. The lowest pH values noted were 3·2 for T. 1 and 2·9 for T. 5. The pH tended to remain neutral in cultures which were incubated without shaking, although in one test it dropped to 2·9 after incubation for 20 days.

Table 2. *The oxidation of thiosulphate by organism T. 2*

					Titration of 5 ml. culture filtrate with 0·01 N-iodine		Polarographie analyses. Waves given by			
Experiment	Method of aeration	Incubation time (days)	Purity of culture	pH of culture filtrate	(a) Difference in titration before and after alkali treatment (ml.)	(b) Difference in titration before and after alkali treatment (ml.) calculated from polarographic data on tetrathionate	thiosulphate	trithionate	tetrathionate*	pentathionate
1	Static	3	nt	7·0	nt	nt	nt	nt	nt	nt
		9	+	6·7	nt	0	+	–	–	–
2	Static	3	nt	7·0	nt	nt	nt	nt	nt	nt
		13	+	nt	2·5	2·7	–	+	+350	–
3	Shaken	2	+	5·3	6·3	6·9	+	?	+885	+
4	Shaken	2	nt	6·5	0	nt	nt	nt	nt	nt
		5	+	4·8	6·4	7·2†	+	?	+920	+
5	Static	3	+	6·7	0	nt	nt	nt	nt	nt
6	Static	6	nt	nt	0·3	nt	nt	nt	nt	nt
		10	+	4·6	7·5	nt	nt	nt	nt	nt
7	Shaken	4	+	6·6	0·2	0	+	–	–	–
		5	nt	6·0	0·5	0·2	–	–	+ 30	–
		6	+	4·5	0·2	0	–	–	–	–
8	Static	3	nt	nt	0	nt	nt	nt	nt	nt
		7	+	4·0	3·0	nt	nt	nt	nt	nt
9	Static	2	nt	nt	1·6	nt	nt	nt	nt	nt
		4	nt	nt	0·2	nt	nt	nt	nt	nt
		6	nt	nt	0·5	nt	nt	nt	nt	nt
		7	+	6·3	1·5	nt	nt	nt	nt	nt

+, Positive; –, not detected; nt, not tested.
* Figures in this column indicate the tetrathionate concentration (p.p.m.) in the culture filtrate.
† See Fig. 7.

Polythionates were demonstrated polarographically in some of the tests with T. 2 and their presence was confirmed using Starkey's method (Table 2 and Fig. 7). Tetrathionate predominated in the filtrate, in concentrations of up to 920 p.p.m. Trithionate and pentathionate were barely discernible in the polarograms and their concentrations were therefore very low. The pH did not fall below 4·0.

In ten experiments with T. 3, polythionates were not detected by Starkey's method but 19 p.p.m. tetrathionate in the filtrate was indicated polarographically on one occasion (Fig. 8 and 9) and 8 and 2 p.p.m. tentatively on two other occasions. In two of eight experiments with T. 4, polarographic analyses revealed tetrathionate at concentrations in the filtrate of 125 p.p.m. (Fig. 10) and *c.* 3 p.p.m.; these amounts were not detected by Starkey's test. The lowest pH values noted were 4·5 for T. 3 and 4·4 for T. 4. Trithionate and pentathionate were not found in cultures of either organism.

In the six experiments with organism C, both methods indicated the accumulation of several hundred p.p.m. polythionate, with tetrathionate predominating in concentrations up to 2300 p.p.m. The concentration was 1460 p.p.m. in Fig. 12. The pH rose; the highest value noted was 8·7.

Sulphur was precipitated in cultures of all the thiobacilli but not in cultures of the heterotroph C. The amount of sulphur was always sufficient to be unmistakable and gave the liquid medium a typical creamy appearance.

Sulphur was also formed abundantly within colonies of the autotrophs on thiosulphate agar.

DISCUSSION

The occurrence of tetrathionate, sulphur and acid in thiosulphate cultures of the autotrophs and the occurrence of tetrathionate and alkali in cultures of the heterotroph suggests that the autotrophs belong to group 1 and the heterotroph to group 5 (Table 1). For reasons given below, the author believes that tetrathionate and sulphur are formed biochemically but that trithionate and pentathionate have a purely chemical origin.

Nobody who has found tetrathionate in bacterial cultures has suggested that it is not a genuine intermediate in the oxidation of thiosulphate and it is of interest that, during investigations involving more than twenty strains of autotrophic thiobacilli, White & Hutchison have not found any strains which do not produce polythionates (White, pers. comm., 1963). An enzyme catalysing the oxidation of thiosulphate to tetrathionate has been found in Thiobacillus X (Trudinger 1961 *a*, *b*), in *T. thioparus* and in autotrophically grown *T. novellus* (Vishniac & Trudinger, 1962), as well as in many other biological systems. However, the failure of some workers to detect tetrathionate has led to the postulation of alternative pathways not involving this compound (Table 1). In the author's opinion, the factors contributing to failure to detect tetrathionate include the inability of some cultures to accumulate polythionate, the lack of frequent testing and the use of tests of low sensitivity. Much the same views have been expressed by Parker & Prisk (1953), who commented on the transient appearance of polythionates in some cultures, and by Vishniac & Santer (1957).

If tetrathionate is a genuine intermediate, the reason for the accumulation of more of it within a given time in cultures of *Thiobacillus thioparus* than in cultures of *T. thiocyanoxidans* is not clear. One possible explanation is that, though the same pathway may be followed, the rates of reaction differ because of inherent differences between the strains in, for example, cell-wall composition and permeability. Pratt (1958) and Jones & Happold (1961) also found that cultures of *T. thiocyanoxidans* produced less polythionate than cultures of *T. thioparus*. Jones

& Happold expressed the view that the type of polythionate which accumulates is influenced by the ratio between potassium and sodium in the medium and that the phosphate concentration also has an effect on polythionate formation.

Although the occurrence of tetrathionate in cultures has been accepted as evidence of its intermediate role, this is not so with trithionate, pentathionate and sulphur. These have been shown to be formed chemically from tetrathionate (Tamiya, Haga & Huzisige, 1941; Parker & Prisk, 1953) and from mixtures of thiosulphate and tetrathionate (Vishniac, 1952). Skarżyński & Szczepkowski (1959) also detected hexathionate and possibly heptathionate in a mixture of thiosulphate and tetrathionate. Although solutions of polythionates are unstable, trithionate, pentathionate and sometimes dithionate and sulphur have nevertheless been considered by some of these workers and by others to be true intermediates in the bacterial oxidation of thiosulphate.

In the present work, trithionate and pentathionate appeared only after tetrathionate had accumulated and once they had formed there was little or no further change in their concentrations over a period of several days. Furthermore, if only small amounts of tetrathionate formed, trithionate and pentathionate were not produced at all or were produced in such low concentrations that they could not be detected.

The sulphur which was precipitated in cultures of the autotrophs appeared most rapidly in shaken cultures and in cultures which had received a large young inoculum; the results indicated that sulphur formation occurred at neutral as well as acid pH values.

If sulphur is produced chemically, it should be associated always with mixtures of polythionates and thiosulphate, but it was not produced in cultures of the heterotroph C or in any of the filtrates which contained abundant polythionates and from which the sulphur and organisms had been removed by membrane filtration. Nor was it produced in some of the polythionate-forming cultures of Parker & Prisk (1953) and of Baalsrud & Baalsrud (1954).

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Structure of Poly-β-hydroxybutyric Acid Granules

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SUMMARY

A membrane surrounding poly-β-hydroxybutyrate (PHB) granules isolated from both *Bacillus cereus* and *B. megaterium* has been demonstrated by the carbon-replica technique and electron microscopy. Some general features and properties of both the membrane and PHB granules are discussed.

INTRODUCTION

The polymeric ester, poly-β-hydroxybutyric acid (PHB) has been studied recently as regards its role as a storage product (Doudoroff & Stanier, 1959; Macrae & Wilkinson, 1958), its enzymic synthesis and breakdown (Merrick & Doudoroff, 1961; Merrick, Delafield & Doudoroff, 1962), its formation in bacteria (Schlegel, Gottschalk & von Bartha, 1961; Sierra & Gibbons, 1962), its assay (Slepecky & Law, 1960), and its chemical and physical properties (Williamson & Wilkinson, 1958). The experimental work pertinent to PHB is expanding rapidly and the reader is referred to the interesting paper of Schlegel & Gottschalk (1962) for more information on the distribution of PHB, its function and biochemistry. The work reported here is the result of a collaborative effort by the authors from the laboratories cited. The likely presence of an envelope or membrane around PHB granules was independently uncovered while studying different properties of PHB. At Syracuse, studies were made of the crystalline structure of PHB isolated from both *Bacillus cereus* and a Rhizobium species (Alper, Marchessault & Lundgren, 1962; Alper, Lundgren & Marchessault, 1963; Alper, Lundgren, Marchessault & Cote, 1963; Hester, 1963), as well as an investigation of the accumulation of PHB in an auxotrophic mutant of *B. cereus* grown with either methionine or cyst(e)ine added to the minimal medium (Lundgren & Bott, 1963). In the laboratories at Buffalo, the enzymic depolymerization of PHB granules isolated from *B. megaterium* has been examined (Merrick *et al.* 1962).

METHODS

Preparation of PHB granules for electron microscopy

Bacillus cereus. Initially PHB granules examined with electron optics were those isolated from 69 hr *B. cereus* C-1 organisms. The complete culture system for this auxotrophic mutant of *B. cereus* ATCC 4342 has been described (Lundgren & Bott, 1963) and both stained and phase-contrast microphotographs of cells containing

polymer were shown. Bacilli containing PHB were centrifuged from 10 ml. of the 69 hr old culture, washed 3 times with distilled water, and subjected to sonic treatment in distilled water for 10 min. at an intensity equivalent to a dial setting '7' with a 20 kcyc. Branson Sonifier (Heat Systems Co., 777 Northern Blvd., Great Neck, Long Island, N.Y.). After sonic treatment the crude extract was centrifuged at 2700 *g* for 15 min. The particulate matter was suspended in 10 ml. distilled water and when examined under phase optics showed individual, slightly refractile granules.

Bacillus megaterium. The PHB granules from *B. megaterium* KM were isolated as follows. The organism was grown as outlined by Merrick & Doudoroff (1961). Five g. bacilli (wet wt.; harvested at the end of exponential growth) were suspended in 30 ml. 0·05 M-potassium phosphate buffer (pH 7·0); 33 mg. lysozyme; 0·5 ml. M-$MgCl_2$ and 0·35 mg. deoxyribonuclease (Worthington Biochemical Corporation, Freehold, N.J.) were added and the mixture incubated for 30 min. The lysate was subjected to ultrasonic treatment with an ultrasonic probe for 2 min. to liberate the PHB granules from the cell membranes. The granules were separated from the crude extract by layering the lysate on glycerol followed by centrifugation in a swinging bucket rotor at 9000 *g* for 20 min. The supernatant fluid was then discarded and the PHB granules which collected on the surface of the glycerol were removed and resuspended in 30 ml. 0·05 M-tris-HCl buffer (pH 8·0). The centrifugation on glycerol was twice repeated, the final preparation of granules suspended in 5 ml. tris buffer system and then dialysed for 24 hr against 0·02 M-tris-HCl buffer (pH 8·0). We have referred to these granules as 'native' polymer granules for they were isolated by procedures less drastic than those used on *B. cereus*. The 'native' granules are readily susceptible to hydrolysis by cell-free extracts of *Rhodospirillum rubrum* organisms that have depleted their own polymer stores. However, these granules, when treated with acetone, ethanol, hypochlorite or heat, will no longer serve as a substrate for the depolymerizing system (Merrick *et al.* 1962). In some experiments the 'native' granules were also subjected to sonic oscillation for various periods of time or were treated with sodium lauryl sulphate.

Specimens for electron microscopy were prepared using the carbon replica techniques of Bradley & Williams (1957). Electron micrographs were taken with an RCA EMU 2D Electron Microscope, with a 50 or 25 μ objective aperture.

RESULTS

Plate 1, figs. 1 and 2, show electron micrographs of a germanium-shadowed carbon replica of PHB granules isolated from *Bacillus cereus* C-1. On many of the granules, fragments of a delicate skin-like structure can be seen. This membranous material, when intact, is apparently wrapped around the granules. This figure also shows the typical surface differences seen in all preparations examined. Some of the granules are smooth in appearance, while others are rough and irregular. These surface differences may, however, reflect the preparative procedures for electron microscope analysis. These differences were readily seen in all specimens examined. Some of the granules appeared to coalesce, a feature which is more demonstrable with granules having intact membrane covers. This property is presumably a result of the drying of the specimen, since synthetic polystyrene pellets coalesced under similar conditions. Differences in the shape and size of the PHB granules are readily

observable in Pl. 1, fig. 2. Although the majority of the granules appeared spherical, some rod-like structures were occasionally seen. These structures also have smooth or rough surfaces and are encased in a membrane. The rod-like granule which possessed the smooth surface appeared to have the membrane coat still intact. PHB granules vary in diameter from about 0·2 to 1·1 μ; there were no noticeable differences in granules isolated from the two bacilli.

Plate 1, fig. 3, is a germanium-shadowed carbon replica of *Bacillus cereus* 4342 spores which is shown for comparative purposes to indicate the similarities and differences between spores and PHB granules. The ridged spores are similar to the *B. cereus* spores shown in the electron micrographs of Bradley & Williams (1957).

Germanium-shadowed carbon replicas of PHB granules isolated from *Bacillus megaterium* are shown in Plate 2. In Pl. 2, figs. 5–8, 'native' granules which were subjected to sonic treatment for 5 min. are shown with broken membranes surrounding the granules. Granules shown in Pl. 2, fig. 5, show the irregularity of the rough-surface granules. Native granules completely enclosed in membranes are seen in Pl. 2, fig. 6. A membrane wrinkle is seen on top of the large PHB granule and the membrane appears to be continuous with the other granules. Native granules shadowed with germanium at an angle of 45° are shown in Pl. 2, fig. 7; the polymer was electron dense with a less dense membrane around it. The other polymer granules in this preparation are clumped together. This is a feature frequently noted in many of the preparations.

Plate 2, fig. 8 to Pl. 3, fig. 11, show electron micrographs of carbon replicas of different PHB preparations from *Bacillus megaterium.* Plate 2, fig. 8, shows the highly dense peripheral portion of a membrane which appears to have more than one layer and which extends to the neighbouring granules as well. A small protrusion is also shown, extending from one of the larger granules. This may represent the early formation of another PHB granule encased within the same membrane. The extraneous material seen as part of the background represents cell debris carried over with the granules during the isolation procedure. Plate 3, fig. 9, shows a carbon replica of a *B. megaterium* with a flagellum and with large PHB granules deposited in (or on) the organism. The granules are still contained within the organism or are at a state just prior to their release after the cell wall was ruptured by sonic treatment and lysozyme. Plate 3, fig. 10, shows intact and enzymically active granules. When such granules were treated with 0·1 % sodium lauryl sulphate for 24 hr with shaking, isolated structures were seen that appeared to be membrane coats (Pl. 3, fig. 11). These were circular bodies with raised edges which were readily torn, as seen in one of the structures; one highly wrinkled coat is shown in this photograph. The raised ring effect is contrasted with a non-disrupted granule which has a significant shadow. The thickness of the membrane shell remaining after the removal of PHB was estimated to be about 150–200 Å thick. Further structural detail relating broken PHB granules, fragments, and loosened membranes may be observed in Pl. 3, fig. 12, and Pl. 4, fig. 13. A close examination of Pl. 3, fig. 11, to Pl. 4, fig. 13 shows material which looks somewhat different from intact granules and is probably extruded crystalline polymer. This material is quite like the 'lath'-shape crystals of PHB formed when a pure solution of PHB in chloroform was treated with ethanol, as shown in Pl. 4, fig. 14. Properties of these crystals have been described elsewhere (Alper *et al.* 1963).

DISCUSSION

The application of the carbon-replica technique to the investigation of PHB granules from Bacillus organisms has made possible the identification of discrete membrane-like structures which encase the granules. This technique of Bradley & Williams (1957) has recently been used by Hopwood & Glauert (1961) to increase the amount of structure observable on the surface of bacteria. Details of the structure of a fibrous coat around spores were detected in *Streptomyces violaceoruber*. Earlier studies of PHB inclusions, using Sudan Black B staining, phase microscopy, and electron microscopy of both gross and ultra-thin sections, have failed to establish clearly the presence of such a membrane. The first two methods could not detect such a structure because the light microscope could not resolve it. Bacteria containing PHB inclusions have been studied with the electron microscope; Chapman (1956) studied *Bacillus cereus*, and Cohen-Bazire & Kunisawa (1963) studied *Rhodospirillum rubrum*. Chapman's micrographs revealed discrete inclusions and instances of coalesced PHB granules; no membranous covering of the inclusions was discussed in that paper. Cohen-Bazire & Kunisawa characterized the reserve material of *R. rubrum* produced under different cultural conditions and reported the absence of a limiting membrane for glycogen and PHB granules. The failure to identify a membrane around PHB granules was probably due to the thinness of such a structure and the method of treatment. The PHB was soluble in the embedding material (Vestopal) which would have destroyed the granules' membrane. Thin sections of bacteria only showed empty areas where the PHB granules were initially present. Electron micrographs of shadowed lipid inclusions (PHB) from *B. megaterium* KM and *B. cereus* were reported by Weibull (1953) and Williamson & Wilkinson (1958), respectively.

Membrane systems have been identified in bacteria in several instances. Outstanding is the example of pigment-bearing particles (chromatophores) in *Rhodospirillum rubrum* which are surrounded by a membrane (Vatter & Wolfe, 1958). Other examples of membranous organelles were cited by Murray (1960) who reviewed the internal structure of the bacterium. Our evidence suggests that the PHB granules are membranous organelles but their nature and complexity needs further investigation. The most likely function for PHB is as a storage material (Macrae & Wilkinson, 1958; Doudoroff & Stanier, 1959; Schlegel *et al.* 1961). How the membrane regulates the metabolism of this storage product is an interesting question; probably even more exciting is the suggestion that perhaps the metabolism of other types of storage materials (i.e. starch, glycogen, polyphosphates) may be regulated by a membranous system. Answers to such questions should prove to be of fundamental importance in understanding the over-all metabolism of bacterial storage substances.

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EXPLANATION OF PLATES

(The scale marks represent 0·5 μ)

Plate 1

Figs. 1–4. Electron micrographs of both carbon replicas of PHB isolated from *Bacillus cereus* and *B. megaterium* and *B. cereus* spores shadowed with germanium.

Fig. 1. Membrane fragments are shown associated with the granules. The surfaces of the granules are varied in smoothness and suggest a laminated structure. × 40.000.

Fig. 2. The rod-like granules show a varied surface as well as evidence of a membrane covering. × 25,000.

Fig. 3. *B. cereus* ATCC 4342 spores showing sculptured surface and a typical loose exosporium. × 33,000.

Fig. 4. Carbon replica of germanium shadowed 'native' granules from *B. megaterium* showing a defined core and outer membranous layer. × 40,000.

Plate 2

Figs. 5–8. Electron micrographs of both carbon replica and germanium shadowed 'native' PHB granules isolated from *Bacillus megaterium*.

Fig. 5. Carbon replica of germanium shadowed 'native' granules showing varied surface structure. Torn membrane fragments are shown around a number of these bodies. × 35,000.

Fig. 6. Carbon replica of 'native' PHB granules showing an entire surface membrane. The large granule shows a definite wrinkle in the membrane covering. × 60,000.

Fig. 7. 'Native' PHB granules germanium shadowed showing a dense core with a well-defined, less dense outer layer. × 28,000.

Fig. 8. Granules with smooth surfaces showing broken membranes and a more dense peripheral structure which may be multi-layered. The peripheral dense appearance is due to its being 'edge-on' in the preparation. × 50,000.

Plate 3

Figs. 9–12. Electron micrographs of carbon replicas of enzymically active 'native' PHB granules both untreated and treated with sodium lauryl sulphate.

Fig. 9. 'Native' granules in association with a vegetative cell. × 38,000.

Fig. 10. Intact 'native' granules which are the type readily susceptible to hydrolysis by cell-free extracts of *Rhodospirillum rubrum*. × 40,000.

Fig. 11. A collection of membrane coats and a single PHB granule showing a rough surface. The coats resemble craters. × 34,000.

Fig. 12. Granules showing fragmented membranes and a coat. × 34,000.

Plate 4

Figs. 13, 14. Electron micrographs showing PHB granules treated with sodium lauryl sulphate and isolated crystals of PHB shadowed with germanium.

Fig. 13. Membrane fragments and coats and suspected crystalline PHB material. × 30,000.

Fig. 14. Single crystals of PHB grown in the test tube. × 30,000.

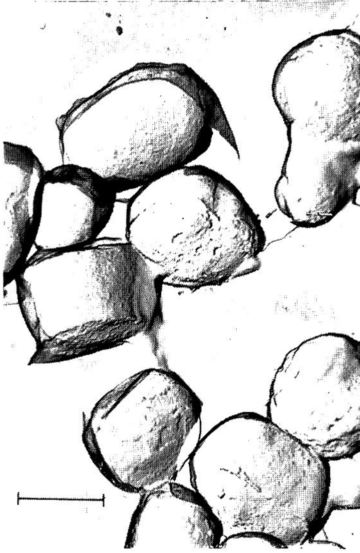


Fig. 1

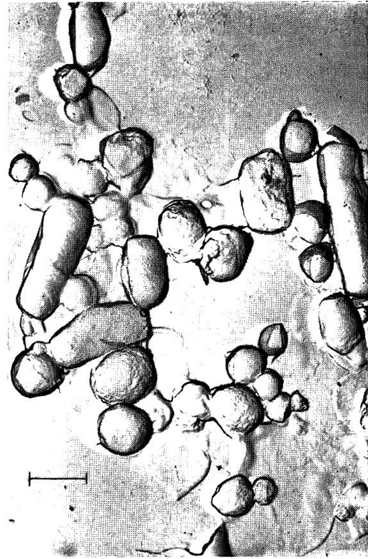
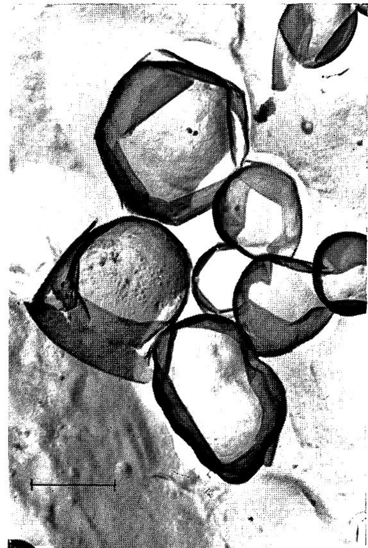


Fig. 2

Fig. 3



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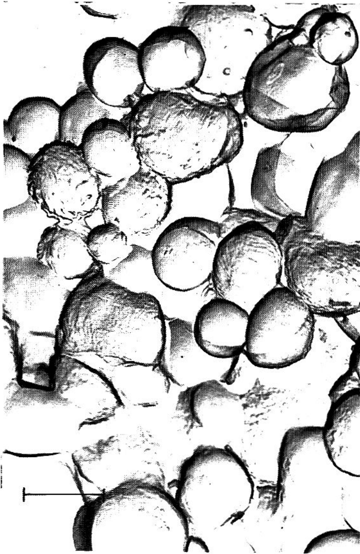

Fig. 5

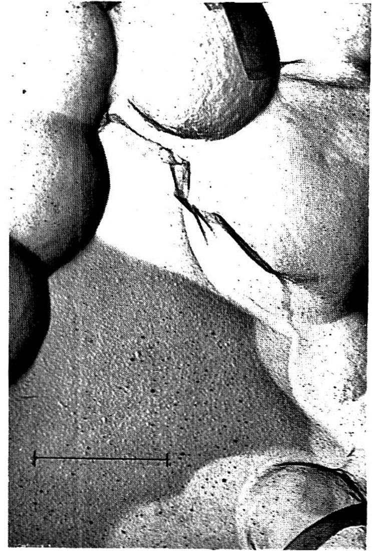

Fig. 6

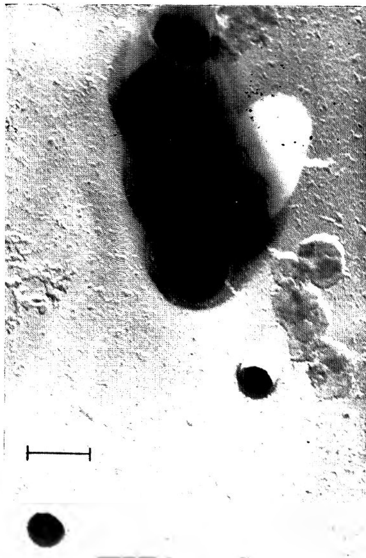

Fig. 7

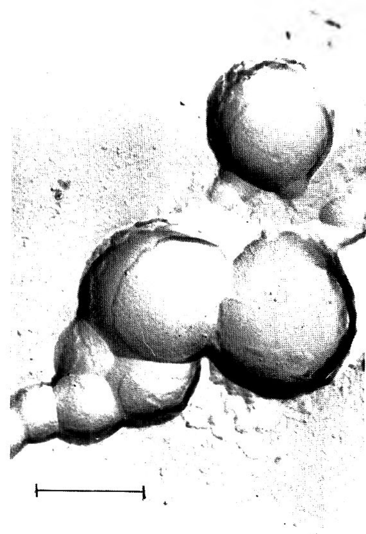

Fig. 8

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Fig. 9

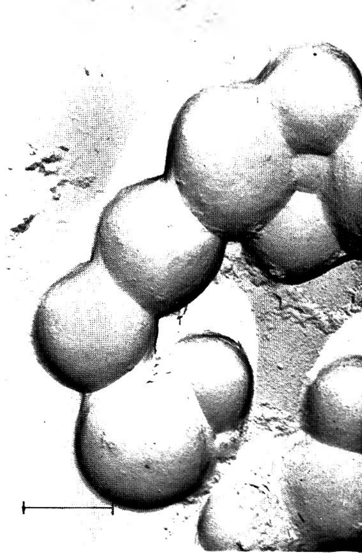

Fig. 10

Fig. 11

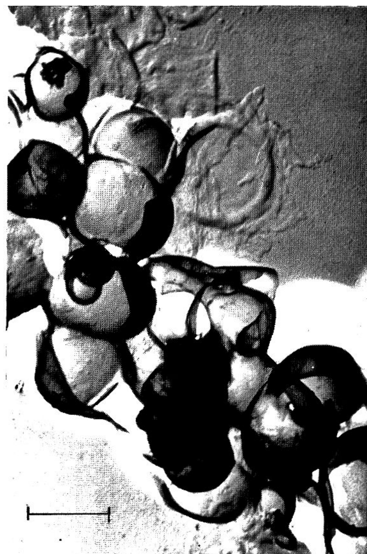

Fig. 12

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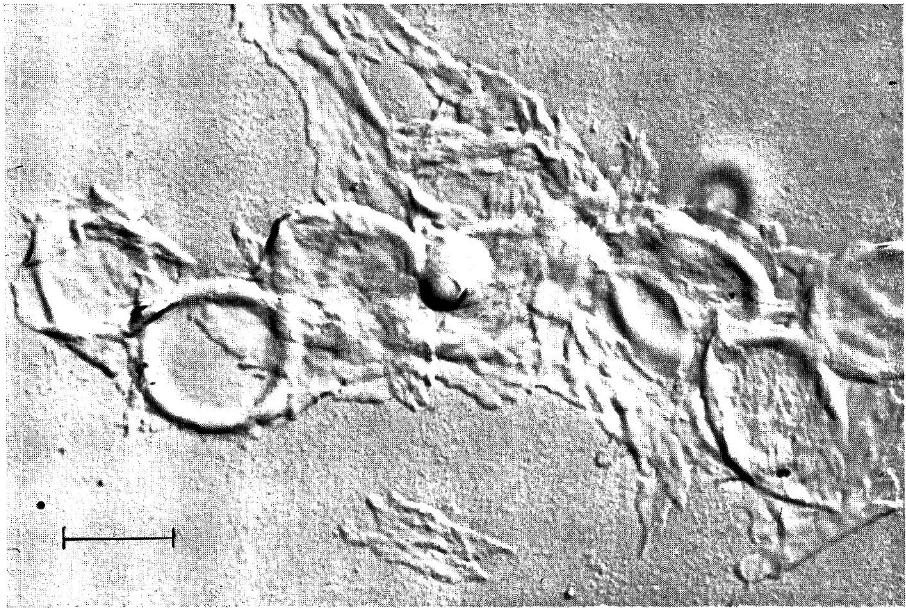

Fig. 13

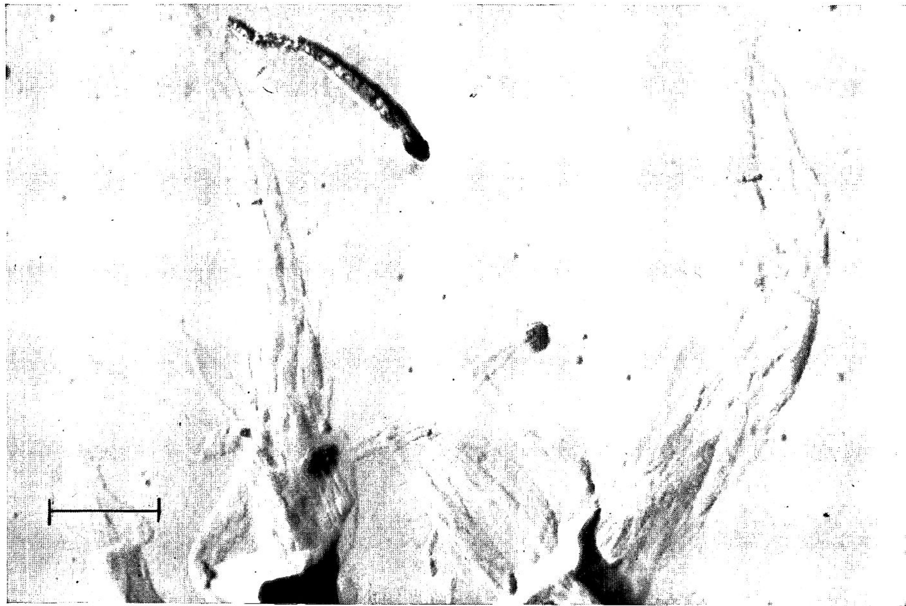

Fig. 14

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Cellulolytic Cocci Occurring in the Rumen of Sheep Conditioned to Lucerne Hay

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SUMMARY

A method is described for the isolation in pure culture of the predominant cellulolytic bacteria which occur in the ovine rumen. Ten isolates of cocci were obtained from the rumen contents of one sheep conditioned to lucerne hay, and were identified as *Ruminococcus albus*. All ten isolates degraded xylan in addition to cellulose and cellobiose and a comparative study of the end-products of fermentation of xylan and cellobiose was made. The rates of growth on cellulose and cellobiose were compared, and the role of these cocci in the breakdown of the fibrous part of the diet assessed.

