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Extracellular Polysaccharide Synthesis by Members of the Genus *Lactobacillus*: Conditions for Formation and Accumulation

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SUMMARY

Members of the genus *Lactobacillus* were examined for their ability to synthesize extracellular polysaccharide from sucrose. Strains physiologically similar to *L. pastorianus* synthesized a glucan. Other strains liberated a complex polysaccharide which contained mannose and glucose. Variants of the glucan-producing strains occurred spontaneously and these lacked the capacity to synthesize glucan; however, extracts of these bacilli contained glucansucrase and invertase. Manometric studies indicated that the latter enzyme functioned in the sucrose metabolism of these non-glucan producing variants.

INTRODUCTION

The extracellular dextransucrases of *Leuconostoc mesenteroides* (Hehre, 1951; Tsuchiya *et al.* 1956) and of *Streptococcus bovis* (Dain, Neal & Seeley, 1956; Bailey, 1959) have been described. A number of reports have also shown that some lactobacilli produce extracellular polysaccharides which yield glucose on hydrolysis. These investigations, some of which were reviewed by Perquin (1940), did not attempt to identify fully or compare the polysaccharides produced by lactobacilli with the dextrans of leuconostoc and streptococcus. In addition, little information is available on the conditions under which lactobacilli produce extracellular polysaccharides except that the synthetic capacity is unstable and cultures stored in the laboratory lose the ability to produce the extracellular material after a period of time.

Pederson & Albury (1955) reported that certain non-dextran-forming leuconostocs and certain lactobacilli could be 'trained' to produce dextran by serial transfer in tomato or orange juice broths at low pH. More recently, Langston & Bouma (1960) found that isolations from silage, which they designated *Lactobacillus brevis* 'variable', produced extracellular polysaccharides when transferred by the method of Pederson & Albury (1955). Niven & Evans (1957) described *L. viridescens* from spoiled meat products which also produced an extracellular polysaccharide from sucrose.

A number of additional reports have dealt with lactobacilli which produce a heterogeneous polysaccharide material from various sugars. Millis (1951) described a slime-forming heterofermentative rod from 'ropy' beer. Shimwell (1949) had

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found an organism earlier which had similar properties. Barker, Bourne, Salt & Stacey (1958) found that the organism which Millis had isolated produced a polysaccharide which contained several sugars. Williamson (1959) also found a slime-producing organism in beer which grew on maltose and produced a heteropolysaccharide.

This paper reports the results of an investigation in which slime-forming lactobacilli from several sources were examined for purposes of comparison. The chemical compositions of the extracellular materials produced by various lactobacilli were studied as well as the conditions under which they were produced. A previous report stemming from the study described the relationship between temperature and dextran production in one of the strains (Dunican & Seeley, 1963).

METHODS

Micro-organisms and media. The lactobacilli used were those which had, at some time, produced extracellular polysaccharide from sucrose. The group contained eleven strains used by Pederson & Albury (1955), *Lactobacillus viridescens* of Niven & Evans (1957), a ropy beer lactobacillus from Carr (1959), five ropy beer strains, including Walker's strain from a collection of Dr M. E. Sharpe (Shinfield), six silage strains from Dr T. Gibson (Edinburgh), five strains from Dr Naylor (Cornell University); nine strains were isolated by the authors.

Culture medium. The medium used was that of Man, Rogosa & Sharpe (1960) without peptone. The basal medium contained no carbohydrate and is referred to as 'MRS' medium. The concentrations of carbohydrates used in various experiments are included in the legends of the Figures or in the text. Stock cultures were maintained by freezing in skim milk or in the lyophilized state. Because of the inhibitory effects of elevated temperatures of growth on the synthesis of polysaccharides, all cultures were incubated at 30° (Dunican & Seeley, 1963).

Purification of polysaccharides. The polysaccharides were precipitated from the cell-free supernatant culture fluid by the addition of 2 vol. chilled 95% ethanol. When low yields of glucan were encountered the flasks containing ethanol were stored overnight in the refrigerator to allow precipitation of polysaccharide. Two additional precipitations with ethanol and one with 2 vol. acetone were sufficient to give relatively protein-free samples of dextran.

The heteropolysaccharide was produced in very low yields and its isolation necessitated the concentration of the cell-free spent media to one-third its original volume by distillation under vacuum at 50° before the addition of the ethanol.

Analysis of polysaccharides. The partially purified polysaccharides were hydrolyzed by the method of Williamson (1959). The hydrolysates were neutralized by the addition of saturated $Ba(OH)_2$ and the solid $BaSO_4$ was removed by centrifugation. Paper chromatograms were prepared by standard techniques with the following solvents: (1) butanol+ethanol+water (2+1+1, by vol.) descending (Chou & Tobias, 1960); or (2) butanol+acetic acid+water (5+1+4, by vol.), ascending (Lederer & Lederer, 1955). Sugars were detected by methods using (*a*) aniline+diphenylamine (Smith, 1960), (*b*) naphthoresorcinol (Partridge, 1948) and (*c*) *p*-anisidine (Hough, Jones & Wadman, 1950). The reagents (*a*) and (*c*) were of particular value because of the different colours developed with the different sugars.

Serological detection of glucan. The supernatant cell-free spent culture fluid was adjusted to pH 6·0–6·2. A portion of this culture fluid was layered over type II pneumococcus serum (Burroughs Wellcome) in a vial. The formation of a ring at the interface within 15 min. constituted a positive test. Types I and III sera were used as controls.

Preparation of cell-free extracts. Organisms were deposited by centrifugation and were washed and suspended in acetate buffer (0·1 M, pH 5·6). The organisms were broken in a Raytheon sonic oscillator, Model DF 101, 10 kcyc. for 30 min. The unbroken organisms and large particles were removed by centrifugation (800*g*) for 30 min. Glucan sucrase and invertase were assayed as described in Dunican & Seeley (1963); protein was determined by the biuret method (Gornall, Bardawill & David, 1949); nucleic acids were measured by the spectrophotometric method of Mitchell (1950).

Manometric studies. These studies were done by conventional techniques. Organisms in the logarithmic phase of growth were washed and suspended in sugar acetate buffer (0·1 M, pH 5·6). Each cup contained 2 ml. organisms, 10 μmole of the sugar in acetate buffer. Total volume was 3·0 ml. The centre well had 0·3 ml. 20% (w/v) KOH. The temperature of the bath was 30·2°.

RESULTS

Two types of extracellular polysaccharides were produced during growth of the cultures. Table 1 shows the distribution of the two types among some of the strains studied. Four organisms which were physiologically similar to *Lactobacillus pastorianus* formed a glucan from sucrose, whereas the majority of the strains produced a polysaccharide which contained mannose and glucose. The latter polysaccharide was designated as 'heteropolysaccharide'.

Table 1. *Extracellular polysaccharides of various lactobacilli*

Organism	No. of strains	Glucan producing	Hetero-polysaccharide producing
L. brevis	5	None	None
L. pastorianus	6	4	None
Unidentified*	26	None	18

* Exhibit similarities to *L. buchneri* and *L. cellobiosus* (Rogosa *et al.* 1953).

