Compartmentation of the Metabolism of Lactose, Galactose and Glucose in *Escherichia coli*

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SUMMARY

Compartmentation phenomena were studied in the course of the simultaneous metabolism of glucose, galactose and lactose by organisms of *Escherichia coli* which were induced for either the *lac* operon, the *gal* operon, both, or neither. Metabolic patterns were investigated in each phenotype by incubating parallel identical cultures with the three sugars in equal chemical concentration but labelled differently with ^{14}C. The four labelled substrates were glucose, galactose and lactose labelled either exclusively in the glucose moiety or exclusively in the galactose moiety.

The metabolites from free glucose in the medium equilibrated with those from free galactose in the medium, but did not equilibrate with metabolic products derived from glucose generated endogenously by the hydrolysis of lactose. Similarly, metabolic products derived from galactose formed in the hydrolysis of lactose equilibrated with those from glucose from the same source, but not with metabolic intermediates formed from either free glucose or free galactose in the medium. Other interpretations of these results, not involving metabolic compartmentation, have been considered and found inadequate to account for the observed results. Some of the implications of compartmentation in bacteria are discussed.

INTRODUCTION

Subcellular compartmentation of pools of metabolic intermediates is known to be a widespread phenomenon (Moses, 1966), and it is probable that compartmentation plays a role in the control of biochemical reactions *in vivo*. Most evidence for metabolic compartmentation so far obtained has come from studies on cells which contain observable barriers to the free diffusion of solutes. The present communication describes an investigation made upon an organism which contains no visible internal membranes, namely, *Escherichia coli*.

Compartmentation is not necessarily a morphological phenomenon; the free diffusion of metabolic intermediates may be inhibited in other ways. At a molecular level, for example, one might envisage compounds bound to the surface of an integrated enzyme sequence, with intermediates along the pathway never being released to equilibrate with their respective pools. Similarly, if the position at which a particular compound is formed and released in the cell is sufficiently distant from the location of its intracellular pool it may undergo reaction before reaching the intracellular pool,

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thus preventing the latter from achieving true equilibrium with the compound at all its locations within the cell.

Experimentally, the study of compartmentation in living cells presents certain problems arising from the difficulty of distinguishing between several reservoirs of a substance without fractionating the cell and thereby causing its death. Those attempts which have been made to investigate compartmentation *in vivo* have relied upon studies of the metabolic behaviour of certain compounds. In some investigations metabolic data is best interpreted by supposing that certain substances exist in two or more non-equilibrating reservoirs. Moses & Lonberg-Holm (1966) extended this approach and sought to expose metabolic compartmentation by designing experiments in which certain metabolites would be generated intracellularly in a number of ways, and would be recognizable as a result of radioactive labelling. This method has been used in the present study.

The area of metabolism chosen for study was that involving the hydrolysis of lactose to glucose and galactose, the subsequent phosphorylation of the monosaccharides, the isomerization of galactose derivatives to glucose derivatives, and the further metabolism of the latter in the cell. *Escherichia coli*, when grown in appropriate media, is able to metabolize lactose, glucose and galactose. During lactose metabolism, the monosaccharides are presumably released within the bacterium, while metabolism of the monosaccharides supplied directly from the medium must involve also the entry of the substances into the bacterium either as free sugars or as derivatives. The question we sought to answer was this: is glucose (or galactose) produced inside the bacterium by the hydrolysis of lactose distinct metabolically from glucose (or galactose) presented to the outside of the bacterium? Or put slightly differently: is the metabolic fate of glucose (or galactose) in any way dependent on its origin?

The problem may be studied by using a radioactive labelling technique. When cells are supplied simultaneously with glucose, galactose and lactose as substrates one might study the fate of various substrates by parallel experiments differing only in the location of the radioactive marker. In the present study it was necessary to use four parallel reaction vessels, each identical chemically, and containing all three sugars. The labelling pattern was then varied so that the four flasks were supplied with labelled glucose, labelled galactose, lactose labelled in the glucose moiety and lactose labelled in the galactose moiety, respectively. It was then possible to study, for example, the fate of glucose derived by the hydrolysis of lactose in bacteria which were simultaneously metabolizing glucose from the medium, and to compare this with the fate of glucose from the medium in a parallel culture of bacteria with an identical history which were also metabolizing glucose derived from lactose. Similar comparisons were also made for galactose, and between glucose and galactose.

Some of the enzymes of lactose and galactose metabolism are inducible and their intracellular levels may be greatly altered by growth in the presence of the appropriate inducers. Glucose metabolism, on the other hand, appears to be phenotypically constitutive in *Escherichia coli*. We included in our studies a comparison of lactose, glucose and galactose metabolism in bacteria induced either for the lactose enzymes, or for the galactose enzymes, or both, or neither.

METHODS

Organism and growth conditions. Escherichia coli strain CAVALLI (*lac⁺gal⁺met⁻thy⁻*) was obtained from Dr A. Simmonds (Dept of Molecular Biology, University of California, Berkeley, Calif., U.S.A.). Stock cultures were maintained on M63 minimal medium (Pardee & Prestidge, 1961) supplemented with glycerol (0·2%, w/v) and specific growth factors. Bacteria were grown in liquid medium with stirring at 37°. Full induction of the *lac* operon was achieved by growing the bacteria for many generations in the presence of 0·5 mM-isopropyl-β-D-thiogalactoside (IPTG). The *gal* operon was induced by growth in the presence of 5 mM-D-fucose. When IPTG and fucose are present together at these concentrations there is no appreciable interaction of either inducer upon the inductive effect of the other (McBrien & Moses, 1966).

Labelled substrates. [G-^{14}C]-D-Glucose (107 μc./μmole) was prepared by the method of Putman & Hassid (1952). [G-^{14}C]-D-Galactose (143 μc./μmole) was prepared according to Bean, Putman, Trucco & Hassid (1953) with minor modifications. It was found necessary to illuminate the algae used for the photosynthetic incorporation of ^{14}CO$_2$ into glycerol-galactoside at a much lower light intensity (11,000 lux) than was previously recommended. Galactose was separated and purified in the manner described by Abraham & Hassid (1957). We are grateful to Dr S. Abraham for assistance in isolating the galactose.

[(G-^{14}C)glucose]Lactose was prepared with lactose synthetase isolated from unpasteurized cow's milk (kindly provided by Challenge Dairies, Berkeley, California, U.S.A.) by the method of Babad & Hassid (1964). [(G-^{14}C)galactose]Lactose was synthesized with a tissue preparation from the mammary gland of a lactating rat (Bartley, Abraham & Chaikoff, 1966). In the preparation of both forms of labelled lactose the labelled hexoses described above were used undiluted by unlabelled sugars, and the products which were isolated and purified by preparative paper chromatography were assumed to have the same specific radioactivities as the parent hexoses. A small sample of each of the labelled lactoses was used to determine the proportion of ^{14}C activity which had been incorporated into the desired moiety. The labelled lactose was hydrolysed with a crude preparation of β-galactosidase obtained from a *lac*-constitutive strain of *Escherichia coli* and the glucose and galactose produced were separated by paper chromatography in ethyl acetate + pyridine − acetic acid + water (5 + 5 + 2 + 3, by vol.). The sugars were located by radioautography, with known radioactive markers on guide strips, the spots excised and the radioactivity measured with opposed end-window Geiger-Müller tubes. In each case at least 97% of the activity was found in the desired moiety.

Labelling experiments. The experimental procedure was adapted from that described by Moses & Lonberg-Holm (1966). Bacteria growing in exponential phase in glycerol minimal medium were harvested at a concentration equiv. 225 μg. bacterial protein/ml. Growth and protein synthesis were halted before harvesting by adding chloramphenicol (100 μg./ml.). The bacteria were washed and resuspended in 0·01 M-phosphate buffer (pH 7·1) containing chloramphenicol (25 μg./ml.). To 20 ml. of this bacterial suspension was added 0·4 ml. of a solution containing 0·7% (w/v) each of lactose, glucose and galactose: after mixing, this gave a concentration of 0·76 mM for the monosaccharides and 0·40 mM for lactose. Four samples of bacterial suspension, each of 2·0 ml., were transferred to 20 ml. flat-bottomed glass vials (as used for

scintillation counting) and placed in a water bath at 37°. The contents of the vials were stirred vigorously by using small polythene-covered magnets over magnetic stirrer motors operating at maximum speed. Fifteen min. after adding the mixed sugars to the bacteria the labelled sugars were introduced as indicated in Table 1. There was no significant contribution to the total sugar content by the addition of the labelled material.

During the following 45 min. after adding the labelled sugars 15 samples of about 30 μl. each were withdrawn from each vial at known times. Each sample was mixed immediately with 0·4 ml. of ethanol (90%, v/v, in water) in pre-weighed stoppered tubes. The tubes were subsequently reweighed to determine the sample size actually

Table 1. *Labelled substrates added to the four parallel vessels of each experiment*

Escherichia coli strain CAVALLI bacteria suspended in 0·01 M-phosphate buffer (pH 7·1) containing chloramphenicol (25 μg./ml.) received 0·76 mM-glucose, 0·76 mM-galactose and 0·40 mM-lactose. Four parallel portions of the suspension (each of 2 ml.) were incubated at 37° with stirring. Fifteen min. later labelled sugars were added as indicated below.

Vessel	Substrate	Vol. of substrate solution (μl.)	Amount of labelled substrate added	
			μc.	μmoles
A	[G-¹⁴C]Glucose	40	8·04	0·075
B	[G-¹⁴C]Galactose	40	6·52	0·046
C	[(G-¹⁴C)Glucose]lactose	30	7·08	0·066
D	[(G-¹⁴C)Galactose]lactose	40	7·80	0·055

taken. The contents of each tube were chromatographed *in toto* by two-dimensional paper chromatography on Ederol no. 202 paper (J. C. Binzer G.m.b.H., Hatzfeld/ Eder, Germany). The solvents were: in the first dimension 90% phenol + water + glacial acetic acid + 0·5 M-K₂EDTA (420 + 80 + 5 + 1, by vol.), and in the second dimension butan-1-ol + propionic acid + water (20 + 9 + 11, by vol.). Radioactive materials on the chromatograms were located by radioautography, and spots so located were excised and counted by using the automatic apparatus described by Moses & Lonberg-Holm (1963). Spots selected for identification were eluted and co-chromatographed with known unlabelled markers. Dicarboxylic acids were located by using bromocresol green reagent (Lugg & Overell, 1948), amino acids with ninhydrin and sugars with AgNO₃ (Smith, 1960).

The experiment was done four times with bacteria in different states of induction for the *lac* and *gal* operons. In bacteria in which neither of these operons were induced the amount of radioactivity incorporated into products from labelled galactose or labelled lactose was so small that no useful information could be obtained. This experiment is therefore not further discussed. The arrangements for the other three experiments are noted in Table 2.

RESULTS

The amount of utilization of the labelled substrates during the period of 45 min. after their addition to the cells is shown in Table 3. It can be seen that in bacteria in which the lactose operon was induced lactose was rapidly hydrolysed to hexose, a large proportion of which accumulated, presumably because subsequent steps in its metabolism were rate limiting. In Table 3 the residual hexose formed from labelled lactose is counted as unused substrate.

Table 2. *Phenotypes of the bacteria used in the labelling experiments*

Escherichia coli strain CAVALLI bacteria were grown in glycerol minimal medium with or without IPTG (0·5 mM) to induce the lactose enzymes or D-fucose (5 mM) to induce the galactose enzymes. The bacteria in exponential growth received chloramphenicol (100 μg./ml.) immediately before harvesting and were then washed and resuspended in 0·01 M-phosphate buffer (pH 7·1) containing chloramphenicol (25 μg./ml.) for the labelling experiments. As a result of the presence of chloramphenicol no enzyme induction could take place during the subsequent metabolism with a mixture of glucose, galactose and lactose. Two ml. of bacterial suspension were used in each reaction vessel.

Experiment	*lac* operon	*gal* operon	Cell concentration (μg. bacterial protein/ ml.)
1	Induced	Induced	198
2	Not induced	Induced	246
3	Induced	Not induced	244

Table 3. *Utilization of labelled substrates by Escherichia coli strain CAVALLI*

Bacteria of the phenotypes shown in Table 2 were allowed to metabolize a mixture of glucose, galactose and lactose in four parallel incubation vessels. Each vessel contained ^{14}C in a different substrate: in free glucose, free galactose, the glucose moiety of lactose or the galactose moiety of lactose. This table presents data on the hydrolysis of lactose and on the utilization of hexoses both from free sugars in the medium and from those produced by lactose hydrolysis, all after 45 min. incubation at 37°.

	Phenotype*		
	$lac^+ gal^+$	$lac^- gal^+$	$lac^+ gal^-$
Extent of lactose hydrolysis (% of total)			
Based on [(G-^{14}C)glucose]lactose	98	9	81
Based on [(G-^{14}C)galactose]lactose	96	17	85
Lactose remaining as free hexose (% of total)			
Based on [(G-^{14}C)glucose]lactose	87	2	19
Based on [(G-^{4}C)galactose]lactose	37	4	92
Glucose from lactose metabolized (μmoles/mg. bacterial protein)	0·214	0·111	0·842
Free glucose metabolized (μmoles/mg. bacterial protein)	0·371	2·175	0·417
Total glucose metabolized (μmoles/mg. bacterial protein)	0·585	2·286	1·259
Galactose from lactose metabolized (μmoles/mg. bacterial protein)	0·996	0·203	0
Free galactose metabolized (μmoles/mg. bacterial protein)	1·078	0·817	0·153
Total galactose metabolized (μmoles/mg. bacterial protein)	2·074	1·020	0·153
Total hexose metabolized (μmoles/mg. bacterial protein)	2·659	3·306	1·412

* + indicates enzymes induced; − indicates enzymes not induced.

With both the lactose and galactose operons induced (Expt. 1) about 78 % of all the hexose used was galactose, 37 % of the total being galactose derived from lactose. When the main source of galactose was curtailed (Expt. 2) by failing to induce the lactose operon, the total consumption of carbohydrate did not decrease, and indeed increased to some extent. The lack of galactose from lactose was entirely compensated by the additional metabolism of glucose from the medium; the metabolism of galactose from the medium did not increase. This suggests that the pathway leading into metabolism from galactose in the medium was saturated in both experiments by galactose
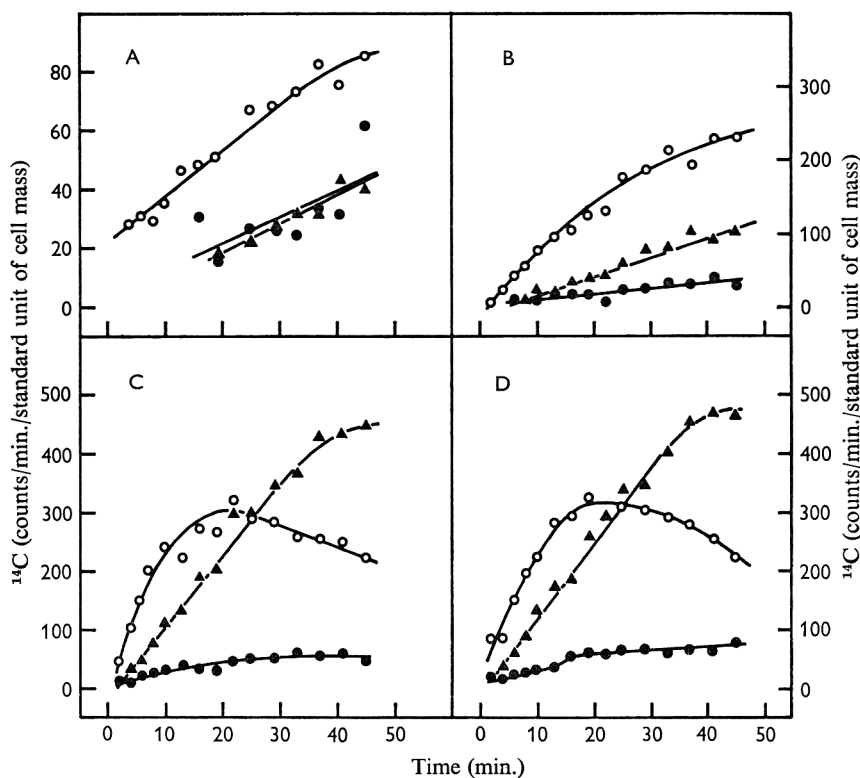

Fig. 1. Formation of glutamate, citrate and succinate in *Escherichia coli* induced for both the *lac* and *gal* operons, and supplied with a mixture of glucose, galactose and lactose labelled in various ways. *A*, [G-^{14}C]glucose; *B*, [G-^{14}C]galactose; *C*, [(G-^{14}C)glucose]lactose; *D*, [(G-^{14}C)galactose]lactose. ○, glutamate; ●, citrate; ▲, succinate.

from this source. The other pathway leading into metabolism from galactose derived from lactose was not accessible to galactose in the medium. However, at a later stage in metabolism, probably the second of these pathways could be entered by glucose from the medium, since the pathway was now largely unoccupied by metabolites from the galactose moiety of lactose. Glucose from the medium could not enter this pathway, however, when it was occupied by galactose metabolites.

This pathway from lactose-galactose was more readily accessible to the glucose moiety of lactose than to free glucose when it was not occupied by galactose metabolites. In Expt. 3 elimination of both galactose pathways by failure to induce the

galactose operon resulted in the greater utilization of lactose-glucose, since this was now available, rather than glucose from the medium. Thus we may conclude that when all the relevant operons were induced the carbohydrate of choice was galactose derived from the hydrolysis of lactose. When it was available, glucose derived from lactose
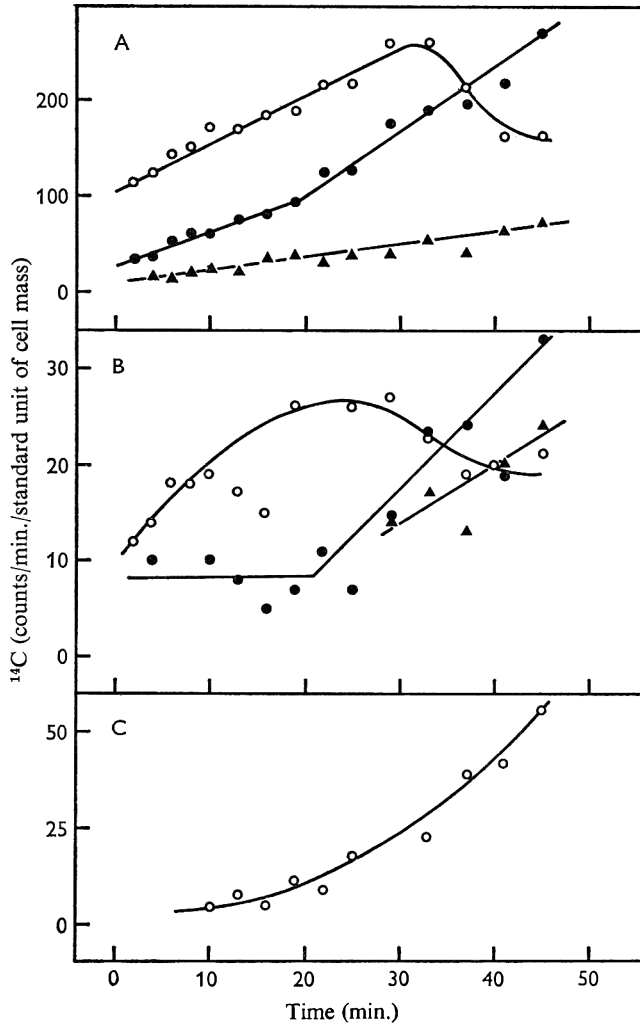

Fig. 2. Formation of glutamate, citrate and succinate in *Escherichia coli* induced for the *gal* operon only, and supplied with a mixture of glucose, galactose and lactose labelled in various ways. *A*, [G-^{14}C]glucose; *B*, [G-^{14}C]galactose; *C*, [(G-^{14}C)glucose]lactose. No activity in these compounds was obtained with [(G-^{14}C)galactose]lactose. ○, glutamate; ●, citrate; ▲, succinate.

was also used in preference to free glucose in the external environment, although the latter could be used when there was sufficient need. These considerations imply that the pools formed inside the organism from externally supplied and internally produced hexose do not mix to any large extent.

Inspection of the radioautographs of the chromatograms produced in these experi-

ments prompted the selection for comparisons of the spots corresponding to citric, succinic and glutamic acids, because they were found on most of the chromatograms from each of the experiments and contained readily measurable ^{14}C activity. For the sake of simplicity data obtained from other spots, labelled to a smaller extent (with a
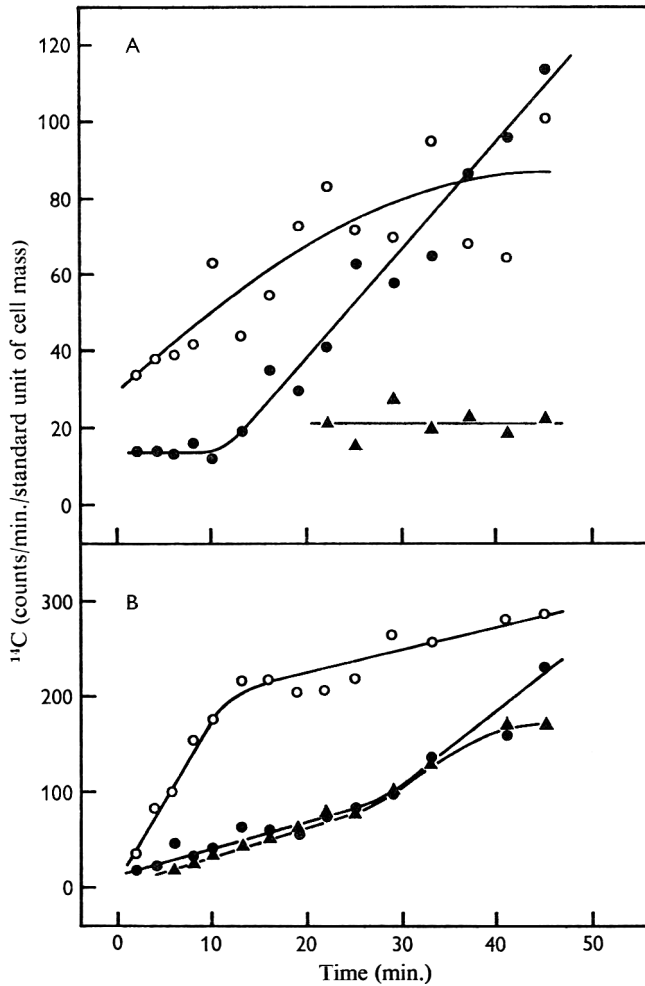

Fig. 3. Formation of glutamate, citrate and succinate in *Escherichia coli* induced for the *lac* operon only, and supplied with a mixture of glucose, galactose and lactose labelled in various ways. *A*, [G-^{14}C]glucose; *B*, [(G-^{14}C)glucose]lactose. No activity in these compounds was obtained with [G-^{14}C]galactose or [(G-^{14}C)galactose]lactose. ○, glutamate; ●, citrate; ▲, succinate.

correspondingly greater scatter of results) and spots which were not subsequently identified will not be presented. Figures 1–3 show the activities of the three selected compounds from all the papers on which their activity was measurable. The results from a single, four-vessel, experiment are shown in each figure.

In considering the behaviour of various compounds shown in Figs. 1–3 it should be borne in mind that quantitative comparisons between experiments performed on

different days and with different batches of bacteria must always be made with caution. One is more confident in comparing parallel portions of the same culture since these certainly had a common history until a few minutes before the experiment began, and considerable effort was expended to maintain chemically identical conditions in each subculture after division of the parent stock.

Examination of Figs. 1–3 shows that there were considerable differences between the relative pool sizes in different vessels of the same experiment. Before these can be discussed in detail one must be quite sure that they reflect aspects of metabolic organization and not merely trivial differences which might by chance have arisen among the four parallel vessels and which alone might account for the metabolic phenomena we observed. Within each experiment it is known that the bacteria and their chemical environment were the same in each vessel, differing only in the nature of the labelled substrate added. The only other possible variation we can conceive between the experimental conditions in each vessel is the degree of aeration. As stated earlier, aeration was achieved by using miniature magnetic stirrers made of stainless steel, sheathed in polythene, and rotated by stirrer motors with the rheostat speed control set at its maximum setting (100 on the dial). The following experiment was designed to determine how critical the speed of stirring was for the rate of respiration of the bacteria.

Warburg flasks with no centre wells were each charged with 0·25 ml. of 20% (w/v) sodium hydroxide placed in the side-arms. A suspension of bacteria in 0·01 M-phosphate buffer (pH 7·1) containing chloramphenicol (25 μg./ml.) was prepared in exactly the same way as for the labelling experiments; the suspension contained equiv. 194 μg. bacterial protein/ml. The bacteria had been grown in the absence of inducers. The external diameter of the Warburg flasks was approximately 30 mm. and of the vials used in the labelling experiments approximately 23 mm. However, the latter were made of thinner glass and the difference between the internal diameters of the two vessels was probably less than between their external diameters.

Three samples (2·0, 1·0, 0·5 ml.) of the bacterial suspension, each diluted to 2·0 ml. with buffer, were placed in the cups of the Warburg flasks. The miniature magnetic stirrers were added, followed by 0·04 ml. of a solution containing 0·7% (w/v) each of glucose, galactose and lactose. The flasks were attached to their manometers, immersed in a water bath at 37° and stirring began with the rheostat speed controls set at 85–90 on the dial. This gave a speed somewhat less than that used in the labelling experiments when it was possible to operate the stirrers at maximum speed. After 10 min. equilibration, measurements of oxygen consumption were begun. For the first 60 min. the rates of oxygen consumption in each flask were constant, the values (μl. O_2/min./μg. bacterial protein) being 4·38, 4·25 and 4·51 for flasks containing 2·0 ml., 1·0 ml. and 0·5 ml. of original bacterial suspension, respectively.

Since the rate of respiration was proportional to the volume of bacterial suspension used over a fourfold range, it must have been maximal. After incubation for 50 min. the rate of stirring was decreased by turning the speed controls to 60. The speed of stirring was not proportional to the potentiometer setting and it was observed that the stirrers appeared to slow down to much less than two-thirds of the original rate. As measured in the next 50 min. the respiration rates were 1·65, 3·92 and 2·89 μl. O_2/min./μg. bacterial protein for the three flasks, respectively. The rate had thus decreased in three vessels to different extents. However, in the labelling experiments the stirrers were always operated at maximum speed. It must also be borne in mind that the total

volume in each vessel during a labelling experiment decreased continuously as samples were withdrawn. It is therefore clear from these results that differences in aeration were not sufficient to account for the differences observed between the labelling patterns in different vessels.

DISCUSSION

If true steady-state conditions had been achieved in the labelling experiments each metabolic pool would gradually have been filled with ^{14}C until equilibrium with the outside solution was achieved; the activity would then have attained a constant maximum value. From Figs. 1 to 3 it can be seen that an approximation to these conditions was achieved in most cases. At the end of 45 min. the rate of increase of radioactivity in each pool tended to decrease. In a few cases the degree of activity in the glutamate pool began to show a decrease after reaching a maximum value, and in some cases no plateau of activity was attained. However, the data presented in Fig. 1 indicate that the size of the succinic acid pool, through which passed the products of metabolism of the glucose and galactose derived from the hydrolysis of lactose, was much larger in comparison to the size of the pool of glutamate than that which received the products of metabolism of exogenous glucose and galactose. Figure 3 indicates that this same effect, although less pronounced, also held true for exogenous and endogenous glucose in bacteria which had a different complement of induced enzymes.

In addition to determinations of the relative sizes of pools, observations on the rates at which pools fill with activity may be helpful in determining whether more than one pool of each compound exists in the cells. For example, in Fig. 1 it can be seen that the pool of glutamate derived from either moiety of lactose began to decrease in activity about 20 min. after the addition of label to the vessels, at which time the activity of the pool of glutamate derived from exogenous glucose or galactose was still increasing. Similarly, from Fig. 3 it will be seen that the size of the labelled pool of citric acid from external glucose became equal to, and subsequently greater than, the pool of labelled glutamate 36 min. after the addition of the labelled material to the cells. However, the pool of labelled citrate derived from endogenously produced glucose did not exceed the size of the labelled glutamate pool within the period of the experiment. If all the labelled substrates are fed into a common pathway (there being only one pool of any intermediate in each cell) as shown in Fig. 4, then the pools of the three acids would behave identically in each vessel regardless of the nature of the labelled substrate. Since in any one of the labelling experiments the bacteria and their chemical environment were ostensibly identical, some explanation must be sought for the differences shown by the behaviour of the labelled pools depending on the identity of the substrate which was labelled.

Although metabolic compartmentation is able to account satisfactorily for the results of these experiments, two other possible interpretations must also be considered: lack of homogeneity in the bacterial population itself, and different environmental conditions in the several incubation vessels comprising each experiment.

The culture used in the present experiments was not synchronous, and it could be argued that bacteria in different stages of the growth cycle might have different enzyme complements (and thus might themselves act as separate compartments). There is considerable evidence that certain enzymes in *Bacillus subtilis*, *Escherichia coli* and yeast are produced discontinuously during the growth cycle of individual cells (Masters,

Kuempel & Pardee, 1964; Kuempel, Masters & Pardee, 1965; Halvorson *et al.* 1964; Masters & Donachie, 1966) although, taken as a whole, protein synthesis is a continuous process (Schaechter, Williamson, Hood & Koch, 1962). The enzymes exhibiting discontinuous (or 'stepwise') synthesis are those whose synthesis is partially or wholly induced or de-repressed. Kuempel *et al.* (1965) proposed a model to explain this stepwise synthesis which involves a cyclic variation in the amount of repressor during one generation period. Halvorson *et al.* (1964) suggested a similar explanation.
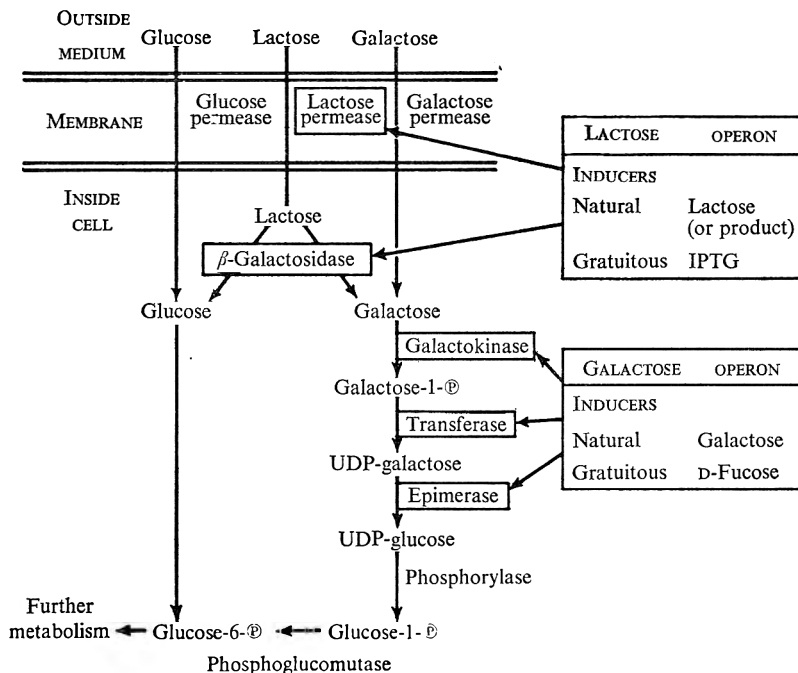

Fig. 4. Initial metabolic interrelations of lactose, galactose and glucose, showing inducible enzymes and some inducers.

They envisaged that transcription is switched on and off sequentially along the DNA molecule during the growth cycle. Thus, if inducers and repressors act by stimulating the rate of messenger RNA synthesis they must only function at given periods of the growth cycle. Halvorson *et al.* (1964) found that the addition of inducer to cells synthesizing enzyme under a repressed regime (e.g. at a basal rate) did not alter the timing of the induced stepwise synthesis of the enzyme. Evidence contrary to this view has been presented by Masters *et al.* (1964) and Masters & Donachie (1966), who found that the synthesis of β-galactosidase under conditions of severe repression did not exhibit stepwise formation but was continuous at a rate which was proportional to the gene dosage (i.e. when the appropriate section of DNA was replicated the rate of synthesis also doubled).

No information is available about the timing of synthesis of enzymes on the central pathways of metabolism. Figure 4 shows the early stages in the metabolism of the sugars used in the present experiments. With the exception of those controlled by the added inducers the enzymes mediating the metabolism of these sugars are found in all normally functioning cells. These enzymes are probably subject to control mechanisms

other than those which act solely at the genetic level, and the presence of any 'cyto-plasmic' control over the enzymic activity of already synthesized protein would tend to counteract the *in vivo* effects of sudden increases in enzyme content caused by step-wise synthesis if this were to occur. For this reason, and because stepwise synthesis has never been demonstrated for these enzymes, it is not considered likely that bacteria in different stages of growth in the cultures used in the present experiments acted as separate compartments due to differences in enzyme content.

Another explanation for the present observations which must be considered is the possibility that the differences in the relative pool sizes observed in different incubation vessels during a single experiment were caused by variation in the immediate environ-ment of the bacteria. Aeration is the only environmental factor which can reasonably be considered as a source of variation; it is difficult to appreciate how other local differences would have arisen. Temperature, mixing, population and medium were undoubtedly identical in each case. Each incubation vessel received a different labelled substrate, but these represented minute chemical quantities of material and we are unable to ascribe our results to differences resulting from unforeseen characteristics of the tracer preparations. Variations in the pool sizes of citric, glutamic and succinic acids have been observed as the result of the growth of *Escherichia coli* under anaerobic conditions. In such circumstances the tricarboxylic acid pathway acts not as a cycle but as two pathways branching at the point of entry of acetyl-CoA (Amarasingham & Davis, 1965). However, the operation of the anaerobic mode of the tricarboxylic acid pathway requires that the formation of α-ketoglutarate dehydrogenase be repressed (Amarasingham & Davis, 1965) and a specific enzyme for the formation of succinate from fumarate (fumarate reductase) be induced (Hirsch, Raminsky, Davis & Lin, 1963). The cultures in the incubation vessels in the present experiments were not growing because of the presence of chloramphenicol. Thus the presence or absence of any enzymes caused by anaerobiosis during the period of growth of the cultures would be observed in each of the incubation vessels to the same extent and so cannot be used to explain differences between them. Variations in the aeration of individual vessels might be expected to cause variations in the ratios of ATP/ADP, NADH/NAD⁺, etc., which would be reflected in the activities of a number of enzymes. However, the respiratory studies reported above indicate that the bacteria in the labelling experi-ments were respiring at a constant and maximum rate, since the specific rate of respiration did not increase when the concentration of bacteria was decreased four-fold. In view of these results we do not consider that there were any differences between the degrees of aeration in different vessels during a single labelling experiment large enough to account for the considerably different metabolic patterns obtained.

The most probable explanation for the observations is that the products of meta-bolism of glucose and galactose, derived from either internal or external sources, feed into pools of citric, succinic and glutamic acid which are at least partially separate. It is, perhaps, surprising that the bacteria can maintain functional separation of the metabolic intermediates from different sources through so many enzymic stages, although the ability to do so is plainly an advantage from the point of view of meta-bolic control. This might be possible, however, if the enzymes on the pathways involved were very closely co-ordinated. The results presented above, particularly those in Fig. 1, suggest that among the metabolic reactions we studied compartmentation plays a role only in distinguishing between hexoses produced within the bacteria and

those presented to the exterior surface. Within each of these two categories the evidence suggests that glucose phosphate produced by epimerization of galactose phosphate equilibrates fully with glucose phosphate formed directly from free glucose. On the other hand, glucose phosphate derived from either of the hexoses in the medium is metabolically distinct from glucose phosphate derived from either of the hexoses produced by the hydrolysis of lactose.

Compartmentation of glycolytic metabolism in *Escherichia coli* has been suggested by Eisenberg & Dobrogosz (1967) as a possible explanation for the fact that bacteria simultaneously oxidizing glucose and gluconate, oxidize the former exclusively by glycolysis and the latter exclusively by an inducible Entner–Doudoroff pathway. Pollock (1966) suggested that micro-organisms might co-ordinate their metabolic activity by incorporating enzymes into an organized structural framework. The growing amount of information on multi-enzyme complexes on the central pathways of metabolism tends to support the view that metabolic compartments do exist in bacteria. It has been known for some time from other systems that the enzymes responsible for the oxidative decarboxylation of pyruvate and α-ketoglutarate exist in such particulate complexes (Reed & Cox, 1966). There is also growing evidence for protein-protein interactions occurring much nearer the beginning of the glycolytic pathway. For instance, the activity of aldolase has been shown to be enhanced by glycerophosphate dehydrogenase (Baranowski & Niederland, 1949) and by glyceraldehyde-3-phosphate dehydrogenase and triose phosphate isomerase (Gulyi, 1960; Gulyi, Dvornikova, Fedorchenko & Pechenova, 1962; Sereda, 1963; Kwon & Olcott, 1965). All these effects were ascribed to protein-protein interactions and not to effects due to the removal of end products. The effects were specific, and proteins not functionally related to the enzyme under study had no effect. These enzymes, which came from a variety of higher animal sources, are found in what is normally regarded as the soluble fraction of the cell, but this evidence suggests that there is in the living cell a high degree of cytoplasmic organization of enzymes functionally related to one another in metabolic sequences. If, in such complexes, an enzyme preferentially accepts its substrate from another enzyme in the complex, rather than from a 'soluble' pool, the situation would be one of compartmentation as described in the Introduction. The concept of metabolic compartmentation in bacteria has rarely been explicitly stated in the past but it is an important one with a significant bearing on the understanding of mechanisms of biological control.

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The Microflora of Fodders Associated with Bovine Respiratory Disease

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SUMMARY

The microflora of samples of fodder fed to cattle on 41 farms where some cattle suffered respiratory disease was comparable with the microflora of hays, previously reported. Of the 59 samples of fodder examined, 30 from 29 different farms were very mouldy and were comparable with farmer's-lung-type hay in being rich in *Micropolyspora faeni* and *Thermoactinomyces vulgaris*. The remaining samples were classified as either good or mouldy in almost equal numbers.

INTRODUCTION

The disease in man called farmer's lung is caused by inhaling dust from very mouldy hay (Pepys *et al.* 1963). Such hay is produced when it is baled at water contents from about 35–45%; such bales may heat spontaneously at 50–70° (Gregory, Lacey, Festenstein & Skinner, 1963; Festenstein *et al.* 1965). In these conditions thermophilic and thermotolerant fungi and actinomycetes grow abundantly. These often include *Micropolyspora faeni* (*Thermopolyspora polyspora*; see Cross, Maciver & Lacey, 1968), the richest known source of farmer's-lung hay (f.l.h.) antigen, and *Thermoactinomyces vulgaris*, another source. Large spore concentrations occur in the air of farm buildings when such mouldy hays are moved (Lacey & Lacey, 1964). Cattle are also subjected to large spore concentrations when feeding on mouldy hay (Austwick, 1963), and actinomycetes have been isolated in large numbers from the lungs of even healthy cattle (Austwick, 1966).

Classically, fog fever is a disease of cattle feeding on aftermath pasture (foggage) during autumn in the west of England, but the name has also been applied to clinically similar diseases, also of unknown aetiology, of housed cattle. Jenkins & Pepys (1965) found precipitins to f.l.h. antigen in the sera of housed cattle said to be affected with fog fever, and in some unaffected cattle. These precipitins resemble those in the sera of farmer's-lung patients, which suggest that respiratory disease in some animals may also be caused by inhaling dust from mouldy hay.

The microflora of fodders sent from farms where cattle had suffered from respiratory disease was examined and compared with that of mouldy hays associated with farmer's-lung disease (Gregory & Lacey, 1963*a*). Some fodders were received during a survey organized by the Veterinary Clinical Observation Unit, for which immunological results have been reported (Jenkins & Pepys, 1965).

METHODS

All the samples of fodder were examined by the wind-tunnel method, by using the cascade impactor and Andersen sampler as described by Gregory & Lacey (1963a), except that grain samples were first put in small muslin bags. Spores blown off the fodders were sampled first with the cascade impactor, operated approximately iso-kinetically with the wind speed of 4·2 m./sec. for 3 min., and then with the Andersen sampler, operated at 25 l./min. for 5 successive runs of 15 sec. each with a fresh set of plates. Two of these sets of plates contained 2 % malt agar with penicillin (20 units/ml.) and streptomycin (40 units/ml.), and 3 contained half-strength 'Oxoid' nutrient agar with actidione (0·5 mg./ml.). Incubation was at 25°, 40° (one set malt agar and one set nutrient agar plates at each temperature) and 60° (nutrient agar only).

RESULTS

In all, 59 samples of hay, straw and grain were examined between September 1962 and February 1966; these came from 41 farms where cattle had suffered respiratory disease. These samples are classified according to type and degree of mouldiness (after Gregory & Lacey, 1963a; Gregory et al. 1963) in Table 1. Most of the species recorded by Gregory & Lacey (1963a) and Gregory et al. (1963) were also isolated from these fodders, with the addition of the following fungi: Aspergillus oryzae, Penicillium piceum, Doratomyces sp., Chaetomium indicum, Dactylomyces crustaceus, Malbranchea pulchella var. sulfurea, Monascus purpureus, Thamnidium elegans, Aspergillus clavatus, A. candidus, Microascus sp., Syncephalastrum racemosum and Thermoascus aurantiacus. The actinomycetes isolated included Thermoactinomyces vulgaris, Micropolyspora faeni, Thermonospora viridis, Streptomyces fradiae, S. thermoviolaceus, S. griseoflavus.

Table 1. *Spore content of fodder samples, and occurrence of*
Micropolyspora faeni and Thermoactinomyces vulgaris

Fodder type		No. farms	No. samples	Actinomycetes (10⁶ spores/g. dry wt)	Fungi	M. faeni (colonies/g. dry wt)*	T. vulgaris
Hay	Good	9	11	0·4–7·3	0·4–5·5	0·1–6·0	0–3·1
	Mouldy	10	10	0·1–22·1	0·1–24·3	0–7·5	0·9–3·2
	Very mouldy	27	27	39·6–817·9	1·0–92·6	0–71·9	0·5–42·6
Straw	Good	2	2	3·4–4·3	0·4–1·2	0–0·5	0·6–1·0
	Mouldy	2	2	7·1–12·8	2·1–21·3	0·3–1·8	0·1–2·7
	Very mouldy	2	2	73·6–515·7	6·6–13·6	8·8–13·1	0·1–74·5
Grain	Good	2	2	5·9–6·0	0·5–0·9	0·8–1·1	5·3–9·1
	Mouldy	2	2	9·9–12·5	0·9–24·8	0–4·2	1·9–5·4
	Very mouldy	1	1	81·5	26·7	12·8	1·1

* See text.

The microflora of the fodder types classified by spore load as good, mouldy and very mouldy agreed approximately with those described by Gregory & Lacey (1963a), but there were a few exceptions. *Micropolyspora faeni* and *Thermoactinomyces vulgaris* were usually abundant in the very mouldy hays, most of which yielded more than 2 colonies of each organism/g. dry wt in a standard Andersen sampler run (equivalent to about 1 % of the blowable, or 0·15 % of the total spore load; Gregory & Lacey, 1963b; Table 2). *Thermoactinomyces vulgaris*, although more abundant than *M. faeni* in most hays,

varied more and was very rare in some samples. However *M. faeni* was not isolated from one very mouldy sample, although this contained 112×10^6 actinomycete and 75×10^6 fungal spores/g. dry wt, and was rich in *T. vulgaris*. Good samples of hay yielded fewer than 2 colonies of *M. faeni*/g. dry wt, and only about one third of the samples classified as mouldy had more than this.

Table 2. *Occurrence of Micropolyspora faeni and Thermoactinomyces vulgaris in fodder of different types*

Fodder type	No. colonies/g. dry wt sample*					
	0–0·5	0·6–2·0	2·1–5·0	5·1–20·0	20·1–50·0	>50·1
	M. faeni: no. samples in each class					
Good	9	5	0	1	0	0
Mouldy	4	5	3	2	0	0
Very mouldy	1	0	5	16	5	3
	T. vulgaris: no. samples in each class					
Good	5	4	4	2	0	0
Mouldy	1	7	4	2	0	0
Very mouldy	3	4	2	1C	6	5

* See text.

Table 3. *Relationship between the occurrence of respiratory disease and the maximum number of Micropolyspora faeni isolated from associated fodder*

	Maximum no. *M. faeni* colonies/g. dry wt sample*					
	0–0·5	0·6–2·0	2·1–5·0	5·1–20·0	20·1–50·0	>50·1
No. farms:	6	4	6	17	5	3

* See text.

Actinomycetes are not the only organisms in mouldy hay which are able to cause respiratory disease in cattle, and other potential pathogens were isolated, sometimes in large numbers. The most common were *Aspergillus fumigatus*, *A. nidulans*, *Absidia* sp. and *Mucor pusillus*. Precipitins to *A. fumigatus* have also been found in cattle sera (Jenkins & Pepys, 1965).

More than one fodder sample was received from some farms and these were usually of different types. Very mouldy fodders came from 29 farms (71 %) and more than 2 colonies of *Micropolyspora faeni*/g. dry wt were isolated from at least one sample from 31 farms (76 %; Table 3). One sample was of particular interest since, unlike all the others, it was associated with fog-fever in grazing cattle. Hay had been cut earlier in the year but had not been removed from the pasture. New grass which had grown through was being grazed by the affected cattle. Samples from this old hay did not contain a large number of spores ($17\cdot7 \times 10^6$ actinomycetes, $3\cdot7 \times 10^6$ fungi/g. dry wt); but 8·3 colonies of *M. faeni*/g. dry wt were isolated which may have been important.

Immunological tests, with sera from 96 cattle on 23 farms showed that 47 of the cattle on 16 of the farms had precipitins to f.l.h. antigen. (P. A. Jenkins & J. Pepys, personal communication).

DISCUSSION

Although only 51 % of the samples from farms where cattle had suffered respiratory disease could be classified as very mouldy, or of farmer's-lung type (after Gregory & Lacey, 1963 a), at least one very mouldy sample came from 71 % of the farms. At least one sample from another 5 % produced more than 2 colonies of *Micropolyspora faeni*/ g. dry wt. The samples from the remaining farms may not have been typical of the fodder being fed before, or at the time of, the outbreak of disease, and so did not reflect the respiratory spore intake of the affected cattle. It has not yet been possible to correlate the results reported here with the clinical and pathological findings in the disease outbreaks at places from which the samples came, but the clinical definition of 'fog-fever' was used as a criterion for the selection of samples. This syndrome is thought to have several causes, of which actinomycete, and possibly mould spores, are only two. The possibility that inhaled spores exacerbate symptoms of concurrent infection by viruses or bacteria must also be considered. However, the presence of precipitins to FLH antigen in sera from the animals suggests a parallel with farmer's-lung disease. This is certainly evidence of exposure to dust from mouldy hay, but is not necessarily proof that this dust caused disease, because precipitins have been found in apparently healthy animals (Jenkins & Pepys, 1965).

Except for the one farm, this survey did not include examples of respiratory disease in grazing animals. Although thermophilic actinomycetes, including *Thermoactino-myces vulgaris*, have been isolated from soil and grass litter (Cross, 1968; Lacey, unpublished), few spores are probably released from these sources as compared with the numbers from hay, perhaps too few to cause disease. The clinical form of fog-fever of cattle grazing aftermath pasture is probably caused by other agents; hypersensitivity to lungworm larvae (*Dictyocaulus viviparus*) is one suggestion (Michel, 1954).

There is a need for a better definition of fog-fever, and possibly the limitation of this name for the classical disease of animals at pasture. Although there are similarities between respiratory disease of some housed cattle and farmer's-lung disease in man, further work is needed to consider all possible causes, with more detailed immunological work with the cattle and information about the microbiology of their fodder. Inhalation tests with extracts of actinomycetes and fungi isolated from hay are needed to see whether they cause disease in cattle as they do in man, and to assess how important mouldy hay is as a cause of allergic respiratory disease in cattle.

Although most very mouldy hays usually produced more, and other hays fewer, than 2 colonies of *Micropolyspora faeni*/g. dry wt in standard wind-tunnel tests, this seemingly small figure is probably an underestimate, for the various reasons given by Gregory & Lacey (1963 a), who could grow in culture only 0·1 % of the spores seen on cascade impactor slides. Although a statistical correction can be applied for multiple impaction by the Andersen sampler jets, it is difficult to allow for the number of spores per particle deposited on the upper stages of the Andersen sampler, or for spore viability. However, the viable particles may not be the only ones that can cause respiratory disease, because dead particles may be allergenic.

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Growth of *Neurospora crassa* in Unstirred Liquid Cultures

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SUMMARY

The growth kinetics of Neurospora in unstirred liquid culture may be accounted for by an exponential and a linear growth phase. If a cubic growth phase exists it is in addition to the exponential and linear phases of growth. A method of measuring the exponential doubling time in unstirred cultures is described which may be usefully applied to fungi which cannot be observed growing exponentially by normal methods. Rate of linear growth is shown to be proportional to the surface area of the culture. Expression of linear growth rates as μg./cm.^{2}/hr would assist in the comparison of data from different laboratories.

INTRODUCTION

Difficulty in analysing the kinetics of growth of filamentous fungi (reviewed by Mandels, 1965) has led to the suggestion that Neurospora (Emerson, 1950) and some other fungi (Machlis, 1957; Marshall & Alexander, 1960) may have a 'cube-root' growth phase corresponding to the logarithmic growth phase of other organisms. During the cube-root phase the mycelium is considered to be a sphere with radius increasing at constant rate, such that the cube root of the mycelial mass would increase in direct proportion to time. Other workers have found clear evidence for an exponential growth phase in fungi (Zalokar, 1959; Pirt & Callow, 1960) but this does not preclude the existence of a cubic growth phase under other conditions. A theoretical discussion of cubic growth has been made by Pirt (1966).

Experiments described in the present paper show that the growth of Neurospora in unstirred cultures may be analysed into an exponential phase followed by a linear phase. No evidence was found to suggest the existence of a cubic phase of growth; experiments of Emerson (1950) may be reinterpreted to support this conclusion.

METHODS

Except where stated, all experiments were made with the St Lawrence wild-type strain STA of *Neurospora crassa* grown at 25° on Vogel liquid minimal medium (Vogel, 1956) with 2 % (w/v) sucrose in unstirred bottles or beakers. The bottles used were 12 oz medical flats containing 100 ml. medium and plugged with cotton-wool. These bottles were sloped at about 10° to the horizontal. Beakers of known diameter were filled with medium to a depth of 1 cm. and covered with two layers of cheesecloth stuck down with autoclavable adhesive tape. The cheesecloth was covered with a sheet of aluminium foil and the beakers were then autoclaved. The aluminium foil was removed and discarded at the time of inoculation. Growth was estimated as dry weight of mycelial pads (collected by filtration through paper on a Büchner funnel) after freeze-drying.

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Inocula consisted of macroconidia counted in a haemocytometer and diluted in distilled water. Cultures were inoculated together by using one inoculum or a series of diluted inocula. Duplicate cultures were harvested at suitable times.

<div align="center">RESULTS</div>

Initial observations of *Neurospora crassa* wild-type strain STA growing in unstirred vessels showed that there was a phase of accelerating growth, followed by a linear phase of growth. The growth rate in the linear phase varied with the type of vessel in which growth occurred, e.g. whether beakers or bottles. Furthermore, the onset of linear growth was delayed when the inoculum was diluted, regardless of the type of vessel used. Results showing these effects are given in Fig. 1. These observations led to experiments specifically designed to investigate the effect of surface area and inoculum size on growth in liquid medium.
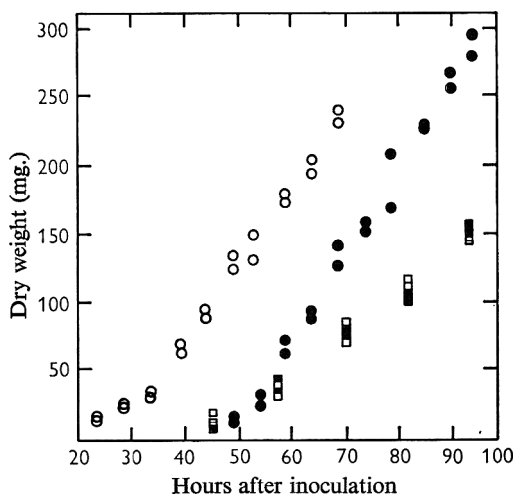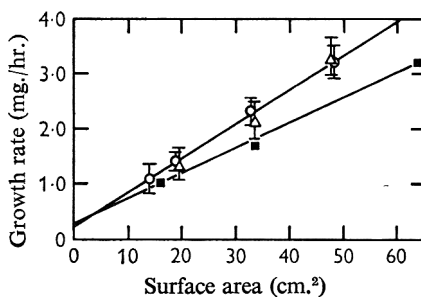

<div align="center">Fig. 1   Fig. 2</div>

Fig. 1. Growth of *Neurospora crassa* wild-type strain STA in various circumstances. O, growth in bottles with an inoculum of 2.6×10^6 conidia per bottle; ●, growth under the same conditions with an inoculum of 2.6×10^3 conidia per bottle; □, growth in 500 ml. beakers containing 135 ml. of medium with an inoculum of 1.5×10^6 conidia; ■, growth under the same conditions except that the beakers contained only 90 ml. medium. Surface area of growth in the bottles was about 80 cm.^{2} and in the beakers 49 cm.^{2}. The data for growth in bottles has been further analysed in Table 1.

Fig. 2. Growth of *Neurospora crassa* wild-type strain STA in beakers of various diameters. Linear growth rate is plotted against surface area of the culture for growth of strain STA in beakers of various diameters. The linear growth rate is calculated from raw data of the type shown in Fig. 1, as a regression of mycelial weight against time. The 95 % confidence interval was calculated from the standard error using Student's t distribution. Data from two separate experiments are plotted as circles and triangles with confidence intervals indicated by vertical lines. ■, data taken from Emerson (1950). (See text for explanation.)

<div align="center">*Linear growth and the surface area of the culture*</div>

Beakers of different sizes were used to vary the surface area of the cultures while the inoculum size was kept constant. Linear growth rates in beakers were measured in two experiments summarized in Fig. 2, which shows that the linear growth rates observed

were directly proportional to the surface area of the culture. Linear growth rate may then be expressed as 61 μg./cm.^{2}/hr for this strain, STA, a measure which should be largely independent of the type of vessel used for cultivation.

Emerson's original experiments purporting to show a cubic growth phase in Neurospora were also made in vessels of different diameters although no explanation of this is given in his paper. The last three growth points of each of his curves fall on a straight line and may be equated with the linear phase of growth identified here. The linear growth rates taken from Emerson's data in this way also show a direct relationship to surface area of the culture (see Fig. 2) and may be used to calculate an overall linear growth rate of 46 μg./cm.^{2}/hr for his strain.

The phase of increasing growth rate

Preceding the linear phase of growth in unstirred cultures there was a phase during which the growth rate increased until it reached the constant linear rate (Fig. 1). Logarithmic plots of this early phase of increasing growth rate give increasing doubling times of 2–5 hr or more. It seems likely that this phase of increasing growth rate is the last part of the logarithmic phase, during which growth is decelerating, before growth rate becomes constant and doubling time ceases to have any meaning. Zalokar (1959) showed, by measuring protein increase after extraction, that germination of conidia was followed by a period of exponential growth with a doubling time of about 2 hr, thus showing that initial growth of an inoculum was exponential. Further indirect evidence for the early exponential growth of inocula is considered below.

The relationship between inoculum size and growth

Theoretical. The relationship between inoculum size and the mass of a culture which is growing exponentially may be deduced from the well-known exponential growth equation $dM/dt = \mu M$, where M = mass of the culture at time t and μ is a constant known as the 'specific growth rate'. This equation may be integrated for initial conditions where M is the mass of the culture at time t, and M_0 the mass of the culture at $t = 0$: $\log_e M - \log_e M_0 = \mu t$. In this equation M_0 is usually considered to be constant and M to be variable. We may consider the case where M_0 (the inoculum size) is varied and M is chosen as an arbitrary constant. Then we find that $-\log_e M_0$ is proportional to t, i.e. in a series of experiments with exponentially growing cultures started from inocula of different sizes the time taken to reach an arbitrary constant mass will be proportional to the logarithm of the inoculum size. If M_0 is plotted against t in such a series of experiments, then the slope will be μ, the specific growth-rate constant. The doubling time, t_d, may be readily calculated since

$$t_d = \frac{\log_e 2}{\mu}.$$

If only two inocula, M_1 and M_2, are being considered where we choose $M_1 = 10 . M_2$ then

$$t_d = \frac{\log_e 2(t_2 - t_1)}{\log_e 10},$$

where inoculum M_1 reaches constant mass M_c at t_1 and inoculum M_2 reaches constant mass M_c at t_2. The value $(t_2 - t_1)$ is called the 'unit time difference' for inoculum dilution of 10^{-1}.

The expressions derived above are only formally true for exponentially growing cultures, but they may also be applicable to cultures which have an exponential growth phase which gradually changes into a linear growth phase, as seems to be found during the growth of Neurospora in unstirred cultures. Indeed, when a constant figure for the doubling time (calculated from inoculum dilution experiments) is obtained under various conditions, then this may be taken to indicate that an early exponential phase of growth exists. If there were a cubic growth phase, then we might expect $-M_0^{\frac{1}{3}}\alpha t$, such that M_0 (inoculum size) is a cubic rather than a logarithmic function of time.