INTRODUCTION

A complete ecological analysis of the rumen population, including information on identity, metabolic functions and nutrition of the individual microbial species is necessary for a more complete understanding of the metabolism of ruminants (Bryant, 1963). Although considerable advances have been made in recent years in knowledge of the most important species of bacteria in the rumen of cattle and sheep (Bryant, 1959; Hungate, 1960, 1963), little work has been directed specifically to study the relationships between the diet of the animal, the predominant types of bacteria in the rumen, and the end-products of fermentation which they make available to their host. A study of this nature with particular reference to typical diets consumed by sheep in South Africa has been undertaken in this laboratory (Gilchrist & Kistner, 1962). It has been shown (Kistner, Gouws & Gilchrist, 1962) that the predominant cellulolytic bacteria in the rumen of sheep fed lucerne hay are cocci which produce rhizoid colonies; these cocci rapidly decrease in number when the animals are changed to a ration of poor quality teff hay and are eventually replaced by rods as the major cellulolytic species (Kistner & Gouws, 1962).

As part of a programme of research to discover the reasons for these changes in the ecology of the rumen, several isolates of cocci from sheep fed lucerne hay were obtained and examined. This aspect of the work is reported in the present paper. Their relationship to the cellulolytic cocci isolated from the rumen of cattle by Bryant, Small, Bouma & Robinson (1958) is discussed. It was found that the organisms degraded xylan, in addition to cellulose and cellobiose, and a comparative study of the end-products of xylan and cellobiose metabolism was made. The role

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of these organisms in the breakdown of the fibrous part of the diet and their contribution to the fermentation products found in the rumen was also assessed.

METHODS

Cultures. The cultures studied included ten isolates of cellulolytic cocci obtained on three dates from counts in the $1/10^6$ dilutions in cellulose agar of rumen fluid from one sheep, K11, which had been conditioned for at least 4 months to lucerne hay, similar in composition to that used by Kistner *et al.* (1962). Strains FD 1, C 94, 7 and 20 were obtained through the courtesy of Dr M. P. Bryant.

Media for isolations. Cellulose agar medium (Kistner, 1960) from which Antifoam B was omitted, and a similar medium in which cellobiose (0·5 %, w/v) and cysteine.HCl (0·05 %, w/v), replaced the cellulose and sodium dithionite, were used.

Phosphate buffer. Sterile phosphate buffer 0·036 M (pH 6·8) containing cysteine.HCl (0·05 %, w/v) and indigodisulphonate (0·0005 %, w/v) was used for breaking up the colonies.

Fermentation tests. The basal medium for fermentation tests was similar to the basal medium of Kistner (1960) except for the omission of agar and Antifoam B, the replacement of sodium dithionite by cysteine.HCl (0·05 %, w/v), and the use of Seitz-filtered rumen fluid to give a clear medium. Thermostable substrates (e.g. cellulose, starch, esculin, sodium lactate) were added to the basal medium in final concentrations of 0·5 % (w/v), after which the medium was distributed in 5 ml. amounts in rubber-stoppered, screw-cap, 1 oz. bottles and autoclaved (121° for 25 min.). Stock solutions of thermolabile carbohydrates were sterilized separately by Seitz-filtration, added to the autoclaved basal medium in final concentrations of 0·5 % (w/v), and distributed with aseptic and anaerobic precautions.

Acetylmethylcarbinol formation. Basal medium containing cellobiose (0·5 %, w/v) was used for the Voges-Proskauer test.

Gelatin liquefaction. Basal medium + cellobiose (0·05 %, w/v) + gelatin (0·4 %, w/v) was used to test for proteolytic activity.

Determination of end-products of metabolism. Medium containing cellobiose (B.D.H., 0·5 %, w/v), or xylan (Nutritional Biochemicals Corporation, 0·5 %, w/v) in basal medium similar to that used for the fermentation tests was used. The complete medium was prepared in 50 ml. quantities in rubber-stoppered, screw-cap, 6 oz. bottles purged with O_2-free CO_2 and sterilized by autoclaving (121°, 25 min.).

Growth rate tests. The medium consisted of basal medium similar to that used for the fermentation tests with added cellobiose (0·5 %, w/v) or cellulose (1·2 %, w/v). The complete medium was prepared in 50 ml. quantities in 6 oz. bottles.

Isolations

Picking colonies. Colonies were picked by using a 2 ml. automatic syringe provided with a 15-gauge stainless steel needle, the bevel of which had been ground away and the front 0·25 in. bent to form a 90° elbow. The stopper of the roll bottle containing the colonies was removed under pressure of sterile O_2-free CO_2 (Kistner, 1960), and the bottle was thus continuously purged throughout the whole operation to protect the colonies against exposure to oxygen. The syringe and needle were

filled with buffer, and the flat end of the needle was located over a selected colony with the help of a slanted beam of light from a microscope lamp. The needle was driven through the agar until it made contact with the glass wall of the bottle over its entire circumference. By allowing the spring-loaded plunger of the automatic syringe to jump back through about 0·25 in., the colony was sucked into the front part of the needle. From here it was washed into the barrel of the syringe by drawing up about 1·5 ml. of buffer. After expelling any gas bubbles trapped in the syringe, the bent needle was replaced by a similar sterile straight one, and the air displaced from this needle by inverting the syringe and spilling a few drops of buffer. The plunger was then brought to the 1 ml. mark.

Dispersion. To break up the colony thus sucked into the syringe into single organisms as far as possible, the blunt needle of the syringe was forced through the vaccine cap of a second roll bottle until it came to rest firmly against the bottom. By depressing the plunger, the colony was forced out between the flat end of the needle and the smooth bottom of the bottle, thus shearing it apart. The buffer was then sucked back into the syringe and the process repeated until all the lumps of agar had been broken up and dispersed. The resulting suspension was drawn into the syringe and the needle withdrawn from the bottle.

Distribution. The blunt needle on the syringe was next replaced by a regular 20-gauge 1·25 in. hypodermic needle, and the air displaced from this by spilling a few drops of the suspension. The rest of the suspension was injected through the rubber stopper into a 1 oz. dilution bottle containing 9 ml. molten cellulose agar medium, and the contents of the bottle mixed by alternately filling and emptying the syringe ten times. One millilitre of this first dilution was withdrawn with the same automatic syringe and injected into the next dilution bottle. With the same automatic syringe this process was repeated until four tenfold dilutions of the original suspension had been prepared. Four roll bottle cultures (Kistner, 1960) were prepared from each dilution and incubated at 38° until growth was observed.

Transfers. The transfers on cellulose agar were repeated until the cultures looked pure on inspection of the colony forms and on microscopic examination of smears from at least three colonies stained by Gram's method. The cultures were then put through at least two successive transfers on cellobiose agar media to show up colonies of any contaminants which might have been overlooked on the opaque cellulose medium. When no contaminants were detected after microscopic examination of about one-third of the colonies in a roll bottle of the fourth dilution, the culture was regarded as pure, and it was transferred back to cellulose agar medium.

Maintenance. Stock cultures were maintained on cellobiose slopes and kept in a box with solid CO_2. They were subcultured every 6–8 months.

Inoculum. The stock culture from the solid CO_2 box was inoculated to a cellobiose slope and incubated for 18 hr at 38°. The resulting growth was suspended in 2 ml. buffer, with anaerobic precautions, and 0·1 ml. portions of the suspension used for all inoculations except for the growth rate tests, for which a denser suspension from a 24 hr culture was used.

Physiological tests

Fermentation tests. The fermentation tests listed in Table 1 were carried out at 38° and read after 1, 2, 3 and sometimes 7 days of incubation.

Growth rate tests. The liquid media mentioned above were warmed to 38°,

inoculated, and, after thorough mixing, samples (1 ml.) of culture were withdrawn for zero-time counts in cellobiose agar (Kistner, 1960). Further counts were made after incubation for 18, 24 and 48 hr.

End-products of metabolism

The end-products of cellobiose or xylan fermentation by the strains of cocci listed in Table 3 were determined after incubating for 48 hr at 38°.

Gas. The gas produced was extracted quantitatively by a Toepler pump and transferred to the chamber of a Van Slyke manometric apparatus. Carbon dioxide was removed by absorption and residual H_2 and CH_4 were determined by combustion (Peters & Van Slyke, 1932).

Total organic acids. The method of Neish (1952) was used to clarify the culture liquid and to determine total organic acids.

Lactic acid. The method of Elsden & Gibson (1954) was used.

Succinic acid was estimated according to Bril (1954).

Ethanol. Neutral volatile compounds were determined according to Neish (1952). These consisted almost entirely of ethanol as found by analysing samples of the distillate by an alcohol-dehydrogenase method based on that of Bücher & Redetzki (1951).

Volatile fatty acids. Total and individual volatile fatty acids were determined by the gas-liquid chromatographic method of James & Martin (1952).

RESULTS

Characteristics of the isolates

The main characteristics of our strains are shown in Table 1.

Colony form. In the thin films of cellulose agar in the roll bottle cultures, all ten of our isolates produced typical rhizoid colonies surrounded by circular zones of cellulolysis which increased in size with the age of the culture. The more colonies there were in a culture, the smaller they remained. Whether this was due to competition for a nutrient present in limiting concentrations or to inhibition by fermentation end-products is uncertain. Surface colonies on cellobiose agar were white, circular and convex; deep colonies in this medium were spindle-shaped.

Morphology and Gram reaction. The cocci occurred mostly in pairs, with a few single ones. Chain formation was never observed. The mean diameter of the cocci from 24 hr cultures on cellobiose liquid medium was 1·0–1·1 μ. Capsule formation was frequently observed on cellulose agar and on cellobiose agar. Spores were never found. The Gram reaction of all our isolates was variable, even in 18-hr cultures. With the exception of isolates 19.08.6 C and 19.08.6 G, the proportion of Gram-positive to Gram-negative cocci in thin smears was usually very low.

Fermentation tests. All ten of our isolates consistently showed growth on cellulose, cellobiose or xylan within 48 hr of incubation. Three isolates fermented no other carbohydrates; the remaining seven isolates fermented D-mannose, though this was not always evident within 48 hr of incubation. Three of the latter isolates also fermented glucose, but with two isolates growth was then obviously slower than on cellobiose medium. Isolate 22.08.6 A showed slow growth on fructose. On cellobiose medium the final pH value was between 5·9 and 6·2, appreciably higher than the

Table 1. *Characteristics of ten isolates of cellulolytic cocci from lucerne hay-fed sheep*

Each sign indicates a separate determination.

Isolate	19.08.6C	19.08.6G	22.08.6A	22.08.6B	22.08.6F	21.09.6A	21.09.6B	21.09.6E	21.09.6F	21.09.6G
Yellow pigment	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −
Gram reaction	Variable	Variable	Variable	Variable	Variable	Variable	Variable	Variable	Variable	Variable
Occurrence in chains	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −
Mean diameter (μ)	1·1	1·1	1·0	1·0	1·1	1·0	1·0	1·0	1·1	1·1
Voges-Proskauer test	+ +	− −	+ +	− −	− −	+ +	+ +	+ −	+ +	+ +
Gelatin liquefaction	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −
Growth on:*										
Arabinose (L)	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −
Rhamnose (L)	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −
Xylose (D)	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −
Glucose (D)	+ − −	− − −	+ + +	− − −	− − −	− − −	− − −	− − −	+ + −	− − −
Fructose	− − −	− − −	− − +	− − −	− − −	− − −	− − −	− − −	− − −	− − −
Galactose	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −
Mannose (D)	+ + −	− − −	+ + +	− − −	+ + −	+ + −	+ + +	− − −	− − −	− − −
Lactose	− − −	− − −	+ + −	− − −	− − −	− − −	− − −	− − −	− − −	− − −
Sucrose	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −
Maltose	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −
Trehalose	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −
Cellobiose	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + −	+ + +
Raffinose	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −
Starch	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −
Xylan	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Cellulose	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Esculin	−	−	−	−	−	−	−	−	−	−
Lactate	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −	− − −

* Within 48 hr of incubation.

values reported by Bryant *et al.* (1958) for their cultures of cellulolytic cocci, probably because the medium used by these authors was less heavily buffered with bicarbonate than was ours. None of the ten isolates fermented arabinose, rhamnose, xylose, galactose, lactose, sucrose, maltose, trehalose, raffinose, starch, esculin or lactate. Gelatin liquefaction was not observed even after extended incubation periods, nor was any yellow pigment produced in any of the media used. Six of the isolates gave a positive Voges–Proskauer reaction, while one, isolate 21.09.6 E, was variable in its reaction.

Rate of growth on cellulose and cellobiose. The rapid growth of isolate 19.08.6 G (Table 2) in liquid media containing cellulose or cellobiose was followed by a steep decline in the number of viable organisms. The form of the growth curve on cellobiose medium shows much resemblance to the curves obtained by Bryant & Robinson (1961) by plotting optical densities of Ruminococcus cultures against incubation time. The decrease in optical density found by these authors immediately after the growth maximum had been reached (whether due to lysis of organisms or to other causes) would coincide with the progressive decline of viability in the present experiment.

Table 2. *Growth rate of cellulolytic coccal isolate 19.08.6 G on liquid media containing cellulose or cellobiose*

Incubation time (hr)	Cellulose liquid medium (Organism/ml.)	Cellobiose liquid medium (Organism/ml.)
0	$4{\cdot}5 \times 10^{4}$	24×10^{4}
18	7×10^{7}	3×10^{8}
24	$1{\cdot}5 \times 10^{8}$	$11{\cdot}5 \times 10^{7}$
48	2×10^{6}	$1{\cdot}5 \times 10^{6}$

Degradation of cellulose. The first sign of activity of the cultures on cellulose liquid medium was a viscidity of the cellulose, similar to that observed by Skinner (1960) in cultures of a cellulolytic soil organism. This was first observed after incubation for about 18 hr. At 24 hr the disappearance of cellulose became obvious while the stickiness decreased; at 48 hr the residual sediment occupied about one-third of the volume of the cellulose present in an uninoculated control and was of a looser and finer structure.

End-products of fermentation

The values for the amounts of end-products from cellobiose and xylan fermentations as reported in Table 3 for our own cultures are the means of determinations on at least two dates, whereas only a single estimation was run on Bryant's cultures in most cases. The concentrations of the end-products found in 48 hr cultures on different dates were very consistent (Table 3).

Xylan fermentation. In the fermentation of xylan the amounts of formic, acetic and lactic acids and ethanol by isolates 19.08.6 C, 19.08.6 G, 21.09.6 A, 21.09.6 B and 21.09.6 E were very similar. These values were of the same order as those found in the corresponding cultures on cellobiose medium. In the case of Bryant's cultures FD 1, C 94, 7 and 20, the agreement between the different strains was not as good. Whereas strains 7 and 20 produced large amounts of ethanol from xylan, strains FD 1 and C 94 produced only insignificant amounts. However, the amounts of formic,

acetic and lactic acids and ethanol produced by each of Bryant's strains on xylan were in fair agreement with the values obtained on cellobiose liquid medium.

Cellobiose fermentation. The fermentation of cellobiose in the liquid medium containing 0·5 % of this substrate did not proceed to completion. Repeated qualitative paper chromatographic tests showed that considerable quantities of cellobiose were still present in the medium when fermentation stopped. It is not known what the limiting factor in the fermentation was, but the different strains of cocci seem to have been affected in much the same way. The recovery of carbon in the end-products was remarkably consistent for our ten isolates, ranging from 30·9 % of the carbon supplied as cellobiose in the case of isolate 19.08.6 C to 37·0 % for isolate 22.08.6 B.

Table 3. *End-products of fermentation (mmole/100 ml.)**

	In rumen fluid-cellobiose medium						In rumen fluid-xylan medium			
			Acids					Acids		
Culture	Hydrogen	Ethanol	Formic	Acetic	Lactic	Succinic	Ethanol	Formic	Acetic	Lactic
19.08.6 C	0·04	0·87	1·38	1·18	0·02	0·0	0·98	1·14	1·06	0·02
19.08.6 G	0·85	1·07	1·18	1·13	0·06	0·0	0·93	1·29	1·14	0·01
22.08.6 A	0·09	1·03	1·58	1·30	0·02	0·0	—	—	—	—
22.08.6 B	0·87	1·24	1·36	1·39	0·02	0·0	—	—	—	—
22.08.6 F	0·64	1·07	1·33	1·39	0·02	0·0	—	—	—	—
21.09.6 A	0·13	1·11	1·26	1·31	0·02	0·0	1·14	1·21	1·10	0·02
21.09.6 B	0·09	1·05	1·42	1·29	0·02	0·0	0·94	1·06	0·91	0·02
21.09.6 E	0·13	1·00	1·35	1·37	0·01	0·0	1·12	1·21	1·14	0·02
21.09.6 F	0·07	1·05	1·38	1·34	0·01	0·0	—	—	—	—
21.09.6 G	0·12	1·05	1·58	1·48	0·02	0·0	—	—	—	—
FD_1	0·05 (0·0)†	0·10 (0·0)	1·27 (3·12)	1·38 (2·38)	0·03 (0·14)	0·80 (1·76)	0·04	0·53	0·83	0·02
C94	0·10 (0·0)	0·01 (0·0)	0·66 (2·72)	0·62 (2·32)	0·07 (0·52)	1·47 (1·85)	0·04	0·98	1·06	0·05
7	0·83 (0·74)	1·20 (3·65)	1·45 (3·74)	1·25 (3·46)	0·02 (0·0)	0·0 (0·11)	1·46	1·29	1·14	0·02
20	0·06 (0·0)	0·80 (1·85)	1·42 (3·14)	1·22 (1·38)	0·06 (0·34)	0·0 (0·0)	0·95	1·29	1·36	0·02

* After subtraction of the blank values for the different metabolites, obtained by analysis of batch of medium used. The range of values for the different batches, expressed as mmole/100 ml., was—ethanol: 0·03–0·09; formic acid: 0·40–0·46; acetic acid: 0·46–0·76; lactic acid: 0·01–0·02; succinic acid: nil.

† Figures in parentheses are values reported by Bryant *et al.* (1958) for the same cultures.

This did not include any fermentation $-CO_2$ formed, since the bicarbonate-carbonic acid buffer system of the medium precluded determination of this end-product. The carbon recoveries which may be calculated from the data of Bryant *et al.* (1958) for members of their Group II vary from 31·4 % for strain $B_3$36 to 102·4 % for strain 7; in comparison, the recovery for strain 7 on our medium was only 36·5 %.

On the whole our ten isolates of cocci were very uniform in their production of ethanol, formic, acetic and lactic acids, but hydrogen production by isolates 19.08.6 G, 22.08.6 B and 22.08.6 F was consistently much higher than by the remaining seven isolates. None of the ten isolates produced succinic acid. The values obtained for two of Bryant's cultures, namely 7 and 20 belonging to his

Group II, were very similar to those obtained for our isolates. However, strains FD 1 and C 94 belonging to Bryant's Group I produced large amounts of succinic acid with lesser amounts of ethanol.

The agreement between the values reported by Bryant *et al.* (1958) for strains FD 1, C 94, 7 and 20 and those found in the present study is not very good. Not only were the amounts of formic and acetic acids produced rather lower on our media, but the clear-cut differences between typical members of Group I and Group II with respect to hydrogen and ethanol production, found by these authors, are not nearly as apparent from the present data. On the other hand, whereas Bryant *et al.* (1958) regarded strain 20 as an atypical member of their Group II because no hydrogen was detected in the culture, a low production of hydrogen was found in the present study, which would bring strain 20 into line with the other members of the group.

The differences between the concentrations of the end-products of strains FD 1, C 94, 20 and 7 reported by Bryant *et al.* (1958) and those found in the present work may stem from differences in the composition of the media used. Both media are derived from that described by Hungate (1950) and are similar in the mineral constituents and buffer system. However, Bryant *et al.* (1958) added 0·5 % trypticase to their medium and decreased the concentration of rumen fluid to 20 % (v/v) as compared to 30 % (v/v) in Hungate's and our media. That the latter change may influence the proportions of end-products of carbohydrate fermentation formed in the culture is shown by the work of Gill & King (1958) who found marked shifts in the fermentation pattern of *Butyrivibrio fibrosolvens* when the proportions of basal medium and rumen fluid supplement were altered. Furthermore, the source of rumen fluid and the time at which it is drawn in relation to the feeding time of the animal are bound to affect the concentration of growth factors in the medium and may, therefore, influence the extent and course of carbohydrate fermentation by the cultures. It is also conceivable that the relative volumes of the culture and the gas space above it may play a part by determining the increase in the pressure of CO_2 in the gas phase during fermentation and consequently determining the concentration of carbonic acid in the culture. This in turn might control the equilibrium of enzymic processes in which CO_2 participates. All these factors hamper the comparison of the characteristics of new isolates with the published results of other workers, and it is possible that some of the nuances in the physiology of isolates described by different authors may have been caused by differences in the environmental conditions rather than by real differences in the enzymic constitution of the organisms. This situation will only be remedied when a medium of known composition and without rumen fluid supplement is found to support the growth of all Ruminococcus strains.

DISCUSSION

The characteristics of all our ten isolates fit the description of one species *Ruminococcus albus* (Hungate, 1957) as amended by Bryant *et al.* (1958). Although the isolates differed in Voges–Proskauer reaction, in fermentation of glucose, mannose or fructose and in the amount of hydrogen produced in the fermentation of cellobiose, these differences did not justify a division into groups.

The seven strains of this species studied in detail by Bryant *et al.* (1958) were all

obtained from the rumen of cows fed lucerne hay, lucerne hay + grain or clover pasture, where they occurred in numbers of 10^8/ml. or more. On the other hand, *Ruminococcus albus* strains examined by Hungate (1957) were isolated from the fifth or sixth dilutions of rumen contents from cows fed timothy hay + concentrates, where they were often outnumbered by other cellulolytic species, especially by members of the genus *Butyrivibrio.* In both cases *R. albus* was not the only species of cellulolytic coccus found, *R. flavefaciens* and intermediate types occurring either in the same animal or in animals on the same diet. Bryant *et al.* (1958) mentioned specifically that widely different strains of Ruminococcus were isolated from the same sample of rumen contents. In contrast, the predominant cellulolytic cocci of our sheep K 3, K 4 and K 8 (Kistner *et al.* 1962), and K 11 conditioned to lucerne hay, were very uniform in morphology, Gram reaction and colony shape on cellulose agar; the ten isolates examined in detail all belonged to the species *R. albus.* Yellow colonies consisting of cocci in chains were never observed in high dilutions of rumen contents of sheep on this diet; only recently have we found organisms which answer to the description of *R. flavefaciens*, in rumen samples from sheep shortly after a change in diet from lucerne hay to poor quality teff hay. It is very difficult to ascribe the uniformity of our predominating cellulolytic cocci to any factors which differed from those operating in the experiments of the above-mentioned workers. It seems unlikely that the absence of colonies of other cellulolytic bacteria in high dilutions of rumen contents from sheep fed lucerne hay was due to selective action of the cellulose agar medium, considering that the same medium regularly supported growth of various Butyrivibrio species from sheep which were fed teff (*Eragrostis tef*) hay. Whether differences in the management of the animals might be responsible for the differences between the findings of different workers who have examined the cellulolytic bacteria of cattle and sheep conditioned to lucerne (or alfalfa) hay is difficult to assess, since the literature contains little reference to such details as exact composition of ration and length of the conditioning period.

From the spectrum of carbohydrates fermented by the ten coccal isolates obtained in the present work, it would appear that these organisms specialize in the fermentation of the polysaccharides associated with the fibrous portion of the diet. In fact, all ten isolates attacked xylan but did not grow on xylose, and seven isolates exhibited the peculiarity reported for aerobic and anaerobic cellulose-digesting bacteria from different habitats (McBee, 1948; Hungate, 1950; Sijpesteijn, 1951; Hall, 1952; Hulcher & King, 1958; Ayers, 1958; Enebo, 1949) namely, preference for cellulose or cellobiose rather than glucose. The efficiency of our cocci in carrying out the function of cellulose degradation is suggested by the fact that the growth rate *in vitro* in a medium containing insoluble cellulose did not lag far behind that in a similar medium with cellobiose.

Of the end-products of cellobiose fermentation found in pure cultures of the ten isolates of cellulolytic cocci, only acetic acid occurs in appreciable quantities in the rumen of sheep fed lucerne hay. Formic acid and hydrogen normally do not accumulate to any extent in the rumen under most conditions 'no doubt because they are very active intermediates and are used in a range of reducing reactions as soon as they become available' (Annison & Lewis, 1959). Beijer (1952) showed that the *in vitro* incubation of sodium formate with goat rumen fluid led to considerable methane formation, while McNeill & Jacobson (1955), with bovine rumen bacteria, concluded

that the reduction of carbon dioxide constitutes the main pathway for methane production in the rumen and that the availability of hydrogen was the chief limiting factor in this reaction. Smith & Hungate (1958) isolated from high dilutions of rumen contents from cattle and one sheep a methanogenic organism, *Methanobacterium ruminantium*, which utilized hydrogen and formic acid, but no other substrates, in the formation of methane.

Ethanol has been found in the rumen of lambs (Cunningham & Brisson, 1955) but normally does not accumulate in the rumen. Moomaw & Hungate (1963) concluded that 'the explanation for ethanol formation in pure cultures may be that it is essential as a repository for hydrogen', whereas 'in mixed culture, such as in the rumen, the hydrogen concentration is kept low (0·05 % in the rumen) by participating in exergonic reactions forming methane, propionic acid, and butyric acid'.

Although it is known that xylans are readily and extensively metabolized in the rumen (Heald, 1953) and that similar concentrations of end-products are formed in the fermentation of cellulose and hemicellulose by mixed suspensions of rumen organisms (Gray & Pilgrim, 1952; Bath & Head, 1961), to our knowledge no work has been done on the end-products of xylan fermentation by pure cultures of cellulolytic rumen bacteria. Hobson & Purdom (1961) compared the end-products of xylose and glucose fermentation by two pure cultures representative of the xylan-degrading bacteria found in two sheep on a diet of hay + grass cubes. In both cultures they found qualitative and quantitative differences in the organic acids produced from these two sugars. The present work has shown that the concentrations of ethanol, and of formic, acetic and lactic acids produced in the fermentation of cellobiose and xylan by fourteen strains of cellulolytic cocci were in good agreement. This suggests that the terminal pathway of fermentation may be the same for both substrates; it is conceivable that the fermentation of xylan proceeds through a pathway which involves hexose synthesis (Wood, 1961).

Reviewing the information about the ten isolates of cellulolytic cocci we obtained from lucerne hay-fed sheep, it is clear that they are functional rumen bacteria in the sense of Elsden & Phillipson (1948). Not only were these cocci capable of attacking cellulose and xylan, which together constitute more than one-third of lucerne hay, but they formed fermentation products which are known to occur in the rumen of sheep fed this diet or which are likely to be converted to such end-products by other rumen bacteria. Furthermore, these cocci occurred at concentrations of about 10^7/ml. rumen fluid and their growth rate on cellulose *in vitro* under conditions aimed to resemble the rumen milieu was such that they could be expected to maintain themselves in the flowing system of the rumen. The significance of these organisms as functional cellulose digesters in the alimentary tract of herbivores is stressed by the fact that they were found to be among the more numerous cellulolytic bacteria in the rumen of several different sheep at different periods.