Substrates for polysaccharide production

The glucan-producing strains RWM-13, RWM-1, RW-119 used sucrose as the sole substrate for glucan formation. Polysaccharide was not produced from glucose, maltose, cellobiose, trehalose, mannose or fructose. These three strains varied in their ability to grow on lactose, melibiose, arabinose, galactose, α-methylglucoside and starch, but none produced glucan from any of these substances.

In contrast to the specificity of sucrose as the substrate for glucan formation, the heteropolysaccharide was produced from several sugars, including maltose, sucrose, glucose, galactose and lactose, but not from fructose. Furthermore, fructose with sucrose prevented the formation of the polysaccharide.

Chemical composition of the polysaccharides formed

The isolated glucans were purified as noted previously by ethanolic precipitation; they were then hydrolysed, and examined by chromatography to identify the component sugars. Glucose was the only sugar found. Its identity was confirmed by reaction with glucose oxidase. The unhydrolysed polysaccharide did not give a colour with iodine and yielded a precipitate with type II pneumococcus serum confirming the view that the material was a glucan.

The purified heteropolysaccharides were examined chromatographically after hydrolysis. Table 2 shows the composition of these materials obtained from several strains; mannose and glucose were the component sugars except in two strains in which rhamnose also occurred. The unhydrolysed material contained substantial amounts of nucleic acid material and protein. The heteropolysaccharide did not give a precipitate with type II pneumococcus serum or stain with iodine.

Table 2. *Chemical composition of heteropolysaccharide synthesized by various strains of lactobacilli*

Strain	Mannose	Glucose	Rhamnose
RW-11	+ + +	+	−
5-144	+ + +	+ + +	−
S-38A	+ + +	+ + +	−
D-18	+ + +	+	−
5-49	+ + +	+	−
D-11	+ + +	+ + +	−
D-16	+ + +	+ + +	−
B-ver	+ + +	+ + +	−
B-g	+ + +	+ + +	−
L-15	+ + +	+ + +	+
2	+ + +	+	+

+ + + = major component; + = minor component; − = absent

It was apparent that while relatively few strains of lactobacilli examined produce a glucan serologically similar to that of *Leuconostoc mesenteroides* and *Streptococcus bovis*, the majority of the strains produced a material which contained mannose and glucose, and so resembled the material reported by Williamson (1959) and Barker *et al.* (1958). The heteropolysaccharide was so similar to the gross chemical composition of the cell walls (Cummins & Harris, 1956) that the material was considered to be the lytic product of the bacteria and was not studied further. This conclusion was supported also by the low yields of heteropolymer found, viz. 250 mg./l. in contrast to the high yields of glucan observed, viz. 15 g./l. by the glucan-producing strains.

Effect of sucrose concentration on the production of glucan

The relationship between the quantity of glucan produced by Lactobacillus strain RWM-13 grown at 30° and concentrations of sucrose is shown in Fig. 1. The results differ from those of Neely & Nott (1962) who observed that *Leuconostoc mesenteroides* did not produce dextran in media with sucrose concentrations below 2%.

Effect of initial pH of the medium on the production of glucan

Adequate control of pH value has been shown to be a requirement for dextran production by cultures of *Streptococcus bovis* (Bailey, Barker, Bourne & Stacey, 1957) and *L. mesenteroides* (Tsuchiya *et al.* 1952). Figure 2 shows that when the initial pH of media was between pH 5·0 and 7·0 there was no profound effect on the glucan production by Lactobacillus strain RWM-13. There was a marked decrease in the production of glucan, however, in flasks adjusted to pH values outside this range.

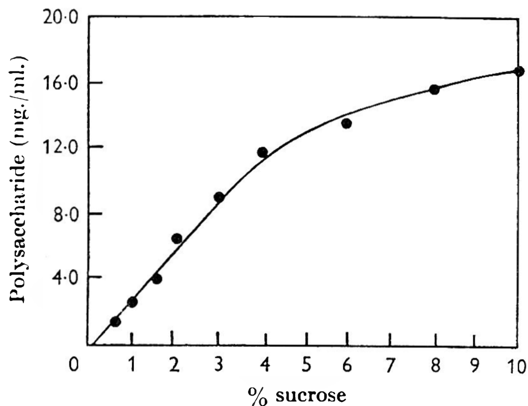

Fig. 1

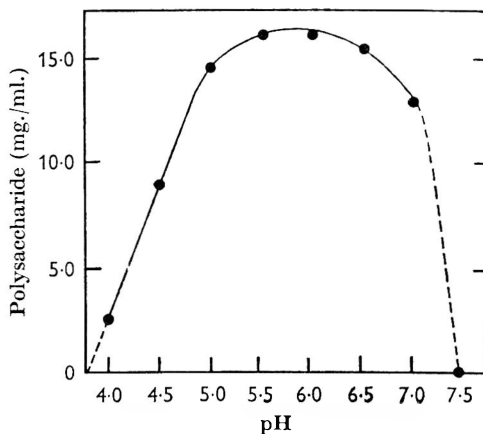

Fig. 2

Fig. 1. The effect of sucrose concentration on glucan production by Lactobacillus strain RWM-13 after 48 hr of growth.

Fig. 2. The effect of initial pH value on the yield of glucan by Lactobacillus strain RWM-13, after 48 hr of growth.

The effect of the omission of various components of the medium on glucan production

The medium of Man *et al.* (1960) was used in this investigation. It contained sodium acetate, diammonium citrate, Tween 80 and Mn^{2+}, in addition to the normal components of a medium for lactic acid bacteria. The results (Fig. 3) show the effects of the omission of each of these components on growth and glucan production by Lactobacillus strain RWM-13. The acetate, citrate and Mn^{2+} were required for optimal growth and glucan production. The effect with citrate was especially noteworthy since the omission of citrate led to a lag in growth and no glucan was produced in the first 24 hr of growth.

The lag in growth due to the omission of citrate was further investigated and the results are presented in Fig. 4. The broken lines show that the omission of citrate had a twofold effect, decreased cell and glucan yield and a greater lag before appreciable growth and glucan production occurred.

The stimulatory nature of these components of the medium on growth and glucan production corresponds to the observations of the requirements for citrate and manganese (Evans & Niven, 1951; MacLeod & Snell, 1947) and acetate (Guirard, Snell & Williams, 1946). The effect of the citrate may be due to its effect as an additional energy source (Gunsalus & Campbell, 1944).

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There was no requirement for Tween 80 or carbon dioxide for either growth or glucan production. *Streptococcus bovis* has been shown to require CO_2 for good dextran production (Dain, *et al.* 1956; Oxford, 1958).

The production of glucan by Lactobacillus strain RWM-13 in relation to cell growth

It was observed early in this work that there was a large increase in the production of glucan towards the end of cell growth. This is shown in Figs. 3 and 4 where it may be seen that the greater part of the glucan was synthesized in the second day of growth.

