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Continuous Culture of Rumen Bacteria: Apparatus

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SUMMARY

A small-scale apparatus for the continuous culture of anaerobic bacteria is described.

INTRODUCTION

The continuous culture of micro-organisms has been studied for a number of years and the most successful designs of apparatus have been based on the 'chemostat' of Monod (1950) and Novick & Szilard (1950), and the 'turbidostat' of Fox & Szilard (1955). As Herbert (1958) pointed out, the chemostat and turbidostat are essentially the same apparatus but they work best over different ranges of growth rate. The chemostat has some advantages over the turbidostat mechanically and for growth at low growth rates, and a chemostat type of apparatus was chosen for our work. Most of the chemostats described have been for the growth of aerobic bacteria and a problem in these designs is to supply sufficient air to the micro-organisms, and this has led to the design of apparatus in which a film of culture flows around the walls of a vessel (e.g. Monod, 1950, and others; Dawson, 1963). This type of apparatus is not so suitable for anaerobic work as the 'stirred fermenter' type. The most important rumen bacteria are strict anaerobes which require special techniques for handling and are unable to initiate growth unless the media used are highly reduced. The apparatus described here was developed after initial experiments some 5 or 6 years ago when a pH-controlled batch culture apparatus was built in which large amounts of *Streptococcus bovis* were grown for the isolation of a polysaccharide. The merits of continuous culture for providing large amounts of micro-organisms for enzyme work and for analysis then became apparent. The rumen may be likened to a kind of continuous culture and it seemed that a better understanding of the behaviour of rumen bacteria might be obtained if they were grown in continuous, rather than batch, culture. The development of a comparatively large apparatus with an external culture circuit in which was incorporated the pH controls proved unsuccessful for strict anaerobes, partly because of the long lengths of silicone tubing involved in the peristaltic pumps. Sufficient air to oxidize the medium can diffuse through silicone tubing. The apparatus was then redesigned on a smaller scale for growth of strict anaerobes. The mechanically stirred fermenter designs such as those of Elsworth, Meakin, Pirt & Capell (1956) were not followed because of expense and the difficulties of sealing the stirrer bearings, and because the bacteria which it was proposed to grow showed signs of being rather fragile and it was felt that a rapidly revolving stirrer might damage the organisms. A magnetic stirrer would overcome the difficulties of sealing bearings, but would still be liable to cause mechanical damage to the organisms. As a

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beginning the pH control was left out, the buffering action of the medium being relied on for pH control, but it has been found possible to incorporate in a larger vessel (250 ml.) an internal electrode system operating, through a pH meter and relay, solenoid-operated alkali and acid inlets. Since experiments at low growth rates were planned to involve doubling times of 7 or 8 hr or more, it was felt that the apparatus must be capable of running for much more than a day or two; and if it were to supply organisms for enzyme work it should run more or less indefinitely with minimum attention. The simplest design was used and all the parts of the present apparatus have been found necessary for its efficient functioning. The apparatus was demonstrated to the Physiological Society (Hobson & Smith, 1962) and a brief description was given in 1963 (Hobson & Smith, 1963).

APPARATUS AND METHODS

The apparatus (shown diagrammatically in Fig. 1) consists of the following parts. All joints are B 5 ground glass joints. Rubber tubing is heavy wall red rubber of internal diameter approx. $\frac{1}{8}$ in. All rubber to glass joints are taped and wired on. A is a plastic balloon which holds about 5 l. of CO_2 under atmospheric pressure. This is attached to the 5 l. flask B through a cottonwool filter and replaces the medium from flask B with CO_2. When the CO_2 in A is at more than atmospheric pressure it forces medium through the pump valves. Flask B is joined by rubber tubing and glass joints to a 2 l. conical flask C which is in a water bath at 39°. Flask C is then joined through a $\frac{1}{4}$ in. rubber tubing to the inlet pump D. The distance between C and D is made as short as possible. The main purpose of heating flask C is to prevent bubble formation as the CO_2-saturated medium is drawn up into the pump. Small bubbles stick to the ball valves of these pumps and stop medium flow at low pumping speeds. The inlet tube between D and F contains a gas-gap (E) which prevents back-growth of the culture up the inlet tube and into C. As this gap tends to fill with medium during long runs it was found most convenient to allow a very small stream of CO_2, controlled by a fine jet and screw clips on the tubing M, to pass through a filter into the side arm of E. Passage of a rapid flow of CO_2 in with the medium tends to suck medium through the pumps. F is the culture vessel, of working volume 120 ml., and it is fitted with a heavy rubber bung through which pass the tubes shown and an inoculation port, a B 5 cone and socket, G. The vessel is in a water bath at 39°. H is a subsidiary pump circuit, helping to stir the culture, but also acting as a sampling point through the two-way tap fitted with a hooded outlet tube. The main stream of CO_2 passes from the 50 lb. cylinder Q through a reduction valve, a 'Rotameter' flow meter P, a furnace containing red hot copper turnings (O), through the branch point N and cottonwool filters to the culture vessel. The tubing of the CO_2 line is pressure tubing which allows a negligible amount of air to permeate through, except for the short branch M which is $\frac{1}{8}$ in. tubing. The CO_2 escapes from the culture vessel by the overflow tubing and carries excess medium with it into the 2 l. flask I which is immersed in a refrigerated tank at 1°. I is fitted with a bunsen valve and cottonwool filter (J) through which excess CO_2 escapes. The outlet tube K is fitted with a tap and ground glass joint to which can be attached the flask L fitted with a cottonwool filter and a glass tube which can be attached to a water vacuum pump. Screw clips are attached at various points in the circuit.

Between experiments the rubber tubing and cottonwool filters are usually replaced and the whole apparatus thoroughly cleaned. The culture vessel head with inlet pump circuit is separately wrapped, as are the circulating pump circuit, the flasks I and L, and the tops of the various flasks. All the separate pieces of apparatus are autoclaved at 120° for 25 min. to ensure thorough heating of pumps, etc. The various joint ends are separately covered under the main wrappings. The sterile apparatus is rapidly assembled as far as the joint below the pump D, the pumps are connected to the motors and connexions made from the CO_2 lines to the filter inlets. The medium is separately prepared in flasks C and B. The basal medium, without bicarbonate, cysteine and carbohydrate, is autoclaved in the flasks, which are

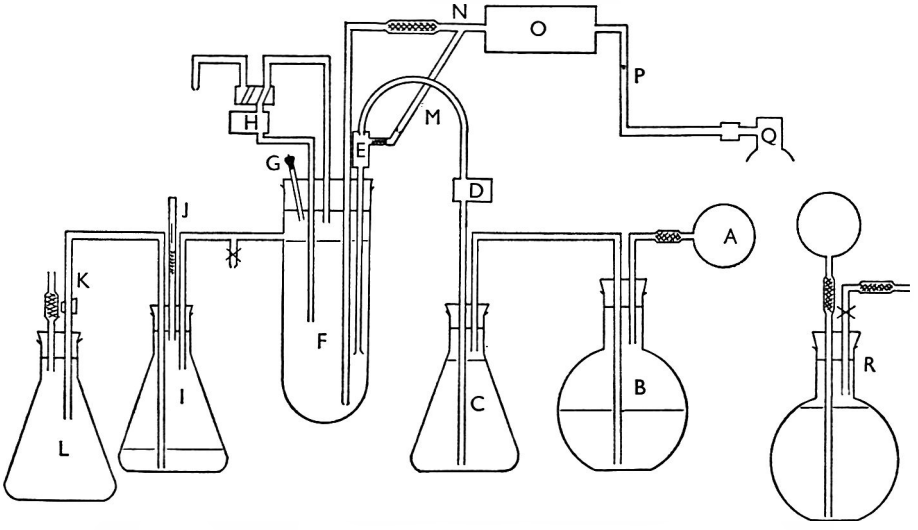

Fig. 1. Continuous culture apparatus. Diagrammatic. The lettered parts are referred to in the text. The hatched areas are cottonwool filters.

closed with aluminium foil, for 25 min. at 120°. On removal from the autoclave, whilst the medium is still hot, the aluminium foil caps are replaced by the bungs carrying the inlet tubes, etc., but with a rubber bladder (a football bladder) of pure CO_2 attached through a filter to the medium outlet tube, as in R. The other tube with the filter is clipped off. Before the bungs are pressed home a strong stream of CO_2 is bubbled through the medium to displace air in the flasks. When the medium has cooled a little, to say 60°, the bung is displaced and a concentrated solution of bicarbonate + cysteine + carbohydrate (filter sterilized) rapidly poured in whilst CO_2 is allowed to escape around the bung. The bungs are then firmly pressed down and sealed with wax. (The top of the culture vessel and other bungs are also sealed with wax.) The medium is then allowed to cool and as it cools CO_2 is drawn in from the attached balloon. (By opening the clips the filters and tube joining B and C are filled with CO_2.) This takes some hours and the flasks are then incubated overnight to test for sterility. When the apparatus has been assembled CO_2 is passed through for at least two hours before the medium flasks are connected to each other and to the inlet pump joint. In connecting up, the tubes are clipped as near to the joints as possible and the joints rapidly connected to allow as little air as possible to get into

the joints. The balloon A is attached to flask B. Carbon dioxide is allowed to flow through the apparatus for a further half-hour and the medium is then slowly pumped from flask C into the culture vessel. Filling the vessel takes an hour or more, and any residual air in the inlet tubing and pump D is pushed out before the advancing medium and flushed out by the CO_2 flow before it can oxidize the medium to any extent. Similarly, any air in the circuit H is driven out by the action of the pump. When the vessel is filled the CO_2 flow is left on at about 50 ml./min., the pumps are stopped and the whole apparatus left overnight to test for sterility. The vessel can then be inoculated through G and the bacteria allowed to grow as a batch culture to a suitable population density before the medium inflow and the pump H are started.

To empty the reservoir I the inlet pump is stopped and the tubing below D closed with a screw clip; otherwise medium is sucked through the pump into the vessel. Flask L is then attached to the tube K and slight suction applied to L. By opening the tap on K liquid is allowed to flow slowly between I and L. I is emptied slowly as otherwise too great a vacuum is caused in the flask and the medium in the culture vessel froths vigorously as the CO_2 flow increases. The flow of liquid from I to L is stopped occasionally to allow the pressure in I to build up again. The cottonwool filter below J stops contamination, if by chance the bunsen valve at J leaks during this process After I has been emptied the apparatus is left to equilibrate for a few minutes before the clip at D is opened and the pump again started.

A 2 l. flask was chosen at C partly because when the medium level in B falls the flow of liquid from B to C does not always fully coincide with the flow out of C and the level in C falls. If during a long run the level in C falls too much it is brought back to normal by pushing a hypodermic needle attached to a cottonwool filter through a sealed-off rubber tubing projecting from the top of C (this is not indicated in Fig. 1) after the tubing has been washed with ethanol, and applying gentle suction. The medium flask B is changed when necessary by closing the rubber tubing near the joint above B and rapidly changing the flask for a similar one.

The pumps have had no parts changed during 2½ to 3 years of experiments, and have only needed regular cleaning and greasing of the gear boxes and bearings; some wear in the stainless-steel piston rods is now becoming apparent though, and these will need changing soon. The copper in the furnace tube is changed occasionally and this can be done during a run when necessary, without getting air into the system. The CO_2 cylinder is always changed well before it is empty as the rate of flow of the last of the gas out of the cylinder is uncertain.

PERFORMANCE OF THE APPARATUS

Since the water bath containing the culture vessel is large and well stirred the temperature of the medium remains very close to 39°, even with the circulating pump H running. The pump H runs continually. The refrigerated bath is thermostatically controlled and cooled by a small refrigerator unit (Grant Instruments, Cambridge). The water in the warm baths is covered with mineral oil to cut down evaporation. Otherwise water tends to distil from the hot baths to the cold glycol solution in the refrigerated bath and raises the level in the latter. When this level gets too high it cools the thermostat unit and water condenses on it and this can

short circuit the motor or thermostat switch. All electrical connexions in the unit have been covered with a plastic film (Holts', 'Damp-Start') to cut down risks of short circuiting. The pumps are Distillers Co. (Great Burgh, Epsom, Surrey) Micro pumps, of the original type. The newer Series II pump is not so suitable because of a number of points in its construction. Aluminium housings have been built over the pump plungers to cut down dust contamination, and a little ethanol is sprayed over the plungers and glands each day. The pump glands need fractionally tightening each day to take up slack due to wear. Occasionally the Teflon gland packing is renewed (after say 6 months' use). The inlet pump will pump a maximum of 175 ml./hr, and the circulating pump is a bigger model set to pump about 650 ml./hr. The calibration curve of the inlet pump varies from one run to another, but is of constant slope. The calibration in the apparatus also differs from the calibration obtained by pumping water from one vessel to another. The rate of pumping is checked during a run by measuring the volume flowing into the refrigerated reservoir. Variations in pumping speed occur during a run at one setting, but the variation is small. Typical results over 200 hr at a setting to give a dilution rate about 0·2 hr^{-1} were D = 0·188, 0·181, 0·181, 0·212, 0·218, 0·207, 0·199, 0·186, 0·184, the volumes pumped being measured at approximately 20-hr intervals.

The flow of carbon dioxide through the culture vessel is 100 ml./min. This mixed a dye solution flowing into the vessel in about 1 sec. The gas stream impinges on the bottom of the vessel and prevents micro-organisms from settling. Foaming in the vessel has never been serious at the gas flow-rates used, the usual result being a layer of large bubbles about 5 mm. thick or less on top of the medium. Evaporation caused by the gas stream is not serious, amounting to not more than about 2% of the volume in an overnight batch culture. When the gas flow is above 100 ml./min. then foaming increases, but flow can drop lower than 100 ml./min., without seriously affecting mixing. The circulating pump alone is not sufficient to stir completely the culture if the gas flow should fail entirely, but this has seldom happened.

Provided that contamination is avoided initially there is little chance of subsequent contamination unless a breakage occurs in one of the glass parts. When contamination has occurred it has usually proved impossible to wash it out completely, even if the contaminating organism (usually a micrococcus) has not swamped the normal growth. Some applications of the apparatus are described in the next paper.

The author wishes to thank Mr W. Smith for assistance during this work.

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Continuous Culture of Some Anaerobic and Facultatively Anaerobic Rumen Bacteria

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SUMMARY

Strains of *Streptococcus bovis*, *Selenomonas ruminantium*, and an anaerobic lipolytic bacterium (5s) have been grown under carbohydrate-limiting conditions in continuous culture for long periods. With *S. ruminantium* and bacterium 5s the fermentation products varied with growth rate. Yield of organism in continuous culture of all three bacteria showed a maximum at a particular growth rate. The yield of *S. ruminantium* was much higher in continuous culture at optimum growth rate than in batch culture, and was also higher than can be explained on present concepts of the energy from fermentation available for growth. The various results are discussed in the light of results obtained with other bacteria and the conditions obtaining in the rumen.

INTRODUCTION

During the development of the culture apparatus described in the previous paper (Hobson, 1965) a strain of *Selenomonas ruminantium* was used because it is an anaerobic rumen bacterium and the strain had been well characterized in previous work (Hobson & Mann, 1961). It has been suggested that selenomonads are not bacteria, but should be classed as protozoa (e.g. Bisset & Davis, 1960). However, apart from other considerations, our own unpublished results show that the cell-wall composition of *S. ruminantium* strain 6 (nitrogen 10·0%; carbohydrate 4·7%; lipid 28%; hexosamine 3·6%; ash 0·7%; phosphorus 0·7%; with twelve amino acids present) is similar to that of other Gram-negative bacterial cell walls. This seems to be an additional reason for regarding the selenomonads as bacteria. When the unusual nature of the results with *S. ruminantium* in the present work became apparent some runs were done with a rumen strain of *Streptococcus bovis*, to examine the behaviour of a facultatively anaerobic organism which ferments the same carbohydrate. Finally, the lipolytic bacterium 5s was used in an extended series of tests; the reasons for this were twofold. First, bacterium 5s is also a strict anaerobe, but it ferments a simpler carbohydrate than does the selenomonad *S. ruminantium*. (This selenomonad lost the ability to ferment glycerol on prolonged growth on glucose.) Secondly, large amounts of organisms were needed for experiments on the lipolytic activity of bacterium 5s. The strains of anaerobic bacteria used would grow only in media containing amino acid mixtures, and not on simpler media containing only ammonia as nitrogen source as do some rumen bacteria. While a simpler medium might have been desirable for some aspects of the work the yields of bacteria have generally been determined for media in which were present most of the monomers of cell constituents.

The symbols used in this paper to describe growth in continuous culture are those of Herbert, Elsworth & Telling (1956). The yield constant, Y_{glc} or Y_{glyc}, is g. dry weight organism/mole substrate utilized, as introduced by Bauchop & Elsden (1960).

METHODS

Organisms used

Streptococcus bovis. This was the strain 18c6 isolated from the sheep rumen described by Hobson & Mann (1955). Stock cultures were kept in Robertson's cooked meat medium or were freeze-dried.

Selenomonas ruminantium var. *lactylilicas.* This was the strain 6 isolated from the rumen and described by Hobson & Mann (1961). On original isolation and for some time after it rapidly fermented glycerol. At the time of the present experiments various cultures had been kept as stocks and continually subcultured on glucose slopes, and other cultures had been kept on glucose and then freeze-dried. On retesting, after the experiments on continuous culture on glucose had been done (some 3 years after isolation of the organism), it was found that neither stock cultures on glucose slopes nor the revived freeze-dried cultures metabolized glycerol to any extent. A little growth was obtained on the basal constituents of the medium, but continued subculture on liquid or solid media containing glycerol did not increase the growth or glycerol utilization.

Lipolytic bacterium. This organism, bacterium 5s, was isolated from a sheep on a hay and concentrate diet. It was similar in all respects, except that it fermented the additional carbohydrate cellobiose, to the lipolytic bacteria previously isolated from sheep (Hobson & Mann, 1961). These bacteria do not ferment glucose. Stock cultures were kept on glycerol slopes at 2°, and were subcultured every 2 weeks. No change in the characteristics of the strain were noted during the period of the experiments.

Media

For selenomonad strain 6. The medium used (CY medium) initially was one containing mineral solutions (*a*) and (*b*), 15 ml. each; Bacto Casitone, 1·5 g.; Bacto Yeast Extract, 0·25 g.; $NaHCO_3$, 0·4 g.; cysteine HCl, 0·05 g.; resazurin, 0·1 ml. of 0·1 % (w/v) solution; water to 100 ml. and glucose in the correct concentration. The constituents of mineral solutions (*a*) and (*b*) and the methods of preparing the media under oxygen-free carbon dioxide were described by Hobson & Mann (1961). This medium was used solidified with agar for stock cultures, and as liquid for batch cultures of the selenomonad. The medium for continuous cultures was originally the CY medium + glucose, but in later experiments this was changed by addition of 0·522 g. K_2HPO_4 and 0·272 g. KH_2PO_4/100 ml. (CYP medium; pH 6·7).

For lipolytic bacterium 5s. Initial experiments were made with medium CY with glycerol added. Later experiments used a medium (AHI medium) of composition: mineral solution (*a*, above), without NaCl, 15 ml.; solution (*b*, above), 15 ml.; acid-hydrolysed casein (Allen and Hanbury, London), 1 g.; Bacto Yeast Extract, 1 g.; $NaHCO_3$, 0·4 g.; cysteine HCl, 0·05 g.; resazurin solution, 0·1 ml. of 0·1 % solution; glycerol; water to 100 ml. The casein hydrolysate gave an acid solution, so the minerals, casein hydrolysate, yeast extract and water were mixed and the solution

adjusted to pH 6·8 with a little 10 N-NaOH. At this pH value a faint opalescence developed; increasing the pH value caused a precipitate to form. The resazurin (and the agar when slopes were being made) was added, and the solution then autoclaved and a filter-sterilized solution of bicarbonate + cysteine + glycerol added in the usual way (Hobson & Mann, 1961). A heavy opalescence formed in the autoclaved medium, but this cleared as the medium cooled; the slight sediment remaining was allowed to settle before the medium, prepared in bulk, was dispensed into smaller portions for batch cultures. In the continuous cultures this sediment settled to the bottom of the medium flasks, leaving a clear supernatant fluid; this sediment was not disturbed as medium flowed between the flasks or into the culture vessel. For the later continuous culture work the sodium bicarbonate concentration was increased to 0·6 %, and the cysteine concentration to 0·1 %. The medium was of pH 6·8, and this is referred to as AH2 medium. The NaCl was omitted from these media as other experiments suggested that a better production of lipase occurred in the absence of NaCl.

Streptococcus bovis. This was grown in the CYP medium.

All cultures were incubated at 39°.

Analytical methods

Glucose was determined by an anthrone method, as was bacterial carbohydrate. Glycerol was determined by the method of Hanahan & Olley (1958). Volatile fatty acids were steam-distilled from the culture media, and the constituent acids identified by paper chromatography. These methods and that for lactic acid were previously described (Hobson & Mann, 1961). The fermentation acids were separated quantitatively on Celite columns by the method of Wiseman & Irwin (1957). This method did not separate lactic and succinic acids completely, so the total lactic + succinic fraction was determined by the column separation and succinic acid determined by subtracting from this a value for lactic acid determined on another portion of the culture.

Organism dry weights were determined by centrifuging 10 ml. of culture in a small conical tube, washing the sediment once with 10 ml. distilled water, again centrifuging, and finally drying the sediment to constant weight at 110° after removing most of the water.

The 'turbidity' of cultures was measured in 1·5 ml. tubes in an EEL colorimeter (Evans Electroselenium Ltd., Harlow, Essex) with an OR1 (640 mμ) filter. Experiments with bacterial suspensions showed that the red filter gave better linearity with amount of organisms, and covered a wider range, than did blue or yellow filters. The turbidity values were read in the EEL colorimeter on a scale 0–10–100–∞. Measurements of organism dry weight/unit volume and turbidity as EEL colorimeter readings were made during batch and continuous cultures. The measurements for each organism fell on the same straight line (Fig. 1). Deviations were found only in cultures where lysis of organisms was taking place or during granular growth. The turbidity was thus routinely used as a measure of the behaviour of the continuous cultures and in some cases to estimate yields of organisms.

The sizes of organisms were measured in wet preparations examined with a calibrated micrometer eyepiece in phase-contrast illumination. The sizes of ten

bacteria taken at random in a field were measured and an average length found; this was repeated at each sampling time.

A Pye pH meter was used to measure pH value as soon as possible after removal of a sample from the culture vessel.

RESULTS

General. Batch cultures were inoculated from overnight cultures, the volume of inoculum usually being about 2% of the culture volume. The continuous culture apparatus was assembled with medium as previously described, inoculated and allowed to grow as a batch culture overnight. This did not usually give the maximum growth attainable in a small batch culture. The culture was then sampled and

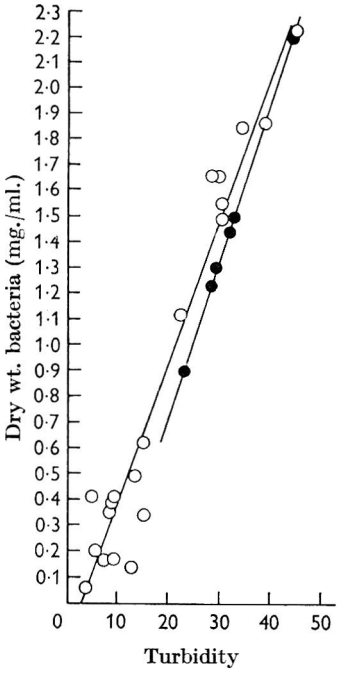

Fig. 1

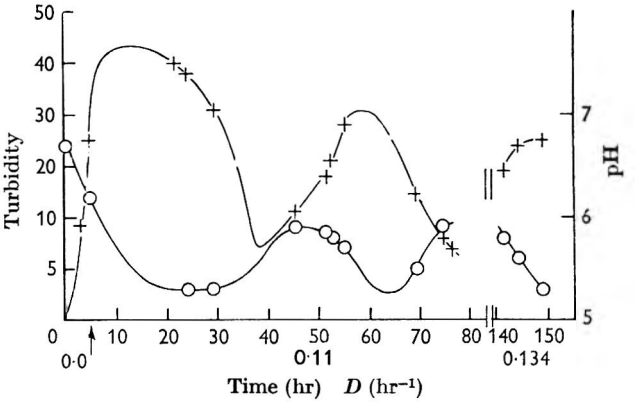

Fig. 2

Fig. 1. The relationship of bacterial dry weight and turbidity in batch and continuous cultures of selenomonad 6 (○) and lipolytic bacterium 5s (●). Turbidity of basal medium = 1–1·5. A turbidity of 30 EEL units = 1·49 mg. dry wt./ml. for selenomonad 6.

Fig. 2. Growth of lipolytic bacterium strain 5s in an insufficiently buffered medium. Glycerol concentration 65·6 μmole/ml. Turbidity measured on an EEL colorimeter, +, culture pH, ○.

the inlet pump started at a suitable rate. The turbidity reading on the EEL colorimeter always decreased in the first few hours after starting the medium flow and then returned to a value corresponding to the dilution rate. Measurements of turbidity were made at intervals and a further sample taken for analysis when the culture had been stable for about 30 hr in most cases at high dilution rates, or about 72 hr or longer at low dilution rates. Since there was a slight variation in turbidity with time the value shown in the figures for turbidity at any dilution rate is an

average of the values found over the steady-state period. As the volume of culture needed to obtain turbidity and pH measurements was about 1·5 ml., this was a little over 1 % of the culture volume and so did not affect the system. When about 10 ml. was taken for analysis the culture was not sampled again for a few hours, depending on the dilution rate, to allow return to steady-state conditions. No difficulties have been experienced with 'wall growth' with the three types of bacterium tested.

Stability of the apparatus. The cultures ran for long periods with a minimum of attention beyond an occasional adjustment of the CO_2 flow, and the apparatus was routinely left to run overnight, and occasionally over a weekend, without attention. The stability of the medium flow has been shown previously. Because of the great dependence of turbidity of the selenomonad culture on dilution rate a rather greater variation in turbidity readings during running at one nominal dilution rate was found than for other organisms, because of slight variations in pumping speed. With the bacterium 5s in a test run of 200 hr at one dilution rate the turbidity readings, measured at about equal intervals, were 23·2, 22·5, 22·4, 21·1, 21·1, 21·1, 21·1, 20·0, 22·6, 22·6, 23·3, 23·6. This period included a change of medium in the 5 l. reservoir.

pH stability. In initial experiments with the bacterium 5s the medium AH1 was not sufficiently buffered. Fig. 2 shows two sections from a graph of the results obtained. A number of dilution rates were tested and in each case the cycle was repeated, the time scale being slightly shorter at higher dilution rates. All the glycerol in the medium had been utilized at the times of maximum turbidity and minimum pH value. It is evident that a comparatively small change in pH value markedly decreased the growth rate of the bacteria and they were washed out until the entry of fresh medium restored the pH value, when growth and fermentation started, and the cycle was repeated. Small changes in pH value may have been partly responsible for the slight variations in turbidity found in other experiments during steady states. Finn & Wilson (1954) suggested that cycling of a yeast culture was due to pH variations. With the medium AH2 the pH value was steadier, the fluctuations over 70 hr being, for example, pH: 5·9, 6·0, 5·9, 5·9, 6·1, 6·0. The selenomonad cultures with medium CYP fluctuated between pH 6·1 and 6·2.

As a further example of the effect of an insufficiently buffered medium the *Streptococcus bovis* strain 18c6 was grown in a yeast medium with glucose (Hobson & Mann, 1955). In batch cultures the pH value fell rapidly and there was little correspondence between turbidity and glucose concentration except at low glucose concentrations. In continuous culture the pH value fell to about 4·9 (from 6·0) and the turbidity (at three dilution rates) to less than half that initially obtained in batch culture. All the glucose was not utilized.

Growth of Streptococcus bovis 18c6

In batch cultures grown in CYP medium with glucose 11·1, 22·2, 33·3, 44·4 and 55·5 μmole/ml. growth was proportional to glucose added between 22·2 and 44·4 μmole/ml. and virtually all the glucose was used; at 55·5 μmole/ml. a divergence of growth and glucose concentration was beginning. At glucose 11·1 μmole/ml., although all the glucose was utilized, growth was higher than expected from the other results because of the contribution of growth on the basal medium. This effect was similar to that found with the selenomonad (Fig. 4). Fig. 3 shows the

effect of dilution rate on growth taken from a run of 450 hr with a medium with glucose 27·7 μmole/ml. The minimum doubling time ($t_d = (\log_e 2)/D$) was about 0·5 hr. No intracellular 'starch' was observed in the organisms from continuous culture. This might be expected from previous experiments on starch formation in this organism (Hobson & Mann, 1955).

Growth of Selenomonas ruminantium strain C

Batch culture experiments were done to find the range over which concentration of organism varied with glucose concentration so that a glucose-limiting system might be used in continuous culture. The results of a number of experiments are shown in Fig. 3 and Table 1. These experiments were not always reproducible for

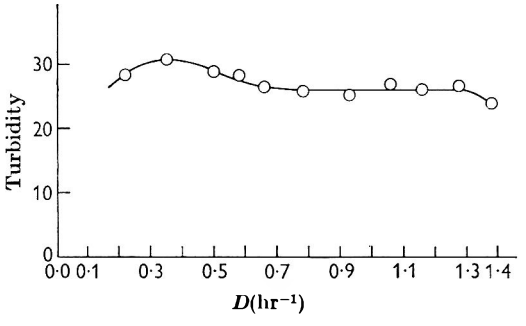

Fig. 3

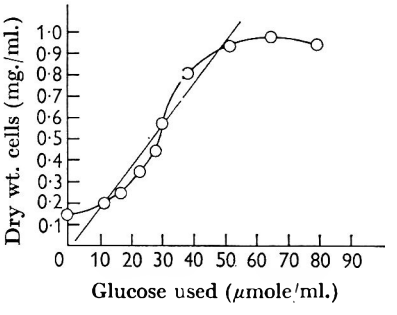

Fig. 4

Fig. 3. Continuous culture of *Streptococcus bovis* 18c6. Steady-state turbidity and dilution rate (D). Glucose-limiting conditions, glucose concentration 27·7 μmole/ml. Maximum turbidity of batch cultures at same substrate concentration *c.* 22.

Fig. 4. Growth of selenomonad 6 in batch cultures at various glucose concentrations.

two reasons. After maximum growth was attained the selenomonads tended to lyse fairly rapidly and the turbidity readings decreased. This rapid decrease in turbidity after maximum growth has been noted with other rumen bacteria in our own laboratories and by others (e.g. Bryant & Robinson, 1961*a*). Also, when growth was slow the cultures often did not attain the maximum yield of a rapidly growing culture, although the glucose was utilized. The values for Y_{glu} given in Table 1 are uncorrected for growth on the basal medium as it does not seem possible to decide, either from Fig. 4 or a similar graph in which concentration of organism (mg. dry wt./ml.) was plotted against glucose added, whether growth on the basal medium was taking place or was suppressed in the presence of glucose. The results shown in Fig. 4 indicated that a glucose-limiting culture was possible with this basal medium at glucose concentrations about 27·7 μmole/ml. At the higher glucose concentrations another nutrient, or possibly the pH value, was becoming limiting in the batch cultures. Volatile fatty acids (VFA), identified as acetic and propionic by paper chromatography, and lactic acid, were produced in the glucose fermentation in the batch cultures. At glucose 27·7 μmole/ml. added, 27·4 μmole VFA and 186·6 μmole lactic acid were produced per 100 μmole glucose utilized. Assuming that the VFA are acetic and propionic acids with CO_2 equivalent to the acetic acid,

this gives a carbon recovery of 107 %. At other concentrations of added glucose up to 83·3 μmole/ml. the proportions of VFA and lactic acid were similar, and carbon recovery on the above basis was somewhat over, or somewhat under, 100 %. At higher concentrations of added glucose carbon recovery was low, being only about 60 % at the highest concentration. The reason for this is not known, unless a large amount of glucose was converted to intracellular polysaccharide. The 'carbohydrate' content of the dried organisms at glucose 27·7 and 33·3 μmole/ml. added was 22 and 26 % measured as apparent glucose by anthrone reagent, but no other determinations were made. If the glucose utilized but not recovered as fermentation products were assimilated then this would increase the low value of Y_{gluc} found at the highest glucose concentration.

Table 1. *Batch growth of selenomonad 6 on glucose*

Glucose added (μmole/ml.)	Glucose used (μmole/ml.)	Cell dry wt. (mg./ml.)	Y_{gluc}*
0	0	0·15	—
11·1	11·1	0·20	18·0
16·6	16·6	0·25	15·0
22·2	22·2	0·35	15·8
27·7	27·7	0·45	16·2
33·3	29·4	0·58	19·7
44·5	37·8	0·81	21·4
83·3	51·1	0·94	18·4
100·0	64·4	0·98	15·2
138·9	78·9	0·94	11·9

Medium as in text. Final pH in cultures containing glucose 5·6–5·7. *Not corrected for growth in basal medium, see text.

In the first continuous culture runs CY medium was used. This was initially at about pH 6·5, but during growth this fell to between pH 5·7 and 6·0. This was felt to be rather low, so further runs were made with CYP medium initially at pH 6·7 and the culture remained at pH 6·1 to 6·2 with glucose 27·7 μmole/ml. and pH 6·4 with 13·9 μmole/ml. However, the results with CY medium and CYP medium all integrated. Figure 5, 6 and 7 show results taken from the steady-state values of five runs of 268 hr, 721 hr, 294 hr, 143 hr and 60 hr. The shortest run was to obtain two points at the higher dilution rates. In all runs the dilution rates examined overlapped with other runs. One run was with CY medium, the others with CYP medium. Points from one run (290 hr) on CY medium with glucose 13·9 μmole/ml. are included, as are points from a run of 130 hr on the basal CY medium. The glucose concentration in the culture vessel, corrected for blank values of the basal medium, averaged 4 % of the incoming glucose at glucose 27·7 μmole/ml. in the medium, and was virtually nil at 13·9 μmole/ml., up to dilution rates (D) of 0·465 hr^{-1}; no measurements were made above this dilution rate. At a number of times during cultivation the total volatile fatty acids were measured (Fig. 7). Only a few determinations of lactic acid were made. The value obtained at $D = 0{\cdot}1$ hr^{-1} was 7 μmole lactic/100 μmole glucose utilized, at $D = 0{\cdot}26$ the value was 87 μmole and at $D = 0{\cdot}286$ the value was 73 μmole. These figures are obviously subject to errors; taking average values for the VFA formed at these dilution rates from Fig. 7 carbon recoveries of 96, 104 and 94 % are obtained. These figures thus show a change

in the fermentation products with change in dilution rate and they also show that little of the glucose was incorporated into cellular material.

Measurement of the size of organisms during runs showed no variation in width, but the length varied somewhat from a minimum of 2·1 μ to a maximum of 4·0 μ, being generally about 3·1 μ. No correlation between length of organism and dilution rate was observed. However, during some of the runs growth became very granular for a day or two. During the granular growth period very long organisms were seen which seemed to be undivided selenomonads. The granular growth returned to normal after a day or two and no reason for it could be found. The selenomonads varied in shape at times, some being almost straight rods, as compared

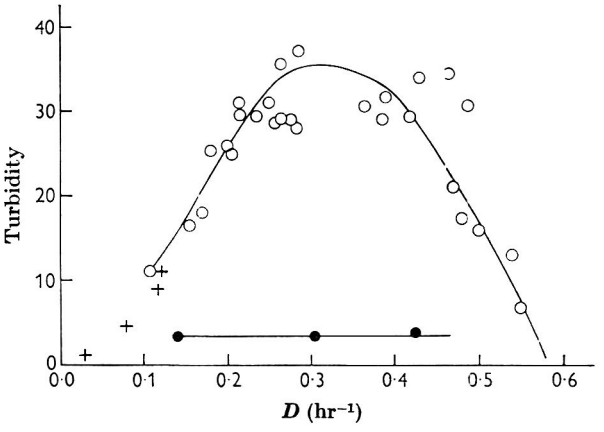

Fig. 5

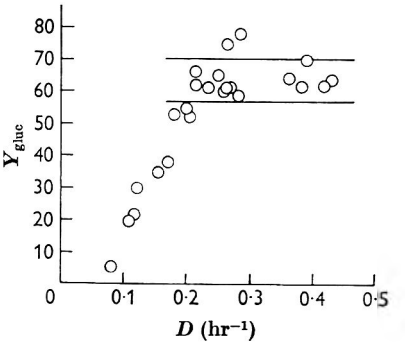

Fig. 6

Fig. 5. Continuous culture of selenomonad 6; relationship between turbidity and dilution rate (D) at different steady states. Glucose-limiting conditions, glucose concentration 27·7 μmole/ml. ○, 13·9 μmole/ml. +, basal medium ●. The values for 27·7 μmole/ml. glucose are from five runs, and with these and the ones for 13·9 μmole/ml. glucose the values are corrected for growth in the basal medium. At dilution rates up to 0·465 hr^{-1} an average of 96 % of the glucose was being utilized. No measurements were made above this dilution rate. Maximum turbidity of batch cultures at 27·7 μmole glucose/ml. *c.* 11.

Fig. 6. Continuous culture of selenomonad 6; relationship between Y_{gluc} and dilution rate (D). Values are taken from five runs at 27·7 μmole/ml. glucose in the medium and one run at 13·9 μmole/ml. glucose.

with the normal curved shape. The percentage of motile organisms was generally much higher in the continuous cultures than in batch cultures. Tests were made at intervals during all runs, especially when the percentage of straight organisms was high, for the reaction of the organisms with antisera specific to the selenomonads (Hobson, Mann & Smith, 1962). In all cases good agglutination was observed.

Culture of lipolytic bacterium 5s

The bacterium 5s was originally grown in both batch and continuous culture in CY medium with various amounts of glycerol. In batch cultures the growth on the basal medium was high and at glycerol 65·2 μmole/ml. only 52 % of the glycerol was used; similar results were obtained in continuous culture. Further experiments showed that an amino acid mixture and yeast extract were needed for good growth

and 1% (w/v) casein hydrolysate + 1% yeast extract medium (see Methods, medium AH1) gave optimum growth and glycerol utilization; but to prevent the pH value cycling in the continuous culture, and to get a more reduced medium, the medium was slightly altered (medium AH2). The results of experiments with batch cultures (AH1 medium) are shown in Table 2; the final pH value was 5·1 to

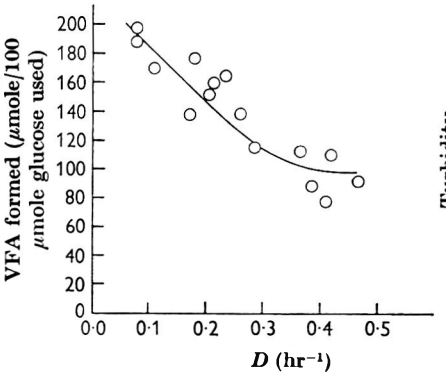

Fig. 7

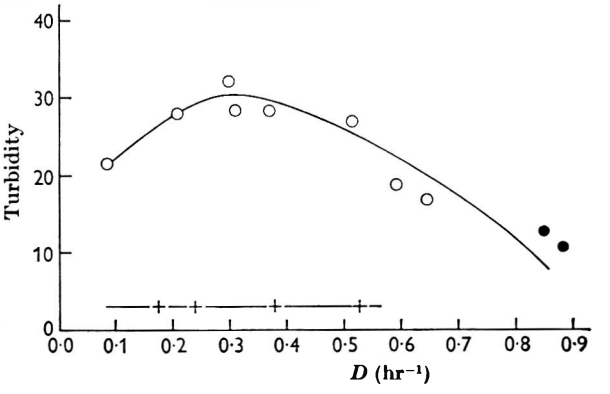

Fig. 8

Fig. 7. Continuous culture of selenomonad 6; relationship between VFA produced and dilution rate (D). Values are taken from five runs at 27·7 μmole/ml. glucose in the medium and one run at 13·9 μmole/ml. glucose.

Fig. 8. Continuous culture of lipolytic bacterium 5s; relationship between turbidity and dilution rate (D) at different steady states. Glycerol-limiting conditions, glycerol concentration 65·6 μmole/ml. ○. At points marked ● a true steady state was not achieved; the density drifted downwards at about 1 unit per 12 hr. Basal medium growth +. At D = 0·083, 98% of the glycerol was used, at D = 0·206, 100%; at D = 0·298, 98·6%; at D = 0·310, 90·7%; at D = 0·370, 88·1%; at D = 0·514, 75·1%; at D = 0·591, 64·3%; at D = 0·850, 53·6%; at D = 0·883, 36·5%. The values are taken from two runs with glycerol and one run with the basal medium. Maximum turbidity of batch cultures at same substrate concentration *c.* 32. A turbidity of 30 EEL units = 1·36 mg. dry wt. bacteria/ml.

Table 2. *Batch growth of lipolytic bacterium strain 5s on glycerol*

Glycerol added (μmole/ml.)	Glycerol used (μmole/ml.)	Dry wt. bacteria (mg./ml.)	Y_{glyc}	VFA formed (μmole/100 μmole glycerol used)
0	0	*	—	—
65·2†	65·2	1·48	22·7	65·5
130·4	67·6	1·36	20·1	61·4
195·6	82·1	1·17	14·3	42·5
260·8	89·1	1·30	14·6	40·3

* Too small to measure accurately.

† Fermentation products (μmole/100 μmole glycerol used): acetic acid, 17·9; propionic acid, 47·4; succinic acid, 31·7; lactic acid, 7·1. Carbon recovery 109%.