Table 1. *Neurospora crassa wild-type strains* STA *and* EMA. *The relationship of growth rate, and time difference, to inoculum dilution for growth in unstirred cultures*

Inoculum dilutions	Linear growth rates (combined)	95 % confidence limits	Unit time difference per 10^{-1} dilution of inoculum	Unit time difference		Calculated doubling time td (hr)	95 % confidence limits for td
				S.E.	95 % confidence limits		
Experiment 1. (Strain STA *grown in bottles, surface area 80 cm.²)*							
0 10^{-1} }	6·9	6·5–7·27	6·02	0·62	4·7–7·3	1·8	1·4–2·2
10^{-2} }	6·54	6·05–7·03	6·75	0·31	6·1–7·4	2·0	1·8–2·2
10^{-3} }	6·76	6·47–7·05	6·74	0·21	6·3–7·2	2·0	1·9–2·2
Experiment 2. (Strain STA *grown in bottles surface area 80 cm.²)*							
0 10^{-3} }	6·12	5·86–6·39	6·06	0·18	5·7–6·4	1·8	1·7–1·9
Experiment 3 (Strain EMA *grown in beakers surface area 42 cm.²)*							
0 10^{-3} }	3·46	3·14–3·78	5·16	0·41	4·3–6·1	1·55	1·3–1·8

An undiluted inoculum of about 10^6 conidia per culture was used in all experiments. The raw data used in Expt. 2 is depicted in Fig. 1.

Results. Table 1 shows the analysis of results of growth experiments in which the inoculum size was varied. Measurements of rate of growth during the linear phase were made and were found not to vary significantly ($P > 0·01$) at different inoculum dilutions. The calculated regressions were used to calculate the 'unit time difference' for a standard inoculum dilution of 10^{-1}. This is the time difference in hours taken between successive inoculum dilutions of 10^{-1} to reach any given weight, and may be used to calculate the doubling time as shown above. Three independent estimates of the unit time differences in experiments with *Neurospora crassa* wild-type strain STA (Expts. 1 and 2, Table 1) were homogeneous (χ^2, $P = 0·5–0·7$), whereas the unit time differences for wild-type strain EMA possibly differed from those for strain STA (χ^2, $P = 0·01–0·05$). Calculation of the doubling time on the basis of these figures, pooled data for Expts. 1 and 2, gives a value of 1·84 hr for strain STA (95 % confidence limits 1·74–1·94 hr). These values are in reasonable agreement with doubling times for strain STA in shaken logarithmic cultures in the same medium at 25°, of 2·3 hr (95 % confidence limits 1·7–2·9 hr: C. F. Curtis, personal communication).

The unit time differences would be expected to decrease by a factor of two for each tenfold dilution of inoculum if growth were 'cubic'. They did not, however, vary significantly with dilution and in fact were entirely consistent with exponential growth and difficult to account for on any other basis.

DISCUSSION

The results presented support the conclusion that the early growth of *Neurospora crassa* wild-type strains in unstirred liquid cultures is exponential and that the exponential growth rate slowly declines until growth becomes linear. The change from exponential to linear growth seems to occur at about the time when the mycelial mass becomes established on the surface of the liquid. At this stage nutrients are still in plentiful supply, since the mass will increase more than tenfold by subsequent linear growth. The rate of linear growth is proportional to the surface area of the culture; this may be interpreted as resulting from the limited rate of diffusion of air or nutrients into the mycelial mat. In fact the mould is growing at a nutrient/air interface and does not grow downwards into the liquid medium probably because of lack of oxygen below the surface. Linear growth may also, however, be obtained in stirred and aerated cultures in which the mycelium occurs in small pieces. In such cultures the constant but limiting supply of air must be responsible for the constant linear growth rate.

The linear growth rate found during growth in beakers was not increased by pumping in sterile air, suggesting that it is the rate of diffusion of air into the mycelial mat rather than into the beaker which was limiting. These results are in agreement with the conclusion of Pirt (1966), based on theoretical considerations of a comparable situation, that the growth-limiting nutrient will almost inevitably be oxygen when fungi grow as spherical pellets in submerged culture.

The results described here throw some doubt on whether a cube-root growth phase exists for *Neurospora crassa*. Although all observations can be accounted for in terms of a linear and exponential growth phase, a cubic growth phase may occur and not be detectable by present methods. However, if a cubic growth phase occurred in addition to an exponential growth phase, it would not be expected that an inoculum dilution experiment of the type described above would give results entirely consistent with exponential growth. It should be pointed out that the evidence of Marshall & Alexander (1960) for a cubic growth phase in fungi and actinomycetes is indirect, depending upon measurements of rate of oxygen uptake. It is difficult to exclude the possibility that such measurements might be affected in a systematic way by changes in metabolic control systems which were growth-dependent.

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Observations on the Tube Method of Measuring Growth Rate in *Neurospora crassa*

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SUMMARY

The relationship between various parameters of growth of *Neurospora crassa* on solid media in tubes is described. These are (*a*) the relationship between culture weight and linear growth rate on various media; (*b*) the relationship between 'lag' in growth and inoculum size on various media. This latter relationship appears to obey a log law, enabling an inoculum doubling-time to be calculated. Adaptation of the arginine auxotroph *arg*-1 (46004) to diminution of arginine in the medium first occurred by decrease in mycelial weight and then by decrease in linear growth rate. The inoculum doubling-time did not appear to vary systematically with arginine concentration.

INTRODUCTION

The tube method of measuring growth of fungi on lengths of solid medium has been widely used in auxanographic studies and bioassays; a comprehensive study of many aspects of this method was published by Ryan, Beadle & Tatum (1943). However, as pointed out by Pirt (1967), the laws which govern the growth of bacterial and fungal colonies remain to be elucidated. Studies of this type of growth are of importance because, not only in the laboratory but also in nature, micro-organisms frequently grow in a colonial or spreading fashion on solid media; furthermore, growth of this type is of interest as it is the simplest model system which approaches the kind of growth which must occur in the tissues of higher organisms.

The purpose of the present work was to search for relationships between the simplest observable variables concerned with growth of fungi on lengths of solid media in tubes: the independent variables were inoculum size and medium composition, and the dependent variables were linear growth rate, lag in growth and mass of growth. The relationship between mass of growth and linear growth rate (outward growth of the colony) was studied for various strains of *Neurospora crassa* grown on various nutrient agar media in tubes. Measurements of lag in growth on tubes were found to be dependent on the inoculum size, and a new parameter called the inoculum doubling-time has been derived from this relationship. The comparison of the way in which these parameters vary together proved helpful in understanding the nature of growth of fungal cultures on the surface of solid media and the way in which growth adapts to dilution of an essential nutrient.

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METHODS

Growth tubes of the type described by Brown & Gillie (1963) were used (Fig. 1). The essential features of these tubes were as follows: the ends were straight enabling a wire to be introduced and used to take a sample of material from any part of the tube, the agar medium (10 ml.) was introduced into the tube through a central chimney (which may also be used for taking samples of conidia in other kinds of experiments) and was retained in the tube by an indented ridge in the glass 4 cm. from the end of the tube. The distance between the indented ridges in which the solid medium lies was 29–30 cm. Soda or Perspex glass may be used with an inside diameter of 12 mm. for the body of the tube and 8 mm. for the chimney. To maintain the tubes in an upright position they have two small glass feet attached at the base half way along the tube. Not only are these tubes more versatile than the classical type, they are also more convenient to handle. They have been made for us by a glassblower at the University at an estimated cost, including overheads of 6 shillings each.
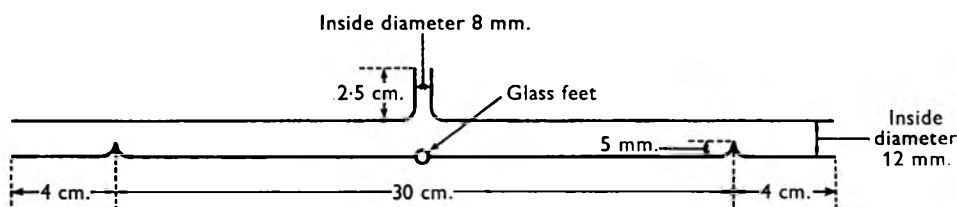
Fig. 1. Growth tube.

The ends of the tubes are plugged with cottonwool and they are dry sterilized before molten agar is pipetted into them. Cleaning of the tubes is greatly facilitated if they are soaked for about 2 days in a mixture of detergent and bleach, after which the agar can usually be easily dislodged by running hot tap water through the tube. A stout wire (with a flattened and bent end) was used to remove sections of the agar and adhering growth of Neurospora from the tube for experimental purposes. The mass of mycelium in an individual section or a number of pooled sections could be determined by weighing after extraction of the agar. To extract the agar 5% trichloroacetic acid was added to the sections (1 ml. to every ml. of agar medium) and the mixture was heated for 10 min. in a boiling water bath. The mycelium was then removed by filtration through paper on a Buchner funnel and could be freeze-dried and weighed. Weighings of less than 2 mg. were made with a Cahn electrobalance. Studies of the extinction of the extraction liquor at 260 and 280 mμ after removal of trichloroacetic acid with ether showed that between 5 and 10% by weight of ribonucleotides were removed by this treatment, but this did not vary sufficiently with the age of the section to influence the results.

Inoculations of the tubes were made by using a calibrated welded wire loop which delivered a mean volume of 1·8 μl. (with a standard error of ±0·6 μl.). The inoculum was suspended in distilled water, filtered through cottonwool, counted in a haemocytometer and then appropriately diluted in distilled water. The point of inoculation was marked, and daily markings of growth were made subsequently.

Standard Vogel (Vogel, 1956 medium N with 2% (w/v) sucrose and 2% (w/v)

Difco Bactoagar were used unless otherwise stated. Various strains were used, including our strains of the Emerson wild type EMA and St Lawrence wild type STA. Strains *arg-1 A* (46004) and *arg-10 a* (B 362) had mixed Emerson-St Lawrence backgrounds. Neither of these arginine auxotrophs was at all leaky on minimal medium. All experiments were done at 25°. Media were supplemented as indicated with arginine HCl obtained from British Drug Houses Ltd.

RESULTS

Measurements of the mass of mycelium growing in tubes

The mass of mycelium growing in 1-cm. long sections pooled from 10 tubes was determined at measured distances from the growing front. Figure 2a and 2b shows
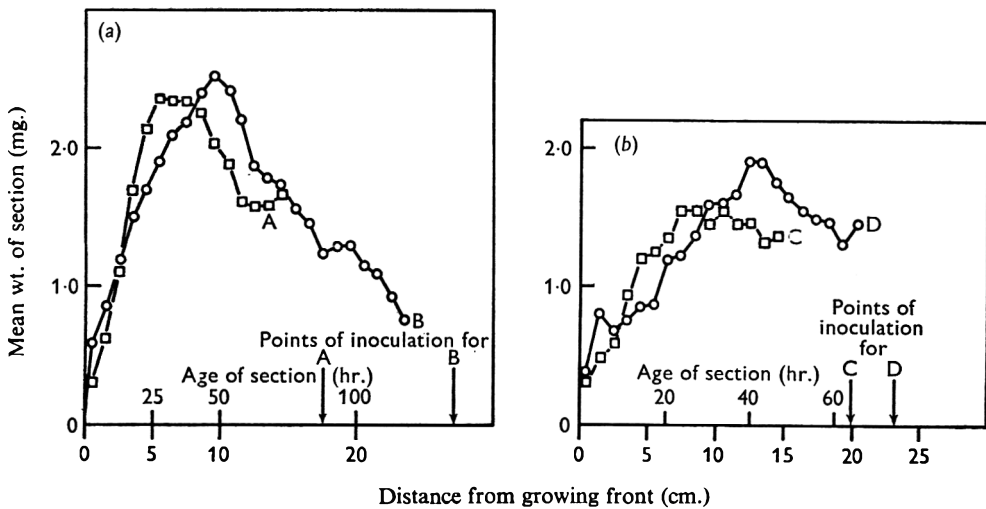

Fig. 2. (*a*) Yield during linear growth in tubes of *Neurospora crassa* wild-type strain EMA. Sections were cut at 1 cm. intervals, starting at the growing front and sections from the same position in each of 10 tubes were pooled and weighed. The mean weight of the section has been plotted at its median distance from the growing front. The mean age of the section is also given as the alternative abscissa. Two experiments (A and B) are shown in which growth occurred over different lengths of tube. These have been superimposed at the growing front to facilitate comparison. The points of inoculation are indicated on the abscissa. (*b*) Yield during growth in tubes of *Neurospora crassa* wild-type strain STA. Experimental procedure as described in (*a*). Two separate experiments, C and D, were made.

that with the Emerson wild-type strain, and less clearly with the St Lawrence wild-type strain, the mycelium increased more or less linearly in mass for 5–10 cm. behind the growing front and thereafter a decrease in mycelial mass was found. The peak of mycelial mass occurred at more or less the same distance from the growing front, regardless of the distance from the point of inoculation. A similar situation was found with all the strains tested, although the peak came a little later and declined less sharply with strain STA (Fig. 2*b*).

Growth of this kind may be visualized as a wave of mass proceeding along the tube and in a sense eventually passing off the end of the tube. This view is supported by measurements of the total extractable mycelial mass in a tube, which was found to

increase until the mycelial front reached the end of the tube, and then decreased. This type of wave-like growth of the perimeter of a 'colony' has obvious similarities to the kind of growth which may in nature lead to the production of 'fairy rings' by certain types of fungi.

The mycelial mass produced varied with dilution of a growth-limiting nutrient. Figure 3 shows growth curves of mycelial mass for the arginine auxotroph, *arg*-1 (46004) grown on different concentrations of arginine. The mycelial mass recovered declined with dilution of arginine in the medium, as might be expected, although at very high concentrations of arginine (5 g. arginine HCl/l.) there was a more gradual increase in mycelial mass along the tube, showing some sort of adaptation. The relation-
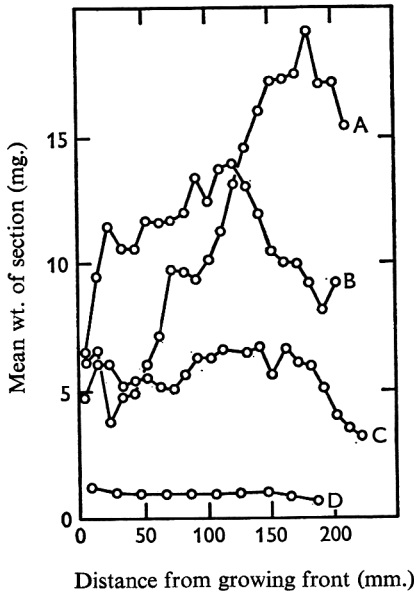

Distance from growing front (mm.)
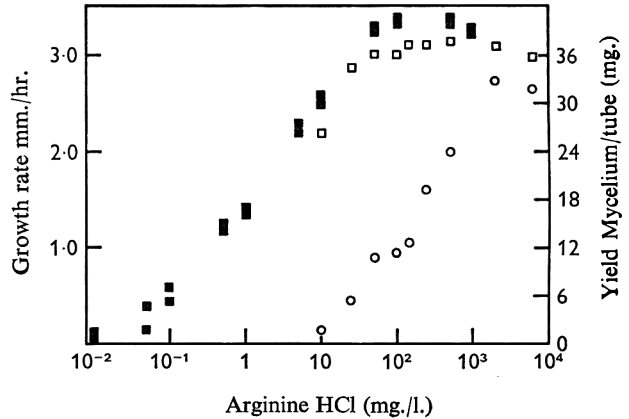
Fig. 3

Arginine HCl (mg./l.)

Fig. 4

Fig. 3. Yield during growth in tubes of *Neurospora crassa* strain *arg*-1 grown on various arginine concentrations. Experimental procedure was as described in the legend of Fig. 1*a*. The arginine concentrations used were: A, 5×10^3 mg./l.; B, 5×10^2 mg./l.; C, $1 \cdot 5 \times 10^2$ mg./l.; D, 10 mg./l.

Fig. 4. The relationship between linear growth rate and yield of *Neurospora crassa* strain *arg*-1 and arginine concentration in the medium. O, measurements of yield of mycelium per tube, averaged from 10 tubes at each arginine concentration. ■, measurements of linear growth rate in individual tubes. □, average measurements of linear growth rate (from 10 tubes) made from the same tubes as the measurements of yield were made.

ship between mycelial mass measured in this way and linear growth rate is shown in Fig. 4. This shows that as the concentration of arginine in the medium decreased there was a 5-fold decrease in yield of mycelial mass before the linear growth rate started to decrease. This type of relationship between yield of mycelial mass and linear growth rate was found with all of several strains grown on various media. Figure 5 shows linear growth rate plotted directly against yield for all the strains and conditions tested, showing that, whether salts, sugar or arginine (in the case of arginine auxo-trophs) were the growth-limiting nutrient, the yield decreased to about 10 mg.

mycelium/tube before the linear growth rate started to decrease significantly. It was not found practical to measure the very low yields obtained at low growth rates and so the relationship between the two parameters was not explored below about 1 mg. mycelium/tube.

The relationship between inoculum size and lag in growth

In a previous paper (Gillie, 1968) the relationship between inoculum size and growth in exponential cultures of micro-organisms was considered and it was shown
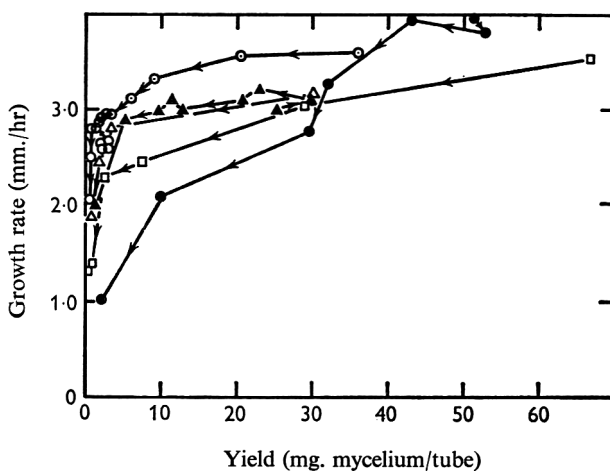

Fig. 5. Linear growth rate is plotted against yield of mycelium per tube for several strains of *Neurospora crassa* growing under widely different conditions. Each experiment, represented by one type of symbol on the graph, was made with one strain growing on a medium in which one constituent was progressively diluted. The direction of progressive dilution is represented on the graph by the arrows on the lines connecting points. The strains and media used were as follows: ●, strain *arg*-10 on 500 mg. arginine/l. and these concentrations of Vogel salt solution: 2·0, 1·5, 1·0, 0·5, 0·1, 0·01 ml./100 ml. medium. ○, strain *arg*-10 on medium with no sucrose and these concentrations of arginine 10, 5, 1·0, 0·5, 0·1, 0·05, 0·01, 0·005 g./l. ☉, strain *arg*-10 grown on 500 mg. arginine/l. and these concentrations of sucrose: 20, 5, 1, 0·5, 0·1, 0·05, 0·01, 0 g./l. △, strain *arg*-10 on these concentrations of arginine: 0·5, 0·04, 0·01, 0·005 g./l. ▲, strain *arg*-1 on these concentrations of arginine: 5·0, 2·0, 0·5, 0·25, 0·175, 0·05, 0·025, 0·01 g./l. □, wild-type strain STA on Roberts medium (Roberts *et al.* 1955) with these concentrations of sucrose: 20, 10, 5·0, 2·0, 0·5, 0·2, 0·1 g./l. Roberts medium contains only inorganic salts with no citrate which was present in the Vogel salt solution and might have acted as a subsidiary carbon source.

that when the inoculum size was varied in a series of cultures and the weight of each culture after growth considered as a constant value (in practice by interpolating between experimental values), then from the exponential growth equation

$$\log_e M - \log_e M_0 = \mu t$$

(where M_0 is mass of inoculum and M is the mass of the culture at time t), it can be seen that if M is treated as constant, $-\log_e M_0$ is proportional to t. A relationship of this type was found to hold between 'lag' (time after inoculation when growth first becomes measurable) and inoculum size for linear growth of Neurospora in tubes. The experimental investigation of this relationship is described below.

Tubes of minimal medium supplemented with different quantities of L-arginine

were inoculated with from 1 to 10⁵ conidia of the *arg-1* or *arg-10* mutant strain of *Neurospora crassa*. The inoculum was diluted in 5-fold steps, and each inoculation at a particular conidial concentration (there were from 5 to 7 such conidial concentrations for each arginine concentration) and a particular arginine concentration was made in duplicate. It was found that at a particular arginine concentration the linear growth rate (mm./hr) did not vary with inoculum size (see Fig. 6). However, as the inoculum became more dilute there was a progressive delay in the initiation of linear growth (see Fig. 6). Extrapolation of the line describing this growth to zero on the length of growth axis served to define the parameter which has been called the lag and which is measured
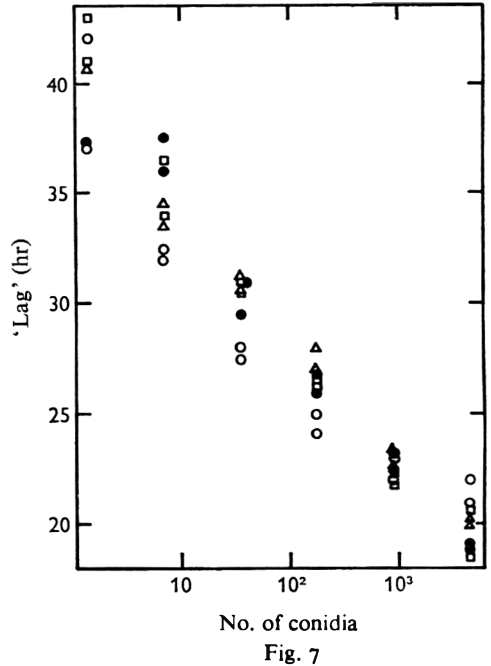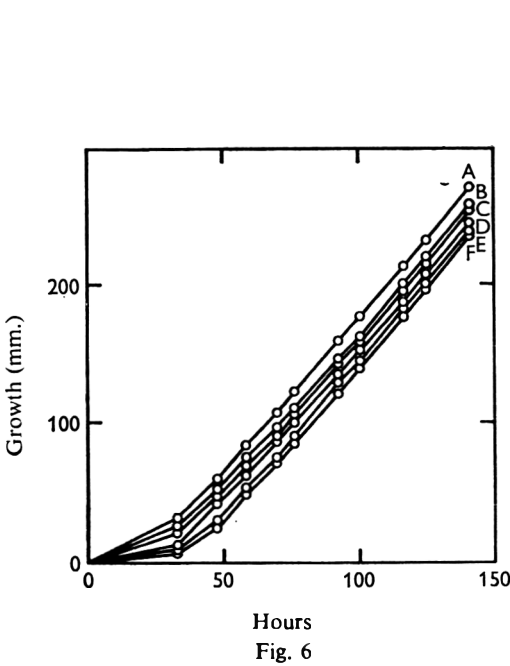

Hours

Fig. 6

No. of conidia

Fig. 7

Fig. 6. Growth of *Neurospora crassa* strain *arg-1* in growth tubes at various inoculum dilutions. Results are shown from only one of two duplicate tubes at each inoculum dilution on 5 mg. arginine/l. Calculated inocula (conidia/tube) were: A, 4·4 × 10³; B, 8·8 × 10²; C, 176; D, 35; E, 7; F, 1. Lags estimated from these data are plotted against inoculum size in Fig. 6.

Fig. 7. Lag is plotted against log inoculum size for *Neurospora crassa* strain *arg-1* grown with various concentrations of arginine in the medium. ● indicate data from medium containing 5 g. arginine/l. △, 2 g. arginine/l. □, 100 mg. arginine/l. ○, 5 mg. arginine/l. These data appear as series D in Table 1.

in hours. The lag then is the time (in hours) taken for growth to be initiated after inoculation, assuming that all growth occurs at the same linear rate. Since growth does occur at the same linear rate, once established, the lag may be readily determined by extrapolation from a graph by using a ruler.

Regressions were calculated of lag against log inoculum size and it was found that there was a linear relationship for arginine concentrations above 3 mg./l. These results are shown in Table 1. The relationship showed a significant non-linearity ($P < 0.01$) in five cases, but this was found to be almost wholly due to the inclusion of inocula

with a calculated number of 10 conidia or less per tube. When inocula of 10 conidia or less were excluded from the calculations then only one out of fifteen regressions showed significant non-linearity ($P = $ 0·001 to 0·01); however, this is not significant when all the experiments are considered together. It is not perhaps surprising that the lag showed greater variability for calculated inocula of 10 conidia or less, since in small samples the percentage variation in actual numbers per tube and the variation in time of germination of individual spores will be greater.

Table 1. *Neurospora crassa strains arg-1 and arg-10 (arginine-dependent). Calculated regressions in lag in linear growth against log number of conidia in inoculum*

Results of the type illustrated in Fig. 7 were collected for strains *arg-1* and *arg-10* grown on various concentrations of arginine in growth tubes, and were used to calculate the slopes of lag in growth (in hr) plotted against log inoculum dilution. The slope is measured by the regression constant *b* which is defined as lag in hr/10^{-1} dilution of inoculum. The experiments were performed in several series (A to F); each series of experiments was done at the same time with the same inoculum dilutions. Inocula with a calculated size of 10 conidia or less have been omitted in calculating these regressions (see text).

Strain	Arginine concentration (mg./l.)	Series	*b*	Standard error of *b*	95 % confidence limits of *b*
arg-1	5×10^3	C	5·94	0·11	6·23 to 5·65
	5×10^3	D	5·33	0·27	6·07 to 4·59
	2×10^3	D	5·15	0·20	5·69 to 4·61
	5×10^2	C	4·72*	0·15	5·11 to 4·33
	10^2	D	5·47	0·33	6·39 to 4·56
	50	F	7·90	0·67	9·62 to 6·18
	10	C	3·97	0·64	5·63 to 2·31
	10	E	6·15	0·38	7·13 to 5·17
	10	F	5·69	0·42	6·77 to 4·61
	8	F	6·26	0·41	7·32 to 5·20
	5	D	2·97	0·29	3·77 to 2·17
	5	F	4·94	1·10	7·78 to 2·16
	3	F	4·69	0·59	6·19 to 3·18
	1	E	−0·07†	4·05	—
	1	F	3·43†	0·96	—
	0·5	E	−0·50†	1·14	—
	0·1	E	−14·70†	8·12	—
arg-10	5×10^2	A	5·11	0·31	5·97 to 4·25
	10	B	7·51	0·90	10·38 to 4·65
	1	A	5·29†	2·30	—

* These figures showed significant non-linearity (0·001 < P < 0·01).
† No significant regression was obtained.

The linear relationship between log of inoculum size and lag was used to calculate the regression constant *b* (Table 1) which is expressed as lag in hours per 10^{-1} dilution of inoculum. An unweighted mean estimate of *b* for *arg-1* grown on 3 to 5×10^3 mg. arginine/l. was calculated as 5·32 hr/10^{-1} dilution of inoculum (95% confidence limits 5·98 to 4·66), and the mean for *arg-10* grown on 10 and 5×10^2 mg. arginine/l. was found to be 5·37 (95% confidence limits 6·25 to 4·49). These mean estimates were compared and found to be homogeneous (0·7 < P < 0·8) and have been combined to give a weighted mean estimate of 5·35 hr/10^{-1} dilution of inoculum (95% confidence limits 5·78 to 4·91). This weighted mean estimate was used to calculate a doubling time

of 1·61 hr (95% confidence limits 1·48 to 1·74 hr). This may be thought of as the time it takes for an inoculum of n conidia to grow to the effective size of an inoculum of $2n$ conidia, where n in practice lies between 10 and 10^5. The method of calculation of doubling time from inoculum dilution data is discussed by Gillie (1967).

Considerable heterogeneity was found amongst the different estimates of b (see Table 2); this was least amongst b values estimated at the same arginine dilution. This suggests that there was an additional source of variability operating between experiments, but the variability found within experiments at particular arginine dilutions was much less than the variability found when the experiments were grouped in any other way. This heterogeneity makes the interpretation of the relationship between b values (or inoculum doubling time) and arginine concentration difficult; however, it seems that there is no clear systematic relationship detectable between them over a range of 3 to 5×10^3 mg. arginine/l. and it is possible that the observed variation in b values is mostly attributable to experimental error. No significant regression was obtained for log of inoculum size against lag for *arg-1* or *arg-10* growing on concentrations of arginine of 1 mg./l. or less, although sustained growth was obtained on these low arginine concentrations.

Table 2. *The homogeneity of b values*

		Degrees of free-dom	χ^2	Probability
Division A	Between series	4	71·80	< 0·001
	Within series	8	60·43	< 0·001
	Total	12	132·23	< 0·001
Division B	Between dilutions	8	116·29	< 0·001
	Within dilutions	4	15·94	0·001 to 0·01
	Total	12	132·23	< 0·001

DISCUSSION

The results described show that the strains of *Neurospora crassa* investigated adapted to decreasing concentrations of an essential nutrient in solid medium by a decrease in the mass of the culture followed by a decrease in linear growth rate. Both these parameters decreased roughly in proportion to the log of the arginine concentration, but the decrease in linear growth rate did not begin until a more than 5-fold decrease in mycelial yield per tube had occurred. The log relationship between yield and arginine concentration cannot continue to be proportional in the same way below 10 mg. arginine/l. since growth occurs even on 10^{-2} mg. arginine/l. and at a measurable rate. It is interesting that it is at about this point (10–50 mg. arginine/l.), at which the rate of decrease in yield with decrease in arginine concentration must level off, that the decrease in linear growth rate begins. This suggests, in conjunction with the results of other experiments in which salts and sucrose as well as arginine were used as growth limiting nutrients, that when the yield of mycelium per tube reaches about 5–10 mg. the linear growth rate starts to decrease. This relationship between yield and growth rate indicates perhaps that there is a control mechanism to maintain the mycelium as near as possible to an optimal density suitable to the habit of growth of the organism.

Growth on solid media has some superficial resemblances to growth in continuous culture in a chemostat, in that the mycelium as it grows is constantly being presented with new food, and also the yield of the organism may vary over a wide range as a result of varying the concentration of nutrients. In a chemostat, however, the yield is directly proportional to the concentration of the limiting nutrient (Monod, 1942), not proportional to the log of the limiting nutrient, as in linear growth in tubes. Linear growth rate also seems to be proportional to the log of the growth limiting nutrient (arginine). A similar non-linear relationship was found by Pirt (1967) between the initial colony radial growth rates of bacteria and the glucose concentration in the medium. Pirt pointed out that the initial concentration of the growth-limiting nutrient cannot be the concentration to which the bacteria are in fact exposed during most of their growth, because a concentration gradient must develop in the nutrient agar. These considerations probably also apply to the linear growth of Neurospora, as here studied, although the linear growth rate is much greater and mycelial 'roots' penetrate right through the agar to obtain nutrients more actively.

Models of spherical growth of fungi in liquid culture (discussed by Gillie, 1968), which assume a constant rate of increase of the radius of the sphere, are based on the linear rate of increase of the radius of fungal colonies on solid media, and tend to assume that the density of the organism is uniform throughout the 'sphere of growth' considered. However, the results described here show that an increase in yield takes place behind the growing front to a maximum value, after which a decrease in yield occurs. Future models of spherical growth of fungi may find it necessary to consider complications of this kind.

The inoculum doubling-time as calculated above is remarkably close to the inoculum doubling-time determined (by using the same reasoning) for growth in unstirred liquid cultures (Gillie, 1968) of 1·84 hr for Neurospora strain STA (95 % confidence limits 1·74 to 1·94 hr) and in reasonable agreement with doubling times for strain STA, grown in shaken logarithmic cultures, of 2·3 hr (95 % confidence limits 1·7–2·9 hr) (C. F. Curtis, personal communication).

In one mutant strain of *Neurospora crassa arg*-11 (30820) which requires either 30 % (v/v) carbon dioxide or arginine + purine + pyrimidine for normal growth (Broadbent & Charles, 1965), an inoculum doubling-time of 15 hr for 'leaky growth' on minimal medium was observed as compared with an inoculum doubling-time of 3 hr on complete medium. Once growth was established after the lag on these media the density of growth and the linear growth rates were in the same range. The slow rate of inoculum doubling in this strain *arg*-11 (30820) on minimal medium may be due to an initial dependence on small quantities of atmospheric carbon dioxide for growth, whereas later growth is able to rely on carbon dioxide generated by the organism itself.

The EMA wild-type strain of *Neurospora crassa* had a consistently lower linear growth rate (1·9 mm./hr) than the STA wild-type strain (3·2 mm./hr) and yet the EMA wild-type strain gave consistently the highest yield of mycelium per tube (42·6 mg. as opposed to 32·4 mg. for strain STA). These results show that it cannot be assumed that a low linear growth rate indicates a lower yield when comparisons are made between strains.

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The Fine Structure of Yeast Regenerating Protoplasts

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SUMMARY

The process of regeneration of *Candida utilis* and *Pichia polymorpha* protoplasts obtained by means of an enzyme preparation from *Streptomyces violaceus MR* and an enzyme preparation from the snail (*Helix pomatia*) was followed with the electron microscope. A reticular network of fibrils making up a tube or surrounding the regenerating protoplasts was observed. The fibrils are glucan fibrils, and in *C. utilis* they are packed in groups of 5 or 6. *P. polymorpha* fibrils occurred singly. The fibrils are the framework for building the new cell wall. The relation between fibrils and plasmalemma particles is discussed.

INTRODUCTION

Regeneration occurs when yeast protoplasts are kept for a time in a suitable isotonic medium. Necas (1956, 1961) studied this process with Saccharomyces strains, and Rost & Venner (1965) with strains of *Saccharomyces fragilis*, *S. willianus* and *Schizosaccharomyces pombe*. Eddy & Williamson (1959) first found that growing protoplasts are covered with a reticular network. Necas (1965) proved that this fibrous network builds the new cell wall. Villanueva (1966) has reviewed this problem. The aim of the present paper is to determine whether the regenerating protoplasts of *Candida utilis* and *Pichia polymorpha* show, in the electron microscope, fibrils or a network which represents the beginning of cell-wall synthesis.

METHODS

Organisms. The strains of *Candida utilis 1016* and *Pichia polymorpha 1132* used in these experiments were obtained from the Colección Española de Cultivos Tipo (CECT).

Culture media. *Candida utilis* was grown in 300 ml. flasks containing 100 ml. of Hansen medium (30 g. sucrose; 10 g. bactopeptone; 0·5 g. $MgSO_4.7H_2O$; 0·5 g. K_2HPO_4; 1000 ml. distilled water). For *Pichia polymorpha* cultures, 100 ml. Winge medium (3 g. yeast extract Difco; 20 g. glucose; 1000 ml. tap water) was used in 300 ml. flasks. Cultures were incubated by shaking at 29°.

Preparation of protoplasts. *Candida utilis* was harvested in the early logarithmic phase of growth and the organisms washed with distilled water. For protoplast formation the organisms were suspended in an enzyme solution from *Streptomyces violaceus* MR (Elorza, Muñoz-Ruiz & Villanueva, 1966), containing M-$MgSO_4$ as a stabilizer; the final concentration of organism was 0·075 % (w/v) dry wt. The mixture was shaken at 30° and the extent of lysis and protoplast formation was measured with a Zeiss phase-contrast microscope. After 2 hr the protoplasts had been free.

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Pichia polymorpha organisms were similarly harvested and washed. Protoplasts were liberated by the snail enzyme preparation (*Helix pomatia*) obtained from the Industrie Biologique Française (Gennevilliers, France) by the Eddy & Williamson (1957) method.

Regeneration of protoplasts. Candida utilis protoplasts were washed three times with M-MgSO$_4$ solution, resuspended in M-MgSO$_4$ solution containing 0·0017% (w/v) of sucrose and incubated at 30°.

Pichia polymorpha protoplasts were washed with M-MgSO$_4$ solution, resuspended in Winge medium containing 20% (w/v) MgSO$_4$ as stabilizer and incubated at 30°. The regeneration process was studied by taking samples of protoplasts and examining them with the Zeiss phase-contrast microscope.

Electron microscopy. Embedding. Samples of regenerating protoplasts taken at 6 and 8 hr were centrifuged down, the deposit washed with distilled water, suspended in 5% (w/v) aqueous potassium permanganate and kept at room temperature for 2 hr. The fixed protoplasts were dehydrated through 25%, 50% and 75% (v/v) acetone in water, then 100% acetone. During dehydration the material was stained overnight in 2% (w/v) uranyl acetate dissolved in 75% (w/v) acetone. The fixed and stained material was embedded in Durcupan ACM from Fluka AG. Buchs, Switzerland, cut with a LKB Ultratome Microscope 4804 Nife (glass knives) and picked up on Formvar-coated grids.

Shadow-casting. Drops of 6 hr and 8 hr suspensions of regenerating protoplasts were applied directly to Formvar-coated grids (after having been washed with water to free them of salts), allowed to dry and shadowed at an angle of 45° with gold + palladium in a Siemens VB 6500 evaporator.

Sections and shadowed preparations were examined with a Siemens Elmiskop I electron microscope and in a EM 9 Zeiss electron microscope.

RESULTS

The regeneration process as seen with the phase-contrast microscope

After incubation for 1 hr the protoplasts showed signs of growth. Budding protoplasts were seen in the medium. After 2 hr the initially spherical protoplasts changed to tubular forms, which grew to form large cytoplasmic masses, from which new yeast cells originated (Pl. 1, fig. 1). These large tubular forms were similar to those obtained by Necas (1956, 1961, 1965), but we produced them in a liquid medium instead of on a solid medium. These forms were not sensitive to osmotic shock, and for this reason could be handled in distilled water.

Thin sections of regenerating protoplasts

Plate 1, fig. 2 and 3, shows sections through regenerating protoplasts of *Candida utilis* and *Pichia polymorpha*. While these protoplasts were not sensitive to osmotic shock, they were very sensitive to the embedding process and the protoplasm is affected (Monreal, Uruburu & Villanueva, 1967). No clear structures were seen in the cytoplasm, but around it was a fairly wide porous layer, more manifest in *C. utilis* than in *P. polymorpha*.

Shadow-casting of regenerating protoplasts

Plate 2, fig. 4–7, and Plate 3, fig. 8, show shadow-casting of regenerating protoplasts. A network of fibrils can be seen building a tube, or surrounding the protoplasts.

In regenerating protoplasts of *Candida utilis* the fibrils are packed in groups of 5 or 6. Each fibril is 250–300 Å wide (Pl. 2, fig. 5, 6). The bundles of fibrils are 3000–4000 Å wide (Pl. 2, fig. 5). Scattered along the tubes are large cytoplasmic bodies which represent the protoplasts (Pl. 2, fig. 7; Pl. 3, fig. 8). Sometimes protoplasts which have not put out a fibrous tube are also surrounded with a fibrous network (Pl. 3, fig. 8).

Regenerating protoplasts of *Pichia polymorpha* show a network of fibrils (Pl. 2, fig. 4). These do not join to form a bundle, however, but remain single, and form a disorderly framework. Each fibril is 200–250 Å wide. Protoplasts can be seen in the fibrous frame-work.

DISCUSSION

The fibrils found in the shadow-casting preparations denote the beginning of new cell walls. Previously, shadow-casting of *Candida utilis* protoplasts showed no fibrils around them (Garcia-Mendoza & Villanueva, 1967). From the chemical composition of the *C. utilis* cell walls (Novaes & Villanueva, 1963) we assume these fibrils are made of glucan. The fact that we were not able to find the fibrils in thin sections is because they do not stain with potassium permanganate. Reports of the fine structure of yeast cell walls have always shown a lack of contrast of the inner glucan layer (Northcote, 1963; Vitols, North & Linnane, 1961). On the other hand, the stained layer in sectioned *C. utilis* regenerating protoplasts is made up of protein, which does react with potassium permanganate. This layer represents the stainable part of the inner layer of the yeast cell wall. While it is believed that protein is located only in the outer cell-wall layer (Mundkur, 1960, 1964), probably it is scattered throughout the glucan framework. Besides, polysaccharides combine with the protein in the form of a glucan-protein (Falcone & Nickerson, 1956). Since protein is not fibrous, one cannot see it in shadowed preparations. Protoplasts have not yet begun to build the outer protein layer.

Moor & Mühlethaler (1963) reported, with frozen-etched yeast cells, that the cytoplasmic membrane was covered with particles which sometimes were concentrated in hexagonal arrangements. From these arrangements grew fibrils which extended into the cell wall. When particles were scattered on the cytoplasmic membrane, no fibrils were seen. The fact that in our pictures the fibrils are sometimes packed in bundles of about six units leads us to assume that perhaps there is a relation between hexagonal arrangements and packed fibrils, and between unarranged particles and single fibrils. This discussion also applies to regenerating protoplasts of *Pichia polymorpha*.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Regeneration process of regenerating protoplasts of *Candida utilis* and *Pichia polymorpha*. Regeneration in *Pichia polymorpha: a, b, c*. Regeneration process in *Candida utilis: d, e, f*. Phase-contrast microscope. × 500.

Fig. 2. Thin section through a regenerating protoplast of *Candida utilis*. × 22,000.

Fig. 3. Thin section through a regenerating protoplast of *Pichia polymorpha*. × 20,000.

PLATE 2

Fig. 4. Shadow-casting of regenerating protoplast of *Pichia polymorpha*. × 13,200.

Fig. 5. Detail of a bundle of fibrils of regenerating protoplasts of *Candida utilis*. Shadow-casting. × 190,000.

Fig. 6. Fibrils of regenerating protoplasts of *Candida utilis*. Shadow-casting. × 25,000.

Fig. 7. Shadow-casting of regenerating protoplasts of *Candida utilis*. × 17,000.

PLATE 3

Fig. 8. Shadow-casting of regenerating protoplasts of *Candida utilis*. × 21,000.

Plate 1

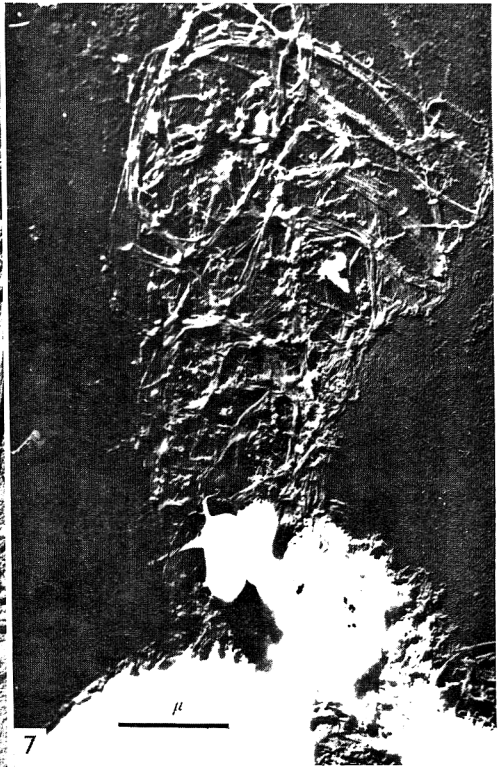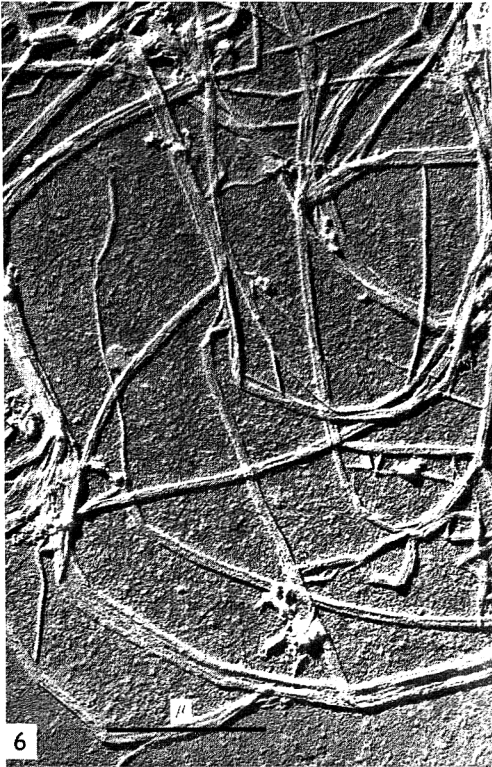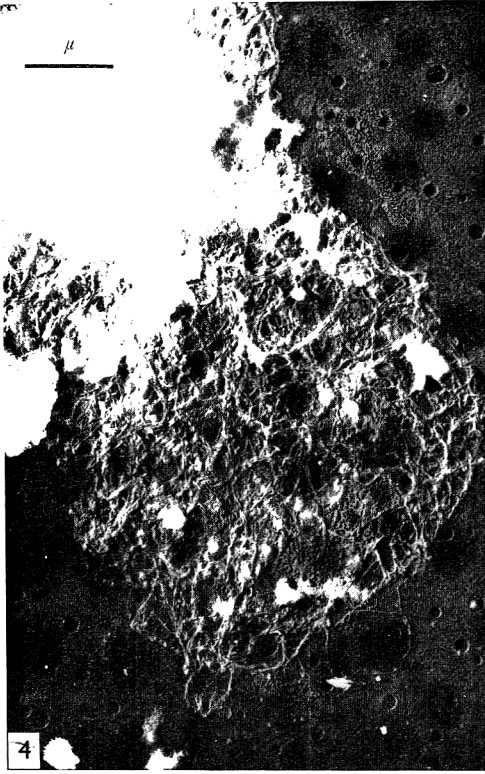F. URUBURU, V. ELORZA AND J. R. VILLANUEVA

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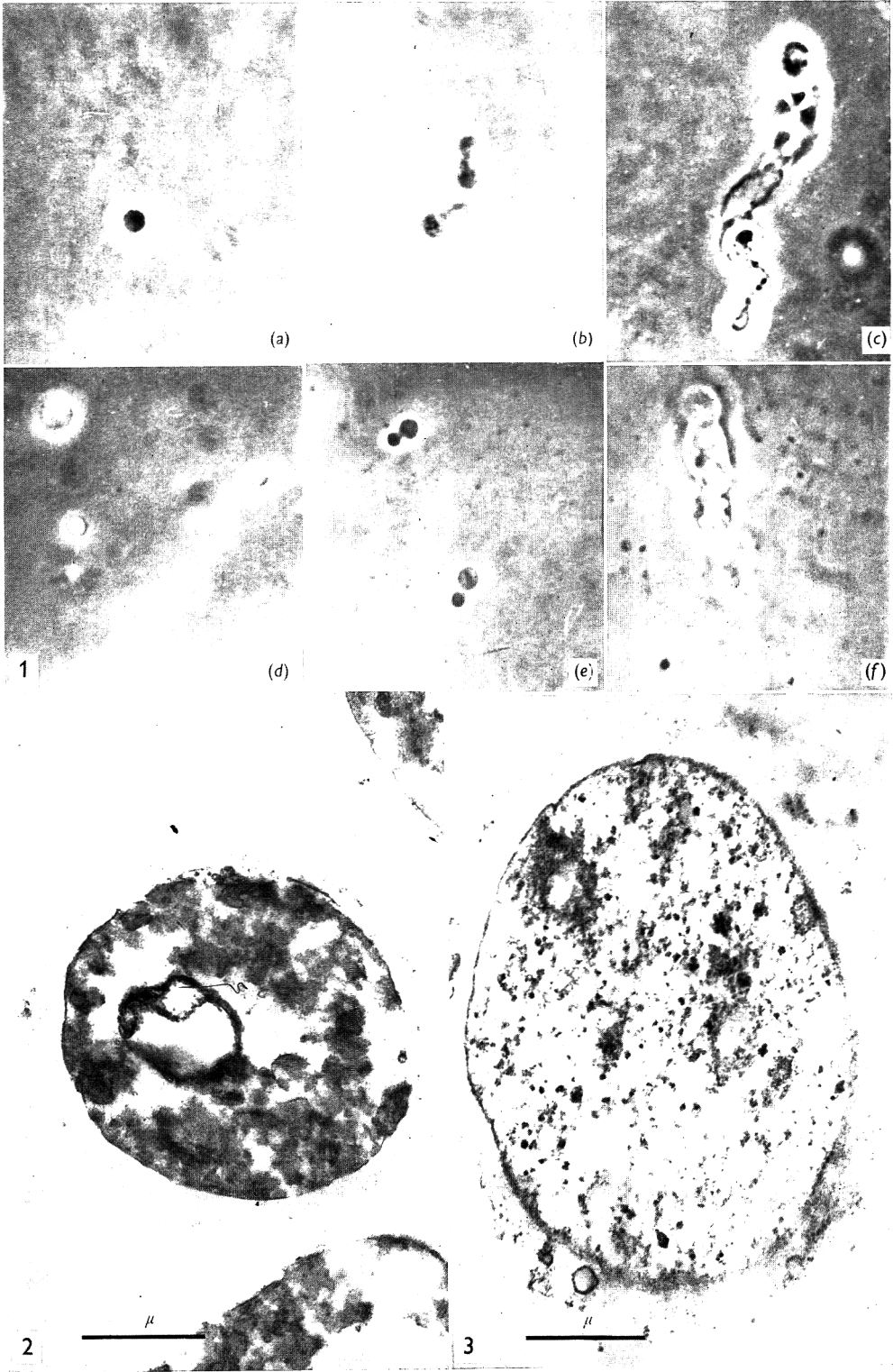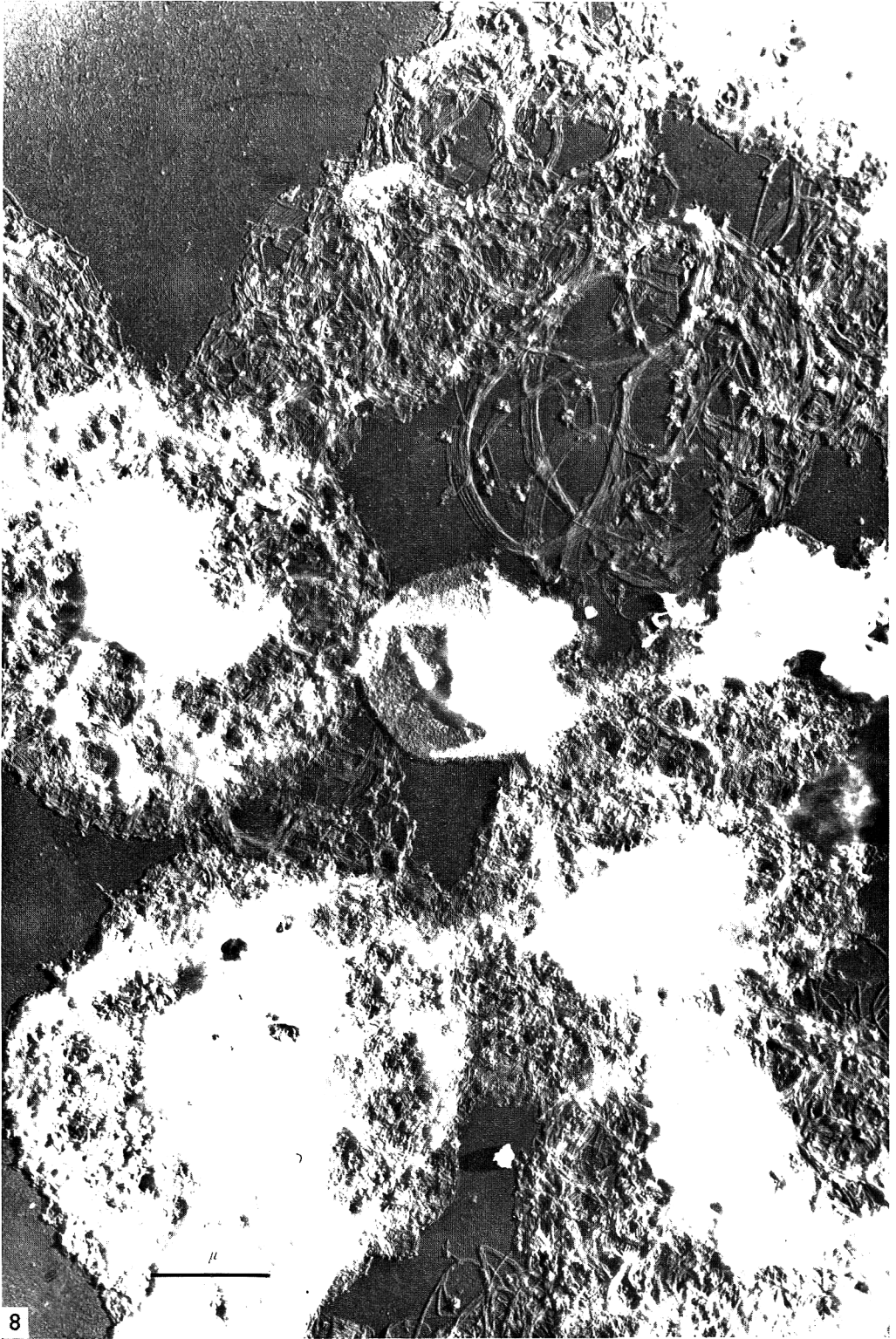
Growth and Division of Some Unicellular Blue-green Algae

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SUMMARY

The growth of microcolonies of six strains of unicellular blue-green algae was studied by time-lapse photomicrography. The four rod-shaped strains divided regularly in a plane perpendicular to the long axis of the cell; one spherical strain divided successively in two planes, and one in three planes, perpendicular to one another.

Anacystis nidulans and the other rod-shaped blue-green algae studied have a very restricted ability to form chains, four-celled elements being the longest ones observed in slide cultures. They are therefore unicellular organisms, in no way different with respect to development from rod-shaped unicellular bacteria. The recent proposal that *A. nidulans* is filamentous and should be reclassified in the genus *Phormidium* is based on a mis-understanding of the nature of unicellularity among procaryotic organisms.

INTRODUCTION

The classification of the unicellular blue-green algae of the family Chroococcaceae is at present controversial. Natural-historical studies have led to the description of a very large number of form genera and species: more than 20 genera and 200 species were recognized in the compilatory work of Geitler (1932). In 1956, Drouet & Daily proposed a greatly simplified taxonomic treatment of the group, largely based on an exhaustive comparison of published descriptions and herbarium specimens; they recognized only six genera and 18 species. Their treatment is, however, not accepted by others (Geitler, 1960; Skuja, 1956).

The simplified generic subdivision of Drouet & Daily (1956) lays major emphasis on the planes of successive cell divisions; but the authors do not mention how this developmental character can be reliably determined by the examination of herbarium specimens. A more fundamental taxonomic ambiguity is revealed by the nomenclatural history of a rod-shaped strain which was purified by Kratz & Allen, and identified originally by Drouet as *Anacystis nidulans* (Kratz & Myers, 1955). This supposedly unicellular organism has since been maintained in many culture collections, and used for various experimental studies. According to Silva (1962), Drouet has more recently re-identified it as *Phormidium mucicola* (family Oscillatoriaceae) on the basis of its ability to form short filaments. This implies that even the familial and ordinal assignment of a blue-green alga is not easily made in some instances.

It should be noted that the classification of this group has not so far been based on the comparative study of pure cultures, grown under reasonably well-controlled conditions. We describe here the growth of *Anacystis nidulans* (strain of Kratz & Allen)

and of five other unicellular blue-green algae, as determined with pure strains examined in slide cultures. Our purpose was to ascertain whether the mode of division is a constant and easily determinable strain property, as implied by the taxonomic treatment of Drouet & Daily (1956); and whether *A. nidulans* and other rod-shaped strains can be most appropriately characterized as 'unicellular' or 'filamentous'.

METHODS

Organisms. Four rod-shaped and two spherical strains were examined (Table 1). Strains 6307 and 6308 were uni-algal when received, and were freed from bacteria by us. Strain 6301 (*Anacystis nidulans* of Kratz & Allen) was received as a pure culture. The other three strains were isolated from nature and purified by us.

Cultivation. Cultures were maintained in tubes of the liquid medium of Hughes, Gorham & Zehnder (1958), modified by increasing the concentration of $NaNO_3$ to 1·5 g./l. The tubes were incubated at 30° in a water bath illuminated laterally with fluorescent lights (intensity at entrance window: 500 ft.-candles).

Table 1. *Unicellular blue-green algae examined*

Strain	Source	Size and shape
6301	*Anacystis nidulans**	Rods, 2·0 × 0·8 μ
6307	*Coccochloris peniocystis*†	Rods, 1·3 × 0·8 μ
6311	Local isolate from water	Rods, 2·0 × 1·3 μ
6312	Local isolate from water	Rods, 2·7 × 1·3 μ
6308	*Gleocapsa alpicola*†	Spheres, 3·4 μ diameter
6501	Local isolate from water	Spheres, 6·8 μ diameter, heavy laminate sheath

* Strain of Kratz and Allen, obtained from Dr M. B. Allen, Kaiser Foundation Research Institute, Richmond, California.
† Obtained from Dr G. P. Fitzgerald, University of Wisconsin, Madison, Wisconsin.

Slide cultures were prepared as follows. A drop of medium containing Difco agar (1 %, w/v) was spread in a thin film over an area of 2·5 × 1·8 cm. on a sterile slide and allowed to solidify. A small drop of a liquid culture was placed on the surface of the agar, two small drops of sterile liquid medium were deposited adjacent to the agar patch, and a sterile coverslip (2·4 × 6·0 cm.) gently lowered on to the agar surface, the edges then being sealed with sterile Vaseline + paraffin (1 + 1). The slide was examined microscopically, and a suitable field for observation selected. The sealed slide was illuminated continuously by two lateral fluorescent lights (intensity at microscope stage: 500 ft.-candles). The growing microcolonies were photographed at intervals with dark-phase contrast illumination.