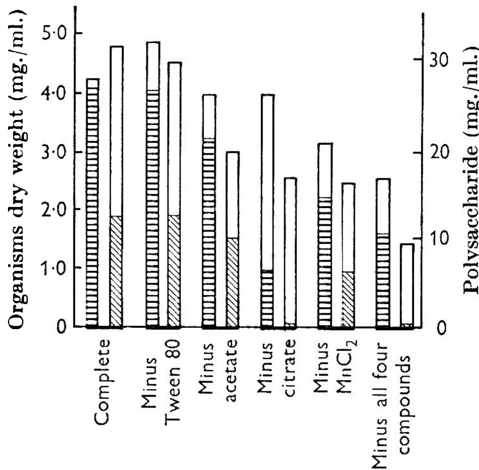

Fig. 3

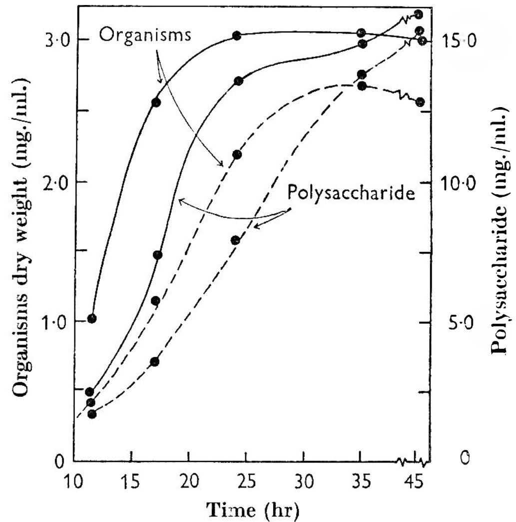

Fig. 4

Fig. 3. The effect of the absence of certain components of the medium on growth and polysaccharide production by Lactobacillus strain RWM-13. Columns represent the total yields of cells or polysaccharide after 48 hr growth. Horizontal shading represents cell yield in 24 hr. Diagonal shading represents polysaccharide yield in 24 hr. Medium contained 8 % sucrose.

Fig. 4. The effect of the omission of citrate from the medium on growth and polysaccharide production by Lactobacillus strain RWM-13. Solid lines = media with citrate; dotted lines = media without citrate. Sucrose concentration. 8 %.

In order to study the relationship of polysaccharide formation to growth over the entire growth cycle, portions of the culture were removed at intervals and the quantities of bacteria and glucan were measured gravimetrically. The results are shown in Fig. 5 in which the plots of both entities are made on a semilogarithmic scale. The bacteria dry weight shows the development of a typical exponential growth curve between 5 and 15 hr. During the same interval the production of glucan is also exponential, suggesting that the processes of glucan production and growth were dependent on one another and that the enzyme glucansucrase was perhaps liberated in relation to the need of the organism to hydrolyse sucrose. The decrease in the quantity of bacteria during the late stationary phase of growth is correlated with increased glucansucrase activity although no agent capable of lysing Lactobacillus strain RWM-13 was observed in the spent medium or in cell extracts of this organism.

The results shown in Fig. 5 indicate that the synthesis of the enzyme or its release were related to growth. Efforts to locate the enzyme in the organism were only partially successful since methods of disintegration of the bacteria by means other than sonic treatment failed so that extracts free from excessive breakage of the particulate fractions could not be obtained. However, the centrifugal fractionation of such extracts of Lactobacillus strain RWM-13 in a Spinco preparative centrifuge into 105,000$g \times 90$ min. soluble and particulate fractions showed that 85% of the activity was contained in the soluble fraction. There was little activity in the cell-wall fraction. The data do not identify the enzyme glucansucrase with any structure but do indicate that the enzyme is located within the organism, necessitating the liberation of the enzyme during growth.

Activity of glucansucrase in cell extracts

Glucansucrase was assayed in extracts of Lactobacillus strain RWM-13 by the method of Dunican & Seeley (1963). Replicate assays were prepared in 10^{-4}M-$AgNO_3$ and invertase activity was computed as reported previously. Figure 6 shows the activity of glucansucrase as a function of time of incubation and a 6 hr period was selected for future experiments as a suitable time to incubate the enzyme assays. This preparation showed negligible invertase activity and this activity was not recorded.

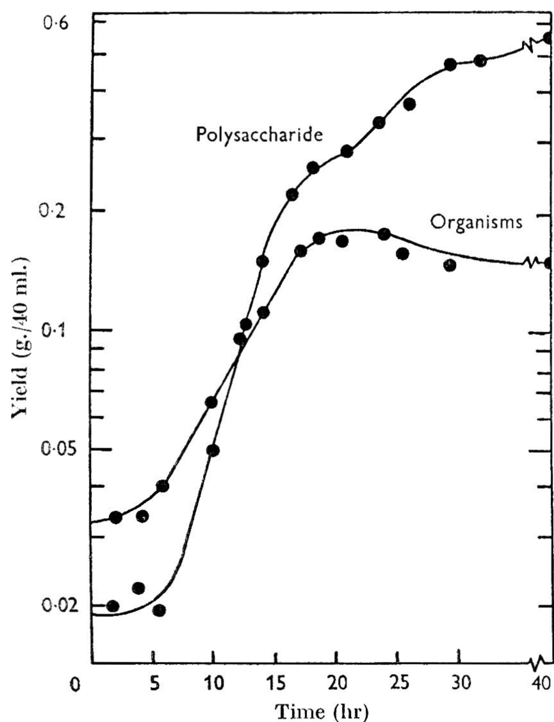

Fig. 5

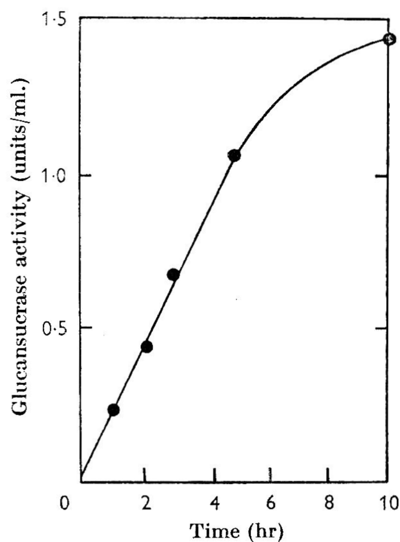

Fig. 6

Fig. 5. The synthesis of glucan in relation to bacterial growth of Lactobacillus strain RWM-13. Yields of polysaccharide and cells are expressed on the same logarithmic scale.

Fig. 6. The activity of glucansucrase from extracts of Lactobacillus strain RWM-13 with respect to time incubation of reaction mixture. Temperature of assay, 37°.