5·3, depending on the glycerol concentration. As with the selenomonad cultures these results were not very reproducible because of the rapid decrease in turbidity after maximum growth, and this was accelerated by increasing the amount of glycerol in the medium. At glycerol 65·2 μmole/ml. all the glycerol was utilized, but

at higher concentrations only a fraction was used. This might have been partly due to the pH value attained in the cultures. The carbon recovery showed that the fermentation products were accounted for and little glycerol carbon was utilized to form cellular material. Except at low values, the turbidity readings were related to mg. dry-weight organism/ml. (Fig. 4) in batch and continuous culture. The results of two continuous cultures, of 320 and 550 hr, are shown in Figs. 8, 9, 10. The turbidity readings are not corrected for growth in the basal medium since Fig. 4 does not show the same relationship between turbidity and dry weight of organisms at low and high turbidities and any correction would be very small and

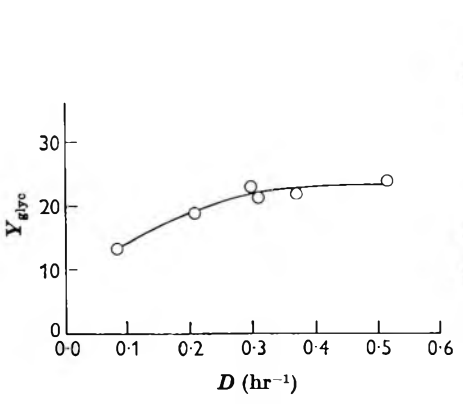

Fig. 9

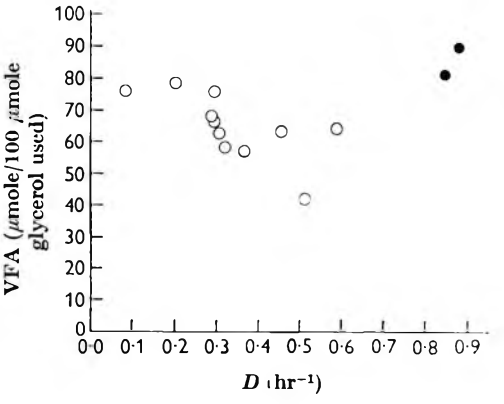

Fig. 10

Fig. 9. Continuous culture of lipolytic bacterium 5s; relationship between Y_{glyc} and dilution rate (D). Values are taken from two runs at a glycerol concentration of 65·6 μmole/ml. in the medium.

Fig. 10. Continuous culture of lipolytic bacterium 5s; relationship between VFA produced and dilution rate (D). Values are taken from two runs at a glycerol concentration of 65·6 μmole/ml. in the medium. At points marked ● a true steady state was not achieved (see Fig. 8).

uncertain in magnitude. Results from other cultures also gave graphs similar to Fig. 8, which is unlike the selenomonad results. The medium remained at about pH 6 at dilution rates up to about $D = 0{\cdot}3$; above that it increased to pH 6·6 at the highest dilution rate. Also included in Fig. 8 is a graph showing growth on the basal medium from one run of 190 hr. As in the case of selenomonad 6, the turbidity in continuous culture of bacterium 5s was always less than in batch culture in the basal medium.

Bacterium 5s never showed granular growth like the selenomonad strain 6; growth was always uniformly turbid. Like the selenomonad the motility of bacterium 5s was much greater in the continuous culture than in batch culture. When the pH value was suitable for growth the 5s organisms were uniform in length and generally single, except at the highest dilution rates, when a few chains began to appear. However, in the cultures where the pH value was uncontrolled and the growth cycled, slowing down of growth as the pH value decreased was accompanied by the appearance of long coiled filaments of uniform diameter and of length up to about ten times the normal. These forms disappeared as the pH value increased

again and growth re-started. Misshapen and spherical forms were also noted at low culture pH values when growth had stopped; these unnatural forms were commonly seen in batch cultures which had passed maximum growth.

DISCUSSION

The results of the present experiments show that it is possible to grow anaerobic rumen bacteria for prolonged periods in continuous culture. The cultivations reported here were done under carbohydrate-limiting conditions, but it would obviously be possible to use nitrogen-limiting conditions. The rumen is a kind of continuous culture, but apart from the fact that there is a wide diversity of microbial types present, it differs from the chemostat type of continuous culture described here in several ways, and, while the present results do give some pointers to the behaviour of rumen bacteria *in vivo*, it was not the purpose of these experiments to try to make an 'artificial rumen'. For instance, the rumen 'culture' has a very low dilution rate *in vivo*, only slightly more than one change of volume per day and, especially in the case of stall-fed animals, the micro-organisms do not attain a true steady state. Nutrient addition to the rumen, and to some extent saliva additions which form the main liquid flowing through the rumen, are intermittent and the concentrations of bacteria rise and fall in correspondence to these additions. Also, because of intermittent nutrient addition, it seems likely that different factors may limit bacterial growth at different times, and that the growth of all organisms will not be limited by the same nutrient. The change in amounts of fermentation products with growth rate, noted with the selenomonad strain 6 and to a lesser extent with the bacterium 5s in continuous culture, suggests that the results of *in vitro* batch cultures may not always be a quantitative guide to fermentation in the rumen. Some change in products with growth rate has been noted with other bacteria, for example *Streptococcus faecalis* (Rosenberger & Elsden, 1960). If the results here are any guide to happenings *in vivo* then the maximum growth rates of the bacteria have some significance. If the maximum growth rate is taken as that at the beginning of the washout portion of the curves, then the selenomonad strain 6 and bacterium 5s have minimum doubling times ($\log_e 2/D$) of about 1·4 hr. This is very much longer than that of *S. bovis* and shows how it is possible in some cases of overfeeding of carbohydrate for *S. bovis* to increase so rapidly as to become the predominant rumen bacterium in a matter of hours. The effect of pH value on the growth of bacterium 5s is interesting (Fig. 2): at pH values below about 5·7 the growth rate markedly decreased, and growth appeared to cease at about pH 5·3. The pH value of rumen contents decreases after feeding and then increases and a value of pH 5·7 is above the minimum found on some rations. For instance, Purser & Moir (1959) and Bryant & Robinson (1961*b*) found minimum values of about pH 5·5 and 5·6 in animals fed on concentrates at 2 to 4 hr after feeding, compared with about pH 7·0 to 7·2 before feeding. Bryant & Robinson's results show that viable bacterial numbers rapidly increased till just before the time of minimum pH value, while the pH remained above 6, and then decreased slowly. If the results of Fig. 2 are typical of the main types of rumen bacteria then it would seem that pH value is probably the factor which controls growth rate of the bacteria, especially in the first few hours after feeding, and not the substrate concentration. The changes in morphology

of the selenomonad strain 6 and bacterium 5s point to a limitation in the techniques of counting morphological types of rumen bacteria at different times after feeding. As previously pointed out (Hobson & Mann, 1961) the selenomonads grown *in vitro* are much smaller than many seen *in vivo*, although there is a serological relationship between the organisms *in vitro* and *in vivo* (Hobson *et al.* 1962). One of the reasons for measuring the size of organisms in the selenomonad cultures was to see whether large forms were produced at high growth rates. Although the size of organism changed, no crescentic organisms of a size similar to that of those seen in the rumen were ever noted.

The theory of continuous culture proposed by Herbert *et al.* (1956) predicts that concentration of organism, equal to the maximum value found in batch culture, will not vary with growth rate until maximum growth rate (equated with dilution rate) is reached, but will then decrease rapidly. The limiting substrate concentration in the culture will generally remain very small until maximum growth rate is approached and will then increase rapidly. Some bacteria appear to behave in the theoretical manner, others diverge from the theoretical, but published data are scanty. A divergence at the higher growth rates, resulting in steady states at dilution rates apparently above the maximum growth rates and a slow decrease of concentration of organism as the dilution rate (D) increases (e.g. Fig. 5) was attributed by Herbert *et al.* (1956) to inadequate mixing in the culture vessel. This is a possibility, but it seems to be a phenomenon generally found and more probably indicates an inadequacy in the present theoretical treatments. Further divergences from the theoretical curve of concentration of organism against dilution rate have been found at low dilution rates. A decrease in organism dry weight/ml. at low dilution rates, found under carbohydrate-limiting conditions by Herbert (1958) with *Aerobacter aerogenes*, was ascribed to endogenous metabolism of cell substance. Herbert suggested a change in the equations for continuous culture which might account for this theoretically. It is also possible to account for this decrease in concentration of organism at low growth rates by supposing that some of the substrate is fermented to provide maintenance energy, thus decreasing the substrate available for cell multiplication. This will have the greatest effect when growth rate is low. All the bacteria grown in the present experiments showed a decrease in concentration of organism at low growth rates. All graphs (Figs. 3, 5, 8) also showed a maximum value for bacterial concentration. Since this peak in the graphs occurred with all the bacteria it seems unlikely to be an artifact. What is very unusual are the graphs for the selenomonad strain 6 (Figs. 5, 6). These show not only a pronounced decrease in bacterial concentration at low growth rates, but a very high yield of organism at optimum growth rate. The yield of organism during an anaerobic fermentation has been linked with the energy available in the 'high-energy' phosphate bonds of ATP. Bauchop & Elsden (1960) found that for some bacteria of known fermentation pathways growing in a medium containing preformed monomers a yield of about 10 g. dry wt. organism/mole ATP (Y^{ATP}) produced during fermentation of the carbohydrate energy source was obtained. One of the bacteria tested by them, however, seemed to have a higher yield than could be accounted for by this method. Gunsalus & Schuster (1961) calculated that the energy available in a mole of ATP is sufficient to polymerize monomers into 33 g. of cell material, so that the experimental value of Y^{ATP} appears to be inefficient.

The theoretical yield of organism per mole of carbohydrate fermented can be calculated for simple fermentations, but calculation becomes difficult for more complex fermentations.

The yield of organism, Y_{glyc}, for bacterium 5s growing on glycerol approximates to 22 at the optimum growth rates in continuous culture and in batch culture where glycerol is limiting. At higher glycerol concentrations in batch culture Y_{glyc} decreases because fermentation of glycerol continues after growth is limited by a nitrogenous, or other, constituent of the medium, or by the decrease in pH value of the culture. The energy available in fermentation at this stage might be released by an ATPase type of enzyme, or might be used for maintenance of the organisms, although in this latter case the pH value (or other conditions in the medium) seems to be such that lysis of organisms takes place more rapidly than their structure can be maintained. The value of Y_{glyc} found for bacterium 5s approximates to formation of 2 ATP molecules during the fermentation (on the basis of $Y^{ATP} = 10$) and is in agreement with the results of Bauchop & Elsden (1960) with a Propionibacterium fermenting glycerol.

The yield of selenomonads (Y_{gluc}), though, is about 62 at optimum growth rates (taking the average of values between the lines in Fig. 6). This would correspond to the formation of about 5–6 mole ATP/mole of glucose fermented. This seems improbable on present concepts of the pathways of fermentation. However, consideration of all the results suggests that the high yield is unlikely to be due to fermentation of the amino acids of the basal medium, or a secondary fermentation of lactate, and it must be concluded that this value is actually Y_{gluc} for this bacterium. There then remain the two possibilities that the selenomonads have sources of energy for growth other than those so far found in known modes of fermentation, or that Y^{ATP} is not universally 10, but is greater for some anaerobes. While this paper was being written Hungate (1963) reported that a strain of *Ruminococcus albus* growing in a continuous culture at a dilution rate of 0·1 hr^{-1} on cellobiose gave a value for Y_{gluc} of 55. This high yield was unexplained. The maximum value for Y_{gluc} calculated from the different batch cultures of the selenomonad approximates to 20, suggesting an ATP formation of 2 mole/mole glucose fermented on the basis of $Y^{ATP} = 10$. This appears reasonable, but since the yield of the selenomonad varies with growth rate in continuous culture it can be shown that the apparent yield of the batch cultures is probably entirely fortuitous, and hears no relationship to the actual yields under optimum growth conditions.

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The Behaviour of Tanned Erythrocytes in Various Haemagglutination Systems

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SUMMARY

Erythrocytes from various species of animals were tanned by exposure to 1/20,000 tannic acid for 10 min. at 37°, and were used in various haemagglutination systems together with untreated red cells as control. The titre of fresh human anti-AB serum was decreased from 4- to 64-fold when tested with tanned homologous red cells, while no decrease of titre was observed in commercially prepared sera of the same group under identical experimental conditions. The titre of anti-sheep red cell serum was not appreciably affected when tested with tanned sheep red cells, but there was a 2- to 4-fold increase in the agglutinin titres of myxoviruses (influenza, mumps) tested with tanned chick or sheep red cells. The haemagglutinin titres of West Nile and Sindbis viruses were not changed when tanned goose red cells were used instead of untreated red cells in haemagglutination tests. Human, guinea pig and fowl red cells did not become agglutinable by West Nile and Sindbis viruses after treatment with tannic acid. West Nile and Sindbis viruses agglutinated sheep red cells to low titres. These titres were twice as high when tanned red cells were used instead of untreated cells. There was a sharp drop in the titres of anti-human cell sera when tested with tanned human group O red cells. It is suggested that tannic acid may act on certain species of red cell to increase or decrease their agglutinability in the presence of immune serum.

INTRODUCTION

Alterations induced by tannic acid on the surface of erythrocytes (red cells) result in attainment by such treated cells of the ability to adsorb protein material and to agglutinate in the presence of specific immune serum (Boyden, 1951). This phenomenon, known as indirect haemagglutination, has been extensively used for detecting antibodies in bacterial, viral and parasitic diseases (Neter, 1956). However, relatively little work has been done to define the behaviour of tanned red cells in direct haemagglutination tests. In a report by Buckland & Tyrrell (1963), changes induced in the agglutinability of red cells after treating them with a variety of substances, including formalin, papain, chymotrypsin, periodate, receptor-destroying enzyme and swine influenza virus, were described. Tannic acid was not included in these studies. Reports of other investigators (Brading, 1956; Hornung & Baer, 1958), as to the extent of agglutinability of tannic acid-treated red cells in human ABO system, have been somewhat contradictory.

Because of the few and conflicting reports of direct haemagglutination with tanned red cells, the problem was thought worth investigating. The experiments

reported here were done to study the behaviour of tanned red cells in various haemagglutination systems, and to learn whether the results obtained would provide any information as to the nature and diversity of receptor sites on the red cell surface. Studies on the effect of tannic acid on surface antigens of HeLa cells have been presented elsewhere (Garabedian & Syverton, 1960)

METHODS

Tanning of erythrocytes. Blood was obtained in modified Alsever's solution (Bukantz, Rein & Kent, 1946), and kept at 8° for 3 days before use. Red cells were washed with 0·85 % NaCl solution and tanned by addition of 1 ml. packed cells to 19 ml. freshly prepared tannic acid (Merck) 1/20,000 (w/v) in 0·85 % NaCl solution. For tanning the sheep red cells, for use in a sheep-anti-sheep red cell haemagglutination system, different concentrations of tannic acid, ranging from 1/1000–1/40,000, were used. Red cell suspensions in the tannic acid were incubated at 37° for 10 min. with frequent agitation (Garabedian, Matossian & Djanian, 1957), sedimented by centrifugation, washed with 0·85 % NaCl solution and diluted in the same solution to a concentration of 1 % according to packed cell volume.

Direct haemagglutination tests. Anti-AB, anti-sheep red cell and anti-cellular titres in sera were determined by making serial dilutions of inactivated serum with 2 % bovine albumin (Armour, fraction V) in phosphate buffer saline (pH 7·0), using 0·5 ml. volumes for each tube. To each dilution of serum, including the control tube, 0·12 ml. of a 1 % suspension of washed red cells was added. Tubes were incubated at 37° for 30 min., after which they were placed at 4° for the red cells to sediment. Readings were taken after gently mixing the contents of the tubes.

Myxovirus haemagglutination tests were done with infectious chick embryo allantoic fluids according to the standard technique described by Jensen (1956). Arthropod borne (arbo) virus haemagglutination tests were performed following the procedure described by Clarke & Casals (1958). Dilutions of immune serum were made in 2 % bovine albumin in buffered saline. This helped the even settling of the tanned red cells but did not interfere with the sensitivity of the test.

Anti-cellular sera. Anti-cellular sera were prepared by intravenous injection of rabbits with human cells (HeLa, KB, primary amnion, and continuous amnion) grown in yeast extract medium supplemented with 20 % (v/v) human serum. The cells were washed repeatedly with 0·85 % NaCl solution, scraped from glass and made into a suspension containing about 2×10^6 cells/ml., frozen and thawed repeatedly and freed from coarse sediment by centrifugation at 500*g*. Each rabbit received four injections of 2 ml. cell suspension at 4-day intervals. After 1 week the rabbits were given a fifth injection of 5×10^6 sonically-disrupted cells in Freund's adjuvant. The dose was distributed at various body sites subcutaneously and intramuscularly. None of the animals showed anaphylactic reactions. Blood was taken from the animals 10 days after the last injection, and the serum was stored at −20°. Before use, samples of test sera were thawed and heated at 56° for 20 min. Control serum was collected from randomly selected uninjected rabbits.

Haemagglutination studies were made with the following systems: (1) anti-AB human serum with human red cells; (2) anti-sheep red cell serum with sheep red

cells; (3) myxoviruses with fowl and sheep red cells; (4) arboviruses with goose, fowl, human, guinea pig and sheep red cells; (5) anti-human cell serum with human red cells.

The human ABO system of haemagglutination was studied by using fresh human sera as well as commercially prepared anti-AB sera. For the sheep-anti-sheep red cell system, rabbit immune serum prepared against sheep red cells was used with sheep red cells. In myxovirus haemagglutination tests influenza A and A1 strains, PR8, FM1, Denver; A2 strain, Jap-305; B strain, Lee, and mumps virus were used as agglutinins against chick and sheep red cells. In arbovirus haemagglutination tests West Nile and Sindbis viruses were used as haemagglutinins with red cells obtained from goose, fowl, man, guinea pig and sheep. Finally, human group O red cells and anti-cellular sera prepared in the rabbit were used in direct haemagglutination tests (Brand & Syverton, 1960). Both tanned and untreated red cells were used in all tests.

RESULTS

Titres of fresh human sera were decreased from 4- to 64-fold when tested with tanned homologous group A, B and AB agglutinogens, while the decrease in titres was no more than 2-fold when commercially prepared sera were used in place of fresh serum in similar experiments (Table 1). These findings explain the discrepancy between the results of Brading (1956) and Hornung & Baer (1958). The latter

Table 1. *ABO system of haemagglutination using untreated and tanned human red cells*

Human red cells		Serum designation and titre* fresh serum				Serum designation and titre commercial serum†	
		Anti-A		Anti-B			
Group	Nature	1	2	3	4	Anti-A	Anti-B
O	Untreated	—‡	—	—	—	—	—
	Tanned	—	—	—	—	—	—
A	Untreated	128	16	—	—	128	—
	Tanned	16	—	—	—	64	—
B	Untreated	—	—	32	64	—	64
	Tanned	—	—	—	—	—	64
AB	Untreated	16	4	16	32	8	64
	Tanned	4	—	—	—	4	32

* Reciprocal of serum titre.
† Ortho Pharmaceutical Lot B9113-3.
‡ Titre less than 1/2.

investigators used commercially prepared sera for their determinations and observed no diminution in titre when tested with tanned red cells. It is possible that commercial serum, which may have been prepared by injecting men with A and B substances, contains accessory factors capable of reacting with tanned red cells. This property may be absent in fresh serum. Nevertheless, the exact mechanism of this phenomenon is not clear.

While 1/1000 and 1/2000 concentrations of tannic acid produced complete or

partial haemolysis and clumping of sheep red cells, and could not be used in tests other concentrations, in the range of 1/5000 to 1/40,000, did not have any appreciable effect on the agglutinability of sheep red cells when these cells were tested with anti-sheep red cell serum prepared in the rabbit (Table 2). Apparently the receptor site of action on the sheep red cells, unlike the human AB red cells, was not affected by any concentration of tannic acid used in this experiment.

Table 2. *Effect of various concentrations of tannic acid on the agglutinability of sheep red cells with homologous immune rabbit serum*

Sheep red cells treated with tannic acid in concentrations of:	Anti-sheep red cell rabbit serum titres			
	1/800	1/1600	1/3200	1/6400
1/1000	haemolysis			
1/2000	partial haemolysis and clumping			
1/5000	+	+	±	−
1/10,000	+	+	+	−
1/20,000	+	+	±	−
1/40,000	+	+	±	−
Sheep red cells, untreated	+	+	±	−

Table 3. *Agglutinability of chick and sheep red cells with myxoviruses before and after treatment with tannic acid*

		Agglutinins						
		Influenza virus strains						
			A1					Controls
Nature of red cells		A PR8	FM1	Denver	A2 Jap-305	B Lee	Mumps	Uninfected allantoic fluid
Fowl	untreated	128*	256	128	128	512	64	< 4
	tanned	512	512	512	512	2048	128	< 4
Sheep	untreated	4	64	128	256	8	32	< 4
	tanned	8	256	256	1024	32	64	< 4

* Reciprocal of haemagglutinin titre.

There was a 2- to 4-fold increase in myxovirus (influenza, mumps) titres, when tested with fowl or sheep tanned red cells (Table 3). This was possibly due to the removal by tannic acid from the red cells of certain elements which were masking the superficial receptor sites of red cells, with resulting exposure of deeper sites capable of reacting with viral haemagglutinins. It is also possible that a slight increase in titre of myxovirus haemagglutinins was due to a change in the surface potential of the tanned red cells. The low titres of influenza viruses A and B with sheep red cells as compared to high titres with other influenza viruses, A1 or A2, are worthy of consideration.

There was no change in haemagglutinin titres of West Nile and Sindbis viruses when tanned goose red cells were used in haemagglutination tests instead of untreated cells. Furthermore, fowl, human and guinea pig red cells did not become agglutinable by West Nile and Sindbis viruses after treatment with tannic acid.

West Nile and Sindbis viruses agglutinated sheep red cells to low titres only. These titres were 2-fold higher when tanned red cells were used instead of untreated cells (Table 4).

Table 4. *Agglutinability of red cells from various species of animal with arboviruses before and after treatment with tannic acid*

Nature of erythrocyte		West Nile virus (pH 6·6)	Control: normal mouse brain (pH 6·6)	Sindbis virus (pH 5·8)	Control: normal mouse brain (pH 5·8)
Goose	untreated	640	< 10	320	< 10
	tanned	640	< 10	320	< 10
Fowl	untreated	< 10	< 10	< 10	< 10
	tanned	< 10	< 10	< 10	< 10
Human	untreated	< 10	< 10	< 10	< 10
	tanned	< 10	< 10	< 10	< 10
Guinea pig	untreated	< 10	< 10	< 10	< 10
	tanned	< 10	< 10	< 10	< 10
Sheep	untreated	40	< 10	10	< 10
	tanned	80	< 10	20	< 10

Table 5. *Haemagglutination of human group O red cells with human anti-cellular serum*

	Anti-cellular sera and titres				
Nature of red cells	Anti-HeLa	Anti-KB	Anti-amnion (primary)	Anti-amnion (continuous)	Normal rabbit
Untreated	256*	64	64	64	8
Tanned	2	< 2	< 2	2	2

* Reciprocal of serum titre.

That tanned HeLa cells lose at least a part of their surface antigens and, when coated on red cells, become non-reactive in indirect haemagglutination tests, was demonstrated earlier (Garabedian & Syverton, 1960). In experiments reported here, the tanned red cells reacted very poorly with anti-cellular serum (Table 5). This was possibly due to the removal from or masking of certain surface antigens of the red cells by the tannic acid treatment. It was also noted that all four anti-human red cell sera reacted similarly by showing either total or drastic decrease of the original titre. This and previous experiments indicate that the action of tannic acid was mainly on human red cells, and only to a slight extent on sheep and chick red cells. A low titre in non-immune rabbit serum against human red cells should be noted. In some experiments irregular settling of erythrocytes, simulating a mild type of haemagglutination, was observed in tubes containing human group O tanned red cells with anti-HeLa serum. Nevertheless, unlike the haemagglutination observed in untreated cells, these erythrocytes were easily dispersed upon gentle shaking of the tubes. A satisfactory explanation for this peculiar phenomenon could not be postulated.

DISCUSSION

The surface structure of the red cell is complex and far from being adequately studied. It has been described as composed of liproprotein (elinin) units located parallel to the cell surface and linked together with ether-extractable lipids. In addition protein (S protein) and some haemoglobin are thought to be associated with this structure (Moskowitz & Calvin, 1952). Springer (1963) has presented an extensive review of work on cell surface antigens.

The reactions in various haemagglutination systems involve an interaction between haemagglutinins and a meshwork of various macromolecules of mucopolysaccharides, mucoproteins, phospholipids and other reactant groups located on the cell surface. Thus, an agent exposed to the intact red cell will react with these various determinant groups as well as with the haemoglobin, if it is capable of doing so. Brading (1956) presented evidence that tannic acid reacts with the iron on the surface of red cells. However, it is possible that beside this simple chemical union, tannic acid also combines with other structures of the red cells, resulting in masking or unmasking of receptors, altering their chemical structure partially or completely and/or changing the surface potential of the cell. For example, considering that ABO blood group activity resides in the 'elinin' fraction of the red cells (Moskowitz & Calvin, 1952), it can be assumed that the action of tannic acid was directed towards this substance, as evidenced by a decrease in agglutinability of tanned AB red cells in the presence of homologous immune serum. It is possible that the decreased agglutinability of tanned group O red cells in the presence of anticellular serum was also due to this same mechanism of action of tannic acid on the red cells. On the other hand it appears that the commercially prepared sera possessed additional factors capable of reacting with other receptors on the red cell surface not affected by tannic acid. It was further apparent that tannic acid had no appreciable effect on the neuraminic acid derivatives. This was shown by the fact that the agglutinability of tanned red cells was not decreased in the presence of myxoviruses. Contrariwise, a slight increase in titre in these tests was possibly due to a change in the surface potential of the red cells, brought about by the tannic acid treatment. Other agents acting on red cells bring about surface alterations which may be different from those caused by tannic acid (Buckland & Tyrrell, 1963).

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Modified Degrees of Streptomycin Dependence and Resistance in *Escherichia coli*

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SUMMARY

For three streptomycin-dependent (S-dependent) strains of *Escherichia coli*, the streptomycin concentration necessary for optimal growth during incubation for 24 hr in a nutrient broth medium was about 10 μg./ml. Less than 5 μg./ml. was sufficient for fairly heavy growth and the minimal streptomycin concentration permitting appreciable growth was 0·40 μg./ml. Division of S-dependent bacteria was inhibited at streptomycin concentrations greater than 20 μg./ml. and small inocula gave no visible growth in 24 hr at more than 30 μg. streptomycin/ml.

Following addition of any of several salts to the growth medium at 0·05 M growth occurred over a wide range of streptomycin concentrations and the optimum was increased from twofold to as much as 1000-fold. Maximal concentration of streptomycin in which growth of S-dependent *Escherichia coli* was possible increased to as high as 20,000 μg. streptomycin/ml. in some instances, and the minimal concentration which supported growth was increased in the presence of several of the salts. Salts also increased the degree of resistance of a streptomycin-resistant *E. coli* mutant from 20 to as much as 10,000 μg. streptomycin/ml. In 0·10 M phosphate-buffered nutrient broth, maximal and optimal concentrations of streptomycin increased with increasing acidity; at pH 5·8 heavy growth of an S-dependent strain of *E. coli* occurred at 200,000 μg. streptomycin/ml.

INTRODUCTION

Growth rates of streptomycin-dependent (S-dependent) bacteria in a mineral medium are proportional to streptomycin concentration (Spotts, 1962) and fairly high streptomycin concentrations, 100 μg. or more/ml., have commonly been used to obtain optimal growth. The activity of streptomycin as an antibiotic is markedly decreased in the presence of salts or in salt-containing defined media however (see Henry & Hobby, 1949; Wasserman, Lessner & West, 1954), and its function as a growth factor for dependent strains may be subject to the same salt effect (Engelberg & Artman, 1962). The 'true' streptomycin optimum for such strains may then be well below reported values. The amount of streptomycin necessary for growth has, in fact, been found to vary with the culture medium (Hashimoto, 1959; Goldschmidt, Matney & Bausum, 1962). We had noted that several S-dependent strains of *Escherichia coli*, routinely grown in nutrient broth at rather low streptomycin concentrations, grew well in the chemically defined minimal medium of Davis & Mingioli (1950) only at concentrations of streptomycin up to 100 times as high. We have therefore attempted to define the optimal streptomycin concentration, as well as the minimal and maximal values for growth of three S-dependent

strains of *E. coli* in an ordinary nutrient medium, and to determine the extent to which these values can be modified by individual salts. Also some study has been made of the influence of salts on the degree of resistance of a low-level streptomycin-resistant mutant.

METHODS

Bacterial strains. Three S-dependent strains of *Escherichia coli* were used: Sd-4 was originally obtained from Dr M. Demerec and has been maintained here as a stock culture for several years; strains HB and D-R (Funk & Plunkett, 1960; Plunkett, 1962) were isolated in this laboratory. The resistant strain, SR, was derived here from *E. coli* strain ATCC 11887.

Media. Difco Nutrient Broth, free of added salt, was the basal growth medium; nutrient agar was used for plating. For studying salt effects the broth was made up in 0·05 M-salt solutions before sterilization. Streptomycin sulphate was added to the medium to give the desired concentrations just before use.

Cultural conditions. S-dependent bacteria for the initial inoculum in each experiment were grown overnight at 37·5° in nutrient broth containing 10 μg. streptomycin/ml., resistant bacteria in nutrient broth alone. Samples (5 ml.) of the various growth media which contained graded concentrations of streptomycin were then inoculated with 2×10^3 to 5×10^3 bacteria of either the S-dependent or S-resistant type. The inoculated cultures, in small flasks, were shaken at 37·5° for 24 hr. The extent of growth in each flask was then determined in terms of light extinction (E) readings at 650 mμ, with uninoculated medium as a reference. For a few experiments larger starting inocula, 10^4 to 10^5 bacteria taken directly from stock cultures, were used. For determining their response to streptomycin on a solid medium, bacteria were spread on series of nutrient agar plates containing different concentrations of streptomycin. Degrees of resistance or dependence were judged by the time at which colonies first became visible, by comparative counts, and by the final size and overall appearance of the colonies on continued incubation.

RESULTS

Optimal, maximal and minimal streptomycin concentrations for growth

In salt-free nutrient broth at streptomycin concentrations increasing in 5 μg./ml. increments from 0 to 60 μg./ml., turbidity at the end of 24 hr was highest at 10 μg./ml. There was, however, nearly equivalent growth at 5 and also at 15 and 20 μg./ml. for strains HB and D-R, and for Sd-4 at 5 and 15 μg./ml. At concentrations higher than 25 μg./ml. extinction readings declined rapidly and there was little or no visible turbidity in 24 hr at streptomycin concentrations above 30 μg./ml. When cultures at higher streptomycin concentrations were incubated for an additional 48 hr growth became evident up to 100 μg./ml., but rarely at concentrations greater than 150 μg./ml. The lowest tested streptomycin concentration that would support appreciable growth in nutrient broth was 0·40 μg./ml. for all three S-dependent strains. Above this concentration the growth response improved rapidly and 2 or 3 μg./ml. were sufficient to produce heavily turbid cultures. Strain SR possessed only a slight resistance to streptomycin; the maximal streptomycin concentration which permitted visible growth in nutrient broth in 24 hr was 20 μg./ml.; on further incubation to 72 hr growth occurred up to 40 but seldom above 50 μg./ml.

On solid media, optimal and maximal streptomycin concentrations were considerably higher than in broth. As judged by the time colonies first became visible on the plates and by their final size, the optimal streptomycin concentration for the S-dependent strains on nutrient agar was 50–100 μg./ml. Outside of this range, rate of growth and overall size of the colonies were diminished. This was true regardless of the concentration of streptomycin in the broth in which the bacteria had been grown originally. S-dependent bacteria which grew well in nutrient broth at the optimal concentration, 10 μg. streptomycin/ml., gave only barely visible colonies in 24 hr on 10 μg. streptomycin/ml. agar while, at the same time, large and clearly distinct colonies appeared on agar containing 50–100 μg. streptomycin/ml. On continued incubation colonies appeared on agar up to 700 μg. streptomycin/ml. Similarly, the resistance of strain SR was higher on agar than in broth, with some colony growth becoming visible at streptomycin levels up to 400 μg./ml.

Effect of salts

Table 1 gives minimal, maximal, and optimal streptomycin concentrations for growth of S-dependent *Escherichia coli* as well as maximal concentrations for the resistant strain (SR) in salt-containing nutrient broth, when streptomycin was present at concentrations between 0 and 20,000 μg./ml. All the salts tested increased the maximal streptomycin concentration which permitted appreciable growth of S-dependent and S-resistant strains; the minimal requirement for S-dependent growth was also increased in several instances. The streptomycin concentration at which the heaviest growth of S-dependent bacteria took place was, in every case, increased. In many of these instances growth was almost equally heavy over a wide range of streptomycin concentrations; this is shown in Fig. 1, which gives specific data for the growth of strain HB in nutrient broth in comparison with growth in media containing each of four of the tested salts. Most salts had the additional effect of stimulating overall growth so that extinction readings at the optimal streptomycin concentrations in the salt-containing media were generally higher than those in nutrient broth controls at the 10 μg./ml. optimum for this medium. Even where overall growth was inhibited, as with K_2HPO_4, the optimal and maximal streptomycin concentrations were again at values higher than those for nutrient broth alone.

Effects of phosphate buffer

Most of the salts had little effect on the pH value of the medium at the concentration used. Nutrient broth alone had pH values between 6·8 and 7·0; the salt-containing broths ranged from pH 6·5 to 7·3 except for K_2HPO_4 with a value near pH 8·0. When optimal and maximal streptomycin concentrations were determined for nutrient broth media made up in 0·10 M-phosphate buffer at three pH values, growth responses corresponding to those shown in Fig. 2 were obtained. The acid-buffered medium was especially effective in increasing growth at high streptomycin concentrations. In one experiment heavy growth of strain HB was observed in this medium even with streptomycin 200,000 μg./ml. At pH 5·8 and 7·0 there was no appreciable growth at streptomycin 10 μg./ml., the optimum for nutrient broth, or even at 50 μg./ml. Optimal growth occurred only at concentrations of the order of several hundred μg./ml. In the alkaline medium growth of S-dependent bacteria,

Table 1. *Minimal, optimal, and maximal streptomycin concentrations for growth of streptomycin-dependent and -resistant strains of Escherichia coli in nutrient broth containing 0·05M-salts*

Streptomycin concentrations: 0, 10, 20, 50, 100, 500, 1000, 5000, 10,000, 20,000 μg./ml. Extinction (E) at 650 mμ was recorded after growth for 24 hr at 37·5°. Minimal and maximal values are, respectively, the lowest and highest concentrations of streptomycin which permitted visible growth (E > 0·03), the optimal concentration is that at which the extinction at 24 hr was highest and is given as a range where turbidity readings were within ± 0·03 of this value.

	Streptomycin concentration (μg./ml.)									
	Strain HB			Strain Sd-4			Strain D–R			Strain SR.
Salt	Min.	Opt.	Max.	Min.	Opt.	Max.	Min.	Opt.	Max.	Max.
NaCl	10	20–100	100	10	10–20	100	10	10–20	500	100
K_2HPO_4	10	50–100	500	20	100	100	50	100–500	500	1,000
$(NH_4)_2SO_4$	50	500–10,000	> 20,000	50	500–10,000	> 20,000	10	500–5,000	> 20,000	10,000
NH_4Cl	10	50–100	1,000	10	20	1,000	10	20–100	5,000	500
$MgSO_4$	50	500–1,000	1,000	50	20–500	1,000	20	50–1,000	1,000	1,000
$MgCl_2$	100	500–1,000	5,000	50	500	5,000	20	500	10,000	1,000
NH_4NO_3	10	20–500	1,000	10	20	1,000	10	20–50	1,000	1,000
Na citrate	500	1,000	1,000	50	500–1,000	1,000	500	500	500	1,000

although limited, occurred again only at streptomycin concentrations well above those required for growth in plain nutrient broth. A phosphate-buffered medium also increased the degree of streptomycin resistance of strain SR. At pH 5·9 heavy growth of strain SR took place at all streptomycin concentrations to 24,000 μg./ml. At pH 7·0 resistance was increased to over 12,000 and at pH 7·8 to 3200 μg. streptomycin/ml.

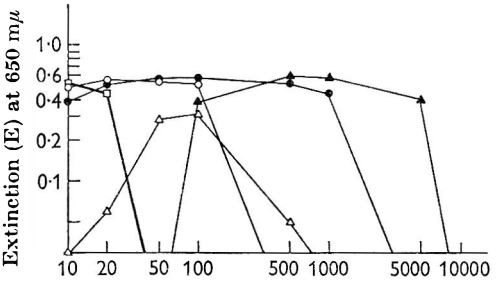

Fig. 1

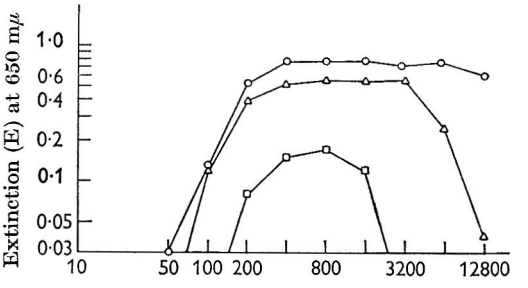

Fig. 2

Fig. 1. Effect of streptomycin concentration on growth of *Escherichia coli* strain HB in nutrient broth in the presence of 0·05 M-salts. □, Nutrient broth control; ○, NaCl; △, K_2HPO_4; ●, NH_4Cl; ▲, $MgCl_2$.

Fig. 2. Effect of streptomycin concentration on growth of *Escherichia coli* strain HB in phosphate-buffered nutrient broth. ○, pH 5·80; △, pH 7·00; □, pH 7·75.

DISCUSSION

The rather common practice of supplementing nutrient broth with 0·5% NaCl may account for some of the high optimal streptomycin values that have been reported by others for streptomycin dependent growth in this medium. Our results indicate that, for three S-dependent strains of *Escherichia coli*, appreciable growth in ordinary nutrient broth was possible even at streptomycin 0·40 μg./ml., and heavy growth at less than 5 μg./ml. In fact, in the absence of added salt, streptomycin actually inhibited the rate of growth of S-dependent bacteria at values only slightly in excess of the optimal 10 μg./ml. This inhibition suggests that streptomycin retains some of its antibiotic action even when it is required for growth and it may give some support to the assumption that streptomycin functions at a site within the cell rather than its surface. It would be of interest in this respect to compare the effects of excess streptomycin on S-dependent bacteria, with those resulting from streptomycin treatment of sensitive bacteria, as a possible means of distinguishing primary from secondary streptomycin effects.

Engelberg & Artman (1961) calculated that as little as 0·60 μg. streptomycin/ml. medium should satisfy the requirement of *Escherichia coli* strain Sd-4, provided all the streptomycin was absorbed. Sodium chloride decreased streptomycin uptake however, and in a defined medium the amount of the antibiotic bound by the S-dependent bacteria was only a small fraction of that required for growth. It seems likely, therefore, that salt interference with streptomycin uptake may be in some part responsible for the highly elevated streptomycin requirements we have found in salt-containing media. However, the effect is of a greater magnitude than might be expected solely on the basis of competition for cell binding sites. The

rather narrow range of effectiveness of streptomycin in nutrient broth alone and the inhibition of growth of S-dependent bacteria that occurred at a streptomycin concentration only 2·5 to 5 times the optimal value is in sharp contrast to the continued effectiveness of streptomycin as a growth factor over a wide range of concentrations, and up to values over 50 times the optimum in the presence of salts. Although the salt effect was a fairly general one, there was considerable variation in the effect of different salts even at equivalent ionic strengths. Specific ions are known to modify the activity of streptomycin against S-sensitive bacteria (Ørskov & Ørskov, 1960; Hurwitz & Rosano, 1958, 1962; Mager, Benedict & Artman, 1962; Bragg & Polglase, 1963; Willick & Polglase, 1963) and similar effects might account in part for this variation. Magnesium, for example, is notably effective as a streptomycin antagonist. Since a function at the bacterial ribosome is postulated both for the magnesium ion (Hershko, Amoz & Mager, 1961) and for streptomycin (Davies, 1964; Cox, White & Flaks, 1964), competition for a binding site here could explain the high streptomycin requirement of S-dependent bacteria in the presence of magnesium salts. Other cations might be expected to have a similar effect.

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The Virulence of Biochemical Variants of *Streptococcus pyogenes*

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SUMMARY

Two biochemical variants were obtained from a strain of *Streptococcus pyogenes*, type 19. One variant, known as 'starch-positive', always produced amylomaltase, readily forming starch from maltose. The other variant, known as 'starch-negative', did not usually form starch from maltose in the same cultural conditions. The starch-positive variant had low mouse virulence and the starch-negative variant had high mouse virulence. The starch positive variant was avirulent for the rat, but the starch-negative variant had some rat virulence. Both variants had plenty of M antigen in precipitin tests and showed good anti-phagocytic power both in bactericidal tests with human blood and in surface phagocytosis tests with isolated human leukocytes. The mouse virulence of the starch-positive variant was enhanced by casein polypeptide and by human plasma. Starch-positive and starch-negative variants obtained from a type 12 strain also had the same properties.