RESULTS

Plate 1 shows the development of microcolonies of Kratz & Allen's strain of *Anacystis nidulans* (6301) on a slide culture over a period of 44 hr, corresponding to approximately three generations of growth. The development of the other rod-shaped strains (6307, 6311, 6312) in slide cultures was similar. Successive divisions took place regularly in planes perpendicular to the long axis of the cell. Although daughter cells

tended to remain associated for a short period following completion of division, the chains produced were never more than four cells in length, since slippage after division caused frequent disorientations.

Plate 2 shows the development of strain 6308 (*Gleocapsa alpicola*) on a slide culture over a period of 36 hr, corresponding to approximately three generations. The cells of this strain are spherical, and not enclosed by a sheath. Division occurred in two planes at right angles to one another; but regular tetrads did not persist, as a result of slight shiftings of the daughter cells in the growing colony.

Plate 3 shows the growth of strain 6501 in slide culture over a period of nearly 80 hr, corresponding to nearly three generations. The spherical cells of this strain were held together in small colonies by a multi-layered sheath, new layers of which were formed at each generation. Reproduction occurred by rupture of the oldest, outer, layer of the sheath through pressure of the growing colony. The sequence shown in Pl. 3 starts with a flat 4-celled colony, consisting of two pairs of recently divided cells: the disposition of the cells and sheath layers indicates that the two preceding divisions had occurred at right angles to one another. The first observed division (24 hr) took place in a third plane, at right angles to the two preceding planes, to yield a three-dimensional, 8-celled colony. The lower layer of 4 cells is indistinct, since it lies somewhat below the plane of focus. Thereafter, constriction by the sheath caused marked displacements of the enclosed cells (34, 46, 57, 75 hr) so that the plane of the next division could not be precisely determined. After 79 hr the colony had become somewhat flattened, as a result of the rupture of the outer layers of the sheath: the lower 8 cells (derived from the lower pair of cells in the first figure of the sequence) are all clearly visible, in the course of a well-synchronized division.

DISCUSSION

The four rod-shaped strains examined multiply by regular binary transverse fission. Attachment between daughter-cells is evidently weak, since even on an agar surface, where the preservation of attachment is favoured, 4-celled chains are the longest elements observed. All these organisms, including the Kratz & Allen strain of *Anacystis nidulans*, show a mode of growth and reproduction that is not significantly different from that of rod-shaped unicellular eubacteria. We cannot therefore accept the contention that they are filamentous blue-green algae, in the sense that an Oscillatoria, with its many-celled structure and hormogonial mode of reproduction, can be described as 'filamentous'. The proposed reassignment of *A. niaulans* to the genus *Phormidium* (Silva, 1962) is evidently based on a misunderstanding of the cellular condition of a growing rod-shaped procaryotic organism, aptly characterized by Robinow (1945) as 'two-cells-about-to-become-four'.

In contrast to the rod-shaped strains, both the spherical strains that we have examined divide in more than one plane. Strain 6308 divides in two planes at right angles to one another, and strain 6501 in three planes at right angles to one another. However, post-divisional changes of cellular orientation can completely obscure the relations between successive planes of division in both these strains. Hence a distinction between genera based on the number and relation of successive planes of division, as proposed by Drouet & Daily (1956), may well lead to determinative difficulties, particularly when identifications are attempted on natural material without cultural

studies. The possible taxonomic value of this character can be adequately assessed only by developmental studies on a large number of spherical strains.

The determinative ambiguities of the traditional form genera appear even more serious. The successive forms assumed by a growing colony of strain 6501 (Pl. 3) might justify assignment to several form genera recognized by Geitler (1932): e.g. *Chroococcus, Microcystis, Gleocapsa* or *Aphanocapsa*. Furthermore, in terms of divisional behaviour (rather than ultimate colony form) strain 6308 (Pl. 2) can be related to the genus *Merismopedia*, in which the spherical cells are disposed in flat arrays of tetrads, while strain 6501 can be related to the genus *Eucapsis*, in which the spherical cells are arranged in cubical packets. In our judgement, a binary subdivision of the Chroococcaceae into two form genera (rods and spheres) would constitute the most useful temporary taxonomic solution. Eventually, a more elaborate subdivision of the group may become possible; but it will not have a solid scientific basis until really extensive comparative studies with pure cultures have been carried out.

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EXPLANATION OF PLATES

PLATE 1

A series of time-lapse photomicrographs illustrating the growth of a rod-shaped unicellular blue-green alga, *Anacystis nidulans* strain 6301, in an illuminated slide culture. The numbers on each figure indicate hours after the preparation of the slide culture. Dark phase-contrast. × 1250.

PLATE 2

A series of time-lapse photomicrographs illustrating the growth of a coccoid unicellular blue-green alga, *Gleocapsa alpicola* strain 6308, in an illuminated slide culture. The numbers on each figure indicate hours after the preparation of the slide culture. Dark phase-contrast. × 1250.

PLATE 3

A series of time-lapse photomicrographs illustrating the growth of an unidentified ensheathed, coccoid, unicellular blue-green alga, strain 6501. The numbers on each figure indicate hours after the preparation of the slide culture; pairs of figures bearing the same number represent photomicrographs taken after the same time interval, but in different focal planes. Dark phase-contrast. × 750.

Plate 2

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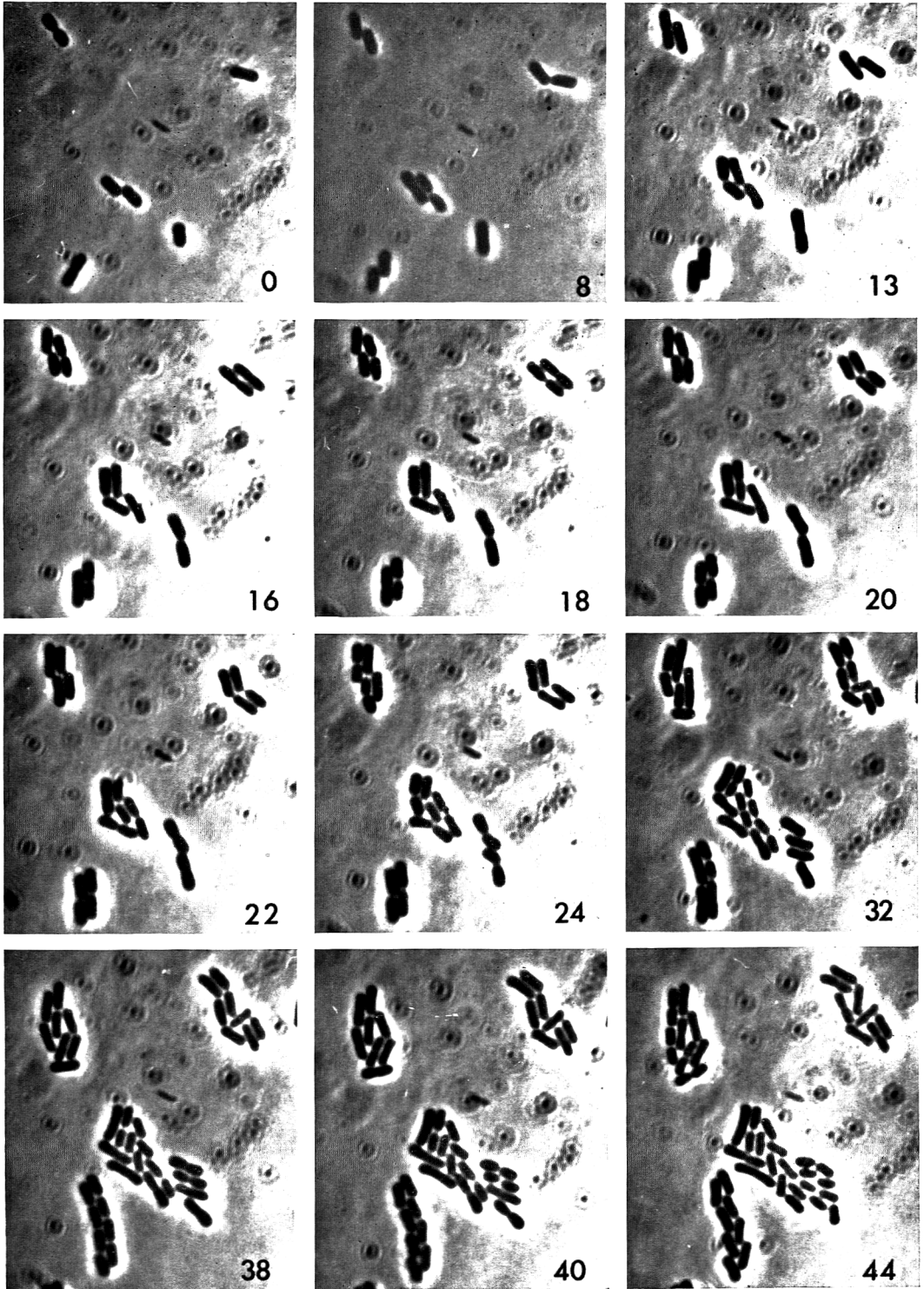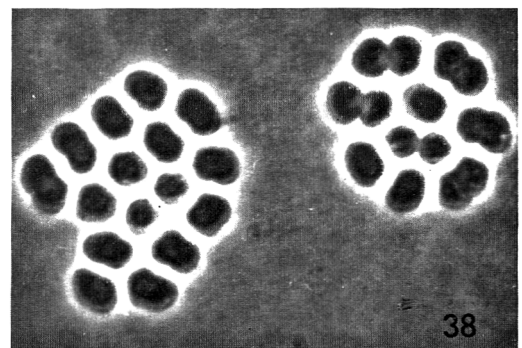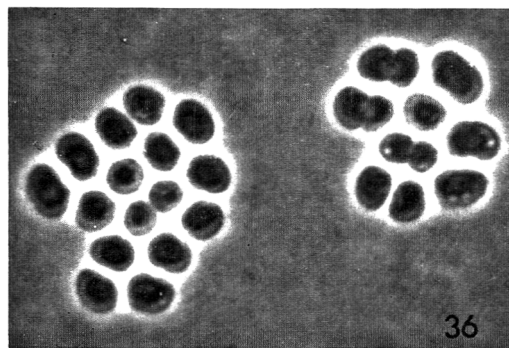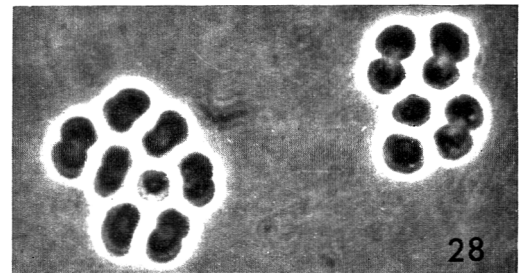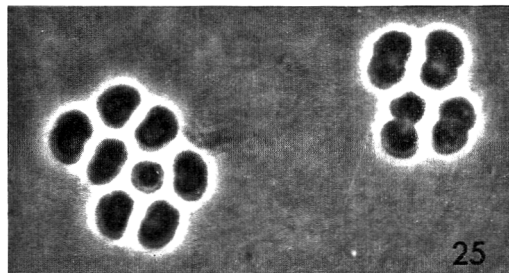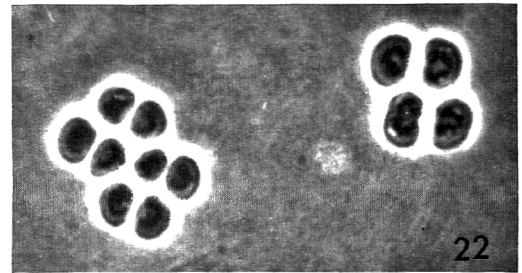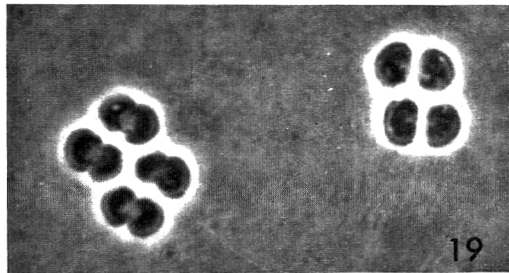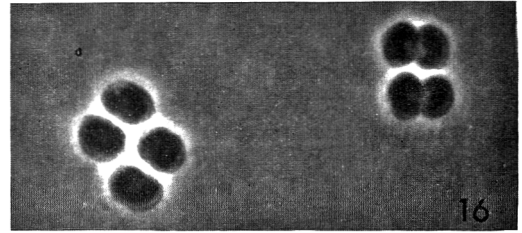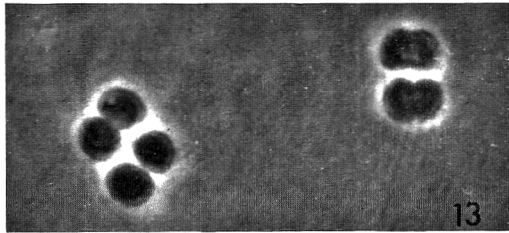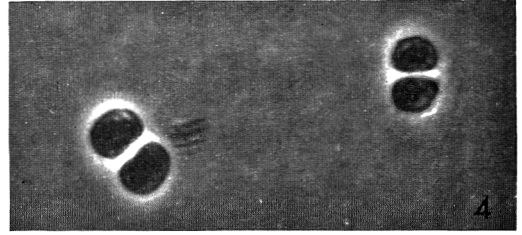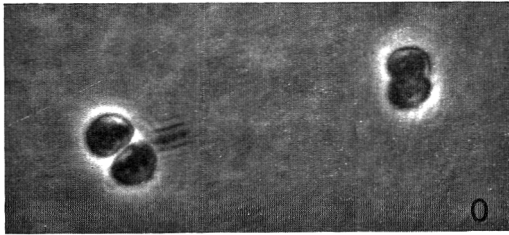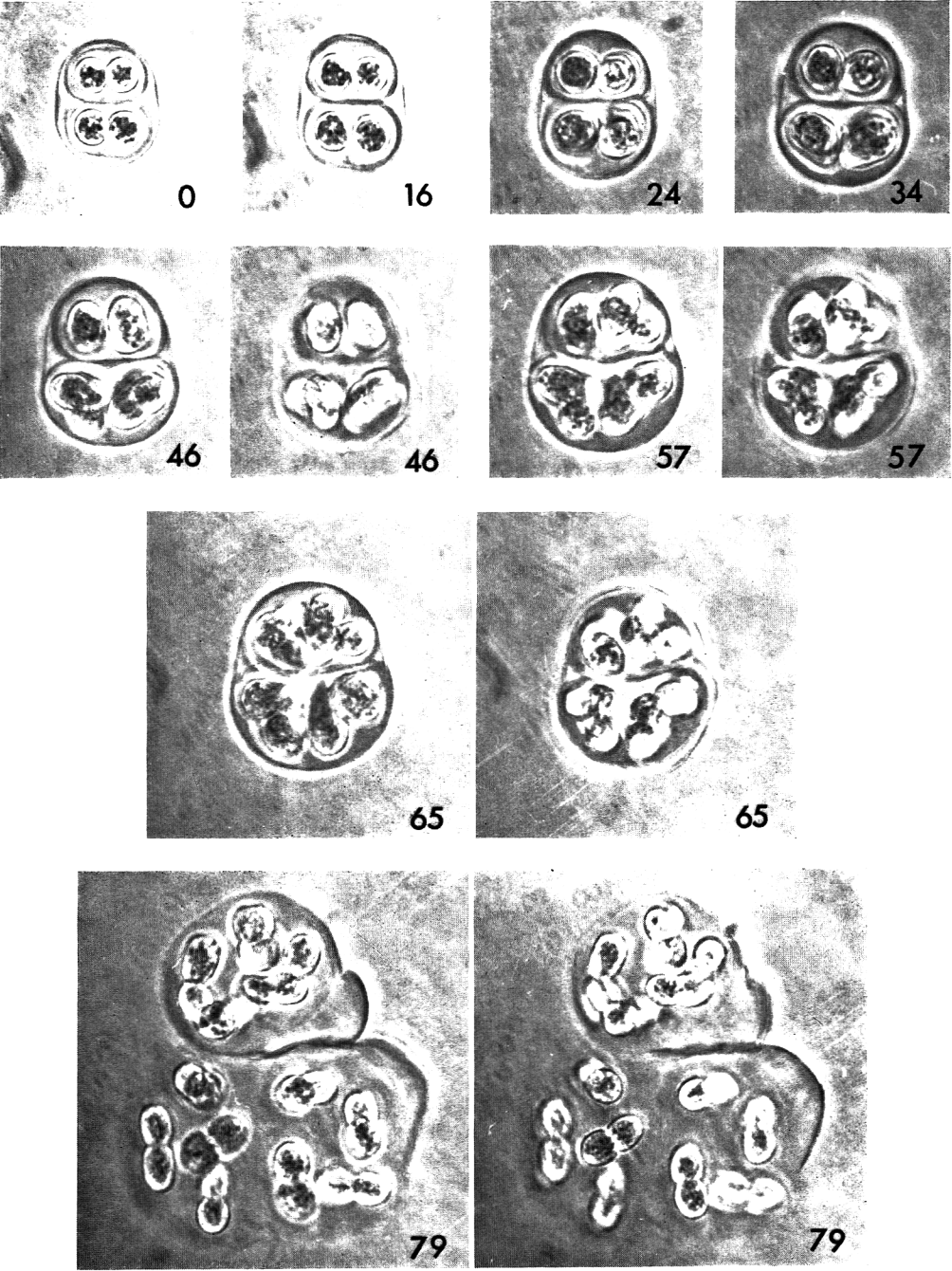
Selective Isolation of Blue-green Algae from Water and Soil

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SUMMARY

For the isolation of blue-green algae from soil and fresh water, temperature is a selective factor of major importance. In a nutritionally non-selective mineral medium, which at 25° supported growth of blue-green algae and of many eucaryotic algae, the development of eucaryotic algae was almost completely suppressed by incubation at 35°. Both the number and variety of blue-green algae recoverable at 35° were greater than those recoverable from parallel cultures incubated at 25°. When a combined-nitrogen source was omitted from the enrichment medium, only blue-green algae developed at both 25° and 35°; the microflora consisted exclusively of heterocyst-forming filamentous types, other groups of blue-green algae being eliminated.

INTRODUCTION

The extension of the techniques of microbiology to the study of blue-green algae has been slow, and relatively few representatives of the group have so far been obtained in pure culture (Koch, 1964). The isolation of these organisms from natural sources would be facilitated if cultures could be enriched in them by methods which effectively counter-selected eucaryotic algae. One such method was discovered by Beijerinck (1902), who showed that blue-green algae of the Nostoc type can be selected by using a mineral medium devoid of a combined-nitrogen source. Subsequent studies (Allen, 1952; Fogg, 1956) suggest that the ability to fix nitrogen is largely restricted to blue-green algae belonging to the Nostocaceae and is by no means universal in the members of this class. Consequently, Beijerinck's enrichment method, although completely effective in eliminating competition from eucaryotic algae, yields an extremely restricted fraction of the total blue-green algal microflora. Apart from the nitrogen-fixers, the blue-green algae seem to have nutritional requirements very similar to those of other algae, so that the possibility of devising other enrichment methods based on the principle of nutritional selection does not appear to be promising. Among algae, thermophily is virtually confined to blue-green algae; and cultures can be effectively enriched in the thermophilic members of the group at temperatures in the range from 60 to 75° (Peary & Castenholz, 1964). However, this physiological property is also a relatively rare one within the group.

A physiological study of a few pure strains (Kratz & Myers, 1955) suggested that even mesophilic blue-green algae may have a temperature range significantly higher than that characteristic of most other algae: the temperature optima were 32·5° for *Nostoc muscorum*; 35° for *Anabaena cylindrica*; and 41° for *Anacystis nidulans*. In commenting on these findings, Fogg (1956) remarked: 'it should be noted that the

temperatures which they [the strains studied by Kratz & Myers] are able to tolerate are much higher than those for algae of other groups obtained from similar environments. Thus, for most species of Chlorella, 25° is the optimum for growth and temperatures above 30° are lethal.'

We have recently examined the temperature ranges of many more pure strains of mesophilic blue-green algae, including members of the Chroococcaceae, Nostococeae and Oscillatoriaceae. Seventeen of these strains were isolated by us from soil and water, at temperatures never in excess of 28°. Twelve other strains were received from other laboratories; although we do not have information about the temperatures used for primary isolation, it is probable that they did not exceed 30°. Every strain in this collection can grow at 35°; eleven can grow at 40°; and two (the strain of *Anacystis nidulans* studied by Kratz & Myers, 1955, and a similar strain isolated by us) can grow at 45°. These results suggested that selection for the ability to grow at a temperature of 35° might provide an effective means for the enrichment of many types of blue-green algae in a nutritionally non-selective medium which was able to support the growth of other kinds of algae at lower temperatures. The experiments described below were undertaken to test this hypothesis.

METHODS

In all the experiments to be described, the mineral base of Hughes, Gorham & Zehnder (1958) was used. It contained (g./l.): K_2HPO_4, 0·039; $MgSO_4.7H_2O$, 0·075; Na_2CO_3, 0·020; $CaCl_2.2H_2O$, 0·027; $Na_2SiO_3.9H_2O$, 0·058; ethylenediaminetetraacetic acid, 0·001; citric acid, 0·006; Fe citrate, 0·006. To this was added 1 ml./l. of microelement mixture A5 (composition, g./l.: H_3BO_4, 2·86; $MnCl_2.4H_2O$, 181; $ZnSO_4.7H_2O$, 0·222; $Na_2MoO_4.2H_2O$, 0·391; $CuSO_4.5H_2O$, 0·079; $Co(NO_3)_2.6H_2O$, 0·0494). Preliminary experiments showed that when supplemented with $NaNO_3$ at 1·5 g./l., this medium supported good growth of all the blue-green algal strains tested. It is also suitable for the growth of many unicellular green algae, diatoms and uglenoids.

For enrichment experiments, 5 ml. samples of this medium, either nitrogen-free or supplemented with $NaNO_3$, were dispensed into test-tubes, which were plugged and sterilized. After inoculation, the tubes were placed in plastic racks in illuminated water-baths, maintained at a temperature of either 25° or 35°, and incubated without gassing or agitation. The illumination was provided laterally by banks of cool white fluorescent tubes, which gave an intensity at the entrance window of approximately 600 ft.-candles.

The sources of inocula for enrichment experiments are listed in Table 1. Water specimens were homogenized by agitation for 30 min. on a rotary shaker before sampling. Soil specimens (10 g.) were mixed with the medium to give a total volume of 50 ml. and agitated for 30 min. on a rotary shaker before sampling.

Serial 10-fold dilutions were prepared from each water sample or soil suspension, and used to inoculate three series of tubes: one of the nitrogen-free medium, and two of the medium supplemented with $NaNO_3$. The nitrogen-free series was incubated at 35°; one of the nitrate-containing series was incubated at 35°, and one at 25°. Algal growth became visible after incubation for 10–14 days; experience showed that the microflora did not change significantly upon longer incubation. Microscopic analysis of the dilution series were, accordingly, undertaken routinely after 14 days of incubation.

Analyses were performed as follows. Each dilution series was initially examined microscopically for the presence of visible algal growth; and the tube furnished with the smallest inoculum which showed growth was first subjected to microscopic examination. When the growth was uniform, a single wet mount was prepared; when dispersed patches of growth were evident, a wet mount was prepared from each patch. The wet mounts were examined with phase-contrast illumination, and the organisms observed were scored in the following categories:

(1) Blue-green algae: (a) unicellular; (b) filamentous, with heterocysts; (c) filamentous, without heterocysts; (d) branching.

(2) Eucaryotic algae: (a) green algae; (b) diatoms; (c) euglenoids.

Table 1. *Source materials used in enrichment experiments*

Water specimens		Soil and mud specimens	
W 1	Shallow courtyard pond, Berkeley, January sampling	S 1	Fresh moist soil under tree, Berkeley
W 1a	Same, May sampling	S 2	Fresh muddy soil, Berkeley
W 2	Shallow courtyard pond, contiguous to 1; January sampling	S 3	Fresh muddy soil, Berkeley
		S 4	Dried soil from rice paddy, Central Valley of California
W 2a	Same, March sampling	S 5	Mud from stagnant ditch, Berkeley
W 2b	Same, May sampling	S 6	Greenhouse soil, Berkeley
W 3	Small open pond, Healdsberg	S 7–S 9	Three different dried tropical soil samples, obtained from Ghana
W 4	Small open pond, Berkeley		
W 5	Freshwater lake, Berkeley		

Within each category, types which could be recognized as distinctly different from one another by virtue of size, form or pigmentation were separately scored. Multiplication by the dilution factor for the tube in question then yielded, for each algal type recorded, the minimal number present in the inoculum.

The tubes in the same series which had received larger inocula were subjected in turn to microscopic analysis, and only those algal types not previously observed were recorded; in each case, the minimal number in the inoculum was calculated. The cumulative data for the tubes in each dilution series were then summed and compared.

It should be specifically noted that the use of 10-fold dilution steps, as well as the fact that only one tube in a series was inoculated from each dilution, make the quantitative aspects of this study relatively imprecise; the true numbers of algae present in the materials examined were, no doubt, often considerably greater than the numbers recorded. However, our primary intention was not to make a precise ecological analysis, but rather to determine whether a satisfactory method for the differential enumeration and selective enrichment of blue-green algae could be developed.

RESULTS

Table 2 shows the outcome of a series of enrichment experiments made with water samples, in most of which no algae could be detected initially by direct microscopic examination. As shown by the data for the dilution series incubated at 25°, all contained appreciable numbers of eucaryotic algae, ranging from 200 to over 20,000/ml. Blue-green algae were also detected in most enrichments at 25°, the numbers ranging from 10 to 2200/ml. Elevation of the temperature of incubation to 35° produced a major change in the outcome of the enrichments. Eucaryotic algae were virtually

eliminated, a very small number of types (all unicellular green algae) developing in the tubes which received the largest inocula; in nearly every case the total number of blue-green algae recovered was appreciably higher than at 25°. The results obtained with sample W 2b are particularly noteworthy. This sample contained a large initial population of eucaryotic algae (over 12,000/ml.), and only 10 blue-green algae/ml. were detected in the dilution series incubated at 25°. Incubation at 35° decreased the count of eucaryotic algae to 1/ml., and revealed a minimal blue-green algal population of 710/ml., 70 times that detected at 25°. Clearly therefore the development of many blue-green algae had been prevented, under non-selective growth conditions, as a result of overgrowth by the large eucaryotic algal population also present in the material analysed.

Table 2. *The influence of temperature of incubation and nitrogen source on the recovery of blue-green and eucaryotic algae from samples of fresh water*

The figures represent minimal numbers/ml. in the sample, as inferred from microscopic analyses of enrichment cultures inoculated with serial dilutions of the material.

Temperature of incubation ...	25°		35°		35°	
Nitrogen source ...	NO_3^-		NO_3^-		N_2	
Type of algae ...	Blue-green	Eucaryotic	Blue-green	Eucaryotic	Blue-green	Eucaryotic
Sample						
W 1	1,220	3,300	1,340	10	1,000	0
W 1a	2,210	7,460	2,310	30	2,030	0
W 2	100	240	110	10	10	0
W 2a	40	220	600	2	132	0
W 2b	10	12,300	710	1	220	0
W 3	30	590	45	5	0	0
W 4	100	200	100	0	100	0
W 5	0	20,000	1	0	1	0

The further selection obtained by using incubation at 35° with a nitrogen-free medium is also shown in Table 2. Eucaryotic algae were totally eliminated; and the number of blue-green algae was typically decreased, though often only to a slight extent.

Quantitative data on enrichments with a series of soil specimens (Table 3) showed that cultivation at 35° was again highly effective in preventing the growth of eucaryotic algae, and in improving the recovery of blue-green algae from materials which contained a large initial eucaryotic algal population (e.g. samples S 7 and S 9).

Table 4 summarizes some qualitative aspects of these experiments. For several source materials, we have listed the total number of different types of blue-green and eucaryotic algae recovered in the parallel enrichment series. It is evident that, as a rule, enrichment at 35° increased the variety (as well as the absolute numbers) of blue-green algae which were detected in any given soil or water sample, and at the same time greatly decreased the variety of eucaryotic algae.

Table 5 shows the qualitative and quantitative effects of omitting a combined-nitrogen source on the outcome of some enrichments for blue-green algae at 35°. The omission of nitrate effectively abolished the development both of unicellular blue-green algae and of filamentous types which do not form heterocysts, and specifically favoured

the growth of some heterocyst-forming filamentous types. These experiments provide additional support for the conclusion that nitrogen fixation is characteristic of certain members of the Nostocaceae, but with other classes of blue-green algae is uncommon or absent.

Table 3. *Influence of temperature of incubation and nitrogen source on recovery of blue-green and eucaryotic algae from soil and mud samples*

The figures represent minimal numbers per gram in the sample, as inferred from microscopic analysis of enrichment cultures inoculated with serial dilutions of the material.

| Temperature of incubation ... | $25°$ | | $35°$ | | $35°$ | |
| Nitrogen source... | NO_3^- | | NO_3^- | | N_2 | |
Type of algae ...	Blue-green	Eucary-otic	Blue-green	Eucary-otic	Blue-green	Eucary-otic
Samples California soils						
S 1	0	10^5	0	0	0	0
S 2	0	10^4	0	0	0	0
S 3	0	10^5	0	0	0	0
S 4	2,000	4,000	3,000	0	3,000	0
S 5	1,100	10^5	1,100	0	1,000	0
S 6	1,000	4,100	2,000	0	100	0
Tropical soils						
S 7	1,000	31,400	11,500	2,000	13,300	0
S 8	2,100	320	5,090	3	370	0
S 9	3,000	4×10^4	14,200	0	2,000	0

Table 4. *The influence of the temperature of incubation and the nitrogen source on the number of types of blue-green and eucaryotic algae which can be detected by enrichment of water and soil samples*

| Temperature of incubation ... | $25°$ | | $35°$ | | $35°$ | |
| Nitrogen source ... | NO_3^- | | NO_3^- | | N_2 | |
Type of algae ...	Blue-green	Eucaryotic	Blue-green	Eucaryotic	Blue-green	Eucaryotic
Samples						
W 1	5	6	9	1	1	0
W 1a	6	21	7	4	7	0
W 2	2	4	2	1	1	0
W 2a	6	6	7	2	6	0
W 2b	1	15	9	1	4	0
W 3	1	25	2	1	4	0
W 4	2	1	1	0	1	0
W 5	0	2	1	0	1	0
S 6	1	5	2	0	1	0
S 7	2	9	7	2	7	0
S 8	5	5	14	3	5	0
S 9	3	4	7	0	2	0
Cumulative totals	34	103	68	15	40	0

We have experimented to a limited extent with other variations on these basal enrichment procedures. From a few mud and water samples, dilution series were prepared at $45°$, as well as at $35°$ and $25°$. No development of blue-green algae was ob-

served at 45°, a finding which concurred with our initial observation that very few strains isolated at temperatures below 30° are able to grow at 45°. Several parallel series of nitrogen-free enrichments were incubated at 25° and at 35°; the results, both qualitative and quantitative, were closely similar, except that growth was more rapid at the higher temperature.

Table 5. *The influence of the nitrogen source on blue-green algal development in enrichments at 35°*

Each figure represents the minimal number of cells of a given type of blue-green alga in the source material, as inferred from the examination of enrichment cultures inoculated with serial dilutions.

Sample	Nitrogen source	Type of blue-green algae — Unicellular	Filamentous with heterocysts	Filamentous without heterocysts
W 1	NO$_3^-$	10	1000, 100, 100, 10	100, 10, 10, 1
	N$_2$	0	1000	0
W 2a	NO$_3^-$	100, 100, 1	100, 100	100, 100
	N$_2$	0	100, 10, 10, 10, 1, 1	0
S 9	NO$_3^-$	1000	10,000, 1000	1000, 1000, 100, 10
	N$_2$	0	1000, 1000	0
S 8	NO$_3^-$	1000	10, 10, 10, 10, 10, 10	1000, 1000, 100, 10, 10, 10
	N$_2$	0	100, 100, 100, 10	10
S 4	NO$_3^-$	1000	1000	1000
	N$_2$	0	1000, 1000, 1000	0

DISCUSSION

A mineral medium which permits the enrichment of many different kinds of algae from soil and water when incubated at 25° becomes highly selective for blue-green algae when incubated at 35°. This fact shows that the temperature maxima of mesophilic blue-green algae are in general higher than those of most eucaryotic algae. There are undoubtedly exceptions to this generalization; for example, a temperature of 35° is too high to permit the growth of certain planktonic blue-green algae which contain gas vacuoles and which can be readily enriched at 25° (G. Cohen-Bazire, personal communication). Nevertheless, temperature selection offers a means for primary isolation of a wide range of blue-green algae, particularly from samples where they constitute a small minority of the total algal population. The utility of this technique might well be increased by varying other parameters, such as light intensity, pH value and nutritional factors.

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Carbon Dioxide as a Growth Factor for Mutants of *Escherichia coli*

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SUMMARY

This report shows that some auxotrophic mutants of *Escherichia coli* can grow on a minimal medium without growth factors when the gas phase is supplemented with carbon dioxide. Mutants which respond to CO_2 are called CO_2 mutants. The CO_2 mutants of *E. coli* resemble those already known in Neurospora. When the gas phase is not supplemented with CO_2 most of the mutants respond to other specific growth factors such as arginine, uracil, adenine, succinate or isoleucine + valine, depending upon the locus of their mutation. One mutant is an obligate CO_2 mutant. The CO_2 effects shown by these mutants are discussed in relation to the general problem cf CO_2 effects in micro-organisms.

INTRODUCTION

Carbon dioxide is necessary for the growth or the initiation of growth of bacteria and other micro-organisms (Valley & Rettger, 1927; Rockwell & Highberger, 1927). This is sometimes referred to as 'the CO_2 effect' (e.g. Wilson & Miles, 1964). CO_2 also has other interesting effects. For example, it causes morphogenetic changes in organisms as different as bacteria (Ivanovics, 1937), fungi (Bartnicki-Garcia & Nickerson, 1962) and coelenterates (Loomis, 1961). Recently, a new kind of CO_2 effect was discovered in which CO_2 acts as a growth factor for mutants of the mould Neurospora (Charles, 1962; Reissig & Nazario, 1962; Charles & Broadbent, 1964; Broadbent & Charles, 1965). The mutants require up to 30 % CO_2 (v/v) in the gas phase for growth to occur, whereas the wild type requires less CO_2 than is present in ordinary air (0·02–0·04 %). When these mutants are not supplied with supplementary CO_2 they behave as normal auxotrophic mutants, each requiring a particular growth factor such as arginine, adenine, uridine or succinate, depending upon the site of the mutation. Mutants which are stimulated by CO_2 are called CO_2 mutants (Charles & Broadbent, 1964). The way in which CO_2 stimulates the growth of the mutants has not been definitely established, and the mechanism may be different in different mutants. A problem arising from the research with the Neurospora mutants was whether the effects observed are peculiar to Neurospora, or whether they are of wider occurrence. This report shows that CO_2 mutants of the bacterium *Escherichia coli* occur. The bacterial mutants are described and compared with those of Neurospora. Apart from their intrinsic interest, CO_2 mutants may be useful because analysis of the mechanism of the CO_2 stimulation may contribute to the understanding of CO_2 effects in other organisms.

The following strains of *Escherichia coli* K 12 were used:

F- AB 1621 (*thi⁻, mtl⁻, xyl⁻, str', ara⁻, gal⁻, lac⁻*)

Hfr AB 2575 (*ilv⁻, str'*) which transfers genes in the order *O–ara, lac, gal, ...*

Hfr P 10 (*thr⁻,leu⁻,thi⁻,T₆', str'*) transferring genes in the order *O–thi–met–mtl...*

Hfr B 7 (*met⁻, str', T₆'*) transferring genes in the order *O–try–his...*

Strains AB 1621 and AB 2575 were kindly provided by Professor E. A. Adelberg, and strains P 10 and B 7 by R. Lavallé.

The minimal media used were those described by Vogel & Bonner (1956), and Davis & Mingioli (1950). Unless stated otherwise the minimal media contained glucose (0·5 % autoclaved separately) and were supplemented with thiamine (2 mg./l.) because the F⁻ strain requires thiamine. The medium was solidified when necessary with Difco Bacto agar (12 g./l.). The complete medium contained the following (g./l.:) tryptone (Oxoid), 10; yeast extract (Difco), 5; KH_2PO_4, 3; glucose, 5; agar, 15 (when required).

Cultures were grown and maintained on slopes of complete agar medium. The temperature of incubation was 37°. Strains to be used in mating experiments were streaked on plates of complete medium to give discrete colonies and stored at 4°. Single colonies were picked for the experiments; their phenotypes were checked before use.

Nutritional requirements of mutants were determined by the auxanographic method (Pontecorvo, 1949); about 3×10^6 bacteria/ml. were dispersed in the minimal agar medium.

When it was required to incubate cultures in a gas phase of air supplemented with CO_2 the procedure was as follows. Cultures in Petri dishes (lids removed) were stacked in 5 l. vacuum desiccators in layers of three with the agar facing downwards, each dish resting equally on 2 dishes below it; CO_2 was generated by the action of hydrochloric acid on marble chips in a Kipp apparatus, and allowed to enter each desiccator after the appropriate amount of air had been withdrawn. Removal of the lids of the Petri dishes was later found to be unnecessary. Equally good CO_2 effects were obtained when the bacteria were dispersed throughout the agar as when they were spread on the surface.

Most CO_2 mutants will grow without a supplement of CO_2 when other specific growth factors such as succinate or arginine are added to the minimal medium; they will be referred to as CO_2 mutants or as succinate, arginine, etc., mutants, as convenient.

The loci of the mutations were determined by the interrupted mating technique (Hayes, Jacob & Wollman, 1956) as follows. Overnight broth cultures of the strains to be mated were diluted 1/10 in fresh broth at 37° and 10 ml. volumes were incubated in a shaking water bath for about 1·5 hr; 0·5 ml. of the Hfr culture and 9·5 ml. of the F⁻ culture were then mixed in a 50 ml. Erlenmeyer flask and the mixture gently rocked in the water bath for the duration of the experiment. Samples (0·1 ml.) of the mating mixture were taken immediately after mixing and then at 5 min. intervals, diluted 100 times in minimal medium without glucose and agitated for 60 sec. by using a rotary mixer to stop the mating process. Samples (0·1 ml.) of the suspensions

were then plated in duplicate on selective media to determine the numbers of recombinants. Transduction experiments were done as described by Glansdorff (1965).

Isolation of CO_2 mutants

Experiments 1–4. Mutations were induced in *Escherichia coli* strain AB 1621 with ultraviolet radiation and the mutants enriched by penicillin treatment (Gorini & Kaufman, 1959). The procedure included a 16–18 hr period of intermediate incubation during which the bacteria multiplied, and their mutations became phenotypically expressed, before they were treated with penicillin. The medium used for this intermediate incubation was varied in Exp. 1–4 (see Table 1) to allow for the possibility that some media might select for or against some kinds of CO_2 mutants. For example, in Expt. 3, CO_2 was used to supplement the minimal medium by slowly bubbling a gas mixture composed of 70 % (v/v) air + 30 % (v/v) CO_2 through the incubating suspension. After penicillin treatment CO_2-requiring mutants were detected as follows. Suitable dilutions of the washed bacterial suspensions were spread on complete medium to give about 200 colonies per plate after incubation. After incubation for 24 hr the colonies on each plate were printed on to two plates of minimal medium by the replica plating technique (Lederberg & Lederberg, 1952). One of each pair of plates was incubated in ordinary air and the other in a gas phase of air + 20 % (v/v) CO_2. After incubation for 24–72 hr the pairs of plates were compared and colonies which had grown only in the presence of 20 % CO_2 were purified by streaking and tested further.

Experiment 5. Mutations were induced in *Escherichia coli* AB 1621 with *N*-methyl-*N*-nitroso-*N'*-nitroguanidine (MNNG) by using a modification of the method of Goodgal, Margolin & Sanderson (1964). Overnight growth in broth (5 ml.) was washed and resuspended in the same volume of minimal medium enriched with 2·5 % (v/v) complete medium. A sample, 0·1 or 0·2 ml., of an unsterilized solution of MNNG (4 mg./ml.) was added and the suspension incubated for 30–60 min. Complete medium (4·5 ml.) was added and incubation continued for about 2 hr. Suitable dilutions were then spread on plates of complete medium and incubated for 3–5 days in a gas phase of air + 20 % (v/v) CO_2. The colonies which grew on each plate were printed on to two plates of minimal medium and CO_2 mutants detected as in Expt. 1–4. Enrichment of mutants was not necessary.

RESULTS

Table 1 shows the results of the mutant isolation experiments. As described in Methods, various supplemented media were used for the period of intermediate incubation in the penicillin selection procedure. The results in Table 1 show that the kinds of mutants obtained depended upon the medium used for the period of intermediate incubation. Thus the only medium which gave a good yield of histidine mutants and isoleucine + valine mutants was minimal medium through which a mixture of 70 % (v/v) air + 30 % (v/v) CO_2 was slowly and continuously bubbled. Mutants requiring arginine, a pyrimidine, or arginine + a pyrimidine, were only obtained when the minimal medium was supplemented with arginine + uridine + adenine.

A gas phase of 80 % (v/v) air + 20 % (v/v) CO_2 was the most stimulatory for the CO_2 mutants of *Escherichia coli*. Higher proportions of CO_2 were not tested because they inhibited the parental strain. All but one of the mutants responded to other growth factors when a supplement of CO_2 was not provided. The mutants were of at

least 10 kinds, distinguishable by their responses to growth factors other than CO_2. As with Neurospora the response of some of the E. coli mutants to CO_2 was annulled by certain metabolites. Mutants responding to a particular growth factor showed the same pattern of response to precursors of that growth factor (when available), suggesting that their mutations were at the same locus. One mutant of each kind was chosen for genetical studies. Nutritional and genetical data are shown in Table 2. Because nutritional experiments were done on solid media, quantitative data for the stimulatory effect of CO_2 and other growth factors are not available, but Table 2 shows the time taken by the various mutants when incubated in 20 % CO_2 to equal the growth of the parental strain after 18 hr. The different kinds of mutants will now be considered individually.

Table 1. *Escherichia coli: CO_2 mutants obtained in mutant isolation experiments*

Experiment no.	Mutagenic treatment	Supplement added to minimal medium for the period of intermediate cultivation	Kinds of CO_2 mutants obtained*	No. of mutants of each kind obtained
1		Nutrient broth (5 % v/v)	Purine	4
			Succinate	2
			Isoleucine + valine	1
2	U.v. irradiation followed by penicillin	Yeast extract + yeast nucleic acid + tryptone (100 mg./l. of each)	Purine	1
			Succinate	1
			Isoleucine + valine	1
3	selection procedure	No supplement, but medium was gassed with a mixture of 30 % CO_2 + 70 % air	Histidine	5
			Succinate	1
			Isoleucine + valine	11
4		Arginine + adenine + uridine (10 mg./l. of each)	Purine	7
			Arginine	1
			Pyrimidine	1
			Arginine + pyrimidine	1
5	MNNG† without penicillin selection	Not applicable	Purine	1
			Succinate	2
			Isoleucine + valine	4
			Methionine,	1
			Obligate CO_2 mutant	1

* The mutants are classified according to their nutritional requirements when incubated in ordinary air.
† MNNG = N-methyl-N-nitroso-N'-nitroguanidine.

Purine mutants

When the gas phase was not supplemented with carbon dioxide the purine mutants were stimulated by adenine, guanine, hypoxanthine or xanthine. This showed that they have a defect in one of the enzymes synthesizing the purine ring. When grown in minimal medium in the presence of 20 % CO_2, or in complete medium, for 3 days, the mutants produced a yellowish brown coloration. The crude culture filtrate of a mutant grown for 24 hr in minimal medium supplemented with a growth-limiting concentration of hypoxanthine (5 mg./l.)s howed a strong ultraviolet absorption maximum at 300 mμ which was not shown by a similar filtrate of the culture in which the parental strain had grown. This pigment is probably 5-aminoimidazole ribonucleotide (AIR).

or a derivative, which has an absorption maximum at 305 mμ (Love & Gots, 1955). Interrupted mating experiments showed that the mutations mapped between the *lac* and *gal* loci in the region of the *ade f* locus (Stouthamer, de Haan & Nijkamp, 1965). Gots (1965) showed that the enzyme specified by this locus catalyses the conversion of AIR to CAIR (5-aminoimidazole-4-carboxylic acid ribonucleotide). This is the only CO_2-incorporation reaction in purine biosynthesis, and the explanation of the CO_2 effect shown by these mutants may be that the carboxylating enzyme in the mutants requires a higher concentration of CO_2 for activity than does this enzyme in the parental strain. The purine mutants were inhibited by aspartate when growing in CO_2, but they were not inhibited by aspartate when any one of the stimulatory purines was present. Some of the Neurospora CO_2 mutants also respond to purines, excrete a similar pigment, and map at a locus which may specify the carboxylating enzyme (Broadbent & Charles, 1965).

Table 2. *Escherichia coli: some characteristics of CO_2 mutants*

Designation of mutants selected for study	Locus of the mutation	Time (hr) required in 20 % (v/v) CO_2 to equal the growth produced by the parent strain after 18 hr	Nutritional requirement when incubated in ordinary air without a supplement of CO_2	Growth in CO_2 inhibited by
A 17	*pur-E (ade-f)*	18	Purines	—
S 19	*suc*	18–36*	Succinate, 2-oxoglutarate, lysine + methionine	Aspartate
L 23	Not known	18–48*	Isoleucine + valine, isoleucine + α-keto-isovalerate	Aspartate, purines
H 13	Not known	36 (18 hr in presence of nicotinic acid)	Histidine	Aspartate, purines
M 12	*pyr-F*	36	Pyrimidines	—
B 22	*cap*	48	Arginine or citrulline	Pyrimidines
AP 21	*cap*	18	Arginine or citrulline + a pyrimidine	Pyrimidines
45-1	Not determined	18	Methionine or cysteine	Aspartate
'Unknown'	Not determined	36	Unknown	Purines, histidine, tyrosine

* Different mutants grow at different rates.

Isoleucine + valine mutants

The isoleucine + valine CO_2 mutants are especially interesting because a CO_2-fixation reaction does not occur in the biosyntheses of isoleucine and valine. The only reaction in the biosynthetic sequences in which CO_2 takes part is a decarboxylation. The genetics and biochemistry of isoleucine + valine mutants have been studied in detail, especially by Ramakrishnan & Adelberg (1964, 1965a, b). The double amino acid requirement arises because the same four enzymes catalyse the last four reactions in the biosyntheses of both isoleucine and valine. The four genes specifying these enzymes are closely linked and are probably adjacent on the bacterial genome. The CO_2 mutants of *Escherichia coli* map in this region in interrupted mating experiments. It was not possible to determine by nutritional experiments which reaction is defective

in the mutants because most of the intermediates are not available. However, the last enzyme of the sequence must be functional because the mutants grow when supplied with isoleucine + α-keto-isovalerate, the latter substance being the immediate precursor of valine. The growth response to CO_2 was inhibited by purines and aspartate, which did not inhibit the parental strain and did not inhibit the mutant when it was growing in the presence of isoleucine + valine. In Neurospora, CO_2 mutants responding to isoleucine + valine were not obtained, despite several large mutant-isolation experiments.

Succinate mutants

Six *Escherichia coli* mutants required succinate when the gas phase was not supplemented with CO_2. These mutants have not all been studied in detail, and they may differ amongst themselves. The important facts are as follows. In the presence of glucose the mutants responded to CO_2, succinate or 2-oxoglutarate. When glucose was omitted from the medium the mutants did not use succinate or 2-oxoglutarate as carbon and energy sources, but the parental strain did. The mutants did not grow when other Krebs-cycle intermediates, or acetate, were supplied as carbon sources, even when succinate (50 mg./l.) was present, or when the gas phase contained 20 % (v/v) CO_2, whereas the parental strain used several of the Krebs-cycle intermediates. These observations show that neither succinate nor CO_2 substituted fully for the product of the defective enzyme(s).

The mutants also grew in minimal medium when supplied with lysine + methionine, instead of succinate or CO_2, whether or not glucose was present. This suggested that the mutants may be defective in the synthesis of succinyl-CoA, which is an intermediate in the biosynthesis of lysine and methionine (Gilvarg, 1957; Rowbury & Woods, 1964). The fact that succinate was stimulatory only in the presence of glucose may indicate that the mutants required glucose to make acetyl-CoA, which might be used in turn for converting succinate to succinyl-CoA.

Succinate mutants similar to those described here have been isolated by other workers, who did not examine the effect of CO_2 on their mutants. Davis *et al.* (1959) described a mutant of *Escherichia coli* which required succinate or lysine + methionine + threonine for growth. It differed from our mutants in requiring threonine and in failing to respond to 2-oxoglutarate. Because it responded to succinate but not to 2-oxoglutarate they suggested that the mutant might be blocked in the conversion of 2-oxoglutarate to succinate. Davis *et al.* (1959) showed that their mutant grew on minimal medium, and did not require succinate or lysine + methionine + threonine when it was incubated anaerobically in glucose minimal medium. They concluded that in the parental strain succinate could be formed from glucose anaerobically by reduction of 4-carbon acids, but aerobically only by oxidation of 2-oxoglutarate. Stouthamer (1967) showed that succinate mutants of an Aerobacter species also grew anaerobically in minimal medium without succinate. The succinate mutants described in the present paper show the same effect. Interpretation of the growth-promoting effect of anaerobic conditions requires caution because some succinate mutants may be CO_2 mutants and growth on minimal medium in anaerobic jars may be a response to CO_2, either added deliberately as a mixture of 95 % (v/v) H_2 + 5 % (v/v) CO_2 (Stouthamer, 1967) or produced by the bacterial cultures themselves. The fact that the gas phase of the cultures was O_2-free may be irrelevant. Experiments which we have done to

determine whether CO$_2$ is responsible for the stimulatory effect of anaerobic conditions have been inconclusive because of technical difficulties.

Stouthamer (1967) found that the Aerobacter mutants were of 2 kinds, one able and the other unable to use succinate as a source of carbon and energy. He concluded that one kind of mutant was blocked in the decarboxylation of 2-oxoglutarate, but that the nature of the lesion in the other kind was unknown. Back & Westaway (1962) also described a mutant of *Escherichia coli* which required lysine + methionine, but they did not state whether it responded to succinate. Various characteristics of the mutants of Stouthamer (1967) and Back & Westaway (1962) are indicative of CO$_2$ effects. Thus the latter authors say that although their mutant required lysine + methionine for growth, it actually synthesized considerably more lysine and methionine than was available from the medium. Stouthamer (1967) found that growing bacteria oxidized glucose completely, but that washed suspensions only partially oxidized glucose. He also found that the greater the number of bacteria in the inoculum then the shorter the lag period before growth began on medium containing lysine + methionine. All these effects may be due to accumulation of CO$_2$ in the growth medium by the bacteria with a resulting stimulation of metabolic activity and growth.

The mechanism by which CO$_2$ stimulates the mutants is not understood. A CO$_2$-fixation step is involved in several reactions leading to the formation of compounds having 4 carbon atoms, and it is possible that high concentrations of CO$_2$ stimulate one of these reactions or bring an alternative one into operation. If this be the case, however, malate and oxalacetate should be stimulatory, because they are products of some of the CO$_2$-fixation reactions.

On the linkage map of *Escherichia coli* (Taylor & Thoman, 1964) the succinate and lysine + methionine loci are shown separately, but close together. The one mutant we have tested also maps in this region, and it seems likely that the succinate and lysine + methionine loci are identical.

Arginine and pyrimidine mutants

The three mutants of *Escherichia coli* to be described in this section were isolated in penicillin selection experiments in which minimal medium supplemented with arginine, uridine and adenine (10 mg./l. each) was used for the period of intermediate incubation (see Methods). Two of the mutants (AP 21, B 22) have defects in the biosynthesis of carbamoyl phosphate (CAP) which is a precursor of arginine and pyrimidines. In *E. coli*, CAP for the biosynthesis of these substances is formed by a CO$_2$-incorporation reaction catalysed by the enzyme glutamino-carbamoyl phosphate synthetase (GCPS-ase; Piérard, Glansdorff, Mergeay & Wiame, 1965). The synthesis of this enzyme is cumulatively repressed by arginine and pyrimidines and its catalytic activity is subject to feedback inhibition by uridine-5'-monophosphate (UMP). Ornithine counteracts the inhibition by UMP, a mechanism which ensures that CAP is available for arginine biosynthesis when required (Piérard, 1966). The enzyme is specified by the *cap* locus (Piérard *et al.* 1965), which is the same as the *pyr* A locus of Jacob & Wollman (1961) and Beckwith, Pardee, Austrian & Jacob (1962).

The *Escherichia coli* mutant designated AP 21 required arginine + a pyrimidine for growth in the absence of supplementary CO$_2$. Arginine was stimulatory in the absence of a pyrimidine when incubation was prolonged for 2–3 days, but growth was never as great as with arginine + a pyrimidine. Arginine was replaceable by citrulline. Pyrimi-

dines which stimulated the mutant were uracil, uridine, cytosine and cytidine; thymidine was weakly stimulatory; orotic acid and thymine were inactive. The pyrimidines were not stimulatory unless arginine or citrulline was also present. The growth rate of the mutant in minimal medium when the gas phase was supplemented with 20 % (v/v) CO_2 was as good as that of the parental strain.

Escherichia coli mutant B 22 grew in minimal medium when supplied with arginine or citrulline without a pyrimidine, but growth was better when a pyrimidine was also provided. The mutant responded slowly to CO_2, 2 days of incubation being necessary for a growth response. Mutant B 22 therefore differed from mutant AP 21 in its rapid response to arginine (or citrulline) and its slow response to 20 % (v/v) CO_2.

The growth response of mutants AP 21 and B 22 to 20 % (v/v) CO_2 was inhibited by uracil, uridine, cytosine, cytidine or thymidine. The inhibitions were annulled by arginine or citrulline. The mutants partially resemble the uracil-inhibited mutants U 28 of Gorini & Kalman (1963) and *ura-s* of Piérard *et al.* (1965) which grow in minimal medium and show inhibition by uracil and annulment of this inhibition by arginine.

Transduction experiments showed that the mutations in *Escherichia coli* mutants AP 21 and B 22 were at the *cap* locus which specifies the only CO_2-incorporation enzyme (GCPSase) in arginine and pyrimidine biosynthesis. Enzymological studies showed that GCPSase activity was present in the mutants but at a lower concentration than in the parental strain (G. A. Roberts, unpublished). The fact that both mutants grew without a pyrimidine when supplied with arginine suggests that CAP was available for pyrimidine synthesis but not for arginine synthesis. Perhaps the CAP-synthesizing enzyme in the mutants was altered at a regulatory site, so that ornithine no longer counteracted the feedback inhibition by pyrimidines, and arginine synthesis consequently failed through lack of CAP. An explanation of this kind was proposed by Piérard (1966) to explain the uracil inhibition of *ura-s*. Carbon dioxide may stimulate growth in minimal medium by increasing CAP synthesis, e.g. by a mass action effect or by antagonizing the pyrimidine feedback inhibition, so that sufficient CAP is available for arginine synthesis. A second and quite different possibility is that the increased CO_2 concentration may bring into action another CAP-synthesizing enzyme such as 'carbamyl phosphokinase', which is present in *E. coli* but does not provide CAP for arginine and pyrimidine synthesis under the usual cultural conditions (Thorne & Jones, 1963; Yashphe & Gorini, 1965). This kind of explanation does not account for the pyrimidine inhibitions unless more assumptions are made.

The *Escherichia coli* mutant M 12 was stimulated by the pyrimidines uracil, uridine, cytosine or cytidine. The growth response to CO_2 was slow and was not inhibited by any common metabolite. Arginine and citrulline were slightly stimulatory to growth in 20 % (v/v) CO_2. Interrupted mating experiments showed that the mutation was in the region of the *pyr C, D* and *F* loci (Beckwith *et al.* 1962; Taylor *et al.* 1964). *Pyr C* and *D* are close together and some distance from *pyr F*, which is nearer to the *try* locus (Taylor 1965; R. Lavallé, private communication). Preliminary experiments suggested that the M 12 mutation may be at the *pyr F* locus which specifies the enzyme orotidylic acid decarboxylase (Beckwith *et al.* 1962; Taylor & Thoman, 1964). This mutation was not at the *cap* locus, which specifies the only CO_2-incorporation enzyme in pyrimidine (and arginine) biosynthesis.

Histidine mutants

The histidine/CO_2 mutants of *Escherichia coli* showed a better response to histidine than to 20% (v/v) CO_2, suggesting either that adaptation was necessary for utilization of CO_2, or that CO_2 only partially compensated for the defects in the mutants. In the presence of supplementary CO_2, nicotinic acid was stimulatory but it was not stimulatory when the gas phase was ordinary air. This relationship between nicotinic acid and histidine biosynthesis requires further investigation. When growing in CO_2 these mutants were completely inhibited by purines. The mutation has not been mapped, and it is possible that it may map outside the *his* operon. A CO_2-fixation reaction does not occur in the biosynthesis of histidine from its precursor adenosine triphosphate (ATP) so that if CO_2 acts by stimulating a CO_2-incorporation enzyme it must act on the enzyme catalysing the formation of CAIR from AIR, in the biosynthesis of ATP. But the mutation did not map at the locus which specifies this enzyme, and it seems unlikely that a defect in this enzyme would cause a requirement for histidine unless there are unexpected complexities in purine and histidine biosynthesis. Histidine/CO_2 mutants are not known in Neurospora.

Methionine mutant

The methionine/CO_2 mutant of *Escherichia coli* responded vigorously to methionine and more weakly to cysteine. It gave a good response to CO_2 within 24 hr. When growing with CO_2 the mutant was partially inhibited by aspartate and completely inhibited by serine; the parental strain was also inhibited by serine, but not as strongly as was the mutant. Methionine/CO_2 mutants are also known in Neurospora. The ability of CO_2 to substitute for methionine in these mutants is not understood.