Effect of incubation in glucose broth on the content of glucansucrase in cell extracts

Lactobacillus strain RWM-13 was grown to the early logarithmic phase in MRS medium containing 4% glucose. Organisms were removed by centrifugation, washed in acetate buffer (0·1 M, pH 5·6) and suspended in MRS medium containing 2% sucrose. Portions of the culture were removed at intervals after the addition of the sucrose and were quickly chilled to 0–5° at which temperature the bacteria were collected by centrifugation. The bacteria were then washed in acetate buffer, broken in the sonic oscillator, and assayed for glucansucrase and invertase. The content of glucan was also determined in each of the portions of the culture. Figure 7 shows the results obtained. There was a significant amount of glucansucrase at zero time which decreased as the time of incubation in the sucrose increased. From the data shown in Fig. 7 it was concluded that the glucansucrase was constitutive in Lactobacillus strain RWM-13 since it was present in the bacteria in the absence of its substrate, sucrose. This enzyme, therefore, resembles that of *Streptococcus bovis* which has been reported to be constitutive (Bailey, 1959). The reservations placed by Bailey on the constitutive nature of the *S. bovis* enzyme apply also in the present investigation since it was not possible to rule out the presence of minute amounts of sucrose in the components of the medium or to exclude the possibility of synthesis of trace amounts of sucrose by the bacteria sufficient to induce glucansucrase. The presence of glucansucrase in extracts of Lactobacillus strain RWM-13 grown on glucose has been reported before (Dunican & Seeley, 1963) where extracts of bacteria grown on 4% glucose or sucrose were shown to contain equivalent amounts of glucansucrase. These findings may be contrasted with those of Neely & Nott (1962) who showed that *Leuconostoc mesenteroides* did not form glucansucrase on a glucose medium, but began to form the enzyme upon the addition of sucrose.

Spontaneously occurring, non-glucan-forming variants of Lactobacillus strain RMW-13

Mayer (1938) first noted variation in the colonies of dextran-forming lactobacilli grown on glucose agar. Smooth colonies which did not grow aerobically were found in the matrix of rough colonies. Perquin (1940) also observed smooth colonies in cultures of *Betabacterium vermiforme* and, in addition, found that the smooth colonies failed to produce dextran. Spontaneously occurring variants of Lactobacillus strain RWM-13 appeared frequently when cultures of this organism were streaked on 8% sucrose agar. The variants were initially very sensitive to oxygen and colonies picked from the agar failed to grow in glucose or sucrose broths unless the broth was boiled before use and the medium was covered by a vaspar seal.

Properties of the variants

The variants existed as long rods in chains. Many organisms in a culture were 10–20 μ long in contrast to the parents which ranged from 1·5 to 3 μ.

Although studies to compare the biochemical characteristics of the parent and the variant strains are not yet completed, it was observed that the variant had a strongly fermentative type of growth since gas production by this organism was vigorous as compared with the nominal amount produced by the parent.

Utilization of sucrose by the variant

Some reports on dextran formation by members of the lactobacilli contain the observation that the capacity of organisms to produce dextran was transient and was often lost when organisms (after isolation from natural sources) were cultivated in the laboratory (Shimwell, 1949; Carr, 1959). The effect of increasing the temperature on the production of dextran by lactobacilli has been discussed (Dunican & Seeley, 1963). Since the variants of Lactobacillus strain RWM-13 were characterized

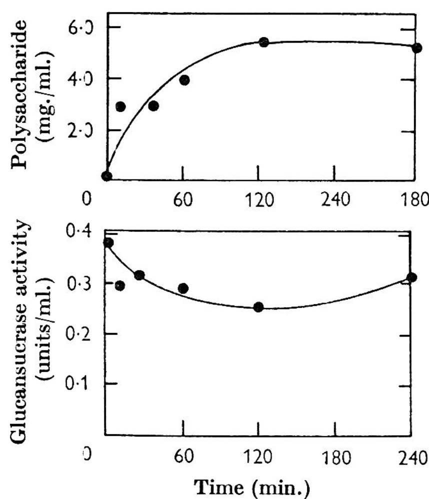

Fig. 7

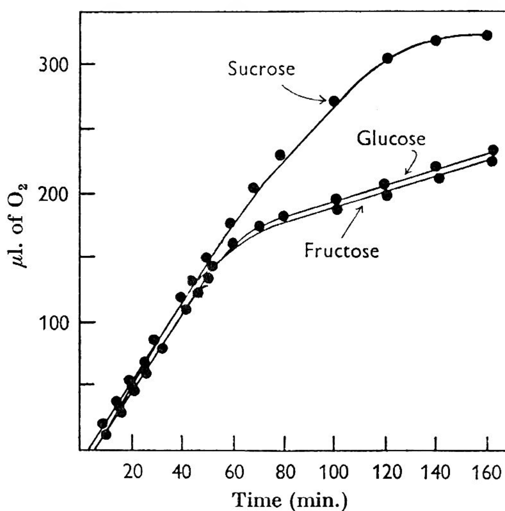

Fig. 8

Fig. 7. Polysaccharide production and glucansucrase activity of Lactobacillus strain RWM-13 in a sucrose-containing medium as a function of time. Time 0 is the point at which sucrose was added.

Fig. 8. Oxygen uptake by glucose-grown organisms of the variant strain of Lactobacillus strain RWM-13 on different sugars. Corrected for endogenous activity.

Table 3. *Sucrose hydrolysis of cell-free extracts of variant of Lactobacillus strain RWM-13*

Organisms grown in MRS 4% sucrose broth for 48 hr at 30°. Assays carried out for 6 hr at 30°. $AgNO_3$ was used at 10^{-4}M; pH of assay mixture was 5·6.

Extract	Reducing sugars in assay mixture	
	mg. net activity	% activity
Without $AgNO_3$ (total activity)	3·07	100
With $AgNO_3$ (glucansucrase)	2·55	83
Difference (invertase)	0·52	17

by their inability to synthesize glucan, it was apparent that a study of the utilization of sucrose, in the absence of glucansucrase, might contribute to the understanding of the loss of the ability of the micro-organism to form glucan.

The variant strain of Lactobacillus strain RWM-13 produced no glucan when grown on sucrose in air, when grown anaerobically, or in various atmospheres of

CO_2 and N_2. Since invertase has been shown to function in sucrose utilization by dextran-forming lactobacilli under certain conditions (Dunican & Seeley, 1963) it was considered probable that invertase was the means by which sucrose was utilized by the variants. To test this hypothesis the variant was grown on an 8% sucrose medium and extracts prepared and assayed for glucansucrase and invertase. The data obtained are shown in Table 3. The content of glucansucrase of the extract was found to be higher than was expected, considering the failure of the whole organisms to synthesize glucan. However, these extracts contained a small amount of invertase (Table 3). Manometric studies (Fig. 8) indicated that the invertase present in the organism was the enzyme primarily acting on the sucrose. The results show that oxygen uptake from sucrose was approximately one-third greater than that from equimolar glucose or fructose. Such a result would be expected from invertase activity which provides both monosaccharide moieties for oxidation, since glucansucrase activity would provide only the fructose portion for oxidation while the glucose was polymerized.

The failure of the variant to form glucan cannot be attributed to the absence of the enzyme but apparently to its unavailability. The enzyme could only be demonstrated with broken organisms, indicating that some liberating mechanism was not functional in the variant. The mechanism for the liberation of the glucansucrase was not elucidated beyond the demonstration that the small invertase content of the organisms was functioning during growth. The slow growth of the variant may result from the inadequate supply of this enzyme.

DISCUSSION

The observations reported here confirm a number of reports that some heterofermentative lactobacilli accumulate two types of extracellular polysaccharide in the culture media in which they are growing (Perquin, 1940; Niven & Evans, 1957; Millis, 1951). Several strains which were physiologically similar to *Lactobacillus pastorianus* produced a glucan which was serologically similar to that produced by *Leuconostoc mesenteroides* and *Streptococcus bovis*. The majority of the cultures studied produced a heteropolysaccharide which contained mannose, glucose, protein and nucleic material, and resembled the substance reported by Williamson (1959). The composition of this material and the small amount produced led to the conclusion that the material was not produced extracellularly but was an autolytic product. No physiological basis could be found to explain why cultures of these particular strains did not always contain this lytic material or why it was not formed on fructose-containing media.