INTRODUCTION

Strains of group A streptococci grown in the presence of maltose or its higher homologues produce an amylose (Crowley & Jevons, 1955; Crowley, 1959; Baum & Crowley, 1960). In thus utilizing maltose, these variant streptococci resemble the mutant strain of *Escherichia coli* discovered by Monod & Torriani (1950) which formed an amylose from maltose through the enzyme amylomaltase. Crowley (1959) found that starch-forming streptococci were present in cultures of certain strains of many different group A serotypes though varying greatly in numbers from strain to strain. Large numbers were found in cultures of strains associated with acute glomerulonephritis and acute rheumatic fever. Because of this possible relationship, of which there is no direct proof, the properties of these amylomaltase-producing streptococci were of some interest. The present work was undertaken to determine whether group A streptococci with the biochemical marker of amylomaltase production were either more or less pathogenic than other streptococcal cells, or possessed of any other unique property which might be related to particular aspects of streptococcal infection in man. This paper describes some properties of starch-positive and starch-negative variants derived from a mixed parent strain, with special reference to the production of the type-specific M antigen, mouse virulence and anti-phagocytic properties.

METHODS

Streptococcal strains and cultures

The group A strain used for the most part was IRP 118, type 19, received in 1957 from Dr R. C. Lancefield, The Rockefeller Institute. Two other strains used were OP,PS, type 12 and type 6 'glossy', both received in 1957 from the Streptococcus Reference Laboratory, Public Health Laboratory Service. All the strains were preserved by freeze-drying but for day-to-day work cultures were deep-frozen at $-10°$ in brain heart infusion broth (Difco), which was the nutrient broth used throughout. The nutrient agar base used in culture plates was prepared with Hartley digest broth supplied by the Park Hospital, Lewisham, unless otherwise stated in the text.

Amylomaltase production, expressed as starch in colonies on maltose-containing medium, was detected by exposing culture plates to iodine vapour, which generally killed the streptococci. The culture plates were, however, always replicated by velveteen imprint (Lederberg & Lederberg, 1952), and colonies chosen for propagation were picked off from another culture plate which had not been exposed to iodine.

Quantitative assay of starch production. Two methods were used, the first being the modification of that of Pucher, Leavenworth & Vickery (1948) described by Crowley (1959) in which streptococcal suspensions were incubated for 5 hr at 37° with 1·6% maltose. The culture deposits were then resuspended in 10 ml. phosphate buffer (pH 7·0) and extinctions were measured at 650 mμ in a Unicam spectrophotometer. The starch was then extracted from the streptococcal cells by perchloric acid and precipitated from this extract by iodine. The starch was then hydrolysed, the glucose estimated and the final result expressed as the % starch/amount of growth, making use of the theoretical factor of 0·90 to convert glucose into starch. The second method also depended on an extraction with perchloric acid, and utilized 18 hr cultures in 20 ml. broth containing 1% (v/v) maltose. The centrifuged culture deposit was first washed with phosphate buffer (pH 7·0) and then suspended in 4 ml. perchloric acid (Analar 72% 1 vol. + distilled water 1 vol.) and stood at bench temperature for 1 hr. The suspension was then centrifuged, and the supernatant containing the starch neutralized with 20% NaOH until yellow with phenol red; N-NaOH was then used to finish neutralization. The glucose in 1 ml. of this extract was then estimated by the anthrone method of Trevelyan & Harrison (1952). In both methods a control culture containing no maltose was subjected to the same procedure.

M precipitin tests were carried out by the method of Swift, Wilson & Lancefield (1943), on crude extracts prepared from 50 ml. broth cultures, and purified M extracts prepared from the crude extracts. Absorbed type-specific anti-M serum for type 19 was supplied by the Streptococcus Reference Laboratory. Anti-M sera against the variants of IRP 118 were prepared by injection of killed suspensions into rabbits and absorbed with heavy suspensions in the usual way.

Regeneration of M antigen in vitro. This was studied by the method of Fox & Krampitz (1956), and casein polypeptide was prepared by their method.

Virulence tests. Six mice/dilution were injected intraperitoneally with 1 ml. broth dilutions (10^{-2} to 10^{-7}) of 2·5 hr cultures. The LD50 was calculated after

7 days by the method of Reed & Muench (1938). Rat virulence was measured in the same way, using an intraperitoneal dose of 2 ml.

Resistance to phagocytosis was studied by three methods. (1) *Direct and indirect bactericidal tests* were carried out by the method of Lancefield (1957, 1958) with fresh heparinized human blood from donors whose sera did not contain the type-specific anti-M antibodies for types 19 or 12. Overnight broth cultures were diluted in broth to provide a series of inocula in the range of 10–100 streptococcal chains, determined by viable counts in blood agar. Each bottle contained 0·1 ml. culture dilution and 0·3 ml. blood. Duplicate cultures were prepared for each dilution, one being stationary and the other continuously rotated during incubation. After 4 hr and again after 24 hr 0·1 ml. of each mixture was seeded into blood agar pour plates for viable counts. In the indirect tests, 0·05 ml. of dilution of rabbit homologous anti-M serum was added to the mixtures. (2) *Surface phagocytosis tests* were carried out using mixtures of streptococci and human leucocytes spread on autoclavable Kleenex squares (1 × 2 cm.). The leucocytes were collected from 30 ml. fresh heparinized human blood by the method of Baron & Roberts (1963) modified by Dr Omar Ahmed (personal communication) as follows. Four vol. blood was diluted with 1 vol. 6 % dextran in Hartman's solution and the tube stood at an angle of 45° for 45 min. The whole plasma layer was then centrifuged at 2000 rev./min. for 5 min. and the supernatant discarded. The pellet of cells was shaken hard for 15 sec. in 1·8 ml. distilled water and 0·6 ml. quadruple-strength Hartman's solution was quickly added and the suspension reshaken and finally centrifuged at 600–800 rev./min. for about 4 min. A leucocyte layer was obtained which when finally suspended in about 0·07 ml. Hartman's solution gave a cell count of about 3×10^6/ml. An overnight culture of the test organism was diluted 1/10 in fresh broth, and gave a count of about 5×10^6 streptococcal chains/ml. About 0·07 ml. was mixed with the leucocytes and spread over three Kleenex squares previously moistened with gelatin + Locke solution, and mounted on a glass slide in a Petri dish lined with moist filter paper. This was then sealed and incubated at 37°. After incubation the squares were transferred to a bottle containing 2 ml. gelatin + Locke solution and shaken vigorously for about 30–45 sec. The suspension obtained was centrifuged for 1 min. at 800 rev./min. The deposit containing leucocytes, phagocytosed streptococci and some free streptococci was suspended in 0·5 ml. physiological saline + 3 % gelatin, and smears of the suspension were prepared and stained with methylene blue. A thousand leucocytes were counted to determine how many contained streptococci. (3) *Phagocytosis in mouse peritoneum* was studied by the method of Foley, Smith & Wood (1959).

EXPERIMENTAL

Isolation of starch-positive and starch-negative variants

Variant streptococci which form starch from low concentrations of maltose (0·1–0·4 %) are present in primary cultures of many group A strains isolated from acute infections, and sometimes all the colonies on maltose screening medium may show some degree of blue staining with iodine. Both resting populations (Crowley, 1955) and lag phase organisms of such strains show amylomaltase activity. Certain strains which appeared to be starch-negative on many occasions even in the presence of very high maltose concentration (> 1·0 %) occasionally showed amylomaltase

activity in the neighbourhood of plate contaminants (author's unpublished observations). An attempt was made to isolate pure cultures of starch-positive and starch-negative variants in order to study their properties separately. The strain IRP 118, type 19, was used because it was a very good starch producer from low maltose concentrations in many different kinds of culture medium. When the freeze-dried culture was first tested on 0·4 % maltose agar, all the colonies of 10^{-3} to 10^{-5} dilutions contained some starch (see Pl. 1, fig. 1).

Using a series of replica platings, strongly starch-positive and weakly starch-positive variants were identified on maltose agar and propagated by indirect selection from either blood agar or glucose agar. Pure cultures of the variants were not obtained, though the differences in starch production between weakly and strongly starch-positive strains began to be more marked especially in overnight cultures. Colonies of streptococci arise from chains of various length, usually four to thirty cocci and occasionally from pairs, so broth cultures were subjected to ultrasonic treatment for 10 min. or longer to break the chains. It was possible that some single cocci might survive to give rise to single-cell cultures. The procedure was repeated six times during another series of replica platings, and eventually produced a heterogeneous population from which the two variants used in this study were finally selected (Pl. 1, fig. 2, 3).

Whether the variant 7s3 designated 'starch-positive' was derived from a single streptococcus must always be uncertain, but the evidence that it was strongly starch-positive is shown in Tables 1 and 2. The variant 191 *b* designated 'starch-negative' was only so-called, for Tables 1 and 2 show that it produced small amounts of starch. In the first test to measure starch production (Table 1) single colonies were seeded into maltose broth, and the starchy streptococci counted after 8, 18 and 48 hr. Only all-blue cocci were counted, for in cultures of the starch-positive variant many cocci contained small spots or traces of starch, which were difficult and tiring to count.

Table 1. *Starch-containing streptococci in maltose broth cultures of variants of organism Group A strain,* IRP *118, type 19*

Hours at 37° ...	8	18	48
Strains	Streptococci starch-positive* (%)		
IRP 118 before selection	18	44	35
Variant 7s3 starch-positive	20	65	61
Variant 191 *b* starch-negative	3	6	16

* Starch-positive = chain containing two or more blue cocci in smears, stained by 1 % Lugol's iodine, of deposit of 1 % maltose broth cultures.

When after 18 hr the culture deposits were suspended in fresh maltose broth and reincubated, the starchy cocci in cultures of the starch-positive strain continued to increase until more than 90 % of the chains contained wholly starchy cells. In cultures of the starch-negative variant on the other hand, the numbers of starchy cocci decreased and disappeared, apparently diluted out. The difference between the two strains was confirmed by the results of quantitative assays of the amount of starch produced in maltose cultures.

Table 2 shows the data for starch production by the parent strain before selection, and by the variants tested by method I. There was never any gross difference between the extinctions of suspensions of the three strains, nor of the growth in overnight cultures. The starch-negative variant always showed slightly more growth. Because the growth of the variants was comparable, no attempt was made to relate starch production to growth when using method 2. Extracts of the starch-positive variant contained 140–160 μg. glucose/ml. extract, whereas comparable extracts of the starch-negative variant contained only 20–40 μg. glucose/ml. extract.

Table 2. *The quantity of starch produced in maltose cultures of variants of group A strain, type 19*

Strains	Extinction (650 μ)	Starch* (%)
IRP 118 parent strain	(*a*)† 45·7	1·5
	(*b*) 46·0	1·27
7S3 starch-positive	(*a*) 47·7	> 2·0
	(*b*) 54·4	> 2·0
191*b* starch-negative	(*a*) 54·4	0·39
	(*b*) 53·1	0·34

* % starch = mg. glucose in perchloric acid extract of deposit of 10 ml. streptococcal cell suspension × 0·90/extinction of 10 ml. streptococcal suspension.

† *a* and *b* = duplicate cultures.

On blood agar both variants were almost indistinguishable from each other and from the parent strain. In this respect different mammalian blood, either horse, rabbit, sheep or human, made no significant difference. On maltose blood agar the colonies of the starch-negative variant appeared translucent in comparison to the opaque starchy colonies. Starch was not formed on maltose + horse blood agar. In broth cultures without added carbohydrate there was no significant difference in rate of growth.

The starch-positive variant formed starch from very low maltose concentrations, 0·1–0·2 %, and starch production was very little affected by changes in culture media, addition of blood or serum (excepting horse) or accessory growth factors such as purines to the least complex media. An occasional batch of pooled human serum caused a temporary suppression of starch production for as long as 48 hr. Crowley (1959) described an inhibitory effect by high serum concentration on starch production by certain group A strains, but increased production by other group A strains, usually amylase producers. The presence of quite small amounts of glucose in the medium, 0·3–0·4 %, caused suppression of amylomaltase activity, and on glucose + maltose agar the colonies were smaller than when either carbohydrate was used alone. The mechanism of the inhibition of streptococcus amylomaltase by glucose is not clear. Numerous compounds are not utilized in the presence of glucose, which prevents the formation of a great many enzymes (Pardee, 1961) though, to be inhibitory, glucose must be metabolized. Glucose may be used in preference to another carbohydrate, the 'diauxie' of Monod (1947). Cohen & Monod (1957), however, suggested that starch-forming bacteria were unable to

utilize glucose through lack of a glucose permease and, though impermeable to glucose, were permeable to maltose through a maltose permease.

The so-called starch-negative variant in contrast showed an increased growth on glucose + maltose agar. It did not usually produce any starch even with very high maltose concentration (> 1·0%) in a great variety of cultural conditions, using differently constituted broths and nutrient agar bases. Starch was sometimes formed, however, in certain circumstances, in the neighbourhood of plate contaminants, for example Pl. 1, fig. 4. These belonged to a number of different bacterial species only occasionally identified because they were often killed by iodine. The effect was not related to maltose concentration, and the finding suggested that amylomaltase was formed in conditions of starvation of some compound utilized by competing bacteria. This suggestion was supported by the finding that the variants ceased to form amylomaltase when growing in fresh culture medium with maltose but without the contaminants. Intermittently the starch-negative variant formed amylomaltase in culture plates prepared with certain batches of nutrient agar, also suggesting that the inducing factor might be a lack of a particular nutrient.

M antigen production

The initiation of infection by group A streptococci in the human or mouse host depends on the type-specific M antigen (Lancefield, 1940; Rothbard, 1948; Morris & Seastone, 1955; Lancefield, 1954, 1957, 1958, 1959; Wiley & Wilson, 1956; Foley *et al.* 1959). The M antigen is anti-phagocytic and differences in mouse virulence are primarily related to its quantity and stability. It enables strains to resist the bactericidal power of fresh human blood, and to grow in blood from very small inocula. Both the parent strain and the two kinds of variant seemed to have plenty of M antigen in precipitin tests, the results of which are shown in Table 3.

Table 3. *M precipitin tests on starch-positive and starch-negative variants of group A streptococcus, strain* IRP *118*

Strains	Cell extracts + absorbed type 19 anti-M serum. Antigen dilutions						
	0	1/2	1/4	1/8	1/16	1/32	1/64
	Starch-positive						
7s3, before mouse-passage	+++	++	+	±	0	0	0
9th mouse-passage	+++	++	+	±	0	0	0
16th mouse-passage	+++	++	+	±	0	0	0
20th mouse-passage*	+++	++	+	+	+	0	0
	Starch-negative						
191*b*, before mouse-passage	−++	++	++	+	±	±	0
8th mouse-passage	−++	++	++	+	±	0	0
23rd mouse-passage†	−++	++	+	+	±	±	0

* LD50 for mice of 7s3, 20th mouse passage, was $3{\cdot}4 \times 10^3$.
† LD50 for mice of 191*b*, 23rd mouse passage, was 2·7.

The M antigen is destroyed by trypsin. Studying the synthesis of M antigen by a type 14 strain, Fox & Krampitz (1956) found that, after its destruction by trypsin, it reappeared on the streptococci after as little as 1·5 hr in favourable cultural

conditions. They also found that, for the maximum synthesis of M antigen, glucose, together with certain amino acids and polypeptides, was an essential growth requirement. It seemed possible that, because of their differences in carbohydrate metabolism, the two variants might produce M antigen at different rates. If, for example, the starch-positive variant failed to metabolize glucose it might be sluggish in manufacture of M antigen at an early phase of growth, and this might affect its survival in the presence of phagocytes. In blood broth without additional carbohydrate both variants, after stripping of M antigen by trypsin, produced it again after 1·5–2 hr. The addition either of glucose or of maltose to the medium had no effect on the M regeneration time of the starch-positive variant, but shortened that of the starch-negative variant to 1–1·5 hr. Both variants also produced M antigen in the 2·5 hr broth cultures used for virulence tests. There was obviously no gross difference between the variants regarding M antigen synthesis *in vitro*, but these findings, together with those in Table 3, suggested that the starch-negative variant produced rather more M antigen than the starch-positive strain.

Capsule production

The second, though lesser, factor in the antiphagocytic complex of group A streptococci is the hyaluronic acid capsule (Rothbard, 1948; Foley *et al.* 1959; Hirsch & Church, 1960). The variants both formed mucoid colonies; both produced well defined but not outstandingly large capsules in 2·5 hr broth cultures. These were also present after 4 hr when there was no difference between the variants. After 6 hr 94 % of the starch-negative variants were still capsulated, compared with 35 % of the starch-positive variants. After 16 hr capsular material had gone from both. Whilst here again there was no gross difference between the strains regarding capsular synthesis, the capsules of the starch-positive variant were less stable. Foley & Wood (1959) showed that in phagocytic tests done in roller tubes streptococci with large capsules, even though they had no M protein, were resistant to phagocytosis. It remained to be seen whether the slight differences between the variants affected mouse virulence.

Mouse virulence

The variants had been cultured for months without mouse passage, and at first both were less virulent than the parent strain, the LD 50 of which was in the range 1×10^2 to 1×10^4. Serial mouse passage quickly restored the virulence of the starch-negative variant, which had an LD 50 of 10 to 100 chains after a few passages. The mouse-history of the starch-positive variant is illustrated in Fig. 1 and shows that after 9 passages it still had a low virulence but, after the 10th passage, the LD 50 was 1 to 5. The streptococci recovered from the 10th mouse (10 B) were, however, starch-negative. The 9th mouse strain still starch-positive was then reinjected into another mouse (10 A), and then the organisms recovered were still starch-positive but again of low virulence. Figure 1 shows that the virulence of all the starch-positive mouse lines was of the same low order, but when starch-negative streptococci were again recovered from certain mice (29 and 37) there was a sudden enhancement of virulence.

Mouse-selected starch-negative strains. Like those selected from culture plates,

the mouse-selected starch-negative variants formed small amounts of starch in certain circumstances. The inducing factors were the same, namely plate contaminants and particular batches of nutrient agar base. An apparently total change from starch-positive to negative happened in three of seventy mice. An obviously mixed culture was recovered from one mouse (12AG not shown) which had an intermediate virulence but was not further studied. If the original starch-positive variant 7s3 was a heterogeneous culture, it was surprising that the change-over did not occur more often, and it seems more likely that the virulent variants arose by mutation in the mouse.

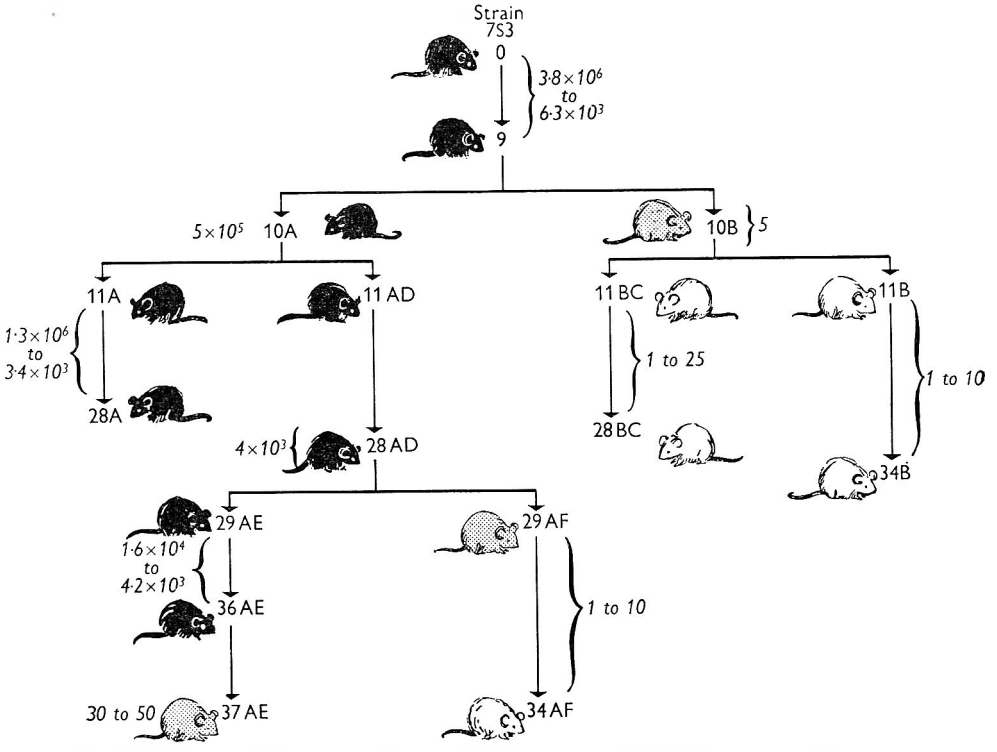

Fig. 1. The recovery of starch-negative variants of high mouse virulence from mice injected with starch-positive variants of low mouse virulence.

Mice

starch-positive organisms injected and recovered.

starch-positive organisms injected but starch-negative organisms recovered.

starch-negative organisms injected and recovered.

Figures in italics—lowest and highest LD50 recorded on strain during course of mouse passage. Figures in ordinary type, number of mouse passages.

Phagocytosis in mouse peritoneum. In mouse peritoneal exudates which were examined at hourly intervals after injection the starch-negative variant was clearly less susceptible to phagocytosis by mouse leucocytes than the starch-positive variant. These results, which are shown in Table 4, were consistent with the differ-

ences in mouse virulence. By M precipitin tests, however, both strains appeared to have plenty of M antigen.

Rat virulence. Foley *et al.* (1959) found that the virulence of group A strains in rats, as in mice, was closely related to their ability to resist surface phagocytosis, and this in turn was related to the amount of M antigen produced by the strains. They also found that two strains with a mouse LD 50 of 1 were considerably less virulent for rats (1×10^3, and $2{\cdot}1 \times 10^7$). The rat LD 50 of the starch-negative variant was $7{\cdot}6 \times 10^5$, though the mouse LD 50 was 1 to 10 chains, which demonstrated that an amount of M antigen which was abundant for resisting phagocytosis in mice was inadequate for the same purpose in rats. The LD 50 for rats of the starch-positive variant was $> 1 \times 10^7$. The rat is of course highly resistant to bacterial infections to which other laboratory animals are susceptible and some other factor may be involved in rat virulence of group A strains.

Table 4. *Phagocytosis of starch-positive and starch-negative variants of group A streptococcus strain in mouse peritoneum*

Strains	Mouse peritoneal exudates	Time after injection (hr) 1	2	3	4
	Starch-positive				
7s3A/20	% phagocytes containing streptococci	> 1	3	8	14
LD 50, $3{\cdot}4 \times 10^3$	Extracellular streptococci	Few	Few	Few	Few
	Starch-negative				
7s3c/20	% phagocytes containing streptococci	< 1	< 1	< 1	8
LD 50, 1	Extracellular streptococci	+	+	±	+ + + +

The starch-negative variant was the mouse-selected strain recovered from a mouse injected with a starch-positive strain. The figure after the stroke = no. of serial mouse passages of variant.

Resistance to phagocytosis by human leucocytes

Man is the natural host of the group A streptococcus, and he may be more susceptible to infection than the mouse, for freshly isolated strains which seem to have plenty of M antigen frequently have a very low mouse virulence. Strains with large amounts of M antigen are resistant to phagocytosis in fresh human blood when the cultures are rotated so that the streptococci are in constant touch with leucocytes (Lancefield, 1957, 1958, 1959). When anti-M serum is added the streptococci become susceptible to phagocytosis (indirect bactericidal test). Lancefield (1959) devised a rough quantitative measure of M antigen of strains by comparing the amount of growth in rotated and stationary cultures, for heavy growth from rotated cultures showed good M antigen production. She also found that when strains had large amounts of M antigen, only neat anti-M serum neutralized the anti-phagocytic effect, whereas diluted anti-M serum was adequate for strains with lesser amounts.

Growth in fresh human blood

Direct bactericidal tests were carried out frequently on the variants before and during mouse passage, and also on the mouse-selected starch-negative variants of high virulence. In rotated blood cultures, growth equal to more than twice the original inoculum, obtained after 4 hr, was considered to be good evidence of anti-

phagocytic power. Heavy growth after 24 hr was considered very good. Neither variant grew out well on every occasion, as shown in Table 5, but both showed good growth in the majority of the tests, and very good growth in about a third. Inconsistencies attributable to technical error or change of blood donor were evenly distributed between the variants. These results, which suggested that there was no significant difference between them with respect to anti-phagocytic power against human leucocytes, were poorly correlated with mouse virulence.

Table 5. *Growth of starch-positive and starch-negative variants of group A streptococcus, strain* IRP *118, in fresh human blood (direct bactericidal tests)*

No. streptococci recovered from rotated cultures	Variants: Starch-positive (28 tests)	Variants: Starch-negative (23 tests)
More than twice the original inoculum	19 (9)*	17 (8)
Equal to or less than original inoculum	9	6

* No. of tests showing growth after 4 hr. In parentheses no. of tests showing heavy growth after 24 hr.

Heavy growth was always recovered from stationary cultures of both variants.

Indirect bactericidal tests were carried out using a homologous anti-M rabbit serum, undiluted and diluted 1/5 in the blood cultures. Very good growth was obtained from both variants using normal rabbit serum, and 1/5 anti-M serum in the cultures. Though the variants grew in the presence of undiluted anti-M serum the bacterial counts of both were reduced in rotated cultures. It was still not possible to demonstrate any significant difference between the M content of the two strains with this anti-M serum; this is being further studied using other anti-M sera.

Surface phagocytosis tests

In the conditions of the human blood bactericidal test as devised by Laneefield, the leucocytes outnumber the streptococci by about a million to one, which would seem to be a stringent trial of the anti-phagocytic power of the organisms. Foley *et al.* (1959), however, found that differences in susceptibility of group A strains to phagocytosis *in vitro* by mouse and rat leucocytes were demonstrable on filter paper when not apparent in tests performed on glass slides or in roller tubes. In surface tests the leucocytes and organisms encounter each other on pieces of freshly excised tissue or on moistened filter paper, which provide a rough surface on which the leucocytes can trap the organisms more easily than in a liquid medium Wood, Smith & Watson (1946). Table 6 shows the results of surface type tests done on the variants using human leucocytes from peripheral blood. The conditions here were weighted a little in favour of the organisms because there were rather more streptococci than leucocytes. The amount of culture medium was negligible so there was no question of growth, as in the bactericidal or virulence tests. A non-virulent strain, type 6 glossy, which has no M antigen and does not survive in direct bactericidal tests with human blood was used for comparison, and proved to be relatively the most susceptible to surface phagocytosis. There was no significant difference between

the anti-phagocytic power of the starch-positive and starch-negative variants in these conditions in spite of their differing mouse virulence. Capsular material had disappeared from the overnight cultures used in these tests, so that the M antigen was the only known anti-phagocytic factor concerned.

Table 6. *Anti-phagocytic power of starch-positive and starch-negative variants against human leucocytes on paper (surface phagocytosis) compared with a virulent strain type 6, glossy*

Variants	Time at 37° (hr)	Leucocytes containing streptococci (% total)	LD 50 (mice)
	Starch-positive		
7s3A/25	2	< 1·0	$1{\cdot}3 \times 10^6$
	4	< 1·0	
7s3A/26	4	< 1·0	
	Starch-negative		
7s3c/25	2	< 1·0	5
	4	3·0	
7s3c/26	4	2·2	
Type 6 glossy	2	4·8	$> 1 \times 10^8$
	4	8·2	

Table 7. *Summary of anti-phagocytic properties of starch-positive and starch-negative variants in different conditions*

Phagocytic test	Survival of variants: Starch-positive	Starch-negative
Human whole blood rotated cultures	+	+
Human leucocytes on paper	+	+
Mouse peritoneum	−	+
Rat peritoneum	−	−

The conflicting results of the *in vitro* and *in vivo* phagocytic tests as a rough quantitative measurement of differences in M antigen content of the variants are summarized in Table 7. It seemed possible that these findings might be explicable if viewed in relation to the growth requirements of the starch-positive variant in addition to its anti-phagocytic powers.

Bacon, Burrows & Yates (1951) found that biochemical mutants of *Salmonella typhi* were avirulent for mice. In particular the virulence of mutants which could not synthesize purines was of a very low order, and mouse peritoneal fluid though rich in other growth factors was low in purines. There is no evidence that the growth *in vitro* of the starch-positive variants is particularly improved by purines, but there is no doubt that compared with starch-negative variants they are dependent on accessory growth factors present in highly complex culture media containing blood or serum. Woolley (1941) found that a mixture of peptides which he called strepogenin was necessary for the growth of certain group A strains and related to protein synthesis. Fox & Krampitz (1956) showed that casein polypeptides greatly increased the rate of M antigen synthesis. The possibility that mouse or rat peritoneal

fluid was a poor culture medium for the starch-positive variants was then investigated, with particular reference to peptide deficiency. The mouse virulence of the starch-positive variant was retested by adding casein polypeptide (1 %, w/v) to each of the test dilutions. The results shown in Table 8 strongly supported the idea that the low mouse virulence of the variant was due to its need for an accessory growth factor in the environment of mouse peritoneum, rather than lack of anti-phagocytic power.

The effect of other peptide preparations on mouse virulence is being further studied. Table 8 shows that an addition of a 5 % human red cell suspension to the test dilutions had no comparable effect on the LD 50, whereas an addition of 20 % human plasma had an effect of the same order as the casein polypeptide.

Table 8. *The effect of casein polypeptide on the mouse-virulence of the starch-positive variant strain 7s3/A29*

Strain diluted in	LD 50 for mice
1. Broth	$3{\cdot}34 \times 10^5$
2. Broth + human red cell suspension 5 %	$3{\cdot}35 \times 10^5$
3. Phosphate buffer (pH 7·0)	1×10^6
4. Phosphate buffer + casein polypeptide 1 %, w/v*	$8{\cdot}3 \times 10^2$
5. Broth + human plasma (20 %)	$8{\cdot}8 \times 10^2$

* The casein polypeptide solution was non-toxic for mice when injected intraperitoneally.

Production of anti-M sera

Really good anti-M sera against either of the variants have not so far been produced, though the most acceptable was prepared with the starch-negative variant. The possible significance of this in relation to the starch-positive variant is being further studied. Cross-absorption experiments did not suggest that there was any antigenic difference between the strains. M antigen extracts and concentrated M antigen extracts were analysed by immuno-electrophoresis by Dr Armine T. Wilson (Wilmington, Delaware personal communication), who could not demonstrate any immunological difference between the M antigens of the two variants.

Variants from another group A strain

A starch-positive variant was isolated by the same methods from another group A strain, OP, PS, type 12, and compared with a starch-negative variant from the same parent. This strain was chosen at random for the second study because it was a good starch producer, very like IRP 118, but a member of another serotype. Both variants grew vigorously in direct bactericidal tests with human blood and resisted phagocytosis in surface tests with human leucocytes. The starch-positive variant (30 B) was non-virulent for mice (LD 50 $> 1 \times 10^6$) whereas the starch-negative variant (29) was relatively virulent (LD 50, 100–300). This finding supported the idea that the properties described apply to starch-positive variants in general and not only to one from a particular parent strain.

DISCUSSION

When working on the pathogenicity of group A streptococci it is customary to mouse-passage strains frequently to keep up their virulence. The present work has demonstrated the futility of this procedure in relation to the particular property being studied for regular amylomaltase production seems to be incompatible with high mouse virulence, when the test dilutions are made in the usual way. Many freshly isolated group A strains which seem to have plenty of M antigen in precipitin tests and are causing infections, may not be mouse-virulent. This puzzling discrepancy may be accounted for by the presence of starch-positive variants in the primary cultures, starch-production from maltose being a marker for streptococci which *in vitro* very quickly become starved of an accessory growth factor. It remains to be seen whether this finding is relevant to human disease.

I wish to thank the Medical Research Council for the grant which supported this work and Josephine Prescott and Clare Notley for their excellent technical help. I also wish to thank Dr M. T. Parker, (Streptococcus Reference Laboratory, Colindale), for supplies of anti-M sera, Dr Armine T. Wilson, (Alfred I. du Pont Institute of the Nemours Foundation, Wilmington), for the immuno-electrophoretic patterns of the M extracts, Dr Omar Ahmed, (Chemical Pathology Department, Royal Free Hospital), for valuable help and advice on collecting human leucocytes and Miss Marjorie Smith, medical artist, for drawing Fig. 1.

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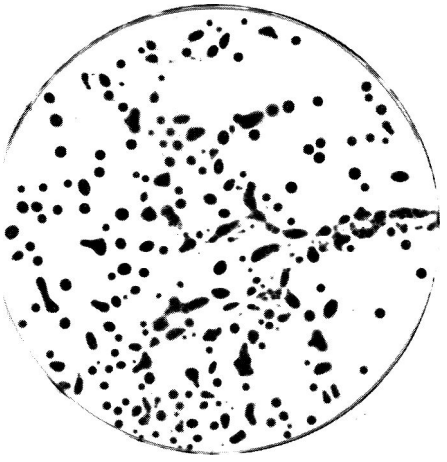
EXPLANATION OF PLATE

Fig. 1. Streptococcal culture before selection of variants; maltose agar, 24 hr after exposure to iodine vapour.

Fig. 2. Heterogeneous culture of starch-positive and starch-negative colonies produced by subjecting broth culture of the strain to ultrasonic vibration.

Fig. 3. Starch-positive and starch-negative variants 7s3 and 191*b* selected from IRP118.

Fig. 4. Starch-negative strain on maltose agar showing starch production in the neighbourhood of plate contaminants.



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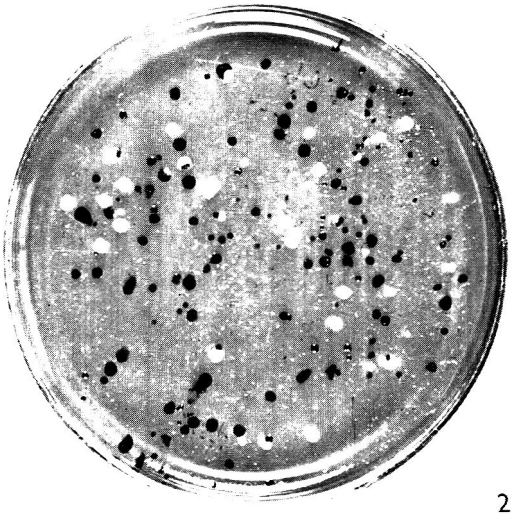

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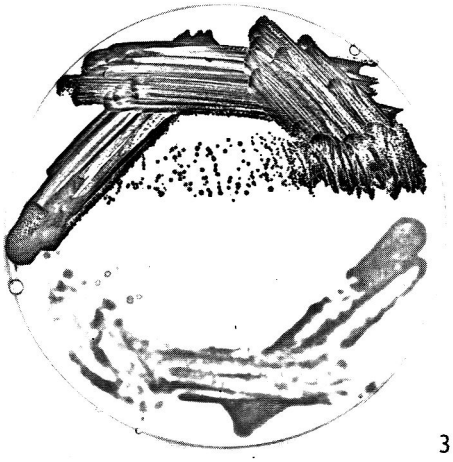

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Kinetics of the Potassium- and Sodium-Activated Infection of a Transforming Deoxyribonucleic Acid in Pneumococcus

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SUMMARY

Study of the kinetics of the effect of KCl and NaCl on the genetic transformation process has shown that an activation of a 3 min.-old DNA-bacterial complex by 0·2 M-salt is responsible for a several-fold increase in the frequency of transformation. It is assumed that this is due to activation of a temperature-dependent metabolic process which is connected with the penetration of DNA into the competent cells. Studies of the kinetics of DNA penetration, by the addition of deoxyribonuclease as a function of time, in the presence and absence of the activating salts, indicate that competence is also a state of increased permeability to macromolecules. The activating effect of both sodium and potassium chlorides bears some relation to the stage of competence achieved and to the dilution or non-dilution of the receptor culture.

INTRODUCTION

Infection of the receptor cell by biologically active transforming deoxyribonucleic acid (DNA) *in vitro* may be prevented or decreased by a number of factors; a much more difficult task for the investigator is, however, to obtain a consistently high degree of infection which eventually would yield transformed progeny. This is because we still do not know the basic mechanism responsible for competence, that is, the ability of the receptor cell to fix DNA, and subsequently to become transformed. Provided there is a saturation concentration of DNA in the transformation system, the limiting factor which determines the number of transformants produced is the number of competent cells (Hotchkiss, 1954). If then, at a saturation concentration of DNA, the addition of salts with monovalent cations (Na^+, K^+; Kohoutová, Kopecká & Koníček, 1965) yields a reproducible many-fold frequency of transformants, one may assume either that the salts participate in the reactions responsible for competence, or that they directly partake in the competent reaction.

On the basis of our earlier findings on the effect of Na^+ and K^+ and, to a lesser extent, of $[NH_4]^+$ ions on transformation, it was suggested that the presence of positive cations might influence the electric potential of the wall of the receptor cell (Kohoutová, 1958*a*, *b*) thus causing a more effective interaction between the negatively charged molecule of DNA and the receptor cell which should also be negatively charged (Zamenhof, 1956). It is also possible that monovalent cations may act as activators of an enzyme in the cell wall. Results are presented in the recent study by Barnhart & Herriott (1963) which suggest that the initial attraction

or interaction of DNA with competent cells is probably ionic in nature, and that the irreversible uptake of DNA has a very specific charge-distribution requirement. For irreversible uptake of DNA in *Haemophilus influenzae*, Barnhart & Herriott found ionic optima which corresponded rather well with our earlier findings as well as with our latest findings, as will be shown in the results presented here. In the present work, a more thorough analysis of the kinetics of the effect of K^+ and Na^+ on the competence of the receptor culture is attempted, with the object of revealing more fully the basic nature of competence, and thus of controlling those mechanisms which lead to infection of the receptor cell with transforming DNA.

METHODS

Most of the procedures and media used in the experiments were worked out originally in the laboratories of Dr H. Ephrussi-Taylor in Gif (France) and only some of them have been modified.

DNA acceptor strain. Unencapsulated streptomycin-sensitive strain (PMS II), obtained from the virulent encapsulated form of *Streptococcus pneumoniae* type II (Kohoutová, 1958*a*) was used as the acceptor of transforming DNA.

Transforming DNA. DNA was isolated from a mutant of the PMS II strain resistant to streptomycin sulphate 2 mg./ml. The freshly subcultured plate culture of the mutant strain was first grown to the logarithmic phase of growth in P-medium (described below) with streptomycin sulphate 2 mg./ml. so as to obtain a pure streptomycin-resistant donor culture. This was then subcultured first into a small and afterwards into a 10 times larger volume of the medium for isolation of DNA, as described by Ephrussi-Taylor (1951). The method of isolation of transforming DNA followed the procedure used at Gif. The DNA preparation was stored at a high concentration in physiological saline at 4°. Good biological activity was preserved even with preparations of DNA more than one year old. Saturating concentrations of DNA were used in all the experiments described here.

Transformation procedure. Single-colony cultures of the PMS II transformable strain were grown in P medium containing 1% Neopeptone (Difco), 0·85% NaCl, 0·8% Difco yeast extract, 0·025% glucose and a very small amount of defibrinated rabbit blood (Ephrussi-Taylor, 1951). A sample (0·5 ml.) of the young culture, with the cocci still in chains, was transferred to 10 ml. of the transforming medium (the charcoal-adsorbed medium without beef extract—Ephrussi-Taylor, 1951) containing 0·2% (w/v) serum albumin (NS medium) so as to give 4 to 6×10^6 colony-forming units/ml.

To follow the appearance of competence and to find its peak, 0·1 ml. samples of the receptor culture were collected, from the 60th to 180th or 240th min. of growth in the transforming medium, into 1 ml. of pre-cooled NS medium, or into NS medium to which KCl or other assayed salt had been added (usually in a final concentration of 0·2 M).

In the transforming assay itself 1 ml. of receptor culture, grown to maximal degree of competence and chilled for 3 min. in ice, was pipetted on to the pre-cooled 0·1 ml. samples of DNA diluted in NS, or NS + KCl medium so as to give the appropriate final concentration of the salt added (0·2, 0·15, 0·1 M approximately). Cultures were then kept for 30 min. at 37°. Streptomycin-resistant transformants

were scored by the technique of delayed selection in agar (Ephrussi-Taylor, 1958). The plates were incubated for 2 hr at 37° in a complete agar medium to allow expression of the streptomycin-resistant phenotype and then overlaid with agar containing 200 μg. streptomycin sulphate/ml. Assays were performed in duplicate or triplicate at each dilution of the mixture tested, and each assay was repeated at least three times.

Deoxyribonuclease. Pancreatic deoxyribonuclease (L. Light and Co. Ltd., Colnbrook, Bucks., England), activity 45%, was used to stop the penetration of excess DNA in some of the experiments.

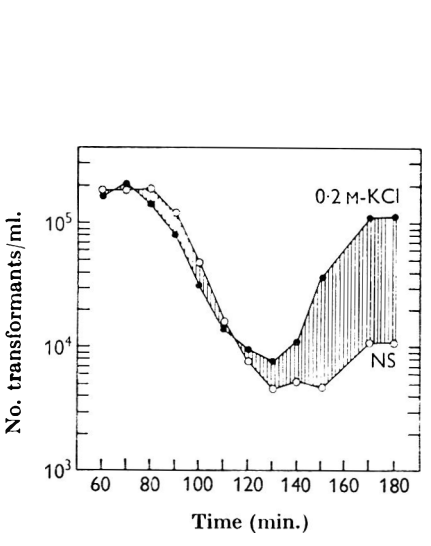

Fig. 1

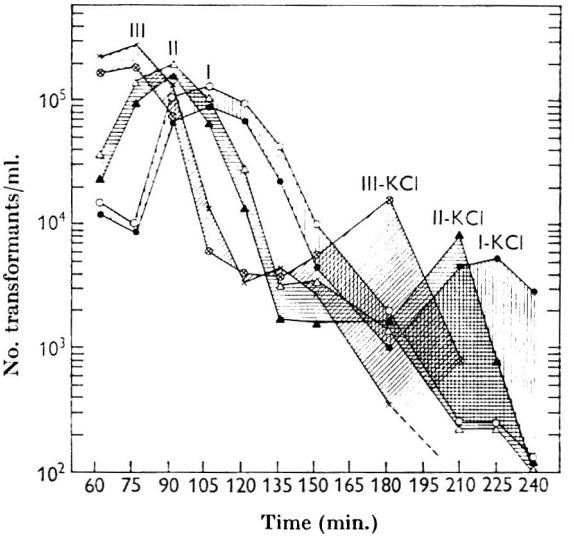

Fig. 2

Fig. 1. Effect of 0·2 M-KCl on cyclic development of competence. Every 10 min. samples (0·1 ml.) of growing receptor culture were suspended into 1 ml. pre-cooled NS medium or NS medium + 0·2 M-KCl transformation medium with DNA, and incubated for 30 min. at 37°. ○—○ = competence in NS medium; ●—● = competence in NS medium + 0·2 M-KCl.