An obligate CO₂ mutant

One mutant of *Escherichia coli* was an obligate CO_2 mutant in that it did not grow in minimal or complete medium except when the gas phase was supplemented with CO_2. In the presence of 20% (v/v) CO_2 colonies formed on complete medium within 24 hr, but on minimal medium colonies took 48 hr to form. This showed that CO_2 itself did not completely compensate for the defect in the mutant. Growth of the mutant in 20% (v/v) CO_2 was inhibited by purines and tyrosine. In some experiments the inhibitions were complete but in others they were incomplete. The reason for this variation from experiment to experiment is not known.

Obligate CO_2 mutants are also known in Neurospora and some are similarly inhibited by purines (Broadbent, 1965; A. Vivian, personal communication.)

DISCUSSION

This investigation shows that CO_2 mutants occur in *Escherichia coli* as well as in Neurospora. The phenomena which they reveal are therefore likely to be of general occurrence. The parental strains of Neurospora and *Escherichia coli* from which the mutants have been isolated differ in their CO_2 requirements. The Neurospora strains grow when incubated in a desiccator containing potassium hydroxide (100 ml., 25%, w/v) but the Escherichia strains do not, showing that Neurospora requires a much lower concentration of CO_2 than Escherichia. A second difference is that growth

of Neurospora is not inhibited when the gas phase contains 30 % (v/v) CO_2, whereas Escherichia begins to show inhibition when the gas phase contains more than 20 % (v/v) CO_2. The differences between the parental strains are reflected in the CO_2 mutants. Neurospora CO_2 mutants do not grow when incubated in minimal medium in the presence of potassium hydroxide, but they grow when the gas phase contains 30 % (v/v) CO_2; when incubated in ordinary air some of the CO_2 mutants grow fairly quickly, probably because of the CO_2 in the environment and because of accumulation of CO_2 by the conidia. In Escherichia, CO_2 mutants do not grow when the gas phase is ordinary air and they grow when the gas phase contains 20 % (v/v) CO_2. The fact that some mutants were obtained only once shows that the samples of mutants isolated were small, and it is likely that more kinds of CO_2 mutants await discovery in both Escherichia and Neurospora. This may explain why the patterns of mutants from the two organisms do not match completely, although there is no reason to expect that they should.

Arginine, purine, pyrimidine and succinate mutants occur in both organisms. The mechanism of the CO_2 stimulation may be fairly easily understood in some of these CO_2 mutants because the relevant biosynthetic pathways all have CO_2-incorporation reactions and some of the mutations which have been mapped fall at the loci which specify the appropriate CO_2-incorporation enzymes. An unexplained fact is that CO_2 mutants occur at three arginine loci in Neurospora. Whether CO_2 mutants occur at several arginine loci in Escherichia remains to be investigated. Mutation at the *arg-11* locus in Neurospora causes simultaneous requirement for arginine, a pyrimidine, a purine and possibly succinate (Newmeyer, 1964; Broadbent & Charles, 1965). The function of this gene is unknown. The facts that the two known *arg-11* mutants are both CO_2 mutants and that CO_2 completely satisfies or annuls all the requirements suggest that the gene may be concerned with a process providing CO_2 for arginine, purine, pyrimidine and succinate biosyntheses. Mutants similar to *arg-11* mutants have not been obtained in Escherichia, but even in Neurospora mutants similar to the *arg-11* mutants were not obtained in CO_2-mutant isolation experiments.

With Escherichia we have the best example of a mutant which is completely dependent on a supplement of CO_2 and unable to use any other growth factor within a reasonable incubation period. The mutant does not grow on complete medium except when extra CO_2 is added to the gas phase. Its requirements may be complex because it responds better to CO_2 on complete medium that on minimal medium. The mutant may have a defect in fatty-acid metabolism, because a small proportion of the bacteria respond to oleic acid and Tween 80 after prolonged incubation.

Isoleucine + valine mutants were obtained with Escherichia but not Neurospora. They are of special interest because there is no CO_2-incorporation reaction in the biosyntheses of isoleucine and valine; this excludes certain proposed explanations of how CO_2 may stimulate mutants.

Tryptophan and acetate mutants are so far known only from Neurospora; the nature of their enzymic deficiencies is not known.

CO_2 mutants which require succinate, or lysine + methionine, when not provided with supplementary CO_2 have been discussed in the Results section. An interesting question arising from the discovery that succinate mutants are CO_2 mutants is whether the reported ability of succinate mutants to grow anaerobically in minimal medium without succinate or lysine + methionine (Davis *et al.* 1959; Stouthamer,

1967), may be due to stimulatory concentrations of CO_2 in the anaerobic jars, rather than to absence of O_2.

The interest of CO_2 mutants lies chiefly in their relevance to problems of nutrition and metabolism. It has long been known that some micro-organisms, and probably all, require CO_2 for initiation of growth (Valley & Rettger, 1927; Rockwell & Highberger, 1927; Rahn, 1941). This phenomenon is often referred to as 'the CO_2 effect'. CO_2 mutants are of special interest in relation to the problems posed by this and other effects of CO_2. When it was realized that generation of a 4-carbon compound from a 3-carbon compound by a CO_2-fixation reaction was necessary for continuous operation of the Krebs cycle, it seemed that the explanation of 'the CO_2 effect' was at hand (Krebs, 1941). However, experiments on nutrition in which attempts were made to replace CO_2 with glutamate, aspartate or Krebs cycle intermediates showed that these substances only partly substituted for CO_2, if at all; and the more stringent the removal of CO_2, or the smaller the inoculum, then the more nutritionally demanding the bacteria became or the longer the lag period before they grew (Lwoff & Monod, 1946, 1947; Wong & Ajl, 1953). With the discovery of CO_2-fixation reactions in other essential processes such as the biosyntheses of arginine, pyrimidines and purines one might have expected the CO_2 effect to be fully explained, but experiments show that in some bacteria it is not possible to replace CO_2 with other growth factors, and in other bacteria growth factors which replace CO_2 do not have a CO_2-fixation reaction in their path of biosynthesis. Other points of interest are: (1) CO_2 requirements are not always satisfied by bicarbonates and carbonates; (2) newly isolated pathogenic bacteria may require relatively high concentrations of CO_2 for growth, which may lessen after serial transfer; (3) effects of CO_2 on morphogenesis are known, for example, high CO_2 concentrations cause some filamentous fungi to adopt a yeast-like mode of growth (Bartnicki-Garcia & Nickerson, 1962), and bring about the development of sex organs in the coelenterate Hydra (Loomis, 1961).

It is sometimes implied that CO_2 is required for the initiation rather than the continuation of bacterial growth. It seems more likely that CO_2 is required both for initiation and growth, and that when growth has started the bacteria may generate far more CO_2 than they use so that it is impossible to demonstrate a net requirement for CO_2. Whether CO_2 must be absorbed from outside the cell during growth or whether intracellular CO_2 may satisfy all the requirements is unknown. It may be that intracellular and extracellular CO_2 are freely exchangeable, in which case the distinction between intracellular and extracellular CO_2 would be false. This possibility is worth investigation because it would help to explain some aspects cf CO_2 effects.

All the problems encountered in the study of 'the CO_2 effect' are encountered in the study of CO_2 mutants; thus bicarbonates and carbonates do not replace CO_2. It is uncertain whether CO_2 is required for initiation and/or continuation of growth. Inoculum size and lag effects are common and some mutants grow without a supplement of CO_2 or growth factors when the inoculum is concentrated or incubation is prolonged. Some mutants do not respond to growth factors other than CO_2. Most interesting of all, some CO_2 mutants respond in the absence of CO_2 from the gas phase to growth factors which do not have CO_2-incorporation reactions in their biosyntheses. It seems likely, for all these reasons, that CO_2 mutants mimic some of the CO_2 effects shown by different bacterial species. CO_2 mutants may be the most satisfactory experimental systems for analysing CO_2 effects because they show well-

defined CO_2 effects expressed against a common genetic background, and because the biochemical problem is considerably simplified, since each CO_2 effect is probably the result of a change in a single enzyme as compared with the parental strain which serves as a control or reference. By contrast, the CO_2 effects shown by wild-type organisms may result from peculiarities in several enzymes and there will be no reference strain available to show which enzymic characteristics are responsible for the CO_2 effect. That mutants may make other contributions to the understanding of CO_2 effects is shown by a recent observation that some mutant strains of Escherichia and Neurospora are inhibited rather than stimulated by CO_2; this raises the possibility that some CO_2 effects may result from inhibition rather than stimulation of enzymic reactions by CO_2 (Roberts & Charles, unpublished).

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Catabolite Repression and the Induction of Amidase Synthesis by *Pseudomonas aeruginosa* 8602 in Continuous Culture

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SUMMARY

Pseudomonas aeruginosa 8602 was grown in continuous culture under steady-state conditions in a carbon-limited medium containing either 20 mM-acetamide or 20 mM-acetamide + 10 mM-succinate. The amidase specific activity was measured at various dilution rates and found to have a sharp peak at a dilution rate of 0·30–0·35 hr^{-1}. Fully constitutive mutants (C 11 and L 9) gave curves for amidase activity with the highest values at very low dilution rates (0·05–0·10 hr^{-1}) and these decreased as the dilution rate increased. A semi-constitutive mutant (C 17) gave a curve intermediate between that of the wild-type strain and the fully constitutive mutant (C 11). Mutants with decreased sensitivity to catabolite repression by succinate gave curves which declined less steeply at the higher dilution rates. Mutant L 9, a fully constitutive mutant with decreased sensitivity to catabolite repression, had higher specific activities than mutant C 11 at the equivalent dilution rates. Mutant L 11, an inducible mutant with decreased catabolite repressibility, had higher amidase specific activities at high dilution rates than the wild-type inducible strain. It is concluded that in continuous culture under steady-state conditions the specific activity of the inducible amidase of *P. aeruginosa* is determined by the balance between induction and catabolite repression and that catabolite repression is directly related to the growth rate of the culture.

INTRODUCTION

Many inducible enzymes are subject to repression by glucose or other cell metabolites, a process termed catabolite repression by Magasanik (1961). This allows an additional control of the synthesis of such an inducible enzyme, since during growth it will be subject to induction by its substrate and repression by the product or products of catabolic pathways. Almost all the studies on catabolite repression of inducible enzymes have been made in batch culture, usually with exponentially growing bacteria synthesizing the enzyme under conditions of gratuity in the presence of non-substrate inducers.

McFall & Mandelstam (1963) examined catabolite repression of three inducible enzymes produced by *Escherichia coli* (β-galactosidase, serine deaminase, tryptophanase) in continuous culture, but the results were mainly used to confirm those previously obtained in batch culture. Gorini (1960), with an arginine-requiring auxotroph of *E. coli* strain w, showed that the extent of repression of the synthesis of

ornithine transcarbamylase by arginine was dependent on the flow rate of the medium. He also found that repression by arginine could be relieved by ornithine and the greater the repression the more ornithine was required to overcome it. Continuous culture experiments on enzyme regulation have been made by Hamlin, Ng & Dawes (1967) with *Pseudomonas aeruginosa* 2 F 32. Hamilton & Dawes (1959) had previously shown that this organism exhibits an unusual diauxie in that when inoculated into glucose + citrate medium the citrate is utilized preferentially to glucose until the citrate concentration has reached a low value. The extent of induction of glucose-metabolizing enzymes in the presence of a constant citrate concentration is directly related to the concentration of exogenous glucose. Ng & Dawes (1967) suggest that there is dual control of the phosphorylative enzymes of glucose metabolism with glucose serving as the inducer and citrate as the precursor of the repressing catabolites.

Pseudomonas aeruginosa 8602 is induced to form an aliphatic amidase (acylamide amidohydrolase, EC 3.5.1.4) during growth in the presence of the substrate acetamide or non-substrate inducers, e.g. *N*-acetylacetamide. This enzyme is subject to catabolite repression and it has been found that succinate is a very effective repressor of amidase synthesis by both inducible and constitutive strains growing in pyruvate medium (Brammar, Clarke & Skinner, 1967). We found previously that when the ingoing medium for a continuous culture growing in a steady state on limiting succinate was changed to succinate + acetamide, the extent of catabolite repression of amidase synthesis was dependent on the dilution rate of the culture (Boddy, Clarke, Houldsworth & Lilly, 1967). At low dilution rates amidase synthesis started after little or no lag, but at a high dilution rate, $D = 0.76$ hr^{-1}, the lag lasted for more than 4 hr after the change in the ingoing medium. We observed that the amidase specific activities of the cultures when they had reached the new steady state of growth on succinate + acetamide also appeared to be dependent on the dilution rate. Amidase synthesis by *P. aeruginosa* 8602 has now been studied under steady-state conditions at various dilution rates by using the wild-type strain, fully constitutive and semi-constitutive mutants, and mutants with altered susceptibility to catabolite repression.

METHODS

Organisms. The strains used were *Pseudomonas aeruginosa* 8602 (wild type) and mutants isolated from it by the techniques described by Brammar *et al.* (1967). These are listed in Table 1. All the strains were made resistant to the lysogenic pseudomonad phage Ps 1. The bacteria were maintained on slopes of Lemco agar, subcultured weekly and stored at 4°.

Media. The minimal salts medium contained (g./l.): K$_2$HPO$_4$, 12.5; KH$_2$PO$_4$, 3.8; (NH$_4$)$_2$SO$_4$, 1.0; MgSO$_4$.7H$_2$O, 0.1; trace element solution 5 ml. (Kelly & Clarke, 1962). This medium was used to prepare succinate + formamide agar (S/F plates) and succinate + lactamide agar (S/L plates) (Brammar *et al.* 1967). When required, sodium pyruvate and sodium succinate were added to the medium before autoclaving. Acetamide and *N*-acetylacetamide solutions were sterilized by membrane filtration and added aseptically to the autoclaved medium.

Continuous culture equipment. An inoculum (70 ml.) grown on succinate + acetamide minimal salts medium, or an acetamide minimal salts medium, was blown from the seed vessel into a 5 l. vessel containing 3 l. of the growth medium at a controlled

temperature of 37°. The pH value of the medium was controlled at pH 7·2 by the addition of 2 M-HCl. Air was supplied through the bottom of the vessel at 1 l./min. Growth medium was fed from a 20 l. aspirator into the culture vessel by a peristaltic pump. Medium left the vessel by an overflow pipe inserted through the bottom of the vessel. Samples (about 3 ml.) were withdrawn through a narrow-bore tube leading to a collecting bottle. Each sample was diluted, part used for the determination of the bacterial concentration, and the remainder cooled rapidly and assayed for amidase within a few minutes.

Dry weight of bacteria. The optical extinctions of diluted bacterial suspensions were measured at 670 mμ with a Unicam SP 600 spectrophotometer, and dry weights of bacteria calculated by using the appropriate conversion factor for each dilution rate.

Enzyme assay. Amidase was measured by the hydroxamate method for transferase activity described by Brammar & Clarke (1964). Except in experiments with *N*-acetyl-acetamide as inducer, the sample was diluted several times before assaying 0·1 ml. samples at 37° for 5 min. The amidase specific activity of each sample was calculated as μmoles acethydroxamate formed/mg. dry wt bacteria/min.

Steady-state conditions. The flow rate of the culture medium was adjusted to the required value and the culture allowed to grow for about 24 hr at the new growth rate, to reach steady-state conditions before sampling. Amidase specific activities of bacteria growing under steady-state conditions at each dilution rate were determined by taking the mean of eight consecutive readings made at 15 min. intervals.

RESULTS

Amidase activities of wild-type bacteria growing under steady-state conditions

Amidase specific activities of cultures of wild-type *Pseudomonas aeruginosa* 8602 growing in continuous culture were determined at dilution rates from 0·05 to 0·60 hr^{-1}. Figure 1 compares the results obtained with cultures growing on acetamide (20 mM) as the sole carbon source, with cultures growing on a mixture of acetamide (20 mM) + succinate (10 mM). In both cases growth was carbon-limited and excess nitrogen was present as ammonium salts. The amidase specific activities of the bacteria increased as the dilution rate was raised, reaching a maximum activity of about 120 at a dilution rate of 0·30–0·35 hr^{-1}. The slopes of the curves were similar at the lower dilution rates for both media. At higher dilution rates the specific activity decreased but in the medium containing succinate + acetamide the amidase specific activity decreased more rapidly until at about $D = 0.60$ hr^{-1} it was about one-third of that in the medium containing acetamide alone. The values given in Fig. 1 were obtained in random order; when amidase activities were determined on more than one occasion no difference was detected whether the new dilution rate had been approached from a previously higher or lower dilution rate. The similarity of the two curves suggested that essentially similar control mechanisms were operating in both media, but since the succinate + acetamide medium produced more dramatic changes in amidase activity at slightly lower dilution rates it was decided to use this medium for the experiments with the amidase mutants.

Amidase activities of constitutive mutants growing in continuous culture

Pseudomonas aeruginosa 8602 mutant C 11 is a magno-constitutive non-inducible strain (Brammar *et al.* 1967). It is as sensitive as the wild type to catabolite repression by succinate (10 mM) in pyruvate medium in batch culture (Table 1). Figure 2 shows that when mutant C 11 was grown in continuous culture in the succinate + acetamide medium the highest specific activity was produced at the lowest dilution rate tested (D = 0·06) and decreased to a value of 10–15 at a dilution rate of 0·42 hr^{-1}. There was no sharp peak of maximum amidase activity and the curve obtained resembled the falling side of the wild-type curve displaced to a lower dilution rate.
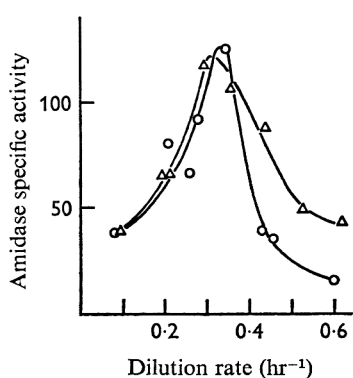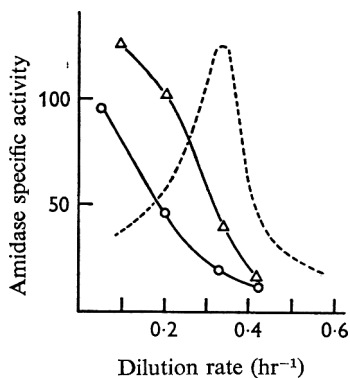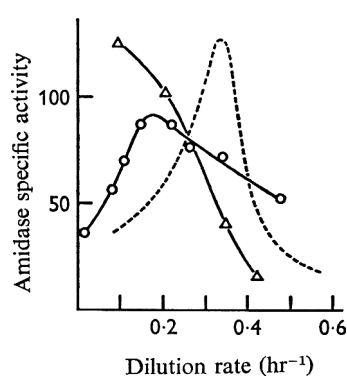
Fig. 1   Fig. 2   Fig. 3

Fig. 1. *Pseudomonas aeruginosa* 8602 wild type, grown in continuous culture under steady-state conditions. Amidase activities of cultures grown at various dilution rates. O——O, Minimal salts medium containing 10 mM-succinate + 20 mM-acetamide; △——△, minimal salts medium containing 20 mM-acetamide.

Fig. 2. The amidase specific activities of fully constitutive mutants of *Pseudomonas aeruginosa* 8602 grown in continuous culture under steady-state conditions. The minimal salts medium contained 10 mM-succinate + 20 mM-acetamide. O——O, Mutant C 11; △——△, mutant L 9; ----, wild type on the same medium.

Fig. 3. Mutants with altered catabolite repressibility. Amidase activities of continuous cultures grown under steady state conditions in minimal medium containing 10 mM-succinate + 20 mM-acetamide. △——△, Fully constitutive mutant L 9; O——O, inducible mutant L 11; ----, wild type on the same medium.

Mutant L 9 was isolated in a single step on succinate + lactamide minimal salt agar (S/L plates) after treatment of the wild-type strain with ethylmethane sulphonate. This mutant also grew on succinate + formamide agar (S/F plates) and when tested for rate of enzyme synthesis in batch culture it behaved as a magno-constitutive non-inducible strain. Mutants isolated from S/L plates have been found to be less sensitive to catabolite repression by succinate. The differential rate of amidase synthesis by strain L 9 in batch culture in pyruvate medium was almost unaffected by the addition of 10 mM succinate (Table 1). In continuous culture the curve obtained for amidase specific activities at various dilution rates resembled that for mutant C 11 but was markedly displaced towards the higher dilution rates (Fig. 2). The result was therefore that until

a dilution rate of about $D = 0.40$ hr^{-1} was reached, the amidase specific activity of the mutant L 9 cultures was always higher than that of the mutant C 11 culture, for any given dilution rate.

Table 1. *Properties of mutant strains of Pseudomonas aeruginosa 8602*

Strain	Amidase phenotype	Mutagen*	Isolation medium†	Catabolite repressibility (%)‡
Wild type	Inducible	—	—	98
C 11	Magno-constitutive	spont.	S/F	85
C 17	Semi-constitutive	u.v.	S/F	80
L 9	Magno-constitutive	ems	S/L	15
L 11	Inducible	ems	S/L	60

* ems = ethylmethane sulphonate.

† Isolation medium: S/F = succinate + formamide agar, S/L = succinate + lactamide agar.

‡ Catabolite repressibility determined with cultures growing exponentially in pyruvate medium, mutants L 11 and C 17 induced with 10 mM-*N*-acetylacetamide; 10 mM-succinate added as catabolite repressor.

Amidase activities of an inducible mutant with decreased sensitivity to catabolite repression

Mutant L 11 was isolated from S/L medium after treatment of the wild-type strain with ethylmethane sulphonate. It differed from mutant L 9 in that it grew only slightly better than the wild-type on S/F plates and behaved in batch culture as an inducible strain. Table 1 shows that, when fully induced by *N*-acetylacetamide as non-substrate inducer in pyruvate medium, it was repressed by only 60 % on the addition of 10 mM-succinate.

Figure 3 compares the results obtained in continuous culture with mutants L 11, L 9 and the wild type. It can be seen that the curve for the amidase specific activity of mutant L 11 has an upward slope at the lower dilution rates. This had been previously observed with the wild-type inducible strain but not for the fully constitutive mutant L 9. The amidase specific activity of mutant L 11 was however significantly higher than that for the wild type between dilution rates of 0.05 and 0.20 hr^{-1}. The maximum amidase activity occurred at a dilution rate of about 0.20 hr^{-1} compared with about 0.30 hr^{-1} for the wild type. At the higher dilution rates the amidase specific activity decreased much more slowly than did that of the wild type and was about 50 at a dilution rate of 0.50 hr^{-1} when that of the wild type and fully constitutive strains had decreased to much lower values.

Amidase activities of a semi-constitutive mutant

Mutant C 17 was isolated on S/F medium after treatment of the wild-type strain by ultraviolet irradiation. It did not grow on S/L plates and in batch culture in pyruvate medium was only slightly less sensitive than mutant C 11 to catabolite repression by 10 mM-succinate. In the absence of inducer, mutant C 17 synthesized amidase at about half the differential rate of the fully induced wild type and could be fully induced by adding substrate or non-substrate inducers to the medium (Brammar *et al.* 1967).

Figure 4 gives the results for amidase activity of mutant C 17 in continuous culture on the succinate + acetamide medium. The general shape of the curve was very like that of the wild-type strain, but the sharp peak of maximum amidase activity occurred at a dilution rate of about 0·2 hr^{-1}. The amidase specific activity at low dilution rates was higher than that of the wild type, as might be expected from a partially constitutive mutant, and it is possible to consider the amidase curve for mutant C 17 as intermediate between that of the fully inducible wild type and the fully constitutive C 11. The rate of decrease in amidase specific activity at the higher dilution rates was about the same as that of the wild type. It was not possible to continue these measurements at dilution rates greater than 0·50 hr^{-1} since this strain deteriorated at higher growth rates and tended to grow on the walls of the culture vessel.
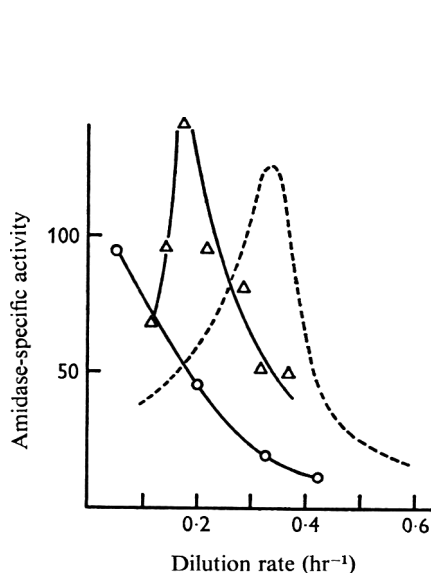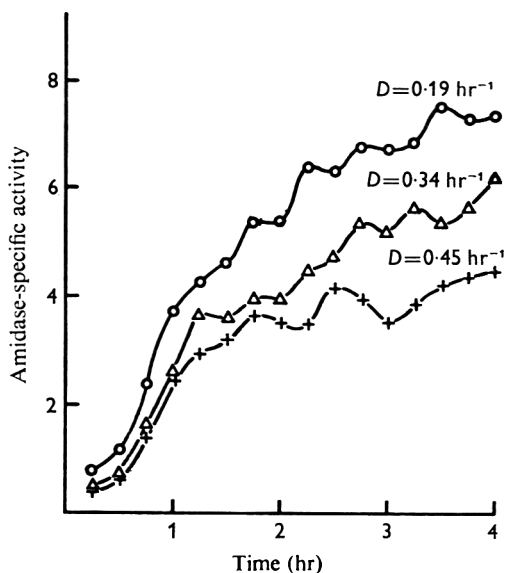
Fig. 4   Fig. 5

Fig. 4. The amidase-specific activities of a magno-constitutive mutant C 11 (O———O) and a semi-constitutive mutant C 17 (△———△) grown in continuous culture under steady-state conditions in minimal medium containing 10 mM-succinate + 20 mM-acetamide. - - - -, wild type on the same medium.

Fig. 5. Amidase synthesis by *Pseudomonas aeruginosa* 8602 wild type in continuous culture following a change of medium from minimal salts + 10 mM-succinate to minimal salts + 10 mM-*N*-acetylacetamide.

Amidase induction by a non-substrate inducer in continuous culture

The non-substrate amidase inducer *N*-acetylacetamide is not sufficiently stable to permit its use for prolonged studies in continuous culture, but we had previously found that it could be used for experiments of at least 4 hr duration and we examined the synthesis of amidase by continuous cultures growing at different rates during the transition period when the ingoing medium was changed from 10 mM-succinate to 20 mM-succinate + 10 mM-*N*-acetylacetamide (Boddy *et al.* 1967). The increase in succinate concentration allowed the bacteria to double in mass and during the transition period to the new steady state the effect of dilution rate on amidase induction

could be studied. At higher dilution rates amidase induction during the transition period was severely repressed and there was a lag of several hours before the rate of synthesis became significant. Similar results were observed during the transition period from a medium containing 10 mM-succinate to a medium containing 10 mM-succinate + 20 mM-acetamide, when the inducing amide was also available as a carbon source. We have now examined the rate of amidase synthesis by the wild type in continuous culture during the 4 hr after the ingoing medium was changed from 10 mM-succinate to 10 mM-succinate + 10 mM-N-acetylacetamide. Under these conditions there was no change in the growth rate and the bacterial mass remained the same. Figure 5 shows that at the three dilution rates tested there was only a short lag ($<$ 0·5 hr) before amidase synthesis started. This corresponds to the building up of a sufficient inducer concentration in the culture vessel and there was no phase of severe repression as had been observed previously when the succinate concentration had been doubled at the time of adding the inducer. However, after 4 hr the specific activities of the cultures at these three dilution rates were significantly different. Under these conditions the mean amidase specific activity of the culture growing at a dilution rate of 0·19 hr^{-1} was greater than that at a dilution rate of 0·34 hr^{-1} and almost twice that at 0·45 hr^{-1}, even though the N-acetylacetamide concentration was rising faster at the higher dilution rates.

DISCUSSION

Brammar & Clarke (1964) showed that in exponentially growing cultures of *Pseudomonas aeruginosa* the substrate inducer acetamide could relieve catabolite repression of amidase synthesis by succinate. With carbon-starved resting bacteria it was possible to demonstrate that N-acetylacetamide, which is a much less effective inducer than acetamide, could also counteract succinate repression of amidase synthesis (Clarke & Brammar, 1964). A similar interaction between induction and catabolite repression of β-galactosidase in *Escherichia coli* is now established (Clark & Marr, 1964; Moses & Prevost, 1966), although this was overlooked for a long time because the necessary quantitative experiments were not done.

We showed previously (Boddy *et al.* 1967) that in continuous culture the synthesis of *Pseudomonas aeruginosa* amidase was markedly affected by changes in the dilution rate of the culture and concluded that the extent of catabolite repression was related to the dilution rate. In studying the events during the transition period after changing the medium from succinate, to succinate + acetamide (doubling the concentration of carbon source), we found that at high dilution rates the synthesis of amidase was severely repressed. We suggested that the very high growth rate which occurred during the time when the culture was adapting to the new steady state resulted in a sufficiently high concentration of intermediary metabolites to prevent enzyme induction by acetamide. At the intermediate dilution rates of 0·30–0·50 hr^{-1} the repression of enzyme synthesis was temporary and amidase synthesis started after a lag period. With the non-substrate inducer N-acetylacetamide this severe repression, with a lag in amidase synthesis, only occurred under conditions in which the concentration of the carbon source for growth was increased, and was not found in the present experiments, in which the ingoing medium was supplemented with N-acetylacetamide, leaving the succinate concentration unchanged. These results support the view that the severe catabolite repression observed during the transition period from one steady state to

another is due to a temporary increase in the concentration of repressing intermediary metabolites. This metabolic adjustment during the transition period may be similar to the metabolic events occurring after the addition of glucose to an exponentially growing culture of *Escherichia coli* synthesizing β-galactosidase. Prevost & Moses (1967) have shown that during transient repression of β-galactosidase synthesis by glucose there is a temporary increase in certain metabolites. The severe transient repression of β-galactosidase synthesis is relieved at the time the concentration of these metabolites falls to the original value.

We have shown now that the balance between induction and catabolite repression of an enzyme is also apparent in the regulation of the synthesis of an inducible enzyme in a culture growing under steady-state conditions. We interpret the curves obtained for the wild-type strain of *Pseudomonas aeruginosa* to mean that at low dilution rates catabolite repression is minimal and the rate of amidase synthesis is dependent mainly on the rate at which acetamide is presented to the bacteria. Thus, with increase in dilution rate the amidase specific activity under steady-state conditions increases. However, above $D = 0.30$ hr^{-1} the growth rate has increased to the point where metabolic intermediates are being formed at a sufficiently high rate to cause significant catabolite repression. At higher dilution rates catabolite repression becomes dominant and at $D = 0.60$ hr^{-1} the enzyme concentration has decreased considerably; but it must be realized that, even at these very high dilution rates, the specific activity of the culture is about the same as that of a batch culture grown overnight with acetamide.

In batch culture, the addition of a number of different carbon compounds to cultures induced by *N*-acetylacetamide results in catabolite repression but succinate appears to be a better source of the actual repressing catabolite than is acetate. In continuous culture, the difference in the curves for the wild-type strain growing in acetamide, or succinate + acetamide, is probably due to the different rates of synthesis of metabolic intermediates from succinate and acetate respectively. This effect may be accentuated by the higher carbon-content of the succinate + acetamide medium used in these experiments. The interpretation of the curves obtained with the wild-type strain is supported by the results with mutants. Genetic experiments have shown that constitutivity markers (C 11, C 17) are co-transduced with the amidase structural gene and we have concluded that a regulator gene is linked closely to the amidase structural gene (Brammar *et al.* 1967). The curves for the two fully constitutive mutants (C 11, L 9) lack completely the part where, in the curves for the wild-type strain, we have suggested that induction is dominant. At low dilution rates the enzyme activities of mutant C 11 approach the maximum value obtained with the wild type. Whereas the effect of catabolite repression is only noticed above $D = 0.30$ hr^{-1} with the wild type, the effect is apparent at much lower dilution rates with mutants C 11 and L 9. This implies that these particular mutations to constitutivity have also made the synthesis of amidase more susceptible to catabolite repression, presumably because the inducer is no longer able to counteract the repressing activity of the catabolite repressor molecule. The curve for the semi-constitutive mutant C 17 falls between the wild-type curve and the curve produced with the fully constitutive mutant C 11. The addition of inducer to this mutant increases the rate of amidase synthesis and might therefore also be expected to affect the catabolite repression.

The mutants with altered catabolite repressibility (L 9, L 11) have not yet been analysed genetically. The genetic lesion in each case may be in the amidase regulator

gene, or in a gene determining an enzyme concerned with succinate and acetate metabolism. The catabolite repressibility values, which were determined under standard conditions, could be correlated with the ability of the mutants to grow on S/L plates. Mutants with catabolite repressibility values of 80 % or more grew no better than the wild type, but both mutant L 11, with catabolite repressibility value of 60 %, and mutant L 9 (15 %) grew equally well on S/L plates. These two mutants also gave curves for amidase activity in continuous culture which differed from those obtained for the corresponding wild-type and constitutive cultures at the high dilution rates where we have suggested that catabolite repression is dominant. The experiments with the wild type under steady-state conditions, with the substrate-inducer acetamide, showed that the maximum effective induction occurred at about $D = 0.35\,\mathrm{hr}^{-1}$. It was not possible to do extended steady-state experiments with the non-substrate inducer *N*-acetylacetamide since it is not sufficiently stable. In the experiments described in this paper the cultures were tending to reach a constant value for amidase activity after about 4 hr. The highest value for amidase activity was observed at the lowest dilution rate ($D = 0.19\,\mathrm{hr}^{-1}$) and the values at 0.35 and 0.45 hr^{-1} were significantly lower even though the *N*-acetylacetamide concentration in the vessel increased more slowly at the low dilution rate. These results superficially resemble those obtained for constitutive cultures. In this case, however, the important factor is the difference in inducer activity of *N*-acetylacetamide and acetamide. With carbon-starved bacteria, values obtained for K_{Ind} were approximately 1 mM for *N*-acetylacetamide and 0.01 mM for acetamide, (Brammar, 1965) and in batch-culture experiments acetamide was much more effective in relieving the effects of catabolite repression. In continuous culture, *N*-acetylacetamide is too weak an inducer to allow any increase in the rate of amidase synthesis by the culture as the dilution rate is increased. With an increase in dilution rate from 0.19 to 0.45 hr^{-1} only the increasing effect of catabolite repression with dilution rate was observed.

These results with continuous cultures confirm the observations made with batch cultures on the relationship between induction and repression of amidase synthesis in *Pseudomonas aeruginosa* 8602. The general conclusion is that the effect of an inducer will depend on the metabolic state of the organism with respect to the production of catabolite repressor molecules. When the internal concentration of the repressing metabolites is high the catabolite repression is severe and changes in inducer concentration have little effect on the rate of amidase synthesis. In a batch culture provided with acetamide and succinate as carbon sources this results in diauxic growth. At the other extreme, with a depleted carbon pool, catabolite repression is minimal and the rate of enzyme synthesis depends on the concentration of inducer and can be increased to the point at which the culture is fully induced. Between these two conditions, there is a partial repression of amidase synthesis by catabolic intermediates which may be increased or decreased by changes in the inducer concentration. The extent to which catabolite repression can be affected by changes in inducer concentration will depend on the relative effectiveness of the inducer and the repressing catabolites. Acetamide is more effective as an inducer than *N*-acetylacetamide, probably because it has a higher affinity for the inducer-binding site of its cytoplasmic repressor. The actual effector molecule is not yet known for catabolite repression in this system, but it is clear from these results that a competitive relationship exists between the inducer and the repressing catabolite. This competitive relationship can only readily be observed

when the inducer is very active, and this probably requires it to have a high affinity for the inducer binding site of its specific cytoplasmic repressor. In continuous culture, variation of the growth substrates and changes in the growth rate of the culture may make it possible to examine in detail this balance of enzyme synthesis.

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Evidence for the Presence of Fimbriae (Pili) on Vibrio Species

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SUMMARY

Three strains of Vibrio were found to agglutinate both guinea-pig and human red cells. The vibrios differed in haemagglutination tests from fimbriated enterobacteria by showing only partial inhibition by mannose and by agglutinating guinea-pig and human red cells equally well. Electron microscopy of the vibrios revealed filamentous appendages which resembled the common fimbriae of *Escherichia coli* in having average diameters between 6 and 10 mμ and in being more numerous on organisms from liquid media than from solid media. The fimbriae on vibrios also had some characteristics of F fimbriae of enterobacteria. In comparative studies fimbriae which resembled common fimbriae of *E. coli* were found on *Pseudomonas multivorans* and *Aeromonas liquefaciens*.

INTRODUCTION

Fimbriae are too narrow to be seen with the light microscope but they can be seen with the electron microscope. For many enterobacteria fimbriae can also be detected by a haemagglutination test, first used for the purpose by Duguid, Smith, Dempster & Edmunds (1955). Although non-fibrial haemagglutination (i.e. haemagglutination by bacteria which is not due to fimbriae) sometimes occurs it differs from fimbrial haemagglutination by occurring only at low temperature, clumps of red cells breaking up as a result of elution of the bacteria as the temperature is raised to 40°–50° (Duguid, 1964). Also, non-fimbrial haemagglutination is not inhibited by mannose, unlike haemagglutination due to type 1 and type 6 fimbriae and the adhesin of type 5 fimbriae (Duguid, 1964; Duguid, Anderson & Campbell, 1966). Some cultural characters such as the formation of pellicles in broth by some enterobacteria (Duguid & Gillies, 1957; Duguid, 1959; Shedden, 1962) are associated with the presence of fimbriae.

During characterization tests of some vibrios it was noticed that pellicles were produced in nutrient broth by some strains but not by others, an observation which suggested that some vibrios might possess fimbriae. We therefore examined some of these vibrios for fimbriae by using the haemagglutination test and by electron microscopy. In this paper we report our findings. We have referred to as fimbriae any filamentous appendages other than flagella found on vibrios, but this does not imply that such

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appendages had all the properties described for fimbriae of other organisms. We use the word 'fimbriae' (Duguid *et al.* 1955) rather than 'pili' (Brinton, 1959) because it has priority and because we accepted the other points (Duguid, 1966) in favour of 'fimbriae'.

<div align="center">METHODS</div>

Organisms. The vibrios studied were: *Vibrio cholerae* NCTC 8021, *Vibrio eltor* NCTC 5395 and *Vibrio* sp. NCTC 4716 (hereafter called Vibrio 4716). Also included in the study were: *Pseudomonas multivorans* NCIB 9691 (strain 104 of Stanier, Palleroni & Doudoroff, 1966), *Aeromonas liquefaciens* NCMB 87 (strain L 417 of Kluyver; see Miles & Miles, 1951), and *Escherichia coli* Microbiology Department, Reading University strain 16 A.

Media. Organisms were grown either on nutrient agar (blood agar base, Oxoid; pH 7·5) sloped in 1 oz. universal screw-cap bottles, or in tryptone water (%, w/v, tryptone, Oxoid, 1·0; NaCl, 0·5; in demineralized water; pH 7·4) in 10 ml. amounts in $6 \times 5/8$ in. test-tubes. Organisms were subcultured to these media from stock cultures on nutrient agar slopes and were incubated for 24 hr or 72 hr at 20°, 25° or 37°.

Haemagglutination tests. Tile tests, using cells from human blood or guinea-pig blood and 2 % (w/v) D-mannose, were performed as described by Cruickshank (1965). Cells separated by centrifugation from fresh citrated blood were washed twice in saline (0·85 %, w/v, NaCl in demineralized water) and made up to a 3 % (v/v) suspension in saline. Organisms grown on solid media were harvested by adding 1·0 ml. saline to each slope and gently emulsifying the growth. The resultant suspension of organisms was deposited by centrifugation; most of the supernatant fluid was poured off and the deposit of each culture then resuspended in the small amount of remaining fluid. Tryptone water cultures were centrifuged and most of the supernatant fluid then poured off. The organisms were resuspended in the small amount of remaining fluid. Each test was performed by adding reagents in the order shown in Table 1 to depressions in a tile previously chilled to 3–5°. The tile was then rocked gently for 5 min. while it warmed to room temperature and was examined each minute. For studies to determine the effect of heat treatment on haemagglutinating ability, each strain was passed through 3 subcultures at intervals of 24 hr in tryptone water. One tube of the final culture was centrifuged and the deposit heated in a boiling water bath for 30 min. The deposit was cooled to room temperature by placing the tube in a water bath at 20° and was then tested for haemagglutinin.

Table 1. *Amounts of reagents (in ml.) added to depressions in chilled tiles in haemagglutination tests with 3 strains of Vibrio and 1 strain each of Escherichia coli, Pseudomonas multivorans and Aeromonas liquefaciens*

Reagent	Depression number					
	1	2	3	4	5	6
(1) 3 % (v/v) guinea-pig red-cell suspension	0·02	0·02	.	.	0·02	.
(2) 3 % (v/v) human red-cell suspension	.	.	0·02	0·02	.	0·02
(3) 2 % (w/v) D-mannose*	.	0·02	.	0·02	.	.
(4) Saline	0·02	.	0·02	.	0·04	0·04
(5) Dense bacillary suspension	0·02	0·02	0·02	0·02	.	.

* Final mannose concentration = 0·7 % (w/v).

Electron microscopy. Organisms grown in tryptone water or harvested from agar were washed twice in sterile distilled water. Because of their potential pathogenicity, cultures of *Vibrio cholerae* and *V. eltor* were fixed before washing by adding formalin to a final formaldehyde concentration of 0·25 % (w/v). Comparison of preparations of Vibrio 4716 fixed with formalin with unfixed preparations indicated that formalin treatment was unlikely to alter the appearance of these organisms. Metal-shadowed preparations were made by placing a loopful of an aqueous suspension of organisms on a formvar-coated copper grid, allowing it to dry in air, and then shadowing with gold-palladium (40:60) at an angle of 20°. Negatively stained preparations were made on carbon-stabilized formvar-coated copper grids. A loopful of an aqueous suspension of organisms was placed on the grid and allowed to dry in air. Then a drop of either aqueous ammonium molybdate (1 %, w/v; pH 7·0) or potassium phosphotungstate (1 %, w/v; pH 7·0) was put on the grid and, after 1 min., the excess removed with filter paper. Preparations were examined in a Siemens Elmiskop I electron microscope using the single condenser, a 200 μ condenser aperture, a 50 μ objective aperture, and an accelerating voltage of 80 kV. Micrographs were taken at initial magnifications of 6000–20,000 on Ilford N 50 thin-film half-tone plates. Measurement of the diameters of fimbriae were made from negatives using a plate microscope and a calibrated eye-piece micrometer ('Filar micrometer'; Nikon, Tokyo). Lengths of fimbriae were measured from prints enlarged from the negatives.

RESULTS

Haemagglutination

Vibrios examined after 3 serial subcultures at 24 hr in tryptone water incubated at 37° consistently agglutinated both guinea-pig and human cells (Table 2). These conditions therefore appeared to be suitable and reliable for demonstrating haemagglutination by vibrios. Vibrios grown under other cultural conditions agglutinated these cells less consistently. Of the vibrios, *Vibrio eltor* gave the strongest haemagglutination reaction. *Escherichia coli*, *Pseudomonas multivorans* and *Aeromonas liquefaciens* when grown in tryptone water or when grown for 72 hr on nutrient agar consistently agglutinated guinea-pig red cells. Haemagglutination by these three organisms was completely inhibited by D-mannose (final conc. 0·7 %, w/v) whereas haemagglutination by the 3 Vibrio strains was only partially inhibited. By partial inhibition we mean that when mannose was included in the mixture which contained organisms and red blood cells, there were fewer, smaller clumps of agglutinated red cells than when no mannose was present.

The 3 Vibrio strains and *Aeromonas liquefaciens* agglutinated human and guinea-pig red blood cells equally well. *Escherichia coli* agglutinated guinea-pig red blood cells strongly and human red blood cells only weakly. *Pseudomonas multivorans* agglutinated guinea-pig red blood cells but failed to agglutinate human red blood cells.

On no occasion did we obtain haemagglutination with supernatant fluids from cultures or with heated cultures.

Electron microscopy

The measurements given here are of negatively stained fimbriae except where it is stated that they are of shadowed fimbriae. Thornley & Horne (1962) reported that the

Table 2. *Results of haemagglutination tests, using human or guinea-pig red blood cells, with concentrated cell suspensions of 3 strains of Vibrio and 1 strain each of Escherichia coli, Pseudomonas multivorans and Aeromonas liquefaciens grown under various cultural conditions*

Growth conditions for organisms tested	Type of red blood cells used	Vibrio el tor	V. cholerae	Vibrio 4716	Escherichia coli	Pseudo-monas multi-vorans	Aeromonas lique-faciens
(1) Organisms grown on nutrient agar							
Harvested after 3 successive subcultures for 24 hr at 20°	Guinea-pig	.	.	.	.	+	+
	Human	.	.	.	.	−	+
Harvested after 3 successive subcultures for 24 hr at 25°	Guinea-pig	V	−	−	V	.	.
	Human	V	−	−	−	.	.
Harvested after 3 successive subcultures for 24 hr at 37°	Guinea-pig	+	−	−	V	.	.
	Human	+	−	−	−	.	.
Harvested after 2 successive subcultures for 72 hr at 20°	Guinea-pig	.	V	−	.	+	+
	Human	.	V	−	.	−	+
Harvested after 2 successive subcultures for 72 hr at 37°	Guinea-pig	V	V	−	+	.	.
	Human	V	V	−	−	.	.
(2) Organisms grown in tryptone water							
Harvested after 3 successive subcultures for 24 hr at 20°	Guinea-pig	.	.	.	.	+	+
	Human	.	.	.	.	−	+
Harvested after 3 successive subcultures for 24 hr at 37°	Guinea-pig	+	+	+	+	.	.
	Human	+	+	+	V	.	.
Harvested after 2 successive subcultures for 72 hr at 20°	Guinea-pig	.	.	.	.	+	+
	Human	.	.	.	.	−	+
Harvested after 2 successive subcultures for 72 hr at 37°	Guinea-pig	−	−	V	+	+	+
	Human	−	−	V	V	−	+

+ = haemagglutination occurred within 2 min.
− = haemagglutination did not occur within 5 min.
V = results were variable.
. = no test performed.

diameter of negatively stained fimbriae was approximately 30 % less than that of shadowed fimbriae. The diameters to be expected for negatively stained fimbriae, calculated from micrographs of shadowed fimbriae using this ratio, are given in parentheses in Table 3. It can be seen that our results agree with those of Thornley & Horne (1962).

Table 3. *Measurements of fimbriae found on 3 strains of Vibrio and 1 strain each of Escherichia coli, Pseudomonas multivorans and Aeromononas liquefaciens*

	Vibrio eltor	V. cholerae	Vibrio 4716	Escheri- chia coli	Pseudo- monas multi- vorans	Aeromonas lique- faciens
(1) Diameter (mμ)						
Metal-shadowed fimbriae	11·3±0·7	10·4±0·5	13·1±0·5	11·1±0·8	8·6±0·3	9·2±0·7
Negatively stained fimbriae	7·9±0·2	7·2±0·3	.	.	.	7·9±0·3
	(7·9)	(6·7)	(9·2)	(7·7)	(6·0)	(6·4)
(2) Length (μ) of longest fimbria seen	3·3	1·0	1·8	1·0	1·5	1·3

Figures in parentheses are the estimated values of diameters of negatively stained fimbriae assuming that these values are 30 % less than those of shadowed fimbriae (Thornley & Horne, 1962).

Values for measured diameters of fimbriae (between 7 and 40 diameters measured for each value) are given as the mean ± the standard deviation.

When determining lengths of fimbriae we measured only those fimbriae which appeared to be clearly attached to the organisms and which were not close to fimbriae from other organisms.

Vibrio eltor. About 50 % of the organisms examined had long, curved fimbriae. These fimbriae did not branch or taper; the average diam. was 7·9 mμ, and the maximum length was more than 2·5 μ. The number of fimbriae per organism and the length increased with the number of subcultures in tryptone water and decreased with the number of subcultures on nutrient agar. The maximum number of fimbriae per organism was 50 (Pl. 1, fig. 1; Pl. 2, fig. 2, 3).

Vibrio cholerae. Unbranched fimbriae which did not taper were found on about 10 % of the organisms. The fimbriae were short and curved and had an average diameter of 7·2 mμ. The maximum length was 1·0 μ and there were up to 9 fimbriae per organism. (Pl. 3, fig. 4).

Vibrio 4716. About 10 % of the organisms examined after 3 serial subcultures at 24 hr intervals in tryptone water had 1, 2 or 3 fimbriae. Measured from shadowed preparations the fimbriae had an average diameter of 13·1 mμ and a maximum length of 1·8 μ (Pl. 3, fig. 5). The fimbriae were not branched and did not taper. However, organisms examined after 1 subculture or 2, 3 or 6 serial subcultures at 24 hr intervals on nutrient agar were surrounded by a mass of branching strands (Pl. 4, fig. 6, 7). These strands increased in quantity as the number of subcultures increased, they tapered at the distal end and they branched. The average diameter of the narrowest strands was 6·8 mμ, which is less than that of the fimbriae seen on organisms after one 24 hr subculture in tryptone water from stock. Furthermore, bearing in mind that shadowed fimbriae appear to be 30 % wider than do negatively stained fimbriae, there was a discrepancy between the values for the diameter of the strands seen in negatively stained preparations (6·8 mμ) and in metal-shadowed preparations (5·7 mμ). The discrepancy could be explained by assuming that the strands were flat, not round like fimbriae

(Brinton, 1965), so that they cast very little shadow. We concluded that the branching strands were unlikely to be fimbriae but were possibly formed in a random way from some material such as slime. These strands would have obscured any fimbriae that were present.

Escherichia coli. About 10 % of the organisms from cultures grown either on solid or in liquid media had numerous short, straight fimbriae which did not branch or taper. The average diameter was 11·1 mμ (in shadowed preparations) and the maximum length was 1·0 μ. (Pl. 5, fig. 8).

Pseudomonas multivorans. About 10 % of the organisms from cultures grown either on solid or in liquid media had numerous, short, straight fimbriae which did not branch or taper. The average diameter was 8·6 mμ (in shadowed preparations) and the maximum length was 1·5 μ. (Pl. 5, fig. 9).

Aeromonas liquefaciens. About 10 % of the organisms from cultures grown either on solid or in liquid media had numerous short, straight fimbriae which did not branch or taper. The average diameter was 7·9 mμ and the maximum length was 1·3 μ. (Pl. 6, fig. 10, 11).

DISCUSSION

Vibrios show a type of haemagglutination which in some ways resembles haemagglutination by fimbriated enterobacteria. Supernatant fluid from tryptone water cultures does not agglutinate red blood cells, thus indicating that the haemagglutinating agent is bound to the organism. Heat-treated organisms also do not haemagglutinate. Haemagglutination by the vibrios differs from haemagglutination by fimbriated enterobacteria in being partially inhibited by mannose: haemagglutination by enterobacteria is either completely inhibited or else is completely unaffected (Shedden, 1962). Similar results to ours were obtained by Barua & Mukherjee (1965) working with strains of *Vibrio eltor* and *V. cholerae.*

Haemagglutination by various enterobacteria usually indicates that these organisms possess fimbriae but haemagglutination can occur without fimbriae being involved (non-fimbrial haemagglutination). Non-fimbrial haemagglutination encountered by Duguid *et al.* (1955, 1966) during studies of various enterobacteria had some distinctive features. These features (the range of species of red cells agglutinated, elution from red cells at high temperature, agglutination not inhibited by mannose) enabled fimbrial and non-fimbrial haemagglutination to be differentiated from one another. It is difficult to know how justifiable it is to apply the information on haemagglutination by enterobacteria to the interpretation of haemagglutination by vibrios. However, we consider that the following points, taken together, indicate that the vibrios we examined are capable of producing fimbriae and that these fimbriae are probably responsible for haemagglutination.

(1) We did not detect elution from red cells when the temperature rose during the tile test or in some other tests we performed in tubes. Observations on elution were complicated by the haemolysis of agglutinated red cells as the temperature was raised to 50°. Doorenbos (1932; cited by Pollitzer, 1959) observed that haemagglutination by el tor vibrios was as good at 37° as at 0°, while Barua & Mukherjee (1965) found that these organisms did not haemagglutinate at 4° but did do so at 30° and 37°. It appears that haemagglutination by vibrios is, with respect to temperature, similar to fimbrial haemagglutination by *Escherichia coli* (Duguid, 1964) and by various Salmonella species (Duguid *et al.* 1966).

(2) Haemagglutination by vibrios was partially inhibited by mannose. Mannose inhibition of non-fimbrial haemagglutination by bacteria has not been reported but haemagglutination due to fimbriae is often mannose sensitive.

(3) The strongest and most reliable haemagglutination by *Vibrio eltor* occurred when organisms were grown under conditions which gave most fimbriae as judged by electron microscopy.

We found, as did Barua & Chatterjee (1964), that the haemagglutination reaction of vibrios was much weaker than was that of *Escherichia coli* in the fimbriate phase. The haemagglutinating activity of enterobacteria varies directly with the degree of fimbriation (Duguid & Gillies, 1957). Barua & Chatterjee (1964) assumed that the same relationship might hold with vibrios and suggested that a smaller percentage of el tor vibrios than of *E. coli* organisms in the fimbriate phase possess fimbriae. In our electron microscope study we obtained some evidence to support the suggestion that the intensity of haemagglutination is related to the percentage of fimbriate organisms and to the number of fimbriae on organisms. Only about 10 % of our *Vibrio cholerae* organisms had fimbriae and the maximum number seen per organism was 9. This strain gave weaker haemagglutination reactions than did *V. eltor*, which had up to 50 fimbriae per organism and which had fimbriae on about 50 % of organisms. It might have been possible for us to obtain a stronger haemagglutination reaction by altering cultural conditions (Bales & Lankford, 1961) or by using a denser bacterial suspension in the test (Finkelstein & Mukerjee, 1963; Barua & Mukherjee, 1965) but we did not make an intensive study of these factors.

Barua & Chatterjee (1964) made an electron microscope examination of el tor vibrios and observed fimbriae with diameters ranging from 6 to 8 mμ. There were a small number of fimbriae per organism and Barua & Chatterjee had some difficulty in detecting them because there was a slime layer surrounding the organisms. This difficulty led Barua & Mukherjee (1965) to conclude that fimbriae had not been convincingly demonstrated in these vibrios. In our work the morphology and the estimated diameters of the fimbriae were similar to those reported by Barua & Chatterjee (1964). There was no slime layer around the cell walls of *V. cholerae* or *V. eltor* but Vibrio 4716, under certain conditions, was surrounded by structures which may represent slime. These structures appear to be distinct from fimbriae but may obscure fimbriae which may be present on the organisms.

The vibrio fimbriae do not appear to correspond to any of the 6 types of fimbriae (designated I–V and F) described by Brinton (1965) or of the 7 types (designated 1–6 and F) described by Duguid *et al.* (1966). The general dimensions are similar to those of types I, IV and F fimbriae of Brinton and to those of types 1, 2 and F of Duguid *et al.* However the vibrio fimbriae are distinct from type IV of Brinton because they do not have a helical structure and from type 2 of Duguid because they are apparently associated with haemagglutinating activity. In the following discussion the term 'type 1 fimbriae' used without qualification means type 1 fimbriae of Duguid *et al.* (1966) and type I fimbriae of Brinton (1965). We compared our electron micrographs of vibrio fimbriae with electron micrographs of type 1 fimbriae (e.g. Brinton, 1965; Duguid *et al.* 1966) and of F fimbriae (Brinton, 1965; Lawn 1966). From this it appeared that: (1) type 1 fimbriae are straighter and more numerous per organism than are vibrio fimbriae; (2) F fimbriae are present in smaller numbers per organism, at least than is the case with fimbriae on *Vibrio eltor*, and are generally longer than are

vibrio fimbriae. It may be that the filaments on the surface of vibrios are filamentous phages. We think that this is unlikely but are unable to comment critically on the possibility at present, especially as it is conceivable that there is an evolutionary relationship between certain fimbriae and filamentous phages (Bradley, 1966).

Brinton (1965) demonstrated that the presence of F fimbriae of *Escherichia coli* is correlated with the presence of the F factor, which confers fertility. Fertility has been demonstrated in vibrios by Bhaskaran (1960) and this makes us wonder if the fimbriae of vibrios are associated with transfer of genetic material in addition to being associated with haemagglutination. So far as we know, the haemagglutinating properties of F fimbriae on *E. coli* and the fimbriation of fertile *V. cholerae* have not yet been determined.

In order to have some known fimbriate organisms for comparison in this study we included a strain of *Escherichia coli*, which gave mannose-sensitive haemagglutination, and two strains of polarly-flagellated organisms, *Pseudomonas multivorans* and *Aeromonas liquefaciens*, previously shown by one of us (W.H.) to possess fimbriae. We found that in their dimensions, morphology and typical number per organism, the fimbriae on these three strains corresponded to type 1 fimbriae. Haemagglutination occurred with all three strains but *A. liquefaciens* and *P. multivorans* showed a minor difference from the haemagglutination chracteristics of type 1 fimbriae (Duguid *et al.* 1966). *Aeromonas liquefaciens* agglutinated human and guinea-pig red cells equally well, while *P. multivorans* agglutinated guinea-pig red cells but failed to agglutinate human red cells. Type 1 fimbriae agglutinate guinea-pig red cells well but only agglutinate human red cells weakly (Duguid, 1964; Duguid *et al.* 1966). So far as we are aware this is the first published report of type 1 fimbriae on *A. liquefaciens* and *P. multivorans*. It has been the experience of one of us (W.H.) during electron microscopy of polarly flagellated organisms that fimbriae are possessed by only a very few species.