One of the objectives of this investigation was to explain the cause of the instability of glucan production. Although Pederson & Albury (1955) had found that dextran-producing strains of lactobacilli which had lost their ability to produce dextran could be 'trained' to synthesize this polysaccharide by serial transfer in fruit juice media, no explanation has yet been found to explain this behaviour. It was assumed in the present work that the ability to lose and regain the capacity to synthesize glucan was a variation of a single process. This assumption was not borne out experimentally. However, two events which resulted in the loss of glucan synthesis were established. The loss of dextran synthesis at temperatures above the

optimum for growth has been reported (Dunican & Seeley, 1963); also, naturally-occurring variants of Lactobacillus strain RWM-13 were found which did not produce glucan. These two events are related in that invertase activity accounted for sucrose hydrolysis in the absence of glucansucrase activity. Glucansucrase was not synthesized at the elevated temperatures but it could be detected in the broken variant. The failure of glucansucrase to be liberated or to function in variant organisms in which it was shown to be present could not be explained.

The loss of the ability of lactobacillus to synthesize glucan can be related to conditions which (1) inhibit the synthesis of the enzyme or (2) inhibit the liberation of the enzyme into the medium where its activity is necessary for sucrose utilization. Under laboratory conditions, it is improbable that the elevated temperatures necessary to cause the inhibition of glucan sucrase synthesis are a factor in the routine growth of glucan-producing lactobacilli. The variability of glucan synthesis would then seem to lie in the accumulation of the spontaneously occurring variants which lacked the synthetic capacity and which utilized sucrose by an invertase-type hydrolysis.

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A Study of the Overall Similarity of Certain Actinomycetes Mainly of Oral Origin

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SUMMARY

Computer techniques were applied to the study of 71 isolates of branching or diphtheroid bacteria from human and bovine sources. Features including morphological and colonial characteristics, nutritional and environmental growth requirements, fermentative abilities and end-products and cell-wall composition were used to determine the relationships between these organisms. The features were determined with aerobic or anaerobic growth conditions or both, according to the nature of the individual bacteria. Relationships between facultatively anaerobic micro-organisms were very similar whether the features were determined aerobically or anaerobically; some apparently close relationships were shown between preferentially aerobic and preferentially anaerobic bacteria when they were compared with facultatively anaerobic micro-organisms.

The analysis separated groups of organisms corresponding to *Actinomyces naeslundii*, *A. bovis*, *Nocardia salivae*, *Bacterionema matruchotii* (*Leptotrichia dentium*) and *Corynebacterium* spp. The members of the various genera showed little affinity to each other. *A. naeslundii* and *A. bovis* were fairly closely related and some unplaced isolates, including typical *A. israelii* strains, showed a lesser relationship to either or both of these species. Isolates of *A. odontolyticus* showed high similarity to *A. bovis* and were included in this species despite their better aerobic growth. Several isolates of *A. naeslundii* grew well aerobically and produced catalase; production of this enzyme is not, by itself, sufficient to remove an organism from the genus *Actinomyces*.

INTRODUCTION

In the study of actinomycosis there have been many disagreements about the nomenclature and taxonomy of the bacteria involved and the identity or otherwise of strains of human and animal origin. The identification and naming of bacteria of this general description found in dental plaques and in carious cavities, has been even more controversial. Part of these difficulties has been due to the changing state of knowledge over the years and part to the varied emphasis placed on certain characteristics as taxonomic criteria by individual taxonomists. The historical background to these problems may be deduced from the monograph by Buchanan (1925) and is dealt with in greater detail elsewhere (Melville, 1964). A wide selection of organisms must be chosen for a classification by overall similarity so that boundary lines are not predetermined, but there are practical limits to the numbers of isolates which can be dealt with at the one time. The present investigation has been confined to parasitic or pathogenic branching or diphtheroid organisms from the human oral

cavity with some bovine oral strains for comparative purposes. It must be borne in mind that all taxonomic conclusions drawn are subject to the proviso that this group of organisms was selected by oral origin.

METHODS

Organisms. The 71 isolates used in this study are listed in Table 1 with their source and the name under which they were received.

Features. Each of the isolates was characterized by using the following criteria: colonial and cellular morphology of well isolated 5-day colonies grown at 37° on brain heart agar plates; microcolony formation examined as soon as growth was evident; cell wall composition (Cummins & Harris, 1956); ability to grow on certain media, in the presence of various inhibitory factors and under various environmental conditions (Table 2); biochemical features (Table 3). Full details of the methods used are given elsewhere (Melville, 1964).

Table 1. *Source of organisms used*

Organisms	Received as	Source
3 strains	*A. naeslundii*	Dr A. Howell Jun., Nat. Inst. Dent. Res., Bethesda, Maryland
3 strains	*A. bovis*	Dr L. Pine, Duke University, N. Carolina
2 strains	*A. bovis*	NCTC 9429 and 4500
2 strains	*A. israelii*	Dr A. Howell Jun.
4 strains	*A. israelii*	Dr J. W. Porteous, Marishal College, Aberdeen
2 strains	*A. israelii*	NCTC 8047 and 6830
1 strain	*A. propionicus*	ATCC 14157*
2 strains	*A. odontolyticus*	NCTC 9931 and 9935*
1 strain	*N. salivae*	NCTC 10207*
1 strain	*B. matruchotii*	Dr Marion Gilmour, Eastman Dental Dispensary, Rochester, N.Y.
1 strain	*Leptotrichia dentium*	NCTC 10206*
7 strains	—	Swabs from patients suspected of having actinomycosis. One from womb, rest oral
14 strains	—	Swabs from patients not suspected of having actinomycosis. Oral origin
5 strains	—	Root canal samples from endodontic cases
2 strains	—	Gingivae in normal mouths
3 strains	—	Tooth surfaces in normal mouths
11 strains	—	Saliva from normal mouths
1 strain	*C. diphtheriae*	Old laboratory stock culture
1 strain	*Lactobacillus* sp.	Saliva from normal mouth

* Original type strains.

The characters of the organisms compared in overall similarity studies are phenetic, the results of testing the organisms under set conditions. When features were studied in liquid or semi-solid media the aerobes and anaerobes could grow and demonstrate their characteristics at the appropriate site in the bottle. Cultures on solid media were grown aerobically, or anaerobically in nitrogen + 5 % (v/v) CO_2. These tests were duplicated for organisms capable of good growth both aerobically and anaerobically.

Certain of the features observed proved to be of no differential value and did not

contribute to the computation of the relationships of the organisms. Thus all the organisms were sensitive to all the antibiotics at the concentration tested; none fermented dulcitol, produced lecithinase, decarboxylated any of the amino acids, produced acetoin or acetic acid in detectable amounts by the methods used to analyse the products of glucose fermentation, hydrolysed coagulated serum, utilized paraffins or grew with inorganic nitrogen as sole nitrogen source.