Fig. 2. Effect of 0·2 M-KCl on three different initial concentrations of receptor culture. ○—○ = competence in NS medium with the initial population density of 3×10^6 colony-forming units/ml.; ●—● = NS medium + KCl (0·2 M); △—△ = 6×10^6 colony-forming units/ml. NS medium; ▲—▲ = NS medium + 0·2 M-KCl; × — × = $1{\cdot}2 \times 10^7$ colony-forming units/ml. NS medium; ⊗—⊗ = NS medium + 0·2 M-KCl.

RESULTS

The effect of KCl on the course of competence

Figure 1 shows the effect of KCl when added in final concentration 0·2 M to the complete NS transformation medium. In the first cycle of competence the effect of KCl was minimal and sometimes rather inhibitory, particularly in the declining phase of competence. In contrast, in the second cycle where, in the control, a rapid decrease in the number of transformants occurred in comparison with the first cycle, the effect of KCl was always a stimulating one, producing as much as a tenfold increase in the frequency of transformants.

While much the same effect was produced by concentrations of 0·15, 0·1 and

0·02 M, the 0·2 M concentration was most effective for the stimulation of transformation.

Figure 2 shows that the size of the inoculum in the range of 3×10^6, 6×10^6 and $1{\cdot}2 \times 10^7$ colony-forming units/ml. of the inoculated transformation medium, under otherwise identical physiological conditions, had no influence on the course of competence in the presence of KCl. Again KCl increased the number of transformants only in the second cycle of competence; in the first cycle it was rather inhibitory.

However, in the actual transformation experiment when 1 ml. of receptor culture, previously chilled for 3 min. in ice, was added to ice-cooled test tubes with 0·1 ml.

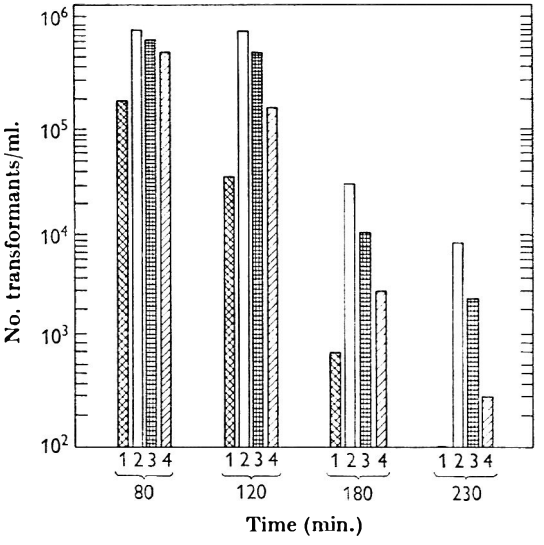

Fig. 3

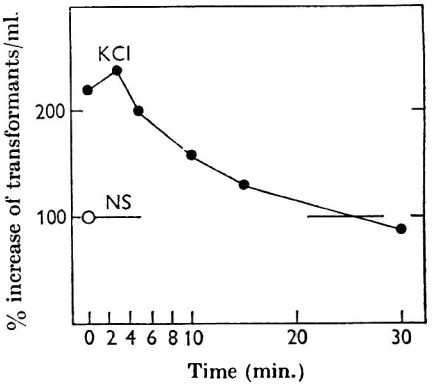

Fig. 4

Fig. 3. Influence of concentration of KCl on the transformation at various degrees of competence. 1 ml. of the receptor culture in its 80th. 120th, 180th, and 230th min. of growth was chilled for 3 min. in ice, and added to ice-cooled test tubes with 0·1 ml. samples of DNA diluted in NS medium or NS medium + KCl, to obtain NS medium + 0·2 M, 0·15 M and 0·1 M-KCl. The peak of competence was at the 90th min. of growth. 1 = NS medium alone; 2 = NS medium + 0·2 M-KCl; 3 = NS medium + plus 0·15 M-KCl; 4 = NS medium + 0·1 M-KCl.

Fig. 4. Kinetics of the effect of KCl as a function of time. 1 ml. of the receptor culture, grown to its maximal competence and then chilled for 3 min., was added to DNA in a series of identically prepared test tubes and immersed into a water bath at 37°. Then KCl to 0·2 M was added to the 0, 3, 5, 10, 15, 20, and 30 min.-old DNA-bacterial complex and after 30 min. incubation (related to the time of immersion of the test tubes into the water bath) the test tubes were put into ice and cultures diluted suitably on plates.

specimens of DNA diluted in NS medium and NS + KCl, there always occurred a substantial increase in the number of transformants in the presence of KCl. The increase was observed irrespective of whether the receptor culture was taken before or after the peak of maximal competence.

Figure 3 shows the number of transformants in 1 ml. of the receptor culture when the culture was taken at the 80th minute of growth, i.e. 10 min. before reaching the

peak of competence. Alternatively, specimens were taken in the 120th, 180th, and the 230th minute, i.e. 30, 90 and 140 min. beyond the peak of competence, and were added to the DNA diluted in the NS medium with 2·0, 1·5, and 1·0 M-KCl to give a final concentration of KCl 10 times lower. The relative increase in frequency of the transformants in 1 ml. of the receptor culture, as compared with the appropriate control culture in the NS medium without added KCl, was approximately 4-, 25-, 40- and 90-fold at the respective times in the presence of 0·2 M-KCl. It can be seen that the highest relative effect on the frequency of transformants was reached with 0·2 M-KCl and all concentrations of KCl were most effective at the lower state of competence, i.e. when the recipient culture was used far beyond the state of maximal competence and when the frequency of the transformants in the NS medium was very low.

The effect of some other salts with monovalent and divalent cations, i.e. 0·2 M-NaCl and NH_4Cl and 0·1 M-$CaCl_2$, was compared with the effect of 0·2 M-KCl on the frequency of transformants. The receptor culture at its maximum competence was used. It was found that NaCl had a similar stimulating effect on the frequency as did KCl, while NH_4Cl had a rather inhibitory effect. No transformants were found in the presence of 0·1 M-$CaCl_2$ ($CaCl_2$ at 0·1 M caused a precipitate in the NS medium).

The kinetics of the effect of KCl

It was of interest to determine at what moment of the transformation reaction the KCl exhibited its stimulating effect on the frequency of transformants. For this purpose KCl in final concentration 0·2 M was added to 0, 3, 5, 10, 15, 20 and 30 min.-old DNA-bacterial complexes. Figure 4 represents the kinetics of the effect of KCl as the function of time. The highest effect of KCl was on the DNA-bacterial complex when 3 min. old. From zero time to 3 min. the effect of KCl increased, being followed by a rapid decrease afterwards; within 30 min. it had decreased to the value of its own control without KCl.

Figures 5*a* and *b* express the kinetics of DNA penetration into competent cells, with and without 0·2 M-KCl (Fig. 5*a*), or NaCl (Fig. 5*b*), assessed by adding pancreatic deoxyribonuclease at different times. It is evident that the number of transformants in 1 ml. gradually increased in both systems during the first 10 min. The increase in NS medium alone, however, was steeper than that observed with the NS medium containing 0·2 M-KCl or NaCl. After 10 min., the competent state of the receptor cells was obviously terminated, since the DNase no longer influenced the penetrated DNA. Between 10 and 20 min. there was a steep increase in the number of transformants in the NS medium + KCl and NS medium + NaCl, to a value about 10- and 40-times higher (in two different experiments) than that observed in the NS medium without salts. This reaction seemed to be completed in 30 min.

The question arises whether KCl and NaCl inhibit the sorption of DNA to the competent cell, since this might have caused the lowering of the frequency of transformants in the first 10 min. of uptake of DNA, in comparison with the control without salts added. Figure 6 gives the results of an experiment to examine this. Instead of ending the penetration of DNA by adding DNase, a 100- to 1000-fold dilution of the DNA-bacterial complex was used to end the competence of the receptor culture in the given time span. It can be seen that in the first 3 min. of

uptake of DNA the course of both curves was similar, in the presence of KCl the frequency being somewhat higher. But after 3 min. there occurred a striking stimulation of the transformation process in the presence of KCl in comparison with the control without KCl; then both curves again followed a similar course. There was no inhibition of adsorption of DNA to the competent cells in the presence of salts. In a similar assay done at 30° instead of 37° there was no activation of the transformation process by KCl.

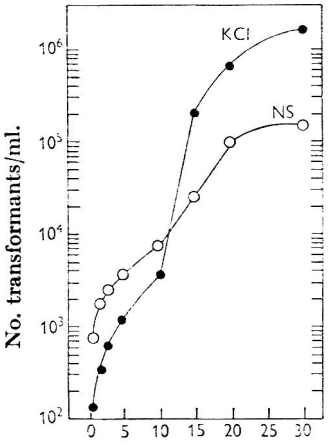

Fig. 5*a*

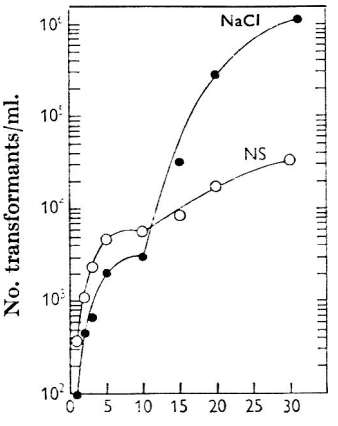

Fig. 5*b*

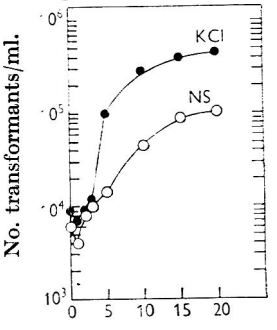

Fig. 6

Figs. 5*a*, *b*. Kinetics of the effect of KCl and NaCl on the penetration of DNA into the competent cells, assessed by means of DNase. Into a series of identically prepared test tubes with DNA, either in NS medium or NS medium with KCl or NaCl (0·2 M final), to which 1 ml. of competent receptor culture was given, DNase was added at zero time, i.e. just before the test tubes with the DNA-bacterial complex were immersed into the water bath at 37° and then after 1, 2, 3, 5, 10, 15, 20, and 30 min. of incubation. After a further incubation of 1 min. with the added DNase, the cultures were put into ice and, after proper dilution, plated as described in Methods.

Fig. 6. Kinetics of the effect of KCl on the penetration of DNA into competent cells by means of dilution of the DNA-bacterial complex. The assay was performed in the same way as indicated for Fig. 5. Instead of adding DNase the respective test tubes were put into ice at the given times, the cultures were diluted 100- to 1000-fold to end the competence of the receptor cultures and then plated as described in Methods.

DISCUSSION

The results presented here indicate that the effect of KCl and NaCl is optimal at a time when the conditions for competence are unfavourable (Fig. 3), rather than at the peak of competence. Activation of this type is also pronounced in the second cycle of competence (Fig. 1) when the receptor cells can no longer go through a second high-competence cycle. Thus by adding KCl or NaCl to 0·2 M, a process is activated which normally proceeds by itself, but with very low efficiency.

The effect of 0·2 M-KCl or NaCl added to the transformation medium at intervals during the progress of the transformation reaction is most intense when the DNA-bacterial complexes are 2–3 min. old (Fig. 4). This would suggest that

these salts do not act at the period in which the maximum adsorption of DNA to cell receptors takes place, but rather at the period of maximum penetration and fixation of DNA by the competent cells. The effect of KCl decreases rapidly when added to DNA-bacterial complexes older than 3 min., though the competent state under our experimental conditions lasted for 10 min. The need to add KCl or NaCl in the first 3 min. and for a temperature of 37° to obtain activation of the transformation process, in addition to the effects mentioned above, suggest that K^+ and Na^+ ions participate as activators of a metabolic process which goes on in, or on, the receptor cell and lasts for only 3 min., but is responsible for 10 to 100 times higher frequency of transformants than can be obtained in the control without KCl or NaCl. Lerman & Tolmach (1957) found that the frequency of transformants was directly proportional to the amount of DNA incorporated, similar results were obtained by Fox (1957). One may therefore deduce that the several-fold increase in the frequency of transformants found in the present work was due to an increase in the irreversible fixation of DNA by some not yet well understood mechanism.

The results presented in Fig. 5*a* and *b* show that, in the presence of 0·2 M-KCl or NaCl (which produce a hypertonic solution when added at this concentration to the complete transforming medium), the DNase added to the DNA-bacterial complex may enter the competent cells along with DNA (Thomas, 1955), because in the first 10 min. in the medium with KCl a substantially lower frequency was obtained than in the control without salt. But when the DNase is added to a 10 min-old DNA-bacterial complex, after termination of the competent state when it can no longer affect the DNA which has already penetrated, a tenfold increase of transformants was found in comparison with the control without KCl; a 40-fold increase with NaCl was obtained in another experiment. It is likely that the hypertonic medium stabilizes the transient, presumptively protoplastic state of the dividing competent receptor cells (Thomas, 1955; Spizizen, 1958, 1959; Kaiser & Hognes, 1960) and thus increases the amount of penetrated macromolecules, including DNA and DNase.

The lower frequency of transformants obtained in the first 10 min. of the transformation process in the presence of KCl or NaCl is difficult to interpret in any other way. The objection might be raised here that DNA is adsorbed to the receptor cell more slowly in the presence of added salt. The results in Fig. 6, where no DNase was given to the system to stop penetration of excess DNA but a 100- to 1000-fold dilution of the DNA-bacterial complex was used instead to end the competent state, are not consistent with such an assumption. The objection that DNase might be more active in the presence of salt cannot be maintained since these salts actually inhibit DNase activity (Kohoutová, 1962; Kopecká & Kohoutová, to be published).

The question remains open why there was no activation, but rather an inhibition, of the transformation yield in the first cycle of competence on diluting the receptor culture with the transformation medium containing DNA and salts. At the same time, activation by salts was observed when the diluting medium was added just after 30 min. of incubation of the DNA-bacterial complex had elapsed. If the recipients went through the second cycle of competence at a time when the population density of the receptor culture increased many times, an increase in transformation yield was observed even with a diluted receptor culture. With the undiluted culture the salts activated the process at any stage of the achieved competence.

This would suggest that K^+ and Na^+ stimulate the transformation process, provided that the so-called competent state is not affected by dilution. This implies that it might be a question of the quantitative relations between substrate and enzyme during contact of DNA with the competent culture.

A possible alternative explanation of our findings might be the effect of ions on DNase. Ottolenghi & Hotchkiss (1962) showed that filtrates of young Pneumococcus cultures contained small quantities of DNase which brought about a loss of capacity of the ageing culture to carry out transformation by added DNA. In our laboratories analogous results have been obtained. Small amounts of DNase are produced by the receptor culture long before the peak of the competence is achieved (Kohoutová, to be published). There is some evidence (Kohoutová, 1960) that DNase at an optimum low concentration, assured by the presence of suitable inhibitors, participates actively in the transformation reaction. Thus salts, apart from the functions already discussed, might inhibit the DNase which is produced in excess by the growing culture and so substantially help to increase the frequency of transformants, even in cultures with an orginally low competence. On the other hand, they might act as inhibitors if there were a suboptimum concentration following dilution of receptor culture from the first cycle of competence. The inhibitory action of KCl or NACl on DNase should support our finding that 0·005 M-sodium citrate had a very similar, although somewhat lower effect than KCl or NaCl, on the frequency of transformants in the second cycle of competence.

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Glyconeogenesis in Growing and Non-growing Cultures of *Tetrahymena pyriformis*

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SUMMARY

The glycogen content of peptone grown cultures of the ciliate *Tetrahymena pyriformis* GL, increased from 6% cellular protein during the exponential growth phase to 30% or more in the stationary phase of growth. The oxygen content in the medium began to decrease at a population of 7500 organisms/ml. in gently shaken cultures and at 80,000 organisms/ml. in well aerated cultures. In either case, depletion of oxygen in the medium was soon followed by cessation of multiplication. The stationary phase was rapidly induced by transfer of log phase organisms to conditions of restricted aeration. Within 3 hr after such a transfer, the rate of glyconeogenesis approximately doubled, both in cultures and in washed suspensions of organisms incubated with 0·1% sodium acetate.

INTRODUCTION

Non-growing cultures of micro-organisms have frequently been shown to accumulate reserves of lipids or polysaccharides (Wilkinson, 1959, 1963). Most commonly found are poly-β-hydroxybutyrate and glycogen or glycogen-like polymers. Accumulation of such polymers usually occurs when growth is limited by factors other than by a carbon source. Thus, with *Escherichia coli*, polysaccharide accumulation was greatest after the onset of the stationary phase of growth, or when the rate of synthesis of nitrogen containing compounds was slow (Dagley & Dawes, 1949; Holme & Palmstierna, 1955, 1956). Similarly, in *Klebsiella aerogenes* the cellular polysaccharide content was higher when growth was limited by a deficiency in nitrogen, phosphorus, or sulphur, rather than a carbon source (Duguid & Wilkinson, 1953).

Manners & Ryley (1952) reported a glycogen content of as much as 23% of the dry weight in stationary phase peptone-grown cultures of the ciliate protozoan *Tetrahymena pyriformis*. The purified product resembled mammalian glycogen in various chemical and physical properties. Further studies by Ryley (1952) showed that this polysaccharide could serve as an energy reserve material for the organisms when anaerobically incubated.

Stationary phase cultures of Tetrahymena when aerated adequately can perform glyconeogenesis at high rates (Hogg & Wagner, 1956; Warnock & van Eys, 1962). Thus, Hogg & Wagner (1956) found that washed stationary phase organisms of

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Tetrahymena pyriformis strain E, doubled their glycogen content at the expense of other endogenous reserves. The observed decrease in the lipid and protein content of the organisms was sufficient to account for the glycogen accumulation. Addition of butyrate, acetoacetate, or acetate to the suspension greatly stimulated the rate of glyconeogenesis. Later, Hogg & Kornberg (1963) invoked the glyoxylate cycle to account for the observed glyconeogenesis.

The present paper describes the glycogen content of *Tetrahymena pyriformis* GL and the oxygen content of the medium during a normal growth cycle, with emphasis on the glyconeogenic capacity during experimentally induced stationary phase. A preliminary report has been presented (Levy, Scherbaum & Hogg, 1964).

METHODS

Organism and growth conditions. Tetrahymena pyriformis strain GL, was grown in a peptone medium of the following composition: 2% (w/v) proteose peptone (Difco), 0·1% (w/v) liver fraction 2 N.F. (Wilson Laboratories, Chicago, Illinois). Sulphates and chlorides were added as in the basal medium A of Kidder & Dewey (1951). In more recent experiments, the only inorganic salt added was 0·1% (w/v) K_2HPO_4. Stock cultures were maintained at 17° in 10 ml. medium in cotton-stoppered upright test tubes (18 × 150 mm.). For inoculation of experimental cultures, 1 ml. portions of the tube cultures, 3–4 days old, were transferred to 50 ml. medium in 250 ml. Erlenmeyer flasks. After incubation for 24–48 hr, samples (3–10 ml.) were used for inoculating each litre of the experimental cultures. To achieve maximum aeration, these cultures were grown in 1 to 1·5 l. batches in a stainless steel tank with surface dimensions of 42 × 80 cm. (Scherbaum, James & Jahn, 1959). The tank was gently tilted about its minor axis (18 strokes/min.) to ensure proper aeration of the 3·0–4·5 mm. layer of culture medium. Sterile water-saturated air replenished the air phase of the culture vessel at a rate of 3 l./min. Organisms were incubated at 28° or at room temperature (24°–28°), In some cases, cultures of 300–1500 ml. were grown in 2·5 l. low-form culture flasks shaken by rotatory movements at 60 to 80 oscillations/min.

Harvesting of organisms. Cultures were centrifuged in 40 ml. conical tubes at 250–300 *g* for 1·5 min., the supernatant fluid removed by suction and the organisms washed at least twice by resuspension in 40 ml. Ringer phosphate buffer (Ryley, 1952). Large samples of suspensions were centrifuged in 600 ml. centrifuge flasks for 5 min. at 250 *g* in an International Centrifuge, model PR2. The flasks were specially designed to facilitate collection of the organisms in a bulb-like extension in the centre of the funnel-shaped bottom. Organisms were then washed as described above. When the glycogen content was to be determined in culture samples, measured volumes of culture were poured directly into cold ethanol to a final concentration of 35% (v/v) ethanol. The organisms were collected by centrifugation at 150 *g* for 5 min., and the supernatant fluid removed by suction and discarded.

Analytical methods. Dissolved oxygen was measured by the volumetric method of Scholander, van Dam, Claff & Kanwisher (1955). To insure rapid fixation of organisms, 4% (v/v) formalin (40%, w/v, HCHO) was added to the buffer solution present in the dead space of the extraction syringe. Glycogen was isolated by the method of Good, Kramer & Somogyi (1933). Radioactive glycogen was purified

either by two precipitations in ethanol, followed by washings, twice each, in 80% (v/v) and 100% ethanol and anhydrous ether, or alternatively by successive precipitation by 50% alkaline ethanol, 67% acidic ethanol, and 75% ethanol. A few milligrams of sodium sulphate were added to each sample to aid in precipitation. Glycogen was estimated by an anthrone method (Morris, 1948) with some modifications. Samples were frozen in a solid CO_2 + ethanol bath, and two volumes of anthrone reagent (2 mg. anthrone/ml. concentrated sulphuric acid) were added to the frozen samples. These were then allowed to melt in a boiling water bath. Protein was determined by the Lowry method, as modified by Chou & Goldstein (1960), on material insoluble after digestion in 3% (w/v) perchloric acid for 15 min. at 90°.

Radioactive samples were counted at infinite thinness on stainless steel or nickel-plated planchet in a thin window gas flow counter (Nuclear Chicago). Counting rates have been corrected for background. ^{14}C labelled acetate was obtained from the California Corporation for Biochemical Research.

Direct counts of organisms were made, in formalin-fixed samples, in a Sedgewick–Rafter counting chamber with the aid of a calibrated ocular Whipple disc in a light microscope at a magnification of $\times 100$ (Scherbaum, 1957).

RESULTS

Glycogen content in log phase and stationary phase organisms

Cultures (1·5 l.) were grown in the stainless steel vessel either shaken and aerated, or not shaken but aerated. All other conditions (composition of medium, age and size of inoculum, etc.) were identical. The generation time of about 3 hr was the same for both cultures during exponential multiplication, but the final population densities in the stationary phase differed significantly. In the shaken culture, it was more than 10^6 organisms/ml.; in the non-shaken culture, it was only between 1 and 2×10^5 organisms/ml. Cultures of the latter type were used for further experiments. Either shaking such cultures or transfer to an inorganic buffer permitted further growth for at least one generation. It appears that in this type of stationary phase, nutrients were not limiting and were still available for conversion into storage products.

The glycogen content of organisms in the exponential growth phase was between 3 and 12% of cellular protein (40 samples; average 6%) and 25 and 40% (17 samples; average 30%) in stationary phase organisms. Calculated per 10^6 organisms, those values were 0·05–0·150 mg. in log phase and 0·8–2·0 mg. in stationary phase.

Culture growth and oxygen content of the medium

Two 1·5 l. cultures were grown in 2·5 l. low-form culture flasks. One flask was shaken vigorously and aerated, while the second was shaken very gently so that no trapping of air could occur at the surface. The second culture was not initially aerated, although air could pass into the vessel by diffusion through a cotton-wool plug. From these cultures, samples were removed at frequent intervals for total counts and determination of dissolved oxygen in the medium. The oxygen content before inoculation was 4·8 μl./ml. culture.

In the well-aerated culture (Fig. 1 A) the oxygen content had decreased to 3 μl./ml.

medium at a population density of about 80,000 organisms/ml. and became undetectable at about 350,000 organisms/ml. The final population density was over 700,000 organisms/ml. In the non-aerated culture, however, the oxygen content was 3 μl./ml. already at a population density of 7,500 organisms/ml. and became undetectable at 30,000 organisms/ml. (Fig. 1B). The maximum population density remained constant at 90,000 organisms/ml. for 20 hr. At this time, flushing of the air phase was started (indicated by the arrow in Figure 1B), but the shaking rate was not altered. After a lag period of 10 hr, multiplication was resumed and continued slowly until the population density had approximately doubled. In another non-aerated control culture, the population density in the stationary phase remained constant at 110,000 organisms/ml. for several days.

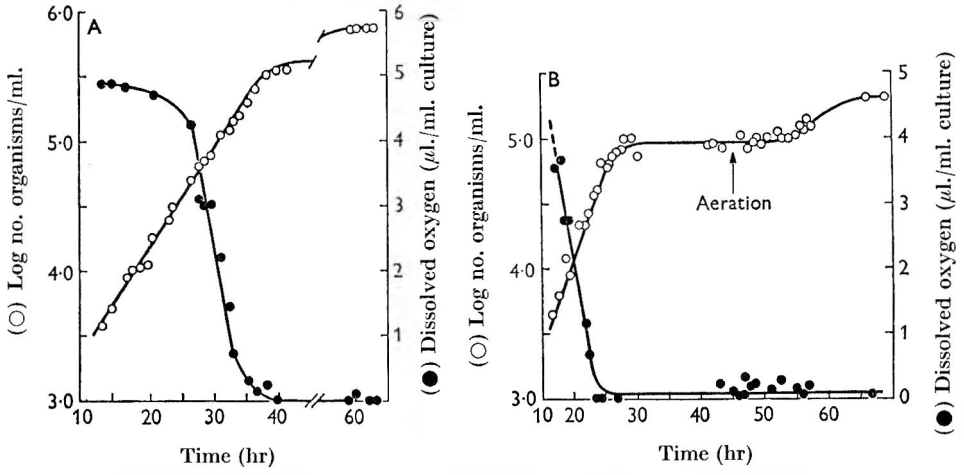

Fig. 1. Oxygen content of the medium during culture growth. Organisms were grown in 2·5 l. low-form culture flasks at 28° ± 1°; surface area approximately 450 cm². A. Flask was vigorously shaken (70–90 oscillations/min.) and air was continuously flushed over the culture. Initial culture volume was 1·2 l. B. Culture was shaken gently so as to prevent surface disturbance. Aeration of the culture was begun at the point indicated by the arrow; however, the rate of shaking was not changed. Initial culture volume was 1·5 l.

The respiratory capacity of the cultures was compared with the availability of oxygen in the non-aerated culture. At a population density of 7,500 organisms/ml. (Fig. 1B) the oxygen content in the medium began to decrease rapidly. Since at this time the culture volume was 1,400 ml., the total number of organisms was about 10×10^6. With a respiratory rate of 5 μl. oxygen/10^6 organisms/min., the respiratory capacity of the culture is about 50 μl. O_2/10^6 organisms/min. for log phase organisms. Ormsbee (1942) and Hamburger & Zeuthen (1959) have reported values of 7·2 and 4·8 μl. O_2/10^6 organisms/min. respectively for log phase organisms. Experimentally determined rates of diffusion of oxygen from air into still or slowly moving water are 0·03 to 0·08 g./m²/hr (= 0·04 to 0·09 μl./cm.²/min.) at 25° and zero oxygen tension (Odum, 1956). The maximum amount of oxygen that could enter the culture (with a surface area of 450 cm.²) would be 20–40 μl./min. Thus already at a population density of 7500 organisms/ml., the respiratory capacity of the culture exceeds the maximum possible rate of diffusion of oxygen. The oxygen content of the

medium was indeed decreasing at this culture density. During stationary phase at a population density of 90,000 organisms/ml., these organisms could respire at only about 5% of their potential, if the rate of diffusion is compared with the respiratory capacity (650 μl. O_2/min.) of the culture. In contrast, the oxygen content in the aerated culture at a population density of 90,000 organisms/ml. had just begun to decrease (Fig. 1A).

Cellular glycogen content and oxygen content of the medium throughout a growth cycle

Fourteen cultures were started simultaneously as described in the legend to Fig. 2. Each of the cultures was used for total count, determination of dissolved oxygen

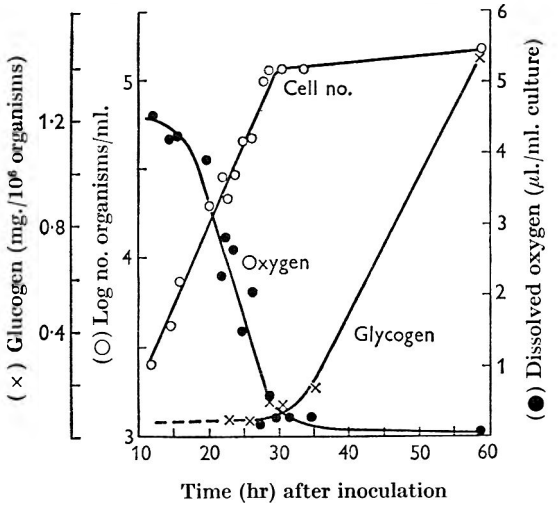

Fig. 2

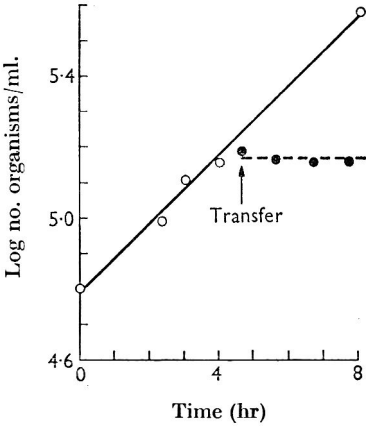

Fig. 3

Fig. 2. Cellular glycogen content and oxygen content of the medium during a growth cycle. Fourteen 500 ml. Erlenmeyer flasks, each containing 160 ml. medium, were inoculated (starting population, 800 organisms/ml.) and maintained at room temperature with very gentle shaking. At the times indicated, the dissolved oxygen in the medium, cellular glycogen, and population density (total count) were determined.

Fig. 3. Induction of stationary phase. Stationary phase was induced by transferring four 29 ml. samples of a well-shaken culture, growing exponentially in thin layer (in stainless steel tank), into four 125 ml. Erlenmeyer flasks, which were then maintained without further shaking. At 0, 1, 2, and 3 hr after transfer, samples were removed for counts (full circles). Shaking was continued in the remainder of the culture. The arrow denotes the time of transfer.

and glycogen content at the times indicated, ranging from 12 hr (6600 organisms/ml.) to 58 hr (210,000 organisms/ml.) after inoculation. At about 30 hr after inoculation, when less than 0·5 μl. O_2/ml. was found in the medium, multiplication ceased and the glycogen content began to increase in those organisms. The stationary phase organisms contained 22 times as much glycogen as the log phase organisms.

Induction of stationary phase

To study possible differences in metabolism between growing and non-growing organisms, a technique was developed for the rapid and reproducible induction of stationary phase. This was accomplished by transfer of well-shaken cultures, growing exponentially in shallow layers in the stainless steel tank, into smaller vessels, followed by maintenance without shaking. The results of such an experiment are shown in Fig. 3. At the time indicated by the arrow, four samples of culture were transferred at a population density of 140,000 organisms/ml. Using this figure and the new surface/volume ratio, it was calculated that respiration was limited to between 5 and 10% of the normal rate. During a 3-hr period following transfer (approximately one generation time in exponential growth phase) there was little increase in total numbers (0–20%), while increase in protein ranged from 10 to 40% and glycogen, 50–300% (5 experiments). Exponential multiplication in the tank was still observed at a population density of 370,000 organisms/ml. (Fig. 3).

Glyconeogenic capacity before and after induction of stationary phase

After transfer to conditions of restricted aeration, samples of culture were taken at the times indicated in Table 1, tracer amounts of sodium acetate-2-^{14}C were added and shaken for 15 min. Glycogen was isolated and the radioactivity determined. The results are shown in Table 1. In the untreated control cultures, the rate of

Table 1. *Incorporation of acetate-2-^{14}C into glycogen during induction of stationary phase*

Stationary phase was induced in part of an exponentially growing culture by transfer of 35 ml. portions of culture into 125 ml. conical flasks, followed by maintenance without shaking. The remainder of the culture was shaken to permit growth to continue. Population density at the time of transfer was 120,000 organisms/ml. At the times indicated, 25 ml. portions of culture were shaken for 15 min. with 1 μc. of acetate-2-^{14}C (sp.act. 20·5 mc./μmole).

	Hours after transfer	Incorporation of acetate-2-^{14}C into glycogen (counts/min./million organisms)
Log phase control	0	727
	1·5	1040
	3	1630
Non-shaken culture	0	727
	1·5	1460
	3	5600

incorporation of the label was 2·2 times in the 3-hr sample as compared to the zero hour sample. In the stationary culture, however, the comparable increase was 7·7 times. In other experiments, values up to 20 times were obtained. Similar results were observed when organisms were washed and resuspended in inorganic buffer before incubation with labelled acetate. However, when incorporation of glucose-^{14}C was tested under the same conditions, the rates at zero and 3 hr after transfer were almost identical.

The results show that limited aeration inhibited growth and division but increased the ability of the organisms to incorporate carbon from acetate into glycogen. Therefore, stationary and log phase cultures were used for the following further comparisons: (1) rates of glyconeogenesis were recorded in cultures as well as in washed suspensions with and without added acetate; (2) the incorporation of acetate-2-^{14}C was studied at tracer concentrations only, and together with acetate at substrate concentrations. Results of these experiments are shown in Fig. 4. All stationary phase cultures tested showed an increased rate of glyconeogenesis in culture (Fig. 4A) were 0·08 and 0·26 μmole glucose/mg. protein/hr in log and

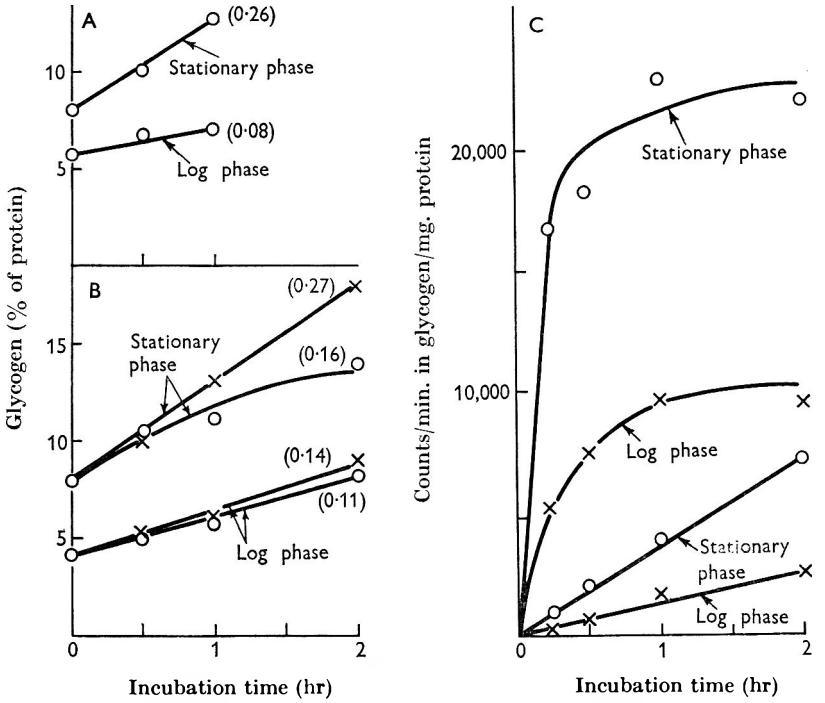

Fig. 4. Rate of glyconeogenesis before and after induction of stationary phase. Stationary phase was induced by transferring 110 ml. portions of an exponentially growing culture into 500 ml. conical flasks, which were maintained for 3 hr without shaking. Population density at the time of transfer was 116,000 organisms/ml. At zero and 3 hr after transfer, glyconeogenic capacity was tested by shaking portions of culture or of washed suspensions. This is referred to as incubation time on the abscissae. A. Glyconeogenesis in culture. Twenty ml. portions of culture were transferred to 250 ml. conical flasks and shaken for 1 hr at 29°. Glycogen values are based on the amount of protein present at the start of the incubation period. The figures in parentheses give μmole glucose/mg. protein/hr measured over a 1 hr period. B. Glyconeogenesis in washed organisms. Organisms were removed from culture medium, washed 3 times and suspended in Ryley's Ringer phosphate buffer. Two and a half ml. portions of the washed suspensions, containing approximately 1 mg. protein/ml., were shaken at 29° in 50 ml. flasks, in the presence (× — ×) or absence (○—○) of 0·1 % (w/v) sodium acetate. Sodium 2-^{14}C acetate (172,000 counts/min. under the test conditions) was added to each flask. The figures in parentheses give μmole glucose/mg. protein/hr measured over a 2 hr period. C. Incorporation of acetate-2-^{14}C into glycogen in washed suspensions. Lower two curves show incorporation in the presence of 0·1 % sodium acetate, upper two curves, the incorporation when acetate was added at tracer concentrations only. × — ×, log phase; ○—○, stationary phase.

stationary phase, respectively. In repeated experiments, comparable values ranged from zero to 0·22 in log phase and from 0·21 to 0·55 in stationary phase. Washed organisms (Fig. 4B) showed the same difference. The rate of glycogen synthesis in log phase organisms was 0·11 μmole glucose/mg. protein/hr and in the stationary phase organisms it was 0·16 μmole glucose/mg. protein/hr. In the presence of 0·1 % (w/v) sodium acetate, however, a marked stimulation was observed in stationary phase organisms, 0·27, but not so in log phase organisms (0·14 μmole glucose/mg. protein/hr). This increased rate of glyconeogenesis in stationary phase organisms is further substantiated by the rate of incorporation of labelled acetate into glycogen (Fig. 4C). After 2 hr of incubation, more than twice the label was found in stationary phase organisms than in log phase organisms. This is the case whether organisms were incubated with trace amounts of sodium acetate (upper two curves, Fig. 4C), or with 0·1 % (lower two curves, Fig. 4C).

DISCUSSION

The results presented in this paper are in agreement with other reports which show that washed stationary phase suspensions of *Tetrahymena pyriformis* may have an appreciable endogenous rate of glyconeogenesis (Hogg & Wagner, 1956; Warnock & van Eys, 1962). The chemical nature of the precursors for glyconeogenesis cannot be given with certainty. Hogg & Wagner (1956) reported a decrease in intracellular protein and lipid with a concomitant increase in glycogen; a respiratory quotient of 0·55 and the ability of fatty acids to stimulate glyconeogenesis suggested that lipids are the main precursors. Warnock & van Eys (1962) showed a considerable increase in glycogen content in washed cells with a significant loss in protein. In cultures grown in proteose peptone, the ultimate precursors for glycogen must be amino acids. It is not known, however, whether these are converted to carbohydrates directly or via fatty acids.

Although the factors which lead to the onset of stationary phase have not been unequivocally determined, a deficiency in the amount of oxygen available to the organisms was clearly related to the onset of stationary phase. In cultures grown with little or no agitation, oxygen became depleted from the medium at low population densities, and this was followed quite soon by cessation of division. In well-shaken cultures, however, oxygen disappeared at much higher population densities and division again ceased, following a somewhat longer transition period. Once detectable oxygen had disappeared from the medium, respiration of the culture remained constant at a rate determined by the rate of diffusion of oxygen into the culture. Consequently, the respiration rate per organism must decrease until culture growth ceases. In some of the stationary phase cultures mentioned here, the respiratory rate was only about 5 % of the organisms' capacity. That Tetrahymena will use all available free oxygen in the medium has already been reported by Baker & Baumberger (1941).

The close correlation between decreased oxygen tension in the medium and the onset of stationary phase does not exclude possible roles other gases might have in the onset of stationary phase and/or the induction of glyconeogenic capacity. The condition used here to limit the exchange of oxygen into the medium would also limit the diffusion of carbon dioxide and ammonia from the culture. Preliminary

experiments indicate that carbon dioxide at high partial pressure (25 %) can rapidly induce stationary phase (Drs A. M. Elliott and I. J. Bak, personal communication) although no increase in glyconeogenic capacity occurred (Levy & Hogg, unpublished data).

A possible biological significance of the results has been suggested by Dr J. F. Hogg (personal communication). Photosynthetic organisms in the natural habitat of Tetrahymena cease oxygen production during the night. Consequently, the falling oxygen tension may lead to an increase in glyconeogenesis from intracellular reserves. This makes possible survival of the organism under temporary anaerobic conditions, where metabolism depends on glycogen fermentation.

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Effect of Ultraviolet Radiation and the Induction of Vegetative Reproduction of Phage Lambda on the Ultracentrifuge Pattern of Extracts of *Escherichia coli*

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SUMMARY

After ultraviolet irradiation, the growth of *Escherichia coli* (non-lysogenic and lysogenic for phage λ) resulted in diminution of the 8S component of water extracts of the bacteria and the loss of its characteristic spike. In the induced lysogenic bacteria, 8S material was synthesized in the second half of the latent period and the characteristic shape of the boundary was regained. The 8S material was free DNA of relatively high molecular weight and these changes were correlated with alterations in the rate of DNA synthesis in the irradiated bacteria. The synthesis of ribosomal material was found to continue during the development of phage λ. After log phase bacteria had stood for a short time in saline, extracts prepared in tris buffer + 0·01 M-magnesium acetate showed that an 85S component developed. The latter was replaced by slower material (73S) when the bacteria (non-lysogenic and lysogenic, irradiated or unirradiated) resumed logarithmic growth in broth. This conversion may reflect a configurational change in the 73S component in environments which do not support growth.