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Plate 2

J. M. TWEEDY, R. W. A. PARK AND W. HODGKISS

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Plate 6
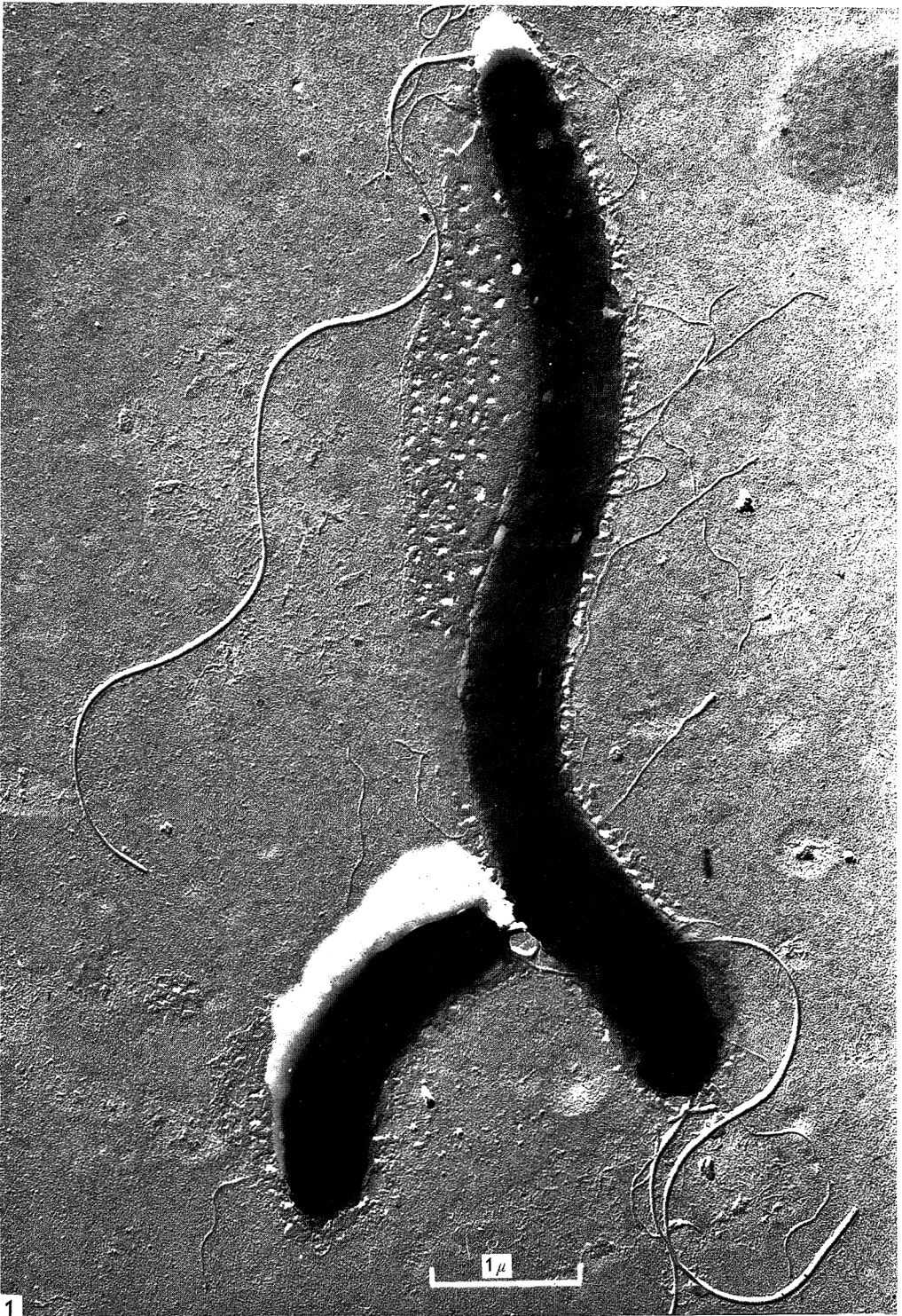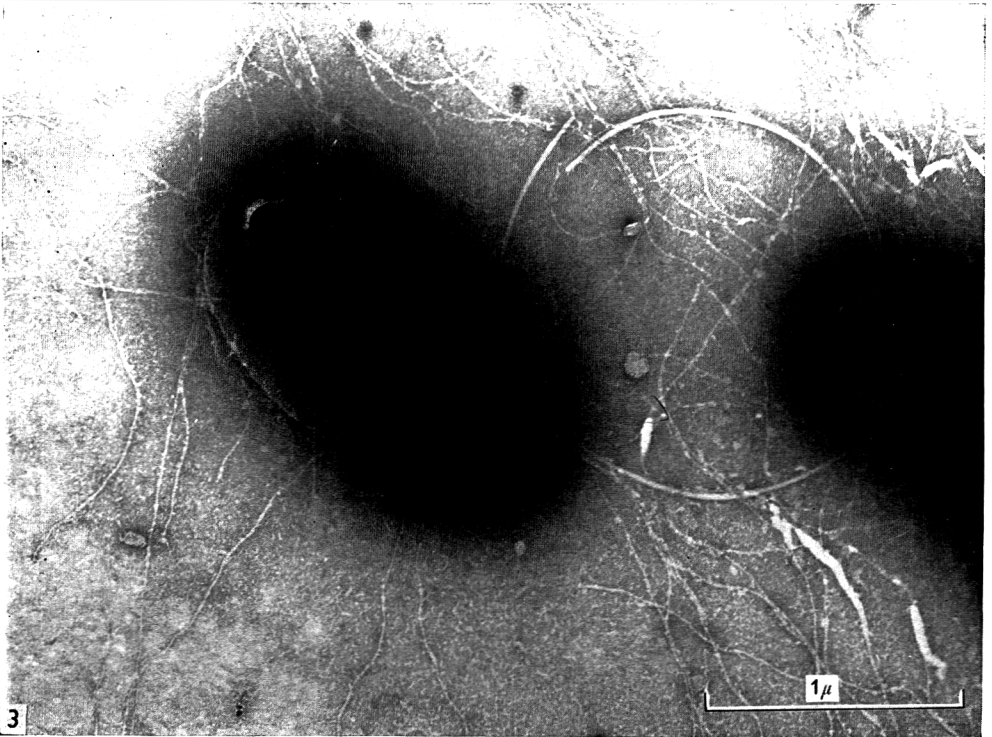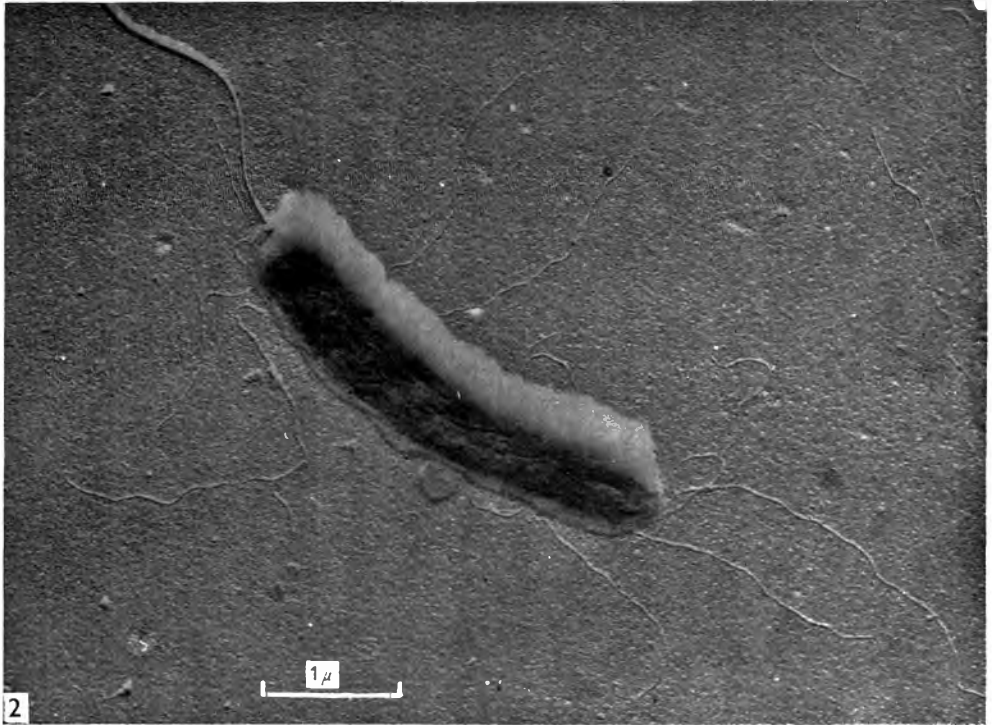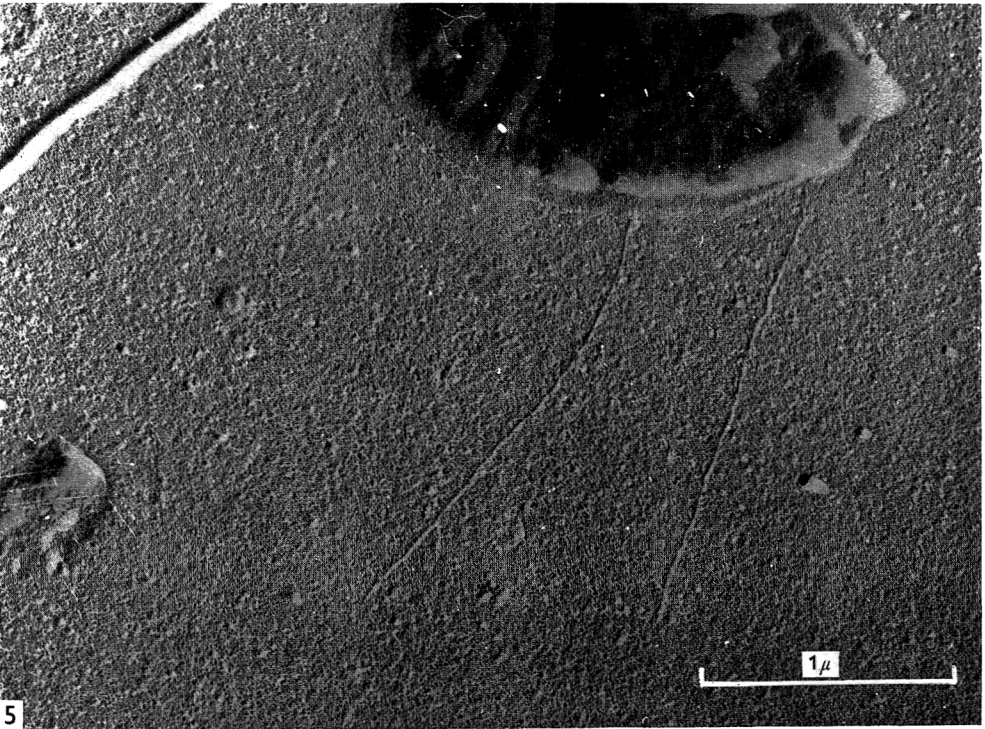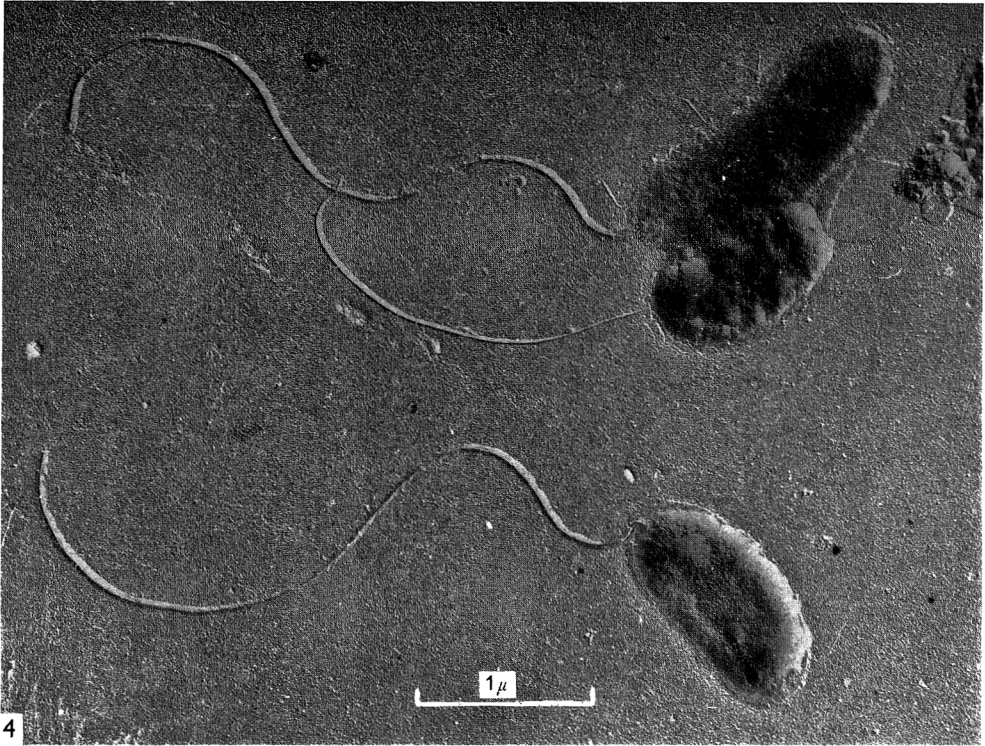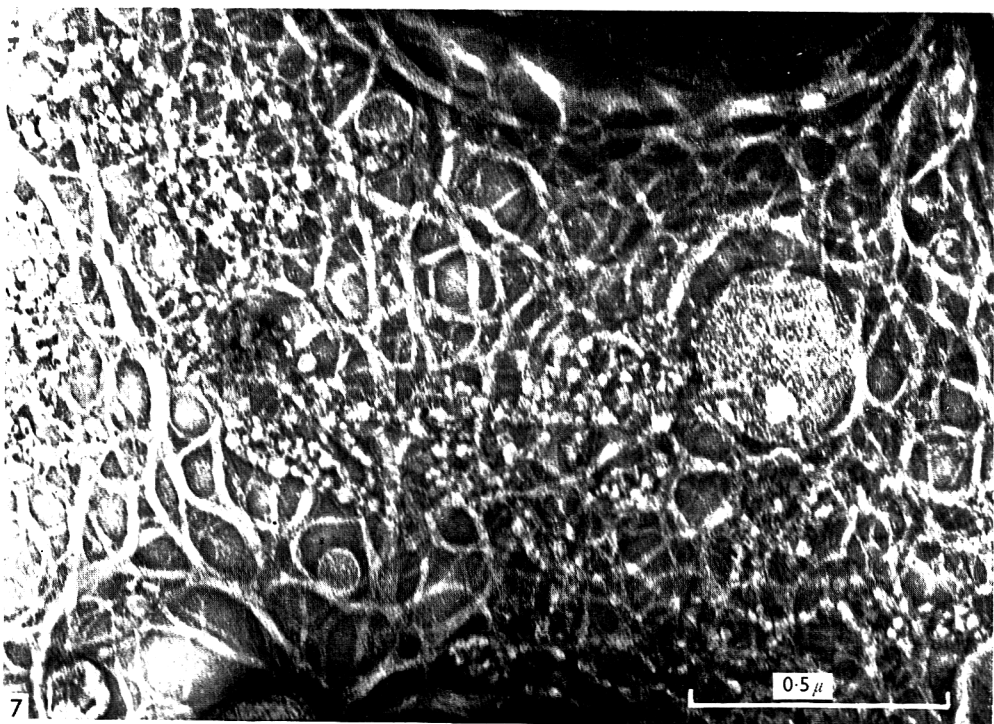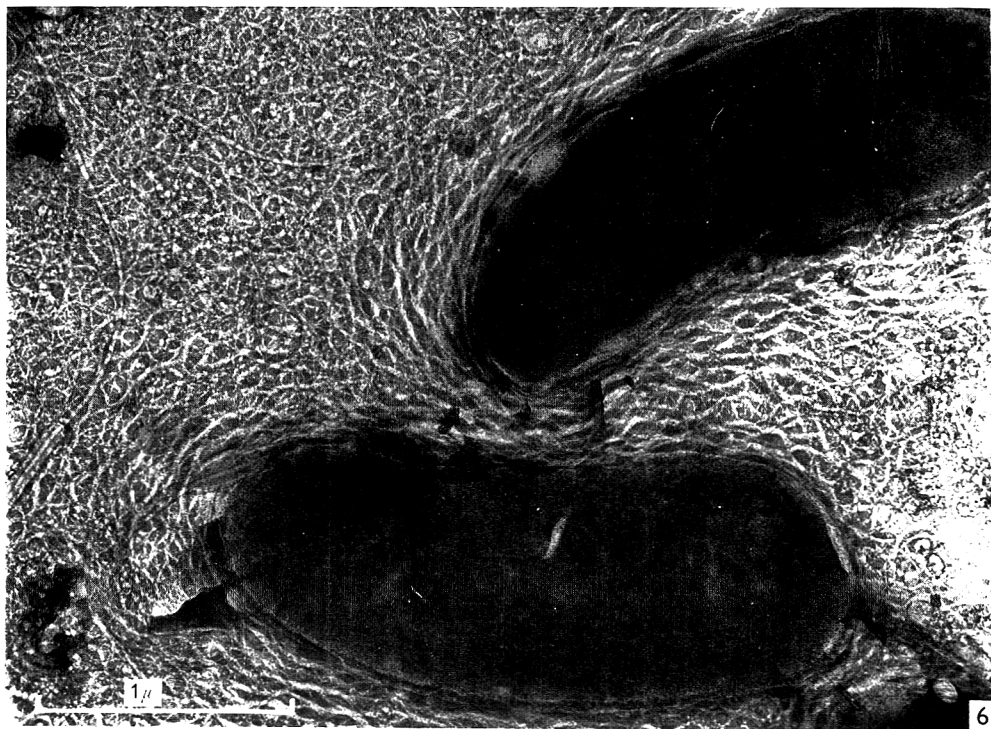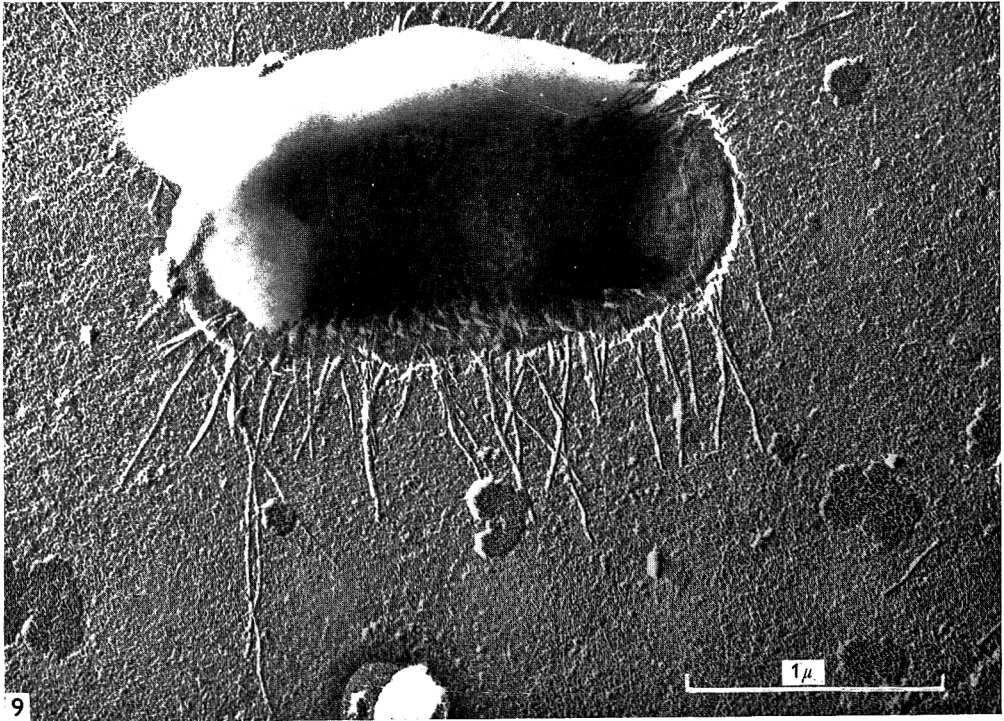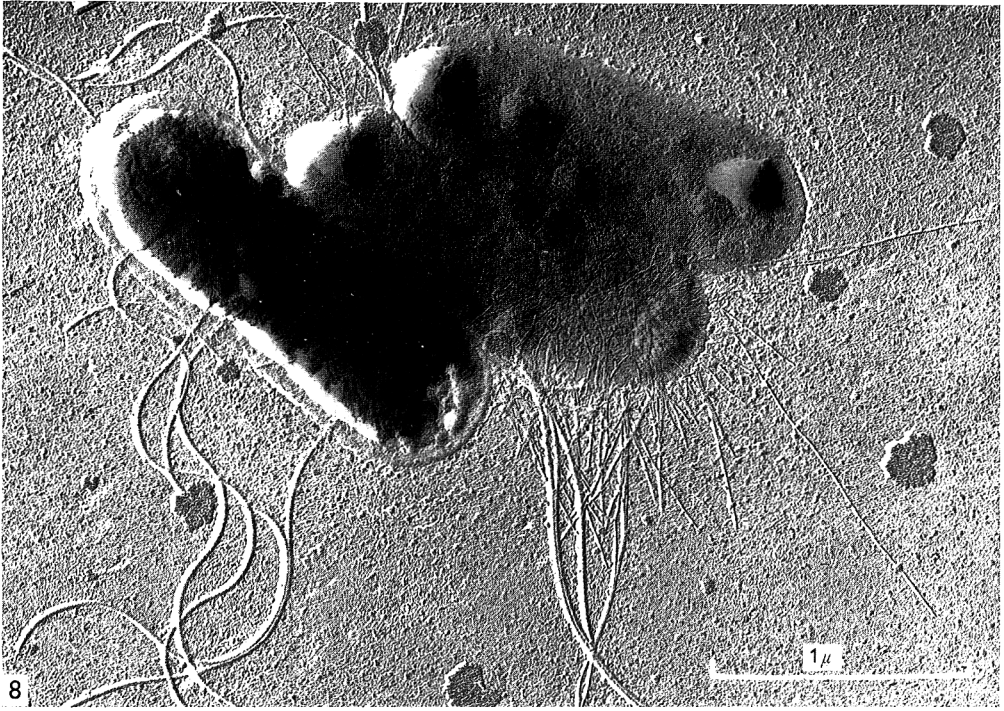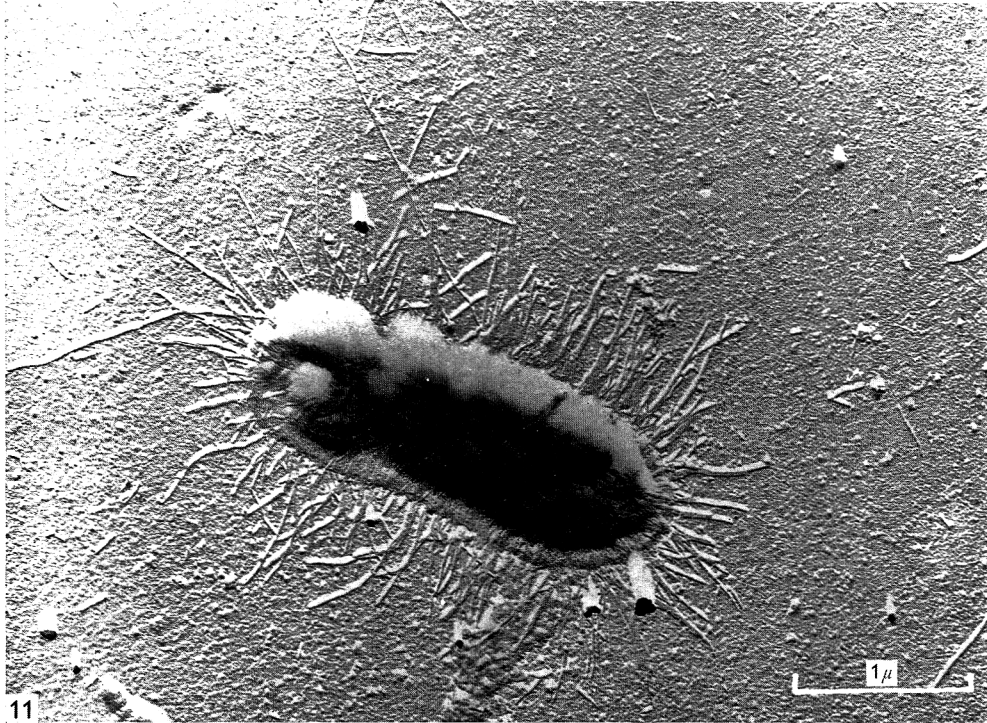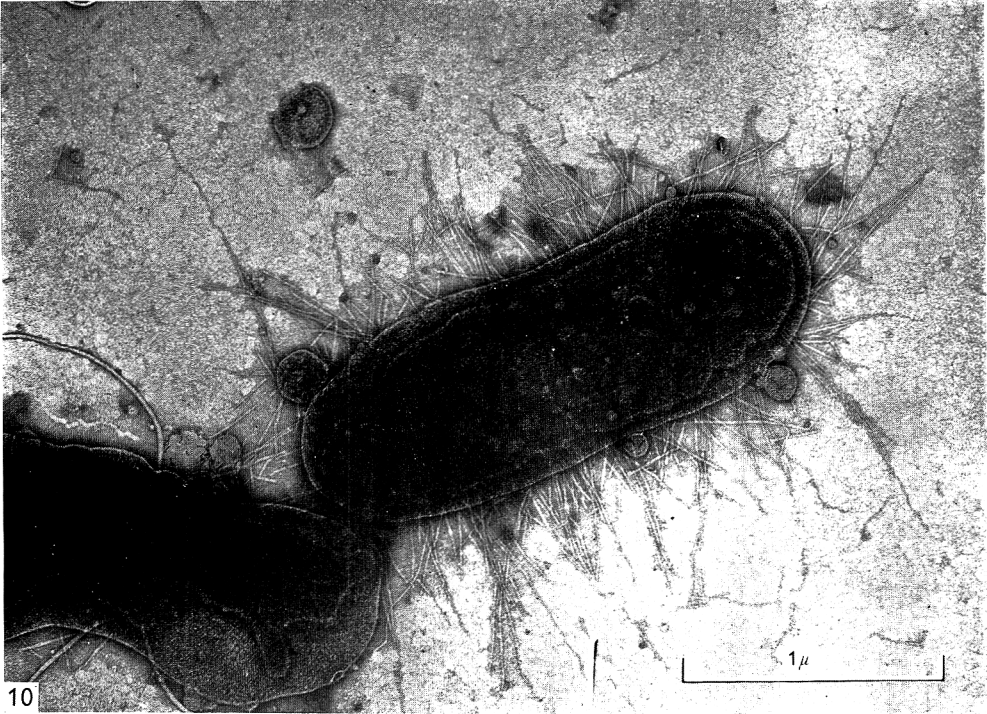
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EXPLANATION OF PLATES

All the figures are electron micrographs of organisms. Shadowed preparations were made of organisms mounted on formvar films using gold–palladium (40:60) at 20°; negatively stained preparations were made of organisms mounted on carbon-stabilized formvar films using ammonium molybdate.

PLATE 1

Fig. 1. *Vibrio eltor* NCTC 5395 after 3 serial subcultures at 24-hr intervals in tryptone water incubated at 37°. The organisms bear long fimbriae which, unlike common (type 1) fimbriae of enterobacteria, are not straight. Formalin fixed, shadowed.

PLATE 2

Fig. 2. *V. eltor* NCTC 5395 after 3 serial subcultures at 24-hr intervals in tryptone water incubated at 37°. Several long fimbriae are visible. Formalin fixed, shadowed.

Fig. 3. *V. eltor* NCTC 5395 after 2 serial subcultures at 72-hr intervals in tryptone water incubated at 37°. Both organisms have numerous fimbriae. Formalin fixed, negative stain.

PLATE 3

Fig. 4. *V. cholerae* NCTC 8021 after 3 serial subcultures at 24-hr intervals in tryptone water incubated at 37°. One organism bears a few short fimbriae. Formalin-fixed, shadowed.

Fig. 5. Vibrio 4716 after 3 serial subcultures at 24-hr intervals in tryptone water incubated at 37°. Two fimbriae are visible. Unfixed, shadowed.

PLATE 4

Fig. 6. Vibrio 4716 after 6 serial subcultures at 24-hr intervals on nutrient agar incubated at 37°. A dense network of strands is present and no fimbriae can be seen. Note the sheathed flagellum. Unfixed, negative stain.

Fig. 7. Vibrio 4716 after growth for 24 hr on nutrient agar inoculated from a stock culture. Any fimbriae which may be present cannot be detected because of the dense network of branched strands. Unfixed, negative stain.

PLATE 5

Fig. 8. *Escherichia coli* after 6 serial subcultures at 24-hr intervals on nutrient agar incubated at 37°. There are numerous straight fimbriae on one of the organisms. Unfixed, shadowed.

Fig. 9. *Pseudomonas multivorans* NCIB 9691 after 3 serial subcultures at 24-hr intervals in tryptone water incubated at 20°. The organism bears numerous straight fimbriae. Unfixed, shadowed.

PLATE 6

Fig. 10. *Aeromonas liquefaciens* NCMB 87 after 3 serial subcultures at 24-hr intervals in tryptone water incubated at 20°. One organism bears numerous straight fimbriae. Unfixed, negative stain.

Fig. 11. *A. liquefaciens* NCMB 87 after 3 serial subcultures at 24-hr intervals in tryptone water incubated at 20 °. The organism bears numerous straight fimbriae. Unfixed, shadowed.

Adansonian Analysis of the Rhizobiaceae

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SUMMARY

One hundred and ninety-one coded features of 21 strains of the genera *Rhizobium*, 18 of *Agrobacterium*, 11 of *Chromobacterium*, selected strains of *Vibrio cholerae*, *Flavobacterium*, and other representative strains of the families Enterobacteriaceae and Pseudomonadaceae were subjected to Adansonian analysis, with the use of a high-speed computer for the establishment of Similarity (S) and Matching (M) coefficients and for the sorting of the strains into taxonomic clusters. From the frequency of occurrence of features computed for each of the clusters, tables were prepared which provided correlated characteristics suitable for the description of the clusters. Hypothetical median organisms were also computed for each of the clusters and actual strains were selected as neotypes for the new genera, *Rhizobium* and *Phytomyxa*. The genus *Rhizobium* includes the fast-growing rhizobia and the agrobacteria and contains at least four species: *R. meliloti. R. leguminosarum, R. radiobacter, R. rubi*. The genus *Phytomyxa* is reserved for the slow-growing rhizobia which have been placed in the species *R. japonicum*. *Agrobacterium gypsophilae* and *A. pseudotsugae* are removed from both *Rhizobium* and *Phytomyxa* as defined in this study. The lack of significantly high intergeneric relationships amongst the members of the family Rhizobiaceae suggest revaluation of the family structure. From the results obtained here, the genera *Rhizobium* and *Phytomyxa* appear more closely related to the members of the Pseudomonadaceae.

INTRODUCTION

Bacterial taxonomy is at present experiencing a renewal of interest due mainly to developments in information science, molecular biochemistry and molecular genetics. Relationships amongst the soil bacteria as presented in the seven editions of *Bergey's Manual of Determinative Bacteriology* were based primarily on brilliant deductions and the intuitive genius of the pioneer bacteriologists such as Winogradsky, Beijerinck and others.

The soil bacteria received a good deal of attention in the earliest days of bacteriology and the taxonomy of the root nodule bacteria traces to the papers of Schroeter (1886) who separated the root nodule bacteria into two species which were based entirely on root nodule structure. Beijerinck (1888) described the isolation and distinct morphological forms of *Phytomyxa leguminosarum* and *Phytomyxa lupini*, the two species established by Schroeter. The root nodule bacteria have been grouped on characteristic legume root nodulation and subgrouped on the range and type of host plants infected. *Bergey's Manual* (1957) accords six of the subgroups (*Rhizobium*

leguminosarum Frank, *R. trifolii* Dangeard, *R. phaseoli* Dangeard, *R. meliloti* Dangeard, *R. japonicum* Kirchner, *R. lupini* Schroeter) specific status. This classification, however, has not fully held up to the tests of time and of new methodologies (Lange, 1961; Graham, 1964; De Ley & Rassel, 1965). Furthermore, the generic relationships within the Rhizobiaceae, i.e. intergeneric relationships of *Rhizobium*, *Agrobacterium* and *Chromobacterium*, as well as the intrageneric relationships require some careful reconsideration (Lange, 1961). The DNA homology studies of De Ley, Bernaerts, Rassel & Guilmot (1966a) also indicate the need for re-evaluation of the family Rhizobiaceae.

An evaluation of certain diagnostic features currently used in identification and classification of Rhizobium species was presented by Graham & Parker (1964) and an Adansonian analysis of Chromobacterium species was made by Sneath (1957), who provided an extended description for that genus. However, no comparative and complete descriptions of the genera of the Rhizobiaceae are available at the present time.

The study reported here is an Adansonian analysis of the Rhizobiaceae and is intended as an extended survey of the *in vitro* features used in the diagnosis of these bacteria. The relationships amongst the strains representing the genera *Rhizobium*, *Agrobacterium*, *Chromobacterium*, *Pseudomonas*, *Enterobacter*, *Escherichia*, *Vibrio* and *Proteus* have been examined on the basis of all available evidence, i.e. computed similarities, nucleic acid data and enzymic similarities. The frequencies of occurrence of each of the characters tested in the present analysis provide an extended description of the species within the family Rhizobiaceae as well as patterns of characters useful in diagnostic applications.

METHODS

Organisms

A total of 59 cultures was used in the study. The species represented and the source of isolates are listed in Table 1. The Rhizobium and Agrobacterium strains, with the exception of isolates 6466 and 5GLY.FE, were maintained on a modification of the basal medium described by Graham & Parker (1964): (in g./l.) $MgSO_4.7H_2O$, 0·25; $CaSO_4.2H_2O$, 0·03; $Na_2HPO_4.12H_2O$, 1·20; KH_2PO_4, 0·55; NaCl, 0·25; $FeSO_4.7H_2O$, 0·0035; $ZnSO_4.7H_2O$, 0·00016; $CuSO_4.5H_2O$, 0·00008; H_3BO_3, 0·0005; $MnSO_4.4H_2O$, 0·0004; Difco yeast extract, 0·25; Bacto agar, 20·0; mannitol, 10; pH 7·0. This medium (YM) with the addition of 1 % proteose peptone (YMP) was used for the maintenance of the remaining isolates included in the study. Each strain was examined for purity before testing was begun. Furthermore, before inoculation of each test medium, all cultures were streaked out to confirm purity. In addition to the strains listed in Table 1, 26 strains, including the genera *Pseudomonas*, *Serratia*, *Alcaligenes*, *Flavobacterium* and *Xanthomonas*, for which coded data from previous studies were in storage on the computer, were compared by retrieving their data.

Characters and methods of testing

A total of 191 characters were studied and coded for computer analysis. These are listed in the text which follows by character sequence number from the computer analysis (numbers in parentheses). The media for examination of physiological features was prepared by using a salts diluent (Graham & Parker 1964); however, the

Table 1. *Source of isolates included in the computer analysis*

Strain no.	Culture	Source
922*	*Rhizobium* sp.	*Glycine javanica*. M. M. Kennedy, Ayr, Queensland, Australia
942*	*Rhizobium* sp.	*Phaseolus aureus* CB 512 D. O. Norris, Taroom, Queensland, Australia
948*	*Rhizobium* sp.	*Phaseolus aureus* CB 121 D. O. Norris, Australia
952*	*R. japonicum*	*Glycine max* CB 1003 D. O. Norris, Thailand
963*	*Rhizobium* sp.	*Lotononis bainesii* CB 376 D. O. Norris, Australia
965*	*Rhizobium* sp.	*Arachis prostrata* CB 530 D. O. Norris, Australia
988	*Rhizobium* sp.	*Leucaena glauca* NGR 8 ex Trinnick, New Guinea
989	*Rhizobium* sp.	*Clitoria ternatea* CB 930 D. O. Norris, Australia
997*	*Rhizobium* sp.	*Dolichos africanus* CB 756 D. O. Norris, Marandellos, South Rhodesia
998*	*Rhizobium* sp.	*Desmodium intortum* CB 627 D. O. Norris, Ex Bonnier, Congo
WU 425†	*Rhizobium* sp.	*Ornithopus compressus* strain S 3 (M. D. Brocx), Esperance, Western Australia (effective on lupins and Ornithopus)
10317	*Rhizobium* sp.	ATCC
WU 7	*R. lupini*	D 27, Swanbourne, Western Australia (isolated by Dr R. T. Lange) authentic 1960
10318	*R. lupini*	ATCC
SU 216‡	*R. meliloti*	*Medicago sativa* strain 107 S.C.W.-5. Wisconsin, U.S.A. (Parker)
SU 277	*R. meliloti*	*Medicago tribuloides*. M. Tr. Perth, Western Australia (seed from Perth but nodulated plant from Leeton Exp. Farm as 271–279)
SU 298	*R. trifolii*	Isolated from effective plant of Crimson Clover L 17 4 A/2
SU 299	*R. leguminosarum*	Vetch and pea culture, New South Wales Department of Agriculture, Australia (originally from U.S.A.)
SU 301	*R. leguminosarum*	Pea B-isolated from effective Field Pea Dunoon, 1952
SU 308	*R. trifolii*	A.G. 14 K. J. Baird, Armidale, New South Wales, Australia
SU 312	*R. phaseoli*	Bean culture, New South Wales Department of Agriculture (1955 Reclassified Nal), Australia
4720	*Agrobacterium tumefaciens*	American Type Culture Collection (ATCC) Washington, D.C.
11156	*A. tumefaciens*	ATCC
11157	*A. tumefaciens*	ATCC
B 6	*A. tumefaciens*	P. K. Chen, Georgetown University, Wash., D.C. (highly virulent strain)
A 6 RI	*A. tumefaciens*	P. K. Chen, Georgetown University, Washington D.C. (virulent strain)
A 66 RI	*A. tumefaciens*	P. K. Chen, Georgetown University, Washington D.C. (attenuated strain)
5 GLY. FE	*A. tumefaciens*	P. K. Chen, Georgetown University, Washington D.C. (avirulent strain)
4718	*A. radiobacter*	ATCC
6466	*A. radiobacter*	ATCC
A. RADIO.	*A. radiobacter*	P. K. Chen, Georgetown University, Wash., D.C.
AR 1001	*A. radiobacter*	P. K. Chen, Georgetown University, Wash., D.C.
AR 1012	*A. radiobacter*	P. K. Chen, Georgetown University, Wash., D.C.
11325	*A. rhizogenes*	ATCC
13333	*A. rhizogenes*	ATCC
13334	*A. rubi*	ATCC
13335	*A. rubi*	ATCC

Table 1 (*cont.*)

Strain no.	Culture	Source
13330	*A. pseudotsugae*	ATCC
13331	*A. pseudotsugae*	ATCC
13329	*A. gypsophilae*	ATCC
12472	*Chromobacterium violaceum*	ATCC
6357	*C. violaceum*	ATCC
12540	*C. violaceum*	ATCC
12541	*C. violaceum*	ATCC
12542	*C. violaceum*	ATCC
553	*C. violaceum*	ATCC
13426	*C. violaceum*	ATCC
6918	*C. viscosum*	ATCC
12473	*C. lividum*	ATCC
6915	*C. amethystinum*	ATCC
16266	*Pseudomonas fluorescens* var. *antirrhinastri*	Pathogenic on antirrhinum seedlings; Queensland, Australia
13430	*P. fluorescens*	ATCC
14216	*P. aeruginosa*	ATCC
14033	*Vibrio cholerae*	ATCC
W1485	*Escherichia coli* K12	S. Falkow, Walter Reed Army Institute of Research, Washington D.C.
4115	*Enterobacter aerogenes*	S. Falkow, Walter Reed Army Institute of Research, Washington, D.C.
9247TR	*Proteus morganii*	S. Falkow, Walter Reed Army Institute of Research, Washington, D.C.
PM-1	*Proteus mirabilis*	S. Falkow, Walter Reed Army Institute of Research, Washington, D.C.
.	*Providence* sp.	S. Falkow, Walter Reed Army Institute of Research, Washington, D.C.

* Strain numbers refer to strains obtained from A. Diatloff, Queensland Department of Primary Industries.

† WU strain numbers refer to strains obtained from Dr C. A. Parker, University of Western Australia.

‡ SU strains received from Dr P. H. Graham, University of Sydney.

media of Simmons and Paton (see below) were made up with distilled water. All tests were read at 1, 2, 7 days and 2, 3, and 4 weeks except for nutritional requirements which were read after 7 days and discarded. Plate inoculations were made by the spot drop (Colwell, 1964) or replica plate technique (Lederberg, 1950). Liquid media were inoculated with 2 drops from a Pasteur pipette of a 3-day culture or, in the case of the slow-growing organisms, 5-day culture.

Morphological characters (1–15) were scored from cultures grown at 25° on agar after 2 days (fast-growing isolates) and 5 days (slow-growing isolates), and examined by phase-contrast microscopy. The characters scored included: rods; curved rods; (oval) spheres; presence of filaments; refractile appearance of organisms; distribution of organisms as predominantly singles, pairs or chains; round or tapered ends; spirals (rods); length 1–3 μ or 3–5 μ; and width 0·6–1·0 μ or 0·2–0·6 μ.

Motility (16) was scored from wet mount preparations examined under phase-contrast microscopy. Examination of broth cultures for motility was done at the same time as the growth characteristics in liquid media were scored (see below).

Flagella stains (17–18) of smears from YM agar slants (Rhizobium species) and

nutrient agar (remaining isolates) were used to distinguish polar from peritrichous flagellar arrangements (Leifson, 1951).

The Gram stain (19–21) used was the Hucker modification (Society of American Bacteriologists, 1957) and cultures were scored as Gram negative, positive, or variable.

Colonial characteristics (22–34) were determined on YM and YMP agar at 3 days and also at 5–10 days for the Rhizobium species. The characters included: small colony size (1–2 mm.); medium colony size (2–5 mm.); convex; rough; opaque; translucent; entire edge, spreading on agar surface; white colony colour; off white colony colour; grey colony colour; butyrous consistency; gum production; mucilaginous colony.

Presence or absence of capsules (35) was determined by using indian ink (Bradshaw, 1964) and *metachromatic granules* (36) with methylene blue (Bradshaw, 1964).

Pigment production (37–40), as a diffusible green pigment, diffusible blue pigment, or visible insoluble pigment, was scored from examination of YM agar, YMP agar, Pseudomonas agar F (Difco), and Pseudomonas agar P (Difco). Fluorescence was determined in a liquid medium (Paton, 1959).

Growth characteristics in liquid media (41–47) included: presence of pellicle or ring; granular or even turbidity; slight, moderate or heavy turbidity with Brown's opacity tubes as reference. Characteristics in liquid media were determined for all isolates in YM broth at 2 and at 5 days, as well as in YMP broth for those isolates which preferentially in a medium with an organic nitrogen source.

Carbohydrate metabolism (48–89). Oxidative and fermentative utilization of carbohydrates was determined by the method of Hugh & Leifson (1953). This medium, without agar and with an inverted inner vial, was also used to detect gas production from carbohydrates. Growth in glucose with and without 10^{-3} M-iodoacetate was determined. The terminal pH value in the glucose fermentation tubes was also measured. Growth and production of acetic acid in ethanol agar was recorded (Shimwell, Carr & Rhodes, 1960). Acid production from carbohydrates (1 %, w/v) and ethanol (5 %, w/v) was determined on agar plates, with the peptone omitted for Rhizobium and Agrobacterium species. The carbohydrates were sterilized by filtration except dulcitol, inulin and dextrin which were steamed for 1 hr on three successive days. The carbohydrates which were tested were: glucose, maltose, lactose, sucrose, galactose, mannitol, adonitol, arabinose, cellobiose, dextrin, dulcitol, fructose, glycerol, inositol, inulin, mannose, melibiose, melezitose, raffinose, rhamnose, salicin, sorbitol, trehalose, xylose, ethanol. Hydrolysis of aesculin and starch, production of dihydroxyacetone from glycerol, and digestion of agar were tested by the methods cited by Colwell (1964). Examination for levan production on YM and YMP agar by the Graham & Parker (1964) modification of the Paton (1960) technique was also done.

The methyl red and Voges-Proskauer tests (90–91) were performed following the techniques described in the *Manual of Microbiological Methods* (1957).

Temperature range of growth (92–102) was determined in YM and YMP broth. Before inoculation for temperature growth tests, the medium was incubated at the given test temperature overnight. Temperatures studied were: 0, 5, 15, 20, 30, 35, 37, 40, 41, 42 and 44°.

NaCl tolerance (103–108) was tested on YM and YMP agar plates (Ionagar, Difco Labs, Chicago, Illinois) adjusted to the required NaCl concentrations: 0, 0·5, 3·0, 5·0, 7·0, 10·0 % (w/v).

The pH range of growth (109–116) was determined in YM and YMP broth adjusted to the required pH values before autoclaving, but in the case of pH 4·0, 4·5, 5·0, 9·0 and 10·0 the media were readjusted and dispensed aseptically after autoclaving. The pH values tested included: 4·0, 4·5, 5·0, 6·0, 7·0, 8·0, 9·0 and 10·0.

The oxidase, cytochrome oxidase, and catalase tests (117–119) were made by the methods cited by Colwell (1964).

The production of phosphatase (120) was detected on YM and YMP agar by the method of Baird-Parker (1963).

Sensitivity to antibiotics and antibacterials (121–133). Sensitivity to the o/129 pteridine compound was tested following the method of Shewan, Hodgkiss & Liston (1954). Antibiotic sensitivities were determined by using BBL (Baltimore Biological Laboratories, Baltimore, Md.) 'Sensidiscs'. Resistance to concentrations of $HgCl_2$ was determined by incorporation of the compound in agar media. YM and YMP agar was used in all instances where sensitivities to antibiotics and antibacterials were tested. The substances and concentrations (per ml.) used were: penicillin, 10 units; dihydrostreptomycin, 10 μg; chloromycetin, 30 μg; erythromycin, 15 μg.; kanamycin, 30 μg.; aureomycin, 30 μg.; novobiocin, 30 μg.; polymyxin B, 300 units; terramycin, 30 μg.; tetracycline, 30 μg.; $HgCl_2$, 1 μg and 10 μg.

Reduction of nitrate and nitrite, gelatin liquefaction and litmus milk tests (134–143) were made following the technique of the *Manual of Microbiological Methods* (1957) and scored as described by Colwell (1964).

Growth on skim milk agar and casein hydrolysis (144–145) were determined with the skim milk agar medium of Gordon & Mihm (1959).

Production of ammonia from peptone at 14 days and hydrogen sulphide from peptone in lead acetate agar (146–147) was measured following the techniques described in the *Manual of Microbiological Methods* (1957). *Hydrogen sulphide production from cystine and cysteine* (148–149) was determined in micro-tubes with lead acetate paper (Colwell & Quadling, 1962).

Urease production (150) was tested according to the method of Christensen (1946) with peptone omitted from the media for the Rhizobium and Agrobacterium species.

Indole production (151) was tested by the Kovacs modification cited in the *Manual of Microbiological Methods* (1957).

Lecithinase (152) production was tested on YM and YMP agar plates in which 5 % (v/v) concentrated egg-yolk emulsion (Oxoid, Colab, Inc., Chicago, Illinois) was incorporated.

Lipolytic activity (153–156) was determined by detection of hydrolysis of Tweens 20, 40, 60 and 80, respectively, in YM and YMP agar following the method of Sierra (1957).

The production of 3-ketolactose (157) was determined by the method of Bernaerts & De Ley (1963).

Ability to utilize citrate (158–159) was determined by growth in Koser's citrate (Koser, 1924) modified by the addition of 1·5 % (w/v) Ionagar (Difco Labs., Detroit, Michigan) and inoculation by the replica plate technique. Reaction in Simmons's citrate (Simmons, 1926) was determined on agar slopes.

Oxidation of calcium lactate through acetate to carbonate and the utilization of 0·3 % sodium malonate, 0·1 % sodium acetate, 0·1 % sodium formate as carbon source and 0·1 % $NH_4H_2PO_4$ as nitrogen source were tested (160–164). For the nutritional

tests, a basal salts agar containing 0·00025 % yeast extract and 0·1 % peptone for strains other than the Agrobacterium and Rhizobium species was used. Details of these procedures were as cited by Colwell (1964). Tests for utilization of the carbon or nitrogen source were always done by comparison with a control plate containing only the basal salts medium or basal salts medium containing ·00025 % yeast extract and +0·1 % peptone where incorporated into the test medium.

The utilization of amino acids as carbon and nitrogen source (165–176) was deter-mined from basal salts agar with 0·00025 % yeast extract and 0·1 % of the test amino acid added. The amino acids were filter-sterilized, except for tyrosine and cystine which were steamed for 1 hr on three successive days and used at a final concentration of 0·05 %. Ionagar (Difco Labs., Detroit, Michigan) was used in the nutritional studies and inoculations were by the replica plating technique. Amino acids tested included: L-ornithine monochloride; L-arginine hydrochloride; L-lysine; L-alanine; L-histidine; L-serine; L-phenylalanine; L-proline; L-leucine; L-glutamic acid; L-cystine; L-tyrosine.

Growth, fluorescence, and production of 2-ketogluconate from potassium gluconate in Paton medium (Paton, 1959) were scored (177–179). Growth in Haynes medium and production of 2-ketogluconate from gluconate (Haynes, 1951) was also recorded (180–181).

Arginine dihydrolase and the arginine, ornithine and lysine decarboxylases (182–185) were tested following the methods of Thornley (1960) and Moeller (1955).

Methylene blue reduction (186) was determined by adding 1 % aqueous methylene blue to a 48-hr (fast-growing organisms) or 4-day (slow-growers) YM and YMP broth culture and incubating for 1 hr.

Pectate hydrolysis (187) was detected by the method of Starr (1947).

Ability to produce penicillinase (188) was determined by the technique described by Foley & Perret (1962).

The methods for *production of phenylpyruvic acid and/or melanin from phenylalanine and production of melanin from tyrosine* (189–191) were as cited by Colwell (1964).

The results were scored for computation by recording positive reaction, i.e. growth present or positive test result, as unit features '1', and negative reaction, i.e. no growth or negative test result as '0'. In this study all organisms were tested for all features; however, the programs for analyisng the data were written so that tests not done or not applicable might be scored '3'. The computer then did not include these entries in similarity comparisons.

An IBM 1620 computer, Model II, with 1311 Disk Pack System, was used in the numerical analyses of the data. The programs where the S value was calculated, com-paring positive matches, and the M value, comparing positive and negative matches, have been documented for the IBM 1620 Computer User's Library (GTP-2 and GTP-5). The frequency of feature occurrence within the major clusters (IBM Program GTP-3) followed the method of Colwell (1964).

The median organism was obtained by computer by using the program GTP-4 based on the method described by Liston, Wiebe & Colwell (1963).
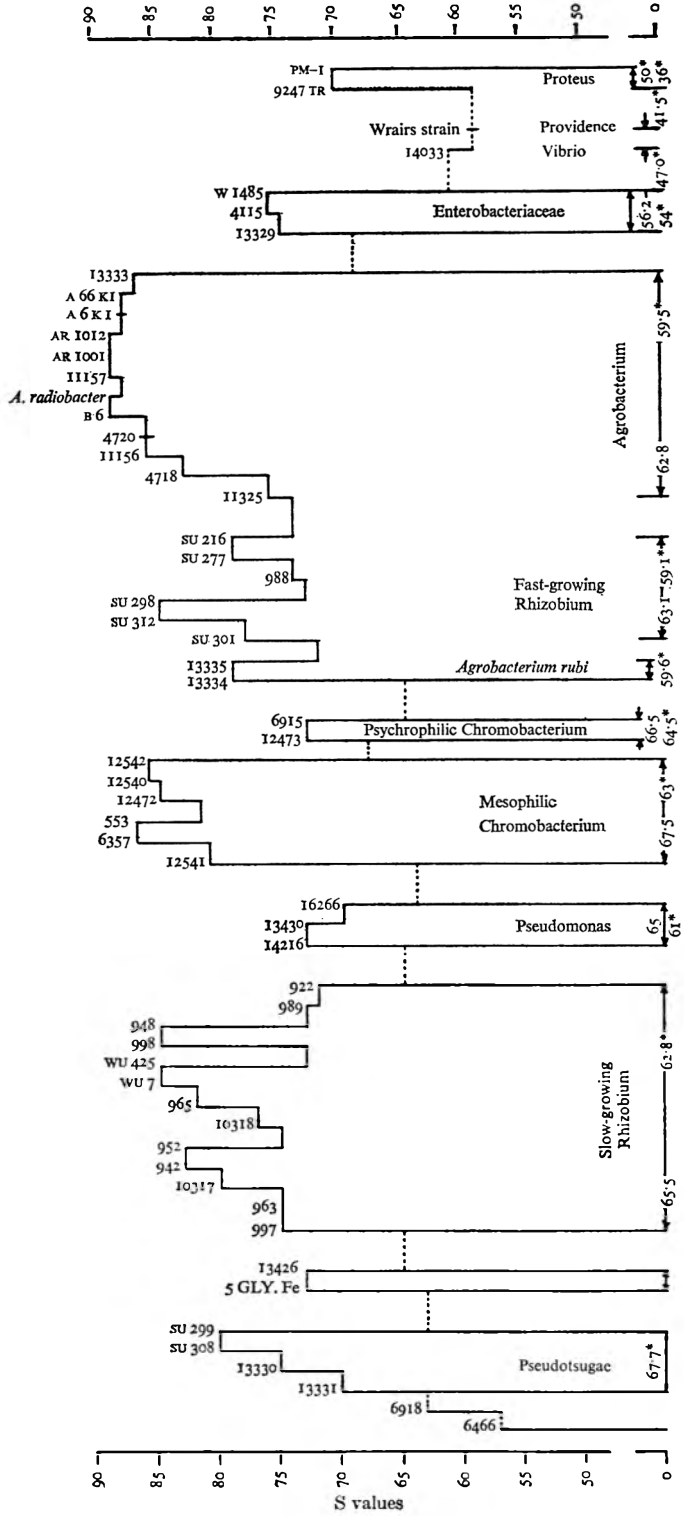
Fig. 1. Sorted clusters from the GTP-2 program output, based on highest linkages.

RESULTS

The groupings obtained by using S values from the computer analysis of the taxonomic data for the 59 strains included in the study are presented in Fig. 1. As seen from Fig. 1, at $S \geqslant 70$, eight clusters were formed. The three major clusters of related strains consisted of: slow-growing Rhizobium strains $S \geqslant 72$; Agrobacterium species and the fast-growing Rhizobium strains at $S \geqslant 72$; and the mesophilic Chromobacterium strains $S \geqslant 81$. The minor clusters were: *Agrobacterium pseudotsugae* and two fast-growing Rhizobium strains at $S \geqslant 70$; strain 5GLY.FE with *Chromobacterium violaceum* strain 13426 at $S = 73$; the Pseudomonas species which grouped at $S \geqslant 70$; psychrophilic Chromobacterium strains grouping at $S \geqslant 73$; and Proteus species, $S \geqslant 70$.

The M-value calculation (Silvestri, Turri, Hill & Gilardi, 1962), in which shared negative features are taken into account in computing strain similarities, was also applied to the taxonomic data for the 59 strains. The clusters which formed were found to be essentially the same as those grouped by means of S-value relationships. The main difference, however, was that from M-value computations, strains clustered at what could be interpreted as a much higher degree of similarity, namely the slow-growing Rhizobium strains clustering at $M \geqslant 85$. Genus or species inter- and intra-relationships were difficult to interpret from inspection of the M-value output. In fact, the strains studied were merged into two major groups at $M \geqslant 80$. In Fig. 2 the groups sorted by shared M-values of 80 % or greater are presented.

Fig. 2. Cluster output from GTP-2 showing groups forming at $M = 80\%$ (M = 'Matching coefficient' of Silvestri *et al.* 1962).

Group 1: (16266) *P. fluorescens* var. *antirrhinastri*+(13430) *P. fluorescens*+(14216) *P. aeruginosa*+(922) *Rhizobium* sp.+(988) *Rhizobium* sp.+(948) *Rhizobium* sp.+(998) *Rhizobium* sp.+(WU 425) *Rhizobium* sp.+(WU 7) *R. lupini*+(965) *Rhizobium* sp.+(10318) *R. lupini*+(952) *R. japonicum*+(942) *Rhizobium* sp.+(10317) *Rhizobium* sp.+(963) *Rhizobium* sp.+(997) *Rhizobium* sp.+(13426) *C. violaceum*+(5 Gly. Fe) *A. tumefaciens*+SU 299 *R. leguminosarum*+(SU 308) *R. trifolii*+(13330) *A. pseudotsugae*+(13331) *A. pseudotsugae*+(6466) *A. radiobacter*.

Group 2: (W 1485) *E. coli*+(4115) *E. aerogenes*+(13329) *A. gypsophilae*+(4718) *A. tumefaciens*+(11156) *A. tumefaciens*+(AR 1012) *A. radiobacter*+*A. radiobacter*+(11157) *A. tumefaciens*+(AR 1001) *A. radiobacter*+(B 6) *A. tumefaciens*+(A 6 KI) *A. tumefaciens*+(A 66 KI) *A. tumefaciens*+(4720) *A. tumefaciens*+(13333) *A. rhizogenes*+(11325) *A. rhizogenes*+(SU 277) *R. meliloti*+(SU 216) *R. meliloti*+(988) *Rhizobium* sp.+(SU 301) *R. leguminosarum*+(SU 312) *R. phaseoli*+(SU 298) *R. trifolii*+(13335) *A. rubi*+(13334) *A. rubi*.