Table 2. *Growth ability features*

Ability to grow:
- Using inorganic nitrogen
- On peptone agar
- On nutrient agar
- On casitone yeast-extract agar
- On brain heart yeast extract agar
- In defined medium (Pine & Watson, 1959)
- At pH values from 5·5 to 8·5
- At 24° and 42°
- Aerobically and/or } Technique of Howell & Pine, 1956
- Anaerobically } Technique of Howell & Pine, 1956
- In various CO_2 concentrations } Technique of Howell & Pine, 1956
- In presence of:
  - Sodium taurocholate (0·1 and 0·25 %, w/v)
  - Potassium tellurite (0·02 and 0·03 %, w/v)
  - Sodium chloride (2·0 and 3·0 %, w/v)
  - Crystal violet (final concentrations 10^{-5}, 10^{-6}, 10^{-7})
  - Antibiotics (1 mg./100 ml. in ditch plates): streptomycin, chloramphenicol, aureomycin, terramycin, penicillin

Table 3. *Biochemical features*

Acid production from carbohydrates
Final pH value and end products of fermentation of glucose
Utilization of organic acids
Utilization of tyrosine, xanthine, paraffins
Production of catalase
Production of cytochrome component (Deibel & Evans, 1960)
H_2S production
Nitrate reduction
Starch hydrolysis
Reduction of methylene blue
Proteolysis of gelatin, serum, casein
Indole production
Decarboxylation of amino acids (Møller, 1955; Stewart, 1963)
Urease and lecithinase production
Phosphatase production (Bray & King, 1943; Howell & Fitzgerald, 1953)
Haemolysis of horse blood

Computer analysis. The analysis was carried out on a Mercury computer according to a programme kindly made available by the University of London Computer Unit. This programme was similar to that of Sneath (1957) except that negative matches were counted as similarities for the reasons presented by Hill, Turri, Gilardi & Silvestri (1961). Quantitative data were scored by the additive method of Sneath (1962) since this appears to be the logical sequel to accepting negative matches. Quantitative data and multiple alternatives for a feature were limited to a maximum of four. Two complete analyses were made, one covering the 49 isolates capable of

good aerobic growth and the other covering the 65 isolates capable of good anaerobic growth: 43 strains of the isolates grew sufficiently well in tests done under both sets of growth conditions to be included in both analyses.

RESULTS

1. Forty-seven of the 71 isolates studied fell into two phenons A and B, each was composed of very similar organisms or of organisms more like the organisms in these two phenons than any others in the series.

Table 4. *Mean percentage similarity values within and between phenons*

	Phenon											
	A		B		C		D		E1		E2	
No. of strains	21	*23*	2	*11*	4	*4*	7	*6*	4	*4*	3	*1*
Phenon A	85	*84*										
B	74	*75*	94	*83*								
C	61	*59*	59	*64*	87	*86*						
D	58	*59*	57	*63*	70	*66*	84	*78*				
E1	69	*70*	72	*71*	72	*71*	64	*65*	83	*82*		
E2	59	*58*	66	*71*	67	*67*	60	*65*	75	*69*	83	*100*

Roman figures indicate similarity aerobically, while italics indicate similarity anaerobically.

Phenon A contained 24 isolates each of which showed a mean similarity value (MSV) to all the other isolates in the phenon in the range 83–88 % aerobic (based on analysis using aerobic results) and 82–87 % anaerobic (based on anaerobic tests). All these organisms grew to some extent under aerobic and anaerobic conditions but one grew too weakly aerobically and three anaerobically to be put through the full series of tests under these conditions. Phenon B contained 11 isolates with a MSV between 82 and 84 % (anaerobic tests). Only two grew aerobically to any degree with a high similarity to each other of nearly 94 %.

A further 12 isolates were more closely related to phenons A and B than to any others established, but the similarity was less close. Phenons A and B showed a mean interphenon similarity value (MIS) of all the organisms in one phenon to all the organisms in the other of 75 % (anaerobic tests).

2. Four isolates formed phenon C and each showed a MSV between 83–89 % (aerobic) and 83–87 % (anaerobic). All grew well both aerobically and anaerobically.

3. Seven isolates formed phenon D and each showed a MSV between 80–88 % (aerobic) and 75–80 % (anaerobic). One isolate grew poorly anaerobically and was not included in this series; the whole group was preferentially aerobic. Two other aerobic strains were less closely related to this phenon but were more like these than other organisms in the series.

4. Two small groups of 4 and 3 isolates formed phenons E 1 and E 2, respectively; these phenons showed a MIS of 75 % (aerobic). The organisms in E 1 showed a MSV

of 81–85% (aerobic) and of 82% (anaerobic); those in E2 showed a MSV of 82–84% (aerobic) but only one isolate was suitable for anaerobic testing. Two other isolates were more like isolates in E1 than the others investigated.

5. Two isolates were completely unplaced and showed low similarity values to all groups; one of these was unidentified, the other was an oral lactobacillus included for comparison.

DISCUSSION

Although similarity values have no absolute correlation with taxonomic rank the phenons suggested by overall similarity values may be compared with previous results arrived at by more classical methods of taxonomy. Phenons A and B and the organisms similar to, but not included in, these phenons appear to correspond to the genus *Actinomyces*. Within this genus, phenon A conforms to the description of *A. naeslundii* as expanded by Howell, Murphy, Paul & Stephan (1959) except that some strains grew more easily under aerobic conditions and, especially under these conditions, produced catalase. Three strains kindly supplied by Dr A. Howell did in fact fall into this phenon.

Phenon B conforms in general to the description of *Actinomyces bovis* and contains several isolates described by Pine, Howell & Watson (1960). Two strains of *A. odontolyticus* isolated by Batty (1958) also fell in this phenon; this supports the close relationship of *A. odontolyticus* to *A. bovis* suggested by Batty on the grounds of similarity of life cycle. It may be an indication that there is insufficient justification for the establishment of *A. odontolyticus* as a separate species, but it should be borne in mind that only two strains of these organisms were included and that they were the only isolates in the phenon which grew well aerobically.

The organisms similar to, but not included in, phenons A and B include all the strains which showed the typical description of *Actinomyces israelii* including the cell-wall composition described by Cummins & Harris (1958), the strain isolated by Buchanan & Pine (1962) and named *A. propionicus* and various isolates not very different from the organisms in phenons A and B.

The other phenons derived from the analysis of similarities each contained very few isolates. Phenon C corresponds to the whip-handle type of Leptotrichia which was named *Leptotrichia dentium* by Davis & Baird-Parker (1959) and *Bacterionema matruchotii* by Gilmour, Howell & Bibby (1961). The present work does not help directly to settle this controversy since the other main oral Leptotrichia (*L. buccalis*) was not included in the survey. The phenon is however more closely related to phenons D, E1 and E2 than it is to the genus *Actinomyces*, while all four isolates in the phenon showed very low similarity values to the lactobacillus included for comparison. This would suggest that the whip-handle type should be placed more nearly with the nocardias or corynebacteria than with the actinomyces and that it bears little similarity to *L. buccalis* if the contention of Richardson & Schmidt (1959) that *L. buccalis* should be placed near to the genus *Lactobacillus* is correct.