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Accelerated Death of *Aerobacter aerogenes* Starved in the Presence of Growth-Limiting Substrates

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SUMMARY

Substrate-accelerated death (Postgate & Hunter, 1963*a*) was observed with glycerol-, glucose-, ribose-, ammonium- or phosphate-limited populations of *Aerobacter aerogenes* grown in defined media and starved in non-nutrient buffer; sulphate- or magnesium-limited organisms did not show this. Glucose or pyruvate accelerated death of starved populations obtained from a complex medium. Lactate-accelerated death of *Escherichia coli* and glucose-accelerated death of *Serratia marcescens* were also observed with populations of appropriate nutritional status. Glycerol-accelerated death of glycerol-limited *A. aerogenes* occurred with organisms from batch or continuous cultures grown at various pH values; it showed a population effect and was particularly pronounced in 0·15 M-NaCl buffered with phosphate. Continued presence of glycerol was necessary and the glycerol was metabolized. Survivors showed prolonged division lags. Tricarboxylic acid cycle intermediates, but not glucose or ribose, also accelerated death. Glycerol-accelerated death was not delayed by malonate, fluoride or fluoraeetate; iodo-acetate delayed its onset but did not affect its rate; 'uncoupling' agents antagonized it though they were themselves toxic. Glycerol-accelerated death was not accompanied by accelerated breakdown of the osmotic barrier, nor by leakage of materials associated with cold shock nor by acquiring sensitivity to cold shock. No catabolism of DNA or protein accompanied it; polysaccharide was synthesized; no change in the rate of degradation of RNA was observed. Coloured substances, pyridine nucleotides, white-fluorescent material and material which absorbed at 220–230 mμ were released during glycerol-accelerated death. Magnesium ions prevented glycerol-accelerated death.

INTRODUCTION

Postgate & Hunter (1963*a*) showed that substrates which had limited the growth of populations of certain Gram-negative bacteria could accelerate their death when they were subsequently starved in non-nutrient buffer. The phenomenon was termed 'substrate-accelerated death' and was shown with nitrogen-, phosphate- and carbon-limited bacterial populations. Sulphate-limited populations differed in that the carbon source, not sulphate, accelerated death. Magnesium-limited populations did not show substrate-accelerated death. The present paper describes substrate-accelerated death in fuller detail and presents data which exclude certain possible interpretations of glycerol-accelerated death of glycerol-limited *Aerobacter aerogenes*.

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METHODS

Organisms and culture. The variant strain of *Aerobacter aerogenes* NCTC 418, described by Postgate & Hunter (1962), was maintained in the same continuous culture apparatus and was in its third and fourth year of growth at a dilution rate of 0·25 hr^{-1}, temperature 40° and pH value 7·0 ± 0·1. Growth was limited to a yield equivalent to 1–1·1 mg. dry wt. organism/ml. (about $2{\cdot}3 \times 10^9$ bacteria/ml.) by the glycerol concentration (2 g./l.) in the defined medium previously described. (Yields of organism and population densities will be expressed in 'mg./ml.', implying 'equiv. dry wt. bacteria/ml.'.) For growth in other nutritional conditions a second chemostat was inoculated from the first 'master' culture and with the modified media and conditions described by Postgate & Hunter (1962; in that publication the sulphate-limiting medium was incorrectly described [see addendum p. 473). For glucose or ribose limitation the regular medium was used with 2 g./l. of these compounds in place of glycerol; the yields were: 0·95–1 mg./ml. from glucose, and 0·85–0·9 mg./ml. from ribose. In a few experiments organisms other than *A. aerogenes* NCTC 418 were used.

Viability was determined by slide culture (Postgate, Crumpton & Hunter, 1961), except where mentioned, on the glycerol medium supplemented with casein hydrolysate, yeast extract and Douglas's meat digest broth (*Medical Research Council*, 1931); with glucose- or ribose-limited organisms the appropriate compound replaced glycerol. Incubation periods of 4–5½ hr were used for slide culture of populations dying in the presence of substrate because the survivors of substrate-accelerated death showed long division lags (see below).

Starvation. For reasons given in the text two main procedures for starving organisms were used during this work. The 'saline tris procedure' was that described by Postgate & Hunter (1962): the organisms were washed twice in saline, suspended in distilled water and diluted to 20 μg./ml. for starvation at 40° in aerated 0·15 M-NaCl buffered to pH 7·0 ± 0·1 with tris and containing a trace of ethylenediamine-tetra-acetate. The 'saline phosphate procedure' avoided exposure to distilled water: organisms were washed only once and the saline was buffered to pH 6·3 (optimal for survival of the strain) or to pH 7·3 with 10 % (v/v) of M/15-sodium potassium phosphate buffer.

Optical and analytical procedures. These were mostly described previously (Postgate & Hunter, 1962). Glycerol was estimated by oxidation with periodate followed by colorimetric determination of formaldehyde with chromotropic acid (Neish, 1950). Magnesium was determined by the Titan Yellow procedure of Garner (1946), scaled down 10-fold and omitting treatment with trichloroacetic acid. Whole bacteria were wet-ashed by repeated evaporation with HNO_3 in the presence of H_2SO_4 before analysis for Mg.

RESULTS

Circumstances in which substrate-accelerated death occurred

Aerobacter aerogenes. When organisms were harvested from continuous culture in the glycerol medium with glycerol limiting growth, and were then starved in saline tris buffer, they showed accelerated death with 10 mM-glycerol; the pH value remained unchanged throughout. Other major components of the growth medium

(sulphate, phosphate, potassium, ammonium) had no influence on the death rate but certain trace elements (Mg^{2+}, Ca^{2+}; and to some extent Fe^{3+}) were protective (Postgate & Hunter, 1962). Further experiments, with the saline tris procedure, showed that glucose (10 mM) or ribose (10 mM) did not accelerate death, though dye-reduction tests indicated that both these substrates were metabolized by the organisms (Table 1). A continuous culture limited by ribose was therefore set up. The population did not show significant ribose-accelerated death when subjected to

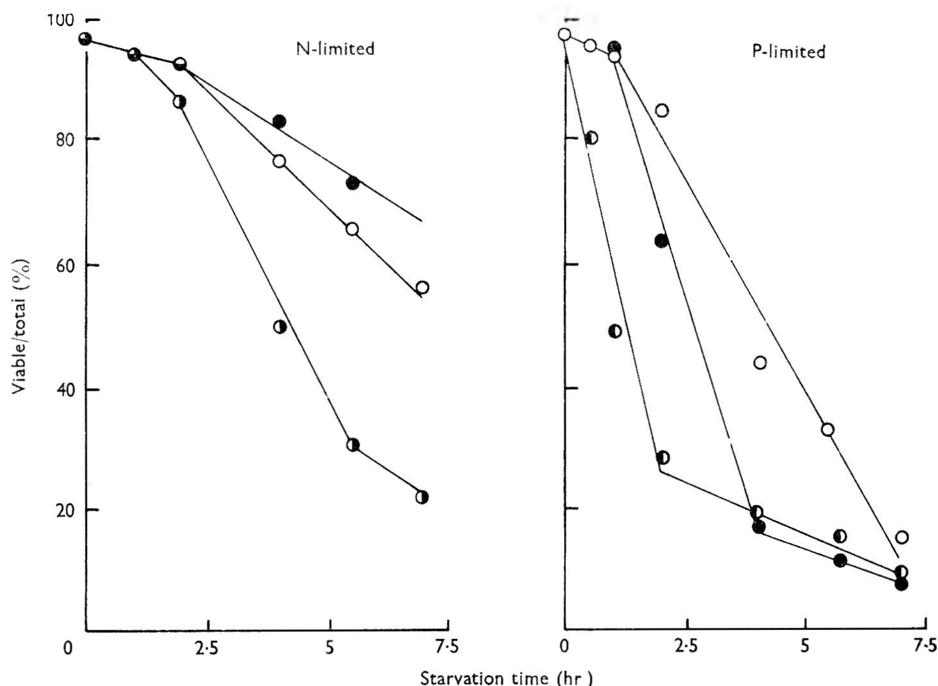

Fig. 1. Substrate-accelerated death of nitrogen- and phosphate-limited *Aerobacter aerogenes*. Organisms harvested from continuous culture, washed and starved at about 20 μg./ml. in aerated saline tris buffer (pH 7·0) at 40°. Viabilities were determined by slide culture (see text). ○, control population; ●, + 10 mM-glycerol; ◑, + 45 mM-NH_4Cl; ◐, + 10 mM-KH_2PO_4.

Table 1. *Reduction of methylene blue by glycerol-limited Aerobacter aerogenes*

Aerobacter aerogenes organisms from continuous culture were aerated for 15 min. at 40° to remove residual glycerol and 2 ml. portions (equiv. 2 mg. dry wt. organisms) added to 4 ml. portions of saline tris buffer (pH 7·0), containing 10 mM substrates, in Thunberg tubes. After evacuation and equilibration for 6 min. at 40°, 0·5 ml. methylene blue was added (final concn. 0·67 mM) and the decolorization times noted. Means of duplicates are quoted.

Substrate	Decolorization time (min.)
Glycerol	1·16
Glucose	1·83
Ribose	4·85
None	30

the saline tris procedure but a pronounced effect occurred with the saline phosphate procedure (e.g. 8·7 % died/hr in saline phosphate (pH 7·3); 83 % were dead in 0·5 hr with buffer + 10 mM-ribose). Glycerol also accelerated death (93 % dead in 0·5 hr with 10 mM glycerol). A glucose-limited continuous culture gave a population that showed slight glucose-accelerated death with the saline tris procedure but a pronounced effect with saline phosphate (death rate: 17 %/hr at pH 7·3; 94 %/hr with 10 mM-glucose). Continuous cultures in which nutrients other than the carbon-*cum*-energy source limited growth were set up to observe whether glycerol or the limiting

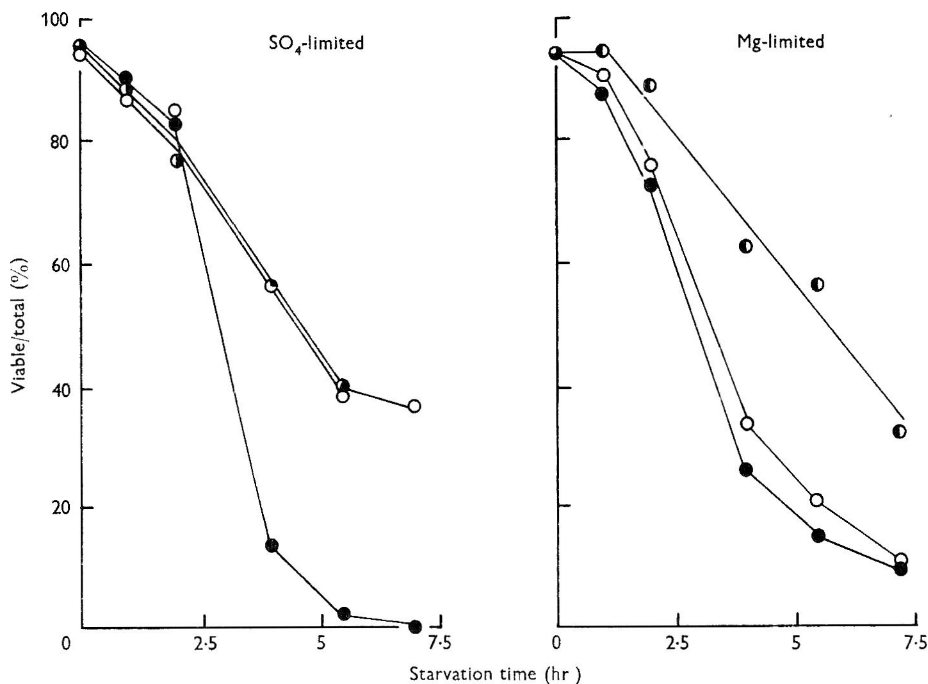

Fig. 2. Effect of substrates on death of sulphate- and magnesium-limited *Aerobacter aerogenes*. Organisms tested as for Fig. 1. ○, control population; ●, + 10 mM-glycerol; ◑, + 10 mM-Na_2SO_4; ◐, + 1·25 mM-$MgCl_2$.

nutrient accelerated death. With the saline tris procedure N-limited organisms showed NH_4-accelerated death, but glycerol delayed death; P-limited organisms showed PO_4-accelerated death and glycerol also accelerated death, though to a lesser extent (Fig. 1). S-limited organisms did not show accelerated death with sulphate but glycerol was active (Fig. 2). Mg-limited organisms showed delayed death with $MgCl_2$ and sometimes with glycerol (Fig. 2). A population of *Aerobacter aerogenes* NCTC 418 was grown in Douglas's meat digest broth and we confirmed the observation of Strange, Dark & Ness (1961) that glucose (10 mM) accelerated its death in saline phosphate at pH 6·5. Sodium pyruvate (10 mM) was also active (Fig. 3); data illustrating the acceleration of death of mannitol-limited stationary phase *A. aerogenes* by mannitol or glucose were quoted by Postgate & Hunter (1963*a*).

Other bacteria. Fig. 4 illustrates accelerated death of lactate-limited *Escherichia coli* (Jepp) by lactate or pyruvate, and of glucose-limited *Serratia marcescens* (M 148) by glucose, both from batch cultures. In general, substrate-accelerated death was less pronounced with stationary phase cultures, and the appearance of the slide cultures of the dying populations suggested to us that cryptic growth (see Postgate & Hunter, 1962) was masking the phenomenon. Cryptic growth was obvious as an increase in optical density of the suspension 'starved' with substrate in unsuccessful attempts to demonstrate substrate-accelerated death with mannitol-limited *Bacillus subtilis*, acetate-limited *Pseudomonas ovalis* and glucose-limited *Candida utilis*.

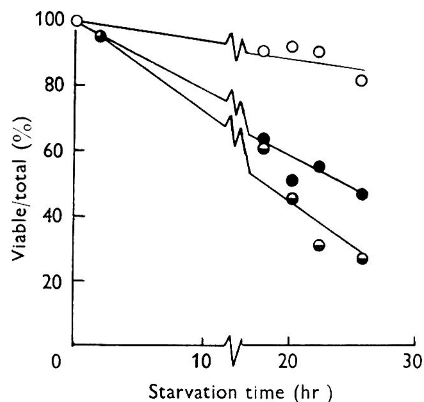

Fig. 3

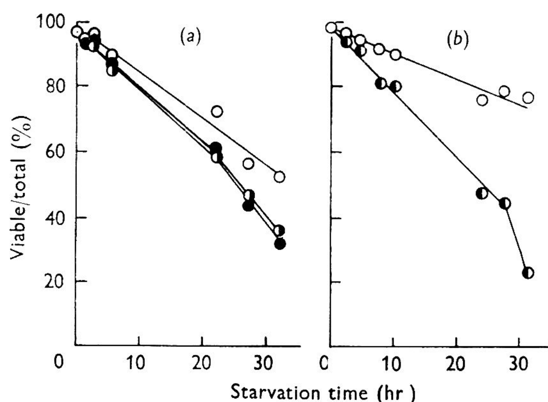

Fig. 4

Fig. 3. Effect of substrates on death of stationary phase *Aerobacter aerogenes* from a complex medium. Organisms grown at 37° in Douglas's meat digest broth were washed twice and starved (at about 20 μg./ml.) in aerated saline phosphate buffer (pH 6·5) at 37°. Viabilities by slide culture on Douglas's meat digest agar. ○, control; ◒, +10 mM-glucose; ●, + 10 mM-sodium pyruvate.

Fig. 4. Substrate-accelerated death of *Escherichia coli* and *Serratia marcescens*. *E. coli* strain Jepp, and *S. marcescens* M148 were grown at 37° in lactate+salts and glucose+salts defined media, respectively, in shaken flasks containing sufficient air to allow complete oxidation of all of the carbon substrate provided. When in the stationary phase, the organisms were washed and starved at about 20 μg./ml. in aerated saline phosphate buffer (pH 6·5) at 37°. Viabilities were determined by slide culture on the medium mentioned in the text with sodium lactate or glucose in place of glycerol as appropriate. (*a*) *E. coli*; (*b*) *S. marcescens*. ○, control population; ◑, +10 mM-sodium lactate; ●, + 10 mM-sodium pyruvate; ◐, + 10 mM-glucose.

Glycerol-accelerated death of Aerobacter aerogenes

Effect of washing and storage procedure. Postgate & Hunter (1962) found that a brief exposure to distilled water experienced during preparation for starvation by the saline tris procedure committed the organisms to a faster death rate than would otherwise have occurred. Omission of exposure to distilled water did not abolish glycerol-accelerated death in saline tris buffer. Postgate & Hunter (1962) also reported that the growth pH value of 7·0 was inferior to pH 6–6·5 for survival during starvation. Since tris does not buffer appreciably at pH values below about 7·0 saline phosphate was adopted for general use unless comparison with earlier work with saline tris was required. At comparable pH values, glycerol-accelerated death was more pronounced in saline phosphate buffer than in saline tris buffer: at pH 7·3,

70 ± 10 % of the populations were dead in 1 hr in saline phosphate buffer + 10 mM-glycerol compared with 50 ± 10 % dead/hr in saline tris buffer + glycerol. Control populations without glycerol died at 12–15 %/hr in either buffer.

Effect of washing procedure. Starvation by the saline phosphate procedure at pH 7·3 or 6·3 allowed pronounced glycerol-accelerated death, despite the absence of a pre-exposure to distilled water. When organisms were pre-exposed to distilled water, as for the saline tris procedure, their rates of glycerol-accelerated death were unchanged in saline phosphate buffers, though pre-exposure to water accelerated death in these buffers if glycerol were absent. Organisms washed exclusively in distilled water showed a small but reproducible decrease in their rate of glycerol-accelerated death compared with organisms washed in saline. Of a 97 % viable population starved with 10 mM-glycerol, 53 % of those washed in distilled water were viable after 30 min. compared with 26 % of those washed in saline; by 60 min. both populations had fallen below 2 % viable.

Growth characteristics of survivors. Casual observation suggested that survivors of death accelerated by ammonium, phosphate, glycerol etc. had long lag periods compared with survivors of starvation in plain buffer. The growth characteristics of survivors of glycerol-accelerated death were measured as follows. Populations in saline phosphate buffer (pH 6·4) were starved for 0·5 or 1 hr with and without glycerol, centrifuged, and re-suspended in liquid medium (that used for slide culture but without agar) warmed to 40°. At this point the viability of the population was determined by slide culture, its optical density (OD) measured, and the culture allowed to grow while aerated through a Pasteur pipette. Viabilities were measured during the lag phase and optical densities during growth. A semi-logarithmic plot of the OD increase was extrapolated to an OD equivalent to that of the viable proportion of the original inoculum (e.g. a population of initial OD of 0·03 and viability 66 % was regarded as having a 'viable OD' of 0·02) and the intercept was taken as a measure of the lag. The lags observed were: 150 min. for survivors of a population which had died to 66 % viable during 30 min., 240 min. for one that had died to 10 % viable over 1 hr. A control population starved without glycerol for 1 hr decreased from 99 to 94 % viable and had a lag period of 36 min. No further death of survivors of glycerol-accelerated death during the lag phase was detected by slide culture, and populations derived from the survivors had mean generation times similar to that of the control population (51–54 min.). In these respects the survivors of substrate-accelerated death differed markedly from the survivors of freezing and thawing (Postgate & Hunter, 1963*c*).

Comparison with plate counts. Slide culture can give false values for the viability if the scatter of individual lags is wide, due to overgrowth of dead organisms by colonies from individuals of short lag (Postgate *et al.* 1961). Though longer incubation times were adopted for the study of glycerol-accelerated death (see Methods), some comparisons with plate counts were made. Figure 5 illustrates that the agreement between slide and plate cultures was adequate; the increased lag of survivors of substrate-accelerated death was reflected in a longer incubation period required for the plates to reach a constant count.

Effect of recovery medium. The presence of glycerol in the recovery medium was not necessary for the expression of glycerol-accelerated death. The organism grew equally well on slide cultures on medium prepared according to the usual recipe but

without glycerol. The survival curves of populations suffering glycerol-accelerated death were indistinguishable from those obtained with the glycerol medium. For these experiments the samples of the dying population were centrifuged and re-suspended in saline before slide cultivation to avoid carry-over of glycerol from the starvation buffer.

Effect of pH value of growth. Continuous cultures of *Aerobacter aerogenes* were grown at pH 6·5, 5·5 and 4·9. Populations from these cultures all showed pronounced glycerol-accelerated death when tested at pH 6·5 by the saline phosphate procedure.

Effect of glycerol concentration. Glycerol-accelerated death of 20 μg./ml. populations occurred to similar extents at glycerol concentrations between 10 mM and 100 μM in saline phosphate (pH 7·3); the phenomenon was less pronounced at 50 μM and undetectable at 10 μM (contrast succinate; see below). Typical values after 1 hr of starvation were: 23 % viable with 10 mM-glycerol, 29 % with 100 μM, 41 % with 50 μM; the control without glycerol was 82 % viable, like the population with 10 μM-glycerol.

Effect of population density. Below a certain maximum, death by starvation is slower the denser the bacterial population (Harrison, 1960; Postgate & Hunter, 1963*b*). Glycerol-accelerated death showed a comparable phenomenon (Table 2).

Table 2. *Effect of population density during glycerol-accelerated death of Aerobacter aerogenes*

Washed organisms were starved in aerated saline phosphate buffer (pH 7·3) at 40° and various population densities, with between 10 mM and 50 mM-glycerol. Initial viabilities: 98 %.

Population density (equiv. mg. dry wt./ml.)	Time: 1 hr	Time: 2 hr
	Viability (%)	
2·0	92	69
0·3	46	3
0·02	18	1

Effect of pre-treatment with glycerol. Aerobacter aerogenes organisms (250 mg./ml.) were aerated for 10 min. at 40° in saline phosphate (pH 6·5) with and without 30 mM glycerol, and then were washed, drained and tested for survival in saline phosphate (pH 6·5) as usual. None died during the pre-treatment, and the death rates were similar when the organisms were subsequently starved in the plain buffer. A control suspension not pre-exposed to glycerol showed glycerol-accelerated death. Hence a brief pre-treatment of the organism with substrate did not commit them to a faster death rate than they would otherwise have shown.

Effect of metabolic intermediates. Glucose or ribose did not accelerate death (see above). Postgate & Hunter (1962) had shown, with the saline tris procedure, that the sodium salts of pyruvate, oxaloacetate, α-ketoglutarate, malate, succinate, or citrate accelerated death when used in place of glycerol. These compounds were retested at 10 mM with the saline phosphate procedure at pH 6·3; all accelerated death, though malate had only a small effect. Succinate appeared more effective than glycerol: like glycerol, it showed a maximum effect at 100 μM or above, but gave a perceptible effect at 10 μM; it had no effect at 1 μM. Pyruvate was active over

a concentration range similar to that of glycerol; citrate was only effective at 1 mM and above.

Effect of metabolic inhibitors. Postgate & Hunter (1962) listed a variety of inhibitors tested for an effect on death of *Aerobacter aerogenes* starved in plain saline tris buffer. Certain of those likely to be involved in terminal oxidative metabolism were re-tested for an effect on death accelerated by 10 mM-glycerol, with the saline tris procedure. Sodium fluoracetate (10 mM) or sodium fluoride (1 mM) had no effect on the accelerated death rate. Sodium malonate (2 mM) further accelerated death with glycerol or succinate; at 400 μM it had no effect. Similar results were obtained when the saline phosphate procedure was used. Sodium iodoacetate (1 mM) hastened glycerol-accelerated death of organisms prepared by the

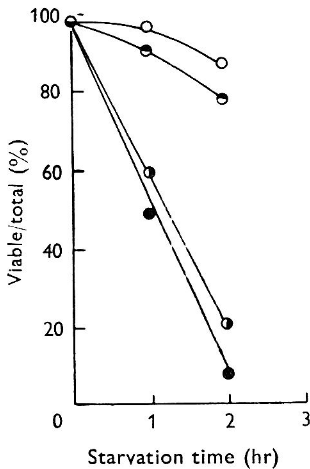

Fig. 5

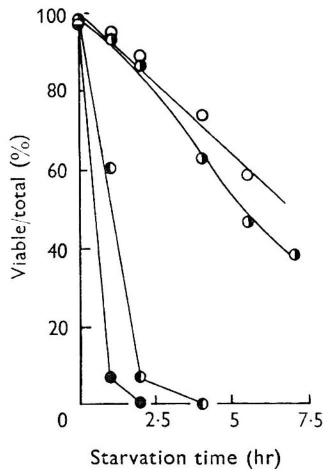

Fig. 6

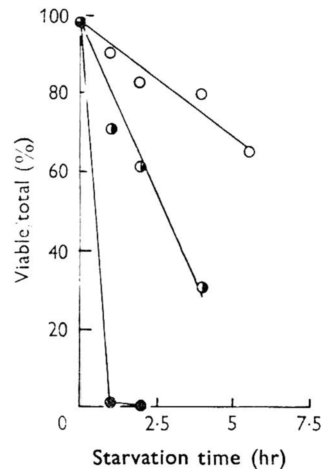

Fig. 7

Fig. 5. Demonstration of glycerol-accelerated death by plate and slide culture. *Aerobacter aerogenes* from a continuous culture was washed and starved at 20 μg./ml. in aerated saline phosphate buffer (pH 6·5) at 40°. Viability by slide culture or by plate count after micropipette dilution (Postgate & Hunter, 1963*c*) in 0·15 M-NaCl; viabilities from plate counts are plotted as % initial value, taken as equal to the initial viability obtained by slide culture. ○, slide culture; ◒, plate count on control population; ●, slide culture; ◑, plate count on population in presence of 10 mM-glycerol.

Fig. 6. Effect of 2,4-dinitrophenol on glycerol-accelerated death of *Aerobacter aerogenes*. *A. aerogenes* prepared as for Fig. 5 and starved in presence of: ●, 10 mM-glycerol; ◐, 1 mM-2,4-dinitrophenol; ◑, 10 mM-glycerol + 1 mM-2,4-dinitrophenol; ○, control population. Samples (1 ml.) were centrifuged and re-suspended in 0·15 M-NaCl before estimating viability by slide culture.

Fig. 7. Glycerol utilization during glycerol-accelerated death of *Aerobacter aerogenes*. *A. aerogenes* prepared as for Fig. 5. ○, viability of control population; ●, + 1 mM glycerol; ◑, proportion of glycerol remaining in centrifuged samples.

saline tris procedure, but with the saline phosphate procedure it delayed the onset of glycerol-accelerated death for up to 1 hr; death subsequently occurred at a rate similar to that of the population without inhibitor. α-Keto-γ-valerolactone-γ-carboxylic acid (KVC) inhibits α-ketoglutarate oxidation (Montgomery & Leyden-Webb, 1954); it was tested at 10 mM with glycerol, α-ketoglutarate or succinate (10 mM) using the saline phosphate procedure and it delayed substrate-accelerated

death with the last two compounds. With glycerol, however, no significant protection by KVC against substrate-accelerated death was observed. These observations suggested a specific protective effect by KVC, but spontaneous hydrolysis of KVC at about pH 7 was found by observing its behaviour alone in saline tris buffer: the pH value dropped from pH 7 to pH 6 in 2 hr, and to pH 5 in 7 hr. Little pH change occurred in the experiments already mentioned, but it is obvious that the agent was not stable in the test conditions. The inhibitors mentioned so far were not unequivocally protective, but two compounds showed clear positive effects with the saline phosphate procedure. Figure 6 shows protection by the uncoupling agent 2,4-dinitrophenol. Though toxic by itself at 1 mM, this substance virtually prevented glycerol-accelerated death at that concentration. A similar effect occurred with azide: it slowed the rate of glycerol-accelerated death but did not prevent it entirely. Azide was tested between 1·5 and 10 mM and showed maximum protection at 5 mM: after 4 hr at pH 6·4, when the populations with azide or glycerol alone were less than 3 % viable, that with azide + glycerol was 26 % viable; controls, with neither substance, were 55 % viable. In all these experiments, azide was more toxic in plain buffer (death rate 30 %/hr with 1·5 mM-azide, and 12 %/hr without azide, in saline phosphate buffer at pH 6·4) than recorded by Postgate & Hunter (1962: 10 %/hr with azide, and 8 %/hr. without azide). Azide was retested with the saline tris procedure and showed an intermediate toxic effect: 20 %/hr with azide, 13 %/hr without.