INTRODUCTION

Siegel, Singer & Wildman (1952) studied the effect of infection with bacteriophage T_2 on the high molecular weight components of the host *Escherichia coli*. Extracts from the infected bacteria were examined in the ultracentrifuge and it was found that the main effect of virus infection concerned the material with a relatively low sedimentation coefficient (of the order of 10S) and containing deoxyribonucleic acid (DNA). The ultracentrifuge diagrams also appeared to show changes in components with higher sedimentation coefficients (29S, 40S), i.e. in the ribonucleoprotein (RNP) particles present in the extracts. These studies were made before the factors which influence the size and therefore the sedimentation coefficients of such particles had been studied. In this respect it is now known that the concentration of Mg^{2+} is especially important. In extracts made in water or dilute phosphate buffer the RNP particles mainly sediment at 29S and 40S (Schachman, Pardee & Stanier, 1952; Dagley & Sykes, 1956, 1959) whilst, in the presence of 0·01 M-Mg^{2+}, particles which sediment at $(S_{20,w})_0 = 70$ predominate (Tissières, Watson, Schlessinger & Hollingworth, 1959).

As part of a programme devoted to phage lambda (λ) and its host *Escherichia coli* an investigation has been made of the effect of vegetative reproduction of 'wild-

type' phage λ on the components which can be detected in cell-free extracts of the host bacteria by analytical ultracentrifugation. Induction of phage development in bacteria lysogenic for phage λ was here achieved by ultraviolet irradiation.

METHODS

Organisms and culture conditions. Escherichia coli strain K112 and the wild-type phage lambda (λ_{22}) were from the collections of Drs F. Jacob and E. Wollman of the Pasteur Institute, Paris. Organisms were grown with aeration at 37° in a nutrient broth medium containing (per litre, w/v) Oxoid tryptone (20 g.) and Lab. Lemco (2 g.) at pH 7.

Irradiation procedure. Cultures were harvested in the mid-logarithmic phase of growth, and then suspended in sterile distilled water at an extinction reading in the range 0·3–1·0 (equivalent to approximately 3×10^8 to 1×10^9 bacteria/ml.). In the experiments where extracts were subsequently made in tris buffer + 0·01 M-magnesium acetate the bacteria were resuspended in 0·9% NaCl. Samples (120 ml.) of the suspension were irradiated for 60 sec. in a sterile enamel dish (11 in. × 14 in.) rocked gently 75 cm. below the ultraviolet lamp (an Hanovia Chromatolite low pressure Hg lamp without the filter). The irradiation of 2 l. or more of suspension took an appreciable time, but control experiments showed that residual growth, percentage induction and phage yield per induced cell were the same in samples transferred to growth medium at 37° immediately after irradiation and in those kept at room temperature for 60 min. before the addition of broth and incubation at 37°. In the experimental conditions used here, the latent period, i.e. the time from the addition of broth until the onset of lysis and the release of mature phage particles, was about 72–75 min. (Fig. 3*a*). Concentrated nutrient broth (10 times usual strength) at 100° was added to the irradiated suspension (200 ml./l.) to help raise the temperature quickly to 37°. The cultures were then aerated in glass bottles immersed in a water bath at 37°. Silicone antifoam emulsion RD (Hopkin and Williams Ltd., England) was added to prevent foaming.

Preparation of extracts. The bacteria were collected by centrifugation and the pellet transferred to a Hughes press (Hughes, 1951) and crushed. The crushed bacteria were extracted for 30–60 min. at 4° with either distilled water or 0·001 M-tris buffer (pH 7) containing 0·01 M-magnesium acetate. After centrifuging the extracted cell paste at 24,000*g* for 45 min. to remove whole bacteria and insoluble debris, the supernatant fluids were collected and diluted with the appropriate solvent (i.e. water or tris + magnesium acetate buffer) so as to contain 10 mg. protein/ml. In this manner variations in other macromolecular components could be viewed against a constant protein background in the analytical ultracentrifuge. After storage overnight in ice, the extracts were examined next day in the Spinco ultracentrifuge (model E with schlieren optics) at room temperature. In each individual experiment, photographs taken at the same time interval after reaching speed (50,740 rev./min.) were taken at the same bar angle. Sedimentation coefficients for the components visible in crude extract sedimentation diagrams are expressed in Svedberg (S) units and are uncorrected values. It is well known that the 40 and 29S components reported here for crude extracts have $(S_{20,w})_0$ values of 50 and 30, respectively, in homogeneous solution (Tissières *et al.* 1959). Area

measurements were made with a planimeter on enlarged photographs taken from comparable diagrams; results are recorded in arbitrary units and have been corrected for radial dilution and differences in bar angle.

Treatment of extracts with enzymes. Extracts and enzymes dissolved in buffer were pre-incubated separately for 10 min. at 37° and then a sample of each mixed together, incubated for the required time and then plunged into crushed ice. Each extract was treated immediately before its addition to the ultracentrifuge cell. In control systems, extracts were incubated with the same amounts of buffer but containing no enzyme. Crystalline ribonuclease (Sigma Chemical Co., St Louis, U.S.A.) was dissolved in the tris+magnesium acetate buffer described above; crystalline deoxyribonuclease (Worthington Biochemical Corp., U.S.A.) was dissolved in 0·05 M-phosphate buffer (pH 7).

Chemical estimations. Protein was estimated by the biuret method (Gornall, Bardawill & David, 1949) with bovine serum albumin (Fraction V, Armour Pharmaceutical Co. Ltd., England) as a standard. DNA was estimated by the method of Burton (1956) with 2-deoxy-D-ribose (L. Light, Colnbrook, England) as standard, and RNA by the method of Schneider (1945) with D-ribose (L. Light, Colnbrook, England) as standard. Results are expressed in terms of 'apparent deoxyribose' or 'apparent ribose', since only the sugar residues bound to purines react in these procedures.

Preparation of samples for determination of protein RNA and DNA. The procedure followed that described by Fry & Gros (1959).

Turbidity of bacterial suspensions. Measurements were made at 610 mμ (with a blue photo-cell).

Spectrophotometry. A Unicam spectrophotometer SP 600 with cells of 1 cm. light path was used.

Ultrasonic treatment. An M.S.E. 60 W. ultrasonic disintegrator fitted with a titanium probe was used and throughout the sonic treatment the extract was cooled in ice.

RESULTS

Ultracentrifugal analysis of cell-free extracts prepared in water

The cell-free extract from unirradiated *Escherichia coli* lysogenic for phage λ gave an ultracentrifuge diagram with components sedimenting at 4, 8, 29 and 40S (Fig. 1*a*); the overall picture was no different from that obtained from the non-lysogenic parent strain (Fig. 1*e*). Immediately after irradiation (i.e. before transfer to growth medium) the ultracentrifuge pattern of extracts of the lysogenic bacteria (Fig. 1*b*) appeared to be the same as for the unirradiated bacteria. Irradiation thus produced no immediate change directly visible in the photographs. However, measurement of boundary areas did reveal a small decrease in the concentration of the 40S component (compare pre- and post-u.v. treatment data in Table 1). Since the bacteria were suspended in water for the irradiation procedure, this result was probably not due to the irradiation as such but to a slow loss of magnesium ions from the bacteria, and, as a result, part of the 40S material became unstable. Dagley & Sykes, 1959, reported a similar phenomenon.

When the irradiated lysogenic bacteria were returned to growth medium, samples taken after 30 and 60 min., showed significant changes in the ultracentrifuge pattern of the extracts. After 30 min., the 8S component had diminished,

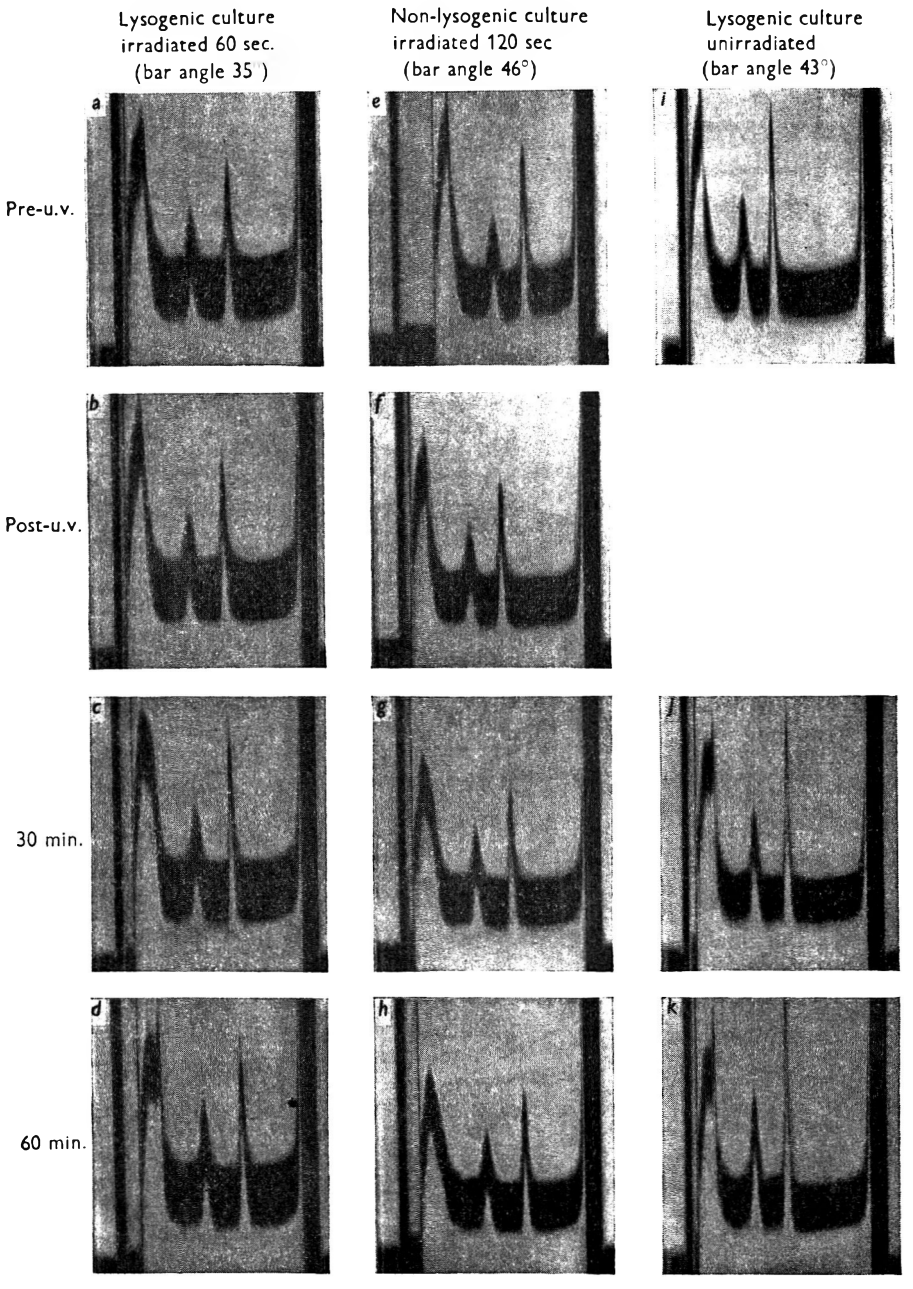

Fig. 1. Ultracentrifuge patterns of extracts from non-lysogenic and lysogenic *Escherichia coli* K112. All photographs were taken 16 min. after reaching 50,740 rev./min. Sedimentation is to the right. From left to right in each diagram the sedimenting boundaries have uncorrected sedimentation coefficients of 4, 8, 29 and 40 S.

and the characteristic spike was absent. On the other hand, in the 60 min. sample this component was again prominent, its characteristic shape had been restored, and, if anything, the spike was more pronounced (Figs. 1*c* and *d*). Siegel *et al.* (1952) concluded that the 8S component in ultracentrifuge diagrams of bacterial extracts was mainly, if not entirely, free DNA. In our experiments, incubation of the extracts with deoxyribonuclease for 3 min. at 37° abolished the 8S boundary but had no effect on the other components (Fig. 2*a*). Similarly, when the extracts were treated ultrasonically for 1 min., the 8S spike disappeared and the 40 and 29S

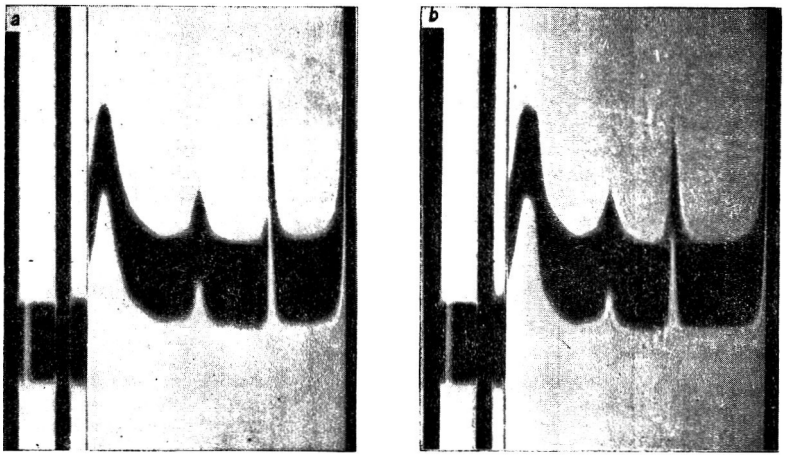

Fig. 2. Effect of (*a*) deoxyribonuclease and (*b*) ultrasonic treatment on the ultracentrifuge pattern of water extracts of induced bacteria harvested after 60 min. in broth. For (*a*), final concentration of enzyme 9 μg./ml. and incubated 3 min. at 37°: picture taken 16 min. after reaching 50,740 rev./min.; bar angle 55°. For (*b*), sonic treatment for 1 min.; picture taken 16 min. after reaching 50,740 rev./min.: bar angle 55°.

components were again unaffected (Fig. 2*b*). Ultrasonic treatment has been shown to disorganize and break large DNA molecules into smaller pieces (Doty, McGill & Rice, 1958). In view of these observations and those of Siegel *et al.* (1952), it seems reasonable to conclude that the 8S component observed in our experiments was free DNA of high molecular weight (perhaps partially depolymerized by the extraction procedures employed). Furthermore, these results show that the presence or absence of a spike sedimenting at 8S in the ultracentrifuge diagrams was an indication of the amount of polymerized DNA in the system. Inspection of the ultracentrifuge diagrams from bacteria 30 and 60 min. after induction (Fig. 1*a–d*), and area measurements of the 40 and 29S components (Table 1), lead to the conclusion that ribosome synthesis continued during the induced development of the temperate phage λ. This result is to be contrasted with the situation in *Escherichia coli* strain B infected with the coliphage T_4, where ribosome synthesis came to a halt immediately after infection (Brenner, Jacob & Meselson, 1961).

The observed changes in the ultracentrifuge diagrams reflected the changes in the synthesis of protein, RNA and DNA in the induced bacteria (Fig. 3). The synthesis of DNA was inhibited during the first 30 min. of the latent period of phage development. On the other hand, protein synthesis continued and in consequence the

DNA:protein ratio fell (from $9{\cdot}0\times10^{-3}$ to $6{\cdot}7\times10^{-3}$), and, as would be predicted, this was shown in the ultracentrifuge picture as a diminution in the 8S component (Fig. 1*c*). In the second half of the latent period, DNA synthesis was resumed at an accelerating rate and became faster than in the control (unirradiated) bacteria (Fig. 3*b*; Waites & Fry, 1964). At the same time there was a falling off in protein synthesis so that the DNA:protein ratio increased (from $6{\cdot}7\times10^{-3}$ to $7{\cdot}8\times10^{-3}$) and this was correlated with the very pronounced 8S component in the bacteria 60 min. after induction (Fig. 1*d*).

Table 1. *Amounts of 40S and 29S ribonucleoprotein components in water extracts of lysogenic and non-lysogenic Escherichia coli*

The bacteria were harvested, irradiated as required, and subsequently incubated in broth as described in the Methods (p. 232). Samples were taken at the times shown and water extracts prepared. Area measurements were made with a planimeter on enlarged photographs of comparable ultracentrifuge diagrams; results are recorded in arbitrary units and have been corrected for radial dilution and differences in bar angle.

Experiment	Sample time (min.)	Corrected area in arbitrary units	
		40S	29S
Lysogenic culture, unirradiated	0	428	253
	20	460	273
	40	525	285
	60	460	360
Lysogenic culture, 60 sec. u.v.-irradiation	Pre-u.v.	482	357
	Post-u.v.	467	357
	30	583	457
	60	610	437
Non-lysogenic culture, 120 sec. u.v.-irradiation	Pre-u.v.	404	242
	Post-u.v.	352	238
	30	260	202
	60	296	239
Non-lysogenic culture, 60 sec. u.v.-irradiation	Pre-u.v.	420	—
	Post u.v.	386	—
	30	477	—
	60	441	—

To determine whether the observed changes in the ultracentrifuge patterns were due to the induced development of phage λ, it was necessary to consider the results obtained in two types of control experiment involving: (*a*) exponential growth of the (unirradiated) lysogenic bacteria, and (*b*) the effect of irradiation on the non-lysogenic parent strain. A culture of *Escherichia coli* lysogenic for phage λ was prepared as described in Methods (except that it was not irradiated) and samples were taken at intervals for chemical analysis and the preparation of extracts for ultracentrifugal analysis. In the first 60 min. after addition of broth, whilst the DNA:protein ratio fell slowly from $9{\cdot}1\times10^{-3}$ to $8{\cdot}4\times10^{-3}$, the RNA:protein ratio rose from $1{\cdot}2\times10^{-1}$ to $1{\cdot}6\times10^{-1}$ at 40 min. and fell to $1{\cdot}3\times10^{-1}$ at 60 min. These chemical data could be correlated with deductions made from the ultracentrifugal diagrams (Fig. 1*i*, *j* and *k*) and area measurements of the 40, 29 and 8S components. There was a marked increase in the 40 and 29S ribonucleo-

protein components (Table 1), a result in agreement with previous workers (Wade & Morgan, 1957; Dagley & Sykes, 1958) who showed that this is a feature of exponential growth. Area measurements of the 8S component lack precision, because of the pronounced self-sharpening and incomplete resolution of the boundary from the 4S peak. None the less, consistent with the chemical analysis, the area of the 8S component slowly decreased during the 60 min. incubation period.

It is known that exposure of non-lysogenic bacteria to u.v. radiation can lead to complete inhibition of DNA synthesis for a period depending upon the amount of radiation received (Kelner, 1953). It was therefore of interest in the present

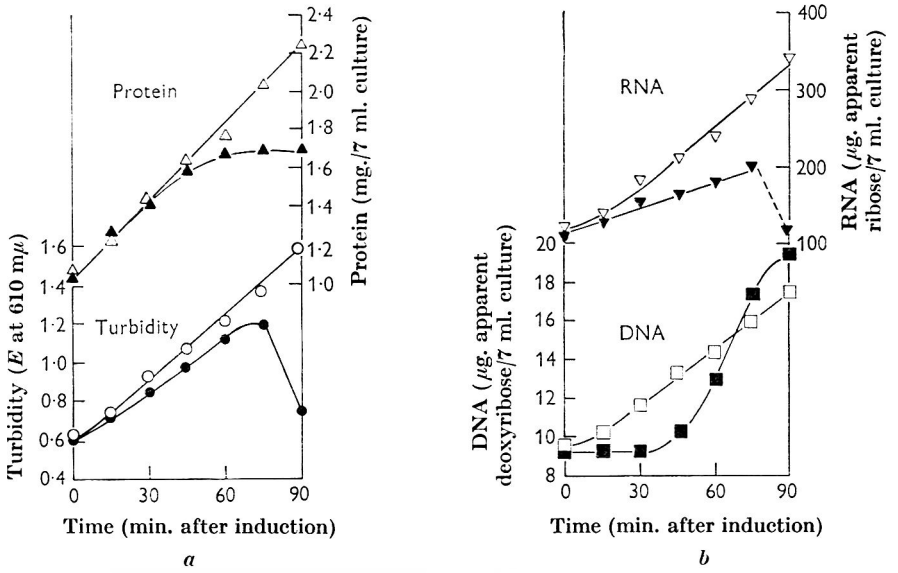

Fig. 3. Changes in (*a*) turbidity (○, ●) and protein (△, ▲), and (*b*) DNA (□, ■) and RNA (▽, ▼) after the induction of phage development in *Escherichia coli* K112 (λ). Initially $5{\cdot}6 \times 10^8$ bacteria/ml. and 96% were induced. Solid symbols refer to irradiated bacteria and open symbols to unirradiated control culture.

context to study the effect of u.v. radiation on the ultracentrifuge diagrams of non-lysogenic *Escherichia coli* strain K112, since, whilst Billen & Volkin (1954) have studied the effect of irradiation with X-rays, there appeared to be no comparable experiments with u.v. radiation. The parent non-lysogenic *E. coli* strain K112 was therefore grown and treated in exactly the same manner as the lysogenic strain, and extracts prepared immediately before and after irradiation and after 30 and 60 min. in the growth medium (Fig. 1*e–h*). The bacteria were irradiated for 120 sec., after which only 4% survived, judged by their ability to form colonies. This dose of radiation was chosen because it gave a population analogous to a fully induced lysogenic culture in which phage development took place in most of the bacteria (90–95%). Once again there was no apparent difference in the ultracentrifuge diagrams of extracts from the bacteria just before and immediately after irradiation (Fig. 1*e*, *f*). However, after the irradiated bacteria had been incubated in complete medium for 60 min., area measurements of the ultracentrifuge diagrams (Fig. 1*e–h*) revealed a decrease in the concentration of the 40S and 8S

components to 73 and 56% of their respective initial (pre-u.v. treatment) values (Table 1). In contrast to the lysogenic strain, chemical analysis showed that DNA synthesis was not resumed during the first 45 min. after irradiation and it was only just beginning after 60 min. (presumably because of the growth of the few surviving bacteria); this explains why the characteristic 8S spike in the ultracentrifuge diagrams was not regained. Since the synthesis of protein and RNA continued at a decreased rate the concentration of the 40S component did not decrease so sharply as the 8S component (by 27% as compared with 44%) during the 60 min. period of the experiment. When the bacteria received u.v. radiation for 60 sec. (the dose used in the induction experiment), 65% were killed; as regards the DNA component, the results were essentially the same as when the bacteria received 120 sec. radiation. The synthesis of DNA was inhibited for 30 min., and with continued protein synthesis the DNA:protein ratio decreased from an initial value of

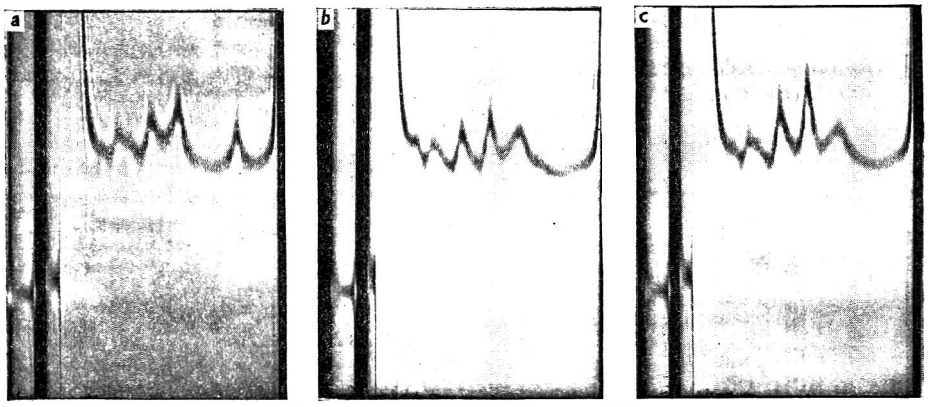

Fig. 4. Ultracentrifuge patterns of extracts of lysogenic Escherichia coli K112 (λ) made in tris + 0·01 M-magnesium acetate buffer: (*a*) 0 min. before induction by u.v.-irradiation, (*b*) 30 min., and (*c*) 60 min. after irradiated cells mixed with tryptone broth and incubated. 8 min. after reaching 50,740 rev./min.; bar angle 30°. In (*a*) the sedimenting boundaries (reading from left to right) have uncorrected sedimentation coefficients of 28, 44, 58 and 85S and, in (*b*) and (*c*), 28, 44, 58 and 73S.

$8{\cdot}5 \times 10^{-3}$ to $5{\cdot}5 \times 10^{-3}$ after 60 min., and the 8S spike again disappeared from the ultracentrifuge diagrams. Ribonucleic acid synthesis continued, the RNA:protein ratio increasing from an initial value of 8×10^{-2} to $1{\cdot}4 \times 10^{-1}$ after 60 min., and there was a simultaneous increase in the 40S and 29S RNP components (Table 1). These observations are therefore in general agreement with those of Kelner (1953) for u.v.-irradiated *E. coli* strain B/r.

Ultracentrifugal analysis of extracts prepared in tris + magnesium acetate buffer

When tris buffer + 0·01 M-magnesium acetate was used to prepare extracts of *Escherichia coli* strain K112 (lysogenic for phage λ and non-lysogenic) the ultracentrifuge diagrams (e.g. Fig. 4*a*) showed components with uncorrected sedimentation coefficients of 28, 44, 58 and 85S (in addition to the 4S boundary due to soluble proteins). These components probably correspond to RNP particles which in

homogeneous solution have $(S_{20,w})_0$ coefficients of 30, 50, 70 and 100S, respectively, as for example in the experiments of Tissières & Watson (1958). Extracts prepared from lysogenic bacteria before and immediately after u.v.-irradiation again showed no difference but after 30 min. in broth the fastest component (85S) was replaced by one sedimenting at an appreciably slower rate (73S) (Fig. 4*b*). This change was apparently not attributable to a change in the viscosity of the extract since the sedimentation coefficients of the other components were essentially unaltered. A similar slower component was also seen in extracts from bacteria harvested 60 min. after induction (Fig. 4*c*) and in extracts of the non-lysogenic parent strain, with or without prior irradiation, after 60 min. growth in tryptone medium. The reason for this change is unknown but was clearly not connected with phage development or the irradiation *per se* received by the bacteria. The faster 85S (uncorr.) component was observed to develop progressively during periods of restricted growth (i.e. while the bacteria were standing in saline during the irradiation procedure) and it was replaced by the slower 73S (uncorr.) boundary when growth was resumed (Fig. 4*b*, *c*). Bacteria harvested in the mid-logarithmic phase and crushed before suspension in saline gave extracts identical in ultracentrifuge pattern to Fig. 4*c*. Although the 28, 44, 58, 73 and 85S components were not isolated and analysed, they all appeared to contain RNA, since incubation of the extracts for 10 min. at 37° with ribonuclease (final concentration 30 μg./ml.) abolished the 28, 44, 73 and 85S components. The 58S component survived this treatment but was partially degraded after 60 min. incubation. Ribonuclease treatment had the same effect on all extracts irrespective of the history of the bacteria from which they were prepared. It therefore seems likely that these boundaries were due to ribonucleoprotein particles as described by Tissières *et al.* (1959).

DISCUSSION

The experiments described here suggest that the induction of temperate phage development by u.v.-irradiation of a lysogenic strain was accompanied by alterations in the RNA and DNA metabolism in the host–phage system. Although Siegel *et al.* (1952) did not make any claims to this effect, their ultracentrifuge diagrams of extracts from phage T_2-infected *Escherichia coli* appeared to indicate that phage development might cause changes in the RNP particles of bacteria. By using more refined labelling and density-gradient techniques Brenner *et al.* (1961) concluded that no new ribosomes were made during the reproduction of phage T_4 in the infected bacteria. The present experiments lead to the conclusion that during the vegetative development of a temperate phage the synthesis of ribosomal material continued. Whilst this stands in contrast to the T-even phage systems it would seem to be consistent with the observation that in induced lysogenic bacteria a limited amount of growth (phase of residual growth) takes place during the latent period of phage development (e.g. Fig. 3*a*). Comparison of the ultracentrifugal and chemical analyses of the induced system with those of the irradiated non-lysogenic control indicates that the observed differences are not simply attributable to the effect of u.v.-irradiation. The inhibition of bacterial DNA metabolism by u.v.-irradiation reported by Kelner (1953) is confirmed here for the irradiated non-lysogenic strain, but in the irradiated lysogenic strain there were additional effects due to the initiation of phage development. In the latter case DNA metabolism was briefly inhibited

and then resumed at an accelerated rate as revealed by chemical analysis (see also Waites & Fry, 1964) and the changes in the 8S DNA component in the ultracentrifuge diagrams. It seems reasonable to ascribe these changes to vegetative development of the temperate phage. When extracts were made in tris buffer + magnesium acetate the bacteria were harvested from cultures in the mid-logarithmic phase of growth and then immediately suspended in saline. This procedure apparently initiated the progressive loss of the slower 73S (uncorr.) component and the appearance of a faster sedimenting species (85S, uncorr.). This was an unexpected change since McCarthy (1960) reported that a similar transition (85S corr. to 100S corr.) was characteristic of *E. coli* passing from the logarithmic phase of growth to the stationary phase. Hence one interpretation of the observations reported here is that simple resuspension of log-phase bacteria in saline rapidly initiated their transformation to a stationary phase. In agreement with McCarthy (1960) we also found that the 73S (uncorr.) RNP component was characteristic of tris buffer + Mg acetate extracts prepared from *E. coli* in the log phase. The reasons for these transitions are unknown but McCarthy (1960) and Horowitz & Weller (1964) have suggested that they may result from a configurational change in the particles in environments of various Mg^{2+} concentrations.

We gratefully acknowledge the kindness of Professor S. Dagley, who allowed us to use the analytical ultracentrifuge in the Department of Biochemistry, University of Leeds, for much of this work. We are indebted to the Department of Scientific and Industrial Research for a grant for the purchase of a similar centrifuge for the University of Sheffield.

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The Base Composition of Deoxyribonucleic Acids of Streptomyces

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SUMMARY

The base composition of DNA preparations from 12 strains of Streptomyces and 1 strain of Nocardia were determined from their denaturation temperature (T_m) and buoyant density.

The % GC (guanine + cytosine) contents ranged from 74·4 to 78·5 (from T_m) and from 69·4 to 73·4 (from buoyant density). The correlation between the two sets of data and the found differences are discussed. The range of pH values which did not affect T_m and the degree of hyperchromic effect have been related to the ionic strength of the solvent. With 0·01 M-phosphate as solvent the pH indifference range was narrower and the hyperchromic effect smaller than with 0·2 M-Na^+ solvent.

Though the difference between the maximum and minimum % GC in Streptomyces is sufficient to distinguish the respective strains, within the extreme values there is a continuous progression of base composition which does not permit taxonomic divisions to be made on the basis of overall % GC alone.

INTRODUCTION

The base composition of bacterial DNA, expressed as % GC (guanine + cytosine), varies widely from one genus to another, but, generally, very little from one species to another in the same genus (Lee, Wahl & Barbu, 1956; Belozersky & Spirin, 1960; Marmur, Falkow & Mandel, 1963). The DNA from any one strain is, however, very homogeneous in base composition, as shown by its narrow banding in caesium chloride density gradient ultracentrifugation, the narrow range of temperature over which it denatures, and its readiness to 'renature' under suitable cooling conditions. Close genetic relationship can be indicated by the formation *in vitro* of hybrid DNA molecules in which the two strands originate from different DNAs. To enable the formation of such hybrids, the two parent DNAs should have, as a first requirement, the same overall base composition and, as a second requirement, similar base sequences, which, in turn, imply similar genetic information, since the latter is held to be coded in the DNA precisely in the sequences of the four bases, guanine (G), cytosine (C), adenine (A), thymine (T). Therefore, we thought it opportune as a first step to determine the base composition of DNAs from strains of Streptomyces and Nocardia that had been included in a previous phenetic classification (Silvestri, Turri, Hill & Gilardi, 1962). For this purpose, two techniques were used: (*a*) to determine for each DNA sample the 'melting temperature' (T_m), which, according

to Marmur & Doty (1962), is linearly related to % GC; (*b*) to determine the buoyant density of the samples in CsCl gradient centrifugation, which according to Schildkraut, Marmur & Doty (1962) is again linearly related to % GC.

METHODS

Organisms. The strains of Streptomyces and Nocardia used were selected from those comprised in the study by Silvestri *et al.* (1962) according to three criteria: (*a*) that they were located in different spheres (or phenetic taxa) in the earlier phenetic classification; (*b*) that they were as near as possible to the respective geometrical centres of their spheres; (*c*) that they were nomenclatural type cultures or, at least, reference strains deposited in recognized collections. The strains chosen as satisfying these criteria are listed in Table 1.

Table 1. *Organisms used in this work*

PSA* no.	Name	Other reference nos.		Sphere	Group
	Streptomyces				
99	*S. kentuckensis*	ATCC*	12691	II	A
107	*S. parvulus*	ATCC	12434	XIV	D
45	*S. griseus*	ATCC	10137	VII	C
145	*S. parvus*	NRRL B	1455	IV	C
101	*S. griseolus*	ATCC	3325	XII	C
108	*S. flaveolus*	ATCC	3319	X	C
148	*S. diastaticus*	NRRL B	1270	III	B
22	*S. bobiliae*	ATCC	3310	VI	B
156	*S. fradiae*	ATCC	10745	IX	B
96	*S. intermedius*	ATCC	3329	XVII	B
60	*S. albus*	ATCC	618	XV	E
61	*S. fradiae*	ATCC	10745	XIII	F
	Nocardia				
165	*N. asteroides*	IMRU	727	XI	E

* ATCC = American Type Culture Collection. NRRL = North Regional Research Laboratory, Peoria, Ill., U.S.A. IMRU = Institute of Microbiology, Rutgers University, N.J., U.S.A. PSA = Progetto Sistematica Actinomiceti, Università Statale, Milano, Italia.

See the catalogue: *The Culture Collection of the Progetto Sistematica Actinomiceti*, 1962, and Silvestri *et al.* 1962.

Cultivation of organisms. Strains were inoculated with a wire-loop from slope cultures into 250 ml. flasks with an anti-vortex baffle containing 70 ml. of a liquid medium composed of: Bacto-Peptone (Difco) 5 g.; Bacto-Yeast Extract (Difco) 3 g.; glucose, 10 g.; Bacto-Casitone (Difco) 1 g.; Bacto-Beef Extract 2 g.; tap water 1 l. (For the Nocardia strain, better growth resulted with Bacto-Peptone and Bacto-Casitone replaced by Peptone and Caseina, 'Costantino', respectively.) Flasks were incubated at 30° on an agitator for 48–60 hr. Subcultures were then made into the same medium by transferring 0·5 ml. samples of fully grown stationary phase culture into several flasks which were re-incubated for 24–30 hr. By this procedure growth as pellets was generally avoided and the cultures were in the logarithmic phase when the mycelia were collected by Buchner filtration or centrifugation. From 5 to 10 g. (wet weight) of mycelia were used for each DNA preparation.

Preparation of DNA specimens. The procedure of Marmur (1961) was followed.

Particular attention had to be paid to render optimal the lysis by lysozyme and sodium lauryl sulphate. Incubation with lysozyme was continued for 2–3 hr. but suspensions, though becoming viscous, did not become transparent until the detergent was added. *Nocardia asteroides* was normally resistant to lysozyme, but partial lysis was induced by pretreating 25 ml. suspension with 0·5 ml. ethane thiol (C_2H_5SH), overnight in the cold with agitation. According to Dr M. Mandel (personal communication) 2-mercapto-ethanol, $CH_2(OH)CH_2SH$, is probably more efficient in uncovering lysozyme-sensitive sites. Final isopropanol precipitates were fibrous in most cases and were stored in the dry state.

Table 2. *Solutions used to determine the effect of ionic strength and pH value on T_m value with DNA from Streptomyces bobiliae*

Soln.	KH_2PO_4 0·2 M (ml.)	Na_2HPO_4 0·2 M (ml.)	EDTA 0·1 M (ml.)	Diluted to (ml.)	Molarity PO_4^{3-} (M)	pH value	Specific conductance $\times 10^{-3}$ mho	T_m (°)
1	2·5	2·5	10	200	0·005	6·7	0·831	75·6
2	2·5	2·5	10	100	0·01	6·8	1·570	80·5
3	2·5	2·5	10	50	0·02	6·7	2·979	85·8
4	2·5	2·5	10	25	0·04	6·7	5·590	90·8
5	4·5	0·5	10	100	0·01	5·85	1·250	76·5
6	3·5	1·5	10	100	0·01	6·40	1·440	79·25
7	1·5	3·5	10	100	0·01	7·10	1·720	81·6
8	0·5	4·5	10	100	0·01	7·50	1·923	81·8

Determination of T_m values. Solutions containing 10–20 μg. DNA/ml. were heated in a Beckman DU spectrophotometer fitted with thermal spacers (Marmur & Doty, 1962) and the increase in extinction on denaturation of the DNA (hyperchromic effect) observed. An initial reading was made at 25° and then, from 3–4° before the onset of denaturation to 2–3° after it had terminated, extinction readings were made at 1° intervals, the temperature being raised at the rate of about 1°/10 min. T_m value is defined as the mid-point (50 %) of the hyperchromic effect.

Solvents. The % GC content of a Streptomyces DNA has been reported to be in the order of 70–74 % (Belozersky & Spirin, 1960; Marmur & Doty, 1962) and therefore the T_m determination in the usual solvent SSC (standard saline citrate; 0·15 M-NaCl + 0·015 M-Na_3 citrate, pH 7·0) requires temperatures higher than 100° (Marmur & Doty, 1962). Use has therefore to be made of more dilute solvents. It is reported in the literature (e.g. Ts'o, Helmkamp & Sander, 1962), that the T_m value is proportional to the logarithm of the ionic strength of the solvent and that, with SSC solvent, the pH value does not influence the T_m value within the range pH 5·5–8·5. Two sets of solutions were prepared (Table 2) to determine the effect on T_m value of changing ionic strength (pH constant) and of changing pH value in a region of an ionic strength lower than that of SSC solvent (molarity being constant). Specific conductance of solutions was determined with a Philips Conductivity Bridge, model GM 4249. By plotting T_m values against the logarithm of specific conductance (Fig. 1) it is seen that the first four solutions, changing ionic strength, pH constant, fall on a straight line. With 0·01 M-PO_4^{3-}, pH changing, the indifference range of pH values was narrower than that reported for SSC solvent, since solutions at pH 6·4 and 7·5 already departed from the straight line. During these experiments

it was noticed that the hyperchromic effect is also influenced by ionic strength. A quadratic relationship was found. The more dilute the solvent the less was the hyperchromic effect (e.g. in solution 1 the hyperchromic effect in duplicate samples was 23·1 and 21·9%, whereas in solution 4 it was 27·2 and 27·9%) (Fig. 2). The degree of hyperchromicity is, however, less reproducible than T_m in replicate experiments. The hyperchromicity varies with conductance according to the following parabolic function:

$$\% \text{ hyperchromicity} = 22{\cdot}40 - 0{\cdot}23 \log x + 9{\cdot}74 (\log x)^2,$$

where x is the specific conductance of the solution.

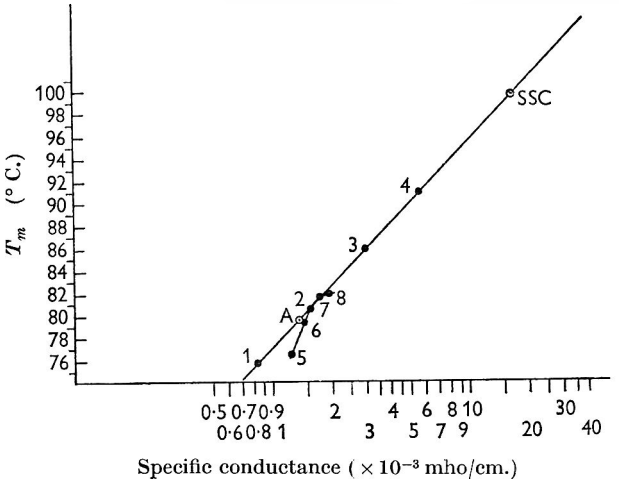

Fig. 1

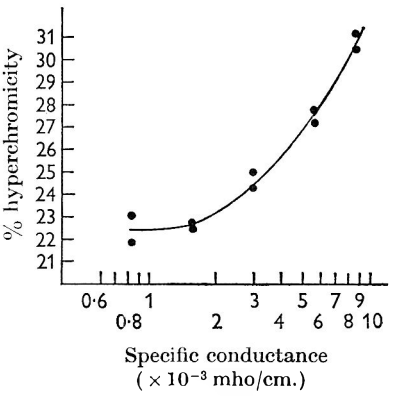

Fig. 2

Fig. 1. The relationship between T_m values and ionic strength (DNA from *Streptomyces bobiliae*). Solvents 1–8, see Table 2. SSC = standard saline citrate solvent. A = phosphate solvent used for T_m determinations.

Fig. 2. The relationship between the hyperchromie effect and ionic strength. DNA from *Streptomyces bobiliae*, duplicate samples.

The solvent finally chosen, as giving T_m values 20° less than the SSC solvent, was: Na_2HPO_4, 0·2 M, 2·5 ml. + NaH_2PO_4, 0·2 M, 2·5 ml. + EDTA, 0·01 M, 10 ml.; diluted to 100 ml.; pH 6·8; specific conductance $1{\cdot}345 \times 10^{-3}$ mho. Under our conditions, the specific conductance of SSC solvent was 16×10^{-3} mho and, from Fig. 1, the above phosphate solution (indicated by A in Fig. 1) should yield T_m values 20·1° less than in SSC solvent.