Phenon D corresponds to, and includes specimens of, the isolates named by Davis & Freer (1960) as *Nocardia salivae* and which differ from typical nocardias in cell-wall composition. No nocardias from other sources were included in the analysis so that the position of *N. salivae* in this genus is neither confirmed or disputed. Phenons E1 and E2 are as closely related as are phenons A and B and from the general properties

of the organisms included in them may well be members of the genus *Corynebacterium*; the number of isolates studied was insufficient to allow further comment particularly in view of the complexity of overall similarity analysis of this group shown by Moore & Davis (1963).

In general the phenons established are too small to allow conclusions to be drawn from a detailed study of the properties of the individual isolates in each group. It is of interest that the facultative isolates fell into the same groups whether their features were determined aerobically or anaerobically, and that certain organisms which were preferentially aerobic or anaerobic appeared in the same group due to their similarity to facultative strains. The experimental evidence also supported the view of Roth & Thurn (1962) that catalase production is adaptive rather than constitutive and indicated that production of this enzyme should not, on its own, be sufficient ground for removing an organism from the genus *Actinomyces*; certainly some micro-organisms placed in phenon A are capable of producing catalase.

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Lysis of Gram-Negative Bacteria by Host-Independent Ectoparasitic *Bdellovibrio bacteriovorus* Isolates

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SUMMARY

Methods of isolation and nutritional requirements of *Bdellovibrio bacteriovorus* strains capable of growth on host-free media are described. Such strains retained their parasitic capacities for various Gram-negative bacteria after many transfers in host-free media. In parasite + host suspensions the spectrum of host specificity of various bdellovibrio strains was considerably wider than that obtained by examination for plaques on host lawns. The growth conditions therefore affect the capacity of the bdellovibrios to attach to and lyse host organisms. A proteolytic exoenzyme formed by certain bdellovibrio strains digested heat-killed, acid-treated or EDTA-treated host organisms, but did not affect intact host organisms. High phosphate concentrations inhibited exoenzyme activity. The morphological sequence of lysis induced by exoenzyme-forming parasitic bdellovibrio strains suggested that this lysis was a two-stage process: the first stage was specific attachment of bdellovibrios with damage to the cell wall of the host organisms; the second stage was non-specific digestion of cellular components by exoenzyme produced by the bdellovibrios.

INTRODUCTION

The description by Stolp & Petzold (1962) and by Stolp & Starr (1963) of an obligately ectoparasitic group of vibrios, *Bdellovibrio bacteriovorus*, highly specific in their ability to attach to and cause lysis of various Gram-negative bacteria and to form plaques on lawns of these host organisms, provided an intriguing new system for the study of mechanisms of bacterial lysis. Because of the ubiquitous occurrence of these bdellovibrios in soil and sewage, these organisms may play an important role in the ecological equilibrium of microbial populations in nature. The present work is concerned with the enrichment of cultures and the isolation of bdellovibrio strains capable of growth on artificial media in the absence of host organisms, and with their nutritional requirements. The host specificity of such bdellovibrio isolates is described and some possible mechanisms for the lysis of host organisms induced by bdellovibrios are suggested.

METHODS

Parasite strains. Three *Bdellovibrio bacteriovorus* strains isolated and described by Stolp & Starr (1963) were used in this work: strain A3.12 (isolated on *Pseudomonas*

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fluorescens ATCC 12633); strain 109 (isolated on *Escherichia coli* B-2262); strain 321 (isolated on *Pseudomonas phaseolicola* ATCC 11355).

Host organisms. The host bacteria used in this work included: *Escherichia coli* B-2262, *E. coli.* K-12, *Aerobacter aeruginosa, Proteus vulgaris, Rhodospirillum spheroides, Caulobacter* sp., *Pseudomonas fluorescens* ATCC 12633, *P. phaseolicola* ATCC 11355, *P. aeruginosa, Bacillus megaterium* (from the stock collection of the Department of Bacteriology, University of California at Berkeley); *Pasteurella pestis* EV 76, *Salmonella typhi* O 901, *S. typhimurium* LT2 and *S. paratyphi B* (from the stock collection of the Department of Bacteriology, Hebrew University-Hadassah Medical School, Jerusalem, Israel). Other host strains used were the *E. coli* UDP-galactose-4-epimeraseless mutants: strains CM7, *Gal*-16 and *Gal*-22; and the *E. coli* strain *Gal*-23 defective in its ability to synthesize UDPG synthetase (UDP-glucose-pyrophosphorylase). The four *E. coli* mutants were kindly given by Dr N. Nikaid Harvard Medical School, Boston, Mass., U.S.A.

Culture media. Nutrient broth (NB) and nutrient agar (NBA) were prepared as described by Stolp & Petzold (1962). Dilute nutrient broth (NB/10) was prepared of 1 vol. NB+9 vol. solution in distilled water containing: $Ca(NO_3)_2$, 200 mg./l.; $FeSO_4$, 10 mg./l.; $MnSO_4$, 10 mg./l.

Medium C was composed of: Hutner's vitamin-less base (Cohen-Bazire, Sistrom & Stanier, 1957), 5 ml./l.; Na_2HPO_4, 1·9 g./l.; KH_2PO_4, 0·9 g./l.; Bacto-peptone, 10 g./l.; Difco yeast-extract, 1 g./l.; thiamine HCl, 2 mg./l. (filter sterilized and added to autoclaved components of medium). For the preparation of solid medium C, 0·85 % (w/v) Ion-Agar (Oxoid) was added. Soft agar was prepared by adding 0·8 % (w/v) Bacto agar to the liquid medium NB/10.

Plaque formation by bdellovibrios on lawns of their hosts. The host inoculum used was a suspension (2×10^7 organisms) of bacteria in the late exponential phase of growth. Samples of 0·2 ml. of the host suspension were placed in tubes containing 2 ml. liquefied soft agar medium together with 0·1 ml. of a selected suspension of the bdellovibrio and then spread evenly on the surface of NB/10 agar plates. The host bacteria formed a confluent lawn on the surface of the plate within 24 hr of incubation at 30°; plaques generally appeared 12–24 hr later.

Lysis of suspension of host bacteria by bdellovibrios. The host bacteria were grown in NB medium for 20 hr at 30° on a shaker. Washed host bacteria and bdellovibrios were suspended in NB/10 medium at the desired host: parasite ratio and the mixtures (10 ml.) in 50 ml. Erlenmeyer flasks fitted with Klett tube side arms incubated in a shaker bath at 30°. The decrease in extinction (Klett–Summerson colorimeter filter 66) of the suspension mixture as a function of time served as the measure of lysis.

Enumeration of particles by use of an electronic particle counter. Particles in the size range of the bdellovibrios were enumerated by the use of an electronic particle counter (Coulter counter, Model A, Coulter Electronics, Hialeah, Fla., U.S.A.) with an orifice of 30μ, and having the following instrument settings: aperture current setting, 5; gain switch, 6; gain trim, 9. To obtain a dilution medium of low particle background, NB/10 medium was repeatedly filtered through packs of Whatman No. 1 filter paper and finally through a Millipore filter ($0{\cdot}22\mu$ pore size).