Utilization of substrate. Glycerol utilization during glycerol-accelerated death was implied by the experiments already described and by later ones (e.g. those on polysaccharide synthesis discussed below). Disappearance of glycerol during glycerol-accelerated death was followed by analysis of centrifuged samples (Fig. 7) and indicated that glycerol utilization continued after all the population was empirically 'dead'. The respiratory quotient of starved organisms declines in parallel with the viability during starvation (Postgate & Hunter, 1962) and the effect of presence of substrate during conventional Warburg respirometry was therefore examined. In a typical experiment, eight Warburg vessels were set up with conventional quantities of organism and glycerol; two without glycerol served as blanks. At intervals, pairs of vessels were removed for viability determination by slide culture. Figure 8 illustrates the most impressive of these experiments; in others glycerol-accelerated death was less pronounced during the 80-min. period but was nevertheless clear. Its extent presumably depended on whether the organisms utilized all the substrate before it 'killed' them.

Effect of magnesium. Magnesium delays death from starvation in saline tris buffer or saline phosphate buffer (Postgate & Hunter, 1962). At 1·25 mM (its concentration in the growth medium) Mg abolished glycerol-accelerated death in both buffers (Fig. 9). Other major components of the growth medium (K_2SO_4, 10 mM; $(NH_4)_2HPO_4$, 4·5 mM) as a mixture had no effect. Experiments were made to determine the minimum effective magnesium concentration: in saline phosphate (pH 6·3), $MgCl_2$ between 6·25 and 12·5 μM brought the death rate of a 20 μg./ml. suspension with 10 mM glycerol back to that of a comparable suspension without glycerol and magnesium; 25 μM-$MgCl_2$ lowered the death rate to that with magnesium but no substrate. $FeCl_3$ (1·6 mM) or $CaCl_2$ (0·1 mM), which had some protective action on organisms starved in plain buffer, did not influence glycerol-accelerated

death at pH 6·4 in saline phosphate buffer. $MnCl_2$ (25 μM), which had no effect on organisms starved in plain buffer, antagonized glycerol-accelerated death to a slight extent. For example, all of an initially 99 % viable population were dead after 1 hr in saline phosphate buffer + 10 mM glycerol (pH 6·4), whereas in a similar solution with $MnCl_2$ they were 27 % viable. The control suspension was 96 % viable.

Polymer catabolism. Death of *Aerobacter aerogenes* by starvation is preceded by the metabolism of certain polymeric cell constituents (Strange *et al.* 1961). Polymer catabolism with and without glycerol was studied, using the saline phosphate procedure and the assay methods cited by Postgate & Hunter (1962). No significant change in the DNA and protein contents of 240 and 300 μg./ml. suspensions,

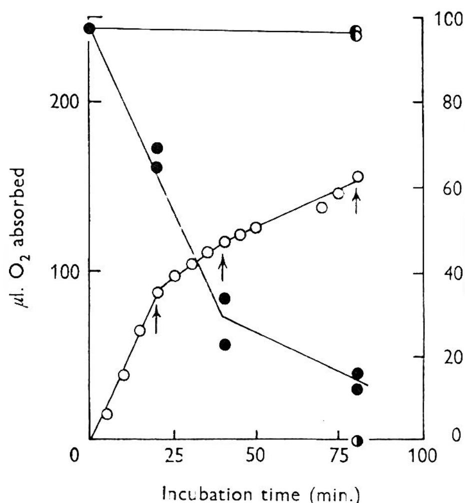

Fig. 8

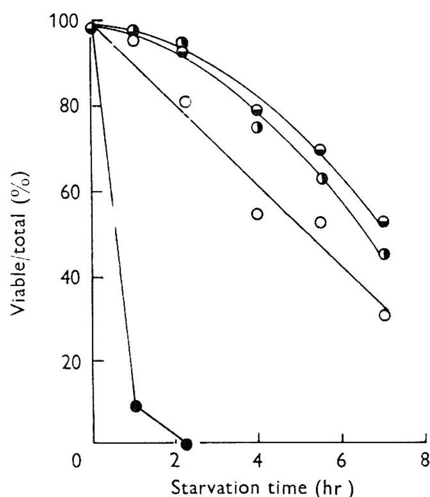

Fig. 9

Fig. 8. Substrate-accelerated death during Warburg respirometry. Warburg vessels containing equiv. 2 mg. dry wt. organism in 2 ml. saline phosphate buffer (pH 7·3); 0·1 ml. KOH (10 M) in centre well; vessels were equilibrated at 40° and, after tipping 3·26 μmole glycerol in 0·2 ml. water as substrate, pairs of vessels were dismantled during the reaction (times indicated by arrows) for viability determination on their contents by slide culture after dilution in 0·15 M-NaCl. ○, mean O_2 uptake values for pairs of vessels with glycerol; ◑, blank pair without glycerol; ●, viabilities of populations in individual flasks with glycerol; ◐, viabilities of blank pair.

Fig. 9. Abolition of glycerol-accelerated death of *Aerobacter aerogenes* by magnesium ions. *A. aerogenes* prepared as for Fig. 5 and starved in saline phosphate buffer (pH 6·3). Viabilities by slide culture. ○, control population; ●, + 10 mM-glycerol; ◒, + 1·25 mM-$MgCl_2$; ◑, + glycerol + $MgCl_2$.

respectively, occurred at pH 7·3 over 2 hr, although the viabilities fell to 2 and 4 % with 10 mM glycerol. Without glycerol, the viabilities were unchanged at 96–98 %. In three experiments the polysaccharide contents of 750 μg./ml. suspensions (pH 7·3) increased 2·1- to 3-fold in 2–4 hr, while the viabilities fell from 99 % to between 60 and 80 % and the control populations without glycerol remained wholly viable. RNA catabolism, indicated by the Bial reaction, was slower with 100 μg./ml. populations at 7·3 and pH 6·4 than recorded by Postgate & Hunter (1962). This may be related to the longer survival of populations treated by the saline phosphate procedure compared with those prepared by the saline tris procedure. At neither

pH value was the rate of RNA breakdown altered when death was accelerated with 25 mM glycerol, though after 4 hr these populations were 85 % to 98 % dead and the viabilities of control populations without glycerol had scarcely changed from the starting value of 97 %. Both showed a 10–15 % decrease in RNA content over this period.

Excretion of materials. Micro-organisms starved in buffer excrete into the medium materials which absorb radiation at about 260 mμ (see Strange *et al.* 1961; Postgate & Hunter, 1962). Differences in the amounts of such materials were not detected during the death of a 60 μg./ml. population dying in saline phosphate buffer, with or without 30 mM-glycerol, because a strong absorption at about 230–240 mμ appeared which obscured the 260 mμ peak. This absorption reached an apparent maximum in 2 hr, during which time the viability of the population decreased from 99 to 2 %; the control population decreased from 98 to 86 % viable. Twenty-fold concentrates of saline phosphate buffer (pH 6·4) in which 20 μg./ml. populations had died during incubation for 7 hr, with or without 10 mM-glycerol, had different appearances. Those from the populations whose death had been accelerated by glycerol were brownish-yellow in colour and had a pronounced white fluorescence in light of 365 mμ wavelength; the controls were colourless and showed little fluorescence. The cyanide reaction (Ciotti & Kaplan, 1957) indicated that, after glycerol-accelerated death, pyridine nucleotides (equivalent to about 5 μM in the original buffer) were present which were absent from the controls. Since loss of these materials may have been a factor in glycerol-accelerated death, mixtures of nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate (10 and 33 μM) were tested with the saline phosphate procedure. These compounds did not influence glycerol-accelerated death. Attempts were made to measure the magnesium contents of such concentrates. Analyses of whole bacteria prepared as for the saline phosphate procedure indicated 0·19 % (w/w) Mg; hence our conventional 20 μg./ml. suspensions corresponded to 1·6 μM magnesium. In our hands the Titan Yellow procedure would just have detected the Mg in 20-fold concentrates of such a solution, but analyses of concentrates of buffers after bacterial death with or without glycerol showed no detectable magnesium. $MgCl_2$ added to such concentrates was recovered quantitatively; hence other materials in the concentrates did not interfere with the analysis. Concentrated buffers in which denser (200 μg./ml.) populations had died did interfere, however; they delayed the appearance of the red colour and lowered the blank reading, as well as diminishing the recovery of added $MgCl_2$. Buffers from populations subjected to glycerol-accelerated death for 6·5 hr interfered to a lesser extent than controls from populations without glycerol. Evaporation of such concentrates with H_2SO_4 followed by NaOH, to remove the volatile acids and NH_3 which interfere with the Titan Yellow reaction (Feigl, 1954), did not prevent this interference. Both types of concentrate interfered with the 'magneson' reaction (Anon. 1949).

State of the osmotic barrier. Postgate & Hunter (1962) used the optical effect of Mager, Kuczynski, Schatsberg & Avi-dor (1956), and permeability to a dyestuff that fluoresced in contact with protein, to show that the osmotic barriers of *Aerobacter aerogenes* organisms starved by the saline tris procedure remained functional after death. Comparable experiments with the saline phosphate procedure showed that glycerol-accelerated death at pH 7·3 did not involve accelerated breakdown of the

osmotic barrier. Some experiments with the saline tris procedure, and measurement of the permeability to anilino-naphthalene sulphonic acid fluorimetrically, confirmed that glycerol- or succinate-accelerated death did not involve accelerated breakdown of the osmotic barrier in these conditions.

State of the 'permeability control mechanism'. 'Cold shock' is characterized by release of undegraded ATP and amino acids into the environment and has been interpreted in terms of inhibition of a permeability control mechanism by abrupt chilling (Meynell, 1958; Strange & Dark, 1962). Since cold shock only occurs among organisms from exponentially growing cultures, it was conceivable that exposure of starving organisms to their limiting substrate might induce a state physiologically analogous to susceptibility to cold shock. Leakage of products comparable to those which appear during cold shock, as well as susceptibility to cold shock, were therefore sought during glycerol-accelerated death. Suspensions of *Aerobacter aerogenes* (2 mg./ml.) dying at pH 6·5 in saline phosphate, with and without 50 mM-glycerol, were sampled after 1 and 2 hr (glycerol-accelerated populations fell to 66 % viable; controls unchanged at 97 %), filtered rapidly into an ice-cold receiver through a membrane filter (Oxoid), the filtrates frozen in liquid nitrogen and stored at -20° for examination next day. ATP was sought by the firefly luminescence technique; none was detected. Cold shock would have released readily detectable amounts of ATP from a susceptible population of this density (Strange & Dark, 1962). Chromatographic examination of the filtrates indicated no enhancement of the amino acid excretion in the presence of glycerol. Our populations of *A. aerogenes* do not ordinarily show cold shock (Postgate & Hunter, 1961). No signs of susceptibility to cold shock (increased death after chilling for 30 min. at 4°) appeared during the death of an 80 μg./ml. suspension accelerated by 40 mM-glycerol in saline phosphate (pH 7·3), nor during starvation of a control population without glycerol. The test population decreased from 98 to 17 % viable during the 60 min. starvation period; the control did not die at all. Strange & Dark (1962) showed that certain concentrations of spermine had a slight protective effect against cold shock. Spermine at 10 μM did not influence glycerol-accelerated death of a 20 μg./ml. population at pH 6·3 in saline phosphate buffer; at 100 μM spermine accelerated death.

DISCUSSION

Ubiquity of substrate-accelerated death. Substrate-accelerated death appears to be a fairly general phenomenon associated with the survival of starved *Aerobacter aerogenes*. It can be observed in organisms harvested from complex media, in which the 'limiting' nutrient is not known and may change as the culture grows. It occurs with other Gram-negative bacteria. Substrate-accelerated death differs in principle from the 'suicidal' behaviour of certain mutants (e.g. inositol-less *Neurospora crassa*, Strauss, 1958; thymine-less *Escherichia coli*, Barner & Cohen, 1956), because 'suicidal' behaviour is provoked by withdrawal of the required nutrient in conditions otherwise favourable to growth, whereas our phenomenon is elicited by providing a previously needed substrate in conditions which, nevertheless, remain unfavourable for growth. Substrate-accelerated death of *A. aerogenes* occurred whether the limiting substrate was the nitrogen, phosphorus or carbon-*cum*-energy source; it was not observed when the sulphur source limited growth, though in this instance

the carbon source continued to accelerate death. It seems possible that the sulphur source limited growth indirectly by influencing the rate of some step in carbon utilization. Magnesium-limited organisms did not show substrate-accelerated death, but this is reasonable since Mg ions protect ordinary starved populations, irrespective of nutritional status (Postgate & Hunter, 1962). Magnesium prevented substrate-accelerated death brought about by glycerol.

The fact that the nutritional status of the population determines which substrate, if any, will accelerate death presumably explains certain conflicting reports in the literature cited by Postgate & Hunter (1963*a*), who pointed out the relevance of substrate-accelerated death to the design of physiological experiments which make use of live 'resting' bacteria; e.g. enzyme induction, respirometry and the replacement of 'maintenance energy' by small additions of substrate. Figure 8 illustrates an extreme case of substrate-accelerated death during Warburg respirometry, and though the existence of such a phenomenon need not necessarily invalidate the conclusions obtained from the respirometric experiments, it seems likely from our data that part of the 'uncoupling' effect of compounds such as azide and 2,4-dinitrophenol in respirometry might be connected with the preservation of viability.

Action of magnesium. The antagonism of glycerol-accelerated death by magnesium showed some differences from the protection these ions ordinarily afford against starvation. Calcium ions had no action in place of Mg ions and Fe^{3+} ions were not protective. Manganese ions, which had no protective effect in ordinary starvation (this observation was re-checked with the saline phosphate procedure), may have antagonized glycerol-accelerated death by sparing the organisms' reserves of magnesium ions. Strange & Shon (1964) showed that organisms washed in NaCl solutions lost more magnesium than those washed in distilled water and became more sensitive to a mild heat stress. A comparable retention of stored magnesium might account for the lower sensitivity to glycerol-accelerated death of organisms washed in distilled water compared with those washed in saline.

Mechanism of substrate-accelerated death. The data presented here do not allow a complete account of substrate-accelerated death and do not establish whether the inorganic substrates, ammonium or phosphate, accelerate the death of susceptible populations by a mechanism similar to that of carbon-*cum*-energy sources such as glycerol, glucose or lactate. For the case of glycerol-accelerated death, the excretion of coloured and fluorescent material, together with the existence of a population effect, implicate a loss, from dying organisms, of materials which, once they reach a threshold concentration, prolong the lives of the surviving neighbours (see discussion of population effects by Harrison, 1960, and Postgate & Hunter, 1963*b*). Magnesium ions may be among the relevant materials excreted, but if so their concentration was below the range of analytical procedures available to us. The release of such materials must be precipitated by the presence of glycerol or some active substitute for it; their release required the continued presence of glycerol in the starvation environment but not in the medium used to assess viability; it did not involve accelerated breakdown of the osmotic barrier, nor did changed permeability characters appear that resembled those which render logarithmic-phase organisms susceptible to cold shock. Accelerated breakdown of the endocellular polymers, DNA, RNA and protein, did not occur and polysaccharide was laid down during glycerol-accelerated death.

The long lags shown by survivors of substrate-accelerated death, which occurred with inorganic and with organic substrates, suggests that reclamation of the lost material after transfer to the recovery medium was a slow process, or that the active substrate influenced the organisms' metabolism in such a way that their behaviour then resembled that of bacteria subject to repression of enzyme synthesis. Recovery from repression is known to involve long lags (Bourgeois, Wiame & Lelouchier-Dagnelie, 1960) and several instances are known in which a substrate may repress synthesis of its own enzyme. For example, β-galactosidase synthesis by partly adapted *Escherichia coli* may be repressed by lactose (Mandelstam, 1961) and in this instance not only are intermediates in the metabolism of lactose also repressive but repression is antagonized by 2,4-dinitrophenol. Hypotheses based on analogy with the repression of enzyme synthesis would be consistent with the fact that glycerol continues to be utilized by operationally 'dead' organisms (Fig. 7), despite the finding of Postgate & Hunter (1962) that metabolism of glycerol declined in parallel with viability during death in a plain buffer, because such hypotheses would envisage repression during turnover of some factor essential for subsequent multiplication. The existing complement of catabolic enzymes would be primarily unaffected by the active substrate. However, the chemical disparity of the agents that induce substrate-accelerated death, together with the variety of intermediates that can replace glycerol, and the capriciousness with which utilizable substrates which are not intermediate metabolites may or may not accelerate death, all make it clear that simple repression of an enzyme sequence involved in the utilization of the substrate provides an inadequate theoretical approach. The active substrate must trigger-off some more central process in the regulatory mechanism of the organism, presumably one concerned with the issue or transfer of genetic information for constitutive biosynthetic reactions.

We are indebted to Miss Patricia Mills for technical assistance, and acknowledge gratefully the help of Messrs F. A. Dark, A. G. Ness, R. E. Strange, H. K. Robinson and H. E. Wade, who performed various analyses for us. Dr S. L. S. Thomas of the National Chemical Laboratory kindly gave the specimen of KVC.

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ADDENDUM

An earlier paper by Postgate & Hunter (1962) contained several errors. We publish the following table of corrections and apologize to anyone who may have been misled.

Reference	*Correction*
Page 236, line 13	*For* $\pm 0{\cdot}01/\text{hr}^{-1}$ *read* $\pm 0{\cdot}01\ \text{hr}^{-1}$
Page 237, line 22	*For* Millipore *read* membrane
Page 240, sketch	*For* 26·7 *read* 96·7
Page 245, last line	*For* $\times 10^{+5}$M *read* $\times 10^{-5}$M
Page 246, table 2	*Insert* no *after* or *in last column*
Page 246, table 2	*For* Fig. 9 *read* Fig. 7 (in note marked †)
Page 248, line 36	*For* uracil *read* uridine
Page 253, table 4; Page 254, line 10	*For* 0·31 mM-Na_2SO_4 *read* 0·031 mM-Na_2SO_4
Page 255, Fig. 12	*Label ordinate* viable/total, %
Page 256, Fig. 13*a*	*Third point in* D = 0·44 *curve should have symbol* ◒
Figs. 2–5, 11, 13–16	*For* % viable total *read* viable/total, %

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Comparative Studies on the Production and Assay of Interferon

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SUMMARY

Interferon was produced in chick embryo cell cultures or in chick embryos by five viruses and in mouse embryo cell cultures or mouse lungs by two viruses. The slopes of the log dose response lines for the five chick interferons were compared by using vaccinia virus as the assay virus. Analysis of variance showed that they did not differ significantly. Partially purified interferon gave the same slope as the crude preparations. These findings allowed a comparison to be made of relative yields of interferon induced by different viruses in the same cell system. Viruses differed widely in their ability to induce interferon. Chikungunya virus induced about 70 times more interferon than did vaccinia or Newcastle disease virus and 2·6 times more interferon than Kumba virus. The slopes of the log dose response lines for two interferon preparations were compared by using Chikungunya virus as the assay virus. Analysis of variance showed that they were not significantly different. A comparison of the vaccinia and Chikungunya assays for interferon showed that the Chikungunya assay was approximately 2·9 times more sensitive when the amount of interferon depressing the plaque count by 50 % (PDD50 doses) were compared. An analysis of variance showed that the difference in slope between the two assays was small but approaching significance. Mouse interferon, induced by two viruses, yielded parallel dose response lines. However, the slope of the curves for mouse interferon was significantly different from that for chick interferon. Because of this difference in slope, interferon production by the same virus in the two cell types could not be validly compared.

INTRODUCTION

Different batches of interferon induced in chick embryos by the same strain of influenza virus gave parallel dose-response curves (Lindenmann & Gifford, 1963), allowing estimates to be made of the relative potencies of different interferon preparations. Comparative dose-response curves for interferon induced by different viruses under similar conditions have not been published. In the present study the dose response lines of five interferons induced by five viruses in chick cells and two interferons induced in mouse cells by two viruses were compared. The sensitivities of two different plaque assays for interferon were compared.

METHODS

Interferon production. Mouse interferon was prepared by the intranasal inoculation of mice with about 600 egg infectious doses of the PR 8 strain of influenza A virus

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as described by Isaacs & Hitchcock (1960). Lungs were removed 3 days later, triturated with Gey's balanced salt solution (BSS), centrifuged at about 1500 rev./min. for 30 min. and the supernatant fluid containing the interferon collected. This interferon preparation was dialysed against 0·1 M-HCl + KCl buffer (pH 2) overnight and then against Gey's BSS to bring to about pH 7·4. A second mouse interferon was produced by infecting mouse embryo cell cultures with Newcastle disease virus at an input multiplicity of about 0·05 plaque forming units (pfu)/cell and incubating at 37° for 24 hr. Since dialysis overnight against pH 2 buffer did not completely inactivate this virus the preparation was treated with perchloric acid, 0·15 N at 5°, as described by Lampson, Tytell, Nemes & Hilleman (1963), centrifuged, and then dialysed against BSS to remove the perchloric acid.

Chick interferon was also produced in two ways. Chick embryos (10-day) were inoculated with the Mel strain of Influenza A virus and incubated for 72 hr at 35°. The allantoic fluid was collected, centrifuged, and dialysed against pH 2 buffer overnight and then against BSS to restore to about pH 7·4. Chick interferon was also prepared in monolayer cultures of chick embryo cells. The same batch of cells and medium, at 35° was used to produce interferon by four different viruses. The four viruses (Chikungunya, Kumba, vaccinia, Newcastle disease) were used at an input multiplicity of 0·06 pfu/cell since this was near the optimal amount of virus for maximal production of interferon by Chikungunya virus in chick cells (Gifford, 1963*a*). Cultures were incubated at 37° for 24 hr and the supernatant fluid removed, centrifuged to remove debris, and treated with perchloric acid as described for the mouse interferon induced with Newcastle disease virus. Fresh medium was placed in these bottles and they were incubated for a further 24 hr. The amount of interferon produced in the second 24 hr incubation period amounted to less than 10 % of the first 24 hr yield.

Cell cultures. Monolayer cultures of chick embryo cells were prepared as described by Porterfield (1960). Cells were suspended in growth medium and, for interferon assay with vaccinia virus, about 9×10^6 cells were dispensed in 3 ml. volumes into 1 oz. 'medical' screw-capped bottles having a rectangular side of 18 cm^2. These cultures were incubated (bottles flat) at 37° and used 42–48 hr later when there were approximately 3×10^6 cells/bottle as a monolayer. For the Chikungunya assays, cells were dispensed into the same type of bottle but at twice the concentration. For interferon production, 64×10^6 cells were dispensed into 8 oz. bottles with a rectangular surface area of 72 cm^2. Twenty-four hr later these cultures contained about 20×10^6 cells attached to the glass. Mouse fibroblast monolayers were prepared by trypsinizing 18- to 20-day eviscerated mouse embryos in a manner similar to the chick embryos. 60×10^6 cells were dispensed in 12 ml. medium into 8 oz. bottles and incubated for 2–6 days. These primary mouse fibroblasts were then re-trypsinized and dispensed into 1 oz. bottles with 3×10^6 cells/bottle in 3 ml. medium. They were used on the following day and contained about 3×10^6 cells as a monolayer.

Media. The same medium, with slight modifications, was used for both the mouse and chick cells. Growth medium for the establishment of chick monolayers consisted of BSS with 0·0025 M-2-amino-2-(hydroxymethyl)-1,3-propanediol (tris), 5 % (v/v) calf serum, 0·25 % (w/v) lactalbumin hydrolysate, and 0·1 % (w/v) proteose peptone. The same medium was used for the establishment of mouse cells except that the serum was increased to 10 % and 0·11 % (w/v) sodium bicarbonate replaced

the tris buffer. Maintenance medium, used for virus and interferon assays with vaccinia virus in chick and mouse cultures consisted of BSS with 0·11 % sodium bicarbonate, 0·25 % lactalbumin hydrolysate, 0·1 % yeast extract, and 0·1 % proteose peptone. For Chikungunya assays of virus and interferon, the overlay consisted of 0·75 % agar (Noble), 5 % calf serum, 0·0025 M-tris, 0·25 % lactalbumin hydrolysate, and 0·1 % proteose peptone in BSS.

Virus and interferon assays. Vaccinia virus and interferon were titrated according to the method described by Lindemann & Gifford (1963) with slight modification. Assay cultures were incubated at 35° instead of 37° since the plating efficiency of vaccinia virus was thus increased by about 15 %. Interferon was also assayed with Chikungunya virus by the method described by Ruiz-Gomez & Isaacs (1963). In all interferon assays, four cultures were used for each interferon dilution.

Protein determinations. Protein was estimated by the Folin phenol method (Lowry, Rosebrough, Farr & Randall, 1951).

Partial purification of interferon. Interferon produced in chick cells with Chikungunya virus was treated with perchloric acid, centrifuged, and precipitated twice with zinc acetate (Lampson *et al.* 1963). About 81 % of the protein was removed.

Viruses. Vaccinia, Chikungunya, Newcastle disease, Kumba (Semliki Forest) and Influenza A (PR 8 and Mel) viruses were used. Chikungunya and Kumba viruses were grown in the brains of newborn mice. Influenza and Newcastle disease viruses were grown in the allantoic cavity of 10-day embryonated chicken eggs. Vaccinia virus was grown on the chorioallantois of 10- to 12-day chick embryos. All viruses were stored in glass capillaries at −60°.

RESULTS

Eleven assays were made and dose response graphs constructed of percent plaque inhibition against log. μl. interferon used (assays 1–11, Table 1). Tests for linearity showed that none of the sets of points deviated significantly from a straight line when plaque inhibition between 10 and 90 % was considered.

Comparison of the dose response of two interferon preparations assayed with Chikungunya virus. Two interferon preparations induced with Chikungunya virus in chick cells were assayed with Chikungunya virus (Fig. 1; assays 8 and 9, Table 1). The difference between the slopes for these two assays was not significant at the 20 % level when examined as the 'departure from parallelism' term in an analysis of variance (i.e. the probability that the difference in slope might be obtained by chance is greater than 1 in 5). This was also shown by the variance of the slope in Table 1. These results indicated that Chikungunya virus would give parallel dose response lines with different preparations of interferon, as previously shown with vaccinia virus (Lindenmann & Gifford, 1963).

Comparison of the Chikungunya and vaccinia assays for interferon. Chikungunya and vaccinia assays were performed on the same batch of cells at the same time using the same interferon preparation. There were marked differences in the assay procedures. The Chikungunya assay was done with overnight pre-incubation of cells with interferon, virus was then allowed to adsorb for 1 hr and an agar overlay containing serum added. In the vaccinia assay, virus and interferon dilutions were added at the same time, the cell concentration was different and no agar or serum

Table 1. *Comparison of the slopes and potencies of different interferon preparations*

Assay no.	Interferon production system		Interferon assay system		No. of dilutions in assay*	Slope†	Variance of slope	PDD 50‡ (μl.)
	Virus	Cell	Virus	Cell				
1§	Chikungunya	Chick culture	Vaccinia	Chick culture	5	51·1	16·2	2·0
2§	Chikungunya	Chick culture	Vaccinia	Chick culture	4	54·8	19·3	12·5
3‖	Kumba	Chick culture	Vaccinia	Chick culture	4	53·0	14·6	17·6
4‖	Newcastle disease	Chick culture	Vaccinia	Chick culture	3	64·1	32·4	472·8
5‖	Vaccinia	Chick culture	Vaccinia	Chick culture	4	56·7	35·4	468·4
6	Influenza	Chick embryos	Vaccinia	Chick culture	4	51·6	7·3	148·6
7‖	Chikungunya	Chick culture	Vaccinia	Chick culture	5¶	67·7	23·3	6·7
8	Chikungunya	Chick culture	Chikungunya	Chick culture	4	52·4	18·4	2·1
9	Chikungunya	Chick culture	Chikungunya	Chick culture	5	48·1	11·0	5·8
10	Newcastle disease	Mouse culture	Vaccinia	Mouse culture	5	30·3	5·4	176.1
11	Influenza	Mouse lung	Vaccinia	Mouse culture	5	31·6	1·9	16·4

* Each dilution was assayed in quadruplicate.

† Slopes represent the change in percentage plaque inhibition for each unit increase of the dose (in logarithms) of interferon preparation.

‡ PDD 50 is defined as the amount of interferon which depresses the plaque connt to 50 % of the control plaque count (Lindenmann & Gifford, 1963).

§ Assay 1 represents a partially purified interferon prepared from the same preparation as the crude preparation used in assay 2. Approximately 81 % of the protein was removed in the purification procedure.

‖ Prepared at the same time on the same batch of cells under comparable conditions. Assay carried out on the same batch of cells. Assay number 6 was carried out at the same time.