Buoyant density. The technique of Meselson, Stahl & Vinograd (1957) was used with *Escherichia coli* B DNA as reference (1710 g./cm.³). CsCl was obtained from Merck (Darmstadt; ELH grade) and used with no further purification. Runs were made in a Spinco model E analytical ultracentrifuge and lasted 24 hr at 44,770 rev./ min.

RESULTS

Thermal denaturation. We previously reported on the reproducibility of T_m values (Hill & Silvestri, 1963); in the present work the largest difference obtained with duplicate samples was 0·25° with the same batch of solvent. T_m values for the various

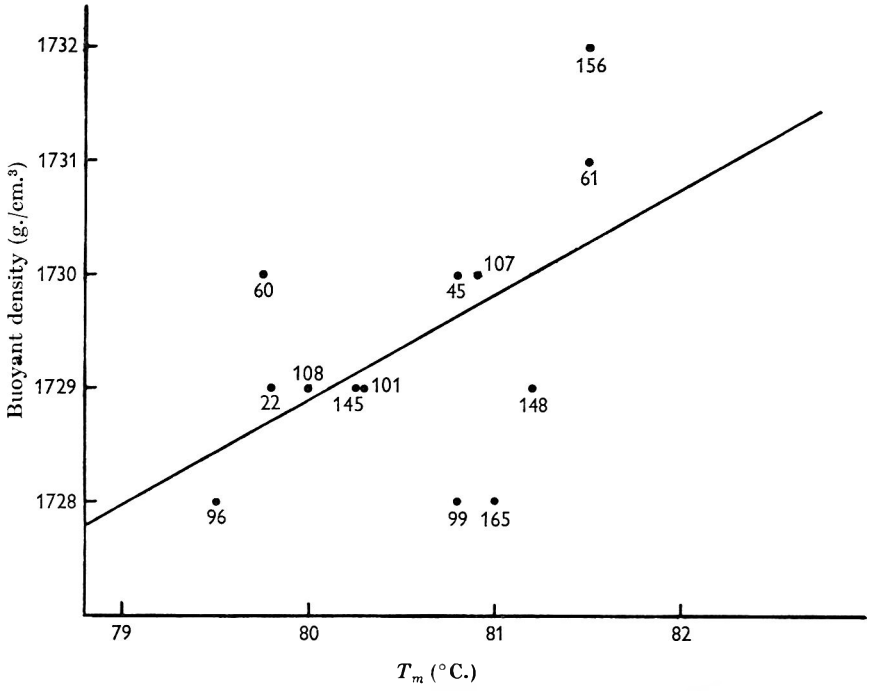

Fig. 3. The correlation between T_m and buoyant density. Numbers in the figure refer to the organisms in Table 1. $r = 0{\cdot}502$, $n = 11$, $P = 0{\cdot}07$.

Table 3. *% GC from T_m and buoyant density (ρ) values of various DNA preparations*

Strain		(1) T_m (°)	(2) % GC from T_m	(3) in g./cm.³	(4) % GC from ρ	(5) Difference 2−4 (%)
Streptomyces						
S. kentuckensis	99	80·8	76·8	1·728	69·4	+7·4
S. parvulus	107	80·9	77·2	1·730	71·4	+5·8
S. griseus	45	80·8	76·8	1·730	71·4	+5·4
S. parvus	145	80·25	75·5	1·729	70·4	+5·1
S. griseolus	101	80·3	75·6	1·729	70·4	+5·2
S. flaveolus	108	80·0	74·8	1·729	70·4	+4·4
S. diastaticus	148	81·2	77·8	1·729	70·4	+7·4
S. bobiliae	22	79·8	74·4	1·729	70·4	+4·0
S. fradiae	156	81·5	78·5	1·732	73·4	+5·1
S. intermedius	96	79·5	73·6	1·728	69·4	+4·2
S. albus	60	79·75	74·3	1·730	71·4	+2·8
S. fradiae	61	81·5	78·5	1·731	72·4	+6·1
Nocardia						
N. asteroides	165	81·0	77·3	1·728	69·4	+7·9

Notes. Col. 1 in 0·01 M-PO_4 + 0·001 M-EDTA (pH 6·8). Col. 2 $T_m = 49{\cdot}3 + 0{\cdot}41$(GC). Col. 3 relative to *Escherichia coli* B NDA = 1·710 g./cm.³ Col. 4 $\rho = 1{\cdot}660 + 0{\cdot}098$ (GC).

samples are listed in column 1, Table 3, together with % GC calculated (column 2) by means of the linear relationship (Marmur & Doty, 1962): $T_m = 49{\cdot}3 + 0{\cdot}41$ (GC).

Buoyant density, ρ. Values are listed in column 3, Table 3, together with % GC calculated (column 4) by means of the linear relationship (Schildkraut *et al.* 1962): $\rho = 1{\cdot}660 + 0{\cdot}098$(GC).

Correlation between T_m and buoyant density values. The correlation coefficient between the two sets of values was calculated, $r = 0{\cdot}502$, $n = 11$, $P = 0{\cdot}07$ (Fig. 3). The correlation coefficient between T_m values (Hill & Silvestri, 1963) in an Na_2HPO_4 solvent, adjusted to pH 7·0 with NaOH, gave the following results: $r = 0{\cdot}6275$, $n = 10$, $P = 0{\cdot}03$.

The better correlation of the previously published data can be attributed to the fact that then determinations were conducted with the same batch of solvent. Changing batches with such dilute solvents introduces another source of variance.

Difference in % GC according to T_m and buoyant density values. In Table 3, column 5, the differences in % GC calculated from T_m and from buoyant density are recorded. Those calculated from T_m were always higher than those calculated from buoyant density, the differences ranging from a minimum of 2·8% to a maximum of 7·9%; the average difference was 5·46% GC.

In the studies of Marmur & Doty (1962) and Schildkraut *et al.* (1962), only one streptomycete DNA (from *Streptomyces viridochromogenes*) was examined for T_m and buoyant density. These authors too found a difference between % GC calculated from T_m (74%) and from buoyant density (70%). Chromatographic analysis of the bases yielded a % GC of 73·8% for this species (Belozersky & Spirin, 1960). Schildkraut *et al.* (1962) discussed several explanations for this discrepancy, perhaps the most important of which was the possible existence of bases other than the usual four.

With respect to our data, the range of % GC calculated from T_m values using the equation of Marmur & Doty (1962) is from a minimum of 73·6% to a maximum of 78·5%.

Range of % GC within Streptomyces. The 2° difference between the minimum T_m value (strain 96 *Streptomyces intermedius*) and the maximum T_m (strains 61 and 156, both *S. fradiae*) corresponds to a % GC range of 4·9%. Again, the % GC from the buoyant density data is 4% (0·004 g./cm.^{3}) difference between the minimum buoyant density (strains 99, 96, 165) and the maximum (strain 156).

DISCUSSION

The range of % GC in Streptomyces seems to be very restricted. Jones & Bradley (1963), with fewer strains, found a yet narrower range. Though the difference between maximum and minimum % GC is large enough to allow a clear distinction between the extreme strains, the fact that there is a continuous progression of base composition precludes an attempt to make taxonomic divisions on the basis of overall % GC composition alone. Only one strain of Nocardia was included in this study, but apparently distinction between the two genera on this basis alone seems impossible. This continuity of DNA composition within a genus was also observed by De Ley & Schell (1963) in acetic acid bacteria. Given that the maximum difference in % GC was about 4%, then even the strains most distant from one

another could have in common many DNA molecules (Sueoka, 1961). Broad-spectrum actinophages are known such as 1091 (Silvestri *et al.* 1962) which are active against strains which differ by as much as 2% GC. Furthermore, reports exist in the literature of transformation between *Streptomyces aureofaciens* and *S. griseus* (Jarai, 1961), of heterokaryosis between *S. cyaneus* and *S. griseus* (Bradley & Lederberg, 1956) and of recombination between *S. rimosus*, *S. coelicolor* and *S. aureofaciens* (Alacevic, 1963). On the basis of the present data concerning DNA base composition, phage sensitivity and genetic exchange, it seems legitimate to doubt of the existence of isolated species within the *Streptomyces* taxon, which is presently considered to merit a generic rank. It is probable that the process of 'splitting' has been carried too far. By phenetic taxometric studies it is easier to resolve the genus *Streptomyces* into distinguishable groups. However, in view of the available facts, we are doubtful whether the phenetic groups (spheres) individuated by electronic computer could be properly considered species deserving distinct binomials. On the other hand, the relative scarcity of molecular and genetic data within the genus does not permit a more definite conclusion at present, neither an evaluation of the taxonomic rank of the phenetic taxa.

It is interesting to note that strain 99 *Streptomyces kentuckensis*, which is in the middle of the % GC range found here within the genus, occupied also a central position in the phenetic hyperspace of our previous taxometric study (Silvestri *et al.* 1962). This organism is near the geometric centre of the second sphere, which is itself located in a very central position in respect to the entire system.

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Use of Antibiotics for Selective Isolation and Enumeration of Actinomycetes in Soil

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SUMMARY

Several antibiotics were tested against a range of actinomycetes, bacteria and fungi representing types found in soil. From these tests four antibiotics, nystatin (50 μg./ml.), actidione (50 μg./ml.), polymyxin B sulphate (5·0 μg./ml.) and sodium penicillin (1·0 μg./ml.), were selected for incorporation into a starch + casein medium to achieve selective growth of actinomycetes on soil dilution plates. This mixture of antibiotics was tested with a number of soils and its efficiency compared with several other methods for selective development of actinomycete colonies. The most suitable mixture for the enumeration of soil actinomycete colonies was starch + casein medium with the two antifungal antibiotics (nystatin, actidione); for isolation of actinomycete colonies the same medium with all four antibiotics was the most satisfactory.

INTRODUCTION

Studies of actinomycetes in soil are usually made with the dilution plate technique. The number of propagules of these organisms in most soils is intermediate between those of bacteria and fungi, so dilutions suitable for colony counting or isolation allow the development of large numbers of bacteria. At such dilutions the numbers of fungal colonies are usually low, but with their large radial spread they can still interfere with the analysis of actinomycetes on the plates. In acid soils, relative numbers of fungal colonies are often high enough to make analysis impossible. Many attempts have been made to overcome these problems, including the use of selective nutrients in media, pre-treatment of soil samples or soil suspensions, and incorporation of selective inhibitors into media.

Several substances have been suggested as selective substrates for actinomycetes. The use of L-arginine as a selective nitrogen source favouring actinomycetes over bacteria was reported by Porter, Wilhelm & Tresner (1960) and El Nakeeb & Lechevalier (1963). Küster & Williams (1964), who examined several carbon and nitrogen sources, found starch (or glycerol) + casein + nitrate to be the most selective mixture. Use of chitin as sole carbon and nitrogen source was recommended by Lingappa & Lockwood (1961, 1962).

Tsao, Leben & Keitt (1960) reported increased selective development of actinomycetes when air-dried soil was re-moistened, mixed with calcium carbonate and incubated at 28°. Agate & Bhat (1963) attempted suppression of bacteria and fungi by pre-incubation of soil at 110° for 10 min. and the use of dried plates. Methods such as these are of little use for ecological studies, where the situation in the soil

at the time of sampling is of prime interest. Treatment of soil suspensions with a 1·4% (w/v) phenol solution was recommended by Lawrence (1956); Řeháček (1959) centrifuged suspensions at speeds selected to separate actinomycete spores from other propagules. In a comparative study of some of these methods, El Nakeeb & Lechevalier (1963) found that the calcium carbonate treatment gave highest colony counts, while the centrifugation and phenol treatments gave counts lower than those from untreated suspensions.

Many workers have used antibiotics in media to achieve selective inhibition of various groups of organisms. The use of antibacterial antibiotics to improve the efficiency of media for isolating fungi has been reported by Martin (1950), Johnson (1957), and others. Antibiotics which inhibit fungi have been found particularly useful in studies on actinomycetes. Actidione (cycloheximide) has been used by Dulaney, Larsen & Stapley (1955), Corke & Chase (1956, 1964), Corbaz, Gregory & Lacey (1963) and Porter *et al.* (1960) who also recommended nystatin and pimaricin. To achieve suppression of bacteria while allowing growth of actinomycetes presents more difficulties since most antibacterial antibiotics also inhibit actinomycetes. Porter *et al.* (1960) reported that attempts to obtain selective inhibition of bacteria with antibiotics were unsuccessful, but Dulaney *et al.* (1955) recommended a mixture of antibacterial and antifungal antibiotics to allow selective development of actinomycetes.

Although many workers have used antibiotics in media to select actinomycetes, few details are available about their relative effects on the various groups of soil organisms, either in pure culture or on soil dilution plates. Therefore it was decided to make a study of the value of the use of antifungal and antibacterial antibiotics for the selective isolation and enumeration of actinomycetes. The antibiotics were tested in the starch casein medium already shown to be selective for actinomycetes by Küster & Williams (1964). The effect of antibiotics, singly and in admixture, on pure cultures of soil-inhabiting organisms was first studied. The antibiotics selected from these tests were then used to prepare soil dilution plates in several comparative experiments.

METHODS

Selection of antibiotics. The antibiotics to be used in detailed tests were selected by a preliminary experiment in which they were tested against a range of actinomycetes. The antibiotics used were Nystatin (Squibb); Actidione (Light); Polymyxin B sulphate (Burroughs Wellcome); sodium benzyl penicillin (Glaxo); Streptomycin sulphate (Glaxo); Chloramphenicol (Allen and Hanburys); Chlortetracycline hydrochloride (Cyanamid).

The test organisms were 45 Streptomyces species, *Micromonospora melanosporea*, *Waksmania rosea* and a *Streptosporangium* sp. These tests and subsequent ones in this work were done with a starch + casein medium adjusted to pH 7·0 (Küster & Williams, 1964). Solutions of the sterile antibiotics were prepared in sterile water; for the insoluble nystatin a suspension was made. Antibiotic solutions were added to molten medium at 45° to give concentrations of 0, 1·0, 5·0, 10·0, 30·0, 50·0, and 100·0 μg./ml. One-ml. samples of spore suspensions of test organisms were mixed with 9 ml. samples of media and plates poured. Spore suspensions were prepared by incorporating one loopful of a mature sporing culture into 10 ml. of sterile water.

Plates were incubated at 25° for 7 days; development of micro-organisms on antibiotic plates were compared with that on control plates and any inhibition of growth noted.

Testing of selected antibiotics against actinomycetes, bacteria and fungi. The four antibiotics selected from the preliminary tests were then tested against cultures of soil-inhabiting organisms comprising 25 bacteria, 30 fungi and 114 actinomycetes. The four antibiotics were tested in various mixtures, the method of testing being as described, except that several tenfold dilutions of the suspensions of test organisms were plated. The dilutions selected for reading were those giving about 100 colonies/plate for bacteria, 50 for actinomycetes and 10 for fungi. By this means, masking of inhibition by over-concentrated suspensions was avoided. Results were recorded after incubation for 14 days at 25°, by comparison of antibiotic plates with control plates. Inhibition was recorded as 'complete' = no growth, 'partial' diminished growth and 'none' = growth identical with controls.

Comparison of soil dilution plates prepared with and without antibiotics. Experiments were done to study the effect of the antibiotics selected from the tests on pure cultures, on the dilution plate colony counts of organisms from several soil samples. The soils used were a garden soil from Ness, Cheshire (pH 5·4), a garden soil from Oxton, Cheshire (pH 6·2) and two horizons from a forest soil at Freshfield, Lancashire, the A horizon being at pH 4·2–5·8 and the C horizon at pH 7·2–8·6. These soils differed considerably in their total microbial content and in the relative proportions of actinomycetes, bacteria and fungi. Thus they were suitable for making an assessment of the various antibiotics for obtaining selective growth of actinomycetes.

Preparation of dilution plates. Soil (1 g.) was added to 100 ml. sterile water in a screw-top bottle and shaken on a reciprocal shaker for 30 min. From the resulting suspension tenfold dilutions were prepared; 10 ml. samples of these were used to seed 90 ml. molten starch + casein medium at 45°, the antibiotics when used being added to the medium at the selected concentrations just before this. After thorough mixing, the medium was dispensed by using a tilting measure to deliver 10 ml. to each plate. Twenty plates were prepared for each dilution, 10 plates with medium unsupplemented and 10 with antibiotics added. This seeding method was found in preliminary tests to be the most satisfactory for decreasing the spread of bacterial colonies developing on the plates. Plates were incubated at 25° for 14 days; the most suitable dilutions for counting were selected and colonies of bacteria, actinomycetes and fungi were counted by using a plate counter with a magnifying lens. Counts were made after 7 and 14 days; whenever possible 14-day counts were selected but in some cases, especially on control plates, spreading fungi made counting after 14 days impossible.

Comparison of starch + casein medium plus antibiotics with some other media selective for actinomycetes. Plate counts of three soils obtained by using starch + casein with all four antibiotics and with the two antifungal ones only were compared with those obtained by using several other suggested media. These media were prepared according to the directions of the various workers and were as follows: 0·2 % colloidal chitin medium (Lingappa & Lockwood, 1962); water agar (Lingappa & Lockwood, 1962); glycerol + arginine medium with antifungal antibiotics (Porter *et al.* 1960); glycerol + arginine + salts medium (El Nakeeb & Lechevalier, 1963); egg

albumin medium (Waksman, 1961). The preparation of dilution plates and counting procedures used were as described for the previous experiment.

RESULTS

Selection of antibiotics

Of the seven antibiotics tested against a range of actinomycetes, the antifungal ones (nystatin, actidione) did not inhibit any strains even at the highest concentration of 100 μg./ml. Similar results were obtained by Okami, Hashimoto & Suzuki (1959) and by Porter *et al.* (1960). Of the antibacterial antibiotics, streptomycin, chloramphenicol and chlortetracycline inhibited 20–50% of the strains even at the lowest concentration of 1·0 μg./ml. The least inhibition by antibacterial antibiotics was obtained by polymyxin B sulphate (no inhibition at 5·0 μg./ml.) and sodium penicillin (10% strains inhibited at 1·0 μg./ml.). Therefore it was decided to use polymyxin B sulphate at 5·0 μg./ml. and sodium penicillin at 1·0 μg./ml. together with the two antifungal antibiotics for detailed testing.

Table 1. *The effect of nystatin and actidione on the growth of the fungi tested*

	Actidione (50 μg./ml.)	Nystatin (50 μg./ml.)	Actidione (50 μg./ml.) + nystatin (50 μg./ml.)
Percentage of fungi completely inhibited	22	69	84
Percentage of fungi partially inhibited	56	22	16
Percentage of fungi not inhibited	22	9	0

Table 2. *The reaction of some soil fungi to actidione + nystatin (each 50 μg./ml.)*

All the fungi tested were inhibited either completely or partially.

Fungi completely inhibited	Fungi partially inhibited
Pythium sp.	*Mortierella alpina*
Mortierella parvispora	*Mortierella marburgensis*
M. vinacea	*Absidia spinosa*
Mucor hiemalis	*Chaetomium* sp.
M. ramannianus	*Paecilomyces carneus*
Zygorhynchus sp.	
Sordaria sp.	
Perisporium vulgare	
Basidiomycetes B1, 2, 3	
Phoma sp.	
Aspergillus sp. (*a*)	
Aspergillus sp. (*b*)	
Cladosporium herbarum	
Gliocladium roseum	
Oidiodendron fuscum	
Penicillium decumbens	
P. lanosum	
P. spinulosum	
Trichoderma viride	
Cylindrocarpon radicicola	
Fusarium culmorum	
Sterile dark forms *a*, *b*	

Testing of selected antibiotics against actinomycetes, bacteria and fungi

Antifungal antibiotics. Nystatin and actidione were tested separately and together at 50 μg./ml. against a range of fungi isolated from soil; the results of these tests are given in Table 1. The highest percentage inhibition was obtained with nystatin and actidione together, none of the test fungi being completely resistant. When used separately, nystatin gave better results than actidione. The efficiency of the two together was largely due to the differences in their antifungal spectra rather than to synergistic action. Results obtained with 100 μg./ml. of each together were identical with those for the 50 μg./ml. concentrations.

Table 3. *The reaction of some soil bacteria to polymyxin B sulphate (5·0 μg./ml.) + sodium penicillin (1·0 μg./ml.)*

Completely inhibited	Partially inhibited	Not inhibited
Achromobacter anitratus (NCTC 8102)	*Arthrobacter* sp.	*Agrobacterium tumefaciens* (NCPPB 397)
Bacillus laterosporus (NCTC 7579)	*B. megaterium*	*B. mycoides* (NCTC 926)
B. licheniformis (NCTC 7589)	*Pseudomonas chlororaphis* (NCTC 7537)	*B. subtilis*
B. pantothenticus (NCTC 8162)		*Chromobacterium lividum* (NCTC 9796)
Bacillus sp.		
Chromobacterium violaceum (NCTC 9757)		
Mycobacterium phlei (NCTC 8151)		
Pseudomonas fluorescens		
Sarcina lutea		
Vibrio percolans		

In Table 2 details of the reaction of individual species of fungi to nystatin + actidione are given. The fungi were chosen to cover a wide taxonomic range and to include species commonly occurring on soil dilution plates. Species such as Penicillium, *Trichoderma viride*, *Cladosporium herbarum* and *Mucor hiemalis*, which often spread widely on soil-dilution plates, were completely inhibited. It is interesting to note that the *Pythium* sp., the only representative of the Oomycetes tested, was not inhibited by nystatin alone. It was suggested by Kinskey (1962) that nystatin acts by altering the permeability of the chitinous cell membrane of sensitive fungi, so that lower Phycomycetes, which have more cellulose in their walls, are resistant.

The efficiency of nystatin + actidione was also compared with two other suggested antifungal agents, 0·4% (w/v) sodium propionate in the medium (Crook, Carpenter & Klens, 1950) and the phenol treatment of suspensions (Lawrence, 1956). The results indicated that the antibiotics achieved a much greater selective inhibition of fungi, and that a mixture of nystatin + actidione (each 50 μg./ml.) added to the medium achieved efficient suppression of fungi on soil dilution plates.

Antibacterial antibiotics. Sodium penicillin (1·0 μg./ml.) and polymyxin B sulphate (5·0 μg./ml.) were tested separately and together against a range of bacteria chosen to include types which commonly occur on soil dilution plates. Their reaction to a mixture of these antibiotics is given in Table 3. Many of the test strains were inhibited, but the results were not as convincing as those obtained with the antifungal antibiotics. Two bacterial species found frequently on soil dilution plates, namely *Bacillus subtilis* and *B. mycoides*, were resistant.

Antifungal and antibacterial antibiotics together. All four antibiotics were next retested against the bacteria and fungi to ensure that their action was not impaired when they were together in the medium. The results were like those previously obtained.

The mixture of antibiotics was finally tested against a series of Streptomyces together with *Micromonospora melanosporea*, *Waksmania rosea* and *Streptosporangium* sp. The reaction of some named strains is given in Table 4; many commonly occurring soil types (e.g. *Streptomyces griseus*, *S. lavendulae*, *S. flavovirens*) tolerated the antibiotics as did also the representatives of the three other actinomycetes.

In Table 5 the overall effects of the antibiotic mixture on actinomycetes is compared with those on fungi and bacteria. These results, with high percentage inhibition of fungi, moderate inhibition of bacteria and low percentage inhibition of actinomycetes, indicated that this mixture of antibiotics might be used to improve the selectivity of the starch + casein medium for development of actinomycetes on soil dilution plates.

Table 4. *The reaction of some actinomycetes to a mixture of the four antibiotics: actidione, nystatin, each* 50 *μg./ml., polymyxin B sulphate* 5·0 *μg./ml., sodium penicillin* 1·0 *μg./ml.*

Completely inhibited: *Streptomyces glaucescens* (ETH*24204), *S. hygroscopicus* (CBS)

Partially inhibited: *S. purpureochromogenes* (CBS)

Not inhibited: *S. azureus* (ETH28555), *S. albus* (ATCC618), *S. californicus* (CBS), *S. cinereoruber* (ETH7451), *S. cinnamoneus* (ETH23996), *S. diastaticus* (CBS), *S. coelicolor* (CBS), *S. flavovirens* (CBS), *S. griseolus* (CBS), *S. griseus* (WC3475), *S. halstedii* (CBS), *S. lavendulae* (WC3440/14), *S. lipmanii* (CBS), *S. netropsis* (ETH24299), *S. phaeochromogenes* (CBS), *S. venezuelae* (CBS), *S. violaceoruber* (WC3030), *S. viridochromogenes* (CBS).

* ETH, Eidige Technische Hochshule, Zurich, Switzerland. CBS, Centraalbureau voor Schimmelcultures, Baarn, Netherlands. ATCC, The American Type Culture Collection, Washington, D.C., U.S.A. WC, The Waksman Collection, The State University, Rutgers, N.J., U.S.A.

Table 5. *Summary of the effects of a mixture of* 4 *antibiotics* (*actidione, nystatin, each* 50 *μg./ml., polymyxin B sulphate* 5·0 *μg./ml., sodium penicillin,* 1·0 *μg./ml.*) *on actinomycetes, bacteria and fungi*

	Strains completely inhibited (%)	Strains partially inhibited (%)	Strains not inhibited (%)	No. of strains tested
Actinomycetes	6	13	81	114
Bacteria	65	15	20	25
Fungi	84	16	0	30

Comparison of dilution plates prepared with and without antibiotics

Results obtained with the starch + casein medium, with and without the four antibiotics used to prepare dilution plates of four soils, are given in Table 6. The pairs of figures marked with an asterisk were significantly different at the 0·01 probability level. It is evident that the presence of the antibiotics had little effect on the numbers of actinomycete colonies. In soils where numbers of fungal colonies on control plates were low (samples from Oxton and Freshfield, C horizon), numbers of actinomycetes colonies decreased slightly on the antibiotic medium, reflecting the inhibition of some propagules by the antibacterial antibiotics. For

soils with higher numbers of fungal colonies on control plates (samples from Ness and Freshfield A horizon), the numbers of actinomycete colonies on the antibiotic plates were slightly higher. On these plates the inhibition of fungi, allowing the development and detection of more actinomycetes, more than counteracted the small degree of inhibition by the antibacterial antibiotics.

In contrast, the numbers of fungal and bacterial colonies were significantly decreased by the presence of the antibiotics. The decrease in bacterial colonies ranged from 55 to 81 %, while fungal colonies were completely eliminated with all the soil samples tested. It was noticed that the antibiotics decreased the rate of growth of some actinomycete colonies, their development lagging 4–5 days behind those on control plates. However, after incubation for 14 days, most colonies were well developed and often sporing heavily.

Table 6. *Comparison of plate counts obtained on starch+casein medium unsupplemented and with 4 antibiotics*

Soil sample	Dilution	Without antibiotics Average no. colonies/plate		With antibiotics Average no. colonies/plate	Change on plates with antibiotics (%)
Freshfield soil, A horizon	Actinomycetes	43		44	+ 2
	Bacteria $1/10^3$	196	*	45	− 77
	Fungi	14	*	0	−100
Freshfield soil, C horizon	Actinomycetes	38		36	− 5
	Bacteria $1/10^3$	121	*	54	− 55
	Fungi	2	*	0	−100
Ness garden soil	Actinomycetes	20		22	+ 10
	Bacteria $1/10^4$	137	*	60	− 56
	Fungi	10	*	0	−100
Oxton garden soil	Actinomycetes	20		18	− 10
	Bacteria $1/10^4$	304	*	58	− 81
	Fungi	3	*	0	−100

* Figures significantly different ($P = 0{\cdot}01$).

Comparison of starch+casein medium+antibiotics with some other media selective for actinomycetes

Plate counts of actinomycete colonies were obtained for three soils on starch+casein medium with all four antibiotics, the same medium with only the two antifungal antibiotics and five other media selective for actinomycetes. Details of these results are given in Table 7. Figures marked with an asterisk differed significantly ($P = 0{\cdot}01$) from figures for the same soil obtained by using the starch+casein medium with all four antibiotics. Starch+casein medium containing the two mixtures of antibiotics and the chitin medium of Lingappa & Lockwood (1962) gave significantly higher colony counts than the other media in all cases except for the figure for the Oxton soil plated on glycerol+arginine medium. The counts for the three soils obtained on the chitin medium and on starch+casein+antifungal antibiotics were very similar, and as might be expected the figures for the latter were higher than those for starch+casein medium with all four antibiotics. Of the two media with very similar composition (glycerol+arginine, Porter *et al.* 1960;

arginine + glycerol + salts, El Nakeeb & Lechevalier, 1963), the Porter *et al.* medium with the recommended addition of antifungal antibiotics gave slightly higher counts.

When the percentage composition of the colonies on the plates was considered, starch + casein medium + 4 antibiotics clearly gave the highest percentage of actinomycetes, with the chitin medium second best. Starch + casein medium + antifungal antibiotics (nystatin, actidione) and the other media gave similar percentages of actinomycete colonies. Thus differences in the total numbers of actinomycete colonies on these media were paralleled by differences in numbers of bacterial colonies (the numbers of fungal colonies being too small to have a significant effect), indicating that the selectivity of these media for actinomycetes over bacteria was virtually the same.

Table 7. *Comparison of results obtained by using several media selective for actinomycetes*

	Average no. actinomycetes colonies/plate				Average % actinomycetes of total colonies/plate			
	Oxton soil dilution ($1/10^4$)	Freshfield soil horizon dilution ($1/10^3$) A	Freshfield soil horizon dilution ($1/10^3$) C	Average of the 3 values	Oxton soil	Freshfield soil horizon A	Freshfield soil horizon C	Average of the 3 values
Starch + casein medium + antifungal† and antibacterial‡ antibiotics	16	36	24	25·3	13	51	75	46·3
Starch + medium + antifungal antibiotics	20	44	27	30·3	6	23	37	22·0
0·2 % chitin medium (Lingappa & Lockwood, 1962)	20	45	21	28·7	9	37	45	30·3
Glycerol + arginine medium (Porter *et al.* 1960)	13	27*	7*	15·7	5	21	33	19·7
Arginine + glycerol + salts (El Nakeeb & Lechevalier, 1963)	11*	25*	5*	13·7	8	18	38	21·3
Egg albumin (Waksman, 1961)	11*	21*	4*	12·0	10	24	25	19·7
Water agar (Lingappa & Lockwood, 1962)	5*	16*	6*	9·0	3	33	37	24·3

* Figures significantly different from those obtained with starch + casein with all antibiotics ($P = 0{\cdot}01$).

† Antifungal = nystatin + actidione, each 50 μg./ml.

‡ Antibacterial = polymixin B sulphate 5·0 μg./ml. + sodium penicillin 1·0 μg./ml.

DISCUSSION

When selecting media for the preparation of soil dilution plates it is important to distinguish between enumeration and isolation of the colonies which develop. For counting colonies, the most suitable media are those which allow development and detection of the maximum numbers of colonies of the organisms under study.

From the results obtained here, the most satisfactory media for counting actinomycete colonies were the starch + casein medium + the antifungal antibiotics, nystatin and actidione, and the chitin-containing medium. These gave similar numbers of actinomycete colonies; starch + casein medium + antibiotics allowed development of more bacteria (an average of 165 colonies/plate compared with 101 for chitin) and hence gave a lower percentage of actinomycete colonies. However, use of the chitin medium had certain disadvantages: the opalescence of the medium made detection and classification of small colonies difficult, particularly when these did not clear the medium. Some actinomycete colonies developed well and cleared the surrounding medium, but others were almost pin-point colonies and were difficult to distinguish from bacteria without microscopic examination. Also the development of pigments, so characteristic of many actinomycetes, did not occur on this chitin-containing medium. It must be noted that use of the chitin medium without antibiotics, as recommended by Lingappa & Lockwood, allowed development of fungi (an average of 5 colonies/plate) which although growing thinly had a large radial spread. Therefore the starch + casein medium + nystatin + actidione, being a clear medium and allowing good development of aerial mycelium and of pigmentation, is considered to be the most suitable for the enumeration of actinomycete colonies.

For isolation purposes, a medium should give a reasonably high total numbers of the organisms under study and these should comprise as high as possible a percentage of the population on the plates to minimize overgrowth by other groups. The media best fulfilling these requirements for actinomycetes were the starch + casein medium + the 4 antibiotics and the chitin-containing medium. Of these, starch + casein + antibiotics gave lower numbers of colonies but a higher percentage of actinomycete colonies due to its greater selectivity against bacteria (an average of 49 colonies/plate compared with 101 for the chitin medium). Most of the disadvantages of the chitin medium already outlined also apply when it is used for the isolation of actinomycetes. Lack of distinguishing pigmentation prevents any preliminary grouping of the colonies on the plates, so all have to be transferred to another medium. Comparison of the number of different types of actinomycetes isolated from the two Freshfield soils by random selection and transfer of colonies showed no significant difference between the numbers obtained from the starch + casein + 4 antibiotics, starch + casein + nystatin + actidione only, and the chitin medium. Therefore the most suitable medium for the isolation of actinomycetes was the starch + casein medium + 4 antibiotics. However, the antibacterial antibiotics in this medium inhibited the development of some actinomycetes, so that the results obtained for a soil depended on the numbers and proportions of susceptible strains present. It is therefore advisable to conduct a preliminary experiment comparing results with and without the antibacterial antibiotics, before using this method in an ecological investigation.

The potentialities of using antibiotics to prepare soil dilution plates have been discussed. However, their use also permits the application to ecological studies of soil actinomycetes of several other techniques which have been shown to have considerable value in studies of the ecology of soil fungi. Most of these are not normally applicable to actinomycetes because of their low competitive ability on plates in the presence of bacteria and fungi. Preliminary work has been carried out on the isolation of actinomycetes from various micro-habitats in soil by using starch + casein medium + the 4 antibiotics. These micro-habitats included mineral and organic particles washed by the technique described by Parkinson & Williams (1961) and Williams, Parkinson & Burges (1965) and macerated washed roots of *Pinus nigra*. Such approaches have theoretical advantages over the use of dilution plates; there is a greater chance of isolating organisms from active mycelium, and separate soil micro-habitats can be studied. From the practical viewpoint, the plates obtained are much more easily and accurately analysed. It is felt that such techniques could be applied profitably in future studies on the ecology of actinomycetes in soil.

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Determination of Kinetic Constants for Nitrifying Bacteria in Mixed Culture, with the Aid of an Electronic Computer

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SUMMARY

An electronic computer was used to integrate differential equations for bacterial growth, and thus to determine simultaneously growth-rate constants, saturation ('Michaelis') constants, and initial numbers of bacteria. In one case numerical methods were necessary to effect the integration and the work would have been prohibitively great if a computer had not been used. By this means curves have been fitted to experimental data on the course of nitrification in water from the Thames Estuary.

Kinetic constants for Nitrosomonas and Nitrobacter at various temperatures were obtained, and the rate of death of these bacteria in the absence of their substrates was estimated.

INTRODUCTION

The activities of ammonia-oxidizing and nitrite-oxidizing bacteria are important in determining both the quality of effluents from biological purification processes for the treatment of domestic and industrial wastes, and the condition of streams, rivers, and estuaries into which such effluents are discharged. To describe quantitatively the influence of these nitrifying organisms in such systems it is necessary to know their growth-constants under the relevant environmental conditions, and the saturation (Michaelis) constants of the substrates which support their growth. It is also desirable to be able to estimate the concentrations of these organisms present in a given sample. A technique for obtaining such information has been developed. This consists essentially in observing the course of nitrification during aeration of appropriate samples when incubated under constant conditions, and then the determination, by trial-and-error, of the values of the kinetic constants and initial concentration of organisms which best fit the observed data. The labour involved would be prohibitive if a desk calculator were used; possibly mainly for this reason the present approach has not received much attention hitherto, but by using an electronic computer most of this labour is eliminated. The method is illustrated below by reference to an examination of the kinetics of nitrification in samples of water from the Thames Estuary.

In the present work it has been convenient to assume that the oxidation of ammonia and nitrite are brought about, respectively, only by Nitrosomonas species and by Nitrobacter species. Nevertheless the existence of at least one other type of nitrifying micro-organism, the ammonia-oxidizing marine organism Nitrocystis, is reasonably well established, and since it is possible that this or other types were active in the samples we examined, the observed growth constants may in fact

represent an average for a mixed population. Since, however, the main object of this work was to develop a general technique for analysis of data from batch cultures, and since for the study of nitrification in rivers, estuaries and treatment plants information on the combined nitrifying ability of the natural population was in itself of sufficient interest to justify the work, the above simplification was considered adequate. A more complete investigation, however, would necessarily involve identifying the types and strains of organisms present.

Kinetics of growth of nitrifying bacteria

The kinetics of growth of both organisms (Nitrosomonas, Nitrobacter) appear to conform fairly closely to the following equation of the Michaelis type, as do those of various heterotrophic bacteria (Monod, 1942; Hinshelwood, 1946; Novick & Szilard, 1950; Herbert, Elsworth & Telling, 1956):

$$dC_m/dt = k_m C_m x/(x+X). \quad (1)$$

In this context C_m is the concentration of Nitrosomonas in mg./l., as dry matter, k_m its growth-rate constant, x the concentration and X the saturation (or Michaelis) constant of the substrate ammonia, both in mg./l. as nitrogen. References to mass and concentration of ammonia, nitrite and nitrate relate throughout to mg. nitrogen and mg. nitrogen per litre respectively. Mass and concentration of bacteria are expressed as dry wt. bacteria (dried at 105°). The corresponding equation for the growth of Nitrobacter is

$$dC_b/dt = k_b C_b y/(y+Y), \quad (2)$$

in which y is the existing concentration and Y the saturation constant of nitrite, C_b is the concentration and k_b the growth constant of Nitrobacter.

It would perhaps be surprising if this relation exactly represented bacterial growth and it should be mentioned that modifications have been proposed. For example, Boon & Laudelout (1962) included an extra term in the equation for Nitrobacter to allow for a slight inhibitory effect of the substrate nitrite. However, at the concentrations of nitrite found in the present experiments this term has a negligible effect.

Oxidation of ammonia. If the oxidation of unit mass of ammonia-N produces a dry mass E_m of Nitrosomonas organisms then

$$C_m - C_{m0} = E_m(x_0 - x), \quad (3)$$

in which x is the concentration of ammonia-N, C_m the concentration of Nitrosomonas, and C_{m0} and x_0 are the initial values of C_m and x. Combining equations (3) and (1) gives a differential equation that can be integrated to a form already published (Downing, Painter & Knowles, 1964), which gives the relation between concentration of ammonia-N and time:

$$k_m t = (1/A)[E_m X \log_e(x_0/x) + (A + E_m X)\log_e\{(A - E_m X)/C_{m0}\}], \quad (4)$$

where

$$A = C_{m0} + E_m x_0.$$

Solutions to this equation, calculated by using an electronic digital computer, were matched against the observations, described later, in order to determine the best values for k_m, C_{m0} and X, the programme for the machine being so devised that trial values for these variables could be chosen over as wide a range as desired. The yield

constant, E_m, was taken as 0·05, this choice being perhaps a little low on evidence quoted by Downing *et al.* (1964) and also on unpublished work at this Laboratory (Painter & Loveless, in preparation). An incorrect value for E_m would, however, affect only the estimate of C_{m0}, but not those of k_m and X. The estimated value of C_{m0} is in fact proportional to the assumed value of E_m. This can be seen from equation (4), which remains unaltered if C_{m0} and E_m are multiplied by the same factor. Figure 1(*a*) shows an example of the agreement between the observed and calculated curves when the values of k_m, C_{m0} and X have been properly chosen.

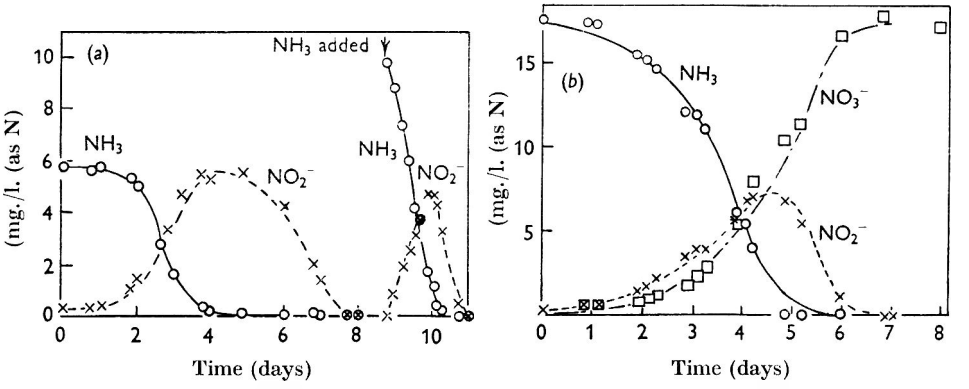

Fig. 1. Examples of computed curves which best fit observations on the course of nitrification during incubation of water from the Thames Estuary. (*a*) An experiment in Series 2, Table 1. Temperature 29·3° until day 8, 30·8° thereafter; more ammonia was added on day 9. (*b*) An experiment in Series 4, Table 1. ○, ammonia observed; ×, nitrite observed; □, nitrate observed; —, ammonia computed; - - -, nitrite computed; -·-·-, nitrate computed.

One of the problems was to decide whether or not any set of values of k_m, C_{m0} and X, giving an apparently satisfactory fit to the observed data, represented a unique solution or whether some other combination of these variables would have given an equally good fit. This problem was, however, made easier by the fact that the value of X appears to be quite low and hardly affected the shape of the ammonia–time curve at concentrations of ammonia-N above about 1 mg./l.