Group 3: (6915) *C. amethystinum*+(12473) *C. lividum*+(P 66) *X. phaseoli*+(12542) *C. violaceum*+(12540) *C. violaceum*+(12472) *C. violaceum*+(533) *C. violaceum*+(6375) *C. violaceum*+(12541) *C. violaceum*.

Group 4: (PM-1) *P. mirabilis*+(9247 TR) *P. morgani*.

Group 5: (17) *Pseudomonas*+ *P. viscosa*+(P 51) *P. atrofaciens*+(P 8) *P. atrofaciens*+(P 9) *P. coronafaciens*+(P 28) *P. lachrymans*+(P 26) *P. pisi*+(P 11) *P. pisi*+(P 33) *P. phaseolicola*+(P 3) *P. phaseolicola*+(P 10) *P. glycinea*.

Group 6: (4358) *F. arborescens*+(8315) *F. flavescens*+(958) *F. suaveolens*+(338) *P. maltophila*+(16) *A. radiobacter*.

Group 7: (BS) *Alc. faecalis*+(14 L) *Pseudomonas*+ *Achr. iophagus*.

Group 8: (SM 10) *S. marcescens*+(SMI) *S. marcescens*+(SM 21) *S. marcescens*.

The S value relationship $(S = NS/N_s + N_D$, where N_s = number of similar matches and N_D = number of dissimilar matches, i.e. features positive for one strain but not the other and vice versa) were used for subsequent analyses of the taxonomic data. In Fig. 3 the formation of the groups can be followed. Eleven groups formed at

Fig. 3. Cluster output from GTP-2 showing groups formed at selected levels of similarity based on S-value calculations (Sokal & Sneath, 1963).

Clustering at S = 80.
Group 1: (948) *Rhizobium* sp. + (998) *Rhizobium* sp.
Group 2: (WU 425) *Rhizobium* sp. + (WU 7) *R. lupini.*
Group 3: (952) *R. japonicum* + (942) *Rhizobium* sp. + (10317) *Rhizobium* sp.
Group 4: (SU 308) *R. trifolii* + (SU 299) *R. leguminosarum.*
Group 5: (SU 312) *R. phaseoli* + (SU 298) *R. trifolii.*
Group 6: (4718) *A. radiobacter* + (11156) *A. tumefaciens* + (4720) *A. tumefaciens* + (B 6) *A. tumefaciens* + *A. radiobacter* + (11157) *A. tumefaciens* + (AR 1001) *A. radiobacter* + (AR 1012) *A. radiobacter* + (A 6 RI) *A. tumefaciens* + (A 66 RI) *A. tumefaciens* + (13333) *A. rhizogenes.*
Group 7: (12542) *C. violaceum* + (12540) *C. violaceum* + (12472) *C. violaceum* + (553) *C. violaceum* + (6357) *C. violaceum* + (12541) *C. violaceum.*
Group 8: (P 51) *P. atrofaciens* + (P 8) *P. atrofaciens* + (P 9) *P. coronafaciens.*
Group 9: (P 11) *P. pisi* + (P 26) *P. pisi* + (P 28) *P. lachrymans.*
Group 10: (609) *Alc. faecalis* + (14 L) *Pseudomonas.*
Group 11: (SM 10) *S. marcescens* + (SMI) *S. marcescens* + (SM 21) *S. marcescens.*

Clustering at S = 75.
Group 1: (948) *Rhizobium* sp. + (998) *Rhizobium* sp.
Group 2: (WU 425) *Rhizobium* sp. + (WU 7) *R. lupini* + (965) *Rhizobium* sp. + (10317) *Rhizobium* sp. + (10318) *R. lupini* + (952) *R. japonicum* + (942) *Rhizobium* sp. + (963) *Rhizobium* sp. + (997) *Rhizobium* sp.
Group 3: (SU 216) *R. meliloti* + (SU 277) *R. meliloti.*
Group 4: (SU 301) *R. leguminosarum* + (SU 312) *R. phaseoli* + (SU 298) *R. trifolii.*
Group 5: (13330) *A. pseudotsugae* + (SU 308) *R. trifolii* + (SU 299) *R. leguminosarum.*
Group 6: (11325) *A. rhizogenes* + (4718) *A. radiobacter* + (11156) *A. tumefaciens* + (4720) *A. tumefaciens* + (B 6) *A. tumefaciens* + *A. radiobacter* + (11157) *A. tumefaciens* + (AR 1001) *A. radiobacter* + (AR 1012) *A. radiobacter* + (A 6 RI) *A. tumefaciens* + (A 66 RI) *A. tumefaciens* + (13333) *A. rhizogenes.*
Group 7: (13334) *A. rubi* + (13335) *A. rubi.*
Group 8: (12542) *C. violaceum* + (12540) *C. violaceum* + (12472) *C. violaceum* + (553) *C. violaceum* + (6357) *C. violaceum* + (12541) *C. violaceum.*
Group 9: (W 1485) *E. coli* + (4115) *E. aerogenes* + (13329) *A. gypsophilae.*
Group 10: (P 53) *P. phaseolicola* + (P ii) *P. pisi* + (P 26) *P. pisi* + (P 28) *P. lachrymans* + (P 8) *P. atrofaciens* + (P 9) *P. coronafaciens* + (P 3) *P. phaseolicola.*
Group 11: (609) *Alc. faecalis* + (14 L) *Pseudomonas.*
Group 12: *P. viscosa* + (17) *Pseudomonas.*
Group 13: (SM 10) *S. marcescens* + (SMI) *S. marcescens* + (SM 21) *S. marcescens.*

Clustering at S = 70.
Group 1: (922) *Rhizobium* sp. + (989) *Rhizobium* sp + (948) *Rhizobium* sp. + (998) *Rhizobium* sp. + (WU 425) *Rhizobium* sp. + (WU 7) *R. lupini* + (965) *Rhizobium* sp. + (10318) *R. lupini* + (952) *R. japonicum* + (942) *Rhizobium* sp. + (10317) *Rhizobium* sp. + (963) *Rhizobium* sp. + (997) *Rhizobium* sp.
Group 2: (13334) *A. rubi* + (13335) *A. rubi* + (SU 301) *R. leguminosarum* + (SU 312) *R. phaseoli* + (SU 298) *R. trifolii* + (998) *Rhizobium* sp. + (SU 277) *R. meliloti* + (SJ 216) *R. meliloti* + (11325) *A. rhizogenes* + (4718) *A. radiobacter* + (11156) *A. tumefaciens* + (4720) *A. tumefaciens* + (B 6) *A. tumefaciens* + *A. radiobacter* + (11157) *A. tumefaciens* + (AR 1001) *A. radiobacter* + (AR 1012) *A. radiobacter* + (A 6 RI) *A. tumefaciens* + (A 6 RI) *A. tumefaciens* + (13333) *A. rhizogenes.*
Group 3: (13331) *A. pseudotsugae* + (13330) *A. pseudotsugae* + (SU 308) *R. trifolii* + (SU 299) *R. leguminosarum.*
Group 4: (12473) *C. lividum* + (6915) *C. amethystinum.*
Group 5: (P 60) *X. phaseoli* + (12542) *C. violaceum* + (12540) *C. violaceum* + (12472) *C. violaceum* + (6357) *C. violaceum* + (553) *C. violaceum* + (12541) *C. violaceum.*
Group 6: (16266) *P. fluorescens* var. *antirrhinastri* + (13430) *P. fluorescens* + (14216) *P. aeruginosa.*
Group 7: (SM 10) *S. marcescens* + (SMI) *S. marcescens* + (SM 21) *S. marcescens* + (13329) *A. gypsophilae* + (4115) *E. aerogenes* + (W 1485) *E. coli.*
Group 8: (PM-1) *P. mirabilis* + (9247 TR) *P. morgani.*
Group 9 (13426) *C. violaceum* + (5 GLY. FE) *A. tumefaciens.*
Group 10: (P 10) *P. glycinea* + (P 33) *P. phaseolicola* + (P 11) *P. pisi* + (P 26) *P. pisi* + (P 28) *P. lachrymans* + (P 8) *P. atrofaciens* + (P 51) *P. atrofaciens* + (P 9) *P. coronafaciens* + (P 3) *P. phaseolicola.*
Group 11: (609) *Alc. faecalis* + (14 L) *Pseudomonas.*
Group 12: *P. viscosa* + (17) *Pseudomonas.*

S $\geqslant$ 80, 13 groups at S $\geqslant$ 75, and 12 groups at S $\geqslant$ 70. Figures 2 and 3 also include the data for other reference strains retrieved from the computer data library. As can be seen from Figs. 1 and 3, separation of the Rhizobia and Agrobacteria from Chromobacterium, Pseudomonas, Serratia, Enterobacter, Escherichia, and Proteus species was at S-value levels of $<$ 70 %.

From examination of Fig. 3 certain results may be noted. The slow-growing Rhizobia and the fast-growing Rhizobia/Agrobacteria clusters are distinct, forming from pairs at S $\geqslant$ 80 into the two clusters at S $\geqslant$ 70. The strains *Agrobacterium pseudotsugae* 13330 and 13331, *Rhizobium trifolii* SU 308 and *R. leguminosarum* SU 299 formed a separate and distinct cluster, unrelated to the two major groups. DNA base-composition data available for these strains (see Fig. 1) indicated a significantly different over-all guanine + cytosine DNA base composition for these strains when compared with strains of the two major groups. Other DNA base composition data for these groups also correlated well with the sorting of the strains obtained from the computer analysis.

Agrobacterium gypsophilae 13329 shared high relationships with the strains of *Escherichia coli*, *Enterobacter aerogenes* and *Serratia marcescens* (S = 70, group '7; Fig. 3). An electrophoretic study of the isozymes and protein patterns of *A. gypsophilae* and other agrobacteria currently underway in our laboratories has provided confirmatory evidence for removing *A. gypsophilae* from the agrobacteria (P. K. Chen, personal communication).

Proteus strains (S = 70, group 8) did not cluster with other strains representing the Enterobacteriaceae (S = 70, group 7). Soil isolates labelled by other investigators as Pseudomonas species (S = 70, group 10) did not cluster with *Pseudomonas fluorescens* and *P. aeruginosa* (S = 70, group 6). These strains and the strains of groups 11 and 12 will be considered in a separate publication.

The Chromobacterium strains formed two major clusters corresponding to the mesophilic (*C. violaceum*) and psychrophilic (*C. lividum*) groups described by Sneath (1960). These two groups joined at S = 68 %. The single strain of *C. viscosum* which was tested did not cluster with the Chromobacterium groups but rather joined at a low level (S = 63 %) to the Rhizobium and *A. pseudotsugae* strains. Also, as noted above, *C. violaceum* 13426 joined only with *A. tumefaciens* 5 GLY. FE and neither of these strains shared significantly high similarities with any of the other isolates tested.

Thus, intra-group relationships for the Rhizobiaceae, and inter-group relationships for the family with the Pseudomonadaceae and Enterobacteriaceae can be observed from Fig. 1. Relationships with Serratia, Flavobacterium, Alcaligenes and Xanthomonas strains tested are given in Figs. 2 and 3.

The characters shared by all strains of the species and genera comprising the family Rhizobiaceae examined in the present study are listed in Tables 2 and 3. Thus, all the strains tested as Gram-negative rods, 1–3 μ length × 0·6–1·0 μ width, round-ended, motile, and occurring mainly as single or paired cells. Colonies on YM and YMP agar were 1–2 mm diameter, convex, opaque, and entire. Growth within the temperature range 20–30°, pH 6–8, and NaCl concentration of 0–0·5 % was noted. All members of the Rhizobiaceae studied were catalase-positive. None of the strains produced a capsule detectable by the method employed. None grew in YM or YMP broth with NaCl added to final concentrations of 5 % or greater. None produced gas from carbohydrates, acetic acid from ethanol, dihydroxyacetone from glycerol, or a urease detectable within 48 hr. The strains were all methyl-red negative and Voges–Proskauer

negative. None of the strains demonstrated starch or pectate hydrolysis, agar digestion, or production of phenylpyruvic acid from phenylalanine. All strains were negative for hydrogen sulphide production in lead acetate agar but positive tests were
recorded when other methods were employed for hydrogen sulphide production (see
Table 4). The characters which were variable for the groups are listed in Table 4.

Table 2. *Positive characters, i.e. feature frequency of 1·00, for the major groups defined
by the computer analysis: (1) slow-growing Rhizobium; (2) Agrobacterium/fast-growing
Rhizobium; and (3) Chromobacterium.*

Character no.	Character	Character no.	Character
1	Straight rods	19	Gram-negative
6	Predominant arrangement as single cells	24, 26, 28	Convex, opaque colonies with entire edge
9	Round ends	95, 96	Growth within temperature range 20–30°
12	1–3 μ length	103, 104	Growth at NaCl concentrations 0–0·5%
14	0·6–1·0 μ width	112–114	Growth within pH range 6·0–8·0
16	Motile	119	Catalase-positive

Table 3. *Negative characters, i.e. feature frequency of 0, for the major groups defined
by the computer analysis: (1) slow-growing Rhizobium; (2) Agrobacterium/fast-growing
Rhizobium; and (3) Chromobacterium*

Character no.	Character	Character no.	Character
2	Curved rods	88	Agar digestion
3	(Oval) spheres	90	Methyl-red reaction
4	Filaments	91	Voges–Proskauer reaction
5	Refractile cells	108	Growth in 10% NaCl
10	Tapered end	147	H₂S produced from peptone in lead acetate agar
11	Spiral (rods)	150	Urease production (48 hr)
35	Capsule	151	Indole production
41	Pellicle formation in YM broth	157	3-ketolactose production
50	Glucose, gas production	184	Ornithine decarboxylase
53	Acetic acid production from ethanol	185	Lysine decarboxylase
86	Starch hydrolysis	187	Pectate hydrolysis
87	Dihydroxyacetone production from glycerol	189	Phenylpyruvic acid production from phenylalanine

The slow-growing *Rhizobium* species cluster demonstrated, in addition to the
characters listed in Tables 2 and 3, the following features: polar flagella; mucilaginous
colonies; metachromatic granules; a slight turbidity in YM broth; little or no detectible utilization of carbohydrates; production of levan; growth in the temperature
range 15–37°; tolerance of NaCl in concentrations up to 3·0%; growth in the pH
range 4–8; oxidase positive; no sensitivity to the 0/129 pteridine compound or to
polymyxin B and marked sensitivity to HgCl₂ at 10 μg. concentration; production of
an alkaline reaction in litmus milk and a detectible penicillinase. Of the characters for
which the strains tested uniformly negative, the following may be noted: phosphatase;
nitrite reduction; gelatin liquefaction; litmus milk peptonization; casein hydrolysis;

Table 4. *Characters with the variable frequency of occurrence amongst the major groups and subgroups defined by the computer analysis*

Characters with frequency of occurrence 0–0·25, scored (−), or 0·75–1, scored (+), were selected and tabulated. The sequence of characters as listed is as coded for the computer. For details of methods for performing tests, scoring, etc., see Methods. ± = Variable occurrence within the set of strains tested. Actual frequency of occurrence is given in parentheses.

					Agrobacterium/fast-growing *Rhisobium*			
Cha-rac-ter no.	Character	Slow-growing *Rhizobium*	*Chromobacterium*		*A. radio-bacter tume-faciens rhizogenes*	*R. meli-loti**	*R. legumi-nosarum trifolii phaseoli*	*A. rubi**
			C. vio-laceum	*C. livi-dum**				
	Morphological and cultural:							
17	Polar flagella	+	+	+	−	−	−	−
18	Peritrichous flagella	−	+	+	+	+	+	+
22	Colony size 1–2 mm dia.	+	+	+	+(0·75)	+	(±0·66)	+
34	Mucilaginous colonies	+(0·92)	−	−	+	+	+	+
36	Metachromatic granules	+	+(0·83)	−	−(0·17)	±(0·50)	−	+
45	Slight turbidity in YM broth	+	+	±(0·50)	−	±(0·50)	±(0·33)	±(0·50)
46	Moderate turbidity in YM broth	−	−	±(0·50)	+(0·92)	+	±(0·66)	±(0·50)
	Carbohydrates:							
48	Glucose (Hugh & Leifson, 1953) oxida-tive, Acid	−(0·08)	+	+	+(0·92)	+	+	±(0·50)
49	Glucose (Hugh & Leifson, 1953) fermen-tative, Acid	−	+	−	+	+	+	+
51	Glucose+iodoacetate, growth	+(0·85)	+	±(0·50)	+(0·92)	+	±(0·66)	±(0·50)
52	Glucose+iodoacetate, acid	−	±(0·50)	−	(+0·75)	+	±(0·33)	−
55	Maltose, acid	−(0·08)	+	±(0·50)	+	+	+	+
57	Lactose, acid	−	−	−	+(0·92)	+	+	+
59	Sucrose, acid	(0·15)	±(0·33)	+	+(0·83)	+	+	+
61	Galactose, acid	±(0·54)	−(0·17)	+	+	+	+	+
63	Mannitol, acid	−	−	−	±(0·58)	±(0·50)	+	+
65	Terminal pH in glu-cose fermentation							
	pH 6·5–7·0	+(0·92)	N.T.†	N.T.	+(0·82)	±(0·50)	−	+
	pH 5·0–6·0	−	N.T.	N.T.	−(0·09)	−	±(0·33)	−
66	Adonitol, acid	−	−	−	+	+	+	+
67	Arabinose, acid	±(0·30)	−	+	+	+	+	+
68	Cellobiose, acid	−	−	+	+	+	+	±(0·50)
69	Dextrin, acid	−	±(0·50)	−	−	−	−	−
70	Dulcitol, acid	−	−	−	+	±(0·50)	+	−
71	Fructose, acid	−(0·15)	+(0·83)	±(0·50)	+(0·75)	−	+	+
72	Glycerol, acid	−	−(0·17)	+	+	±(0·50)	+	+
73	Inositol, acid	−	−	+	+(0·92)	−	+	±(0·50)
74	Inulin, acid	−	−	−	−(0·25)	±(0·50)	+	−
75	Mannose, acid	−(0·15)	±(0·66)	+	+	+	+	+
76	Melibiose, acid	−	−	−	+(0·75)	+	+	±(0·50)
77	Melezitose, acid	−	−	−	+(0·92)	+	−	±(0·50)
78	Raffinose, acid	−	−	−	−(0·17)	−	+	−
79	Rhamnose, acid	−(0·23)	±(0·50)	−	+	+	+	±(0·50)
80	Salicin, acid	−(0·08)	±(0·33)	−	+	+	±(0·33)	+

Table 4. (*Continued*)

Character no.	Character	Slow-growing Rhizobium	C. violaceum	C. lividum*	A. radiobacter tumefaciens rhizogenes	R. meliloti*	R. leguminosarum trifolii phaseoli	A. rubi*
			Chromobacterium		Agrobacterium/fast-growing Rhizobium			
81	Sorbitol, acid	−	−(0·17)	+	+(0·75)	+	+	±(0·50)
82	Trehalose, acid	−	+(0·83)	−	+	+	+	+
83	Xylose, acid	±(0·38)	−	±(0·50)	+(0·92)	+	+	±(0·50)
84	5 % Ethanol, acid	−(0·08)	−(0·17)	−	±(0·33)	−	−	−
85	Aesculin hydrolyzed	±(0·61)	−	+	+	±(0·50)	±(0·33)	+
89	Levan produced	+(0·77)	−	±(0·50)	+	+	+	−
	Physiological:							
93	Growth at 5°	−	−	+	−	−	−	±(0·50)
94	Growth at 15°	+(0·77)	+	+	+	+	+	+
97	Growth at 35°	+	+	+	+	+	±(0·33)	+
98	Growth at 37°	+(0·85)	+	−	+(0·92)	+	−	+
101	Growth at 42°	−	±(0·66)	−	−(0·08)	±(0·50)	−	−
105	Growth in 3·0 % NaCl	+	−	−	+	+	+	+
106	Growth in 5·0 % NaCl	−	−	−	±(0·67)	−	−	−
109	Growth at pH 4·0	+	−	−	±(0·58)	−	±(0·33)	+
110	Growth at pH 4·5	+	+	+	+	±(0·50)	+	+
116	Growth at pH 10·0	±(0·30)	+	±(0·50)	+	+	−	+
117	(Kovacs) oxidase	+	+	±(0·50)	±(0·83)	+	±(0·66)	+
120	Phosphatase	−	+	+	±(0·50)	−	−	±(0·50)
	Antibacterials:							
121	Sensitivity to o/129 pteridine compound	−	−	−	−	−	−	±(0·50)
	Sensitive to:							
122	Penicillin, 10 units	−(0·15)	−	±(0·50)	−(0·17)	−	±(0·66)	+
123	Dihydrostreptomycin, 10μg.	±(0·61)	+(0·83)	+	−	±(0·50)	−	+
124	Chloromycetin, 30 μg.	−(0·23)	+(0·83)	+	+(0·92)	+	+	±(0·50)
125	Erythromycin, 15 μg.	−(0·23)	+(0·83)	+	±(0·33)	−	+	+
126	Kanamycin, 30 μg.	±(0·69)	+	+	+	+	+	+
127	Aureomycin, 30 μg.	+(0·77)	+(0·83)	+	+	+	±(0·66)	+
128	Novobiocin, 30 μg	±(0·69)	+	+	+(0·83)	+	±(0·66)	±(0·50)
129	Polymyxin B, 300 units	−	+	+	±(0·50)	+	+	+
130	Terramycin, 30 μg	±(0·46)	+	+	+	+	+	±(0·50)
131	Tetracycline, 30 μg.	+(0·85)	+	+	+	+	+	+
132	HgCl₂, 1 μg.	+(0·92)	−	−	−(0·08)	−	−	−
133	HgCl₂, 10 μg.	+	+	±(0·50)	+	+	+	+
	Biochemical tests:							
134	Nitrate reduced to nitrite	+(0·85)	+	+	+	−	+	+
135	Nitrite reduced	−	±(0·50)	±(0·50)	±(0·58)	−	−	−
136	Gelatin liquefied	−	+	±(0·50)	−	−	−	−
137	Litmus milk, peptonized	−	+	−	+	−	+	+
140	Litmus milk, alkaline	+	−	+	+(0·92)	+	+	+
145	Casein hydrolysed	−	+	−	−	−	−	−
146	Ammonia produced in peptone water	+	+	+	±(0·42)	±(0·50)		
148	Hydrogen sulphide produced from cystine	±(0·46)	+	+	+	+	±(0·66)	+
149	Hydrogen sulphide produced from cysteine	±(0·69)	+	+	+	+	+	+

Table 4. (*Continued*)

Character no.	Character	Slow-growing Rhizobium	Chromobacterium		Agrobacterium/fast-growing Rhizobium			
			C. vio-laceum	C. livi-dum*	A. radio-bacter tume-faciens rhizogenes	R. meli-loti*	R. legumi-nosarum trifolii phaseoli	A. rubi*
150	Urease (1 week)	±(0·69)	±(0·50)	±(0·50)	−	+	+	+
152	Lecithinase	−	+	±(0·50)	−	−	−	−
153	Lipase (Tweens 20, 40)	+(0·85)	+	+	−	−	−	+
154	Lipase (Tween 60)	+(0·85)	+	±(0·50)	−	−	−	−
155	Lipase (Tween 80)	−	+	±(0·50)	−	−	−	−
158	Growth in Koser's citrate	−(0·23)	+	+	±(0·66)	±(0·50)	−	±(0·50)
159	Simmons citrate	−	+(0·83)	±(0·50)	−	−	−	−
160	Oxidation of calcium lactate through acetate to carbonate	−	+	+	−	+	±(0·66)	+
	Nutrition:							
161	Utilization of 0·3 % sodium malonate as C source	+(0·77)	−	+	−(0·25)	+	±(0·66)	+
162	Utilization of 0·1 % sodium acetate as C source	+	−	±(0·50)	−	+	+	+
163	Utilization of 0·1 % sodium formate as C source	+(0·92)	±(0·33)	±(0·50)	+	+	±(0·66)	+
164	Utilization of $NH_4H_2PO_4$ as N source	−	−(0·17)	+	+(0·92)	+	±(0·33)	±(0·50)
	Utilization as C and N source:							
165	Ornithine mono-chloride	±(0·69)	+	−	+	+	±(0·66)	−
166	Arginine hydro-chloride	±(0·31)	±(0·67)	+	+	+	±(0·66)	−
167	L-lysine	±(0·69)	+	+	+(0·83)	+	−	−
168	L-alanine	±(0·69)	+	+	+	+	+	−
169	L-histidine	−(0·23)	+	+	+	+	±(0·66)	+
170	L-serine	±(0·38)	+(0·83)	+	+(0·75)	+	+(0·66)	−
171	L-phenylalanine	+(0·77)	+	−	−(0·08)	+	±(0·33)	−
172	L-proline	+(0.77)	+(0·83)	+	+	+	±(0·66)	−
173	L-leucine	±(0·69)	−	+	−	±(0·50)	−	−
174	L-glutamic acid	±(0·54)	+	±(0·50)	±(0·42)	−	+	−
175	L-cystine	±(0·54)	−	−	−(0·08)	±(0·50)	−	−
176	L-tyrosine	+(0·77)	+	+	−(0·17)	±(0·50)	+	−
	Other:							
179	Production of 2-keto-gluconate from potassium gluconate	−	−	−	−(0·08)	−	−	−
182	Arginine dihydrolase	−	±(0·33)	−	−	−	−	−
183	Arginine decarboxylase	−	+	−	−(0·08)	−	−	−
186	Methylene-blue reduction	−	−(0·17)	+	+(0·75)	−	−	−
188	Penicillinase	+	±(0·33)	+	−	+	±(0·66)	+
190	Melanin produced from phenylalanine	−	+(0·83)	−	−	−	−	−
191	Melanin produced from tyrosine	−	±(0·50)	+	+(0·83)	−	−	−

± = Variable occurrence with the set of strains tested. Actual frequency of occurrence is given in parentheses.

* Only two strains tested.

† N.T. = Not tested.

lecithinase; utilization of $NH_4H_2PO_4$ as nitrogen source; production of 2-keto-gluconate from gluconate; arginine decarboxylase; and methylene-blue reduction.

The Chromobacterium strains formed two clusters corresponding to the mesophilic (*C. violaceum*) and psychrophilic (*C. lividum*) strains described by Sneath (1960). These two groups joined at S = 68 %. There were only a few characters in each group which varied from the descriptions provided by Sneath (1960). The data are presented in Table 4. The differences may be cited as follows. Mesophilic strain ATCC 12541 produced acid from sorbitol. Chromobacterium strain no. 12540 did not produce acid from trehalose. Failure to produce acid from salicin, lactose and inulin, by the psychrophilic group agreed with Leifson's results (Sneath, 1960). However, the two strains in this group did not produce acid from mannitol. Growth was not detected in either

Table 5. *S value of Agrobacterium strains with the hypothetical median organism*

Strains		S value	No. of tests*
11157	*A. tumefaciens*	94·4	107
A. RADIO	*A. radiobacter*	92·9	112
AR 1001	*A. radiobacter*	92·9	112
B6	*A. tumefaciens*	90·1	111
A6RI	*A. tumefaciens*	87·7	114
4720	*A. tumefaciens*	87·4	111
A66RI	*A. tumefaciens*	86·2	116
AR 1012	*A. radiobacter*	85·2	115
11156	*A. tumefaciens*	84·2	114
4718	*A. radiobacter*	83·9	112
13333	*A. rhizogenes*	82·2	118
11325	*A. rhizogenes*	75·4	118

* Number of tests $(n_s + n_d)$ on which S value (S = $n_s/n_s + n_d$) is based. Total number of coded tests was 191.

group with 3 % NaCl but all strains grew in the range pH 4·5–10·0 except strain no. 6915 which did not grow at pH 10·0. Pigment description was excluded from the tables because although all strains characteristically produced a dark blue non-diffusible pigment, this character was not stable, as non-pigmented or palely pigmented colonies frequently were present on agar plate streaking of a pure culture. De Ley (1964) made similar observations concerning the pigmentation of Chromobacterium.

Neither Chromobacterium cluster produced acid from adonitol, dulcitol, melibiose, melezitose or raffinose. Both groups possessed lipase (Tween 20, 40) and were sensitive to $HgCl_2$, 10 μg. The two groups differed in several respects. The mesophiles were fermentative in glucose metabolism, were not sensitive to dihydrostreptomycin 10 μg., peptonized milk, hydrolysed casein, did not grow in a minimal medium with malonate or acetate as carbon source, and tested positive for arginine decarboxylase. The mesophiles utilized ornithine and phenylalanine as a nitrogen and carbon source while the psychrophiles utilized leucine. These features may be worth further analysis to determine their usefulness in diagnostic applications, since they represent metabolic functions most probably governed by gene complexes, and would therefore, as a combination, or plexus of features, provide a determinative key.

The Agrobacterium/fast-growing Rhizobium cluster, although divisible into four subgroups consisting of *A. radiobacter* + *A. tumefaciens* + *A. rhizogenes*, *R. meliloti*,

R. leguminosarum + *R. trifolii* + *R.*'*phaseoli*, and *A. rubi* (Fig. 1), have many features in common. Several differ from those scored for the slow-growing Rhizobium and the Chromobacterium clusters. In contrast to the slow growers, the Agrobacterium/fast-growing Rhizobium cluster was peritrichously flagellated, slightly fermentative in glucose medium (decrease in Ph in fermentation tube) and produced acid in a number of the carbohydrates tested. The strains also were sensitive to Polymyxin B and lipase negative. These and other characters useful in distinguishing the subgroups of this cluster are listed in Table 4.

Table 6. *S value of slow-growing Rhizobium strains with the hypothetical median organism*

Strain		S value	No. of tests*
965	*Rhizobium* sp.	84·2	76
998	*Rhizobium* sp.	82·3	79
952	*R. japonicum*	82·3	79
10318	*R. lupini*	81·8	77
942	*Rhizobium* sp.	80·5	82
997	*Rhizobium* sp.	80·5	82
WU7	*R. lupini*	79·7	74
948	*Rhizobium* sp.	78·3	83
10317	*Rhizobium* sp.	77·8	81
WU425	*Rhizobium* sp.	75·6	78
963	*Rhizobium* sp.	73·2	82
989	*Rhizobium* sp.	73·2	82
922	*Rhizobium* sp.	71·3	80

* Number of tests ($n_s + n_d$) on which the S value (S = $n_s/n_s + n_d$) is based. Total number of coded tests was 191.

Table 7. *S value of Chromobacterium violaceum strains with the hypothetical median organism*

Strain		S value	No. of tests*
553	*C. violaceum*	90·0	100
12472	*C. violaceum*	88·3	103
12542	*C. violaceum*	87·6	105
12541	*C. violaceum*	86·8	106
6357	*C. violaceum*	85·6	104
12540	*C. violaceum*	82·2	107
13426	*C. violaceum*	55·9	118

* Number of tests ($n_s + n_d$) on which the S value, S = $n_s (n_s + n_d)$, is based. Total number of tests employed was 191.

Hypothetical median organisms (Liston *et al.* 1963) were calculated for the Agrobacterium, slow-growing Rhizobium, and *Chromobacterium violaceum* groups. A measure of homogeneity of the clusters can be obtained from the tabulation of strain S-values of a cluster with the hypothetical median organism for that cluster. The Agrobacterium strains each share S-values > 75 % with the hypothetical median organism (Table 5). Thus a suitable representative or neotype strain for this group, for example, would be *A. tumefaciens* 11157 (S = 94 %).

Tables 6 and 7 contain S-value calculations of each strain with the respective hypothetical median strains for the other two groups which were analysed. *Chromobacterium*

violaceum 13426, not a member of the *C. violaceum* cluster from the initial data results (Fig. 1), yielded a very low S-value (S = 56%) with the hypothetical median organism for the major *C. violaceum* cluster, reaffirming its lack of membership in the group.

Fig. 4. Inter-S-values computed from the hypothetical median organisms for the Agrobacterium, *Chromobacterium violaceum*, slow-growing Rhizobium and the *A. pseudotsugae* clusters.

		1	2	3	4
Slow-growing Rhizobium	1	100	.	.	.
Agrobacterium	2	45	100	.	.
A. pseudotsugae	3	51	60	100	.
Chromobacterium violaceum	4	46	48	53	100

Fig. 5. Inter-S-values for the median organisms of the Rhizobiaceae clusters and for other representative strains included in the study.

	965	SU308	11157	553	13430	16266	14216	W1485	4115	PM-1	9247TR
Rhizobium sp. 965	100										
R. trifolii SU308	48	100									
A. tumefaciens 11157	45	54	100								
Chromobacterium violaceum 553	43	53	46	100							
Pseudomonas fluorescens 13430	51	51	49	55	100						
P. fluorescens 16266 var. *antirrhinastri*	46	58	55	52	70	100					
P. aeruginosa 14216	42	54	53	57	73	68	100				
Escherichia coli W1485	42	46	57	52	49	52	55	100			
Enterobacter aerogenes 4115	35	51	62	52	53	62	64	76	100		
Proteus mirabilis PM-1	41	46	50	54	45	49	51	53	57	100	
P. morgani 9247TR	42	45	50	53	46	49	49	55	52	70	100

By using the computed hypothetical median organisms for the four groups Agrobacterium, Chromobacterium (mesophilic), slow-growing Rhizobium and *A. pseudotsugae*, an S-value table of inter-group relationships was obtained (see Fig. 4). The Agrobacterium and the *A. pseudotsugae* groups shared S-value relationship of 60% but the other values for the groups were much lower. An ordering of the groups on the basis of hypothetical median organism similarities would thus be as given in Fig. 4.

An S-value table for actual median organisms (selected on the basis of highest computed relationship with the hypothetical median strain for the given group) was computed for 11 of the species from the analysis (Fig. 5). The intra-S-value for the three Pseudomonas strains was c. 70% and for *Escherichia coli* W1485 and *Enterobacter aerogenes*, 4115, 76%, S-value levels which, from earlier studies (Colwell & Liston, 1961), would be considered at least a generic level of similarity. The two Proteus species, showed a similar S-value, 70%. The order in which the strains are listed in Fig. 5 represents a final arrangement based on the computed similarities amongst the strains as given in the triangular matrix.

DISCUSSION

The relation of the Rhizobia to other bacteria, the relationships amongst the crown gall organisms, the root nodule organisms, *Agrobacterium radiobacter*, and the hairy-root lesion organism (*A. rhizogenes*), and the division of the root nodule bacteria into two broad groups were problems facing the early students of soil bacteriology and remain to the present as points of debate amongst the workers in the field. As early as 1903 the Rhizobia were divided into two groups on the basis of cultural characteristics, including carbohydrate reactions, gelatin reaction, etc. (Fred, Baldwin & McCoy, 1932). Löhnis & Hansen (1921) established both the so-called fast-growing Rhizobium group, characterized as producing rapid profuse slimy growth, appreciable acid formation and a 'serum zone' in milk, and the slow-growing group which exhibited slower, less profuse growth and an alkaline reaction in carbohydrate media and no change in milk. As a result of the work of Fred *et al.* (1932), 6 species were established for the nodule bacteria and the species were placed into the new genus *Rhizobium* (*Bergey's Manual*, 1957).

Lange (1961) clearly demonstrated that the cross-inoculation group separations of the rhizobia for species designation was not, in practice, satisfactory. Distinctly different host groupings were apparent from the data collected during his study of nodule bacteria associated with the indigenous legumes of south-western Australia. As pointed out by Lange, the economic importance attached to agronomic legumes and the resulting disproportionate attention to these has resulted in a skew classification of the nodule bacteria.

Lange's suggestion that an Adansonian analysis of the root nodule bacteria should be done was taken up by Graham (1964). Graham's analysis of strains of the genera *Rhizobium*, *Agrobacterium*, *Chromobacterium*, *Beijerinckia* and *Bacillus* provided some valuable new data. From the results of his analysis, Graham concluded that *Chromobacterium*, *Bacillus* and *Beijerinckia* showed little affinity with the nodule bacteria studied. The results of the analysis reported in the present work provide strong support for the separation of *Chromobacterium* from the other genera of the Rhizobiaceae. In fact, there seems little reason to retain the family as presently composed. Of the several genera included in the present analysis, the highest relationship of the *Chromobacterium violaceum* was to *Pseudomonas aeruginosa* (57 % S; see Fig. 5). The root nodule bacteria might better be placed in the family Pseudomonadaceae, a conclusion also arrived at by De Ley *et al.* (1966*b*) from DNA homology studies. Furthermore, the *C. violaceum* and *C. lividum* inter-species relationships were so low as to suggest treatment of these clusters as different genera.

Graham also pointed out that the genus *Rhizobium* required major revision and suggested, from his results, that *R. trifolii*, *R. leguminosarum* and *R. phaseoli* be consolidated into a single species, that *Agrobacterium* and *A. tumefaciens* be included as *R. radiobacter* in the genus *Rhizobium* and that the genus *Phytomyxa* be applied to the strains of slow-growing root-nodule bacteria. We find all of these suggestions most useful after examination of the results of our analyses. From Fig. 1 it is obvious that the major separation of the Rhizobium species is into the slow growers and the fast growers as originally described by Löhnis & Hansen (1921). Generic level of separation, at the least, is clearly indicated. Since Buchanan (1926) concluded that the two names, Phytomyxa and Rhizobium, were available for

the bacteria of leguminous plants, it seems logical for historical reasons to retain the fast-growers within the genus *Rhizobium* and include the slow-growers in the new genus *Phytomyxa*. The intra-generic relationships of the fast-growers and the Agrobacterium strains are not as clear-cut as Graham (1964) would imply. It is clear, however, that the results reported here support the consolidation of the remaining Rhizobium into two or more species. Our analysis would support retention of *R. meliloti* (strains SU 277 and SU 216, Fig. 1) and *R. leguminosarum* (SU 301, SU 312, SU 298, Fig. 1). However, besides *Rhizobium radiobacter*, into which we would place *A. tumefaciens*, *A. rhizogenes* and *A. radiobacter*, we would also propose the retention of species *Rhizobium rubi*, into which strains presently designated *A. rubi*, 13334 and 13335, should be placed.

Agrobacterium gypsophilae strain 13329 should not be included in the Rhizobiaceae; that is, the results of the computer study indicate that it may more logically be placed in the family Enterobacteriaceae (see Fig. 1).

The *Agrobacterium pseudotsugae* strains included in the present study would be not considered members of the species *R. radiobacter*, as were the other Agrobacterium species. *Agrobacterium pseudotsugae* appears to warrant a separate generic designation. De Ley, Park, Tijtgat & Van Ermengem, 1966b) also questioned the relationship of *A. pseudotsugae* to the other agrobacteria on the basis of DNA base composition.

Genera, other than those of the Rhizobiaceae, which were studied provide some interesting information. Members of the Enterobacteriaceae formed two clusters linked via the *Vibrio cholerae* type strain 14033 (Hugh, 1965) and the four isolates of *Serratia marcescens*, data for which were included in the computations as they were available from an earlier study (Colwell & Mandel, 1965). The genus *Vibrio* might better be placed in the family Enterobacteriaceae. Strains of the genus *Vibrio* are being studied in our laboratory in order to resolve this point.

The clustering together of the two strains 5 GLY.FE, a non-virulent *Agrobacterium tumefaciens* strain and *C. violaceum* 13426, and the rather nebulous position of *A. radiobacter* strain 6466 and *Chromobacterium viscosum* 6918, were not readily explicable. In the case of *A. radiobacter* 6466 and *A. tumefaciens* 5 GLY.FE, misidentification by the persons isolating these strains may be the explanation for the anomalous results. Sneath (1960) also concluded that *C. viscosum* does not rightly belong in the genus *Chromobacterium*. The following facts should be made clear. In our hands, *C. violaceum* 13426 did not produce pigment under any of the conditions tested and *C. viscosum* produced a purple water-soluble pigment. None of the other Chromobacterium strains tested in this analysis produced a water-soluble pigment, the pigment being in every case restricted to intracellular location.

From the data in Table 7 it appears that *C. violaceum* 12472, the proposed neotype strain (Sneath, 1960) was a suitable choice (S = 88 % with the hypothetical median organism of the mesophilic cluster of the genus *Chromobacterium*). Other neotype strains, as indicated from Tables 5 and 6, would be 11157 for *Rhizobium radiobacter* and 965 for *Phytomyxa japonicum*.

Family relationships, as determined from Figs. 4 and 5 would result in the elimination of only *Phytomyxa* (represented by Rhizobium 965) and *Proteus*, if an inter-S level of 50–60 % were selected as the demarcation. The lack of over-all similarity of Proteus species one to the other, as well as of the genus to the rest of the genera of the Enterobacteriaceae, was noted by Krieg & Lockhart (1966). Perhaps, more

logically, it would be better simply to order the genera, as was done in Fig. 1, according to inter-group S-values, since the over-all DNA base compositions correlated extremely well with the intra- and inter-group arrangements thus obtained.

The feature frequency of occurrence for *Rhizobium radiobacter* and *R. meliloti*, *R. leguminosarum* and *R. rubi* for *Phytomyxa japonicum* and for *Chromobacterium violaceum* and *C. lividum* (Table 4) permit establishment of characteristics useful in forming descriptions for each of these species, independent as in the case of the Rhizobium and Phytomyxa species, of plant inoculation studies. These characteristics thus provide reference markers, or base-points, for purposes of comparison with new data obtained in future or may be employed in a determinative key for identifying the species of the genera listed.

It is important to emphasize that bacterial taxonomy is in a dynamic situation, a fluid state with new information constantly becoming available. As more knowledge concerning the biochemical pathways and genetic control mechanisms operating in these organisms is accumulated, it is to be expected that the descriptions and limits of the taxa proposed here will be appropriately altered.

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Relationship between Bacteria and Ciliate Protozoa in the Sheep Rumen

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SUMMARY

Two sheep were reared together and then kept on a standard diet over a period of 9 months. The rumen of one sheep contained a mixed population of ciliate protozoal species; the other was initially unfaunated, then faunated with *Entodinium caudatum* and then with Entodinium and *Polyplastron multivesiculatum*. For each sheep diurnal and day-to-day variations in total concentration, and concentration of particular types, of bacteria, in viable counts of bacteria on non-selective and differential media and in concentrations of the different protozoal species, were determined. Measurements were also made of *in vitro* rates of fermentation, rumen pH and ammonia and volatile fatty-acid concentrations. The presence of protozoa decreased rumen bacterial concentrations and increased ammonia and volatile fatty-acid concentrations. However, these changes were not always completely clear-cut since the protozoa changed the patterns of diurnal variation not only of bacterial numbers but of some metabolic activities and metabolites and also altered the balance of bacterial types. These results are discussed in the context of interactions between bacteria and protozoa and between protozoal species. The main effects of faunation of the rumen seem to be caused by non-selective ingestion of small bacteria by the protozoa.

INTRODUCTION

The rumen contents of sheep and other ruminants contain a population of bacteria and protozoa. Whilst the rumen microbial population exists symbiotically with the host animal, there are also symbiotic relationships between the various micro-organisms in that, taken over a (long) period, the micro-organisms form a stable population in which in many cases one organism exists because of its relationship to another, e.g. it may depend on synthesis of growth factors by another organism. The relationship between the larger protozoa, the ciliates, and the bacteria and the relationship between protozoa and the host have been of interest since the protozoa were first observed. The latter relationship is outside the scope of the present work as too few animals were used to make statistically useful observations. However, it may be mentioned that recent work shows that although the overall processes of rumen metabolism are similar in the presence or absence of protozoa, the presence of protozoa may cause some slight improvement in the growth of the animal (Abou Akkada & El Shazly, 1964). The first relationship has been the subject of some direct and indirect experiments. Indirectly, biochemical tests, principally on washed suspensions of pro-

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tozoa, have shown that the protozoa possess hydrolytic and fermentative enzyme systems of their own and do not depend on bacterial enzyme systems for degradation of carbohydrate nutrients, as had been at one time suggested. More directly, observations on suspensions and growing cultures of a number of species of rumen protozoa have shown that they ingest bacteria and the ingested bacteria probably serve as a nitrogen source for the protozoa (see, for instance, Coleman, 1964). These experiments were done *in vitro*. *In vivo* observations show that the concentration of bacteria in the rumen contents of a faunated sheep is less than that in an unfaunated sheep, and that inoculation of an unfaunated sheep with ciliate protozoa leads to a rapid decrease in concentration of bacteria as the protozoal population develops (Eadie & Hobson 1962). In the present work an attempt has been made to analyse the interactions between some protozoal and bacterial populations of the rumen.

METHODS

Sheep. The animals used (nos. 27, 28) were two Cheviot females from a set of triplets. The general basis of rearing ciliate-free sheep has been described by Eadie (1962*a*), and the animals were bottle-fed cows' milk, given dried grass (*ad lib.*) from 4 weeks old and weaned at 13 weeks. They were then fed a concentrate mixture (100 g.; 4 parts ground maize, 1 crushed oats, 1 wheat bran, $\frac{1}{2}$ linseed cake meal, $\frac{1}{2}$ white fish meal) three times a day, with dried grass *ad lib.* for 4 months, and then were given 300 g. dried grass and 150 g. concentrates fed at 8 a.m. and 4 p.m. This was varied slightly in quantity but not in proportions (2 dried grass:1 concentrates) over the next 2 months and then throughout the experimental period (from about 9 to 18 months old) the feed was standardized at 210 g. dried grass + 140 g. concentrates (3 dried grass:2 concentrates) fed at 8 a.m. and 4–4.30 p.m. The feed was generally consumed within 1 hr, and water, though available during the day, was also taken mainly at this time. The animals were fitted with rumen cannulae at 7 months old. They were kept in the same pen for the first 12 weeks, when the rumen of 27 was inoculated with mixed protozoa (Eadie, 1962*a*). The inoculation was repeated at 13 weeks, after which all species in the inoculum became established. After this the sheep were kept separated, but still in the same room as other faunated and unfaunated animals so that they had equal opportunities for airborne inoculation with the normal rumen bacteria and flagellate protozoa. Sheep 28 did not at times eat all of its feed, but these periods of greatly decreased intake did not coincide with any of the observations described.

Protozoal populations. The inoculum of mixed protozoa was a suspension concentrated from sheep rumen contents and contained bacteria (Eadie, 1962*a*). Sheep 27 was inoculated with a mixture of *Isotricha prostoma, I. intestinalis, Dasytricha ruminantium, Polyplastron multivesiculatum, Ophryoscolex tricoronatus, Diploplastron affine* and various Entodinium species including *E. caudatum* and the large *E. bursa* Stein. Sheep 28 was inoculated at 12 months old with *Entodinium caudatum* and at 14 months old with *Polyplastron multivesiculatum.* These ciliates were obtained from isolated sheep containing limited protozoal populations. The same populations had been used previously for bacterial experiments (Abou Akkada & Howard, 1960; Abou Akkada, Eadie & Howard, 1963). The inocula (introduced through the rumen cannula) were approximately 1000 ciliates contained in 1 ml. of a well-washed, buffered suspension in which only a few bacteria adherent to the protozoa were present. Only *E. caudatum*

was present in the first sheep population, but after the second inoculation some other small Entodinia, which were present in the inoculum, developed along with *P. multivesiculatum*.

Sampling procedures. Samples were obtained via the rumen cannula by gentle suction through a wide-bore glass tube from the same position in the rumen each time.

Total counts of bacteria and protozoa. Samples (5 ml.) of rumen contents were removed and immediately diluted to 25 ml. with 10 % (v/v) formalin in 0·9 % (w/v) saline, and later diluted according to the size and number of organisms to be counted. 'Small' bacteria were counted in a 0·02 mm. deep chamber observed under phase-contrast illumination, and 'large' bacteria in a 0·1 mm. deep chamber after staining of the bacteria with methylene blue. Protozoa were counted in a 1 mm. deep chamber made from glass slides cemented together with Araldite (I.C.I. Ltd.) and similar to the chamber described by Boyne, Eadie & Raitt (1957). A known area of the chamber was defined by a standardized microscope eyepiece grid. Portions of the formolized samples were diluted with aqueous glycerol solution (50 %, v/v) to give a suitable concentration for counting protozoa (Purser & Moir, 1959; Boyne *et al.* 1957). Preliminary investigations of all counting methods ensured that the techniques gave reproducible counts. 'Small bacteria' included all morphological types not counted as 'large bacteria'. These latter were defined as Quin's organism and large selenomonads (see for instance Moir & Masson, 1952, for pictures of these.) Oscillospira and the unidentified large oval organism described by Eadie (1962a) ('Eadie's oval') were counted as seperate groups in the same manner as large bacteria. In counting small bacteria indeterminate clumps of organisms and debris were ignored and the few short chains of bacteria were counted as one organism. Protozoa were identified as far as genera and were grouped as 'large' (Polyplastron, Isotricha, Ophryoscolex), 'medium' (*Entodinium bursa*, Diploplastron, Dasytricha), and 'small' (small Entodinium species including *E. caudatum*).

Viable counts of bacteria. All media were prepared, inoculated and incubated under oxygen-free carbon dioxide, using techniques based on those of Hungate (1950) (Hobson & Mann, 1961). The basal constituents of all three media, per 100 ml.: mineral solutions *a* and *b*, 15 ml. each; cysteine hydrochloride, 0·05 g; resazurin, 0·0001 g.; agar, 2·0 g.; $NaHCO_3$, 0·4 g.; centrifuged rumen fluid, 20 ml. Medium 1 contained in addition (per 100 ml.), Bacto Casitone, 1·0 g.; Bacto Yeast Extract, 0·25 g.; sodium lactate (70 %, w/v), 1·0 ml.; glucose, 0·2 g.; cellobiose, 0·2 g.; maltose, 0·2 g. Medium 2 contained Bacto Casitone, 1·0 g.; Bacto Yeast Extract, 0·25 g.; soluble starch (B.D.H.), 0·5 g. Medium 3 contained Bacto Casitone, 0·5 g.; Bacto Yeast Extract, 0·125 g.; cellulose powder (MN 300, Camlab (Glass) Ltd., Cambridge), 1·0 g. The constituents of mineral solutions *a* and *b* and the methods of heat- or filter sterilizing the constituents of the media were described by Hobson & Mann (1961).

One-millilitre portions of the samples of rumen contents were taken by a wide-mouth pipette and successive tenfold dilutions prepared under CO_2 in a diluting fluid of the composition of medium 1 with the agar omitted, 0·1 % (v/v) Tween 80 (Honeywill and Stein, Ltd., London) added, the glucose concentration raised to 0·5 % and the other sugars omitted. Half or one millilitre portions of the diluted rumen contents were added to suitable amounts of the media in 6 $\times \frac{5}{8}$ inch test-tubes and roll tubes prepared. Three or four tubes for each of the appropriate dilutions (10^6, 10^7, 10^8 for media 1 and 2, and 10^3, 10^4, 10^5 for medium 3) were inoculated for counting. Cellulo-

lytic colonies were identified by clearing of the cellulose powder around the colonies. All cultures were incubated at 38°; media 1 and 2 for 3 days; medium 3 for 4 weeks.

Morphological types of bacteria. Representative colonies were picked from the 10^7 dilution cultures in media 1 and 2 and Gram films prepared. Wet and dry preparations of the whole rumen contents were also examined at intervals.

In vitro fermentation rates of rumen contents were measured by a small-scale adaption of the manometric method described by El Shazly & Hungate (1965) in which fermentative activity was measured by production of gas from a suspension of rumen contents (in the present case, 10 g.) in minerals (*a* and *b*)+sodium bicarbonate (1·5 %, w/v, solution, 20 ml.). An excess of substrate (1 g. MN 300 cellulose powder, rice starch or glucose) was added and gas production measured over 20 min. incubation at 38°. A control without substrate was also included. Measurements were started within 10 min. of removing the sample from the rumen.

Rumen pH values were determined from the sample immediately after collection.

Total volatile fatty acids were determined on samples, fixed in an $H_2SO_4 + MgSO_4$ mixture, by steam distillation and titration with standard alkali.

Ammonia nitrogen was determined on samples, fixed in HCl, by a micro-diffusion method.

General procedure. Samples were taken from the various animals at intervals of 1 or 2 hr during the 8 hr period between the 8 a.m. and 4 p.m. feeds, usually starting at 9 a.m., to record 'diurnal' variations. 'Diurnal' in this text is used to describe the period between the morning and afternoon feeds, and not the daily period of 24 hr. The procedures were repeated at approximately weekly intervals to determine longer-term variations during any particular part of the experiments.

RESULTS

Diurnal variations in numbers of organisms

Total counts of small bacteria. Curves showing the diurnal variation in bacterial numbers are shown in Fig. 1. Each curve is an average of a number of determinations. Six sets of observations were made over a period of 6 weeks on sheep 28 unfaunated (curve *a*). The maximum concentration of bacteria always occurred 4–5 hr after feeding, but the number at this time varied from 47×10^3 to 58×10^9 ml. over the 6 weeks. At 1 hr and 7 hr after feeding the variation in numbers was less. The presence of entodinia decreased the diurnal variation; curve *b* is an average of three, weekly, observations where the number of bacteria at 5 hr after feeding varied from about 35 to 40×10^9/ml. The addition of Polyplastron to the rumen (Fig. 1, curve *c*, which is an average of 4 sets of measurements) changed the whole pattern of diurnal variation to give a minimum bacterial concentration 3–4 hr after feeding (the minimal numbers varied from about 21 to 24×10^9/ml. in the 4 samples). This pattern was similar to that found in sheep 27 with a mixed population of protozoa (Fig. 1, curve *d*, which is an average of 5 observations, with minimum numbers varying from about 16 to 24×10^9/ ml.). These results also show variations in the average concentration of small bacteria taken over the whole feeding cycle in the presence or absence of protozoa.

Viable counts of bacteria. Figure 2(*a–c*) shows the diurnal variation in bacterial count obtained using three media. These counts can be most nearly correlated with the total counts of small bacteria, as it is known that Quin's organism, Oscillospira and
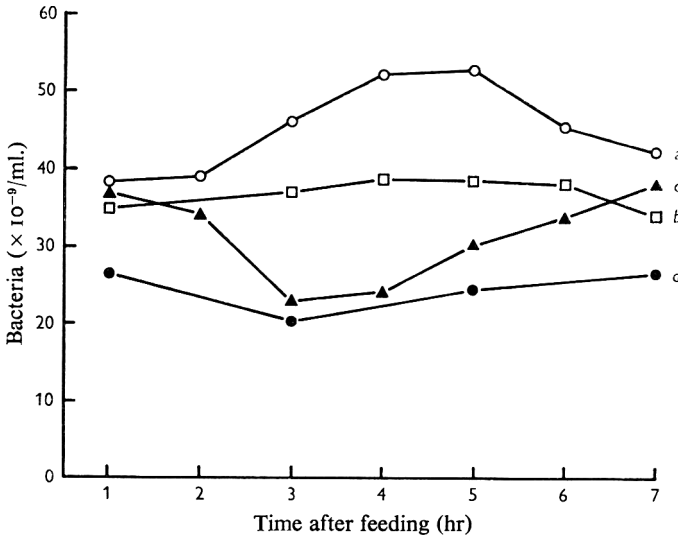
Fig. 1. Diurnal variation in total small bacteria concentration in differently faunated sheep Sheep 28 (unfaunated), ○, curve *a*. Sheep 28 (Entodinium), □, curve *b*. Sheep 28 (Entodinium + Polyplastron), ▲, curve *c*. Sheep 27 (mixed protozoa), ●, curve *d*.

Fig. 2. Diurnal variation in viable counts of bacteria. (*a*) Medium 1 (non-selective). Sheep 28 (unfaunated), ○, curve *a*. Sheep 28 (Entodinium), □, curve *b*. Sheep 28 (Entodinium + Polyplastron), ▲, curv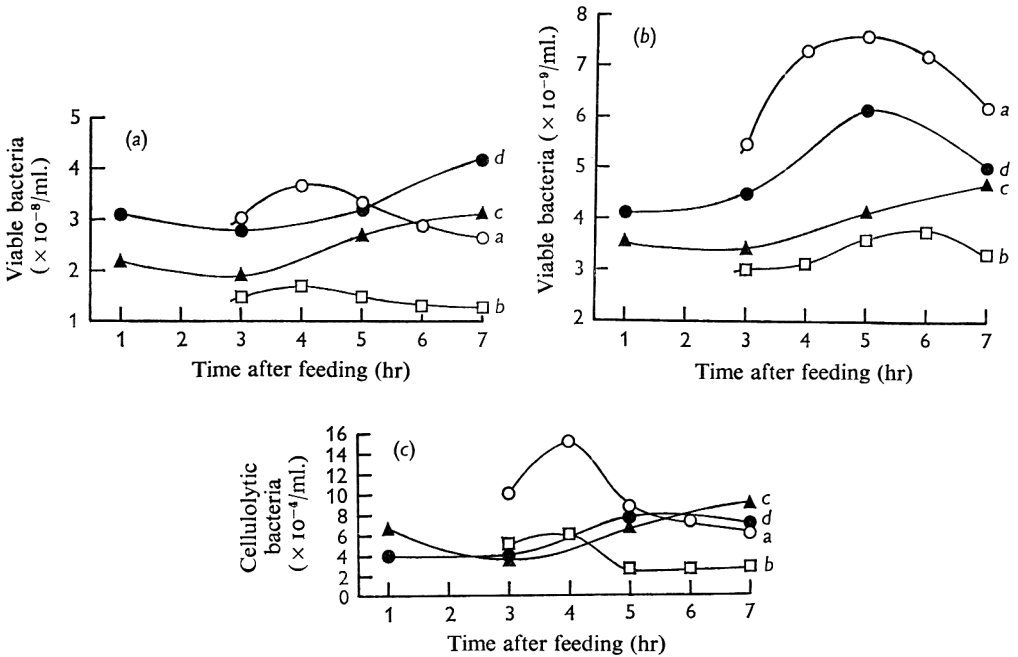e *c*. Sheep 27 (mixed protozoa), ●, curve *d*. (*b*) Medium 2 (starch-containing), symbols as above. (*c*) Medium 3 (cellulose-containing), symbols as above.