¶ Only three dilutions used in calculations since one point resulted in 98 % inhibition and one in 100 %.

was used (Fig. 2; assays 7 and 8, Table 1). Analysis of variance showed the difference in slope between these two log dose response lines to be just significant at the 5 % level. As this is generally considered to be on the borderline of significance the result is difficult to interpret, but it may be noted that the slope for the vaccinia assay is the largest of all the slopes in the vaccinia series (assays 1–7). The slopes represent the change in percentage plaque inhibition for each unit increase of the dose (expressed in logarithms) of interferon preparation. The weighted mean slope for all vaccinia assays was 55·1 and the corresponding weighted mean slope for Chikungunya assays was 49·7, a difference which is approaching significance ($t = 1{\cdot}75$; $0{\cdot}10 > p > 0{\cdot}05$). If the lines for the Chikungunya and vaccinia assays could be considered parallel and therefore a valid comparison made, then the

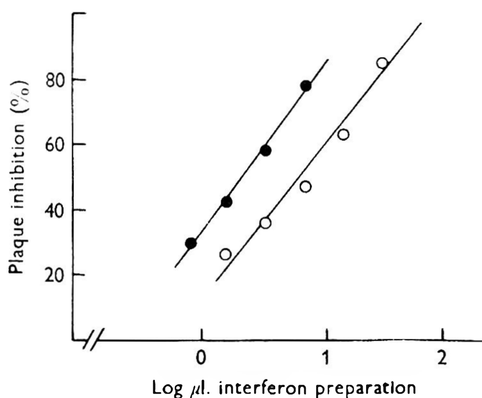

Fig. 1

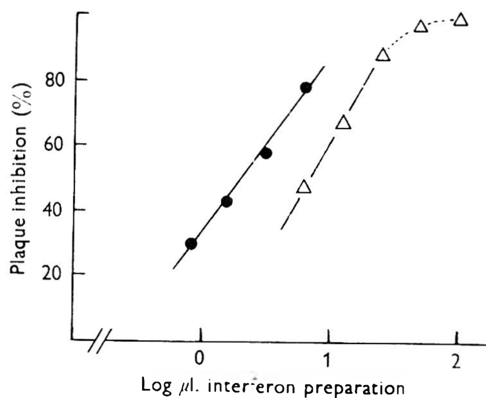

Fig. 2

Fig. 1. Comparison of the dose response of two interferon preparations assayed with Chikungunya virus. ○ and ● represent two interferons induced with Chikungunya virus in chick cell cultures.

Fig. 2. Comparison of the Chikungunya and vaccinia assays for interferon. Both assays were done with the same interferon preparation and on the same batch of cells. × = Chikungunya assay; △ = vaccinia assay.

Chikungunya assay would be 2·9 times more sensitive than the vaccinia assay when the log PDD50 doses were compared. However, the vaccinia virus assay was used in all subsequent studies because of its simplicity and because induction of interferon by the assay virus was less of a complication.

Comparison of crude and partially purified interferon. Interferon induced in chick cell cultures with Chikungunya virus was partially purified. The dose responses of the crude and purified preparations were assayed using vaccinia virus as the assay virus (Fig. 3; assays 1 and 2 Table 1). The difference between the slopes for these two assays was not significant and the partially purified and concentrated material showed about 6 times the activity of the crude preparation.

Comparison of the yield of interferon by four viruses in chick cells. Interferon production was induced in chick cells with four different viruses (Kumba, Newcastle disease, vaccinia, Chikungunya) using the same conditions of culture and the relative potencies compared. Another interferon produced in chick embryos infected with influenza virus was assayed at the same time (Fig. 4; assays 3–7 Table 1). The data

for the five assays were considered in a single analysis of variance which showed that the slopes calculated for the five lines might all be regarded as estimates of the same slope, for the variance due to non-parallelism was only 1·7 times the residual error of the experiment, a value which might be expected to occur by chance between 1 in 5 and 1 in 10 times. About 70 times more interferon was produced by Chikungunya virus than by Newcastle disease or vaccinia viruses and 2·6 times more than by Kumba virus.

Comparison of the dose response of mouse interferon. Two mouse interferons, produced by different viruses, were assayed in mouse cells with vaccinia virus (Fig. 5; assays 10–11 Table 1). Analysis of variance gave a probability of greater than 20 % for the departure from parallelism term. The weighted mean slope for these two assays (31·24) was significantly less than the slopes for the chick assays at the 0·1 % level. A valid comparison of the amount of interferon produced by a given virus in these different cell types could not be made.

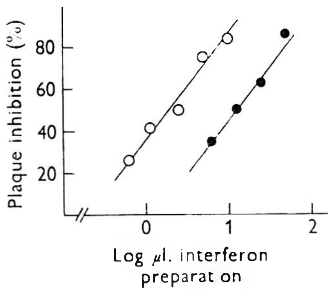

Fig. 3

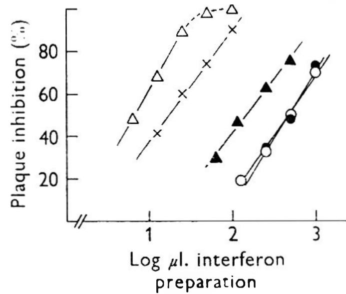

Fig. 4

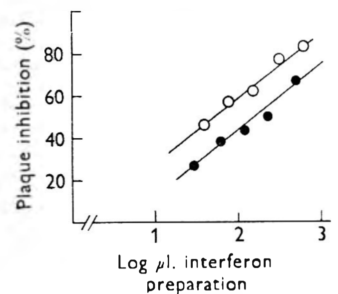

Fig. 5

Fig. 3. Comparison of crude and partially purified interferon. ○ = partially purified; ● = crude preparation from which the partially purified was derived.

Fig. 4. Comparison of the yield of interferon by different viruses in chick cells. Interferons induced by the different viruses are represented by: △ = Chikungunya; × = Kumba, ▲ = influenza; ● = vaccinia and ○ = Newcastle disease.

Fig. 5. Comparison of the dose response of two mouse interferon preparations. ○ = interferon induced in mouse lungs with influenza virus; ● = interferon induced in mouse cell cultures with Newcastle disease virus.

DISCUSSION

Plaque inhibition assays of interferon have been used by many workers. The measure of interferon activity employed has usually been the dose depressing the plaque count to 50 % of the controls for which the abbreviation PPD 50 (50 % plaque depressing dose) has been suggested (Lindenmann & Gifford, 1963). Relative potencies can only be estimated for preparations yielding parallel dose-response curves when the response is plotted against the logarithm of the dose. Factors which alter the shape of the dose-response curve for interferon are unknown. It was thought that variations might be expected with interferon preparations induced with different viruses, since it was possible that in some preparations of interferon there might be materials which exert an antagonistic or potentiating effect or affect stability of interferon. However, it was interesting that the dose-response curves obtained with interferon induced with several different viruses, demonstrated

parallel lines. These findings give further evidence that the same interferon is induced by different viruses. The reasons for the quantitative differences in interferon production by different viruses remains unknown. The same slope was consistently found for many interferon preparations produced over several months and also with partially purified preparations. Variations were encountered in potency estimations when the same interferon preparation was used on various batches of cells as previously reported (Lindenmann & Gifford, 1963). Changes in pH, temperature, and volume of overlay medium did not appear to markedly alter the shape of the dose response curves (Gifford, unpublished observations).

Although the significance of the dose-response slopes with respect to the mechanism of action of interferon is unknown, it is of interest that the slopes for mouse and chick interferon were markedly different. It has been shown (Gifford, 1963*b*) that the slopes of chick and mouse interferons, when assayed on both homologous and heterologous cell systems, were characteristic of the cell type in which interferon had been produced and not the cell type in which it was assayed.

We wish to acknowledge the excellent technical assistance of Mr C. Gilchrist and Mrs Barbara Knight. We also wish to thank Miss Hilary Prince for performing the protein determinations. This investigation was supported in part by a Public Health Service Fellowship (ESP-17932) from the National Institute of Allergy and Infectious Diseases, Public Health Service, U.S.A. (to G.E.G.) and a Fellowship from the Leukemia Society, Inc., New York, U.S.A. (to E.H.).

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The Uptake and Utilization of Histidine by Washed Amoebae in the Course of Development in *Dictyostelium discoideum*

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SUMMARY

The characteristics of L-histidine uptake by *Dictyostelium discoideum*, after removal of the food supply, are described. There were only small differences in the rate of histidine uptake and incorporation into protein with changing pH value. It is concluded that the site of histidine stimulation of the rate of development must be at the cell surface or outside the cell. The production of urocanic acid from histidine is described. At the concentrations produced, urocanate had little or no effect on the rate of morphogenesis. However, at higher concentrations, urocanate and a variety of imidazole-containing compounds were stimulatory.

INTRODUCTION

In a previous communication (Krichevsky & Wright, 1963), the ability of a variety of materials to stimulate the rate of development in *Dictyostelium discoideum* was described. The stimulant most studied was L-histidine. The action of this amino acid was shown to be not due to such phenomena as buffer capacity, tonicity, chelation, direct energy generation, or to stimulation of protein synthesis. The ability of histidine to inhibit the uptake of radioactive amino acids was shown; glucose did not possess this ability. In view of the foregoing, a study of the fate of exogenously supplied histidine was undertaken. Since the stimulatory effect of histidine was apparent within 4 hr of the removal of residual bacteria from vegetative myxamoebae, it was decided to concentrate on this initial period.

MATERIALS

The procedures for growing and harvesting myxamoebae, determining the effect of materials on the rate of morphogenesis, isolating protein, and determining ^{14}C specific activity of protein were as described previously (Krichevsky & Wright, 1963).

Imidazole-containing compounds were assayed in triplicate by the Pauly diazotization reaction as described by Macpherson (1942). It was found that urocanic acid has a molar extinction coefficient about two-thirds that of histidine.

Thin-layer chromatography was done as described in *Operating Manual 103* of Brinkman Instruments, Inc., Westbury, New York. The layers were 250 μ thick and composed of cellulose with $CaSO_4$ (Type MN 300 G).

Uniformly ^{14}C-labelled histidine (specific activity = 1·13 microcuries/mg.) was

obtained from Nuclear Research Corporation, Orlando, Florida. Urocanic acid was a gift from Dr Alan H. Mehler (NIDR, NIH, Bethesda, Md.). Filtration to remove cells was done with Millipore filters of pore size 0·45 μ and a disc diameter 47 mm. (Millipore Filter Corporation, Bedford, Mass.; catalogue number HAWPO 4700).

RESULTS

To determine the fate of exogenous histidine in *Dictyostelium discoideum*, initially three parameters were considered: the concentration of extracellular histidine; the uptake of histidine; the metabolism of histidine.

As shown in Fig. 1, the imidazole content of the Millipore filtrate of cell suspen-

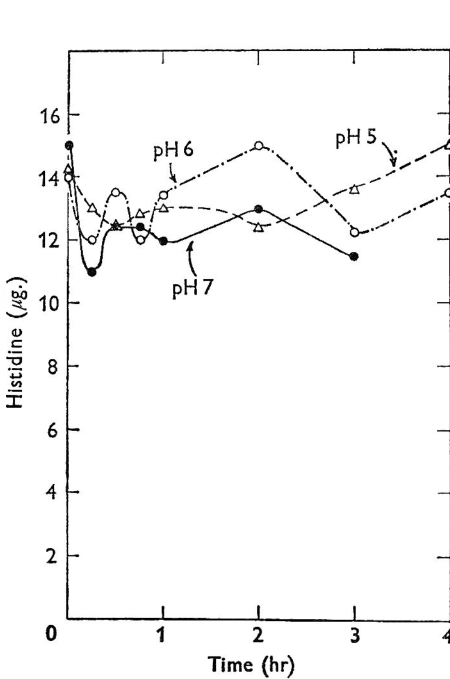

Fig. 1

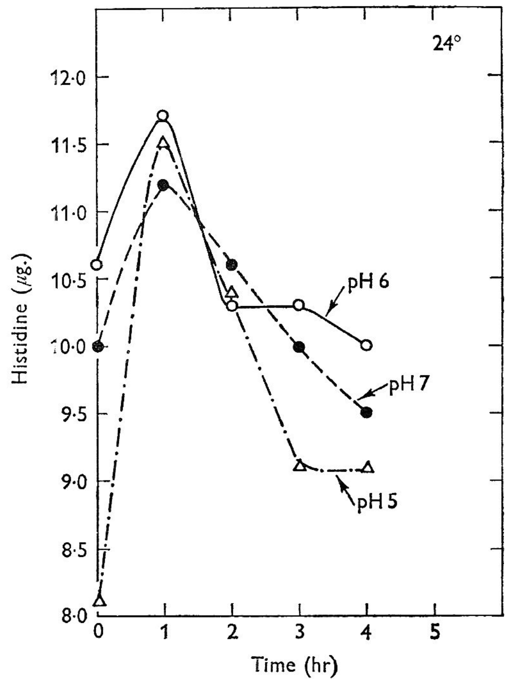

Fig. 2

Fig. 1. Extracellular histidine concentrations in cell suspensions. Amoebae were harvested from two cookie sheets, washed and finally suspended in Bonner's salt solution (Bonner, 1947); final volume 30 ml. A 4·0 ml. reaction mixture for each time and pH variable was prepared by adding, to a standard 9 cm. Petri dish, 2 ml. of 0·08 M-histidine of the appropriate pH value; 1 ml. Bonner's salts ($\times 4$); 1 ml. cell suspension. At the times shown in the figure the incubation mixtures were filtered through Millipore filters. The filtrates were diluted 1/50 and 0·1 ml. samples assayed in triplicate for imidazole-containing material.

Fig. 2. Histidine uptake into the soluble pool at 24°. Amoebae were washed from two cookie sheets with cold distilled water, washed free from residual bacteria with cold distilled water and resuspended, final volume 9·0 ml. Samples of the cell suspension (0·4 ml.) were placed on standard 9 cm. diam. Petri dishes containing 0·4 M-histidine in 2·5 % Noble agar at the pH value shown. The dishes were incubated at 24° for the times shown. After incubation, the cells were washed from each Petri dish with 15 ml. cold distilled water, centrifuged, and washed once. Each cell sample was finally suspended in 5 ml. of distilled water and placed in a boiling water bath for 15 min. After centrifugation, the supernatant fluids were assayed in triplicate for histidine.

sions did not change appreciably with time. Possibly, the imidazole content of the filtrate at pH 5 was slightly higher overall than at pH 6 or 7. However, the total range of variation was only about 20 % of the total imidazole-reacting material initially present. Conversely, the imidazole content of the supernatant fluid of washed and boiled cells (representing the internal pool) incubated at initial pH 5 was lower than that at pH 6 or 7 (Fig. 2). This was especially true at the earliest time shown, which was not a true zero but was in the range of 2–4 min. Krivanek & Krivanek (1959) detected no histidine in the soluble pool of *Dictyostelium discoideum* at any stage of development. In addition, we found that there was little or no endogenous histidine under the conditions used here (Fig. 3). The foregoing experiment was performed at

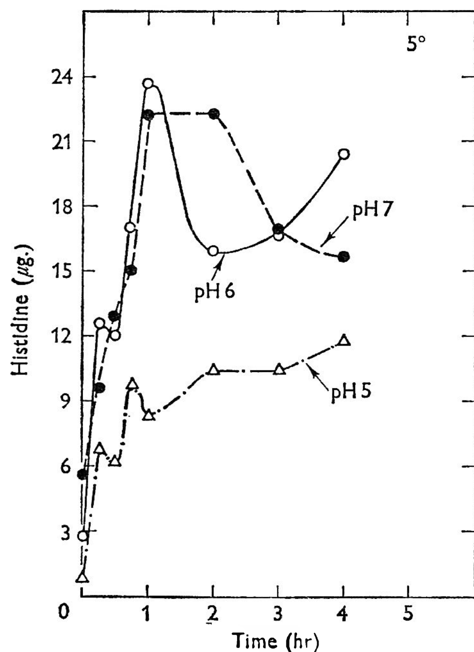

Fig. 3

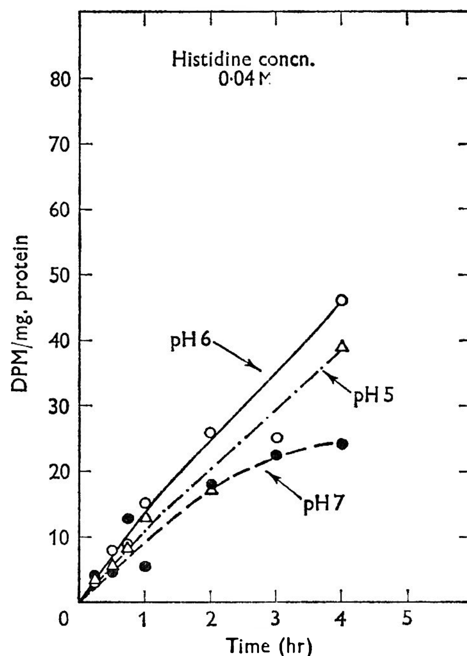

Fig. 4

Fig. 3. Histidine uptake into the soluble pool at 5°. The conditions of this experiment were like those of Fig. 2 with the exception that the incubation temperature was 5°.

Fig. 4. Incorporation of exogenously supplied histidine-^{14}C (uniformly labelled) in protein. Amoebae from two cookie sheets were harvested, washed, and finally suspended in cold distilled water to final volume 9·0 ml. To Petri dishes containing 3·6 ml. Bonner's salt solution with 0·04 M-histidine-^{14}C (uniformly labelled; 0·05 μC/ml.) at the pH value given, 0·4 ml. samples of the amoeba suspension were added. After incubation at 24° for the times listed, 2 ml. of 12 % (w/v) trichloroacetic acid were added. The protein specific activities were determined. Protein concentration was determined by reading the purified materials at 220 mμ, with bovine serum albumin as standard, by the method of Tombs, Souter & Maclagan (1959). DPM = ^{14}C disintegrations per min.

24°. When it was repeated at 5° in order to slow any metabolism of histidine, the results shown in Fig. 3 were obtained. In this case, the maximal concentration of internal histidine, when supplied exogenously at pH 5, was about 50 % of the value observed with external pH 6 or 7.

Since the amount of exogenous amino acids incorporated into protein is a reflexion

of the internal pool concentrations (Krichevsky & Wright, 1963), the kinetics of incorporation of exogenous ^{14}C-labelled histidine into protein at 24° was investigated (Fig. 4). There were only small differences in the rates of incorporation at the pHs studied.

During experiments on the leakage of ultraviolet-absorbing materials from amoeboid cell suspensions, it was noted that, whenever histidine was added, the Millipore filtrate of such suspensions contained material which absorbed maximally at about 300 mμ in 0·5 M-NaOH and at about 267 mμ in 3 % trichloroacetic acid or 0·1 N-HCl (unpublished data). When the filtrate of an amoeboid cell suspension, incubated with 0·04 M-histidine at pH 5, was chromatographed on a column of

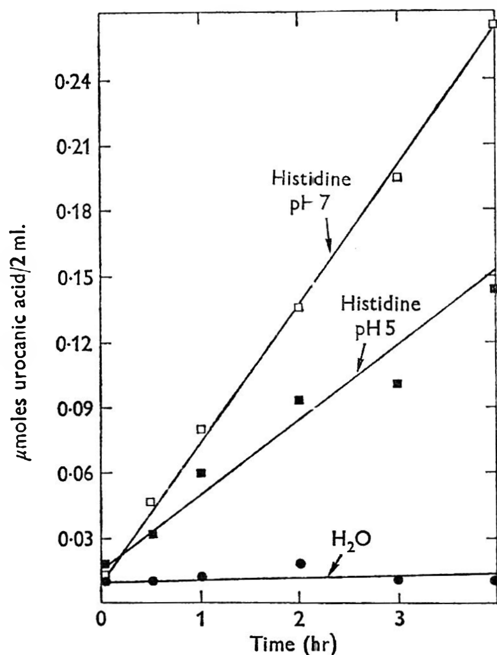

Fig. 5

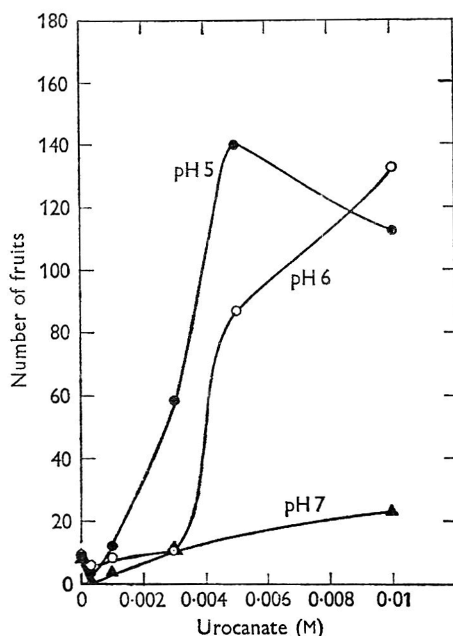

Fig. 6

Fig. 5. Urocanic acid production from histidine by washed myxamoebae. Amoebae were harvested from four cookie sheets, washed and resuspended in cold distilled water to final volume 20 ml. Beakers (1 l.), containing 42·3 ml. of either 0·04 M-histidine (pH 7 or pH 5), or distilled water, were used as incubation vessels. To each beaker was added 4·7 ml. of cell suspension to a final volume of 47 ml. The incubation was carried out at room temperature for the times shown. At the various time intervals, 1·0 ml. samples were withdrawn and pipetted into 1·0 ml. of cold 6 % perchloric acid. The initial values were obtained by adding the perchloric acid to the incubation mixture before adding the cells. The samples obtained were centrifuged at 5° and the supernatant solutions retained for analysis. Urocanic acid measurements were done on a 1/10 dilution, with 0·5 M-KOH as the diluent. The number of μmole urocanate/2·0 ml. original incubation mixture was determined by measuring the absorption at 290 mμ.

Fig. 6. Stimulation of morphogenesis by urocanate. Amoebae from one 9 cm. diam. Petri dish were harvested, washed, and resuspended in 10 ml. cold distilled water. Six 0·01 ml. samples were placed on the surface of each 2·5 % Noble agar plate containing the materials noted in the figure. The plates (unspread) were incubated for 26 hr and the number of fruits on each plate determined. For a discussion of this assay see Krichevsky & Wright, 1963.

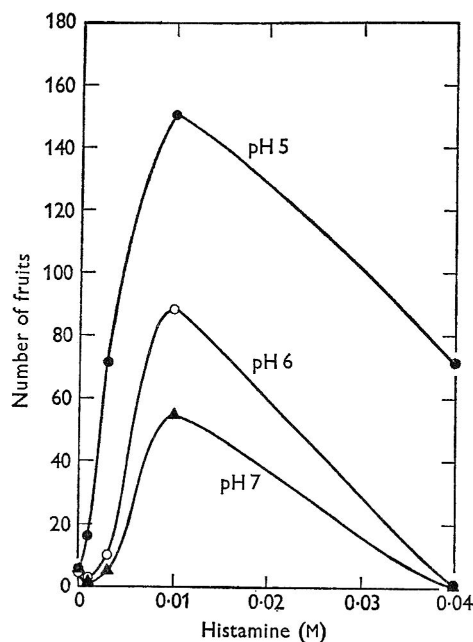

Fig. 7

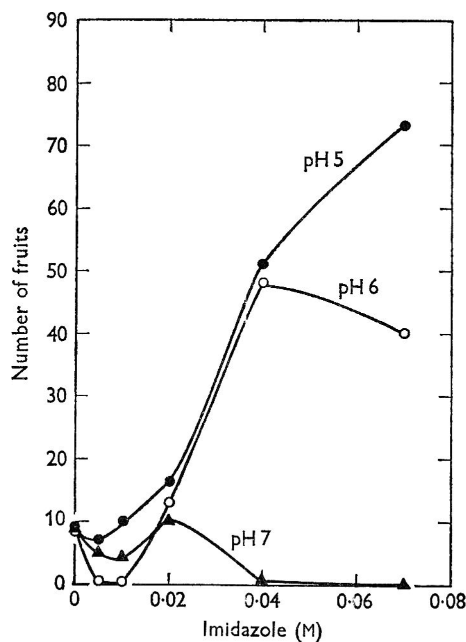

Fig. 8

Fig. 7. Stimulation of morphogenesis by histamine. All conditions were the same as those listed in Fig. 6 except that the materials incorporated in the test plates were as indicated.

Fig. 8. Stimulation of morphogenesis by imidazole. All conditions were the same as those listed in Fig. 6 except that the materials incorporated in the test plates were as indicated.

Table 1. *Thin-layer chromatography of unknown material (isolated from Dowex-1-formate columns) on cellulose-CaSO$_4$*

Solvent	Unknown R_f* values	Urocanic acid R_f* values
Distilled water + NH_4OH to pH 10	0·76	0·75
n-Propanol + 0·2 M-NH_4OH solution, 3 + 1, by vol.	0·52	0·52
Phenol + water, 75 + 25, wt. by vol.	0·78	0·80
n-Butanol + acetic acid + water (60 + 20 + 20, by vol.)	0·62	0·61

* Quenching of fluorescence under 253·7 mμ used to visualize materials.

Dowex-1-formate, only one major peak of u.v.-absorbing (260 mμ) material was eluted. The incubation mixture was identical with that described in Fig. 5. This peak was eluted almost immediately after beginning a gradient elution pattern of 0–1 N-formic acid, thus indicating a very weak acid. After lyophilizing the fractions containing the 260 mμ-absorbing material, the sample was taken up in a small volume of distilled water and spectra were determined under the seven conditions given by Mehler & Tabor (1953). The shift in the absorption maximum with different

pH values was identical with that of authentic urocanic acid (4-imidazole-acrylic acid). Furthermore, the unknown material and urocanic acid had the same mobilities in thin-layer chromatograms on cellulose-$CaSO_4$, developed in four different solvent systems (Table 1). Combinations of these solvent systems in two-dimensional chromatography also showed the identical mobility of the unknown with urocanic acid. As shown in Fig. 5, urocanic acid was produced linearly with time by myxamoebae incubated in the presence of 0·04 M-histidine, and almost twice as fast at pH 7 as at pH 5. Even at pH 7, however, the urocanic acid formed after 4 hr

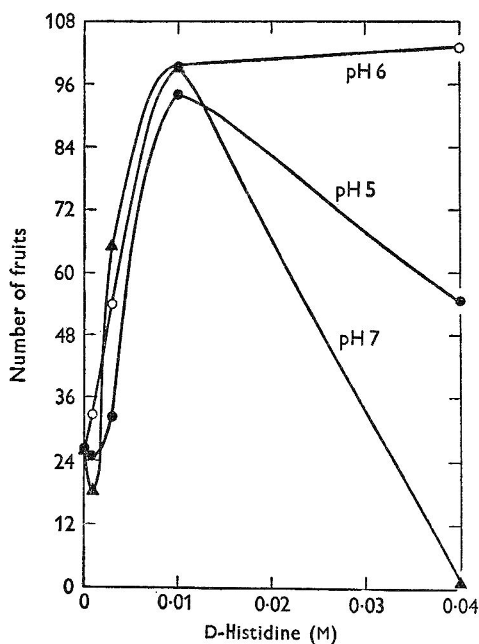

Fig. 9

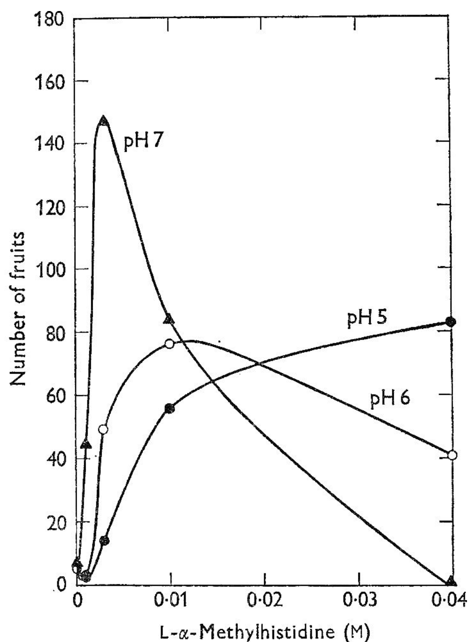

Fig. 10

Fig. 9. Stimulation of morphogenesis by D-histidine. All conditions were the same as those listed in Fig. 6 except that the materials incorporated in the test plates were as indicated.

Fig. 10. Stimulation of morphogenesis by L-α-methylhistidine. All conditions were the same as those listed in Fig. 6 except that the materials incorporated in the test plates were as indicated.

represented only approximately 0·3 % of the histidine supplied. This amount of urocanic acid is equivalent to a concentration of $1\cdot3 \times 10^{-4}$ M, if it be assumed that the urocanic acid is evenly distributed between the cells and their suspending medium.

Control incubations were performed with *Escherichia coli* grown under the same conditions as the amoebae, in a final concentration of 0·1 absorbancy at 660 mμ, which is more than 1000 times the concentration of *E. coli* organisms carried by the amoebal suspensions used. In no case was the production of any urocanic acid by *E. coli* detected.

To test the significance of urocanic acid for the morphogenetic process, the experiment illustrated in Fig. 6 was performed. It may be seen that urocanate was

stimulatory in a manner analogous to L-histidine; i.e. the lower the pH value at which the stimulant was supplied, the greater was the degree of stimulation. Similarly, histamine (Fig. 7) and imidazole (Fig. 8) were more effective stimulants at lower pH values.

While 0·04 M-D-histidine and 0·04 M-L-α-methylhistidine were not stimulatory of the rate of morphogenesis at pH 7 (Figs. 9, 10) and were so at pH 5 and 6, the usual relationship of pH value to stimulation did not obtain for lower concentrations of these materials.