The idea that any combination of values which gave a good fit would be unique was supported by the fact that a second such combination which fitted equally well was never obtained, and also by the general consistency of the results, both among themselves and also as compared with other published data.

Apart from such general support, it can be shown from equation (1) (which after substituting $-E_m dx/dt$ for dC_m/dt gives the slope, dx/dt, of the ammonia–time curves) that only the correct value of k_m will give a curve passing through two experimental points, provided that the first of these points was so chosen that (*a*) the concentration of ammonia oxidized since the beginning of the experiment was sufficient to make C_m large compared with C_{m0} and (*b*) X had been correctly chosen or was small compared with x. (For any particular value of x, condition (*a*) ensured that all curves through one point applied to substantially the same concentration of Nitrosomonas and condition (*b*) that all such curves had substantially the same value for $x/(x+X)$.) Suppose, for instance, the correct curve was drawn through two

such points, and that k_m was of value k, then any other curve through the points must, somewhere between them, have a greater slope than the first curve, and somewhere else have a smaller slope. Where the curve has a greater slope than the correct curve at the same ammonia concentration then equation (1) shows it has a k_m value *greater* than k, for by equation (3) and condition (*a*) above C_m is the same for both

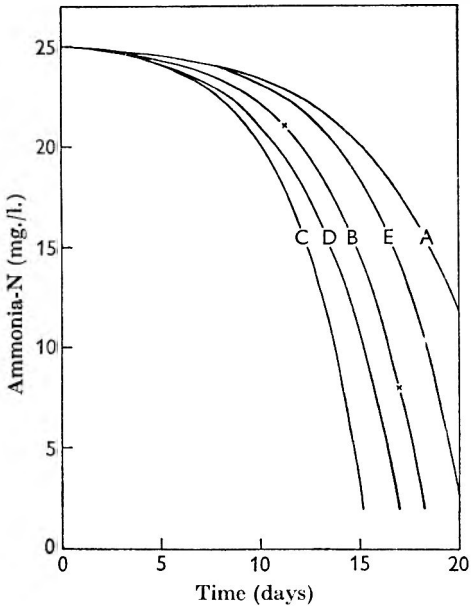

Fig. 2

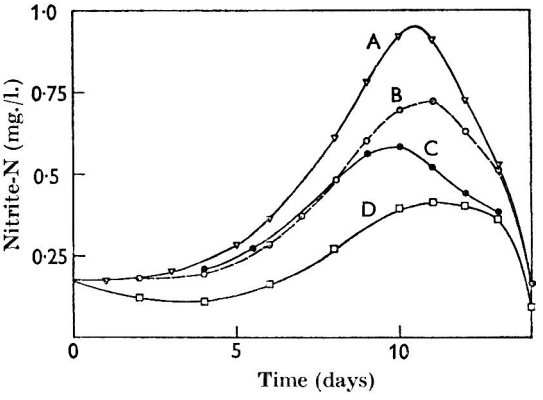

Fig. 3

Fig. 2. Effects of values of growth constant and initial concentration of Nitrosomonas on shape and position of computed ammonia–time curves.

A	Growth constant	0·20	day^{-1}; initial concentration of Nitrosomonas	0·016 mg./l.
B		0·25		0·016 mg./l.
C		0·30		0·016 mg./l.
D		0·25		0·022 mg./l.
E		0·25		0·010 mg./l.

Saturation constant, 0·36 mg./l. in each case; ×, points referred to in text under the heading 'Oxidation of ammonia'.

Fig. 3. Effects of initial concentration and saturation constant of Nitrobacter on shape and position of computed nitrite–time curves.

A	Initial concentration	0·001	mg./l.; saturation constant	2 mg./l. N
B		0·002		2 mg./l. N
C		0·001		1·5 mg./l. N
D		0·004		1·5 mg./l. N

Growth constant 2 days^{-1} in each case.

curves, and by condition (*b*) so is $x/(x+X)$. Similarly, where the curve has a smaller slope it has a k_m value *smaller* than k. Therefore it does not have a constant value for k_m and so cannot be a solution. Such points, in Fig. 2, are 21 mg./l. at 11·3 days and 8 mg./l. at 17 days; only curve *B* goes through both points. Thus, when a fit is obtained to a number of points along an appropriate part of the ammonia–time curve it is very probable that the correct k_m value has been used. Having established

a value of k_m by the middle part of an ammonia–time curve then an argument similar to the above shows that the values of X obtained from the fit to the later part of the curve are correct, subject to the accuracy of the determinations of low concentrations of ammonia.

For estimating C_{m0}, the initial concentration of Nitrosomonas, it is assumed that no lag-phase intervened between taking the sample and continuance of growth with a steady growth-rate constant. This assumption is made on the ground that, except when the incubation temperature was different from that in the estuary, the bacteria probably did not necessarily undergo any great change in their chemical environment or physical conditions on being transferred from the estuary to the sample bottle. When the incubation temperature was low the changes in concentration of ammonia in the early stages were too small to provide evidence of a lag-phase. There is certainly no indication of such a lag where the incubation temperature was of the order of 30°, since the concentration of ammonia decreased rapidly from zero time, giving an experimental curve that can be well fitted throughout by equation (4), using fixed values of k_m, C_{m0} and X. When in any experiment a lag-phase did occur, the effect would be that the estimate of C_{m0} would be too low, but that the estimate of k_m, essentially based on fitting those parts of the experimental curve which show definite curvature, would be correct. In passing it may be noted that while only one set of values of k_m, C_{m0} and X would genuinely fit perfect data, the precision with which these values can be selected is, of course, in practice dependent on the accuracy of the observations.

Oxidation of nitrite. In extending the calculations to the changes in concentration of nitrite with time the concentration of Nitrobacter is given by an equation like equation (3), viz.:

$$C_b - C_{b0} = E_b\{y_0 + f_m(x_0 - x) - y\}, \tag{5}$$

where y is the concentration of nitrite-N, C_b the concentration of Nitrobacter, C_{b0} and y_0 are the initial values of C_b and y, f_m is the ratio of the mass of nitrite-N formed to that of ammonia-N oxidized, and where the oxidation of unit mass of nitrite-N produces a dry mass E_b of Nitrobacter.

The approximate constancy of the total amount of inorganic nitrogen present throughout the period of incubation (as shown, for example, in Fig. 1*b*) indicates that the value of f_m is close to unity; it has been taken as 0·99 in the present calculations.

Substituting the value of C_m given by equation (3) into equation (1) we then have a differential equation for the variation of ammonia-N with time, which may be summarized as

$$dx/dt = \phi_1(C_{m0}, x_0, x, X, k_m, E_m), \tag{6}$$

the integrated form of which is equation (4). Similarly, substituting the value of C_b given by equation (5) in equation (2) gives a differential equation for the variation of nitrite-N with time, summarized as

$$dy/dt = \phi_2(C_{b0}, x, y_0, y, Y, k_b, E_b). \tag{7}$$

$\phi_1(\ )$ and $\phi_2(\ )$ are general expressions for functions. The occurrence of x in the expression for dy/dt, together with the involved relation between x and t given in equation (4), makes it impossible to integrate equation (7) to an explicit relation between nitrite concentration, y, and time, t. Accordingly equation (7) was treated

by 'numerical integration' to obtain a predicted curve for concentration of nitrite against time for any chosen set of values for C_{b0}, k_b, and Y. From the evidence quoted by Downing *et al.* (1964) E_b, the mass of Nitrobacter formed per unit mass of nitrite-N oxidized, was taken as 0·02; choice of a wrong value for E_b would affect the estimation of the initial concentration of bacteria (the result being proportional to the value chosen for E_b) but would not affect the estimates of growth-rate and saturation constants.

By combining differential equations (6) and (7) with a 'subroutine' based on the Runge–Kutta–Merson procedure (Fox, 1962), and supplied by the makers of the digital computer used, a single program was formed, which gave predicted curves for many sets of C_{b0}, k_b, and Y at the rate of about one every 2 min. (with a desk calculator about 20 hr was required for each curve). The values used for C_{m0}, k_m and X were those that had been found, as described earlier, to give the best fit to the ammonia–time curve.

The availability of such a numerical integration 'subroutine' is of great assistance in writing a programme for solving simultaneous differential equations. Makers of computers are usually able to supply a version of the Runge–Kutta process programmed in a language suitable for their machines, while for workers using the 'universal' computer language, Algol, a programme published by Lukehart (1963) for the Runge–Kutta–Merson method is suitable.

In this way were determined values of C_{b0}, k_b and Y for Nitrobacter that gave the best fit to the experimental points; examples of such best fits are shown in Fig. 1. From the many trials made, it seemed that, as for Nitrosomonas, a good fit could be obtained only with a particular set of values of these three unknowns except in one or two cases in which a completely satisfactory fit could not be obtained with any combination. The predicted curves were much altered by variation in any of the unknowns, the nature of the change depending on the unknown concerned. For example, in Fig. 3 the change from A to C is produced by decrease of the saturation constant from 2 to 1·5 mg./l., from A to B by increasing the assumed initial concentration of Nitrobacter from 0·001 to 0·002 mg./l., and from C to D by increasing this from 0·001 to 0·004 mg./l. As in the case of Nitrosomonas, when a lag-phase were present the estimates of C_{b0}, the initial concentration of Nitrobacter, would be too low.

The prediction of the change in concentration of nitrate with time was made from the best fit kinetic constants and values of C_{m0} and C_{b0}, assuming that unit mass of nitrite-N forms 0·99 unit mass of nitrate-N on oxidation. This assumption appears to be justified by the close fit so obtained, as illustrated for example in Fig. 1*b*.

METHODS

Five series of experiments were made, the objectives being to study the way in which the kinetic constants of nitrifying bacteria were affected by temperature, by aeration in the absence of the energy source (ammonia or nitrite) and by concentration of dissolved oxygen, and to examine the possibility that a relation exists between estimated concentrations of nitrifying bacteria in Thames water and the concentration of suspended matter. The first four series were made in 1960 and the fifth in 1963.

Series 1. Subsamples from 50 (British Imperial) gallons (about 227 l.) of water,

from the Thames Estuary near London Bridge, were incubated, without dilution, in 10 l. aspirators in three constant-temperature rooms at about 7·5°, 14° and 20°. Two experiments were made in each room, one with the water as taken, and the other with the addition of ammonium sulphate to raise the initial concentration of ammonia-N by about 6 mg./l. Solids present in each sample were kept in suspension with magnetic stirrers. Each aspirator was completely filled and the mouth of the vessel closed by a rubber stopper, care being taken to exclude air when the stopper was inserted. Samples were generally taken daily from each vessel by removing the stopper and allowing water to flow through a side arm near the bottom. Concentrations of dissolved oxygen, NH_3-N, NO_2-N and total oxidized-N (NO_2-N plus NO_3-N) were measured in each sample.

Complete filling of the aspirator was not essential for the aspects now under discussion but was done for the purpose of measuring the rate of consumption of dissolved oxygen. When necessary the contents of the aspirator were aerated by shaking in contact with air, an additional small sample being taken for determination of dissolved oxygen. The aspirator was then replenished from another that was open to the air and contained a further portion of the original sample of Thames water. In calculating the results of the experiments, allowance was made for the slightly different composition of the replacement water. This allowance was a very small one because the analyses for ammonia, nitrite and nitrate gave similar figures for aspirator contents and replenishment water (which indicated similar growth of nitrifying bacteria), and especially because the volume of water used per day for replenishment was only about 2 % of the total volume of the sample. From these considerations it was clear that the replenishment would not significantly change either the concentration of the nitrifying bacteria or the general biological conditions.

The same analytical methods were used in all series of experiments.

Ammonia-N was determined by distillation of the sample from a borax buffer solution, nesslerization of the distillate, and measurement of the extinction in a Spekker absorptiometer.

Total oxidized-N was determined by reduction of a 50 ml. sample with Devarda's alloy in the presence of 5 ml. of 8 % (w/v) sodium hydroxide solution, followed by distillation and then nesslerization of a suitable sample of the distillate. A weighed amount (1 g.) of Devarda's alloy was used, and a blank determination was made for each series of analyses.

NO_2-N was measured by using Griess–Ilosvay reagent according to the procedure recommended by the Ministry of Housing and Local Government (1956).

Dissolved oxygen was measured by the azide modification of the Winkler method, as recommended by the Ministry of Housing and Local Government (1956).

Series 2. Thames water containing nearly 3 mg./l. ammonia-N (taken from about the same position as the water used in Series 1) was incubated without dilution, at about 28° in three vessels with the addition of ammonium sulphate equivalent to 0, 2·5, and 5 mg./l. ammonia-N. On the ninth day (by which time nitrification was complete) a further quantity of ammonium sulphate was added to each vessel to give concentrations of 2, 10, and 20 mg./l. ammonia-N, respectively, and the incubation continued. The techniques used in Series 1 were again used, except that the oxygen content of the water was replenished as necessary by bubbling oxygen through it for short intervals to give initial concentrations of 12–15 mg./l.

Series 3 *and* 4. It was evident from the results of the tests made in Series 1 and 2 that the quantity of nitrifying bacteria in the Thames water was fairly small, so before starting Series 3 and 4, in which the effect of the concentration of dissolved oxygen on the nitrification process was examined, the sample of Thames water was stored at 19° (the temperature of the subsequent incubation) for several days to obtain a larger population of nitrifying organisms by the oxidation of the ammonia-N present initially. The water was then fortified with ammonium sulphate equivalent to 18 mg./l. ammonia-N, and incubated in six vessels. A different oxygen content was maintained in each vessel by aerating with mixtures of air, nitrogen, and carbon dioxide. The rate of flow of each gas was measured by a rotameter, the total rate of flow being about the same in each vessel. The partial pressure of the carbon dioxide in the gas mixture was adjusted to a value that would maintain the concentration of free carbon dioxide that was present in the water at the beginning of the experiment. The required rate was obtained in a subsidiary experiment by adjusting the rate of flow to maintain the initial pH value of the water. Subsequent experiments, and work reported elsewhere (*Department of Scientific and Industrial Research*, 1964) indicate that loss of ammonia by volatilization during the experiments was unlikely to be of significant proportions.

Series 5. In these experiments the progress of nitrification was measured in a series of five samples taken from the Thames Estuary at London Bridge on the same day and from the same depth but at different states of tide; the concentration of suspended matter in the samples ranged from about 100 to 1000 mg./l., and the object was to examine whether or not the initial concentration of nitrifying bacteria bore any relation to that of suspended solids. It was thought that such a relation might exist if nitrifying bacteria became bound up in the estuary with suspended mud particles, which tend to be flocculent in nature, and that since the concentration of suspended mud varied with the state of the tide so too might that of the nitrifying bacteria; however, no significant relation was found. The procedure was similar to that in the experiments of Series 3 and 4, except that normal air was used for aeration.

RESULTS

Table 1 gives details of the results of the five series of tests.

Growth constants

Effect of temperature. It will be seen from Table 1 and also from Figs. 4 and 5 that the growth constants of the organisms present in Sample 1 increased considerably with increasing temperature in the range from 8 to 23°. The equation of the regression line fitted to the data for Nitrosomonas is

$$\log_{10} k_n = 0{\cdot}0413T - 0{\cdot}944$$

(with k_n in day^{-1} and T in °C.), the implication of which is that the growth-rate constant increased by about 9·5 % of the existing value per degree Centigrade increase in temperature. The corresponding equation for Nitrobacter is

$$\log_{10} k_b = 0{\cdot}0255T - 0{\cdot}492$$

(with k_b in day^{-1} and T in °C.), implying that the temperature coefficient for this organism was about 5·9 %/deg. It is also evident that at a given temperature the value

Table 1. *Growth-rate constants, saturation constants and initial concentrations for Nitrosomonas and Nitrobacter in samples from estuary of River Thames*

In Series 2, 3 subsamples are continuations of incubations (see text).
In Series 3 and 4 samples were incubated before start of experiments (see text).

Series no. and date of sampling	Original sample no.	Temperature of incubation (°)	Chloride (Cl^- mg./l.)	Suspended solids (mg./l.)	Dissolved oxygen during incubation (mg./l.)			Range of pH values	Initial ammonia-N (mg./l.)	Nitrosomonas			Nitrobacter		
					Min.	Average	Max.			k_m (day^{-1})	X NH_3-N (mg./l.)	C_{m0} (mg.*/l.)	k_b (day^{-1})	Y NH_3-N (mg./l.)	C_{b0} (mg.*/l.)
1; 11 Jan. 1960	1	8·3	280	69	1·3	5·6	9·7	7·5–7·6	8·0	0·2	0·2	0·0028	0·5	0·2	0·0008
	1	8·6	280	67	1·3	5·3	8·3	7·5–7·7	2·7	0·27	0·2	0·001	0·6	0·18	0·00022
	1	13·9	280	74	1·4	5·5	9·4	7·4–7·6	8·5	0·45	0·4	0·0013	0·7	0·18	0·0003
	1	14·5	280	67	2·3	5·9	9·2	7·5	2·7	0·58	0·4	0·0004	0·7	0·25	0·0006
	1	22·2	280	68	1·0	4·9	7·5	7·6–7·7	2·7	1·02	1·0	0·002	1·2	1·0	0·0013
	1	23·2	280	71	0·5	4·2	7·6	7·7–7·8	8·2	0·85	1·2	0·003	1·3	1·3	0·0008
2; 28 Mar. 1960	2	28·5	—	125	1·2	5·5	11·9	7·4–7·7	2·9	2·0	1·1	0·00065	2·0	2·0	0·0001
	2	29·2	—	125	1·5	10·4	18·0	—	1·7	1·8	1·8	0·085	1·5	3·0	0·05
	2	29·3	—	143	1·0	5·6	11·4	7·7	5·7	1·95	2·0	0·003	2·0	3·0	0·0002
	2	30·8	—	143	2·3	8·4	14·6	—	9·8	1·9	2·0	0·10	2·0	7·0	0·10
	2	28·8	—	137	1·6	5·6	11·0	7·7	8·3	2·0	1·6	0·0025	1·9	5·0	0·0005
	2	29·8	—	137	2·0	8·2	19·0	—	19·6	2·1	8·0	0·17	2·5	8·0	0·15
3; 8 Nov. 1960	3	19·0	32	—	8·1	8·3	8·4	7·3	17·2	0·7	0·6	0·055	1·2	2·0	0·03
	3	19·0	32	—	6·5	6·6	6·8	—	17·2	0·68	0·6	0·05	1·2	1·5	0·02
	3	19·0	32	—	4·4	5·1	5·9	—	17·3	0·65	0·6	0·038	1·1	1·5	0·01
	3	19·0	32	—	2·6	3·4	4·0	—	17·7	0·75	0·6	0·06	1·0	1·7	0·015
	3	19·0	32	—	1·4	2·2	2·6	—	17·7	0·57	0·6	0·11	0·7	1·6	0·016
4; 6 Dec. 1960	4	18·8	33	—	7·6	8·4	8·8	—	18·0	0·7	0·6	0·015	0·9	1·5	0·02
	4	18·8	33	—	4·0	4·7	4·9	—	17·9	0·7	0·6	0·049	1·1	1·7	0·01
	4	18·8	33	—	3·2	3·4	3·8	—	17·4	0·7	0·6	0·04	0·8	1·7	0·03
	4	18·8	33	—	1·9	2·0	2·3	7·4–8·6	17·8	0·7	0·6	0·044	0·9	2·0	0·01
	4	18·8	33	—	1·2	1·4	1·9	—	17·6	0·6	0·6	0·026	0·67	1·9	0·012
	4	18·8	33	—	0·5	0·6	0·7	—	17·8	0·5	3·0	0·04	0·6	2·5	0·035
5; 9 Sept. 1963	5	27·0	—	103	7·9	7·9	7·9	—	18·9	1·5	1·7	0·017	—	—	—
	6	27·0	—	253	7·9	7·9	7·9	—	19·1	1·5	1·7	0·017	—	—	—
	7	27·0	—	980	7·9	7·9	7·9	—	19·3	1·5	1·7	0·017	—	—	—
	8	27·0	—	750	7·9	7·9	7·9	—	19·5	1·5	1·7	0·019	—	—	—
	9	27·0	—	470	7·9	7·9	7·9	—	19·7	1·5	1·7	0·017	—	—	—

* Mg. bacteria after drying at 105°.

of the growth-rate constant for Nitrobacter was about 50% greater than that for Nitrosomonas. Data for the remaining Samples 2–5 appear to be in good agreement with the trends of those from Sample 1.

Buswell, Shiota, Lawrence & van Meter (1954) studied the effect of temperature on the growth of pure cultures of Nitrosomonas and obtained values of k_m increasing from about 0·5 day^{-1} at 15° to about 2 days^{-1} at 32°; the temperature coefficient

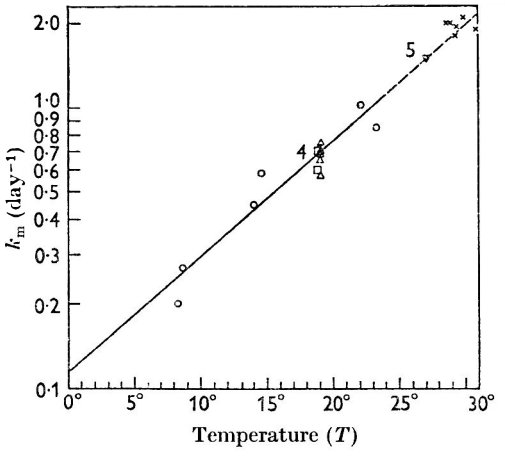

Fig. 4

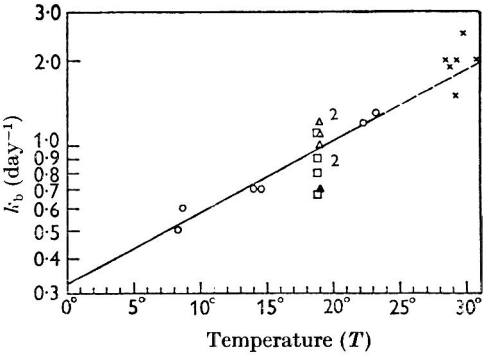

Fig. 5

Fig. 4. Effect of temperature on the growth-rate constant for Nitrosomonas. Regression line is fitted to the points for Sample 1, Table 1. ○, Sample 1; ×, Sample 2; △, Sample 3; □, Sample 4; ▽, Sample 5. A number alongside a point shows the number of determinations giving a value represented by that single point.

Fig. 5. Effect of temperature on the growth-rate constant for Nitrobacter. Regression line is fitted to the points for Sample 1, Table 1. ○, Sample 1; ×, Sample 2; △, Sample 3; □, Sample 4. A number alongside a point shows the number of determinations giving a value represented by that single point.

calculated from these data is 8·2%/deg. A similar effect of temperature on the growth of Nitrosomonas in activated sludge from a sewage-treatment plant was reported by Downing *et al.* (1964) and by Downing & Hopwood (1965). The magnitude of k_m at a given tempeturera is known to depend on the pH value of the medium and its composition. At temperatures of about 30°, for which k_m in the present work ranged from about 1·6 to 2·0 days^{-1}, the following values (all in days^{-1}) were reported for pure batch cultures grown in medium containing mineral solids: 1·19 (Engel, 1930), 1·03 (Bomeke, 1946), 0·46 (Lees, 1952), and 1·5 (Engel & Alexander, 1958). Skinner & Walker (1961) obtained a figure of 3·1 days^{-1} during continuous culture, with a clear medium of composition adjusted to give maximum growth rates. Painter & Loveless (unpublished work in this Laboratory) obtained maximum values of k_m of about 0·7 to 0·9 day^{-1} at 25° and pH 7·6–8·0 in batch cultures in a clear medium; beyond this range they found that growth rates decreased markedly. For comparison with the growth-rate constants found for Nitrobacter there appears to be only a figure of 1·39 day^{-1} at 32° obtained by Boon & Laudelout (1962) with a pure culture; this is only about half the corresponding value in the present work.

Effect of concentration of dissolved oxygen. Results for Series 3 and 4, Table 1, indicate that k_m varied little with concentration of dissolved oxygen above 2 mg./l. but decreased with decrease in concentration below 2 mg./l.; k_b appeared to decrease with decreasing concentration of dissolved oxygen below 4 mg./l. The effect on k_m was only qualitatively consistent with the effect of dissolved oxygen on the instantaneous rate of respiration of Nitrosomonas in activated sludge (Downing *et al.*

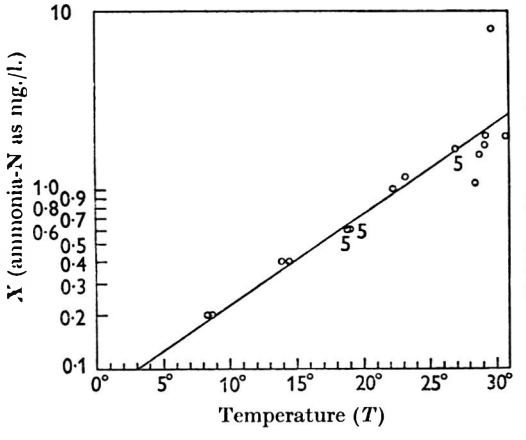

Fig. 6

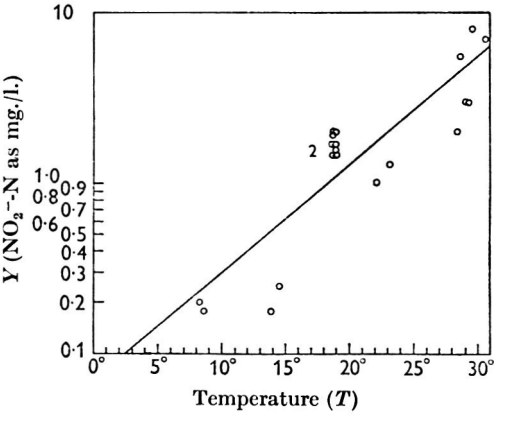

Fig. 7

Fig. 6. Effect of temperature on saturation constant for Nitrosomonas. Regression line is fitted to all points; Samples 1 to 5, Table 1. A number alongside a point shows the number of determinations giving a value represented by that single point.

Fig. 7. Effect of temperature on saturation constant for Nitrobacter. Regression line is fitted to all points, Samples 1 to 5, Table 1. A number alongside a point shows the number of determinations giving a value represented by that single point.

1964) and in soil (Greenwood, 1962). The difference in detail may indicate a genuine difference in the long-term effect on growth as compared with the short-term effect on function. With regard to the effect on k_b, Boon & Laudelout (1962) found the saturation constant for oxygen was 0·25 at 18° and 0·5 mg./l. at 32°. These data are not necessarily inconsistent with those in Table 1, although they are perhaps lower than the latter might lead one to expect.

Saturation constants

All the estimates of the saturation constants of ammonia-N (for Nitrosomonas) and nitrite-N (for Nitrobacter), other than values obtained in experiments in which the concentration of dissolved oxygen was below 1 mg./l. for a significant period, are plotted against temperature in Figs. 6 and 7. It is seen that at temperatures below about 20° the values for ammonia and nitrite were of the same order but, whereas the values for both substrates increased with temperature, the rate of increase in the values for nitrite appeared to be higher than in those for ammonia.

As regards the saturation constant of ammonia-N for Nitrosomonas there is little to afford a comparison with the values shown in Fig. 6, other than a value of 0·2 mg./l. at 21° obtained at this Laboratory by Dr H. A. Painter (but this was for activated sludge from a sewage-treatment plant) and an approximate value of

10 mg./l. at 30° calculated by Downing *et al.* (1964) from data by Hofman & Lees (1953). Accordingly, Fig. 6 appears to be the first evidence for a large temperature coefficient of the saturation constant for ammonia (Nitrosomonas). The equation of the regression line in Fig. 6 is

$$\log_{10} X = 0{\cdot}051T - 1{\cdot}158 \qquad (8)$$

(with X in mg./l. N and T in °C.); this corresponds to an increase in saturation constant of about 11·8% of the existing value per degree Centigrade increase in temperature.

The saturation constants found for nitrite-N are in good agreement with the figure for pure culture of Nitrobacter (6 mg./l. at 30°) calculated by Downing *et al.* (1964) from the data of Lees & Simpson (1954), and of 8·4 mg./l. at 32° found by Laudelout & van Tichelen (1960), though the latter is different from the value of 22 mg./l. at 32° given by Boon & Laudelout (1962). Laudelout & van Tichelen found an appreciable temperature coefficient (though not so large as that determined in the present experiments), their values being 1·4 mg./l. at 14°, 2·1 mg./l. at 18°, 8·4 mg./l. at 32°. The equation of the regression line fitted to our values in Fig. 7 is

$$\log_{10} Y = 0{\cdot}063T - 1{\cdot}149 \qquad (9)$$

(with Y in mg./l. N and T in °C.); this corresponds to a temperature coefficient of about 14·5% per degree.

The actual values of X and Y (particularly X) for Series 4, last subsample, in which the concentration of dissolved oxygen was maintained at a very low value, are higher than expected from the results on other subsamples from the same and other series; this may represent not a true change in X and Y but the existence, in the growth-rate equation, of a term containing dissolved-oxygen concentration but having a significant effect on growth rate only at low concentrations of dissolved oxygen.

Initial concentrations, C_{m0}, C_{b0}

It will be seen from Table 1 that for each separate group of subsamples derived from Samples 1 to 4 there was considerable variation in the estimated initial concentrations of nitrifying bacteria, but whereas the average values for each series varied by about a factor of 30, the range of variation of the estimates within each group was between limits only of about 2 and 0·5 times the mean value (neglecting one very low figure in the group of subsamples derived from Sample 1); furthermore the ratio of the mean values of C_{b0} to those of C_{m0} for each series varied between comparatively narrow limits of 0·30 and 0·68. The second, fourth and sixth subsamples of Sample 2 showed high values of C_{m0} and C_{b0} because they had already undergone one period of incubation (see earlier).

It is not yet known whether the observed variability in the values of C_{m0} and C_{b0} in some groups of subsamples was due to a variable lag-phase or to real variations, resulting perhaps from a failure to subdivide reproducibly the suspended matter in the original sample. Thus the question of the accuracy of the estimates must remain in doubt. On the other hand, the degree of variation is not particularly high and there seem to be no definite grounds for believing that the values were necessarily greatly in error.

At present it is not possible to offer any explanation of the large differences in the

Table 2. *Effect of absence of substrate on apparent concentrations of Nitrosomonas and Nitrobacter remaining viable during aeration of water from the Thames Estuary*

	1st phase				2nd phase		
Temperature of incubation (°)	Initial concentration Nitrosomonas (mg.*/l.)	Ammonia-N oxidized (mg./l.)	Nitrosomonas formed (mg.*/l.)	Days without ammonia	Expected initial concentration Nitrosomonas (mg.*/l.)	Actual initial concentration Nitrosomonas (mg.*/l.)	Nitrosomonas survival (%)
Nitrosomonas							
29	0·00065	2·9	0·15	4·4	0·15	0·085†	57†
29	0·0025	8·3	0·42	5·6	0·42	0·17	40
30	0·003	5·7	0·29	4·8	0·29	0·1	34
Temperature of incubation (°)	Initial concentration Nitrobacter (mg.*/l.)	Nitrite-N oxidized (mg./l.)	Nitrobacter formed (mg.*/l.)	Days without nitrite	Expected initial concentration Nitrobacter (mg.*/l.)	Actual initial concentration Nitrobacter (mg.*/l.)	Nitrobacter survival (%)
Nitrobacter							
29	0·0001	3·15	0·063	0·6	0·063	0·05†	79†
29	0·0005	8·6	0·172	0·8	0·172	0·15	87
30	0·0002	5·95	0·12	0·8	0·12	0·1	83

* Mg. bacteria after drying at 105°.

† Values for this sample approximate owing to scatter of experimental points.

initial concentrations of nitrifying micro-organisms estimated to be present in samples taken from the estuary at different times, other than those due to pre-aeration of the samples, to which reference has already been made. On the other hand, it would not be surprising if such large variations did occur. Quite apart from the fact that the estuary is far from completely mixed, it would appear that, as indicated below, the organisms did not survive for long periods in the absence of substrate. Moreover, the population density at a given place was also considerably dependent on the concentrations of ammonia, nitrite, and dissolved oxygen, and the variations in these along the estuary. All these factors change markedly with freshwater flow, temperature, and tidal conditions.

Survival of nitrifying organisms. In the case of the subsamples from Sample 2 to which further ammonia was added a day or two after all ammonia originally present had been completely oxidized to nitrate, it appears from data summarized in Table 2 that in this second phase, although the growth constant for both organisms remained substantially unchanged, a good fit to the experimental data could be obtained only by assuming initial numbers of nitrifying bacteria much lower than those calculated to have been formed from the oxidation of the ammonia originally present. Thus for one sample (observed and calculated curves for which are shown in Fig. 1*b* and other data in Table 2) the concentration of Nitrosomonas at zero days was estimated at 0·003 mg./l. From the concentration of ammonia oxidized this would have increased to 0·29 mg./l. at 5 days, but at the start of the second part of the experiment (8·8 days) the concentration of Nitrosomonas was estimated to be only 0·10 mg./l. (E_m was 0·05 for these calculations but assumption of any other value, E, would merely multiply each estimated value by $E/0{\cdot}05$). It thus appears that a substantial amount of Nitrosomonas may have died during the period when dissolved oxygen was present but ammonia was not; two other experiments of the same kind, results of which are included in Table 2, lead to the same conclusion. Although the data are somewhat variable, the proportion of Nitrobacter apparently surviving during the interval when nitrite was absent, was higher than the corresponding proportion of Nitrosomonas; possibly this is connected with the fact that this period was much shorter than that for which the Nitrosomonas was deprived of substrate (ammonia).

DISCUSSION

The work shows the usefulness of electronic computers for the rapid integration of differential equations for bacterial growth, particularly those which describe sequential growth of interdependent bacteria and which can only be solved numerically. By these means, estimates of kinetic constants and initial concentrations of bacteria can be obtained quite rapidly from experimental data about the changes in concentration of substrates and products with time in batch cultures. Application of the same method to sequences of enzyme-catalysed reactions could no doubt be made for the purpose of obtaining the velocity-constant for each stage.

The quite accurate fitting of the curves for variations with time in concentrations of ammonia and nitrite in incubated samples containing nitrifying bacteria (assumed to be species of Nitrosomonas and Nitrobacter), using the Michaelis type of growth-rate equation, is a further example of the applicability of this type of relation to bacterial growth. Originally developed for enzymic reactions, for which it is widely successful, the Michaelis rate-equation is theoretically based on the idea of reversible

formation of any enzyme + substrate complex. It is perhaps not surprising that this rate-equation, developed for enzymic reactions in homogeneous solution, applies well to Nitrobaeter, in which the nitrite-oxidizing system may be almost in the external medium possibly being situated, according to Boon & Laudelout (1962), near or at the outer surface of the cell-wall. The ammonia-oxidizing system of Nitrosomonas (involving two stages, the first yielding hydroxylamine) may be similarly situated; if, on the other hand, it is located within the cell, then it can be shown (Rashevsky, 1960) that the Michaelis form of rate-equation would still apply, provided that the rate of transport of ammonia by simple diffusion through the cell-wall to the interior is so high that the concentration of ammonia at the site of reaction is little below that in the external medium. Even this condition need not be met if, instead, transport through the cell-wall is by formation of an intermediate complex or compound, as shown to be the case for phosphate transport into *Staphylococcus aureus*, for which a Michaelis type expression represents the rate of transport (Mitchell, 1959). Transport by formation of an intermediate complex appears to apply also to amino acids entering *Escherichia coli* (Britten & McClure, 1962) and glucose absorption by mammalian cells (see Hober, 1947), and has been suggested for calcium, potassium and sodium entering mammalian cells (Hasselbach, 1962).

The results of the present work support data reported by Buswell *et al.* (1954) indicating a large temperature coefficient for the growth-rate constant for Nitrosomonas, and show, further, that the saturation constant of ammonia for this organism is somewhat similarly dependent on temperature. The results also show a similar relation between growth-rate constant and temperature for Nitrobaeter and confirm qualitatively the previously reported high temperature coefficient for the saturation constant for Nitrobaeter. It would appear from the present work that both types of organism tend to die fairly rapidly when aerated in the absence of the relevant energy source. The growth-rate constants of the surviving bacteria were, however, little changed by such treatment, in agreement with Buswell *et al.* (1954) who showed that it was necessary to keep Nitrosomonas aerated for 12 days without ammonia to produce an alteration in growth-rate constant.

Mr A. L. H. Gameson was responsible for planning the sampling and experimental work in 1960; most of the determinations at that time were made by Mr C. G. Ogden. Sampling and determinations in the 1963 work were done by Mr L. J. Scragg.

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The Differentiation of *Streptococcus faecalis* and *S. faecium*

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SUMMARY

Enterococci mainly from laboratory collections were separated by established and new or modified tests into two distinct species, *Streptococcus faecalis* and *S. faecium*. Of the established tests only reducing activity and potassium tellurite tolerance completely differentiated the organisms. Of the additional tests the most useful were fermentation of glycerol in a soft agar medium containing fumarate, H_2O_2 formation from polyhydroxy alcohols and the ability to ferment them, H_2O_2 formation on a basal medium containing no added specific energy source, citrate fermentation and dissimilation of malate in the presence of glucose. Catalase activity and the ability to use malate as an energy source were also useful tests but did not completely differentiate the organisms.

INTRODUCTION

The enterococci (from which are excluded the *Streptococcus bovis–equinus* complex) appear to be divisible into two major physiological and biochemical groups, one represented by *S. faecalis* and the other by *S. faecium*. Evidence for such a division can be found in the work of Orla-Jensen (1919, 1943); Olsen (1949); Skadhauge (1950); Sharpe & Shattock (1952); Shattock (1955); Barnes (1956); Lake, Deibel & Niven (1957); Deibel, Lake & Niven (1963). The practical necessity of being able to distinguish the two species has become increasingly evident in relation to the detection of faecal pollution of water supplies and foods. Recent work on the distribution of these species by Kjellander (1960), for instance, revealed that *S. faecalis* was the more numerous species in human faeces and *S. faecium* in animal faeces. Tests for separating *S. faecalis* from *S. faecium* before those devised by Deibel *et al.* (1963) were few and not always satisfactory. The purpose of the present work was to develop new tests which, in conjunction with those described earlier, would assist in the identification of the enterococci. Since the completion and publication of this work in thesis form (Whittenbury, 1961), Deibel *et al.* have reported a comprehensive study of this group of organisms, reviewing the development of its taxonomy and describing new tests which differentiate *S. faecalis* from *S. faecium*. Some of the tests described in the present work are similar to those devised by Deibel *et al.*, but as they were devised independently and differ in certain respects from those of Deibel *et al.* it is considered that they are still worth reporting. Among the organisms studied were strains thought to be typical of *S. faecalis*, *S. faecium* and *S. durans*, and strains which possessed unusual characteristics.

METHODS

Organisms. The strains and their sources are listed in Table 1.

Media. Basal medium agar was: meat extract (Lab-Lemco), 0·5 g.; peptone (Evans), 0·5 g.; yeast extract (Difco), 0·5 g.; Tween 80, 0·05 ml.; $MnSO_4.4H_2O$, 0·01 g.; agar (Davis), 1·5 g.; in 100 ml. tapwater, adjusted to pH 6·5 and autoclaved at 121° for 15 min.

Table 1. *Source and identification of cultures used*

Identification	Designation when received	Source
Streptococcus faecalis	*S. faecalis* 581	NCDO
	,, 370, 775, 2707	NCIB
	,, B8, H69D5, N83, S161	Dr P. M. F. Shattock
	S. liquefaciens 588, 598	NCDO
	,, Z93, Black	Dr T. Gibson
	,, N97, EIV, GB122	Dr P. M. F. Shattock
	S41	Silage
	S. zymogenes 585, 587	NCDO
S. faecium	*S. faecium* 942, 943, 944, 945	NCDO
	,, HGH511, 1MEC, P_5, Hobbs 2678	Dr P. M. F. Shattock
	,, 2703 (former NCTC no.)	Dr T. Gibson
	S. faecalis 580, 582	NCDO
	,, 8274	NCIB
	S1, S2, S3, S4, S5, S6, 1, 60, 73, S32, Motile S43	Silage
	T83, SD1, SD20, JMA3	Dr T. Gibson
	S. durans 498	Dr P. M. F. Shattock
	,, Sherman 98D	Dr T. Gibson

NCDO = National Collection of Dairy Organisms.
NCIB = National Collection of Industrial Bacteria.
NCTC = National Collection of Type Cultures.

Liquid medium was similar to the basal medium agar but without agar.

'Inoculum' medium was similar to basal medium agar but with the agar omitted and glucose 0·5% (w/v) added.

Soft agar medium, which was used for showing oxygen relationships and fermentative activities, was prepared and inoculated as described previously (Whittenbury, 1963) with the exception that the bromcresol purple content was doubled. Sugars and polyhydroxy alcohols were prepared as Seitz-filtered distilled water solutions and added to give a final concentration in the media of 0·5% (w/v).

Arginine hydrolysis. The method of Niven, Smiley & Sherman (1942) was routinely used with the exception that Tween 80 was included in the medium. In some studies liquid medium containing L-arginine monohydrochloride 0·3% (w/v), with or without glucose 0·1 or 1% (w/v), adjusted to pH 6·5, was used. Ammonia production was determined with Nessler's reagent on a spot plate and glucose disappearance with Benedict's reagent. Cultures were held at 37° in a water bath Growth was measured nephelometrieally at 10–15 min. intervals at the end of the lag phase; pH values were measured electrometrically.