Eadie's oval organism do not grow on any of the media used, and although a sero-logical relationship has been shown between the small selenomonads cultured on media such as number 1 and the large selenomads seen in the rumen (Hobson, Mann & Smith, 1962), it has not been shown uniquivocally that the large selenomonads can be cultured. Since only 2, or 3, sets of viable counts were done for each medium and each

Table 1. *Viable counts of bacteria in different media*

	Medium			Medium	
	2	1	3	1	2
		Viable count*		% of total count†	
Sheep					
28 unfaunated	100	55·3	0·018	0·7	1·2
28 + Entodinium	100	49·6	0·017	0·4	0·8
28 + Polyplastron and Entodinium	100	56·1	0·011	0·9	1·5
27 + mixed protozoa	100	62·1	0·009	1·3	2·1

* Medium 2, starch containing; 1, mixed sugars; 3, cellulose (see text). Count expressed as per-centage of count in medium 2. Averages of all determinations at 3 hr after feeding.

† Representative figures, 3 hr after feeding, from total and viable counts done on same sample. Other samples gave similar results.

Table 2. *Percentage frequencies of counts of 'Eadie's oval' and Oscillospira in sheep with different fauna*

	Organisms							
	Eadie's oval*				Oscillospira*			
Cell numbers (× 10⁻⁶/ml.	28†	28E‡	28PE§	27‖	28†	28E‡	28PE§	27‖
80	8·6	—	—	—	—	—	—	—
70–80	5·7	—	—	—	—	—	—	—
60–70	5·7	—	—	—	2·9	—	—	—
50–60	8·6	—	—	—	2·9	—	—	—
40–50	8·6	—	—	—	14·7	38·5	—	—
30–40	11·4	15·4	—	—	11·8	30·7	—	—
20–30	14·3	15·4	—	—	26·5	7·7	15·4	—
10	37·1	15·4	46·2	14·3	41·2	23·1	84·6	46·4
≤ 10⁶/ml.	—	53·8	53·8	85·7	—	—	—	53·6

* Percentage of total number of counts done on different days and at different times of day having a value as in left hand column. 70 counts of each organism were made.

† Sheep 28, no protozoa.   ‡ Sheep 28, + Entodinium.   § Sheep 28, + Entodinium and Poly-plastron.   ‖ Sheep 27, + mixed protozoa.   — Nil.

sheep and the variation in counts was comparatively large, avarage counts would not be very significant, so one representative curve is shown in Fig. 2 for each sheep and each medium. The shapes of the curves were in all cases similar to those shown and the relative positions of the curves, with respect to counts, were similar. The maximum variation between counts done on different days was about $1·5 \times 10^8$ bacteria/ml. for media 1 and 2 and for medium 3 about 1×10^4/ml. Medium 1 was designed to culture all types of rumen bacteria and similar media have been used by other workers to obtain complete viable counts (e.g. Bryant & Robinson, 1961). However, the starch-containing medium (medium 2) gave consistently higher counts than did medium 1. It

should be noted that this is a count of all bacteria growing in the medium, not just amylolytic bacteria. As zones of amylolysis spread rapidly throughout this medium (in the cultures examined the starch was completely hydrolysed after 3 days of incubation) it is usually impossible to say that a particular colony is amylolytic. This
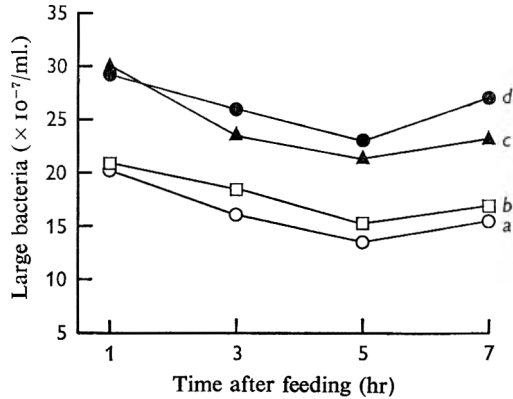
Fig. 3. Diurnal variation in total counts of large bacteria. Sheep 28 (unfaunated), ○, curve *a*. Sheep 28 (Entodinium), □, curve *b*. Sheep 28 (Entodinium + Polyplastron), ▲, curve *c*. Sheep 27 (mixed protozoa), ●, curve *d*.
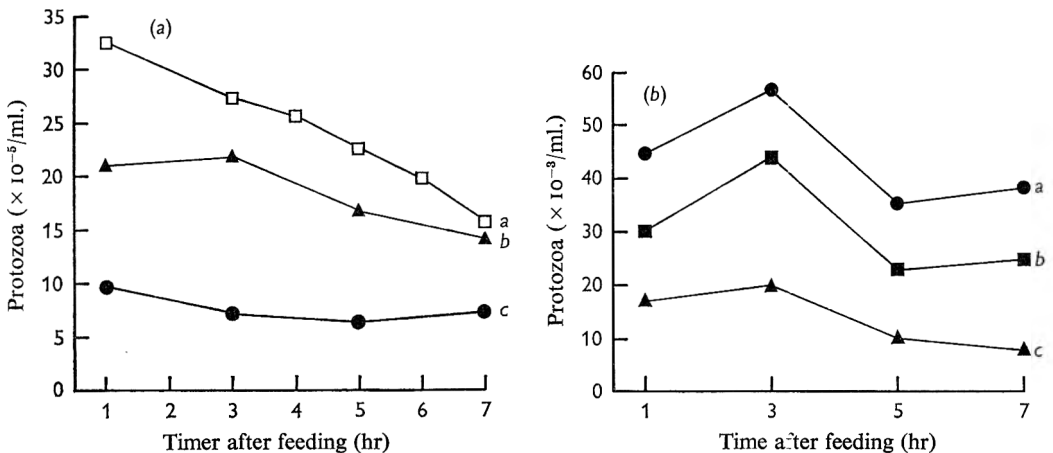
Fig. 4. (*a*) Diurnal variations in concentration of Entodinium. Sheep 28 (Entodinium), □, curve *a*. Sheep 28 (Entodinium + Polyplastron), ▲, curve *b*. Sheep 27 (mixed protozoa), ●, curve *c*. (*b*) Diurnal variations in concentration of 'large' (●, curve *a*) and 'medium' (■, curve *b*) protozoa in sheep 27 (mixed protozoa) and Polyplastron (▲, curve *c*) in sheep 28 (Entodinium + Polyplastron).

discrepancy in counts has been recently noted in investigations of sheep on other diets in these laboratories; but why it should be so cannot be stated at the present time. Theoretically any bacterium which will grow only on starch or starch hydrolysis products (such as *Bacteroides amylophilus*) should grow on the maltose in medium 1, and the many non-amylolytic rumen bacteria should grow on medium 2 only by utilization of the maltose or glucose formed by the amylolytic bacteria or the small amount of glucose transferred with the diluting fluid inoculum. Also some bacteria,

such as strains of ruminoccocci, cannot ferment either glucose or maltose, but should grow on the cellobiose in medium 1. A more definite change in pattern of diurnal variation on introduction of ciliate protozoa into the rumen is shown in medium 1 than medium 2, and the pattern of medium 1 is repeated in the counts of cellulolytic bacteria in medium 3. In our experience counts of cellulolytic bacteria in sheep have always been only a small fraction of the total viable count of bacteria and it may be that all cellulolytic bacteria are not counted in solid media such as number 3. Zones of celluloysis vary from distinct to indefinite and the numbers of these latter are especially difficult to determine. Table 1 shows the number of bacteria growing in each medium when the number growing in the starch medium is counted as 100, and also the viable count in media 1 and 2 as a percentage of the total count of small bacteria in the same samples.

Total counts of large bacteria. Figure 3 shows the diurnal variations in concentrations of 'large bacteria' (large selenomonads and Quin's organisms). In general the large bacteria varied in numbers from week to week to a much greater extent than did the small bacteria, for instance Fig. 3, curve *a* is an average of 7 sets of observations where the minimum numbers varied from about 7 to 20×10^7/ml. However, although the absolute concentrations varied, the shape of the curve was similar for each set of measurements.

Oscillospira and 'Eadie's oval'. This latter oval organism was described by Eadie (1962*a*) and independently by Warner (1962) who counted it among the 'flagellates'; it was also seen by Abou Akkada & El Shazly (1964). Following Warner (1966) it is referred to as 'Eadies' oval'. These organisms fluctuated considerably in numbers during the day and from day to day so that a good curve for diurnal variation could not be obtained. However, Table 2, which records the frequencies of appearance of the two organisms at different levels of concentration, shows that faunation, especially with more than one genus of protozoan, tended to decrease the average ruminal concentration of these organisms. Counts were made hourly during the day at similar times to those shown for other organisms, and were repeated on a number of days.

Total counts of ciliate protozoa. Although there was day-to-day variation in the concentrations of protozoa the curves for diurnal variations had a pattern characteristic of the state of faunation of each animal. The diurnal variations were determined during the periods when the protozoal populations had become stabilized. Figure 4(*a*) shows curves of diurnal variations in numbers of entodinia. Since the variation in numbers of protozoa from day to day was sometimes large, average numbers were not plotted and the curves are representative of 3–5 sets of observations at each stage of faunation. However, the positions of the curves relative to the numbers of protozoa are correct. The presence of other types of protozoa modified the curve obtained when entodinia were the only ciliates present in the rumen. Figure 4(*b*) shows the diurnal variations in numbers of the other groups of protozoa in the sheep at different times. In these cases the curves are similar in shape, and again they are representative of 3–5 sets of observations at each stage of faunation.

Day-to-day variations in numbers of organisms

The day-to-day variations in numbers during the periods when the previous measurements were made are shown in the figures. Figure 5(*a–c*) shows the numbers of small bacteria, large bacteria and entodinia in the rumen of sheep 28 at 4–5 hr after feeding in

the period after inoculation of the rumen with entodinia. A determination of numbers of bacteria was made 1 week before the inoculation, but the next determination was made 3 days after the inoculation. At 3 and 5 days after inoculation no entodinia were found in the counting chamber, although some were seen in undiluted rumen fluid, at 7 days an approximate count gave 0.2×10^5 entodinia/ml. The count at 9 days represents the first reliable count. It was not found possible to obtain accurate counts of entodinia in numbers lower than about 10^5/ml. by using lower dilutions of the original
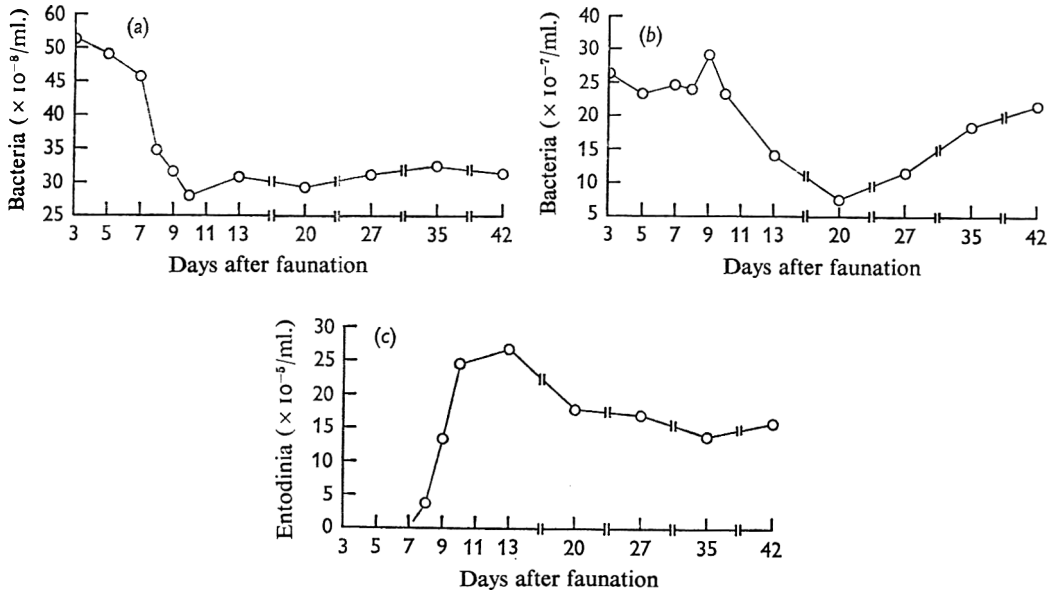

Fig. 5. Concentrations of small bacteria (*a*), large bacteria (*b*) and entodinia (*c*) in sheep 28 after faunation with Entodinium. Counts at 4–5 hr after feeding.
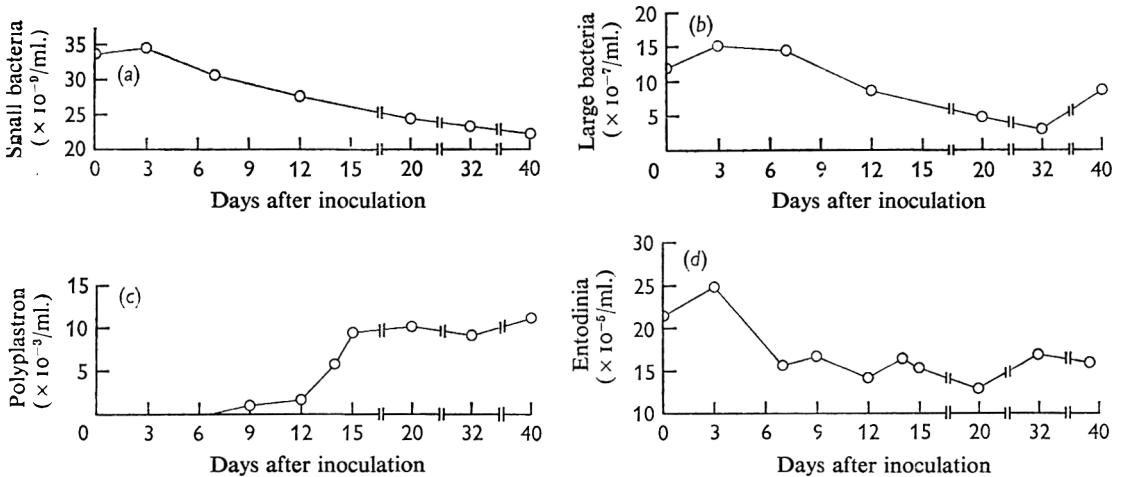

Fig. 6. Concentrations of small bacteria (*a*), large bacteria (*b*), Polyplastron (*c*) and Entodinium (*d*) in sheep 28 after faunation with Polyplastron. Counts at 4–5 hr after feeding.

rumen contents in the 1 mm. deep cell as the background debris made it difficult to see the small protozoa.

Figure 6(*a–d*) shows the variations in numbers of small bacteria, large bacteria, Polyplastron and Entodinium in the period after inoculation of the rumen of sheep 28 with Polyplastron. Since Polyplastron is larger than Entodinium it is possible to see and count it in smaller dilutions of rumen fluid. However, Polyplastron, although seen at 7 days after inoculation, was not present in sufficient numbers for counting. One month elapsed between the last counts shown in Fig. 5 and the first counts in Fig. 6 and during this period the number of entodinia increased from about 16×10^5 to 21×10^5/ml. During the same period the number of small bacteria also increased slightly whilst the number of large bacteria decreased.

During the period shown in Fig. 5 the numbers of Oscillospira fell during the first 7 days from 16×10^6/ml. to $1\cdot2 \times 10^6$/ml., then began to increase again until by the 35th to 42nd days they reached 18×10^6/ml. By the beginning of the period shown in Fig. 6, 1 month later, the number was down to $9\cdot4 \times 10^6$/ml. and from then it rose to 36×10^6/l. at day 20 of this period and it fell then to $7\cdot2 \times 10^6$ by day 40.

Changes in morphological types of bacteria

Gram-stained films of whole rumen contents show such a large number of bacteria of varied morphological types that it is often difficult to determine more than gross changes in the types of bacteria in films from different animals. Some 2000 Gram films of colonies growing in the 10^7 dilution of rumen contents in media 1 and 2 were therefore examined to see whether these gave a better indication of changes in bacterial population under the different conditions of faunation of the sheep. Tables were prepared showing the frequency of appearance of eleven morphological types of bacteria in the cultures. The 11 groups were as follows.

(1) Gram-negative: selenomonad types (crescent-shaped 'rods'); bacteroides types (pleomorphic rods); slender curved rods (Butyrivibrio, etc.); other Gram-negative rods; large and small cocci.

(2) Gram-positive: large and small rods; small, often pointed, streptococci, like *Streptococcus bovis*; tetracocci; large cocci; Gram-positive, or variable, small cocci (other than *S. bovis*).

(3) Spirochaete-type organisms.

Of these groups the most frequently encountered were the Gram-negative rods, *Streptococcus bovis*-type cocci and the small Gram-variable cocci, and these are mentioned here, but all the above morphological types were encountered in small numbers, although not in every culture. In general the bacteria growing in medium 1 and medium 2 were similar in types and numbers so the overall picture described below does not differentiate between media. In sheep 28 (without protozoa) the slender Gram-negative curved rods predominated with smaller numbers of bacteroides-like organisms, *S. bovis*-types and small Gram-negative and Gram-variable cocci. Few selenomonads were found. However, in sheep 28 (with entodinia) all four types of Gram-negative rods were present in similar numbers, with *S. bovis*-types being the only other frequently encountered organisms. When Polyplastron was added to the rumen of sheep 28 the four groups of Gram-negative rods were still present, and there was a large increase in Gram-variable small cocci, while *S. bovis*-type cocci were less frequently encountered. Spirochaete-type organisms were seen slightly more frequently.

In sheep 27 the four types of Gram-negative rods were always present with bacteroides-types tending to predominate. The only other group occurring at all frequently was the Gram-variable small cocci. *S. bovis*-types were present in only very small numbers. There was no obvious diurnal change in numbers of the various morphological types except for bacteroides-types in sheep 27 which tended to increase in numbers from the first to the seventh hour after the morning feed.

In vitro fermentation rates

The rates of *in vitro* fermentation of glucose, cellulose and starch were compared for each sheep under the different conditions of faunation. Again a number of observations were made at weekly intervals during the periods when the rumen populations
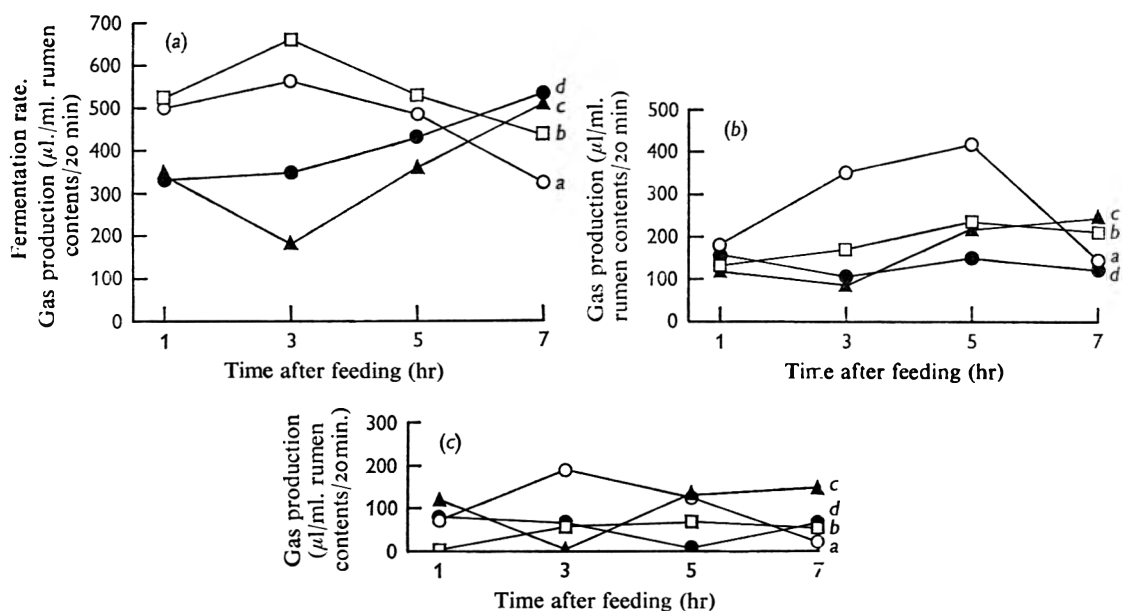
Fig. 7. *In vitro* fermentation rates of glucose (*a*), starch, (*b*) and cellulose, (c). Sheep 28 (unfaunated), ○, curve *a*. Sheep 28 (Entodinium), □, curve *b*. Sheep 28 (Entodinium + Polyplastron), ▲, curve *c*. Sheep 27 (mixed protozoa), ●, curve *d*.

of sheep 28 had become stabilized (e.g. from about 1 month after inoculation with entodinia) and average curves are shown in Fig. 7 (*a–c*). In each case the gas production by the rumen contents alone has been subtracted. These rates represent short-term fermentations under conditions of substrate saturation and should give an indication of the maximum fermentative (or hydrolytic, if this is the rate-governing factor in starch or cellulose fermentation) capacities of the micro-organisms at any one time. The substrates are artificial in that they are not constituents of the feedstuffs and so do not represent the rates of fermentation of the similar feed constituents, but they enable a comparison to be made between the different rumen contents. Similar substrates have been shown to be degraded *in vitro* by rumen organisms, including the protozoa.

Rumen ammonia concentrations

Diurnal changes in rumen ammonia concentrations are shown in Fig. 8. Each point represents an average value from a number of determinations made at intervals over the periods when the microbial populations had become stabilized. Sheep 27 was tested 7 times over a period of 9 months (i.e. near the beginning and end of the experiments on sheep 28). The shapes of the diurnal variation curves were similar at each time of sampling but the ammonia concentrations varied. The variation in concentration in sheep 27 at 3 hr. after feeding was typical of all points and was from about 17 to 29 mg. N/100ml. In sheep 28 (unfaunated) at 3 hr after feeding 8 determinations varied from 8 to 13 mg./100 ml. In sheep 28 (with Entodinium) the variation at 3 hr was from 18 to 24 mg./100 ml. and in 28 (with Entodinium and Polyplastron) from 21 to 27 mg./100 ml.
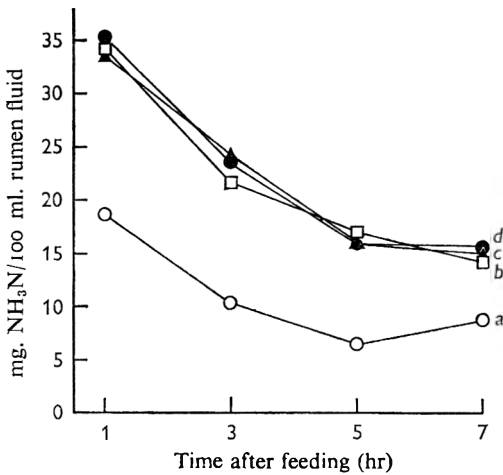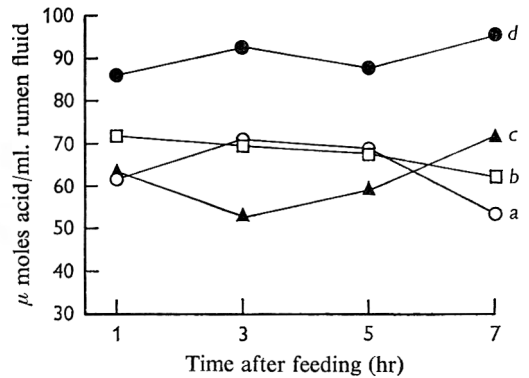
Fig. 8. Diurnal variations in rumen ammonia concentration. Sheep 28 (unfaunated), ○, curve *a*. Sheep 28 (Entodinium), □, curve *b*. Sheep 28 (Entodinium + Polyplastron), ▲, curve *c*. Sheep 27 (mixed protozoa), ●, curve *d*.

Fig. 9. Diurnal variations in rumen volatile fatty-acid concentration. Sheep 28 (unfaunated) ○, curve *a*. Sheep 28 (Entodinium), □, curve *b*. Sheep 28 (Entodinium + Polyplastron) ▲, curve *c*. Sheep 27 (mixed protozoa) ●, curve *d*.

Rumen volatile fatty acid concentration

Figure 9 shows the diurnal changes in total volatile fatty acids in the different sheep. Again the points are avarages of a number of determinations which gave curves of similar shape. Taking the variation in concentration at 3 hr after feeding as typical, in sheep 28 (unfaunated) this was from about 60 to 83 μmole/ml. In sheep 28 (with Entodinium) it was 64-75 μmole/ml. In sheep 28 (with Entodinium and Polyplastron) it was from 48 to 61 μmole/ml., and in sheep 27 from 83 to 97 μmole/ml.

Rumen pH values

In each animal, or at each stage of faunation, the rumen pH values varied little during the period between feeds. There was a slight tendency for a minimum pH value

to occur 4–5 hr after feeding. In the case of sheep 28 (unfaunated) a number of determinations gave maximum and minimum average values of pH 6·6 and 6·4. In sheep 28 (with Entodinium) the values were pH 6·6 and 6·5. In sheep 28 (with Entodinium and Polyplastron) they were pH 6·8 and 6·6, and in sheep 27, 6·5 and 6·3.

<div align="center">DISCUSSION</div>

Although the rumen provides a form of continuous culture this probably never attains a true steady state in the sense in which the term is used of the laboratory continuous culture of one organism. However, the rumen flora is built up by degrees by inoculation of the animal from external sources—air-borne organisms, contaminated feedstuffs, and contact with other animals—and these inocula form a population in which a large number of the organisms form a more or less stable population and the others form only a transient population of insignificant number. The constituents of this population are to a large extent conditioned by the feed of the host animal. However, even when the feed is of constant composition the organisms are subject to several influences which cause variations in their concentrations. Nutrients for the organisms are added intermittently in the form of the feed of the host animal. The flow of saliva, which acts as a diluent and buffer in the rumen, fluctuates, partly in response to the feeding regime. The influences of the type of feed and the frequency of feeding on the concentrations and kinds of rumen organisms have been the subject of a number of investigations (for a review see, for instance, Hungate, 1966). Less attention has been paid to interactions between the organisms themselves, although it is known that such interactions do occur. For instance, Eadie (1962b) noted antagonism between species of ciliate protozoa. For example, in an animal on a constant feeding regime *Polyplastron multivesiculatum* will become dominant over other large Ophryoscolecids, and this has been shown to be due to predation. This dominance, however, has not been shown towards Entodinium species. Similarly, *in vitro* experiments have shown that many, perhaps all, species of rumen ciliates ingest bacteria (reviewed by Hungate, 1966), although here the comparatively rapid division rate and large numbers of bacteria present in the rumen make it unlikely that one species of bacterium would be completely eliminated by protozoal predation. However, it has been shown that there is a significant decrease in the total bacterial concentration in the rumen when a mixed population of protozoa is established in a previously unfaunated rumen (Eadie & Hobson, 1962). This decrease in bacterial numbers may be caused by the above-mentioned predation of protozoa on the bacteria, or it may be caused by the competition for nutrients (or 'space') between the organisms. The experiments described here were done in an attempt to throw further light on the relationship between the ruminal bacteria and protozoa. The animals used were reared in an identical manner and were fed the same ration at the same time each day in order to eliminate as far as possible any variations in the rumen organisms caused by external influences. The volumes of rumen digesta should have been similar in all animals so that variations in concentrations of micro-organisms or metabolic products should reflect changes in the total rumen activity. Of the two major possibilities of interaction between the bacteria and protozoa, bacterial ingestion by the protozoa might be either selective or unselective. The early experiments of Gutierrez on bacterial ingestion by the holotrich and Entodinium protozoa suggested that ingestion was selective on morphological grounds.

(Gutierrez, 1958; Gutierrez & Hungate, 1957; Gutierrez & Davis, 1959). However, later experiments by Coleman (1964) on *Entodinium caudatum* cultured *in vitro* suggested that ingestion was non-selective and that the number of bacteria ingested was proportional to their size. The number of species of bacteria in the rumen is large, and there is very great strain variation amongst the known species. It was thought that it would be an almost impossible task to determine whether a particular species of bacterium was being ingested by the protozoa, but since the only suggestion of selective bacterial feeding by the protozoa has been selection on morphological grounds it was thought that the study of the broad morphological groups of bacteria in the rumen with and without protozoa might show selective bacterial ingestion if it occurred, or might indicate a completely non-selective ingestion. Ingestion of bacteria by the protozoa might take place only at certain stages in the protozoan feeding or metabolic cycle and so diurnal variations in numbers of organisms were studied.

The case of competition for nutrients between bacteria and protozoa is more difficult to substantiate. As comparatively little is known about the nutritional requirements of the protozoa, and especially of their nitrogen metabolism, it seemed that competition might only be established on the basis of the carbohydrate metabolism of the organisms. *Entodinium caudatum* was the first protozoan established, as this is known to ferment mainly starch. It might therefore compete with starch-fermenting bacteria for substrate and this it was hoped might be reflected in viable counts of bacteria on a starch-containing medium. Entodinia have also been said to ingest Gram-positive streptococci (*S. bovis*) (Gutierrez & Davis, 1959) or to ingest completely non-selectively (*in vitro*) (Coleman, 1964). It was hoped that the experiments might distinguish between these extremes under *in vivo* conditions. The second protozoan added to the rumen was *Polyplastron multivesiculatum*. This ferments starch (Abou Akkada *et al.* 1963) and might be expected to compete both with the entodinia and with starch-fermenting bacteria, if nutrient competition existed. It also ferments cellulose (Abou Akkada *et al.* 1963) and so a second possibility for competition existed, with the cellulolytic bacteria. Finally the rumen flora of a sheep containing a mixture of many protozoal species, such as is found normally in the rumen, was studied to see if further interactions of the organisms could be determined. Other measurements were made to see whether the presence or absence of protozoa had any gross effect on some aspects of the rumen metabolism of most importance to the overall metabolism of the host animal. All measurements were started 1 hr after the morning feed as at that time any residual food was removed and weighed back. Also the main increase in salivary flow due to eating would have ceased by the end of the hour. Rumen conditions therefore would by then have settled.

Figure 1 shows the changes in diurnal pattern of variation of concentration of small bacteria. The pattern (curve *a*) in the unfaunated sheep shows a distinct maximum in concentration at 4–5 hr after feeding. The presence of entodinia decreases the average number of bacteria and also markedly decreases the peak concentration. Coleman (1964) found that *Entodinium caudatum, in vitro*, could ingest 12,000 *Escherichia coli* per protozoan per hr. If the rate of uptake of bacteria by the number of *E. caudatum* present in sheep 28 (Entodinium), which is about 25×10^5 per ml., were of this same order, then it could be calculated that the protozoa could when most active almost completely nullify the increase in rumen bacterial population due to division of the bacteria. The changes in concentration of viable bacteria cultured on medium 1

which should be non-selective, are similar in pattern (Fig. 2(*a*), curves *a*, and *b*). However, viable bacteria are a smaller percentage of the total in sheep 28 (with entodinia) than they are in the unfaunated sheep (Table 1). Since the numbers of viable bacteria are greatest during the period 3–5 hr after feeding these results taken together suggest that a selective ingestion of viable bacteria by the entodinia was taking place, although the fact that the concentration of entodinia falls during the whole inter-feeding period (Fig. 4*a*) suggests that the protozoa were not actively dividing at this time, and were being washed out by saliva flow through the rumen. Warner (1962) found in sheep fed once a day that maximum concentrations of dividing entodinia were present at about 16 hr after feeding. If conditions in the present experiments were similar this suggests that the period under consideration was too short for entodinia to divide and that most division would occur during the early morning period before 8 a.m. feed. Ingestion of viable bacteria by the entodinia may not be selection of viable bacteria as such, but may be due to ingestion of starch granules with a large number of adherent viable bacteria (rapid ingestion of starch granules by the entodinia was observed microscopically in the first 1–2 hr after feeding). Minato, Endo, Ootomo & Uemura (1966) showed that, as might be expected a somewhat higher proportion of viable (i.e. culturable) bacteria was attached to the solids part of the rumen contents of a hay and concentrate-fed sheep than was present in the bacteria 'free' in the rumen fluid. When Polyplastron were added to the rumen organisms there was a greatly altered pattern of diurnal variation of total and viable bacteria, the curves for both these functions showing a definite minimum at 3–4 hr after feeding (Fig. 1, curve *c*, Fig. 2(*a*), curve *c*). At 3 hr after feeding the concentration of Polyplastron was slightly greater than at 1 hr (Fig. 4(*b*), curve *c*), which would indicate that multiplication of the organisms was taking place. Microscopic examination showed rather larger numbers of dividing Polyplastron at about 4 hr after feeding than in the first hour or two. The coincidence of this maximum in numbers of Polyplastron and minimum in numbers of bacteria suggests that Polyplastron were ingesting bacteria, but mainly at the same time when Polyplastron were dividing, since later the numbers of Polyplastron decreased and the numbers of bacteria increased. The percentage of viable bacteria (compared with the total numbers) at this time returned to a similar figure to that in the unfaunated sheep (Table 1). It might be inferred that Polyplastron ingested both live and dead bacteria, and possibly more dead than live ones, (by ingestion of more of the 'free' bacteria than 'fixed' ones) as the entodinia would still have some effect, although they were in smaller numbers than when present alone (Fig. 4*a*). Since the total concentration of bacteria at the beginning and end of the inter-feeding period is similar whether entodinia alone or entodinia and polyplastrons are present, although there were much lower numbers at 3–5 hr in the latter case, it would appear that the polyplastrons are, over a limited time, much more active in ingesting bacteria than are entodinia. Although the smaller effect of entodinia alone on the concentration of bacteria (Fig. 1, curve *b*) might be partly explained by the fact that the numbers of entodinia are constantly decreasing over the inter-feeding period. Competition for nutrients alone would appear to be ruled out, especially in the case of Polyplastron, as the decrease in numbers of bacteria between 2 and 3 hr after feeding (Fig. 1, curve *c*) is much greater than could be accounted for by a decrease in growth rate of the bacteria and consequent washout from the rumen. No figures were obtained for the rate of washout of a marker substance from the rumen of this sheep, but

Blackburn & Hobson (1960) found, for a sheep fed on a more artificial roughage-concentrate diet, that polyethyleneglycol washed out of the rumen exponentially at a rate of 7·3 %/hr between 1 and 7 hr after feeding. The fall in concentration of Entodinium when alone was of the same order as this (Fig. 4(a), curve a), suggesting that this was due to washout of non-dividing organisms. In no case can the change in concentration of protozoa either diurnally or between different states of faunation be due to pH effects as the pH values were never low enough to affect growth of the organisms.

When a population of a number of species of protozoa was present (sheep 27), then the total counts of bacteria were lower than in the other sheep at all times during the inter-feeding period, and the curve for diurnal variation showed a small decrease between 1 and 3 hr (Fig. 1, curve d). Polyplastron in sheep 27 were present in smaller numbers than in sheep 28 (Entodinium+Polyplastron), (about 10·6 × 10³/ml. 3 hr after feeding, see Table 3 and Fig. 4(b), curve c), and so would not have as great an effect as they had in sheep 28, assuming that they were here the main cause of the rapid fall in concentration of bacteria (Fig. 1, curve c). In the experiments of Eadie & Hobson (1962) the average difference in total small bacterial count (at 3 hr after feeding) between two unfaunated and one faunated sheep over a period of 16 months was 1·3 × 10¹⁰/ml. The present counts in sheep 27 and 28 agree quite well with these figures, allowing for the fact that the ration fed to the previous sheep with its rather different ratio of hay to concentrates may have given a different diurnal variation in counts. In the graphs representing the diurnal variations in protozoal numbers it is of interest that the presence of other protozoa modified the diurnal variation in numbers of entodinia (Fig. 4a), and it would appear that the division cycle altered as these protozoa no longer washed out during the inter-feeding period. There appears to be no obvious explanation of this. In mixed populations of protozoa the concentrations of entodinia were considerably smaller than when entodinia were present alone. This was probably not due to predation on entodinia by other protozoa, but may be a reflexion of competition for nutrients. Results from sheep 28 (Polyplastron+Entodinium) showed that Polyplastron does not ingest Entodinium to any great extent. *Entodinium caudatum* appear to live largely by fermentation of starch and this might be competed for by the other protozoal species although these have other sources of fermentation energy. The fall in numbers of Polyplastron between sheep 28, where it was present with Entodinium, and sheep 27, where it was in a mixed population, is not so great as that of Entodinium. Polyplastron can attack a diversity of carbohydrate substrates. Of course, other possibilities of protozoal competition may exist. The shape of the curve for diurnal variation in concentration of Polyplastron was similar whatever other protozoa were present and in turn was similar to the curves for all the protozoa (other than entodinia in sheep 28 and sheep 27) in that a maximum concentration occurred about 3 hr after feeding. The curves do vary, though, round about 5–7 hr after feeding, and occasional counts done at 8 hr (just before the second feed) showed that the upward or downward trend at 7 hr (Fig. 4a, b) was continued.

In Fig. 10 are plotted some of the figures obtained for concentration of small bacteria in the sheep at different states of faunation, against the approximate volumes of the protozoal populations at these same times. It will be seen that the numbers of bacteria decreased with increasing volume of ciliates, and it would seem most likely that this was due to ingestion of bacteria. The figures of Coleman (1964) suggest that

Entodinium caudatum can *in vitro* ingest bacteria to a very large proportion of the total volume of the protozoal cell. Coleman obtained maximum and minimum volumes for bacteria ingested in a number of experiments of $2 \cdot 2 \times 10^4 \mu^3$ and $8 \times 10^3 \mu^3$ per *E. caudatum* cell. Assuming that the volume of a small bacterium is $1 \cdot 5 \, \mu^3$ then the decrease in bacterial volume is similar to the increase in protozoal volume when the
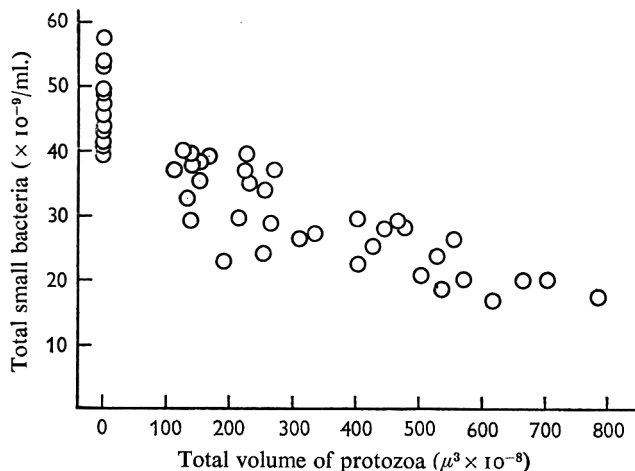
Fig. 10. Comparison of small bacterial concentrations and estimated approximate volumes of protozoa in differently faunated sheep taken from counts at 3, 5 and 7 hr after feeding, and on different days. Volumes of protozoa taken as: small protozoa (including Entodinium), $10^4 \mu^3$; medium protozoa, $5 \times 10^4 \, \mu^3$; large protozoa, $10^6 \mu^3$.

Table 3. *Diurnal variations in concentration of certain species of protozoa in the mixed protozoal population of sheep 27*

	Time after feeding (hr)			
	Number of protozoa ($\times 10^{-3}$/ml.)			
Protozoan	1	3	5	7
Isotricha	11·3*	13·3	4·7	6·1
Dasytricha	21·4*	21·9	11·0	11·3
Polyplastron	9·0†	10·6	3·2	5·8

* Averages of 4 sets of counts.   † One count.

latter is small, and in this case the protozoal volume figures are mainly from sheep 28 with only entodinia present. At higher protozoal volumes (when mixed protozoal populations were present) the decrease in bacterial volume is much less than the increase in protozoal volume. This suggests that (in conformity with Coleman's, 1964, results) entodinia can ingest bacteria to a volume almost that of the protozoan, while in the larger protozoa the volume of bacteria ingested is much less than the cell volume.

However, if the viable counts on the starch-containing medium 2 reflect the numbers of starch-hydrolysing bacteria (see later; *in vitro* fermentations) then there is some suggestion of preferential ingestion of starch-hydrolysing bacteria by Entodinium and

Polyplastron, as the maximum concentration of bacteria growing in medium 2 is moved from about 5 hr to 6 hr or more after feeding when Entodinium or Polyplastron are added to the rumen (Fig. 2b) and these latter times correspond to the lowest inter-feeding concentrations of the protozoa. This 'selective' ingestion may not be selective for bacteria as such but may reflect the ingestion by the protozoa of starch granules with adherent starch-hydrolysing bacteria. Although almost all the species of protozoa in sheep will ingest starch granules the reversal of the trend to a later peak of starch-hydrolysing bacteria shown in sheep 27 may be a reflexion of the much decreased numbers of very actively starch-ingesting Entodinia in this sheep as compared to sheep 28.

The changes in diurnal rhythm of the cellulolytic bacteria are similar to those of the total viable bacterial count (medium 1) (Fig. 2a, c) and presumably reflect non-selective ingestion of bacteria by the protozoa. There is no suggestion of competition between cellulolytic Polyplastron and the cellulolytic bacteria.

The identification of the principal morphological types of bacteria present with the different protozoal populations does not on the whole suggest any selection by the protozoa of bacteria on morphological grounds. In fact the presence of rather more Streptococcus bovis-type bacteria in sheep 28 (with entodinia) than in sheep 28 (un-faunated) would appear to be evidence against the previously reported ingestion of S. bovis by Entodinium (Gutierrez & Davis, 1959). However, S. bovis largely disappeared in the rumens containing Polyplastron + Entodinium and the mixed protozoal population. In the case of the Gram-negative rod-type bacteria, they appear to be more evenly distributed in cultures from the faunated sheep than the unfaunated. Small selenomonads, in particular, were more prominent in cultures from the faunated sheep. The fact that curved Gram-negative rods became less prominent after faunation may be a reflexion of their initially larger proportion of the flora. The generally more frequent appearance of the other Gram-negative rods may be a reflexion of better rumen conditions for growth, although it is difficult to say what these conditions are. Coleman (1964) found that when Entodinium caudatum ingested Escherichia coli labelled with ^{14}C amino acids, the amino acids were incorporated into protozoal protein after digestion of the bacteria, and also that some of the hydrolysed bacterial protein was 'washed out' of the protozoa into the surrounding medium. If this process takes place in the rumen then this would not only recycle amino acids for use in bacterial growth but also provide more amino acids for bacterial deamination, and thus tend to increase the ruminal ammonia concentrations. Our own experience with selenomonads suggests that all strains need animo acids for growth and they could be provided in this way, whilst the increased concentration of rumen ammonia in the faunated sheep might tend to favour growth of Bacteroides-type bacteria, as, for instance, Bacteroides amylophilus utilizes mainly ammonia as nitrogen source (Blackburn, 1965; Hobson & Summers, 1967) and B. succinogenes utilizes ammonia in preference to amino acids (Bryant, Robinson & Chu, 1959). It seems unlikely that the higher volatile fatty acid concentration in the faunated sheep would stimulate growth of the Gram-negative rods. The increased propionic acid concentration found in faunated sheep (Abou Akkada & El Shazly, 1964; Christiansen, Kawashima & Burroughs, 1965; Luther, Trenkle & Burroughs, 1966) may be related to the increased proportions of selenomonads and Bacteroides-type bacteria found in the present faunated sheep. Selenomonas ruminantium strains produce lactic and, sometimes, succinic acids.

Bacteroides ruminicola, B. amylophilus, B. succinogenes and *Succinimonas amylolytica* (which morphologically would come in this group) all produce large amounts of succinic acid and both lactic and succinic acids are precursors of propionic acid in the rumen fermentation.

The numbers of 'large bacteria' (large selenomonads and Quin's ovals) in these sheep were on average similar to those previously found by Eadie & Hobson (1962) However, in both cases large day-to-day variations in numbers were found. In the present experiments the only reproducible result was the shape of the curve for diurnal variations in numbers. The curves in Fig. 3 are typical and are averages from a number of determinations where the population of organisms seemed stabilized for some days, but consideration of Fig. 5 and 6 will show that they do not truly indicate the relative concentrations of the large bacteria over a long period, and in fact when the concentrations of large bacteria at any one time after feeding are plotted against days on the experiment for sheep 28 it is found that the concentrations increase and decrease periodically without reference to the protozoal population. For instance in sheep 28 (with Polyplastron and Entodinium) the concentration of large bacteria continued to increase after 40 days (Fig. 6b) reaching about 23×10^7/ml. at 50 days, 24×10^7/ml. at 57 days, and 20×10^7/ml. at 64 days. These numbers were similar to those found at the peaks of concentration during the period preceding faunation with Entodinium. There is thus no discernible influence of faunation on the concentration of large bacteria. What appear to be interactions in Fig. 5 and 6, e.g. (Fig. 5b, c) the rapid decrease in numbers of large bacteria from days 9 to 13 where the concentration of entodinia is increasing rapidly, are entirely fortuitous, and this does emphasize the fact that even in a comparatively constant environment such as the rumen observations should be continued over a prolonged period to provide reasonable data on the state of the microbial population.

However, the curves for small bacteria and protozoa (Fig. 5a, c; 6a, c) do show definite evidence of interaction in that, although there are some day-to-day variations the number of bacteria is decreased by the presence of protozoa, and this decrease coincides with the development of the protozoal populations. The 'overshoot' of numbers of entodinia at about 12 days after their introduction, before they settle down to a stable population, is similar to that found when a bacterial population is developing from a small inoculum in an *in vitro* continuous culture.

The *in vitro* fermentation rates of different substrates are shown in Fig. 7. These should give an indication of the maximum fermentative capacity of the bacteria and protozoa, but this need not be strictly correlated in magnitude with either the total numbers of organisms or the numbers of viable bacteria. Viable bacteria will be fermentative in these experiments, but the status of the 'non-viable' ('non-culturable') bacteria is obscure. Some non-culturable bacteria amy still ferment sugars. The shapes of the curves for fermentation of glucose (Fig. 7a) are quite similar to the diurnal variation curves of the total small bacteria and the viable counts in medium 1 (Fig. 1; 2a). This suggests that most of the fermentative capacity of the rumen organisms under these conditions is due to the bacteria. The *in vitro* fermentation of starch (Fig. 7b) gives a different pattern of diurnal variation and this corresponds quite well with the viable counts on the starch medium 2 (Fig. 2b). This supports the suggestion made earlier that the viable counts in medium 2 are related to the numbers of starch-fermenting bacteria, and also again suggests that the *in vitro* fermentation is mainly a product of

bacterial metabolism. *In vitro* cellulose fermentation is small, as are the viable counts of cellulolytic bacteria, and it seems unwise to make any deductions from the shape of the curves for diurnal variation in this case. In all fermentations all species of the protozoa remained motile at the end of the incubation period.

The curves for diurnal variation in rumen ammonia and volatile fatty acid concentrations are shown in Figs. 8 and 9. Although there was day-to-day variation in these concentrations the ammonia concentration in the faunated animals was always higher than in the unfaunated animals (Fig. 8). This agrees with the observations on faunated and unfaunated sheep made by Abou Akkada & El Shazly (1964). Luther, Trenkle & Burroughs (1966) have also demonstrated an increase in ammonia production when a washed suspension of mixed rumen protozoa was added to an *in vitro* fermentation system containing only rumen bacteria. However, the shape of the diurnal variation curve at each state of faunation does not vary, and is not altered to coincide with diurnal variation curves for any of the protozoa, also there is no difference in ammonia concentrations between the animals with different protozoal populations. If the protozoa produce ammonia then it must be concluded that all species do so; or that, perhaps in the way previously suggested, bacterial ammonia production is enhanced by the presence of protozoa. This latter possibility was also suggested by Purser & Moir (1966). The gradual decline in ammonia concentration from about 1 hr after feeding is probably a reflexion of the presence of substrate leading to ammonia formation in the period after feeding and then the gradual utilization of this ammonia by bacterial growth and removal of it from the rumen by absorption or passage to the omasum.

The curves for volatile fatty acid concentrations (Fig. 9) support the observations of Abou Akkada & El Shazly (1964) and Christiansen *et al.* (1965) in that volatile fatty acid concentration was higher in the sheep (27) with a mixed ciliate population than in the unfaunated sheep (28). The diurnal variations are also smoothed out. The much increased concentration of VFA in sheep 27 is somewhat difficult to understand though, unless the protozoa produce more VFA than an equivalent volume of bacteria, which does not seem likely. The proportion of viable bacteria is greater in sheep 27 than 28 (unfaunated; Table 1), although the total numbers are less. This may indicate some stimulation of bacterial metabolism by the protozoa, such as was suggested for ammonia formation. If substrates are not limiting then ingestion of bacteria by protozoa may, in effect, increase the 'dilution rate' of the bacterial population and so increase bacterial growth rate. However, this does not seem to occur in the rumens with only Entodinium or Entodinium + Polyplastron, as the VFA concentrations here were similar to those in the unfaunated sheep, and the proportions of viable bacteria are not increased by the presence of the protozoa (Table 1). In sheep 28 (Polyplastron + Entodinium) the concentrations of VFA tended to decrease at 3 hr after feeding and this correlates with a decrease in bacterial numbers (Fig. 1), and with a slight increase in numbers of Polyplastron and Entodinium. This suggests that the bacteria are mainly responsible for VFA formation.

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The Oxidation of Fatty Acids by Mycelium of *Penicillium roqueforti*

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SUMMARY

Low concentrations of fatty acids with less than 14 carbon atoms were oxidized without a lag phase over a wide range of pH values by mycelium of *Penicillium roqueforti*. The effect of the fatty acids upon oxygen uptake by a given weight of mycelium, and the nature of the products of oxidation, were dependent upon the concentration and chain length of the fatty acid and the pH value of the system. The C_9–C_{12} fatty acids which showed the greatest inhibitory effect were not oxidized to the corresponding methyl ketone with one less carbon atom in such high yields as the less toxic C_6–C_8 acids. The C_6–C_8 fatty acids markedly inhibited endogenous respiration at low pH values but this inhibition was reversed by increasing the pH value. The toxic effect associated with some fatty acids was less pronounced against mycelium which had been previously shaken over an extended period in phosphate buffer. It is suggested that the cellular regulation of fatty acid oxidation and methyl ketone formation involves deacylation of β-oxo acyl thiolester which provides an alternative means of recycling coenzyme A when oxidation of acetyl CoA is impaired.

INTRODUCTION

The ability of growing cultures of fungi to oxidize fatty acids of medium chain length to methyl ketones was first noted by Stärkle (1924), who showed that *Penicillium roqueforti* and two species of the genus *Aspergillus* produced methyl ketones when grown for several weeks on fatty acids of intermediate chain length. Moreover, when single fatty acids were used as substrates (Stärkle, 1924; Stokoe, 1928; Hammer & Bryant, 1937) these and certain other species of fungi only synthesized methyl ketones with one less carbon atom. It is not clear however whether the oxidation of fatty acids in the above investigations was the result of spore or mycelial activity. A decline in methyl ketone formation as spores germinate to mycelium has been reported (Thaler & Geist, 1939; Franke & Heinen, 1958). Gehrig & Knight (1963) concluded that the capacity of spores of *P. roqueforti* to form methyl ketones disappeared rapidly and progressively as the spores germinated. On the other hand Lawrence (1965b, 1966) found that the slow rate of formation of heptan-2-one from octanoic acid by resting spore suspensions of *P. roqueforti* was markedly increased by the addition of those amino acids and sugars that stimulate germination of the spores. Reports that low concentrations of fatty acids were oxidized by washed mycelium of various Penicillium species (Rolinson, 1954; Girolami & Knight, 1955; Vinze & Ghosh, 1962) would also appear to be in disagreement with the conclusions reached by Gehrig & Knight (1958, 1961, 1963) that hyphal cells of *P. roqueforti* were unable to oxidize fatty acids, regard-

less of the type of medium in which they were grown. It was not clear whether the uncertainty concerning the ability of fungal mycelium to oxidize fatty acids had arisen from the inhibitory effect of the fatty acids on respiration under certain conditions (Rolinson, 1954). The present work was undertaken in an attempt to clarify the situation.

METHODS

Gehrig & Knight (1963) grew mycelium of *Penicillium roqueforti* on a chemically defined medium developed by Meyers & Knight (1958), which contains both acetate and oleate initially at pH 4·0. Preliminary work in the present investigation, however, indicated that mycelium grown at pH 4·0 was about 5 times less active in oxidizing fatty acids than that grown at pH 6·5. As acetate at pH 4·0 has also been reported to be inhibitory towards respiration by yeasts (Neal, Weinstock & Lampen, 1965), a new chemically defined medium without fatty acids was developed.

Growth medium. After preliminary trials the following defined medium was used: ammonium nitrate, 2 g.; magnesium sulphate ($MgSO_4.7H_2O$), 0·15 g.; potassium chloride, 0·25 g.; ferrous sulphate ($FeSO_4.7H_2O$), 0·0125 g. These four components were dissolved in 400 ml. water and autoclaved for 20 min. at 12 pounds/sq.in. (118°). The brown precipitate which formed was allowed to settle and after 24 hr the supernatant fluid was decanted. A Seitz-filtered solution of 10 g. glucose, 0·6 g. potassium dihydrogen phosphate and 0·4 g. dipotassium hydrogen phosphate in 100 ml. water, supplemented with 1 ml. of a trace element solution (mg./l.) — zinc sulphate, $ZnSO_4.7H_2O$, 180; copper nitrate, Cu $(NO_3)_2.6H_2O$, 15; manganese nitrate, Mn $(NO_3)_2.6H_2O$, 80; ammonium molybdate, $(NH_4)_6Mo_7O_{24}.4H_2O$, 10 — was then added. This growth medium was initially at pH 6·5.

Growth of mycelium. Spores of *Penicillium roqueforti*, ATCC 6989 (American Type Culture Collection, Washington) were grown on slopes of Czapek-Dox agar (Oxoid) at 22°. After 5–6 days the spores were transferred to 100 ml. of the medium in 500 ml Erlenmeyer flasks. The suspensions were incubated overnight, without shaking, to allow spore germination and then shaken at 20° for 24 hr. The mycelium was harvested, washed and gently blended with a 'Polytron' homogenizer (Mobil Aaran A.G., Luzern, Switzerland) to free trapped ungerminated spores. The suspension was reinoculated in fresh medium and incubated for a further 24–48 hr. This resulted in a uniform and homogenous suspension, providing all organisms with comparable nutritional conditions and equal access to oxygen. The growth was harvested by filtration on a Buchner funnel, resuspended several times in about 2 l. distilled water, refiltered and finally suspended in phosphate buffer. The concentration of blended mycelium was adjusted to approximately 10 mg./ml. dry wt, the exact concentration being determined by dry weight measurement. The mycelium was finally examined microscopically to ensure that it was free of spores.

Oxygen uptake. Measurements were made manometrically at 27° in a standard Warburg apparatus (Braun, Model 'V8'). 0·2 ml. 20% (w/v) KOH was used in the inner compartment of the Warburg flasks unless otherwise stated.

Radioactive measurements. These were done by liquid scintillation techniques as previously reported (Lawrence, 1966).

Estimation of methyl ketones and methyl carbinols. Methyl ketones were estimated by pipetting 1 ml. of the contents of the Warburg flasks into 2 ml. of 2,4-DNP hydra-

zine (2 g./l. 2N-HCl) in a stoppered test-tube and the hydrazones estimated as detailed previously (Lawrence, 1965a). For the estimation of methyl carbinols the pH value of 10 ml. medium was adjusted until red to phenolphthalein and then extracted with diethyl ether. This extract was concentrated to about 0·5 ml. by carefully removing the ether with a stream of air. Samples (1 μl.) of the concentrate were analysed in a gas chromotograph (Aerograph Hy-Fi, Model A-600-B, Wilkins Instrument and Research. Inc.). Columns normally used were either 20 % UCON 50 HB 2000 or 20 % Carbowax 1540 on Chromosorb W (Mesh 30–60), although initially 20 % diethylene glycol succinate (DEGS) on firebrick was used.

Identification of the peaks was accomplished by co-chromatography with authentic samples of heptan-2-ol and heptan-2-one. The retention times of heptan-2-ol relative to heptan-2-one were 1·45 on the DEGS column (115°), 1·78 with Carbowax (90°) and 2·02 with UCON (100°) columns. Quantitative measurements were made by measuring the peak areas on the chromatograms with a planimeter. Since the concentration of heptan-2-one was known accurately, that of heptan-2-one could also be determined. Recovery trials of known amounts of heptan-2-one and heptan-2-ol carried out using the procedure above were quantitative (± 5 %) over a range of concentrations of the alchohol and ketone from 0·25–10 μmoles/ml.

Chemicals. Solutions of the salts of the fatty acids (Applied Science Laboratories, Inc., State College, Pa., U.S.A.) were prepared by adding potassium hydroxide and adjusting to the required concentration. [1-^{14}C] octanoic acid was obtained from The Radiochemical Centre, Amersham, Bucks. [2-^{14}C] octanoic acid was synthesized by a micro adaptation of the method of Reid & Ruhoff (1944). The purity of the acid was checked directly by co-chromatography with a pure sample of octanoic acid on a gas chromatograph using a column packing of 20 % (w/w) DEGS and 2 % H_3PO_4 on 60–80 mesh, acid-washed 'Celite 545' (Metcalf, 1960).