DISCUSSION

The production of urocanic acid (4-imidazole-acrylic acid) from histidine is a common catabolic reaction in mammalian liver (Mehler & Tabor, 1953) and various bacteria (Barker, 1961). *Dictyostelium discoideum* also produces urocanic acid from histidine, as shown by the spectrum and chromatographic behaviour of isolated material. It appears that histidine does not stimulate the morphogenesis of *D. discoideum* by being converted in small amounts to urocanic acid, because equivalent amounts of urocanic acid alone do not stimulate morphogenesis as a whole. Bradley, Sussman & Ennis (1956) stated that urocanate did not stimulate aggregation, but gave no details. The stimulation by L-histidine may simply be due to the imidazole portion of the molecule alone. The facts that all the imidazole compounds tested are stimulatory in the same general range of concentrations and that they all exhibit similar pH dependence (at least at the higher concentrations) would support this conclusion.

The effect of histidine on morphogenesis can be observed as soon as 4 hr after separation of the amoebae from all sources of food. When 0·01 ml. samples of suspensions of amoebae are placed on 2·5 % agar gel containing 0·04 M-histidine, a definite change in the nature of the spot may be seen which is not apparent in controls without added histidine. The periphery of the cell mass (large population densities are used) becomes irregular while the central area becomes 'lumpy'. These changes indicate the very early stages of aggregation. They are as readily apparent to inexperienced as to experienced observers. Thus, any action of histidine which results in stimulation of rate of morphogenesis must at least be initiated during the first 4 hr. Furthermore, a phenomenon which results from histidine addition which bears a casual relationship to the increased rate of development must be more efficient with greater hydrogen ion concentrations, since stimulation by histidine increases at lower pH values (Krichevsky & Wright, 1963). That is, little or no stimulation is observed with histidine at pH 7, but, as the pH value is lowered, the stimulation by histidine increases (at least to pH 5).

When the data here reported are considered in the light of the above considerations it seems that the stimulation of morphogenesis is due, at least in part, to the histidine outside the cell. If the internal histidine were important, the rate at which histidine entered the pool should be higher at pH 5 than at pH 6 or 7; this was clearly not the case; histidine disappeared from the external environment at very much the same rate regardless of the hydrogen ion concentration.

Though they did not test the effect of different hydrogen ion concentrations, Bradley *et al.* (1956) reported that histidine was taken into the internal pool from that supplied extracellularly in the form of 0·01 M-histidine agar at pH 6·2. Furthermore, these workers found that the pool concentrations decreased

quite slowly from their initial values. We have observed essentially the same phenomenon (Fig. 2). In addition, histidine enters the free pool more slowly at pH 5 than at pH 6 or 7. That the observed smaller pool concentrations at pH 5 (as against pH 6 or 7) are due primarily to decreased entry rather than increased metabolism is shown by the following observations. (*a*) At 5° the differences in pool concentrations were greatly amplified while metabolism would be greatly slowed. The maximum histidine concentration, once achieved, was maintained (Fig. 3), in contrast to the shape of the curves obtained at 24° (Fig. 2). (*b*) Histidine incorporation into protein was slower when the external pH value was 5·0 than at pH 7·0. Urocanate production was similarly affected by the pH difference. Therefore, both anabolic and catabolic utilization of histidine was slower under conditions where the amino acid was supplied at a pH value which greatly enhanced its ability to stimulate the rate of development.

The fact that histidine stimulates the rate of morphogenesis in a manner not correlated with its ability to penetrate the amoeboid cell leads to the conclusion that the rate of synthesis of even a single protein is not the rate-limiting step. If the synthesis of even one protein were rate limiting, its rate of synthesis would be correlated with internal histidine concentration. This would be true even if the histidine were acting not directly as a monomer of peptide chains; the same would apply if the histidine were acting to provide other intermediates required for the synthesis of a given protein (e.g. ammonia or carbon skeletons from urocanic acid). Any hypothesis which requires the internal presence of histidine seems to be eliminated.

The simplest mechanism for histidine stimulation of the rate of morphogenesis appears to involve the ability of histidine to decrease the permeability of the amoeboid cells. If histidine is capable of inhibiting the exit of one or more essential metabolites, then it would control the rate of development by controlling the internal concentration of that metabolite. That histidine can affect permeability was shown previously (Krichevsky & Wright, 1963), where the entry of ^{14}C-labelled amino acids into cells was markedly decreased by the presence of histidine externally. It is reasonable to assume that histidine also can inhibit the exit of materials from the amoebae into the environment.

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With 1 plate
Printed in Great Britain

Phosphotungstate Staining of Vaccinia Virus

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(*Received* 23 *September* 1963)

SUMMARY

Vaccinia virus subjected to various repeated treatments and then negatively stained with potassium phosphotungstate was examined with the electron microscope. To obtain reproducible results it was necessary to standardize the preparation procedure. The standarized method now recommended is that the virus should be mixed with stain before being placed on the electron microscope grids and the grids then immediately examined or dried for 1 hr in a vacuum of at least 1×10^{-3} torr. The staining procedure may be altered to vary the degree of penetration of stain so as to reveal either internal or external detail.

INTRODUCTION

Negatively stained preparations of purified vaccinia virus appear to contain two types of particle. This has been reported by Nagington & Horne (1962), Noyes (1962) and Westwood *et al.* (1964). Westwood and co-workers showed that in fresh preparations between 80 and 90 % of the virus had a beaded surface like a mulberry (M form, Pl. 1, fig. 1), whilst the remainder were larger capsulated particles (C form, Pl. 1, fig. 2). They also showed that by various treatments it was possible to alter the ratio of the two types of particle in a given preparation by converting M forms to C forms. During this part of that investigation, quantitative discrepancies in the results of repeated conversion treatments indicated the necessity of standardizing the procedure for preparing specimens. This paper describes the standardization found to be necessary for reliable quantitative studies and demonstrates the errors in structural assessment that can occur if standardization be omitted.

METHODS

Virus. Dermal strains of vaccinia virus were grown on rabbit skin as described by Hoagland, Smadel & Rivers (1940) and purified by density-gradient centrifugation by the method of Zwartouw, Westwood & Appleyard (1962). The final deposit in each case was suspended in distilled water and its infectivity destroyed by ultraviolet (u.v.) irradiation.

Negative staining. Solutions of 2 % (w/v) potassium phosphotungstate (PTA) were used. Solutions of either 2 % (w/v) ammonium molybdate or 1 % (w/v) uranyl acetate were used in some experiments to confirm the results obtained with PTA solution.

Electron microscopy. Except where otherwise stated, carbon-covered grids were used and the virus (with or without stain) applied as drops from a pipette. Specimens were examined with a Siemens Elmiskop I operated at 80 kV, with double condenser illumination.

RESULTS

Effect of allowing virus to dry before staining

Brenner & Horne (1959), who originally described the negative staining method, recommended mixing together virus and stain and spraying the mixture on to coated grids. In the investigation by Westwood *et al.* (1964) where structure in shadowed specimens was investigated, it was considered necessary, for direct comparison, to examine virus which had been allowed to dry before staining. It was soon observed that different specimens prepared from the same virus batch showed wide variations in the ratio of C to M types of particle. Since the staining time had been standard for all specimens, it appeared that the ratio might depend upon the time for which the specimens were allowed to dry before staining.

Table 1. *Vaccinia virus: variation in ratio of C:M particle forms with time of drying before staining with PTA solution*

Virus batch	Drying time	M form (%)	C form (%)
1	Control	82·5	17·5
	10 min.	61	39
	2 hr	40	60
	4 hr	20	80
	24 hr	19	81
2	Control	89·3	10·7
	30 min.	71	29
	1 hr	52	48
	2 hr	17	83
	4 hr	13	87

Table 2. *Vaccinia virus: effect of type of support film on rate of conversion to C form*

Support film	Drying time (hr)	M form (%)	C form (%)
Formvar	Control	84	16
	2	76	24
	6	32	68
Carbon	Control	77	23
	2	46	54
	6	11	89

Table 1 gives the results of experiments with two batches of virus in which the period of drying on the grid before staining was varied. The dried virus specimen was stained by immersion of the grid in PTA solution for 5 min. followed by draining and drying on the laboratory bench. The ratio of C to M forms clearly depended on the initial drying period and reached a maximum at 2–4 hr, when 15–20 % of M forms remained. Further extension of the drying period apparently caused no additional conversion. The rate of conversion was decreased by preparing the specimens on grids coated with 'Formvar' instead of carbon (Table 2). Whereas evaporated carbon films are virtually non-wettable with water, freshly made Formvar films are readily wettable and apparently absorb and retain moisture for some time, so that drying takes place more slowly than on a carbon film.

Effect of delay in examination after staining

Variations in the C:M ratio were also observed in replicate specimens from the same virus batch (mixed with stain) prepared by the spray method of Brenner & Horne (1959). In this case the only variable factor was the delay between preparation of the specimen and its examination with the electron microscope. The effect of varying the period of delay was therefore investigated.

Drops of virus suspension and PTA solution (mixed in equal volumes) were placed on carbon-covered grids. Some specimens were examined immediately and some left for 18 hr before examination. It is seen from the results in Table 3 that delay in examination caused conversion of M to C forms. In addition to the typical C form, an intermediate form had also been produced (Pl. 1, fig. 3) in which the stain had apparently penetrated the surface 'thread' layer (Westwood *et al.* 1964) of the M form but had not reached the nucleoid. The continued slow penetration of stain shown by this experiment implied that sufficient residual moisture remained after drying on the laboratory bench for diffusion of PTA to take place and suggested that specimens should be stabilized by rapid complete drying.

Table 3. *Vaccinia virus: conversion to C form (including intermediate form) brought about by delay in examination after staining with PTA solution*

	Examined immediately		Examined after 18 hr delay	
Virus batch	M form (%)	C form (%)	M form (%)	C form (%)
1	79·5	20·5	35	65
2	80	20	41	59
3	80	20	34	66

Table 4. *Vaccinia virus: results showing stabilization of virus form by vacuum drying*

Virus batch		M form (%)	C form (%)
1	Immediate examination (control)	82·5	17·5
	On bench for 24 hr before examination	48	52
	Under vacuum for 24 hr before examination	81	19
	Under vacuum for 1 hr, then on bench for 24 hr before examination	81·7	18·3
2	Immediate examination (control)	80	20
	On bench for 24 hr before examination	29	71
	Under vacuum for 24 hr before examination	75	25
	Under vacuum for 1 hr, then on bench for 24 hr before examination	81·5	18·5

Table 4 shows the results of an experiment in which replicate specimens were prepared as before. Some were examined immediately, others left standing on the bench for 24 hr before examination and the remainder vacuum-dried at 1×10^{-3} torr. After 1 hr half of the vacuum-dried specimens were removed from the desiccator and allowed to stand on the laboratory bench for a further 24 hr. The others were kept under vacuum for 24 hr and then immediately examined with the electron microscope. It is apparent from the results that stained specimens can be completely stabilized by rapid vacuum drying.

Reconversion of C to M forms in specimens dried before staining

As already shown, desiccation of the virus before staining leads to conversion of M to C forms. This conversion, which appears to be due to increased penetration of stain into the virus particle, may be brought about by chemical agents (Peters, 1962; Westwood *et al.* 1964) in which case it is irreversible. To test the possibility that the conversion following desiccation was reversible, specimens which had been allowed to dry on the laboratory bench for 2 hr were soaked in water for 1 hr before staining. Table 5 shows that there was a complete reconversion to give the original C:M ratio of the specimen.

Table 5. *Vaccinia virus: effect of reconstituting dried virus before staining with PTA solution*

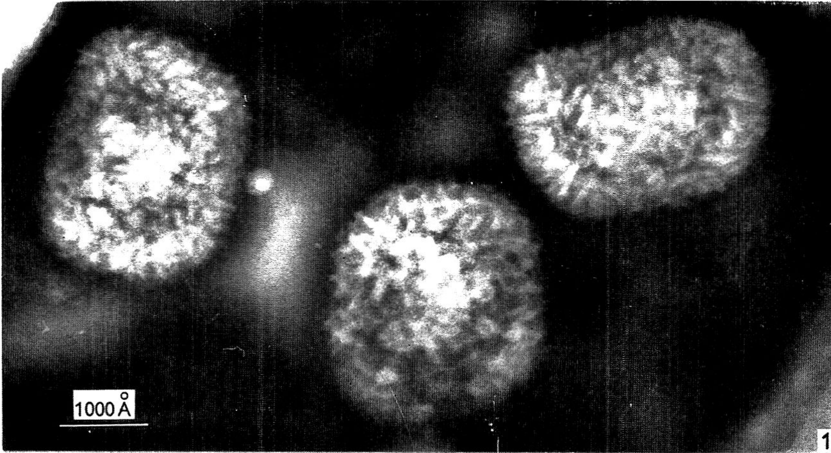
	M form (%)	C form (%)
Control specimen stained and examined immediately	90	10
Specimen allowed to dry for 2 hr before staining	54	46
Specimen allowed to dry for 2 hr, reconstituted for 1 hr before staining	92	8

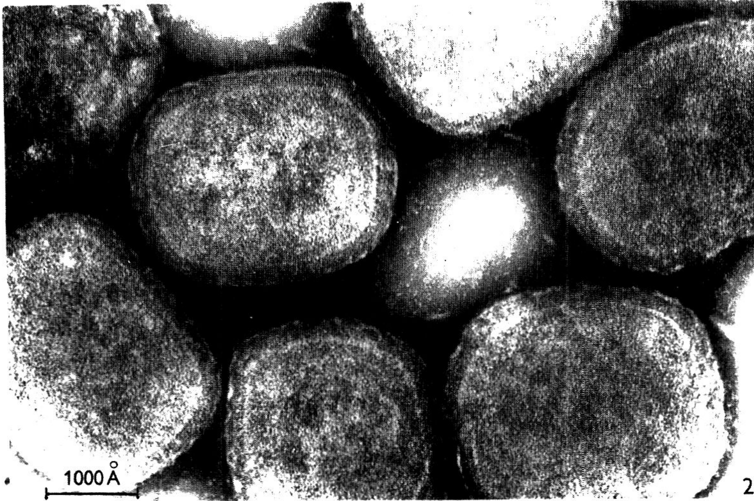
DISCUSSION

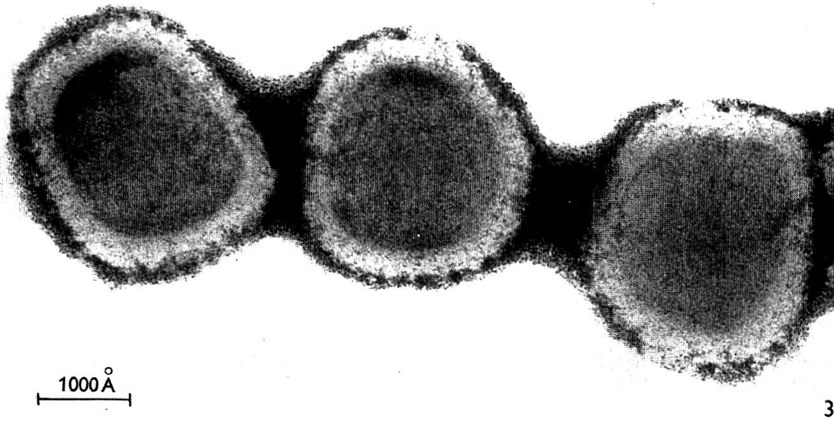
The experimental results clearly show that to obtain repeatable quantitative results and reliable structural assessment when examining preparations of vaccinia virus negatively stained with PTA, it is essential to standardize the preparation procedure. Differences in virus-type ratios brought about by drying before staining are clearly best eliminated by staining the virus in the wet state. Experiments have shown that the time of staining above a minimum of 30 sec. is not critical but, for some reason, optimum spreading of the PTA on carbon-covered grids appears to be obtained with a minimum staining time of 5 min. and this was adopted as the standard procedure.

Westwood *et al.* (1964) concluded that the C form of virus appears to be a particle deficient or damaged in some way which permits penetration of stain into the nucleoidal region and reported that the proportion of C forms could be increased by several treatments of the virus, including exposure to ethanol. It was therefore suggested that the 'thread' layer of the M form might be normally impermeable to stain because of the incorporation of some lipid component. However, as it is possible to reconstitute and reconvert dried C form virus to M form, perhaps the state of turgidity of the 'thread' (or some other layer) also determines whether the virus is of the M or C form, for when turgid the layer may act as a barrier to the penetration of stain.

There appear to be two disadvantages of leaving PTA stained specimens for any length of time before examination. First, as already pointed out, there appears to be a gradual diffusion of stain into the virus; the results in Tables 3 and 4 clearly show the errors that can be encountered and the way in which they can be satisfactorily prevented. We believe that PTA on a grid quickly forms a partially







W. J. HARRIS AND J. C. N. WESTWOOD

(Facing p. 495)

impervious skin so that it becomes increasingly difficult with time to wash away excess PTA from around stained virus. Consequently the virus particle is held in slowly drying PTA enclosed in a 'shell'; thus with the loss of turgidity in the virus due to drying, stain is slowly able to enter the virus. Intermediate forms with confusing appearance are then frequently produced. The second disadvantage of long delay before examination of the specimens is that the PTA in such specimens always appears to be very granular (Pl. 1, fig. 3). The granularity of the PTA appears to undergo change with time because in control specimens examined immediately after staining it is very much finer (Pl. 1, figs. 1, 2).

In the results quoted, the control specimens were prepared under standardized conditions which gave consistent results in replicate experiments. These conditions are: (1) virus mixed with stain for 5 min.; (2) drops of mixture from a pipette placed on carbon-covered grids; (3) immediate drying of prepared specimens either by examination with the electron microscope or by exposure for 1 hr to a vacuum of 1×10^{-3} torr.

The results not only stress the need for standardization of preparation technique if consistent results are to be obtained, but also suggest that the staining procedure may be varied for different purposes according to whether exclusion of stain to reveal external detail or its penetration for the study of internal structure is desired. It may well be that similar standardization is desirable for the reliable examination of viruses other than vaccinia.

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EXPLANATION OF PLATE

Fig. 1. M form of vaccinia virus.

Fig. 2. C form of vaccinia virus.

Fig. 3. Intermediate form of vaccinia virus.

Books Received

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Parasitism. By J. F. A. SPRENT. Published by Baillière, Tindall and Cox, Ltd., 7 and 8 Henrietta Street, London, W.C. 2. 145 pp. 22*s*. 6*d*.

The Ecology of Water Waste Treatment. By H. A. HAWKES. Published by Pergamon Press, Headington Hill Hall, Oxford. 203 pp. Price 42*s*.

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its thirty-ninth General Meeting at University College London, on Thursday and Friday, 2 *and* 3 *January* 1964. *The following communications were made:*

SYMPOSIUM: 'THE BIOSYNTHESIS OF BACTERIAL CELL STRUCTURES'

The Fine Structure of Bacterial Cells. By Audrey M. Glauert* (*Strangeways Research Laboratory, Cambridge*)

Many different bacteria have now been studied in the electron microscope and the main outlines of bacterial fine structure are well established.

The cytoplasm of bacteria is much denser than that of other cells due to the presence of large numbers of closely packed ribosomes. The ribosomes have a similar appearance to those in cell fractions but are usually too close together for specific aggregates (polysomes) to be distinguishable within the cells.

The cytoplasm is bounded by a plasma membrane which appears as a typical 'unit membrane' in thin sections. Small membranous bodies near the periphery of the cell are often present in Gram-positive bacteria and appear to be attached to, and to arise from, the plasma membrane. These peripheral bodies (Chapman & Hillier, 1953) or mesosomes (Fitz-James, 1960) are associated with ingrowing and completed cross walls (Chapman & Hillier, 1953; Glauert, Brieger & Allen, 1961; Imaeda & Ogura, 1963) and with the developing spore membrane (Fitz-James, 1960). Larger and more complex membranous bodies are observed in mycobacteria (Brieger, Glauert & Allen, 1959; Koike & Takeya, 1961), corynebacteria, nocardia and streptomycetes (Glauert & Hopwood, 1960*a*, *b*). These bodies may fill a large proportion of the cytoplasm and sometimes extend into the nuclear regions (Van Iterson, 1961). Numerous connexions with the plasma membrane indicate that the larger membranous bodies may also arise as complex infoldings of the membrane.

Photosynthetic bacteria contain chromatophores which are frequently in the form of small vesicles. The membranes of these vesicles appear to be continuous with the plasma membrane (Boatman & Douglas, 1962). In contrast, vesicular 'chromatophores' are not found in *Rhodospirillum vanielli* (Boatman & Douglas, 1961) and *R. molischianum* (Giesbrecht & Drews, 1962) but peripheral lamellae and lamellated inclusions are present.

The cell wall of a Gram-negative bacterium, such as *Escherichia coli*, has a similar appearance to the plasma membrane and is about 75 Å thick. In contrast the walls of Gram-positive bacteria are 150 to 800 Å thick; the thickness depends upon the age of the bacterium. Little fine structure is seen within any of the walls in thin sections, but an examination of isolated cell walls by the negative-staining technique reveals complex hexagonal patterns in *Spirillum serpens* (Murray, 1963) and *Micrococcus radiodurans* (Glauert, 1962).

Some bacteria have capsular layers on the outside of their walls. These layers may be thick as in corynebacteria and pneumococci, or they may be so thin (*c.* 50 Å) that they are only visible in the electron microscope, as in mycobacteria and nocardia (Glauert, 1962) and are termed microcapsules (Wilkinson, 1958).

Rapidly moving bacteria possess flagella; the flagella of *Salmonella typhimurium* are about 120 Å in diameter and are attached to the cell by a 'hook'. An examination of isolated flagella indicates that they are composed of globular molecules of flagellin arranged

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in five parallel rows to form a hollow tube (Kerridge, Horne & Glauert, 1962). Cells of *Spirillum serpens* have a tuft of flagella at one end; these flagella appear to be similar to those of *S. typhimurium* and the hooked ends are associated with a specialized layer, or 'polar membrane', just inside the plasma membrane (Murray & Birch-Andersen, 1963). The core of the sheathed flagellum of *Vibrio metchnikovii* penetrates the cell wall and seems to be attached to a small basal disc, about 300 Å in diameter (Glauert, Kerridge & Horne, 1963).

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A Micellar Model for the Lipids of Membranes. By J. A. Lucy and Audrey M. Glauert (*Strangeways Research Laboratory, Cambridge*)

The ability of lipid molecules to orientate themselves in an aqueous environment is generally believed to provide a physico-chemical basis for the formation of biological membranes that are composed of lipoprotein. For example, lecithin in water yields layered, myelin figures which are usually interpreted in terms of bimolecular leaflets, and the bimolecular leaflet is often regarded as the only configuration of lipid that is relevant to the structure of biological membranes.

Electron microscopy of negatively stained preparations of artificial mixtures of lipids reveals lamellar, hexagonal and helical structures which cannot be interpreted in terms of bimolecular leaflets. In this paper, the macromolecular structures of three different artificial mixtures of lipids will be discussed, and the possible significance of these structures to the structures of biological membranes will be considered.

Dispersion of a mixture of lecithin and cholesterol in water produces micro-myelin forms that are observable in electron micrographs of samples negatively stained with potassium phosphotungstate. These lamellar structures often appear to be composed of rows of discrete subunits, each about 40 Å in diameter. Tubular structures have also been observed that have an appearance very like that of certain bacterial flagella. It is thought that the subunits in the lipid structures are globular micelles of lecithin, containing cholesterol. These micelles apparently associate spontaneously to yield the observed lamellar and tubular arrangements in a similar manner to the formation *in vitro* of flagella from globular molecules of flagellin. Negatively stained preparations of cholesterol and saponin, the second mixture, reveal hexagonal and lamellar structures each of which is composed of small globular subunits. These subunits, about 35 Å in diameter, are thought to be globular micelles of saponin that contain cholesterol. Finally, helical and hexagonal structures are observed in preparations containing lecithin, cholesterol and saponin that

can be interpreted in terms of specific arrangements of lecithin-cholesterol micelles and saponin-cholesterol micelles. Micrographs of helices prepared using a short-chain synthetic lecithin, in place of ovolecithin, reveal helices containing subunits of correspondingly reduced dimensions.

A natural extension of the interpretations of these various macromolecular assemblies of lipids as specific arrangements of globular micelles leads to the idea that the lipids of biological membranes may, under certain circumstances, be present in the form of globular micelles. Consideration of the structure and properties of a micellar model for the lipids of membranes indicates that this kind of membrane might well play an important role in biological systems. In the theoretical micellar model, the micelles have radii of approximately the lengths of the phospholipid molecules of which they are composed. Within the plane of the membrane, these globular micelles would probably be arranged in a hexagonal lattice but in continuous random motion about their mean positions. The stability of the structure would depend on hydrogen bonding and electrostatic interactions between adjacent micelles, and also on interactions with extended protein molecules on the surfaces of the membrane.

Transitions to and from a micellar type membrane that may be in dynamic equilibrium with a bimolecular leaflet structure may be particularly important in relation to the transportation of molecules and to membrane permeability. The micellar model contains water-filled pores between the micelles; these pores are about 5 Å in radius and extend through the thickness of the lipid layer. Replacement of individual micelles of lipid by globular molecules of specific proteins could localize enzymic activities within the plane of the lipid layer of the membrane, while replacement of a number of adjacent lipid micelles by different enzyme molecules in a specific two-dimensional array would enable several enzymes to participate in an organized sequence of reactions.

The Biosynthesis of Polysaccharide Capsules. By G. T. MILLS (*Department of Organic Chemistry, School of Chemistry, The University, Newcastle upon Tyne*)

Of the micro-organisms having polysaccharide capsules, the pneumococcus presents the field of widest interest, extending from carbohydrate structural studies through immunochemistry to the genetic control of capsule production. It is of great interest therefore to have some knowledge of the pathways of capsular polysaccharide biosynthesis in this organism. Some eighty immunologically distinct capsular types of pneumococci are known, but of these, the structure of the capsular polysaccharide is partly or completely known in not more than ten although the monosaccharide components of the polysaccharides are known for another 25 types. The simplest of the pneumococcal capsular polysaccharides is that of type 3 which is a linear polymer with a repeating unit of

–3-β-D-glucuronosyl-1–4-β-D-glucosyl–.

In view of the now well-known role of the nucleoside diphospho-glycosyl compounds in the interconversion and transfer of glycosyl residues it was considered likely that uridine diphosphoglucose (UDPG) and uridine diphosphoglucuronic acid (UDPGA) would serve as the glycosyl donors in the synthesis of type 3 capsular polysaccharide (S3). It has been found that many nucleoside diphosphoglycosyl compounds can be isolated from various types of pneumococci and that the enzymes necessary for the synthesis of these compounds are present in extracts of the cells. Enzyme extracts prepared from type 3 pneumococci grown in the presence of the capsular depolymerizing enzyme would incorporate glucose and glucuronic acid, from ^{14}C-labelled UDPG and UDPGA, respectively, into S3. The corresponding sugars and sugar-1-phosphates were inactive in this system. In these experiments there was net synthesis of S3 which was identified by (*a*) isolation, (*b*) precipitation with specific type 3 pneumococcal anticapsular serum and recovery from the antigen–antibody complex and (*c*) hydrolysis with the specific capsular depolymerizing enzyme. The distribution of ^{14}C in the S3 indicated that UDPG and UDPGA were the direct donors of the glucose and glucuronic acid, respectively.

Further experiments have shown that the synthesis system has an optimum pH of 8·3, an optimum temperature of 32° and requires Mg^{2+}. The system is present in particulate material sedimenting between 30,000 *g* and 100,000 *g*. and with this particulate material, which may be fragmented cell membrane, the reaction rate was proportional to the amount used. The activity of this particulate material was not affected by treatment with RNase, DNase or the S3 depolymerizing enzyme and when the substrates UDPG and UDPGA were not present in great excess, complete utilization for S3 formation was demonstrated.

The question of a requirement for a primer molecule has not been settled, but S3 depolymerized by the S3 depolymerizing enzyme until only 3% of its antibody precipitating power remained, caused some stimulation of S3 formation. Preliminary experiments suggest a progressive elongation of the polysaccharide molecules during the course of synthesis since at an early phase the molecular size appears to be smaller than at completion. Measurements of the viscosity of the synthesized S3 in water and NaCl solution and its behaviour in immuno-electrophoresis suggests that its molecular size approximates that of native S3.

The synthesis system appears to have a considerable specificity in that if UDPG is replaced by TDPG the rate of synthesis is decreased 10-fold and does not proceed to completion. ADPG is completely inactive in the system.

Similar experiments on the biosynthesis of type 8 capsular polysaccharide (S8) which has a repeating unit of –4-β-D-glucuronosyl–4β-D-glucosyl–4-α-D-glucosyl-1–4-α-D-galactosyl–, have shown that a particulate fraction will utilize UDPG, UDPGal, and UDPGA for the formation of S8.