Serine hydrolysis. Liquid medium containing L-serine 0·3% (w/v) was adjusted to pH 6·5. Ammonia production was determined by Nessler's reagent and acetoin

(indicating dissimilation of pyruvate, the deamination product) by Barritt's (1936) modification of the Voges–Proskauer test.

Ability to initiate growth at pH 9·6. The method of Chesbro & Evans (1959) was used. Additional experiments with the same medium converted to a soft agar were also done.

Tolerance of potassium tellurite. Basal medium agar containing glucose 0·5% (w/v), tellurite 0·04% (w/v) (the concentration recommended by Skadhauge, 1950), adjusted to pH 6·5, was used. The results, erratic at first, became constant when the concentration of the meat extract in the medium was doubled.

Reduction of 2,3,5triphenyltetrazolium chloride (tetrazolium). This test was adapted from that described by Barnes (1956). Liquid medium containing glucose 0·5% (w/v) was adjusted to pH 6·0. Tetrazolium, 0·01% (w/v) final concentration, was added from a stock solution heated at 100° for 15 min. A heavy inoculum, 3 capillary pipette drops of turbid culture, was added to 4 ml. medium in a test tube. The cultures, incubated at 30°, were examined at 8 hr. Positive cultures were coloured a deep magenta, whilst cultures regarded as negative were either colourless or faintly pink.

Reduction and tolerance of methylene blue in milk and the reduction of litmus in milk. A heavy inoculum, 3 capillary pipette drops, was added to the milk media which were incubated at 30° and read at 8 hr.

Haemolysis. Brown's (1919) method was followed, with nutrient agar containing NaCl 0·5% (w/v) and defibrinated horse blood 3% (w/v). Poured agar plates were incubated for 2 days at 30° and refrigerated overnight. Further tests were made with ox blood in the basal medium agar containing NaCl 1·0% (w/v).

Hydrogen peroxide formation, pseudocatalase and catalase tests. Methods and media (heated blood *o*-dianisidine agar, heated blood agar and glucose agar) described previously (Whittenbury, 1964) were used. In testing for H_2O_2 formation it was essential that the inoculum be taken from aerobically incubated slope cultures.

Malate and citrate dissimilation. Ability to dissimilate malate in the presence and absence of glucose was determined. Liquid medium was used containing DL-malic acid 4·0% (w/v); bromcresol purple, 2·8 ml. of a 1·6% (w/v) ethanolic solution/l. (unless acetoin was being tested for); glucose 1–2·0% (w/v); adjusted to pH 6·0 with KOH and distributed in 4 ml. amounts in $5 \times \frac{1}{2}$ in. test tubes containing Durham tubes. Water agar seals were added to the inoculated tubes; the function of the Durham tubes was to support the seals. Action on malate was judged by increase in pH value, gas (CO_2) production, and, in the absence of glucose, by increase in the amount of growth over that in the basal medium and by acetoin production.

Citrate dissimilation was examined in similar media to those used in the study of action on malate. Citrate (potassium citrate 3·0%, w/v) was added and the medium adjusted to pH 8·0 for routine use. Action on citrate in the absence of glucose was judged by gas production, acetoin formation, increase in pH measured electrometrically, and by increase of growth over that in the basal medium. In the presence of glucose (medium adjusted to pH 6·0) gas (CO_2) production indicated action on citrate.

To examine for gas production from citrate and malate, each tube was sharply tapped to initiate effervescence. In cultures above pH 6·5 bromcresol purple was

frequently bleached except when the culture was in contact with the atmosphere (the top 2 mm. of the agar seal) where it remained oxidized, so allowing an estimate of pH change to be made in these circumstances.

A relatively high concentration of potassium in the medium is essential for active dissimilation of citrate and malate (Keddie, 1959). Consequently pH adjustments of malate media were always made with KOH and potassium citrate was used in the citrate media.

Temperature of incubation. Unless otherwise stated, incubation was at 30°.

RESULTS

The identification of the organisms as enterococci was confirmed by established physiological and biochemical tests which collectively separate the enterococci from other streptococci. These tests are: ability to tolerate 6·5% NaCl, to hydrolyse

Table 2. *Differentiation of the organisms by currently used tests*

	Streptococcus faecalis (no. positive out of 18 strains tested)	*Streptococcus faecium* (no. positive out of 29 strains tested)
Fermentation of:		
Arabinose	4	20
Melibiose	0	18
Melezitose	12*	0
Sorbitol	18	2†
Glycerol (in the absence of fumarate)	18‡	24 (aerobic)
Tolerance of:		
Tellurite	18	0
Reduction of:		
Methylene blue (1·0%)	18	0
Tetrazolium (0·01%)	18	0
Litmus	18	0

* Three strains gave melezitose-fermenting mutants.

† Both strains gave sorbitol-fermenting mutants.

‡ Three strains fermented glycerol aerobically and gave anaerobically fermenting mutants; one strain fermented glycerol aerobically only.

arginine, to withstand heating at 63° for 30 min., to initiate growth at pH 9·6 and to grow at 10° and 45°. Only the ability to grow at pH 9·6 and at 10° and 45° was common to all strains. Where growth at pH 9·6 was tested by the modification of the method of Chesbro & Evans (1959) in which the medium was made into a soft agar, most strains formed a uniform dense growth but *Streptococcus durans* and similar strains gave rise only to a few colonies, suggesting that most of the organisms had not survived. The 'survivors' did not seem to be mutants adapted to alkaline conditions since inocula prepared from them gave only a few colonies, as before. Of the remaining tests arginine hydrolysis was the most useful since only one strain (the motile enterococcus) was negative. This strain was at one time able to hydrolyse arginine but has lost this property within a 4-year period.

The organisms were next subjected to tests commonly used for the differentiation of the enterococci. The results (Table 2) distinguished two main groups which will be discussed as *Streptococcus faecalis* and *S. faecium*. Only the tellurite and reduction tests differentiated the groups completely and the reduction tests (see Methods) had to be modified (Whittenbury, 1956) to achieve a separation. The rate at which tetrazolium, methylene blue and litmus were reduced proved to be a more useful criterion than the ability to reduce these substances; this also seems to have been the view of other workers including Deibel *et al.* (1963). The additional tests that were used are described below and the results are given in Table 3.

Table 3. *Differentiation of the organisms by additional tests*

	Streptococcus faecalis	*Streptococcus faecium*
Fermentation of glycerol in the presence of fumarate	+	*A*
H_2O_2 from:		
Basal medium	−	+
Glycerol	−	+*
Sorbitol	−	+*
Mannitol	−	+*
Malate as an energy source	±†	−
Gas from malate + glucose	−	+
Citrate as an energy source at pH 8·0	+	−
Catalase (in presence of heated blood)	±‡	−

+ = all strains positive, − = all strains negative, ± = some strains positive and some negative, *A* = negative or aerobic only.

* If substrate used.

† $\frac{2}{18}$ strains negative.

‡ $\frac{16}{18}$ strains positive.

Action on polyhydroxy alcohols

Glycerol. A test separating *Streptococcus faecalis* from *S. faecium* by their action on glycerol, the former fermenting it anaerobically and the latter fermenting it aerobically or not at all, was developed from the work of Gunsalus (1947). He noted that organisms able to ferment glycerol anaerobically did so more rapidly when fumarate was added to the medium as an exogenous hydrogen acceptor. Experiments with a soft agar medium confirmed this finding and revealed that a separation based on glycerol fermentation could only be achieved by adding fumarate (sodium salt 0·5 %, w/v) to the medium. In the absence of fumarate one strain of *S. faecalis* (NCIB 370) fermented glycerol aerobically only; three other strains fermented it aerobically and gave anaerobically fermenting mutants. Deibel *et al.* (1963) had found fumarate useful in this test but observed that one strain of *S. faecalis* fermented glycerol aerobically only while one strain of *S. faecium* fermented glycerol anaerobically, so limiting the value of the test. In these instances a further test based on glycerol metabolism might prove useful, namely the ability to form hydrogen peroxide from glycerol. Gunsalus (1947) observed that streptococci which fermented glycerol only aerobically, formed H_2O_2 in the process. Of the strains studied here all those identified as *S. faecium* and able to use glycerol formed H_2O_2 on glycerol heated blood *o*-dianisidine agar. No *S. faecalis* strain,

including the one that showed a strictly aerobic action in the absence of fumarate, formed H_2O_2 on this medium.

Mannitol and sorbitol. Both these hexitols were fermented aerobically and anaerobically by all the *Streptococcus faecalis* strains and by some of the *S. faecium* strains. The hexitol-fermenting *S. faecium* strains, however, formed H_2O_2 on hexitol heated blood *o*-dianisidine agar and the *S. faecalis* strains did not. Therefore inability to ferment these hexitols, or the production of H_2O_2 from them when they were used, proved useful in separating *S. faecium* from *S. faecalis*.

Hydrogen peroxide formation on heated blood o-dianisidine agar

All the organisms identified as *Streptococcus faecium* but none identified as *S. faecalis* formed H_2O_2 on heated blood *o*-dianisidine basal agar. The H_2O_2 reaction by *S. faecium*, although distinct, did not cover such a wide area around the growth as it did when a utilizable polyhydroxy alcohol was present. The addition of glucose to the medium inhibited the production of an H_2O_2 reaction by all but three strains of *S. faecium*. Consistent results for H_2O_2 formation were only obtained when the inocula used had been grown on aerobically incubated slopes. Some strains of *S. faecalis* formed traces of H_2O_2 when the inocula were not fully adapted to aerobic conditions as, for instance, when they were taken from static broth cultures.

Greening on blood agar

Deibel *et al.* (1963) differentiated the non-haemolytic enterococci on sheep blood agar: *Streptococcus faecium* but not *S. faecalis* gave a strong greening reaction. A similar finding was made in the present work with ox blood and horse blood agars. The clearest results were obtained with surface inoculated cultures.

The addition of glucose (1 %, w/v) to the blood media had the effect of lysing red cells under and around the growth of *Streptococcus faecium* but not those under and around the *S. faecalis* growth. In all but three instances the *S faecium* strains did not cause greening. Since these strains were those which formed H_2O_2 on heated blood *o*-dianisidine glucose agar, the relationship of the greening reaction with H_2O_2 production with unlysed blood agar, with and without added glucose and *o*-dianisidine, was examined. The areas of greening corresponded with the H_2O_2 areas and glucose had a similar effect on both. This circumstantial evidence suggests that greening by the *S. faecium* strains is caused by the H_2O_2 they form.

Catalase activity

Catalase activity on heated blood agar was of some differential value in that 15 of 18 strains of *Streptococcus faecalis* were positive whilst all strains of *S. faecium* were negative. No organism produced pseudocatalase.

Malate and citrate dissimilation

The following tests were developed from methods used to identify strains of *Streptococcus faecalis* and *S. faecium* from silage (Whittenbury, 1956).

Malate. All but two of the strains of *Streptococcus faecalis* used malate as an energy source; the two exceptions had no action on malate. All the strains of *S. faecium* dissimilated malate but did not use it as an energy source. With both types

of organism there was an increase in pH value, indicating decarboxylation; gas (CO_2) production was frequently observed (always when the tubes were heated in boiling water); acetoin was detected, with *S. faecalis* there was a marked increase in amount of growth over that in the basal medium.

A more precise test was obtained when glucose (2·0%, w/v) was included in the medium. In this case *Streptococcus faecalis* acidified the medium and had no effect on the malate. *Streptococcus faecium*, on the other hand, fermented the glucose and simultaneously dissimilated the malate so that initially the pH value decreased but finally rose to pH 7·0 or higher. Gas production was vigorous; a light tapping of the tube at 2–3 days frequently resulted in an effervescence which forced the seals out of the tubes. Acetoin formation from malate was not investigated in this medium since many strains form acetoin from glucose.

Measurements were made of glucose disappearance, pH value and growth in various concentrations of malate and glucose. This showed that the vigorous dissimilation of malate by *Streptococcus faecium* in the presence of glucose reflected a great increase of growth over that developing in the medium containing malate but no added glucose. With *S. faecalis* glucose had a diauxie effect on malate utilization; glucose-grown cocci adapted to malate only when glucose had disappeared from the medium. Consequently in a medium containing excess glucose growth conditions became unfavourable before adaptation was possible. These results indicate that *S. faecalis* and *S. faecium* metabolized malate by different pathways. There is evidence (Whittenbury & Playne, unpublished) which shows that *S. faecium* carries out a non-energy-yielding dissimilation of malate under anaerobic conditions; acetoin, lactate and carbon dioxide are the principal end-products. *Streptococcus faecalis*, on the other hand, carries out an energy-yielding fermentation of malate; acetoin, lactate, formate, acetate, carbon dioxide and ethanol are the major end-products. Acetate and formate are probably the final products of a phosphoroclastic split of pyruvate to formate and acetyl phosphate, the acetyl phosphate giving rise to adenosine triphosphate and acetate.

Citrate utilization. Deibel *et al.* (1963) found the ability to use citrate a valuable test; all their strains of *Streptococcus faecalis* used citrate as an energy source, but only a few strains of *S. faecium* did so. A similar finding (Whittenbury, 1956) led to the development of a medium in which only *S. faecalis* used citrate. It was observed that as the initial pH value of the medium was increased from pH 6·0 to 7·0 fewer strains of *S. faecium* were able to use citrate and in a medium initially at pH 8·0 none did. The changes observed in the absence of glucose were acetoin production, increase in pH value, gas (CO_2) production and increased growth over that in the basal medium. Glucose (2·0%, w/v) had a diauxie effect on citrate fermentation by both types of organism.

Other tests. No other differential tests were devised. An attempt, however, was made to separate the organisms on their metabolism of serine and arginine. Deibel & Niven (1960) reported that *Streptococcus faecalis* and its varieties, but not *S. faecium*, deaminated serine and used it as an energy source. In the present work some strains of both species were found to use serine whilst others did not. Deibel (1960) noted that whereas both species deaminated arginine, only *S. faecalis* used it as an energy source. Strains of both species studied here, however, derived benefit from arginine (0·3%, w/v) in the complex basal medium. Strains of *S. faecium*

obtained least; these gave twice the amount of growth observed in the basal medium, whilst *S. faecalis* gave 4 to 5 times more growth. One strain of *S. faecium* did not hydrolyse arginine; it proved a useful control since the addition of arginine to the basal medium did not affect its growth. Glucose had a diauxic effect on the arginine metabolism of the several strains of *S. faecalis* tested, glucose-grown inocula using arginine only when glucose was no longer detectable. Arginine-grown inocula, however, used both glucose and arginine simultaneously. This diauxie effect was not observed with *S. faecium*.

DISCUSSION

The enterococci were conveniently separated into two species, *Streptococcus faecalis* and *S. faecium*. Among the organisms identified as *S. faecalis* were those recognizable as *S. faecalis* (non-haemolytic and non-proteolytic), *S. zymogenes* (haemolytic and sometimes proteolytic), and *S. liquefaciens* (non-haemolytic and proteolytic); apart from differences in haemolytic, proteolytic and one or two fermentative properties these organisms constitute a fairly homogeneous group. Of the organisms identified as *S. faecium* some were regarded as 'typical'—those fermenting arabinose, melibiose and mannitol and, aerobically only, glycerol—and some were identified as *S. durans*—those fermenting glycerol aerobically only but failing to ferment arabinose, melibiose and mannitol. Haemolysis did not seem to be a reliable characteristic of *S. durans* as one strain (498) lost its haemolytic activity. Deibel *et al.* (1963) also observed the instability of this character among their strains of *S. durans*. Other organisms identified here as *S. faecium* possessed various ranges of properties and occupied intermediate positions between the 'typical' *S. faecium* and *S. durans*. The one motile strain examined and identified as *S. faecium* appears to be similar to the yellow pigmented, motile streptococci described by Graudal (1952), to the motile enterococci described by Langston, Gutierrez & Bouma (1960), possibly to those described in the references listed by Hugh (1959) and to the motile Group D streptococcus mentioned by Sherman (1938). Apart from its ability to ferment inulin and loss of ability to hydrolyse arginine within a 4-year period, it is very similar to the 'typical' *S. faecium*.

Of the established tests used in this study only tellurite tolerance and reducing activity completely differentiated the organisms; the other commonly used tests were, for various reasons, of limited value. Of the new or modified tests used, fermentation of glycerol in a soft agar medium containing fumarate, hydrogen peroxide formation from polyhydroxy alcohols and ability to ferment them, hydrogen peroxide formation on a medium containing no added sugar or polyhydroxy alcohol, citrate fermentation and dissimilation of malate in the presence of glucose seem to be the most useful. Catalase activity and the ability to use malate as an energy source were also useful but did not differentiate the organisms completely. The practical value of these tests in the routine identification of enterococci remains to be assessed from studies of larger collections of enterococci from divers sources.

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Spore Surface Depsipeptides in Some Pithomyces Species

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SUMMARY

Spicules morphologically similar to those present on the spores of *Pithomyces chartarum* invest the spores of several other *Pithomyces* species. Depsipeptides are quantitatively removed from dried mycelial felts by brief chloroform washing, and their amount is proportional to the number of spores present. Three pure spore-coat depsipeptides have no demonstrable antibiotic activity.

INTRODUCTION

The fungus *Pithomyces chartarum* produces a group of metabolic products, the sporidesmolides. These compounds, which have physical properties similar to those of lipids, are cyclic depsipeptides (Russell, 1962). Consideration of analytical data prompted the conjecture (Russell & Brown, 1960) that these materials formed the spiculate spore-coat visible in electron micrographs of spores of this species, a suggestion later confirmed by several independent lines of evidence (Bertaud, Morice, Russell & Taylor, 1963). We have now extended this work by a chemical and electron microscopical examination of some other fungi of the genus *Pithomyces*.

METHODS

Organisms. Not all the species of Pithomyces listed by Ellis (1960) could be obtained for this study, but all of those available were examined. Isolate c of *Pithomyces chartarum* (Dingley, Done, Taylor & Russell, 1962) was obtained from Dr Drew Smith, Ruakura Animal Research Station, Hamilton, New Zealand. Dr M. B. Ellis, Commonwealth Mycological Institute, Kew, England, kindly supplied other isolates, which were as follows: *P. maydicus* (IMI 98084, IMI 46232); *P. sacchari* (IMI 102686); *Pithomyces* 'species v' (Dr M. B. Ellis, personal communication; IMI 102682); *Pithomyces* species unknown (IMI 101184). Other *P. chartarum* isolates, kindly supplied as dried mycelial felts by Miss Joan M. Dingley (Plant Diseases Division, D.S.I.R., Auckland, New Zealand), were PDD 36 c, PDD 6324 and PDD 6323.

Cultivation of organisms. Reasonably stable high-sporing cultures were obtained by single spore or single hyphal tip transfers from high-sporing sectors of the isolates. These were maintained on plates of potato glucose agar (Oxo Ltd., London)

at 25° in constant light of approximately 3 ft.c. provided by 'Osram' White fluorescent tubes (Osram, (G.E.C.) Ltd., North Wembley, Middlesex) for 8 hr alternating with 16 hr of complete darkness. For electron microscopy, spores were taken from potato glucose agar plates at 10–14 days. For isolation of depsipeptides, the organisms were grown on potato glucose agar slopes, and spores washed from these with sterile 0·05% (v/v) Lissapol N (Imperial Chemical Industries Ltd., Millbank, London) were used for inoculum. The medium for *Pithomyces chartarum* was potato + carrot extract (Done, Mortimer, Taylor & Russell, 1961), and that for IMI 101184 and *P. maydicus* IMI 46232 was the salts + glucose + asparagine medium of Ross (Butler, Russell & Clarke, 1962). For *P. maydicus* IMI 98084 and for IMI 102682 this medium was enriched with yeast extract (0·1%, w/v; 'Difco' brand, Baird & Tatlock, Ltd., Chadwell Heath, Essex). The medium (100 ml.) was placed in 1 l. Roux bottles or one-pint milk bottles (44 mm. internal neck diameter), plugged with cottonwool. The vessels were autoclaved at 120° for 15 min., cooled, and inoculated with a spore suspension (0·1 ml.) containing 10^5 spores/ml. The bottles were incubated in a horizontal position for 2 weeks at 25°, while being illuminated intermittently as above. At the end of the incubation the fungus was collected, washed and dried as previously described (Done *et al.* 1961).

Electron microscopy. Whole spores were examined by using the technique described to us by Mr W. S. Bertaud (personal communication). A dry specimen of spores was obtained on carbon-coated collodion films on specimen grids by inverting a mature plate culture over the grids, and tapping it until a sparse sprinkling of spores had been released. The preparation was then shadowed at an angle of $\tan^{-1} 1/3$ (with either carbon or gold + palladium wire) and examined in a Philips EM 100 electron microscope.

Analytical methods

Spore number, mycelial dry weight and *reducing sugars* were determined as before (Done *et al.* 1961), and *total nitrogen* was estimated by a micro-Kjeldahl procedure (Chibnall, Rees & Williams, 1943). *Specific rotations* for the D line of the sodium spectrum were measured for 0·5–2·0% (w/v) solutions in chloroform at room temperature in a 1 dm. tube. *Infrared spectra* were determined for paraffin mulls with a Perkin-Elmer 'Infracord' spectrophotometer, and *ultraviolet spectra* were measured for ethanol solutions with a Unicam S.P. 500 spectrophotometer. *Melting points* were determined on a Kofler block and are corrected.

Chromatography. Thin-layer plates (250 μ) of Kieselgel G (E. Merck A. G., Darmstadt, Germany) were washed chromatographically in methanol and concentrated hydrochloric acid (9 + 1, by vol.) to remove traces of iron, and dried for 1 hr at 110°. Depsipeptide solutions (4 μl.; 2%, w/v, in chloroform) were applied and the solvent allowed to evaporate. The plates were developed in chloroform + ethyl acetate (4 + 1, by volume) and dried for 5 min. at 100°. Spots were revealed by spraying the dried plates with iodine (0·25%, w/v) in diethyl ether, and stabilized by further spraying with an aqueous solution of soluble starch (1%, w/v) and potassium iodide (1%, w/v).

Depsipeptides. The dried fungal tissue (5 g.), coarsely powdered, was stirred with chloroform (50 ml.) for 5 min. and collected by filtration. Two further extractions were made. The combined filtrates were evaporated to dryness *in vacuo* at 50°.

To the residue was added diethyl ether (20 ml.) which had previously been saturated with the appropriate depsipeptide and filtered. After 16 hr the insoluble residue was collected at the centrifuge, washed with depsipeptide-saturated ether (10 ml.) and allowed to dry at room temperature. The crude depsipeptide was dissolved in chloroform (4 ml.) and the solution decolorized by passage through activated carbon as previously described (Done *et al.* 1961). It was advantageous to support the carbon on a similarly sized pad of aluminium oxide (British Drug Houses Ltd., Poole, Dorset; 'for chromatography'). The effluent and washings (3×2 ml.) were mixed, the volume adjusted to 10·0 ml. and the optical rotation determined. The chloroform was evaporated on a steam bath and the residue dried to constant weight at 110°. The technique used on the preparative scale (30–40 g. dried mycelial felts) was identical except that pure diethyl ether was used.

Residues obtained in this way were usually electrostatically charged. The charge was completely removed by moistening the solid with diethyl ether and allowing the ether to evaporate.

Tests for antibiotic activity

Depsipeptides were screened for antimicrobial activity by an agar plate technique. They were dissolved in chloroform to give solutions of 10 mg./ml. Paper discs, 7 mm. diameter, cut from Whatman no. 1 filter paper, were moistened with 0·01 ml. of solution by means of a micropipette, and sterilized by dry heat at 160°. The impregnated discs, each containing 100 μg. depsipeptide, were then applied to the surfaces of agar plates previously seeded with *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus cereus*, *Escherichia coli*, *Candida albicans* or *Trichophyton mentagrophytes*. The type and depth of medium used, the size of the inoculum and other conditions were adjusted to afford maximum sensitivity in the tests. After appropriate incubation periods the plates were examined for the presence of zones of partial or complete inhibition around the discs. Discs containing 10 μg. Framycetin sulphate (Laboratoires Roussel, Boulevard des Invalides, Paris 7e, France) were used as positive controls in the antibacterial tests, and discs containing 100 μg. Episol (Crookes Laboratories, Gorst Road, London, N.W. 10) were used as positive controls on the Candida and Trichophyton test plates.

RESULTS

Spores from all isolates grown on potato glucose agar and examined in the electron microscope were found to bear spicules similar to those present on *Pithomyces chartarum* spores (Bertaud *et al.* 1963; Pl. 1, figs. 1–5). The spicules were apparently easily detached, even by the relatively gentle methods used to prepare the specimens, and frequently the grid around a spore showed numbers of free spicules (Pl. 2, fig. 6). These often looked more obviously crystalline than the undetached spicules, which frequently, particularly in early experiments with carbon shadowing, appeared to have melted partially and then resolidified (Pl. 2, fig. 7). This may perhaps be explained by the greater rate of heat loss from a small crystal when resting on a grid, as compared with that from a spicule surrounded by many others and suspended in a vacuum.

On potato+carrot extract at 25° the degree of sporulation varied widely from isolate to isolate and from batch to batch of the same isolate. It was therefore

necessary to determine, for each isolate, conditions for obtaining satisfactory sporulation. We were thus led to a more general study of environmental effects on sporulation in this genus, the results of which will be described in a later publication. The *Pithomyces sacchari* isolate spored only sparsely under all conditions tried. The other isolates were grown at 25° for 2 weeks on defined or partially defined media, except for the *P. chartarum* isolates which were grown on potato+carrot broth. Early in this work we found that sporulation of *P. chartarum* was affected by light, and thereafter we adopted standard conditions of illumination for all cultures.

Dried felts of each of the other isolates were first analysed by the technique used for determining sporidesmolides in *Pithomyces chartarum* (Done *et al.* 1961), but with the use of pure ether in place of sporidesmolide-saturated ether. In every case but that of *P. sacchari*, materials were isolated whose properties were similar to those of sporidesmolides: they were insoluble in water, very difficult to wet, very sparingly soluble in ether and readily soluble in chloroform. Shaken with chloroform+methanol+water (10+7+3, by vol.) they were recovered quantitatively from the lower phase, and it was therefore clear that they could be isolated quantitatively in the same way as the sporidesmolides. However, this technique includes a preliminary overnight extraction with hot methanol, and can give no indication of the possible location of the substances extracted. Compounds located on the spore surface and readily soluble in chloroform should be removed from dried mycelial felts by brief washing with this solvent. Sporidesmolides were accordingly extracted from dried *P. chartarum* felts by brief washing with chloroform, and were readily and quantitatively isolated from the extracts in pure form. When the chloroform-washed fungal tissue was dried and further extracted with hot methanol (Done *et al.* 1961), no more sporidesmolides were obtained.

Dried felts of the other isolates were examined in the same way, except that the ether used was saturated with the material isolated from the appropriate organism by the earlier method. Occasionally a final overnight stirring with chloroform replaced the extraction with hot methanol. Crystalline ether-insoluble materials were completely removed by three chloroform washes, and were shown by infrared spectroscopy to be identical with the products isolated previously. Spicules were absent from chloroform-washed spores. The accessibility of the isolated substances, as shown by the results in Table 1, was similar to that of the sporidesmolides, and this was consistent with their being similarly located. Such results would also be expected if three chloroform washes sufficed to extract all chloroform-soluble material completely. This was not the case, however, for, in other routine experiments, stirring the residue overnight with chloroform invariably removed considerable amounts of extractive. This material, which was readily and completely soluble in ether and may have been lipid derived from the cell wall (Russell, Sturgeon & Ward, 1964) or cytoplasm, was not examined further.

The chloroform-soluble ether-insoluble materials isolated in this way were examined by thin-layer chromatography (Table 2). Use of the non-specific detecting reagent iodine allowed the purity of the isolated materials to be assessed. The *Pithomyces* species IMI 101184 and IMI 102682 both gave residues which produced a single spot when examined in this way. The R_F values were identical, and recrystallization from ethanol gave, in both cases, the same compound, angolide (Russell

& Ward, 1964), $C_{22}H_{38}N_2O_6$, m.p. 261–262°, $[\alpha]_D$ −83°. A single spot was also given by the residue obtained from *P. maydicus* IMI98084, and recrystallization from pyridine and water (7+3, by vol.) furnished a second compound (Bishop & Russell, 1964), $C_{34}H_{60}N_4O_8$, m.p. 227–228°, $[\alpha]_D$ −195°, for which we propose the name sporidesmolide IV. The residue from *P. maydicus* IMI46232 was indistinguishable from that obtained from *P. maydicus* IMI98084 in chromatographic behaviour, melting point and infrared spectrum. However, it had a lower specific rotation ($[\alpha]_D$ = −183°) even after two recrystallizations, and appears therefore to be a very closely related compound, or a mixture (Table 3).

Table 1. *Removal of depsipeptides by chloroform washing of dried powdered mycelial felts of some Pithomyces species*

Mycelial felts were stirred with chloroform for 5 min. and filtered. After three such extractions, either a fourth overnight extraction was performed, or depsipeptides were determined in an extract prepared by continuous overnight extraction with hot methanol.

			Wt. of depsipeptide (mg.) extracted by				
			chloroform			or	methanol
Batch no.	Isolate	Wt. (g.)	1	2	3	4	
2	*P. chartarum*	21·6	453	98	25	—	0
27	IMI101184	30·3	206	35	11	—	0
34	IMI101184	10·9	107	31	0	—	—
29	*P. maydicus* (IMI98084)	35·2	236	7	5	—	0
36	IMI102682	8·4		18	0	—	—
54	IMI102682	7·2		21		0	—
49	IMI101184	23·8		173		—	0
32	*P. maydicus* (IMI98084)	35·2		354		—	0
55	*P. maydicus* (IMI46232)	22·1		962		0	—

Table 2. *Behaviour of some Pithomyces depsipeptides on thin-layer chromatography*

The chromatography was done with Kieselgel G, and solvent chloroform + ethyl acetate (4+1, by vol.).

Compound	R_F
Sporidesmolide III	0·26
Sporidesmolide I	0·54
Sporidesmolide IV	0·62
Angolide	0·70

Thin-layer chromatograms prepared from the total sporidesmolide fraction of *Pithomyces chartarum* isolate 'C' revealed with iodine two spots corresponding to the known constituents, sporidesmolide I (Russell, 1962) and sporidesmolide III (Russell, Macdonald & Shannon, 1964). The spot due to sporidesmolide I possessed a 'beard' which may have been due to the presence of a higher homologue of sporidesmolide I since its position coincided approximately with that of sporidesmolide IV. The same chromatographic pattern was also given by residues from other isolates of *P. chartarum*, and the physical and chemical properties of the sporidesmolide mixtures were the same (Table 3).

That the two new compounds were cyclic depsipeptides was suggested by their high melting points and specific rotations, by their neutral character, and in particular by their infrared spectra, in which absorptions due to ester and amide linkages were present. Neither had other than end-absorption in the ultraviolet region. Preliminary chemical studies, briefly reported elsewhere, have confirmed this identification (Russell & Ward, 1964; Bishop & Russell, 1964).

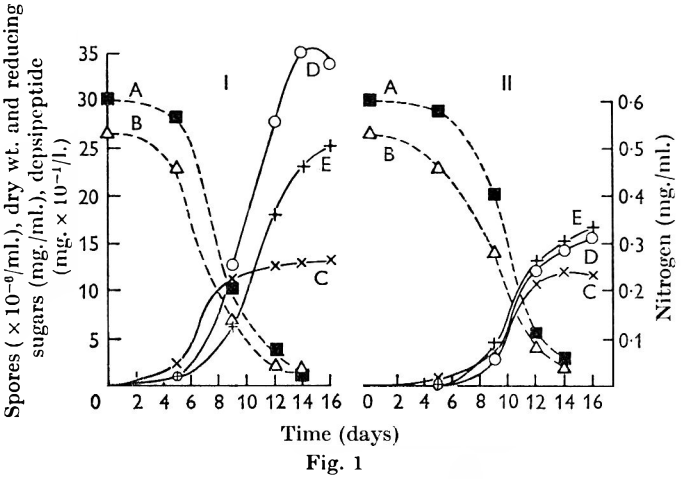

Fig. 1

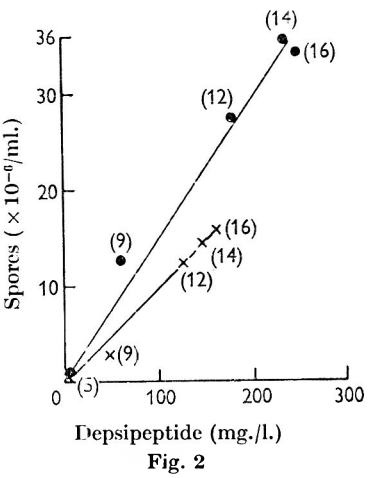

Fig. 2

Fig. 1. Utilization of medium constituents and production of depsipeptides by *Pithomyces maydicus* IMI 98084 (I) and Pithomyces IMI 101184 (II). A, Reducing sugar; B, nitrogen; C, mycelial dry weight (all in mg./ml.); D, spore count × 10^{-6}/ml.; E, depsipeptides, mg. × 10^{-1}/l.

Fig. 2. Spore count/ml. medium as a function of depsipeptide production. ●—●. *Pithomyces maydicus* IMI 98084; × — ×, Pithomyces IMI 101184. Figures in parentheses indicate the age of culture (days) at which the particular analysis was made.

Table 3. *Spore-surface depsipeptides of some Pithomyces species*

Isolate	Species	Depsipeptides identified	Yield (mg./l. medium)
C, PDD 36c, PDD 6324, PDD 6323	*P. chartarum*	Sporidesmolides I and III	100–300 (15); 9 (1)*; 68 (1)*; 138 (1)*
IMI 98084	*P. maydicus*	Sporidesmolide IV	77–230 (17)
IMI 46232	*P. maydicus*	Unknown	481 (1)
IMI 101184	Not assigned	Angolide	20–160 (9)
IMI 102682	'Species v'	Angolide	11–15 (2)

Figures in parentheses refer to the number of experiments performed.
* Identified by physical properties of mixture and thin-layer chromatography only.

The isolate of *Pithomyces sacchari* examined formed few spores and yielded little extractive to chloroform. The extract gave two slow-moving spots on a thin-layer chromatogram, and an acid hydrolysate of the material present was ninhydrin-positive. There was too little of this material for further investigation; it seems very likely that the extract contained peptide-like substances.

Cultures of IMI 101184 and *Pithomyces maydicus* IMI 98084 were serially harvested and analysed, with results shown in Fig. 1; repetition of the experiments gave similar

results. The two species produced, respectively, angolide and sporidesmolide IV, in amounts proportional to the number of spores present in the cultures at harvest (Fig. 2).

Antibiotic tests. Sporidesmolides I and IV and angolide, tested for antibiotic properties, produced no inhibition zones on the test plates, indicating that they possessed little or no activity. The positive control discs all produced zones of inhibition, ranging in diameter from 12 mm. (*Streptococcus pyogenes*) to > 50 mm. (*Trichophyton mentagrophytes*).

DISCUSSION

Bertaud *et al.* (1963) gave very complete evidence that the spores of *Pithomyces chartarum* were covered with spicules of sporidesmolides. Earlier isolations of sporidesmolides from dried pasture samples (White, 1958; Perrin, 1959; Sandos, Clare & White, 1959; Russell, Synge, Taylor & White, 1962) and their correlation with numbers of *P. chartarum* spores (Thornton & Sinclair, 1960) make it clear that this is not an *in vitro* phenomenon only, but that these compounds are also produced by the organism under natural conditions in the field. The present work provides analytical and electron-microscope evidence for a similar feature in each of the Pithomyces isolates examined. We infer that the possession of a crystalline depsipeptide spore coat is a general feature of fungi of the genus.

It has been suggested (Bishop & Russell, 1964) that depsipeptide analysis in this genus is a simple and useful taxonomic aid. Thus, all isolates of *Pithomyces chartarum* examined in the present work gave depsipeptides whose pattern of spots on thin-layer chromatograms was identical, and was readily distinguished from the single spot given by both the isolates of *P. maydicus* examined. Since it has been reported (Dingley, 1962) that spore morphology is not reliable in distinguishing between the two species, the simple technique described here could be helpful in cases where differentiation was important, as in studies on the pathogenesis of facial eczema.

It was suggested (Bertaud *et al.* 1963) that because of their chemical similarity to certain antibiotics the *Pithomyces chartarum* depsipeptides might confer a selective advantage on this organism in its natural habitat by inhibiting the growth of bacteria. To demonstrate conclusively the absence of antibiotic activity is impossible, but neither the present findings nor those of other workers (Shemyakin *et al.* 1963) provide any evidence to support this suggestion. However, as pointed out to us by Dr A. Taylor (personal communication), none of the organisms against which the compounds were tested is a likely neighbour of Pithomyces spp. in the field.

It was also suggested earlier that the spicules might contribute to the water-repellent properties of the spores. This effect, if present, might be of more general interest, for spores of Aspergillus and Penicillium contain cyclic peptides that may be on the surface (Sumiki & Miyao, 1952), and these spores are also water-repellent. The experiments of Davies (1961) suggested that water-repellent properties may be important in spore dispersal; this is supported for *Pithomyces chartarum* by the observations of Crawley, Campbell & Smith (1962). However, spores of this organism also bear a surface layer of lipid (Bertaud *et al.* 1963). Whether the depsipeptide spicules significantly augment water-repellency is therefore open to doubt, particularly as they are so easily detached. There is no compelling reason to suppose

that the spore-surface depsipeptides possess a 'function', i.e. that their presence confers a selective advantage. They may be no more than excretory products formed by an aerial structure. In the absence of more efficient competing forms, the apparent wastefulness of such a situation has no selective significance.

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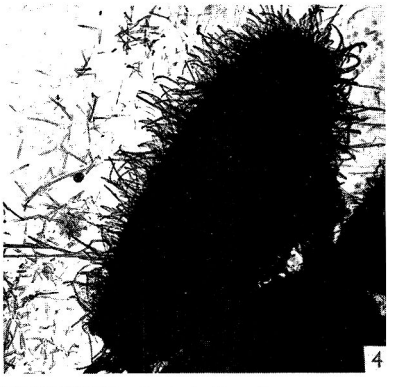
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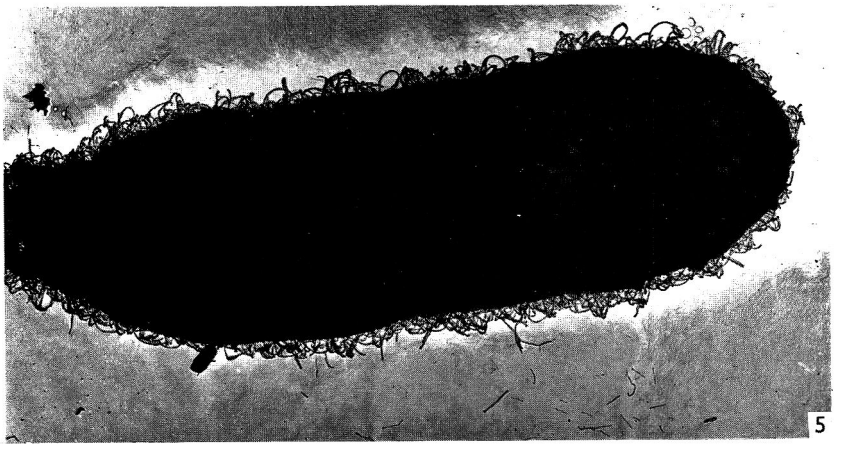
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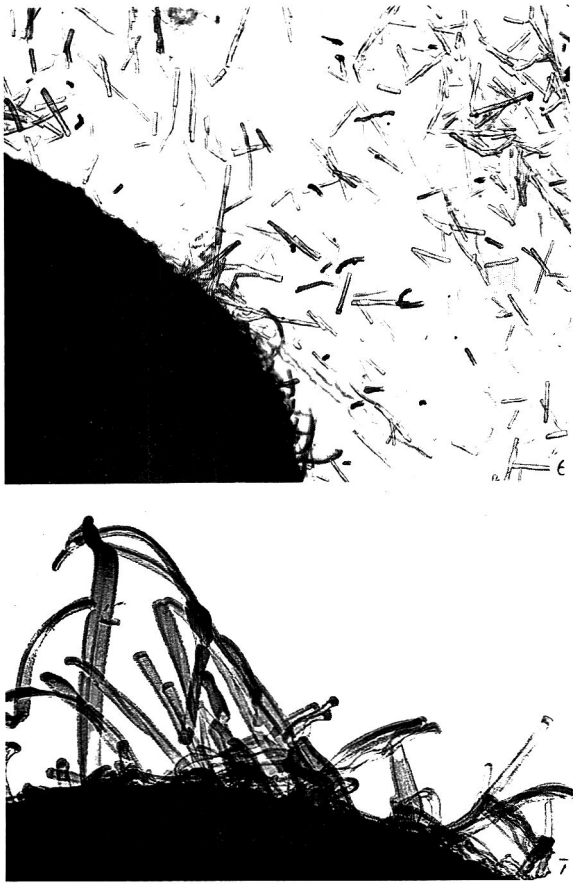

EXPLANATION OF PLATES

Plate 1

Electron micrographs of whole spores of *Pithomyces* species shadowed with gold + palladium, all × 4950.

Fig. 1. *Pithomyces maydicus* IMI98084.

Fig. 2. *P. maydicus* IMI46232.

Fig. 3. *P. sacchari* IMI102686.

Fig. 4. *Pithomyces* 'species v', IMI102682, with detached spicules.

Fig. 5. *Pithomyces* IMI101184.

Plate 2

Fig. 6. Profile electron micrograph of a spore of *P. sacchari* IMI102686, showing detached spicules; × 7200.

Fig. 7. Profile electron micrograph of a spore of *Pithomyces* 'species v', IMI102682, showing partially melted appearance of spicules; × 30,000.