Heptan-2-ol was synthesized by reducing heptan-2-one with sodium and ethanol (Whitmore & Otterbacher, 1944). It was purified from unchanged heptan-2-one by shaking with 2,4-DNP hydrazine + HCl reagent and fractionally distilling under reduced pressure until the alcohol was completely free of ketone. The purity was checked by gas chromatography using a column of 20 % DEGS on chromosorb W (30–60 mesh).

RESULTS

Effect of growth medium on ability of mycelium to oxidize fatty acids

Lower yields of methyl ketones from fatty acids were obtained with mycelium grown in Casamino acids (3 g./500 ml.) than in the defined medium described above. The latter medium was therefore used in all subsequent work. The addition of C_{12} acid (2 μmoles/ml.) to the growth media increased mycelial growth but the mycelium subsequently showed no enhanced ability to oxidize C_{12} acid.

Effect of age of mycelium

The age at which the mycelium was harvested markedly affected the ability of the mycelium to oxidize fatty acids. Maximum oxygen uptake and methyl ketone formation in the presence of fatty acids was obtained with mycelium harvested after 48–60 hr. Oxidation of fatty acids was slower with mycelium grown for less than 30 hr, in agreement with similar observations by Vinze & Ghosh (1962).

Endogenous respiration of mycelium

In the course of most experiments a change in the rate of oxygen uptake could be clearly shown and unambiguously interpreted as a transition from endogenous respiration to the oxygen of the substrate. Moreover the basal rates of oxygen uptake before the addition of substrate and after its complete utilization were not significantly different. When allowance was made for the amounts of methyl ketone formed, almost quantitative balances ($\pm$ 10 %) were usually obtained by assuming that endogenous respiration was proceeding simultaneously and subtracting its value from the oxygen uptake in the presence of substrate. It was therefore concluded that endogenous respiration was not suppressed by the addition of non-toxic concentrations of fatty acids, which supports similar findings by Blumenthal (1963) with other *Penicillium*. spp. The validity of this assumption was supported by the finding that concentrations of 2,4-dinitrophenol up to 10^{-3}M had no effect upon oxygen uptake, indicating that no significant oxidative assimilation was taking place. (Throughout this communication the term 'toxic' acid is defined as one that suppresses the oxygen uptake of mycelium relative to endogenous. It does not necessarily mean complete inhibition. Similarly a 'non-toxic' acid is, in this context, one whose presence does not inhibit oxygen uptake by mycelium.)

Different batches of mycelium grown under apparently identical experimental conditions sometimes showed markedly different rates of endogenous respiration and also oxygen uptake in the presence of the same amount of substrate. The rates were, however, similar in duplicate experiments using the same batch of mycelium.

Metabolism of heptan-2-one

As long as octanoic acid was available, it appeared that any heptan-2-one formed was not oxidized by the mycelium. Thereafter, however, the ketone was metabolized at a rate which apparently depended upon the age of the mycelium. A typical experiment showed that mycelium harvested after 72 hr almost completely metabolised 0·4 μmoles of heptan-2-one in 4 hr, whereas the same batch of mycelium harvested and tested 24 hr earlier was only one tenth as active.

It was thought possible that heptan-2-one might be reduced to heptan-2-ol but it was found that less than 5 % of the heptan-2-one was reduced to the methyl carbinol after 32 hr. On the other hand 90 % of heptan-2-ol, at the same concentration, was oxidized to heptan-2-one in the same time.

The effect of pre-shaking mycelium in buffer for extended periods

Although endogenous respiration of mycelium was not usually markedly decreased, pre-shaking in buffer enabled the mycelium to oxidize fatty acid at concentrations which inhibited the oxygen uptake of freshly harvested mycelium (Table 1). The oxygen uptake by pre-shaken mycelium in the presence of non-toxic concentrations of octanoic acid (1 μmole) was also higher than with fresh mycelium but the yield of heptan-2-one was lower.

Effect of phosphate on fatty acid oxidation

Both oxidation of substrate and endogenous respiration were inhibited in the absence of phosphate during incubation of mycelium with fatty acids. Optimum phosphate concentration for oxygen uptake in the presence of fatty acids was about

0·01 M with fresh mycelium and about 0·03 M for mycelium shaken in phosphate buffer for 36 hr. This difference may be associated with the observed decrease in toxicity of fatty acids towards preshaken mycelium.

Effect of metabolic carbon dioxide on fatty acid oxidation

Decreases of up to 50 % in the yield of methyl ketone were obtained by the removal of metabolic carbon dioxide as it was formed during incubation. The absorption of respiratory CO_2 by alkali was not therefore a satisfactory measure of fatty acid oxidation since such methods of determining respiration are valid only if the cells follow the same pathways in the presence or absence of CO_2 (Dixon, 1951).

Table 1. *The effect of preshaking the mycelium of Penicillium roqueforti on its ability to oxidize octanoic acid at pH 2·5*

Each Warburg flask contained 10 mg. dry wt equiv. mycelium, harvested after 65 hr, 50 μmoles phosphate, (pH 2·5) and distilled water to 3 ml. Time of incubation 120 min. The mycelium was either used immediately after harvesting or after being shaken in phosphate buffer, pH 6·0, for 15 or 20 hr.

	Oxygen Uptake (μl.)			Heptan-2-one (μmoles)	
	Endogenous	1 μmole acid	3 μmoles acid	1 μmole acid	3 μmoles acid
Mycelium					
Fresh	155	285	22	0·45	0·32
Pre-shaken 15 hr	140	351	104	0·19	0·50
Pre-shaken 20 hr	145	350	150	0·14	0·70

Effect of concentration of fatty acid

At any one pH value and for a given weight of mycelium it was found that there was an optimum concentration of acid for maximum oxygen uptake and optimum conversion to methyl ketone. Thus at pH 6·5 and with 4 mg. dry wt mycelium maximum rates of oxidation were obtained with 3·5 μmoles octanoic acid (Fig. 1). Lower concentrations of this acid (1 μmole) were completely oxidized with almost no trace of heptan-2-one formation and in general increased oxygen uptake was found to occur at the expense of methyl ketone formation. Increasing the octanoic acid concentration above 3·5 μmoles at this pH value decreased the rate of oxidation. A similar inhibitory effect was also obtained by using less mycelium, showing that the ratio of the number of hyphal cells to the fatty acid concentration determined the rate of oxidation. For the following studies, therefore, the weight of mycelium (10 mg.) used was that which would oxidize 3 μmoles of octanoic acid completely at pH values between 5·0 and 7·0 in 2–3 hr.

Effect of pH value

The relative amounts of methyl ketone and respiratory carbon dioxide formed during fatty acid metabolism were dependent upon the pH of the incubation medium. At pH 2·5 oxygen uptake was inhibited by 3 μmoles octanoic acid, whereas at pH 6·0 the same concentration of acid was rapidly oxidized with a lower yield of heptan-2-one. With increasing concentration of octanoic acid, the pH value at which oxygen uptake was a maximum also increased. Optimum pH values for the oxidation of 1, 3 and 25 μmoles of octanoic acid by 10 mg. dry wt mycelium were pH 5·0, 5·5 and 6·0, respectively.

Oxidation of fatty acids with an even number of carbon atoms

The rate of oxygen uptake by the mycelium increased immediately on the addition of C_4–C_{12} fatty acids but formation of the corresponding methyl ketone was detected only after about 30 min. (Fig. 2). The rate and pattern of oxidation was markedly dependent upon the pH value (Figs. 3a–d). At pH 2·5 the rate of respiration by the mycelium in the presence of the C_6–C_{12} fatty acids was less than its endogenous respiration, the maximum inhibitory effect being shown by decanoic acid (Fig. 3a); high yields
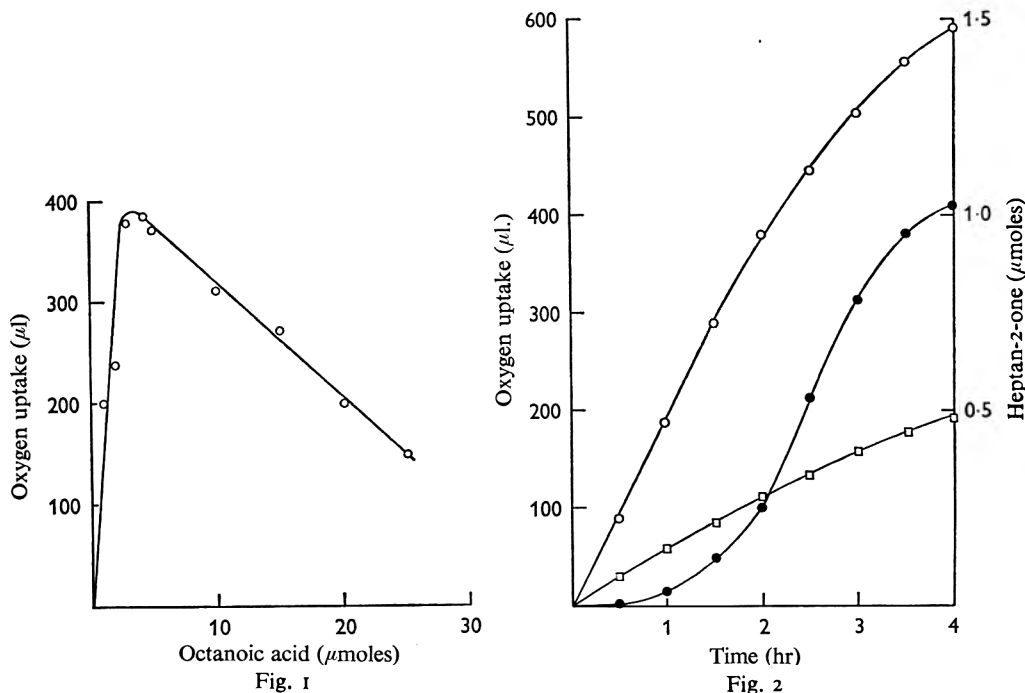
Fig. 1. The effect of concentration of octanoic acid on the rate of oxygen uptake by mycelium of *Penicillium roqueforti*. Reaction conditions: 4 mg. dry wt equiv. mycelium harvested after 48 hr, 50 μmoles phosphate buffer (pH 6·5).

Fig. 2. The rate of oxygen uptake (O-O) and methyl ketone formation (●—●) by mycelium of *Penicillium roqueforti* incubated with 3 μmoles octanoic acid. □—□, endogenous oxygen uptake. Reaction conditions: 10 mg. dry wt equiv. mycelium harvested after 48 hr, 50 μmoles phosphate buffer (pH 5·2).

of the corresponding methyl ketone with one less carbon atom were nevertheless obtained with the C_6–C_8 acids. At pH 5·2 the pattern of oxygen uptake and methyl ketone formation was essentially the same as at pH 2·5, except that C_6 and C_8 no longer partially inhibited endogenous respiration and that C_{12} acid was most toxic (Fig. 3b). At pH 6·8 rates of oxygen uptake in the presence of all fatty acids were greater than that of endogenous respiration, maximum uptakes being obtained with C_8 and C_{10} acids as substrates. The yield of pentan-2-one from hexanoic acid was considerably less than at pH 5·2 (Fig. 3c). At pH 8·0 oxygen uptake in excess of endogenous respiration and the yields of the corresponding methyl ketones were relatively low in the presence of all fatty acids. The rate of oxidation was greatest with C_{10} acid as substrate (Fig. 3d) and least with C_{14} fatty acid.

Shaking the mycelium in buffer for 48 hr considerably increased the subsequent rate at which the mycelium oxidized the C_6–C_{12} fatty acids at all pH values between 2·5 and 6·8, although the relative yields of methyl ketone from each acid were not significantly different. The most toxic acid towards fresh mycelium at any one pH value was also the most inhibitory towards pre-shaken mycelium. The C_{14}–C_{18} fatty acids were not oxidized to any significant extent by either fresh or pre-starved mycelium at any pH.
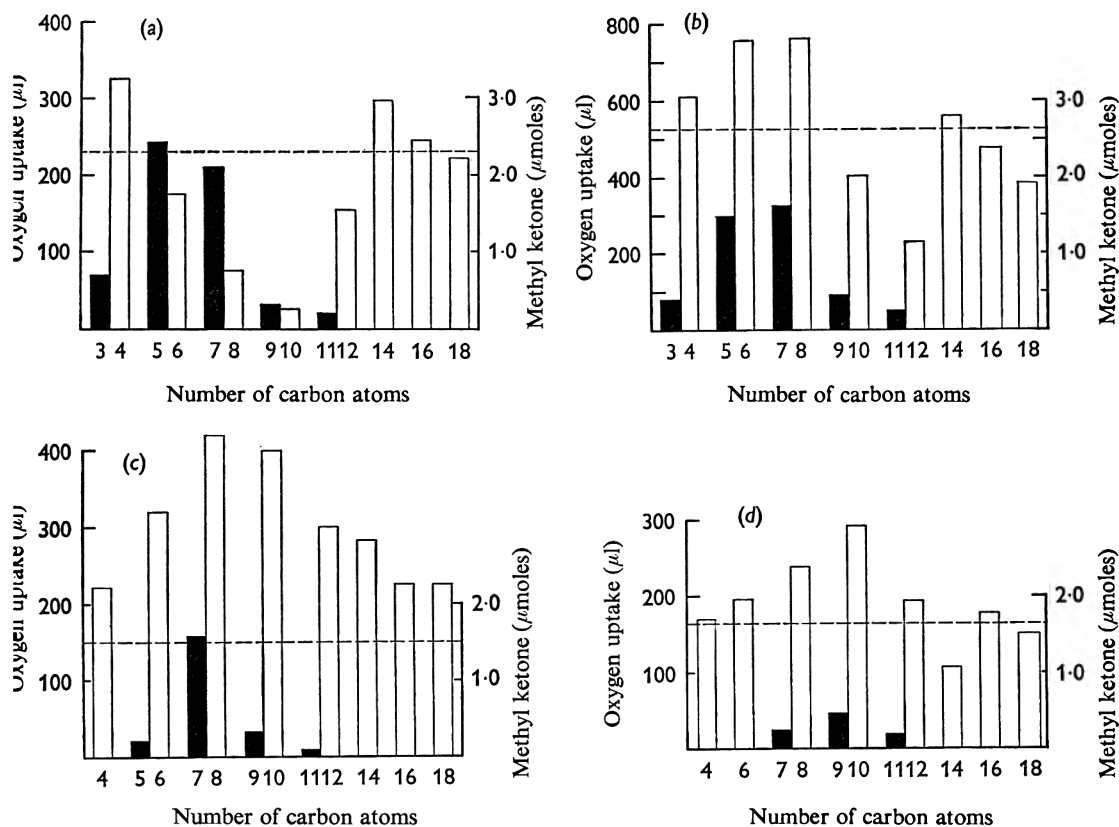
Fig. 3. The influence of chain length of fatty acids on the oxygen uptake (unshaded bars) and formation of methyl ketones (shaded bars) by mycelium of *Penicillium roqueforti* incubated with even numbered fatty acids in the range C_4–C_{18} at (a) pH 2·5, (b) pH 5·2, (c) pH 6·8, (d) pH 8·0. Reaction conditions: equiv. 10 mg. dry wt mycelium harvested after 66 hr, 3 μmoles of fatty acids, 50 μmoles phosphate and distilled water to 3 ml. Endogenous respiration of the different batches of mycelium is indicated by the dotted horizontal lines. Even carbon numbers refer to fatty acid substrates and odd carbon numbers to the corresponding methyl ketone with one less carbon atom.

Oxidation of fatty acids with an odd number of carbon atoms

The pattern of oxygen uptake and of methyl ketone formation was very similar for fatty acids containing both an even and an odd number of carbon atoms from C_3 to C_{11} (Fig. 4), suggesting that the oxidative pathway for all fatty acids is similar.

Mechanism of toxicity

The mechanism by which the C_6–C_{12} fatty acids inhibited oxygen uptake was examined with particular reference to a possible relationship between this toxic effect and the production from each acid of only the corresponding methyl ketone with one less carbon atom. The addition of toxic concentrations of C_{10} or C_{11} acid markedly suppressed the oxidation of non-toxic concentrations of C_5 acid by freshly harvested mycelium, whether added at the same time or after oxidation of the non-toxic acid was proceeding normally (Fig. 5). In the latter case C_{11} acid was more immediately effective in inhibiting oxidation of the C_5 acid than the less toxic C_{10} acid but in both cases the formation of butan-2-one from the C_5 acid was markedly increased, showing that oxidation proceeded as far as the β-oxo acid (Table 2).

A considerably higher yield of methyl ketone, however, was obtained from a non-

Table 2. *The effect of toxic acids on the formation of methyl ketones from a non-toxic acid by mycelium of Penicillium roqueforti*

Each Warburg flask contained 10 mg. dry equiv. wt mycelium harvested after 60 hr, 50 μmoles phosphate buffer (pH 5·2) and 3 μmoles of each acid. Where indicated a second acid was added 30 min. later and incubation continued for a further 160 min.

	Methyl ketone (μmoles)		
Fatty acid	C_4	C_9	C_{10}
C_5	0·19	—	—
C_{10}	—	0·40	—
C_{11}	—	—	0·16
$C_5 + C_{10}$	0·39	0·37	—
C_5 followed by C_{10}	0·39	0·35	—
C_5 followed by C_{11}	0·90	—	0·20

Table 3. *The effect of pretreating mycelium of Penicillium roqueforti with different amounts of C_{10} acid for 10 or 40 min. on its ability to oxidize C_8 acid to heptan-2-one*

Each Warburg flask contained 10 mg. dry wt equiv. mycelium (pretreated with 2–10 μmoles C_{10} acid for 10 or 40 min. and then washed with phosphate buffer and distilled water) harvested after 48 hr, 2 μmoles C_8 acid, 50 μmoles phosphate buffer (pH 5·2). Period of incubation 120 min.

Pretreatment (min.)	C_{10} acid (μmoles)	C_8 acid (μmoles)	Heptan-2-one (μmoles)	Oxygen uptake (μl.)
10	—	—	Nil	179
	—	2	0·27	414
	2	2	1·53	68
	4	2	0·50	33
	6	2	0·21	32
	8	2	0·10	30
	10	2	0·09	36
40	—	—	Nil	202
	2	—	Nil	10
	4	—	Nil	3
	—	2	0·80	438
	2	2	1·30	75
	4	2	0·24	15
	6	2	0·12	15

toxic acid by pretreatment of the mycelium with low concentrations of C_{10} acid for short periods. This pretreated mycelium was collected on a Millipore filter and washed with 0·05 M-phosphate buffer (pH 7·0) and distilled water. The mycelium was resuspended in 3 ml. phosphate buffer (pH 5·2), 2 μmoles C_8 acid were then added and oxidation allowed to proceed for 2 hr. The use of mycelium pretreated with 2 μmoles C_{10} for 10 min. resulted in a 6-fold increase in yield of heptan-2-one (Table 3). Preincubation of the mycelium with 10 μmoles C_{10} acid for 10 min., however, almost completely inhibited its subsequent ability to oxidize octanoic acid to heptan-2-one.
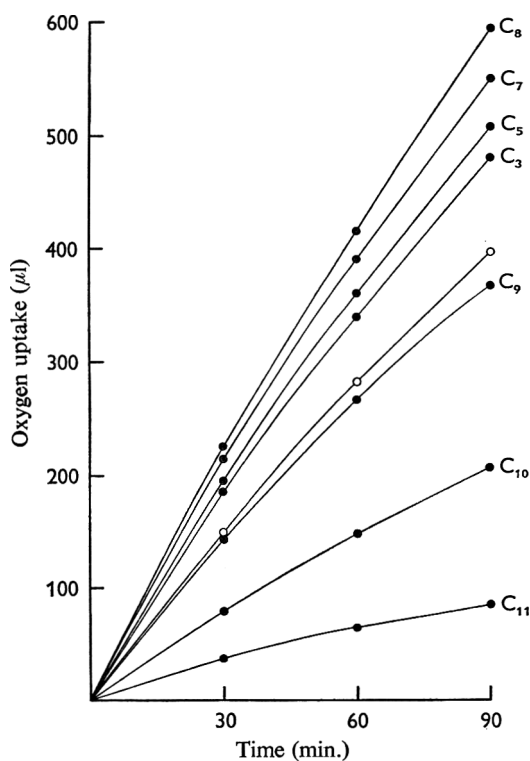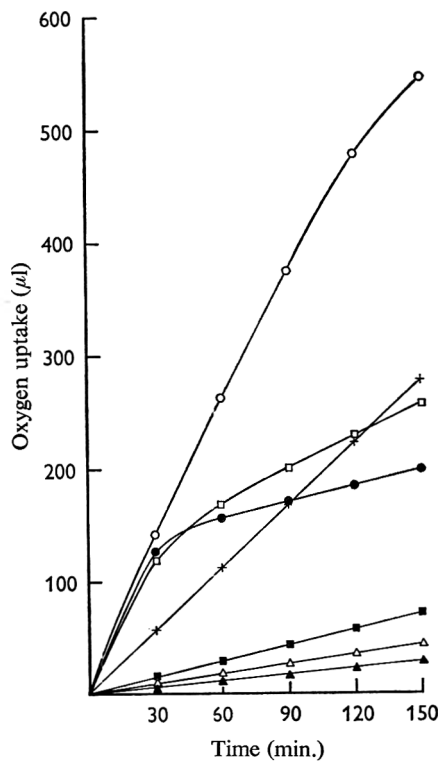
Fig. 4. The oxygen uptake at pH 5·2 by mycelium of *Penicillium roqueforti* incubated with fatty acids with an odd number of carbon atoms in the range C_3–C_{11}, compared with rates for C_8 and C_{10} fatty acids. Reaction conditions: 10 mg. dry wt equiv. mycelium, harvested after 66 hr, 3 μmoles of acid and 50 μmoles phosphate buffer. E = endogenous respiration (○—○).

Fig. 5. The inhibition of oxygen uptake by the addition of C_{10} or C_{11} fatty acids to mycelium of *Penicillium roqueforti* incubated with C_5 fatty acid. Reaction conditions: 10 mg dry wt equiv. mycelium harvested after 48 hr., 3 μmoles of each acid, 50 μmoles of phosphate buffer and distilled water to 3 ml. Oxygen uptake in the presence of C_5 acid (○—○), C_{10} acid (△—△), C_{11} acid (▲—▲), C_5 and C_{10} acid added simultaneously (■—■), C_5 acid followed after 30 min. by C_{10} acid (□—□) or C_{11} acid (●—●); endogenous respiration (×—×).

The extent of damage to the enzyme systems of the mycelium was also dependent, to a lesser extent, on the length of time the cells were subjected to the toxic effect of the C_{10} acid. Preincubation of the mycelium with C_{10} acid for 40 min. resulted in a slightly lower rate of oxidation than mycelium pretreated with the same concentration of acid for 10 min. (Table 3.)

No $^{14}CO_2$ was detected when mycelium that had been pretreated with a toxic concentration of unlabelled C_{10} acid for 20 min. was subsequently incubated with [2-^{14}C]octanoic acid for 2 hr, whereas about 7 % of the radioactivity was converted to $^{14}CO_2$ by untreated mycelium. The yield of [1-^{14}C]heptan-2-one was, however, three times greater with mycelium that had been pre-treated with C_{10} acid.
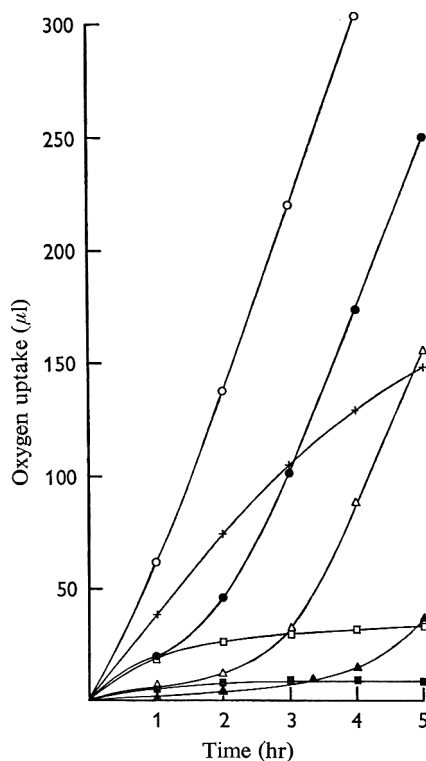
Fig. 6. The annulment of the inhibitory effect of octanoic acid at low pH value on the oxygen uptake of mycelium of *Penicillium roqueforti* by an increase in pH value. Reaction conditions: 10 mg. dry wt equiv. mycelium harvested after 48 hr, 50 μmoles phosphate buffer and distilled water to 3 ml. Oxygen uptake of mycelium pretreated at pH 2·5 for 1 hr with 4 μmoles ($\bullet$—$\bullet$), 6 μmoles ($\triangle$—$\triangle$) and 8 μmoles C_8 acid ($\blacktriangle$—$\blacktriangle$) after addition of sufficient KOH to raise to pH 5·5; Oxygen uptake in presence of 4 μmoles C_8 acid at pH 2·5 ($\square$—$\square$) and pH 5·5 ($\bigcirc$—$\bigcirc$ and of 6 μmoles C_8 acid at pH 2·5 ($\blacksquare$—$\blacksquare$) also given; endogenous respiration ($\times$—$\times$).

Reversibility of toxic effect of fatty acids at low pH values

Inhibition of respiration was almost instantaneous at pH 2·5 when toxic concentrations of C_6–C_{12} fatty acids were added to freshly harvested mycelium. This effect could be annulled, after a lag phase, by increasing the pH value of the reaction mixture to 5·5 for C_6 and C_8 acids and to pH 6·8 for C_{10} and C_{12} acids. The length of the lag phase before oxidation proceeded depended on the concentration of fatty acid added (Fig. 6) and to a lesser extent upon the length of time the cell was in contact with the acid at pH 2·5.

Rates of formation of $^{14}CO_2$ from $[1\text{-}^1C^4]$ and $[2\text{-}^{14}C]$octanoic acids

The rate of appearance of ^{14}C in respiratory CO_2 was $1\cdot7$–$3\cdot1$ times faster from $[1\text{-}^{14}C]$octanoic acid than from $[2\text{-}^{14}C]$octanoic acid, over 21 % of the former and 7 % of the latter being oxidized to $^{14}CO_2$ in 150 min. at pH $6\cdot0$ (Table 4). No heptan-2-one was detected in the first 120 min. and only $0\cdot05$ μmoles after 150 min. A correction for the $0\cdot05$ μmoles $^{14}CO_2$ formed in the decarboxylation of β-oxo octanoic acid had therefore to be applied in the latter instance.

Table 4. *A comparison of the rate of appearance of* ^{14}C *in respiratory carbon dioxide during the oxidation of* $[1\text{-}^{14}C]$- *and* $[2\text{-}^{14}C]$*octanoic acids by mycelium of Penicillium roqueforti.*

Each flask contained equiv. 10 mg. dry wt mycelium harvested after 48 hr, 50 μmoles phosphate buffer (pH $6\cdot0$), $1\cdot5$ μmoles of either $[1\text{-}^{14}C]$octanoic acid (43,500 counts/min.) or $[2\text{-}^{14}C]$octanoic acid (44,750 counts/min.).

	$[1\text{-}^{14}C]$octanoic acid: $^{14}CO_2$ formed		$[2\text{-}^{14}C]$octanoic acid: $^{14}CO_2$ formed	
Time (min.)	Counts/ min.	Total activity (%)	Counts/ min.	Total activity (%)
30	1150	$2\cdot7$	500	$1\cdot1$
60	1955	$4\cdot5$	1495	$3\cdot3$
90	4380	$10\cdot1$	2234	$5\cdot0$
120	6365	$14\cdot7$	2575	$5\cdot7$
150	9430	$21\cdot7$	3185	$7\cdot0$

Effect of malonate

It was expected that the addition of malonate would inhibit the oxidation of fatty acids to carbon dioxide and thus possibly lead to an increase in methyl ketone formation. Various concentrations of malonate up to $0\cdot1$ M, however, did not inhibit respiration of mycelium either in the presence or absence of fatty acids between pH $2\cdot5$ and $6\cdot0$. Low concentrations of malonate at pH values less than $5\cdot0$ actually increased the rate of oxygen uptake, indicating ability of the hyphal cells to metabolize malonic acid. Malonate was, however, an effective inhibitor of the high succinic dehydrogenase activity in cell-free extracts obtained by disintegrating the mycelium in a Hughes press. Similar findings have been reported with preparations from higher plants (Young & Shannon, 1959; Hatch & Stumpf, 1962).

DISCUSSION

The rate of oxygen uptake by mycelium of *Penicillium roqueforti* increased immediately in the presence of low concentrations of C_4–C_{12} fatty acids. The length of time for which the mycelium had been grown, however, greatly affected its ability to oxidize fatty acids to respiratory carbon dioxide. This is in agreement with the finding that the oxidation of acetate by the mycelium of *P. urticae* was markedly dependent upon the phase of growth at which it was harvested (Bu'Lock *et al.* 1965).

The rate of appearance of ^{14}C in respiratory carbon dioxide was $1\cdot7$–$3\cdot1$ times greater for $[1\text{-}^{14}C]$ than for $[2\text{-}^{14}C]$octanoic acid, indicating the existence of a β-oxidative

pathway in mycelial respiration (Katz & Chaikoff, 1955), although carbon dioxide production from both acids was slow. The thiolase and deacylase reactions involved in the metabolism of fatty acids to respiratory carbon dioxide and methyl ketones respectively have a common intermediate, namely the β-oxoacylester. By analogy with other regulatory mechanisms it would appear that this first divergent step in fatty acid oxidation is the most likely point of cellular regulation. It is possible that the lag phase which occurs in methyl ketone formation, and the observed slow formation of $^{14}CO_2$ from [2-^{14}C]octanoate, may result from a product inhibition of thiolase activity and consequent induction of either deacylases or β-oxo decarboxylases. In this event deacylation of the β-oxo acyl ester and subsequent decarboxylation of the β-oxo acid to methyl ketone would provide an alternative source of coenzyme A for further fatty acid activation when the oxidation of acetyl CoA formed in the thiolase reaction is impaired. Such a mechanism would explain why only a single methyl ketone with one less carbon atom was detected in the oxidation of a single fatty acid as substrate and would support the hypothesis that secondary metabolites are formed as a result of induced enzyme mechanisms (Bu'Lock & Powell, 1965).

The lowest yields of methyl ketone were obtained with the C_9–C_{12} fatty acids which were the most inhibitory to oxygen uptake by the mycelium. A possible explanation for this finding is that the β-oxo acyl esters of the C_9–C_{12} acids are much less readily deacylated than those of the C_4–C_8 acids and that less coenzyme A is therefore available in the system. This is supported by the marked decrease in formation of carbon dioxide and increase in production of heptan-2-one when octanoic acid was added to cells which had been shaken with low concentrations of the C_9–C_{12} acids. It may also be significant that the C_{14}–C_{18} fatty acids were not oxidized by the mycelium to any significant extent at any pH and that no corresponding methyl ketones were detected from these acids.

The present investigation has emphasized the importance of pH value on the toxic effects of the homologous series of fatty acids upon the mycelium of *Penicillium roqueforti*. C_{10} and C_{12} acids were the most toxic acids at pH 2·5 and 5·2 respectively, whereas at pH 6·8 none of the acids from C_4 to C_{18} inhibited oxygen uptake. The most toxic acid at pH 8·0 was C_{14} acid but it is possible that at alkaline pH values the toxicity is a result of a surface effect as distinct from the toxicity observed at pH values less than 6·0. The inter-relationship between the toxic action of fatty acids and pH allows a ready explanation of the apparent contradiction between the results of Rolinson (1954), who reported C_8 and C_{10} fatty acids to be toxic to *P. chrysogenum* at pH 6·3, and those of Girolami & Knight (1955), who found that at pH 7·3 these two acids stimulated oxygen uptake by *P. roqueforti* to a greater extent than other acids. Similarly in this investigation the C_9–C_{12} acids more actively inhibited the respiration of mycelium than other fatty acids at pH values below 6·0 but were the most rapidly oxidized at pH 8·0. Conversely the C_3–C_5 acids were most readily oxidized at pH 2·5 but least rapidly at pH 8·0.

The present work supports the hypothesis that the primary inhibitory effect of toxic compounds on fungal cells occurs after they have penetrated the cell wall (Cochrane, 1958). Cells pretreated with a toxic concentration of fatty acids and then thoroughly washed with buffer were capable of oxidizing non-toxic concentrations of fatty acids to the corresponding methyl ketone but not to carbon dioxide. Similarly the C_6–C_{12} fatty acids inhibited endogenous respiration of the mycelium markedly at pH values below

6·0 but were nevertheless oxidized to methyl ketones in high yield. This suggests that the toxic effect was not due to adsorption of the fatty acids on the cell wall but that the acids actually entered the hyphal cell. The observed variation with pH of the inhibitory effect of fatty acids on oxygen uptake by mycelium is most easily explained by assuming that only undissociated molecules of fatty acids are transported into the cell and that, at a certain concentration, they or their oxidation products are toxic.

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The Esterases of *Mortierella ramanniana* in Relation to Taxonomy

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SUMMARY

The esterase profiles of 9 strains of *Mortierella ramanniana* grown on glucose and olive-oil media were determined by starch-gel electrophoresis. The enzymes produced on the oil medium were in most instances produced also on the glucose medium, but on the latter some additional esterase components were produced. The esterase patterns of the different strains varied widely; only one esterase was common to all strains grown on glucose medium, and a second esterase was common to all grown on the olive-oil medium. The data available do not suggest that esterase profiles are of taxonomic significance for these fungi.

INTRODUCTION

Previous studies of some species of the genus *Mortierella* showed that the synthesis of fat and the utilization of extracellular fats were important aspects of their metabolism (Chesters & Peberdy, 1965). This indicated that esterases (carboxylic ester hydrolases, E.C. 3.1.1.) are important enzyme systems in these fungi. Several workers have shown that gel electrophoresis is a suitable technique for studying esterases and in many cases the system has been found to consist of a group of molecular species, not a single enzyme (Aranson & Pantelouris, 1966; Robinson, 1966). Gel electrophoresis has been used as a taxonomic aid by several workers. Protein patterns were found useful with various fungi (Chang, Srb & Steward, 1962; Clare, 1963; Durbin, 1966) and actinomycetes (Gottlieb & Hepden, 1966). Esterase patterns have also been found to be a useful taxonomic criterion for bacteria (Norris, 1964; Cann & Willox, 1965; Robinson, 1966). In view of the possible importance of esterase systems in Mortierella it was thought to be of interest to extend the electrophoretic study to these fungi.

Characteristics of primary importance in fungal taxonomy include measurements of spores and spore-bearing structures, as well as characters of the vegetative mycelium and the colour of mycelium and spores. In the Mucorales the mode of branching of the sporangiophores is important, and the production of characteristic resting bodies may have secondary significance. The group of species typified by *Mortierella isabellina* Oudemans & Koning, in which *M. ramanniana* can be placed, differs markedly in morphology from the rest of the genus. Within this group, 5 major species can be distinguished on morphological characters (Turner, 1963) but the distinction is not always clear-cut. Any biochemical characters which might help to define these species, or to separate this group from the rest of the genus, could be of value; but it is neces-

sary first to ascertain whether the character under consideration is constant within a given species, and whether any variations show sufficient correlation with morphological variation to make comparison with other species of value.

METHODS

The organisms. Nine strains of *Mortierella ramanniana* (Moëller) Linnemann were used (designated 20, 23, 29, 119, 138, 163, 187, 212, 213). Cultures were maintained on potato glucose agar (Oxoid), and for morphological examination were grown at room temperature (about 20°) for 5–7 days on soil extract agar (pH 6·0) containing (g./l.): glucose, 1·0; KH_2PO_4, 0·2; yeast extract (Oxoid), 0·1. All measurements were made on unstained material, freshly mounted in 0·9 % NaCl. Spores were measured by the camera-lucida method.

Composition of media for esterase production. The basal medium contained (g./l.): $(NH_4)_2HPO_4$, 1·0; KCl, 0·2; $MgSO_4.7H_2O$, 0·2; yeast extract (Oxoid), 10·0. To this solution glucose 20 g./l., or olive oil B.P. (Boots Pure Drug Co. Ltd.) 20 ml./l. were added as carbon source. The medium was adjusted to pH 6·5 before autoclaving at 108° for 30 min. The olive oil was sterilized separately by heating at 160° for 2 hr and added aseptically to the culture medium.

Inoculation and incubation. Cultures were grown in squat 1 l. conical flasks containing 300 ml. medium, inoculated with 5 ml. of spore suspension prepared from 7-day cultures on potato glucose agar. The flasks were incubated at 25°, on a reciprocal shaker. After 5 days, cultures were harvested by filtration and the mycelial pads thoroughly washed with cold distilled water before extraction of enzymes.

Preparation of mycelial extracts. The washed mycelium was ground at 4–5°, in a pre-cooled mortar, with an equal quantity of acid-washed sand, for about 10 min. In early preparations, about 3 ml. cold McIlvaine buffer (pH 6·4) was added during the final stages of grinding; later the buffer was replaced by cold distilled water with no detrimental effect. Sand and mycelial fragments were removed by centrifugation at 2500 *g* for 10 min. The supernatant fluid was distributed into small tubes and stored at −20°. Samples have been stored for up to 6 months, during which time some of the component enzymes deteriorated until finally they became inactive.

Electrophoresis. The mycelium homogenates were analysed by the starch-gel technique of Smithies (1955) with hydrolysed starch (Connaught Medical Research Laboratories) and the discontinuous buffer system of Poulik (1957) as modified by Baillie & Norris (1963). Electrophoresis was done at room temperature (about 20°) at constant voltage (7–10 V./cm. gel). The initial current applied to the gels was 25 mA. The buffer front was allowed to run 10 cm. from the starting line towards the anode. In some gels the brown line, which marked the position of the buffer front, was not always clear. This difficulty was overcome by using bromothymol blue as a marker dye, which ran with the buffer front. Horse serum was used as a standard with all gels.

Detection of esterases. On completion of electrophoresis the gels were sliced into 4 layers, 1·5 mm. thick. The middle two slices were stained for esterases by the method of Lawrence, Melnick & Weimer (1960). The gel slices were flooded with a freshly prepared solution of tris maleate buffer (0·1 M, pH 6·4) 200 ml.; 1 % (w/v) α-napthyl acetate in 50 % (v/v) acetone in water, 8 ml.; fast blue salt B (Gurr) 0·1 g. The presence of esterases was indicated by the appearance of red bands after 1 hr at room temperature (20°).

Photography. The stained gels were photographed by reflected light, with Kodalith ortho type 3 film and D 19B or D 163 developer.

RESULTS

Esterase preparations were obtained from 3 cultures of each strain on each medium. The positions of the esterase bands were measured and the E_F values given have been corrected to a horse serum standard of 0·68.

Data of the electrophoretic analyses is given in Tables 1 and 2 and the esterase profiles are presented graphically after the manner of Gottlieb & Hepden (1966) in Fig. 1. Measurements of morphological structures are summarized in Table 3.

These data indicate that there were 15 different components in the esterase systems of the strains examined; the maximum number for any 1 strain was 10, in strain 213 grown on olive-oil medium. Thus the esterase systems in these fungi are also multi-molecular systems. It is sometimes difficult to decide the correct location of a band, e.g. strain 20 on both media has a component with an E_F value in the range 0·84–0·88, which has been designated band *l*, but may well be identical with band *k* produced by other strains. The E_F values for any one band in a given strain may also vary, e.g. band *k* in strain 29 on glucose medium. It is intended to submit these and additional data to statistical treatment and so give a more precise picture of the profiles.

The effect of the medium on the esterase system of these fungi was not very marked. In some strains more enzymes were produced by the cultures when grown on glucose medium than on oil medium. The esterases present in the mycelium produced on oil medium were almost always produced also on the glucose medium.

The esterase patterns of the nine strains showed little resemblance, though 8 of them had at least 1 band in common (band h, mean E_F 0·75, on glucose medium, and band j, mean E_F 0·80, on oil medium). Strain 163 was unique in showing 3 components, 3 of which were broad bands about 1 cm. wide. Attempts to analyse the preparations from this strain by vertical starch-gel electrophoresis, in the hope of separating these broad bands into further components, were unsuccessful.

DISCUSSION

Expression of the esterase profile in terms of E_F values has obvious advantages over a purely qualitative description. Chang *et al.* (1962) and Gottlieb & Hepden (1966) published quantitative data (R_F or E_F values) but only the latter give evidence of variation in the position of particular protein bands in the gels. In comparison with the data of Gottlieb & Hepden our results show a little more variation. This might well be a reflexion of the different electrophoresis methods used. One serious problem with the horizontal starch-gel method is the frequent uneven running of the buffer front; this can make measurements difficult particularly when the brown line marking the buffer front in the gel is not clearly visible.

As yet we have no evidence of the role of the esterases produced by these fungi. In cultures grown on glucose medium the enzymes may function at a later stage of development if the exogenous energy source is depleted and the intracellular fat utilized. Whether these same enzymes are used in the breakdown of extracellular oils when supplied as a carbon source is also unknown. There is no doubt that the olive oil is

Table 1. *Average E_F values* for esterase components from* Mortierella ramanniana *strains grown on basal medium + olive oil*

Strain	Extract	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o
											Esterase bands					
20	i	—	0·50 (20%)†	—	—	0·68 (10%)	0·72 (20%)	—	—	—	0·81 (100%)	—	0·84 (20%)	—	—	—
	ii	—	0·47 (57%)	—	—	—	—	—	—	—	0·79 (100%)	—	0·87 (28%)	—	—	—
	iii	—	0·47 (100%)	—	—	—	—	—	0·76 (100%)	—	0·82 (100%)	—	0·87 (28%)	—	—	—
23	i	—	0·49 (36%)	—	—	—	—	—	0·74 (14%)	—	0·80 (93%)	0·84 (50%)	—	—	—	—
	ii	—	0·48 (64%)	—	—	—	—	—	0·72 (14%)	—	0·80 (100%)	0·83 (50%)	—	—	—	—
	iii	—	0·47 (75%)	—	—	—	—	—	0·78 (50%)	—	0·80 (92%)	0·86 (83%)	—	—	—	—
29	i	—	0·46 (64%)	—	—	—	—	—	0·76 (91%)	—	0·79 (73%)	0·84 (54%)	—	—	—	—
	ii	—	0·47 (80%)	—	—	—	—	—	0·76 (70%)	—	0·79 (90%)	0·84 (90%)	—	—	—	—
	iii	—	0·46 (89%)	—	—	—	—	—	0·75 (100%)	—	0·78 (100%)	0·83 (100%)	—	—	—	—
119	i	—	—	—	—	0·72 (9%)	—	—	—	—	0·79 (100%)	0·82 (9%)	—	—	—	—
	ii	—	0·51 (8%)	—	—	0·68 (8%)	—	—	—	—	0·79 (100%)	0·86 (17%)	—	—	—	—
	iii	—	0·52 (17%)	—	—	0·65 (25%)	—	—	0·76 (100%)	—	0·79 (100%)	0·82 (58%)	—	—	—	—
138	i	—	—	0·55 (36%)	—	—	—	—	0·74 (54%)	—	0·79 (54%)	—	—	—	—	—
	ii	—	—	0·54 (27%)	—	—	—	—	0·74 (100%)	—	0·78 (100%)	0·83 (45%)	—	—	—	—
	iii	—	—	0·54 (30%)	—	—	0·72 (50%)	—	0·76 (100%)	—	0·81 (91%)	—	—	—	—	—

Table 1 (cont.)

Strain	Extract	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o
163	i	—	0·49	0·57 (100%)	—	—	0·71	—	(100%)	—	0·79	—	—	—	0·92 (100%)	—
	ii	—	0·49	0·58 (100%)	—	—	—	0·73	(100%)	—	0·79	—	—	—	0·93 (100%)	—
	iii	—	0·49	0·58 (80%)	—	—	—	0·73	(100%)	0·77	—	—	—	—	0·91 (80%)	—
187	i	—	0·49 (37%)	—	—	—	—	—	0·78 (100%)	—	0·82 (94%)	—	—	—	—	0·96 (6%)
	ii	—	0·52 (40%)	—	—	—	—	—	0·76 (100%)	—	0·81 (100%)	—	—	—	—	0·95 (7%)
	iii	—	0·48 (36%)	—	—	—	—	—	0·76 (100%)	—	0·82 (100%)	—	—	—	—	0·96 (7%)
212	i	—	—	—	—	0·73 (45%)	—	—	—	0·77 (100%)	0·80 (64%)	0·83 (9%)	—	0·89 (9%)	—	—
	ii	—	—	—	—	0·70 (44%)	—	—	—	0·77 (100%)	0·80 (89%)	0·83 (33%)	—	0·89 (10%)	—	—
	iii	—	—	—	—	0·68 (23%)	—	—	—	0·76 (89%)	0·80 (100%)	0·83 (10%)	—	—	—	—
213	i	0·20 (18%)	—	—	—	—	—	—	0·75 (36%)	0·77 (45%)	0·79 (64%)	0·82 (91%)	0·85 (36%)	0·88 (18%)	—	—
	ii	0·19 (15%)	0·49 (18%)	—	—	0·66 (9%)	—	—	0·75 (27%)	0·77 (45%)	0·79 (100%)	0·82 (54%)	0·85 (27%)	0·88 (54%)	—	—
	iii	0·18 (9%)	—	—	—	—	0·72 (18%)	—	0·75 (36%)	0·77 (100%)	0·80 (73%)	0·82 (73%)	0·84 (54%)	0·89 (9%)	—	—

Esterase bands

*Each extract was analysed at least 5 times.

†Figures in parentheses are % of gels in which the band was present.

Table 2. Average E_F values* for esterase components from Mortierella ramanniana strains grown on basal medium + glucose

Strain	Extract						Esterase bands									
		a	b	c	d	e	f	g	h	i	j	k	l	m	n	o
20	i	—	0·50† (17%)	—	—	—	—	—	0·76 (34%)	—	0·82 (100%)	—	0·85 (17%)	—	—	—
	ii	—	0·51 (100%)	—	—	—	—	0·73 (40%)	0·76 (80%)	—	0·82 (100%)	—	0·88 (100%)	—	—	—
	iii	—	0·48 (86%)	—	—	—	—	—	0·75 (100%)	—	0·81 (100%)	—	0·86 (100%)	—	—	—
23	i	—	0·48 (50%)	0·54 (17%)	—	—	—	0·73 (33%)	0·75 (33%)	0·76 (50%)	0·80 (100%)	—	0·85 (66%)	—	—	—
	ii	—	0·45 (57%)	0·53 (14%)	—	—	—	0·73 (86%)	0·75 (43%)	—	0·79 (100%)	—	0·84 (86%)	—	—	—
	iii	—	0·46 (57%)	—	—	—	0·71 (28%)	0·73 (57%)	0·75 (28%)	0·78 (14%)	0·81 (100%)	—	0·85 (71%)	—	—	—
29	i	—	0·44 (80%)	—	—	—	—	—	—	0·77 (100%)	—	0·84 (60%)	—	—	—	—
	ii	—	0·44 (100%)	—	—	—	—	—	0·73 (100%)	0·78 (100%)	—	0·83 (80%)	—	—	—	—
	iii	—	0·44 (100%)	—	—	—	—	—	0·75 (100%)	0·78 (100%)	—	0·82 (100%)	—	—	—	—
119	i	—	—	—	—	—	—	—	0·75 (28%)	0·78 (86%)	0·81 (57%)	—	—	—	—	—
	ii	—	0·55 (40%)	—	—	—	—	—	0·75 (100%)	0·79 (86%)	—	—	0·85 (100%)	—	—	—
	iii	—	0·52 (15%)	—	—	—	—	—	0·76 (100%)	—	0·81 (86%)	—	0·86 (100%)	—	—	—
138	i	—	—	—	—	—	—	—	0·75 (28%)	—	0·80 (100%)	0·83 (28%)	—	—	—	—
	ii	—	0·54 (83%)	—	—	—	—	—	0·74 (100%)	—	0·79 (100%)	0·84 (100%)	—	—	—	—
	iii	—	0·53 (28%)	—	—	—	—	—	0·74 (100%)	—	0·79 (100%)	0·84 (86%)	—	—	—	—

Table 2 (cont.)

Esterase bands

Strain	Extract	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o
163	i	—	0·49	0·58 (100%)	—	—	—	—	0·75	(100%)	0·80	—	—	—	0·92 (100%)	—
	ii	—	0·48	0·57 (100%)	—	—	—	0·73	(100%)	0·78	—	—	—	—	0·92 (100%)	—
	iii	—	0·46	0·56 (100%)	—	—	—	0·73	(100%)	—	0·79	—	—	—	0·92 (100%)	—
187	i	—	0·49 (100%)	—	—	—	0·72 (100%)	—	0·75 (83%)	0·77 (33%)	0·80 (100%)	0·84 (83%)	—	—	—	—
	ii	—	0·48 (100%)	—	—	—	0·72 (100%)	—	0·75 (100%)	0·77 (83%)	0·81 (83%)	0·84 (83%)	—	—	—	—
	iii	—	0·48 (71%)	—	—	—	0·73 (100%)	—	0·75 (71%)	0·77 (86%)	0·80 (86%)	0·83 (71%)	—	—	—	—
212	i	—	—	—	—	—	0·71 (33%)	0·73 (83%)	0·75 (75%)	0·78 (50%)	0·80 (33%)	—	—	—	—	—
	ii	—	—	—	—	—	0·71 (33%)	—	0·75 (50%)	0·78 (83%)	—	—	—	—	—	—
	iii	—	—	—	0·60 (83%)	—	0·71 (33%)	—	0·75 (50%)	0·78 (50%)	0·82 (17%)	—	—	—	—	—
213	i	0·19 (33%)	0·44 (17%)	—	—	—	—	—	0·75 (33%)	0·79 (66%)	0·81 (83%)	0·83 (50%)	0·85 (50%)	0·89 (17%)	—	—
	ii	0·19 (66%)	0·44 (66%)	—	—	0·66 (66%)	—	—	0·75 (100%)	0·79 (66%)	—	0·83 (66%)	0·85 (66%)	0·89 (17%)	—	—
	iii	0·19 (66%)	0·43 (66%)	—	—	0·66 (83%)	—	—	0·75 (100%)	0·79 (100%)	—	0·83 (66%)	0·85 (50%)	0·89 (17%)	—	—

*Each extract was analysed at least five times.

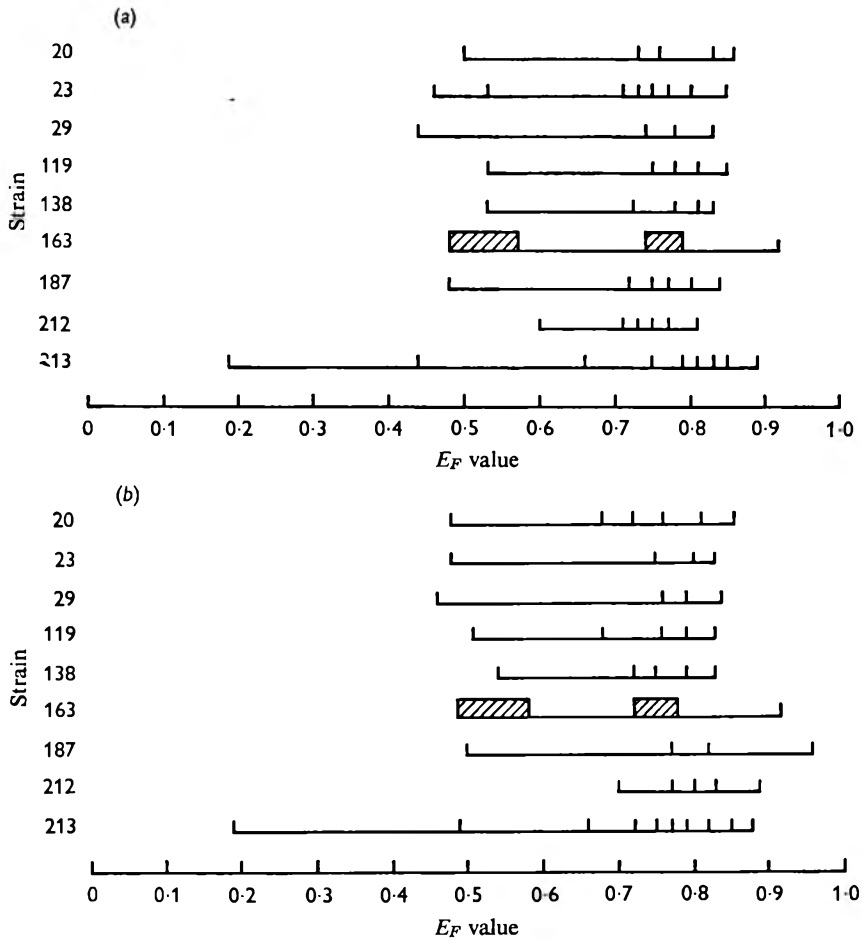†Figures in parentheses are % of gels in which the band was present.

Fig. 1. Esterase profiles of *Mortierella ramanniana* strains grown on (*a*) basal medium +
glucose and (*b*) basal medium + olive oil. The E_F values given are the average values from
all analyses of three cultures of each strain. The cross-hatched blocks refer to wide bands.

Table 3. *Measurements of reproductive structures and giant cells of the Mortierella*
rammaniana strains examined. All measurements in microns

Strain	Sporangiophore length (μ)		Sporangium diameter (μ)		Spore size (μ)		Giant cells (μ)	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
20	466·0	150–625	16·9	11–26	2·4 × 1·8	1·5–3·0 × 1·0–2·5	43·3 × 40·3	28–60 × 28–56
23	476·5	250–720	19·7	12–26	2·7 × 1·7	1·5–3·5 × 1·5–2·5	43·7 × 41·9	30–60 × 30–60
29	680·5	500–1000	20·2	15–27	4·1 × 1·9	3·0–5·0 × 1·5–2·5	31·5 × 23·0	13–60 × 13–34
119	736·0	480–1300	19·5	12–28	2·7 × 1·7	2·0–3·5 × 1·5–2·5	—	—
138	641·0	340–1200	19·0	12–24	3·6 × 2·4	2·5–4·5 × 1·5–3·0	—	—
163	301·5	180–480	13·1	10–20	2·2 × 1·9	1·5–3·0 × 1·5–3·0	—	—
187	620·0	420–1250	20·4	12–26	3·2 × 1·9	2·0–4·5 × 1·5–2·5	31·1 × 28·2	10–54 × 10–44
212	486·5	300–650	18·6	12–24	2·7 × 2·4	2·0–3·5 × 1·5–3·5	24·3 × 22·7	18–36 × 18–34
213	411·6	240–600	16·7	10–28	2·9 × 2·4	2·0–4·0 × 2·0–3·0	29·7 × 27·7	18–40 × 18–38

utilized during growth. At the start of the growth period the medium was opaque because of the emulsification of the oil with shaking. After 4 days the emulsion had been broken completely and the medium was clear. The fact that some components of the system produced only faint bands on the gels may reflect (i) that they were minor components of the multimolecular system, or (ii) that they were truly extracellular enzymes, in which case only small amounts would be expected in the mycelium, or (iii) that they were intracellular but not so active against the substrate used in these experiments. Of course if these esterases are intracellular they may still function in the break-down of extracellular lipids. It is generally believed that the older parts of fungal hyphae soon undergo autolysis and release their contents into the medium. Evidence relating to the criteria of Pollock (1962) is necessary before a definite statement on the nature of these enzymes may be given. Further studies on these systems are being made.

The variation in esterase profiles found between the different strains was surprising. Only strains 23 and 29 produced exactly the same pattern and then only when grown on olive-oil medium. If some of the assumptions mentioned earlier, relating to the location of certain bands, were made, then at least 4 strains (20, 23, 29, 119) would have basically the same pattern when grown on olive-oil medium. It has been stated that certain esterases are constant in all strains when grown on the 2 media. Whether the presence of these enzymes, when the fungi are grown under defined conditions, can be used as taxonomic criteria can only be assessed when other species of the genus are investigated.

Of the 9 strains examined, 20, 23, 212 and 213 are morphologically indistinguishable, and may be regarded as 'typical' *Mortierella ramanniana*. Strains 119 and 138 lack the large chlamydospores ('giant cells') which are normally found in these species. The sporangiophore of these two isolates, also those of 29 and 187 (both of which possess 'giant cells') are larger than those of the other strains; the spores of 138 and 187 are slightly larger and more oval; those of 29 are larger still and definitely elliptical. Since the columella is also unusually well developed, and the colony colour slightly different, this isolate 29 may represent a variety of the species (Turner, 1963), but the esterase pattern provides no further evidence for this. In fact for these 8 strains there seems to be little or no correlation between the variations in the esterase pattern and the morphological differences.

The only strain which shows any marked morphological differences, and which also shows a markedly different esterase pattern is strain 163. This strain, in addition to the normal type of sporangiophore (rather shorter than usual but still well within the range of variation for the species), produces in parts of the colony a number of very short sporangiophores (30–60μ long), unbranched or branching in a manner resembling *Mortierella vinacea*. It also resembles this species in being thiazole-independent, and in lacking the 'giant cells' of *M. ramanniana*. However, the characteristically angular spores of *M. vinacea* have not been seen with this isolate 163. This was at first suspected of being a mixed culture, but repeated re-isolations from single spores or hyphal tips continue to present the same picture. According to Dr E. A. Evans (personal communication) such strains are not uncommon, and they can mutate spontaneously to produce normal, thiazole-dependent *M. ramanniana* strains. The difference in esterase pattern may be of significance here. Comparison with other similar isolates and with typical strains of *M. vinacea*, should prove interesting.

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