In the case of type 1 capsular polysaccharide (S1) the fine structure is not known but the polysaccharide contains galacturonic acid (70%), acetylglucosamine (10%), galactose and fucose. It has been found that a particulate enzyme fraction prepared from type 1 cells will form a polymer which precipitates antibody from type 1 anticapsular serum when uridine diphosphogalacturonic acid is used as substrate. When the UDPGalA is labelled with ^{14}C the incorporation of ^{14}C galacturonic acid is about 10%. The amount of material precipitating with type 1 antiserum when UDPGalA is used as substrate is increased by 100% when UDPGalA and uridine diphosphoacetyl glucosamine are present together.

It is clear from these experiments that the nucleoside diphosphoglycosyl compounds are the immediate precursors of the component units of the pneumococcal capsular polysaccharides and also that the enzyme system from a particular pneumococcal type is capable of synthesizing only its specific capsular polysaccharide.

An investigation of a number of non-capsulated variants of pneumococci has shown that the deficiency in polysaccharide production can be related in most cases to a single enzyme deficiency. In the case of a number of non-capsulated type 3 strains (S_{-3}), each of which is genetically distinct, all have the same biochemical lesion, namely, a deficiency of UDPG dehydrogenase—these strains producing virtually no UDPGA. In those S_{-3} strains which have been tested, the capsular polysaccharide synthesizing system is active and can form S3 from UDPG and UDPGA although at a somewhat slower rate than the enzyme from wild type 3 cells. It would appear from transformation experiments that these S_{-3} cells possess non-identical mutations involving the same cistron since any one S_{-3} strain may be transformed by DNA from any other S_{-3} strain to produce a cell with normal capsular polysaccharide production (Austrian *et al.* (1959), *J. exp. Med.* **110**, 585).

In the case of non-capsulated type 1 strains the biochemical lesion can be either a lack of UDPG dehydrogenase or a lack of UDPGA-4-epimerase.

It is evident from a study of the metabolic pathways involved in capsular polysaccharide synthesis, that the explanation for the formation of binary capsulated type 1–3 cells observed by Austrian and Bernheimer ((1959), *J. exp. Med.* **110**, 571) when an S_{-3} cell is transformed with DNA from a capsulated type 1 cell, lies in the interaction at the metabolic level between the pathways controlled by the simultaneous presence of the mutated type 3 genome and by normal type 1 genome in the binary capsulated type 1–3 cells.

The Formation of Bacterial Flagella. By D. KERRIDGE (*Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

There are two major stages in the formation of bacterial flagella; the synthesis of the constituent macromolecules and the aggregation of these molecules to form the functional flagellum. Most bacterial flagella studied consist almost entirely of protein, although with *Vibrio metchnikovii* the flagellar sheath is continuous with the bacterial cell wall and may have a related chemical composition; the flagellar core is probably protein (Glauert, A. M., Kerridge, D. & Horne, R. W. (1963), *J. cell. Biol.* **18**, 327).

The synthesis and functioning of flagella in Salmonella are controlled at a number of genetic loci. There is no evidence to suggest that formation of flagellin molecules differs fundamentally from the synthesis of other cell proteins. Flagellins from certain Salmonella strains (Ambler, R. P. & Rees, M. W. (1959), *Nature, Lond.* **184**, 56; Stocker, B. A. D., McDonough, M. W. & Ambler, R. P. (1961) *Nature, Lond.* **189**, 556) and possibly *Spirillum serpens* (Martinez, R. J. (1963), *Biochim. biophys. Res. Comm.* **12**, 180) are unusual in that they contain the amino acid Σ-*N*-methyllysine (NML). Stocker *et al.* (1961) have suggested that NML is not incorporated as such into the polypeptide chain, but is formed by methylation of lysine residues in flagellin (cf. the methylation of sRNA, Fleissner, E. & Borek, E. (1962), *Proc. nat. Acad. Sci., Wash.* **48**, 1199). Isotope labelling studies have provided some support for this idea. L-[Me-^{14}C]methionine and L-[U-^{14}C]lysine are precursors of flagellar NML in *Salmonella typhimurium*, but ^{14}C-labelled NML cannot be detected in the amino acid pool after incubation of *S. typhimurium* with either of these amino acids. Nor has it been possible so far to detect methylation of lysine by cell free systems from *S. typhimurium.*

Flagellin has been detected in the soluble protein fraction of *Proteus vulgaris* (Weinstein, D., Koffler, H. & Moskowitz, M. (1960), *Bact. Proc.* p. 63) and *Salmonella typhimurium* (Kerridge, D. (1963), *J. gen. Microbiol.* **33**, 63). However, there is no evidence that this material is utilized for flagellar synthesis. There is no detectable lag after the addition of a ^{14}C-labelled amino acid to a culture of *S. typhimurium* before the incorporation of the amino acid into flagella reaches its maximum rate. Kinetic studies suggest that the flagellin in the cell cannot all be acting as a precursor pool although a small fraction may do so.

Biochemical studies have provided no information on the site of flagellar synthesis in bacteria. However, the synthesis of flagellin molecules might be expected to occur in the vicinity of the base of the flagellum. Cytological studies by Murray & Birch-Andersen, (Murray, R. G. E. & Birch-Andersen, A. (1963), *Canad. J. Microbiol.* **9**, 393) and Glauert *et al.* (1963) have demonstrated structures in *Spirillum serpens* and *Vibrio metchnikovii* that may he associated with the synthesis and attachment of flagella in these organisms. Martinez (1963) separated and isolated the 'hooks' from flagella of *S. serpens.* These structures contain RNA and he suggested (personal communication) that this RNA could be concerned with either synthesis or aggregation of flagellin molecules. It will be interesting to explore the possibility of the existence of a group of ribosomes or a polysome at the base of the flagellum.

Bacterial flagella break down at acid pH values into the constituent flagellin molecules and under suitable conditions this process can be reversed when the flagellin molecules re-aggregate to form 'flagella' (Prof. H. Koffler, personal communication; Ada, G. L., Nossal, G. J. V., Pye, J. & Abbot, A. (1963), *Nature, Lond.* **199**, 1257). The ability of flagellin molecules to aggregate spontaneously may play an important role in the formation of bacterial flagella but it would be naïve to discount other factors that may be involved. In *Salmonella typhimurium*, the 'flagellar synthesizing systems' have a limited life (Kerridge, D. (1961), *Symp. Soc. gen. Microbiol.* **11**, 41) suggesting that the bacteria have some mechanism whereby they can control the aggregation of flagellin molecules at particular sites within the bacterium. Formation of spheroplasts from *S. typhimurium* does not impair the ability of the organisms to incorporate labelled amino acids into their proteins, but flagellar synthesis has not been detected in spheroplasts. Formation of flagella can result from the inherent ability of flagellin molecules to aggregate spontaneously

but in bacterial cells, the localization of a polysome synthesizing flagellin at or near the base of the flagellum and some structural configuration of the plasma membrane–cell wall complex might be necessary to provide suitable conditions for this aggregation.

Nucleotide Precursors and Bacterial Cell-wall Synthesis. By PAULINE MEADOW *(Biochemistry Department, University College London)*

Among the complex nucleotides isolated from bacteria are cytidine, uridine and thymidine derivatives containing the cell-wall constituents muramic acid, glucosamine, ribitol phosphate, rhamnose and D-amino acids. Transfer of the sugar or mucopeptide moiety from these nucleotides to a pre-existing primer has now been shown in several systems. In Gram-positive bacteria, polymers containing ribitol- and glycerol-phosphate, rhamnose and mucopeptide have been synthesized, while in Gram-negative organisms studies have been limited mainly to the synthesis of lipopolysaccharides.

Cell-free preparations of *Staphylococcus aureus* and *Lactobacillus plantarum* convert CDP-ribitol into a ribitolphosphate-polymer (Ishimoto, N. & Strominger, J. L. (1963), *Fed. Proc.* 22, 1820; Glaser, L. (1963), *Biochim. biophys. Acta*, **71**, 237), while *Bacillus subtilis* uses CDP-glycerol to synthesize a polymer of glycerolphosphate (Burger, M. M. & Glaser, L. (1962), *Biochim. biophys. Acta*, **64**, 575). The preparations (105,000 *g*-precipitates from sonicates) act as both enzyme and primer for the transferases. UDP-*N*-acetylglucosamine is the source of the α- and β-linked *N*-acetylglucosamine side chains of the polyribitolphosphate backbone of the teichoic acid in *S. aureus* (Nathenson, S. & Strominger, J. L. (1962), *J. biol. Chem.* 238, 3161).

The role of TDP-rhamnose as precursor of the streptococcal C polysaccharide has recently been demonstrated by Zeleznick and co-workers (*Science*, **140**, 400 (1963)). Lysed protoplast membranes of Group A *Streptococcus pyogenes* transfer rhamnose from TDP-rhamnose into a polymer which is coprecipitated with Group A polysaccharide. Pazur & Anderson ((1963) *Biochim. biophys. Acta*, **74**, 788) have shown similar addition of rhamnose to cell-wall fragments from sonicated *Strep. faecalis*.

A particulate system from *Staphylococcus aureus H* catalyses a reaction in which UDP-*N*-acetylmuramyl-ala-glu-^{14}C-lys-ala-ala and UDP-*N*-acetylglucosamine (UDP-GNAc) are utilized to form a lysozyme-sensitive polymer (Meadow, P. M., Anderson, J. S. & Strominger, J. L., *Biochim. biophys. Res. Comm.* in the Press). The reaction requires, and is specific for, both substrates. Experiments with ^{32}P-labelled substrates indicate that they contribute only the GNAc and the acetylmuramyl-peptide moieties to the product. The amount of ^{14}C-labelled polymer formed from doubly labelled UD^{32}P-*N*-acetylmuramyl-ala-glu-^{14}C-lys-ala-ala and unlabelled UDP-GNAc is approximately equal to the UDP-GNAc-dependent release of UM^{32}P and ^{32}Pi. The presence of phosphatases has prevented the direct demonstration of UD^{32}P formation. The ^{14}C-polymer formed is degraded by lysozyme into a low molecular weight fragment which can then be further degraded by β-*N*-acetylglucosaminidase to yield an *N*-acetylmuramyl-^{14}C-peptide. The reaction is inhibited by penicillin, but only at high concentrations and the physiological significance remains to be established.

Some mutants of Salmonella and Escherichia, that are incapable of synthesizing particular sugars, form defective lipopolysaccharides. The lipopolysaccharide of *Salmonella typhimurium* consists of a heptose core with side chains of glucose, galactose, rhamnose, and abequose; that from mutants lacking phosphoglueose-isomerase contains only the core; while that from UDP-galactose-epimerase-less mutants contains only glucose in addition to the heptose core. Extracts from isomerase-less cells transfer glucose from UDP-glucose to lipopolysaccharide isolated from these but no other strains (in which available glucose sites are already filled). Similarly epimerase-less strains transfer galactose from UDP-galactose to their own lipopolysaccharide (Nikaido, H. (1962), *Proc. nat. Acad. Sci., Wash.* 48, 1542; Osborn M. J. *et al.* (1962), *Proc. nat. Acad. Sci., Wash.* 48, 1831 and (1963), *Bact. Proc.* 117). Mutants of *Escherichia coli* carry out similar reactions (Heath, E. C. (1963), *Bact. Proc.* 118).

Eosomes, Ribosomes and Polysomes. By J. SYKES *(Department of Biochemistry, University of Sheffield)*

A complete description of the biosynthesis of the bacterial ribosome has yet to emerge. Nonetheless, pulse-labelling experiments and studies on RNA accumulation during various growth-inhibited states are revealing a strong precursor-product relationship between ribonucleoprotein (RNP) material sedimenting at 10–20S and the larger ribosomes (30–100S) in bacterial extracts.

Material sedimenting at approximately 14S in ultracentrifuge diagrams of bacterial extracts has been described as 'eosomal' (McCarthy, B. J., Britten, R. J. & Roberts, R. B. (1962), *Biophys. J.* 2, 57) since, in addition to establishing its direct precursor-product relationship with all the larger ribosomes, this group have shown that 67% of the RNA associated with this fraction has a ribosomal type of base composition. The residual 33% of the RNA has a base composition approximating that expected for messenger RNA (Midgley, J. E. & McCarthy, B. J. (1962), *Biochim. biophys. Acta*, **61**, 696). Chloromycetin or puromycin inhibition of *Escherichia coli* growth and methionine starvation of the *E. coli* mutant 58–161 result in an accumulation of RNP material in the 10–20S fraction (Dagley, S., White, A. E., Wild, D. G. & Sykes, J. (1962), *Nature, Lond.* **194**, 25). The chloromycetin-accumulated RNA has a base ratio similar to ribosomal RNA and, in the case of the mutant material, the protein associated with the RNA has a cyst(e)ine content similar to that reported for the larger ribosomes; these values are characteristically lower than the general cell protein content of this amino acid. The chloromycetin and puromycin RNP is rapidly broken down when the drug is removed, 260 mμ absorbing material does not leave the cells and the 30S RNP fraction sharply increases. The RNP accumulated in the starved *E. coli* mutant is also lost on adding methionine to the culture and, as growth resumes, the 50S ribosome content increases (Dagley, S., Turnock, G. & Wild, D. G. (1963), *Biochem. J.* 88, 555). A precursor-product relationship is therefore suggested by these results but not established. Simultaneous ^{14}C-uracil labelling experiments with these inhibited systems have also shown that the existing ribosomal material is neither synthesized nor degraded during the phase of inhibition and the accumulated labelled material is transferred to the larger ribosomes when the inhibition is released.

The RNA components of the eosome and ribosome fractions are also indicative of the mode of biosynthesis of the mature ribosome. The RNP accumulated during chloromycetin inhibition and methionine starvation has been shown to contain the 16 and 23S RNA species characteristic of ribosomal RNA (Dagley *et al.* (1963), and Kurland, C. G., Nomura, M. & Watson, J. D. (1962), *J. molec. Biol.* 4, 388). A 4–8S fraction has been reported in RNA preparations from all classes of ribosomes (McCarthy, B. J. & Aronson, A. I. (1961), *Biophys. J.* 1, 215). This component is (i) rapidly labelled with ^{14}C-uracil, and (ii) a major fraction of the 20S RNP particle in which the 16 and 23S RNA species do not appear. Once again kinetic, pulse-labelling experiments have shown that the 4–8S material progressively aggregates to form the 16 and 23S RNA of the larger ribosomes without first equilibrating with the nucleotide pool. The further observation that the 16 and 23S RNA species can be non-randomly degraded to a 4S unit reinforces the idea that ribosomes may arise by the aggregation of RNA (and protein) subunits; the units formed then give rise to the larger 70, 85 and 100S ribosomes—a process partly depending upon the prevailing concentration of Mg^{2+}.

In addition to the well-documented size variation of ribosomes with the magnesium ion concentration, a compositional variation has also been reported (Sykes, J. & Tempest, D. W. (1963), *Bact. Proc.* P109, 122). A *Pseudomonas* sp. growing at the same rate in the chemostat under conditions of (i) carbon and (ii) magnesium limitation gave 50S ribosomes with 48 and 31% RNA, respectively, the remainder being protein. The relative amount of 50S material in the Mg^{2+} limited cells was significantly lower than that in the carbon limited state, but the amount of trichloroacetic acid insoluble, non-ribosomal RNA was considerably higher; the latter material did not appear in the 10–20S region of ultracentrifuge diagrams of extracts from these cells. The mature ribosome may therefore be in a dynamic state with respect to both its size and composition throughout the changing environmental conditions of the normal growth cycle.

Multiple ribosomal structures (polysomes) have recently been identified in sucrose gradients when labelled T2-messenger RNA or poly-uridylic acid are added to *E. coli* ribosomes (Barondes, S. H. & Nirenberg, M. W. (1962), *Science*, **138**, 813). Polysomes appear to be simple aggregates of 70S ribosomes, the size of the aggregate and its stability apparently being determined by the messenger RNA involved.

The Pigment-bearing Structures in Photosynthetic Bacteria. By June Lascelles

(*Microbiology Unit, Department of Biochemistry, University of Oxford*)

The term 'chromatophores' was originally applied by Schachman, H. K., Pardee, A. B. & Stanier, R. Y. ((1952), *Arch. Biochem. Biophys.* **38**, 245) to the relatively homogeneous pigmented fraction obtained from extracts of *Rhodospirillum rubrum* and which consisted of particles about 600 Å in diameter. Such preparations contain the entire complement of photosynthethic pigments and the cofactors and enzymes required for photophosphorylation. Electron microscopy of sections of photosynthetic bacteria have revealed within the cytoplasm membrane-bound vesicles similar in size and appearance to that of isolated chromatophores.

Recent observations have suggested that chromatophores arise from, or are part of, the cytoplasmic membrane, rather than being hollow membrane-bound spheres that exist free in the cytoplasm. Thus, the photosynthetic apparatus is located in the membrane fraction of osmotically lysed protoplasts of *Rhodospirillum rubrum* (Tuttle, A. L. & Gest, H. (1959), *Proc. nat. Acad. Sci., Wash.* **45**, 1261) and, in some electron micrographs of whole cells and lysates, continuity between the vesicles and the cytoplasmic membrane has been shown (Cohen-Bazire, G. & Kunesawa, R. (1963), *J. cell. Biol.* **16**, 401; Giesbrecht, P. & Drews, G. (1962), *Arch. Mikrobiol.* **43**, 152). Also, the composition of chromatophores (apart from the photosynthetic pigments) is similar to that of the respiratory particles from non-photosynthetic bacteria, which are derived from the cytoplasmic membrane. It seems likely, therefore, that chromatophores prepared from extracts arise by comminution of the cytoplasmic membrane. The homogeneity of such preparations remains to be explained.

The pigment content of photosynthetic bacteria varies considerably with the environment and the number of vesicles in electron micrographs of cell sections is correlated with the pigment content (Cohen-Bazire, G. & Kunisawa, R. 1963). How, then, is the cytoplasmic membrane modified to accommodate the variable quantities of pigment? The problem is clearly seen in Athiorhodaceae adapting from the non-pigmented to the pigmented state, for instance, upon transfer from highly aerobic to conditions of low aeration. This transformation involves not only the formation of the photosynthetic pigments and their integration into the chromatophore structure but also the elaboration of additional cofactors and enzymes, including some that catalyse steps in the formation of the pigments.

In an attempt to see if pigment formation is accompanied by synthesis of chromatophore protein, adaptation to *Rhodopseudomonas spheroides* from the non-pigmented to the pigmented state has been studied with suspensions incubated under low aeration (Bull, M. J. & Lascelles, J. (1963), *Biochem. J.* **87**, 15). Pigment synthesis under these conditions is obligatorily linked to protein synthesis, since it depends on a source of cell nitrogen, and is inhibited by amino acid and nucleotide base analogues. Incorporation of ^{14}C-phenylalanine into the soluble and chromatophore protein fractions occurs during the adaptation, but the incorporation into the latter occurs at about twice the rate of that into the soluble protein. This preferential incorporation into chromatophore protein is apparently associated with pigment synthesis; it does not occur under high aeration, which represses pigment synthesis, nor does it occur in a mutant strain that lacks the ability to form the photosynthetic pigments. Direct analysis of the protein formed in each fraction during pigment synthesis reveals, however, that *both* the soluble and chromatophore fraction increase by about 20 % in 3 hr. incubation. This is to be compared with an apparent increase of 37 and 64 %, respectively, in the soluble and chromatophore fractions as measured by ^{14}C incorporation. The experiments suggest that pigment synthesis by the adapting organisms is linked to preferential turnover of protein in the chromatophore fraction (presumably

derived from the cytoplasmic membrane). The pigment-bearing structure may therefore arise, at least to some extent, by turnover of pre-existing membrane and re-organization in a new form which incorporates the pigments and associated factors.

The formation of these structures by photosynthetic bacteria in response to the environment bears a striking similarity to respiratory adaptation in yeast, which involves formation of cytochromes and other components of the electron transport chain as well as enzymes of the tricarboxylic acid cycle. Electron microscopy of cell sections and analysis of extracts by density gradient centrifugation suggest that mitochondria are developed by the adapting yeast from pre-existing cytoplasmic membrane (Schatz, G., Tuppy, H. & Klima, J. (1963), *Z. Naturf.* **18**b, 145; Schatz, G. (1963), *Biochem. biophys. Res. Commun.* **12**, 448.

ORIGINAL PAPERS

The Effect of 2:4-Dinitrophenol on CO_2-fixation and ATP Content of *Thiobacillus thioparus*. By D. P. Kelly* and P. J. Syrett (*Department of Botany, University College London*)

Peck & Fisher ((1962), *J. biol. Chem.* **237**, 190) have proposed, from studies with cell-free extracts, that the oxidation of thiosulphate by *Thiobacillus thioparus* proceeds by its reduction to sulphide and sulphite, followed by the oxidation of sulphite to sulphate by reactions linked to a substrate-level phosphorylation insensitive to dinitrophenol (DNP). Using a newly isolated strain of *T. thioparus* that couples CO_2-fixation to the oxidation of sulphide, thiosulphate, tetrathionate or trithionate, we have investigated the inhibition of CO_2-fixation by DNP concentrations ranging from 10^{-5} to 10^{-3} M. CO_2-fixation linked to sulphide oxidation was more sensitive to DNP inhibition than that linked to the oxidation of thiosulphate or tetrathionate. Assuming that CO_2-fixation is a measure of high energy phosphate formation, these results suggest that both DNP-sensitive and DNP-insensitive phosphorylations accompany substrate oxidation by this organism, the DNP-sensitive phosphorylations being relatively more important during sulphide oxidation. Calculation from the results shows that if all the phosphorylations linked to sulphide oxidation are of the DNP-sensitive type, thiosulphate oxidation is accompanied by sensitive and non-sensitive phosphorylations in the ratio of 2:1.

The addition of either sulphide or thiosulphate to cells was followed by an immediate increase in their ATP content. When sulphide was the substrate, the increase in ATP was abolished by 10^{-4} M DNP, but with thiosulphate as substrate the rise was unaffected. Thus these results, too, suggest that a DNP-insensitive phosphorylation is linked to the oxidation of thiosulphate but not to that of sulphide.

Sulphur Metabolism of *Chromatium* Strain D and Rhodanese Activity in Extracts. By A. J. Smith (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

The purple photosynthetic sulphur bacterium *Chromatium* photometabolizes certain organic compounds in addition to growing autotrophically in the presence of reduced sulphur compounds that are oxidized to sulphate. Thiosulphate, sulphide, exogenous and endogenous sulphur, and sulphite have been shown to support autotrophic growth of *Chromatium*. Growth yields on these inorganic sulphur compounds, in terms of protein, can be correlated with the number of reducing equivalents made available on oxidation to sulphate. Tetrathionate has been suggested as the initial product of thiosulphate oxidation in the Thiobacilli (Vishniac (1952), *J. Bact.* **64**, 363; Trudinger (1959), *Biochim. biophys. Acta*, **31**, 270). These chemoautotrophs also oxidize thiosulphate to sulphate. *Chromatium* does not grow on tetrathionate or on tetrathionate plus thiosulphate. Tetrathionate also inhibits non-competitively and reversibly thiosulphate oxidation by illuminated suspensions of *Chromatium*.

Rhodanese is present in most animal tissues and has been crystallized from beef liver

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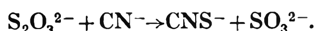
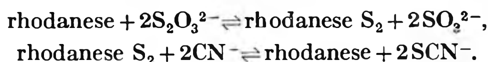
(Sorbo (1953), *Acta chem. scand.* **7**, 1129). It catalyses the cleavage of the S—S bond in thiosulphate and thiosulphonates



$$S_2O_3^{2-} + CN^- \rightarrow CNS^- + SO_3^{2-}.$$

Recently Green ((1961), *J. biol. Chem.* **236**, 3047) and Westley ((1962), *J. biol. Chem.* **237**, 547), using polarographic and isotopic techniques, have proposed a double displacement mechanism



$$\text{rhodanese} + 2S_2O_3^{2-} \rightleftharpoons \text{rhodanese } S_2 + 2SO_3^{2-},$$
$$\text{rhodanese } S_2 + 2CN^- \rightleftharpoons \text{rhodanese} + 2SCN^-.$$

Sonic extracts of thiosulphate-grown *Chromatium* contain a soluble heat-labile factor which catalyses the cyanolysis of thiosulphate. The activity of extracts was measured using a standard rhodanese assay (Cosby & Sumner (1950), *J. biol. Chem.* **185**, 669). The stoichiometry of the cleavage has been established in terms of thiosulphate and thiocyanate.

Villarejo & Westley ((1963), *J. biol. Chem.* **238**, PC 1185) have reported that beef liver rhodanese catalyses the reductive cleavage of thiosulphate

$$\text{lipoic acid } (SH)_2 + S_2O_3^{2-} \rightarrow \text{lipoic acid } (S)_2 + HS^- + H^+ + SO_3^{2-}.$$

A reductive cleavage of thiosulphate by *Chromatium* extracts has not yet been demonstrated. It is possible that such a reaction may be catalysed by the same factor that mediates the cyanolysis of thiosulphate.

Thiosulphate reductase activity has been reported in yeast (Kaji & McElroy (1959), *J. Bact.* **77**, 630) and also in a *Thiobacillus* (Peck (1960), *Proc. nat. Acad. Sci., Wash.* **46**, 1053). This type of reaction may be a key step in the oxidation of thiosulphate by *Chromatium* as suggested by Peck (1960) for Thiobacilli. The fact that *Chromatium* grows on sulphide and sulphite but not on tetrathionate is consistent with the key role envisaged for thiosulphate reductase.

Growth of Dihydrostreptomycin-treated *Escherichia coli* after Removal of Extracellular Antibiotic.

By MARGOT KOGUT and J. W. LIGHTBOWN (*National Institute for Medical Research, Mill Hill, London*)

Treatment of *Escherichia coli* B in the early logarithmic phase of aerobic growth in a mineral salts-glucose medium with low concentrations of dihydrostreptomycin—conditions where intracellular accumulation of the drug is known to occur (Hancock, R. (1962), *J. gen. Microbiol.* **28**, 493)—produced gradually increasing inhibition of growth. When samples were removed at various times after addition of the antibiotic and freed from extracellular dihydrostreptomycin by filtration and washing and then resuspended in the same volume of warmed, antibiotic-free medium, they continued to grow at apparently exponential, but inhibited rates. The extent of inhibition was a function of the duration of prior aerobic growth in dihydrostreptomycin and of the initial antibiotic concentration (Kogut, M. & Lightbown, J. W. (1963), *Biochem. J.* **89**, 18P).

Calculations showed that the apparently exponential nature of this inhibited growth in the absence of extracellular antibiotic could not be the result of large inequalities in the growth rates of different portions of the population. Direct microscopic observations showed that during periods equivalent to three generation-times, the proportions of dividing cells (Postgate, J. R., Crumpton, J. E. & Hunter, J. R. (1961), *J. gen. Microbiol.* **24**, 15) in such cultures did not differ from those of controls. However, conventional plate counts showed reductions in the proportion of cells giving rise to visible colonies.

After approximately 3–4 hr of inhibited exponential growth, the growth rates increased and gradually returned to those characteristic of controls. This 'recovery' was due neither to outgrowth of streptomycin-resistant cells, nor to continued uninhibited growth of a proportion of cells present at the time of filtration. It appeared to be due to genuine increases in the growth rates of inhibited cells. The time of onset of this recovery was independent of the increase in optical density which had occurred during the phase of inhibited growth.

Methionine Effect in the Fission Yeast *Schizosaccharomyces pombe*. By C. H. Clarke (*Mutagenesis Research Unit, Institute of Animal Genetics, Edinburgh*)

In the haploid fission yeast *Schizosaccharomyces pombe* the presence of L- or DL-methionine in the plating medium leads to an inhibition of the phenotypic expression of newly induced mutations to adenine-independence ($adn^- \rightarrow adn^+$) (Clarke, C. H. (1962), *Z. Vererbungsl.* **93**, 435; (1963), *J. gen. Microbiol.* **31**, 353). Further results obtained for reverse mutations of *adenine*-1 auxotrophs indicate that the degree of inhibition by methionine depends on (*a*) the particular mutant studied, (*b*) the mutagen used to induce the reverse mutations, (*c*) methionine concentration.

In doubly auxotrophic strains requiring both adenine and methionine, the inhibitory effect of methionine upon adenine reversions can lead to an apparent reduced reverse mutability of the adenine allele, and to distorted ratios of adn^+ to met^+ reverse mutations ('mutagen specificity'). Adenine-independent revertants due either to unlinked suppressor mutations, or to reverse mutation at or very close to the *adenine*-1 locus, are both inhibited in their expression by methionine. There is no effect on the growth of adenine revertants which are already phenotypically expressed before exposure to methionine medium.

Methionine has the additional effect of reducing the residual divisions undergone by adenine-requiring cells when incubated on a medium lacking adenine. However, this does not seem to be the underlying reason for the inhibition of adn^+ revertants' expression, since conditions allowing increased residual growth do not lead to an abolition of the methionine effect on reverse mutations.

The phenotypic expression of some slow growing suppressor revertants of leucine, arginine and uracil auxotrophs is not affected by the methionine in the plating medium, nor is that of the HNO_2-induced revertants of several lysine auxotrophs. Methionine has been shown, however, to interfere with the expression of revertants of mutants at several different adenine loci, to affect the growth of some complementing diploids, and to inhibit the growth of certain adenine double mutants on 6-methyl aminopurine (Heslot, personal communications).

Rough Mutants of *Salmonella typhimurium*; Genetical, Chemical, Immunological and Enzymic Studies. By T. V. Subbaiah and B. A. D. Stocker (*Lister Institute of Preventive Medicine, London, S.W.* 1), Ilse Beckmann (*Max-Planck Institute for Immunobiology, Freiburg*), H. Nikaido and Kishiko Nikaido (*Massachusetts General Hospital and Harvard Medical School, Boston, Mass.*)

In colicine-factor-mediated crosses (Smith & Stocker, *Brit. Med. Bull.* (1962), 18, 46) of typical rough mutants of *Salmonella typhimurium* strain LT2 the rough character in twelve (*rouA* class) mapped near *ile* (isoleucine-requirement); in six (*rouB* class) near *try* (tryptophane-requirement) and *his* (histidine-requirement).

Phenol extraction of thirteen mutants (at Freiburg) yielded typical rough lipopolysaccharides, lacking the smooth-specific sugars mannose, rhamnose and abequose; in haemagglutination and inhibition tests (Beckmann *et al.*, *Biochem. Zf.*, in the Press) five of the seven *rouA* lipopolysaccharides showed antigen R_I, all of six *rouB* showed antigen R_{II}. Additional, non-sedimentable, material containing glucose, galactose, mannose, rhamnose and abequose (but not heptose) was present in every *rouA* but no *rouB* extract; this material reacted with O 4,12 serum by precipitation and haemagglutination, i.e. had some smooth antigenic specificity.

Sonicated extracts of six *rouA* and six *rouB* mutants were assayed at Boston for enzymic activity in eleven reactions leading from glucose to the presumed sugar donors for polysaccharide synthesis: UDP-glucose; UDP-galactose; TDP-rhamnose; GDP-mannose; and CDP-abequose. All mutants had normal activity in all reactions except one *rouB* mutant, TV208, unable to convert TDP-4-keto-6-deoxy-D-glucose to TDP-rhamnose. The rough character of TV208 probably results from inability to add rhamnose to R_{II} lipopolysaccharide, through lack of activated rhamnose; the other *rouB* mutants, also making R_{II}

lipopolysaccharide, are presumably unable to add rhamnose from TDP-rhamnose to R_{II} through lack of a transferase.

As the R_{II} lipopolysaccharide of *rouB* mutants lacks mannose and abequose a rhamnose unit is probably the deepest of the smooth-specific sugars in the smooth side-chain. The unidentified *rouA* lesion perhaps prevents conversion of R_I core lipopolysaccharide to R_{II}; it seems to permit synthesis, at least partial, of smooth side-chains, but not their attachment to the core.

Exopolysaccharide Depolymerase Produced by Phages in Association with *Escherichia coli* K12. By I. W. Sutherland and J. F. Wilkinson (*Bacteriology Department, Edinburgh University*)

The production of exopolysaccharide depolymerases following phage infection of bacteria has been studied in *Klebsiella pneumoniae* B (Adams, M. H. & Park, B. H. (1956), *Virology*, 2, 719) and in *Azotobacter vinelandii* (Eklund, C. & Wyss, O. (1962), *J. Bact.* 84, 1209). Some properties of phage-bacterium systems producing depolymerases for *Escherichia coli* K12 extracellular polysaccharides are now described.

Phages were isolated from raw sewage by standard methods. Those which showed haloes surrounding the plaques were examined for the production of a depolymerase. From over 30 phage isolates active against *Escherichia coli* K12, five were found to produce an enzyme capable of breaking down the extracellular polysaccharide produced by this group of bacterial strains. As judged by reduction in viscosity of polysaccharide solutions, the phage enzymes were also capable of depolymerizing the extracellular (slime) polysaccharide of *Aerobacter cloacae* NCTC 5920, a substance of the same chemotype as that produced by *E. coli* K12. Two phages isolated using *A. cloacae* as the host bacterium produced depolymerases active against the polysaccharides of both bacterial species. Further tests revealed that one of the five phages isolated originally on *E. coli* could also multiply in *A. cloacae* cells. This phage, designated F1, was of particular interest in that the depolymerase was only produced following infection of mucoid *E. coli* cultures. Variants of K12 which did not produce detectable capsular or slime material supported bacteriophage multiplication but not depolymerase production. The enzyme from the phage F1 system has been partially purified by ammonium sulphate precipitation and by chromatography on DEAE-cellulose.

Cell Wall Composition in *Corynebacterium acnes*. By C. S. Cummins (*London Hospital Medical College*)

The cell wall compositions of 6 strains of *Corynebacterium acnes* have been examined. The principal components found by chromatography were glucose, galactose, alanine, glutamic acid, glycine, LL-diaminopimelic acid and hexosamines; arabinose was not detected. The results suggest that *C. acnes* is more closely related to propionibacteria than to corynebacteria of the type of *C. diphtheriae*.

A Study of L-Forms of *Staphyloccocus aureus* by Acridine Orange Fluorescence Microscopy. By B. C. Pratt and G. W. Csonka (*Department of Bacteriology, Wright-Fleming Institute of Microbiology, St Mary's Hospital Medical School, London*)

Acridine orange has been used to stain the L-forms of *Staphylococcus aureus*. Under the conditions employed, DNA fluoresces green and RNA fluoresces red-orange. Cultures of the organisms grown for 48 hr in a special serum and high salt medium yield a mixture of L-form elements staining for DNA and RNA in varying degrees. These elements differ from the parent staphylococcus in size and also in the apparent proportions of DNA to RNA in individual cells. By differential centrifugation (modified from Mandel, P., Terranova, T., Sensenbrenner, M. & Feo, F. (1959), *Nature, Lond.* 183, 194) the heterogeneous

culture can be separated into four distinct fractions. Fractions 1 and 2 give similar staining reactions in that the elements appear to consist entirely of DNA, there being no visible RNA; the only apparent difference between these two fractions is in the size of the elements. The cells in fraction 3, though not differing markedly in size from those in fraction 2, stain for both DNA and RNA. The amount of RNA present in the cells varies and appears as isolated areas on the surfaces of the cells. In some of these cells the RNA forms a continuous layer on a DNA core. The fourth fraction contains small 'particles' staining for RNA.

Growth of these fractions in liquid medium yields the same mixture of elements as that in the unfractionated culture. Growth on solid medium gives rise to colonies with the typical 'fried-egg' form, common to L-forms and some PPLO. Stained with acridine orange, these colonies show the dense granular centre to be predominantly RNA, whereas the periphery consists of large vesicles containing wholly or predominantly DNA.

FILM

Continuous Culture: A Simple Explanation. By G. C. Ware, L. B. Quesnel and L. W. Greenham (*Department of Bacteriology, University of Bristol*)

This film was made in the Department of Bacteriology for the specific purpose of pictorially presenting to students the fundamentals of continuous culture. The film opens with a discussion of the laws and conditions governing the nature and rate of bacterial growth and division in batch culture. The effect of the addition of fresh medium dropwise to an 'exhausted' culture is then examined, and gradually evolved to a continuous process of medium replenishment and accompanying bacterial growth. An introduction to the formal mathematical proof is included in animated form and the film ends with a demonstration of some actual devices at the Microbiological Research Establishment, Porton.

The film which was made on 35 mm. Eastman colour negative type 5250 by xenon flash exposure was processed by Humphries Limited. The photographing of about 30,000 individual frames for the animation necessitated some degree of automation.

The authors would like to thank the Peter Hunt Recording Studios Ltd., Dublin, for generous loan of studio facilities for recording the sound track.

Index of Authors

Small roman numerals refer to pages in the Proceedings of the Society for General Microbiology

AJL, S. J., *see* MONTIE, T. C. 249
ALLEN, M. B., FRIES, L., GOODWIN, T. W. & THOMAS, D. M. The carotenoids of algae: pigments from some Cryptomonads, a heterokont and some Rhodophyceae 259
ANDERSON, T. F., *see* WILDY, P. 273
APPLEYARD, G., *see* WESTWOOD, J. C. N. 67

BADDILEY, J., *see* SHARPE, M. E. 333
BERNHEIM, F. The effect of pyruvate and acetate on the rate of decrease in optical density of suspensions of *Pseudomonas aeruginosa* in sodium, potassium or sodium-potassium phosphate buffers 327
BOBO, R. A. & FOSTER, J. W. The effect of enzyme treatments on *Brucella abortus* cell walls 1
BROCKMAN, H. E. Effects of amino acids on the utilization of tryptophan and indole for growth by a mutant of *Neurospora crassa* 31
BUTLER, M. & LEACH, R. H. A mycoplasma which induces acidity and cytopathic effect in tissue culture 285

CHARLES, H. P. Relationships between certain pyrimidine and arginine mutants of Neurospora, as revealed by their response to carbon dioxide 131
COLLINS, F. M. Composition of cell walls of ageing *Pseudomonas aeruginosa* and *Salmonella bethesda* 379
COLLINS, J. F. The distribution and formation of penicillinase in a bacterial population of *Bacillus licheniformis* 363
COLLINS, J. F., MASON, D. B. & PERKINS, W. J. A microphotometric method for the estimation of penicillinase in single bacteria 353
COOK, A. M., ROBERTS, T. A. & WIDDOWSON, J. P. Gamma irradiation of *Bacillus subtilis* spores in the presence of sugars 185
CROSSE, R., MCWILLIAM, R. & RHODES, A. Some relations between chemical structure and antifungal effects of griseofulvin analogues 51
CSILLAG, A. Growth of a form 2 mycobacterium and various bacillus species on Löwenstein-Jensen medium 79
CSILLAG, A. The mycococcus form of mycobacteria 341

DANFORTH, W. F., *see* MARZULLO, G. 9
DANFORTH, W. F., *see* MARZULLO, G. 21
DAVIS, G. H. G. Notes on the phylogenetic backgroumd to Lactobacillus taxonomy 177
DAVISON, A. L., *see* SHARPE, M. E. 333
DE LEY, J. On the unity of bacterial ribosomes 219
DIXON, B. & ROSE, A. H. On the synthesis of ornithine carbamoyltransferase in biotin-deficient *Saccharomyces cerevisiae* 229

FOSTER, J. W., *see* BOBO, R. A. 1
FRANKLIN, J. G. & SHARPE, M. E. Physiological characteristics and vitamin requirements of lactobacilli isolated from milk and cheese 143
FRIES, L., *see* ALLEN, M. B. 259
FRY, B. A., *see* WAITES, W. M. 413

GIBSON, T., *see* SMITH, N. R. 269
GIFFORD, G. E., MUSSETT, M. V. & HELLER, E. Comparative studies on the production and assay of interferon 475
GORDON, R. E., *see* SMITH, N. R. 269
GOODWIN, T. W., *see* ALLEN, M. B. 259
GOUWS, L., *see* KISTNER, A. 447

HARRIS, W. J. & WESTWOOD, J. C. N. Phosphotungstate staining of vaccinia virus 491
HARRIS, W. J., *see* WESTWOOD, J. C. N. 67
HELLER, E., *see* GIFFORD, G. E. 475
HORVÁTH, I., VARGA, J. M. & SZENTIRMAI, A. Control of valine and isoleucine metabolism in *Pseudomonas aeruginosa* and *Escherichia coli* 241
HUNTER, G. D. & MILLSON, G. C. The intracellular location of the agent of mouse scrapie 319
HUNTER, J. R., *see* POSTGATE, J. R. 459

KAPLAN, I. R. & RITTENBERG, S. C. Carbon isotope fractionation during metabolism of lactate by *Desulfovibrio desulfuricans* 213
KAPLAN, I. R. & RITTENBERG, S. C. Microbiological fractionation of sulphur isotopes 195
KELLY, D. P. & SYRETT, P. J. The effect of uncoupling agents on carbon dioxide fixation by a thiobacillus 307
KISTNER, A. & GOUWS, L. Cellulolytic cocci occurring in the rumen of sheep conditioned to lucerne hay 447
KLEIN, D. T. Time-temperature interaction in the induction of pleomorphism in *Trichophyton mentagrophytes* 125
KRICHEVSKY, M. I. & LOVE, L. L. The uptake and utilization of histidine by washed amoebae in the course of development in *Dictyostelium discoideum* 483

LEACH, R. H., *see* BUTLER, M. 285
LOVE, L. L., *see* KRICHEVSKY, M. I. 483
LUNDGREN, D. G., PFISTER, R. M. & MERRICK, J. M. Structure of poly-β-hydroxybutyric acid granules 441

MCCOMB, A. J. The preparation of ^{14}C-labelled gibberellic acid 401
MCWILLIAM, R., *see* CROSSE, R. 51
MASON, D. B., *see* COLLINS, J. F. 353
MARZULLO, G. & DANFORTH, W. F. Composition of ethanol-insoluble assimilatory products of oxidative assimilation of acetate by *Euglena gracilis* 21

MARZULLO, G. & DANFORTH, W. F. Kinetic studies of the oxidative assimilation of acetate by a non-photosynthetic strain of *Euglena gracilis* 9
MERRICK, J. M., *see* LUNDGREN, D. G. 441
MEYNELL, G. G., *see* MEYNELL, E. 153
MEYNELL, E. & MEYNELL, G. G. The role of serum and carbon dioxide in capsule formation by *Bacillus anthracis* 153
MILLSON, G. C., *see* HUNTER, G. D. 319
MONTIE, T. C. & AJL, S. J. The anatomical distribution of murine toxin in spheroplasts of *Pasteurella pestis* 249
MUSSETT, M. V., *see* GIFFORD, G. E. 475

O'KANE, D. J., *see* WILKINS, P. O. 389

PANKHURST, E. S. Polarographic evidence of the production of polythionates during the bacterial oxidation of thiosulphate 427
PATEMAN, J. A., *see* ROBERTS, D. B. 295
PERKINS, W. J., *see* COLLINS, J. F. 353
PFISTER, R. M., *see* LUNDGREN, D. G. 441
POSTGATE, J. R. & HUNTER, J. R. Accelerated death of *Aerobacter aerogenes* starved in the presence of growth-limiting substrates 459

RHODES, A., *see* CROSSE, R. 51
RITTENBERG, S. C., *see* KAPLAN, I. R. 195
RITTENBERG, S. C., *see* KAPLAN, I. R. 213
ROBERTS, D. B. & PATEMAN, J. A. Immunological studies of amination deficient strains of *Neurospora crassa* 295
ROBERTS, T. A., *see* COOK, A. M. 185
ROSE, A. H., *see* DIXON, B. 229

SHARPE, M. E., DAVISON, A. L. & BADDILEY, J. Teichoic acids and group antigens in lactobacilli 333
SHARPE, M. E., *see* FRANKLIN, J. G. 143
SHATTOCK, P. M. F., *see* SMITH, D. G. 165
SHON, M., *see* STRANGE, R. E. 99
SMITH, D. G. & SHATTOCK, P. M. F. The cellular location of antigens in streptococci of groups D, N and Q 165
SMITH, N. R., GIBSON, T., GORDON, R. E. & SNEATH, P. H. A. Type cultures and proposed neotype cultures of some species in the genus *Bacillus* 269
SNEATH, P. H. A., *see* SMITH, N. R. 269
STEWART-TULL, D. E. S. & WHITE, R. G. The occurrence of muramic acid in wax D preparations of mycobacteria 43
STRANGE, R. E. & SHON, M. Effects of thermal stress on viability and ribonucleic acid of *Aerobacter aerogenes* in aqueous suspension 99
SUTHERLAND, R. The nature of the insensitivity of Gram-negative bacteria towards penicillins 85
SYRETT, P. J., *see* KELLY, D. P. 307
SZENTIRMAI, A., *see* HORVÁTH, I. 241

THOMAS, D. M., *see* ALLEN, M. B. 259
TITMUSS, D. H. J., *see* WESTWOOD, J. C. N. 67

VARGA, J. M., *see* HORVÁTH, I. 241

WAITES, W. M. & FRY, B. A. Effect of infection with phage lambda on the synthesis of protein, RNA and DNA in *Escherichia coli* 413
WESTWOOD, J. C. N., HARRIS, W. J., ZWARTOUW, H. T., TITMUSS, D. H. J. & APPLEYARD, G. Studies on the structure of vaccinia virus 67
WESTWOOD, J. C. N., *see* HARRIS, W. J. 491
WHITE, R. G., *see* STEWART-TULL, D. E. S. 43
WIDDOWSON, J. P., *see* COOK, A. M. 185
WILDY, P. & ANDERSON, T. F. Clumping of susceptible bacteria by bacteriophage tail fibres 273
WILKINS, P. O. & O'KANE, D. J. Accumulation of glucose and galactose by *Streptococcus faecalis* 389

ZWARTOUW, H. T. The chemical composition of vaccinia virus 115
ZWARTOUW, H. T., *see* WESTWOOD, J. C. N. 67

Index of Subjects

Small roman numerals refer to pages in the Proceedings of the Society for General Microbiology

Acetate assimilation in Euglena (Marzullo & Danforth) 9
Acetate, effect on *Pseudomonas aeruginosa* (Bernheim) 327
Acetate, products of oxidative assimilation by *Euglena gracilis* (Marzullo & Danforth) 21
Acidity induced in tissue culture (Butler & Leach) 285
Aerobacter aerogenes, substrate accelerated death of (Postgate & Hunter) 459
Aerobacter aerogenes, thermal stress on viability and ribonucleic acid of (Strange & Shon) 99
Algae, carotenoids of (Allen, Fries, Goodwin & Thomas) 259
Amination deficient mutants of *Neurospora crassa* (Roberts & Pateman) 295
Amino acid effect on Neurospora (Brockman) 31
Amoebae in *Dictyostelium discoideum*, histidine uptake by (Krichevsky & Love) 483
Antifungal effects of griseofulvin analogues (Crosse, McWilliam & Rhodes) 51
Antigens in lactobacilli (Sharpe, Davison & Baddiley) 333
Antigen location in streptococci (Smith & Shattock) 165
Arginine and pyrimidine relationship in Neurospora (Charles) 131
Assay of interferon (Gifford, Mussett, & Heller) 475
Assimilation of acetate by *Euglena gracilis*, products of (Marzullo & Danforth) 21

Bacillus anthracis, capsule formation by (Meynell & Meynell) 153
Bacillus genus, cultures of (Smith, Gibson, Gordon & Sneath) 269
Bacillus licheniformis, distribution and formation of penicillinase (Collins) 363
Bacillus subtilis, gamma irradiation of (Cook, Roberts & Widdowson) 185
Bacteriophage tail fibres, clumping of susceptible bacteria by (Wildy & Anderson) 273
Biotin-deficient *Saccharomyces cerevisiae*, ornithine carbamoyltransferase in (Dixon & Rose) 229
Brucella abortus cell walls, effect of enzyme treatment (Bobo & Foster) 1

Capsule formation by *Bacillus anthracis* (Meynell & Meynell) 153
Carbon dioxide fixation by Thiobacillus (Kelly & Syrett) 307
Carbon dioxide in capsule formation by *Bacillus anthracis*, role of (Meynell & Meynell) 153
Carbon dioxide, pyrimidine and arginine relationship, response to (Charles) 131
Carbon isotope fractionation by *Desulfovibrio desulfuricans* (Kaplan & Rittenberg) 213
Carotenoids of algae (Allen, Fries, Goodwin & Thomas) 259
Cell-wall composition of *Pseudomonas aeruginosa* and *Salmonella bethesda* (Collins) 379
Cell walls of *Brucella abortus*, effect of enzyme treatments on (Bobo & Foster) 1
Chemical composition of vaccinia virus (Zwartouw) 115
Chemical structure of griseofulvin analogues (Crosse, McWilliam & Rhodes) 51
Clumping of bacteria by T-even tail fibres (Wildy & Anderson) 273
Cocci, cellulolytic, in rumen of sheep (Kistner & Gouws) 447
Composition of ageing cell walls (Collins) 379
Cultures of Bacillus species (Smith, Gibson, Gordon & Sneath) 269
Culture, tissue, mycoplasma inducing acidity and cytopathic effect in (Butler & Leach) 285

Desulfovibrio desulfuricans, carbon isotope fractionation by (Kaplan & Rittenberg) 213
Development of dictyostelium (Krichevsky & Love) 483
Dictyostelium discoideum, histidine uptake and utilization by amoebae in (Krichevsky & Love) 483
Distribution of penicillinase in *Bacillus licheniformis* (Collins) 363
Distribution of murine toxin in *Pasteurella pestis* (Montie & Ajl) 249

Enzyme treatments on brucella cell walls (Bobo & Foster) 1
Escherichia coli, effect of lambda phage on protein, RNA and DNA in (Waites & Fry) 413
Escherichia coli, valine and isoleucine metabolism in (Horváth, Varga & Szentirmai) 241
Euglena gracilis, kinetics, of acetate assimilation in (Marzullo & Danforth) 9
Euglena gracilis, products of oxidative assimilation of acetate by (Marzullo & Danforth) 21

Formation of penicillinase in *Bacillus licheniformis* (Collins) 363
Fractionation of sulphur isotopes (Kaplan & Rittenberg) 195

Galactose accumulation by *Streptococcus faecalis* (Wilkins & O'Kane) 389
Gamma irradiation of *Bacillus subtilis* (Cook, Roberts & Widdowson) 185
Gibberellic acid, preparation of ^{14}C-labelled (McComb) 401
Glucose accumulation by *Streptococcus faecalis* (Wilkins & O'Kane) 389
Gram-negative bacteria, insensitivity to penicillins (Sutherland) 85
Griseofulvin analogues, chemical structure and antifungal effects of (Crosse, McWilliam & Rhodes) 51

Growth, effect of amino acid on Neurospora (Brockman) 31
Growth of form 2 mycobacterium on Lowenstein-Jensen medium (Csillag) 79

Hay, lucerne, cellulolytic cocci in sheep rumen conditioned to (Kistner & Gouws) 447
Histidine uptake and utilization in *Dictyostelium discoideum* (Krichevsky & Love) 483

Immunology of *Neurospora crassa* (Roberts & Pateman) 295
Indole, effect of amino acids on, for growth of Neurospora (Brockman) 31
Induction of pleomorphism in Trichophyton (Klein) 125
Interferon, studies on production and assay (Gifford, Mussett & Heller) 475
Isoleucine metabolism in *P. aeruginosa* and *E. coli* (Horváth, Varga & Szentirmai) 241

Kinetics of acetate assimilation in Euglena (Marzullo & Danforth) 9

Lactate, metabolism of, by *Desulfovibrio desulfuricans* (Kaplan & Rittenberg) 213
Lactobacilli from milk and cheese (Franklin & Sharpe) 143
Lactobacillus, taxonomy of (Davis) 177
Lactobacilli, teichoic acids and group antigens in (Sharpe, Davison & Baddiley) 333
Lowenstein-Jensen medium, growth of mycobacterium form 2 (Csillag) 79

Metabolism of lactate by *Desulfovibrio desulfuricans* (Kaplan & Rittenberg) 213
Metabolism of Neurospora mutants (Charles) 131
Metabolism, valine and isoleucine, in *P. aeruginosa* and *E. coli* (Horváth, Varga & Szentirmai) 241
Methods for estimating penicillinase in single bacteria (Collins, Mason & Perkins) 353
Methods, preparation of ^{14}C-labelled gibberellic acid (McComb) 401
Microscope, electron, structure of vaccinia virus (Westwood, Harris, Zwartouw, Titmuss & Appleyard) 67
Mouse scrapie, intracellular location of (Hunter, Millson & Meek) 319
Muramic acid, in wax D preparations of Mycobacteria (Stewart-Tull & White) 43
Mutants of *Neurospora crassa*, amination deficient (Roberts & Pateman) 295
Mycobacteria, muramic acid in wax D preparations of (Stewart-Tull & White) 43
Mycobacteria, mycococcus form of (Csillag) 341
Mycobacterium, form 2, growth on Lowenstein-Jensen medium (Csillag) 79
Mycococcus form of Mycobacteria (Csillag) 341
Mycoplasma inducing acidity and cytopathic effect in tissue culture (Butler & Leach) 285

Neurospora crassa, effect of amino acids on growth of (Brockman) 31
Neurospora crassa, immunology of (Roberts & Pateman) 295
Neurospora, pyrimidine and arginine relationship (Charles) 131

Optical density of *Pseudomonas aeruginosa* in phosphate buffers (Bernheim) 327
Ornithine carbamoyltransferase in *Saccharomyces cerevisiae* (Dixon & Rose) 229

Pasteurella pestis, distribution of toxin in (Montie & Ajl) 249
Penicillinase distribution in a bacterial population (Collins) 363
Penicillinase estimation in single bacteria (Collins, Mason & Perkins) 353
Penicillins, insensitivity of Gram-negative bacteria to (Sutherland) 85
Phosphate buffers, *Pseudomonas aeruginosa* in (Bernheim) 327
Phosphotungstate staining of vaccinia virus (Harris & Westwood) 491
Phylogeny of lactobacillus (Davis) 177
Physiology of lactobacilli isolated from milk and cheese (Franklin & Sharpe) 143
Pleomorphism in *Trichophyton mentagrophytes* (Klein) 125
Polarographic evidence of thionate production (Pankhurst) 427
Poly-β-hydroxybutyric acid granules, structure of (Lundgren, Pfister & Merrick) 441
Polythionate production during oxidation of thiosulphate (Pankhurst) 427
Protein in *Escherichia coli*, effect of infection with phage lambda (Waites & Fry) 413
Pseudomonas aeruginosa, composition of cell walls (Collins) 379
Pseudomonas aeruginosa in phosphate buffers, effect of pyruvate and acetate (Bernheim) 327
Pseudomonas aeruginosa, valine and isoleucine metabolism in (Horváth, Varga & Szentirmai) 241
Pyrimidine and arginine relationship in Neurospora (Charles) 131
Pyruvate, effect, on *Pseudomonas aeruginosa* (Bernheim) 327

Ribonucleic acid of *Aerobacter aerogenes*, effect of thermal stress on (Strange & Shon) 99
Ribosomes, bacterial, unity of (De Ley) 219
Rumen cellulolytic cocci (Kistner & Gouws) 447

Saccharomyces cerevisiae, OCT in biotin-deficient (Dixon & Rose) 229
Salmonella bethesda, composition of cell walls (Collins) 379
Scrapie, mouse, intracellular location of (Hunter, Millson & Meek) 319
Serum in capsule formation by *Bacillus anthracis*, role of (Meynell & Meynell) 153
Staining of vaccinia virus (Harris & Westwood) 491
Streptococci, groups D, N and Q, cellular location of antigens (Smith & Shattock) 165
Streptococcus faecalis, accumulation of glucose and galactose by (Wilkins & O'Kane) 389
Structure of poly-β-hydroxybutyric acid granules (Lundgren, Pfister & Merrick) 441
Structure of vaccinia virus (Westwood, Harris, Zwartouw, Titmuss & Appleyard) 67
Substrate accelerated death of *Aerobacter aerogenes* (Postgate & Hunter) 459

Sugar accumulation by *Streptococcus faecalis* (Wilkins & O'Kane) 389
Sugars, gamma irradiation of *Bacillus subtilis* in presence of (Cook, Roberts & Widdowson) 185
Sulphur isotopes, fractionation of (Kaplan & Rittenberg) 195
Synthesis of ornithine carbamoyltransferase in *Saccharomyces cerevisiae* (Dixon & Rose) 229
Synthesis of protein, RNA and DNA in *Escherichia coli*, effect of infection with phage lambda on (Waites & Fry) 413

Taxonomy of lactobaeillus (Davis) 177
Teichoic acids in lactobacilli (Sharpe, Davison & Baddiley) 333
Temperature interaction of pleomorphism in Trichophyton (Klein) 125
Thiobacillus, carbon dioxide fixation by (Kelly & Syrett) 307
Thiosulphate, production of polythionates during oxidation of (Pankhurst) 427
Toxin location in *Pasteurella pestis* (Montie & Ajl) 249
Trichophyton mentagrophytes, pleomorphism in (Klein) 125
Tryptophan, effect of amino acids on, for growth of Neurospora (Brockman) 31

Valine metabolism in *P. aeruginosa* and *E. coli* (Horváth, Varga & Szentirmai) 241
Viability of *Aerobacter aerogenes*, effect of thermal stress on (Strange & Sohn) 99
Virus, vaccinia, chemical composition of (Zwartouw) 115
Virus, vaccinia, staining of (Harris & Westwood) 491
Virus, vaccinia, structure of (Westwood, Harris, Zwartouw, Titmuss & Appleyard) 67
Vitamin requirements of lactobacilli isolated from milk and cheese (Franklin & Sharpe) 143

Wax D preparations of Mycobacteria, muramic acid in (Stewart-Tull & White) 43