RESULTS

Isolation and properties of host-independent Bdellovibrio bacteriovorus mutants

By using the parasitic *Bdellovibrio bacteriovorus* strain A3.12 isolated on *Pseudomonas fluorescens* as starting material, several mutants were obtained which were capable of growth on artificial media in the absence of host bacteria, but which, nevertheless, retained their capacity to attach to and to lyse living host organisms. Enriched suspensions of such mutants were obtained by methods which included physical separation of the small bdellovibrios from their larger hosts by filtration through a Millipore filter (0·45μ pore size). Large initial populations ($\sim 10^9$ organisms/ml.) of Bdellovibrio A3.12 (wild-type) were required since heavy losses of organisms occurred during the filtration process. The host-free parasite-containing filtrates were transferred into media NB or C or into NB/10 (containing heat-killed [120°, 15 min.] *P. fluorescens*, $\sim 10^8$ organisms/ml.) and incubated at 30° with vigorous shaking. The several *B. bacteriovorus* A3.12 isolates which grew under these conditions were maintained for many months in transfers on host-free media (NB or C). The predacity of such isolates was retained and was not lost upon lyophilization.

The generation time of these host-independent mutants (on media NB or C, shaker incubated at 30°) was 170–180 min. In the exponential phase of growth, the bdellovibrios were actively motile and of uniform size. As the culture entered the stationary phase, the number of motile bdellovibrios decreased and spirals and long rods appeared. Several days after the cessation of growth, degenerate forms became evident, characterized by cytoplasmic spheres at the end of the spiral-like organism (Pl. 1, fig. 1).

The vitamin requirement of a host-independent *Bdellovibrio bacteriovorus* A3.12 mutant is shown in Table 1. The yeast extract in medium C could be partially replaced by thiamine; however, the yeast extract seemed to contain at least one other factor essential for growth of *B. bacteriovorus* A3.12.

On solid media C and NB agar, excessively large inocula of the bdellovibrios were required for growth initiation; minimal inoculum for colony formation of host-independent mutants, under these conditions, was found to be on the order of 10^4 organisms/plate. The addition of 1% (v/v) autoclaved culture supernatant fluid of the same bdellovibrio strain grown in NB medium provided a growth-initiating factor, making possible regular production of isolated colonies from suitably diluted bdellovibrio suspensions. The growth-initiating factor was present in supernatant fluids of bdellovibrio cultures after 24 hr of incubation in NB medium; in 8-day cultures the concentration of this factor seemed to be markedly higher. In the presence of bdellovibrio culture supernatant fluids, the colonial growth of bdellovibrio mutants was rapid at 30° (within 48 hr) and the ratio of total counts estimated in a Petroff–Hauser chamber (American Hosp. Supply Corp., Phila., Pa., U.S.A.) to colony numbers approached unity.

The host-independent *Bdellovibrio bacteriovorus* A3.12 mutants, when mixed with their pseudomonad host immediately attached to the host bacteria (Pl. 1, fig. 2). The typical morphological changes in the host, leading to lysis, consisted in the formation of V-like and ring structures, when induced by the wild-type *B. bacteriovorus* A3.12; these were also observed with the host-independent mutants (Pl. 1, figs. 3–5). Upon lysis of the pseudomonad host, ghosts of similar shape remained and no

formation of an intermediate spheroplast stage was observed. The host-independent isolates attached to susceptible host bacteria only during the exponential and early stationary phases of the growth of the bdellovibrios.

Plaques were formed on lawns of *Pseudomonas fluorescens*, and among the various isolates, a number of types forming plaques of different size and opacity were observed (Pl. 1, figs. 6–8). Size of such plaques ranged from 1 to 10 mm. after 4–5 days' incubation at 30°.

Table 1. *Vitamin requirements of Bdellovibrio bacteriovorus* A 3.12 *host-independent mutant*

Basal medium consisted of 0·02 M-phosphate buffer (pH 7·0), 5 ml./l. Hutner's base and peptone (10 g./l.) as carbon source. The amount of each vitamin (μg./l.): riboflavin 0·2; thiamin HCl, 2·0; folic acid, 0·004; nicotinic acid, 0·8; calcium pantothenate, 0·8; cobalamin (B_{12}), 2·0; biotin, 0·004; *p*-aminobenzoic acid, 0·2; pyridoxal phosphate, 0·8; pyridoxamine HCl, 0·8.

riboflavin	thiamin HCl	folic acid	nicotinic acid	Ca pantothenate	cobalamin (B_{12})	biotin	*p*-aminobenzoic acid	pyridoxal-PO_4	pyridoxamine	1 % (w/v) peptone	1 % peptone; 0·1 % (w/v) yeast extract	Turbidity units (Klett; 40-hr culture)
+	+	+	+	+	−	+	+	+	+	+	−	230
+	+	+	+	+	+	+	−	+	+	+	−	230
+	+	+	+	−	−	+	+	+	+	+	−	230
+	+	−	+	+	+	+	+	+	+	+	−	240
+	+	−	+	+	+	+	−	+	+	+	−	230
+	+	+	+	+	−	−	−	−	−	+	−	200
+	+	+	+	−	−	−	−	−	−	+	−	230
+	+	−	+	+	+	−	−	−	−	+	−	230
+	−	+	+	+	−	+	+	+	+	+	−	50
+	−	+	+	+	+	+	−	+	+	+	−	40
−	−	−	−	−	−	+	+	+	+	+	−	50
−	−	−	−	−	+	+	−	+	+	+	−	50
+	−	+	+	+	−	−	−	−	−	+	−	50
−	−	−	−	−	−	−	−	−	−	+	−	50
+	+	+	+	+	+	+	+	+	+	+	−	230
−	+	−	−	−	−	−	−	−	−	+	−	260
−	−	−	−	−	−	−	−	−	−	−	+	320
−	+	−	−	−	−	−	−	−	−	−	+	360

To determine whether host-independent bdellovibrio isolates retained their predacity after cultivation in the absence of host bacteria, the plaque-forming efficiency of several of the *Bdellovibrio bacteriovorus* A 3.12 isolates on host lawns of *Pseudomonas fluoroscens* was tested. At different growth phases, the bdellovibrio cultures were tested for plaque-forming capacity on NB/10 and compared to the colony-forming ability on solid medium C. In addition, the most probable number of viable organisms in the bdellovibrio suspension was estimated by the dilution tube technique (McCrady, 1918) in liquid medium C. For determination of the total number of parasites in suspension, particles in the size range of the parasite were counted in an electronic particle counter, and these results were corroborated in a number of experiments by direct counts in a Petroff–Hauser chamber.

Figure 1 summarizes the results of an experiment comparing numbers of plaques formed by a host-independent isolate to numbers of organisms counted by the other methods. In the early growth stage (first 5 hr) every particle in the suspension was viable and capable of forming a plaque. In these cultures, therefore, predacity was retained by practically every organism. After 10 hr of incubation in this medium, nearly every particle in the bdellovibrio size-range was viable, but only one out of every five organisms was capable of plaque formation.

Similarly, the number of viable cells from the lysed, 8 hr culture of a host-independent *Bdellovibrio bacteriovorus* A3.12 mutant grown continuously in two-membered cultures with its host *Pseudomonas fluorescens* was determined by plaque count and by

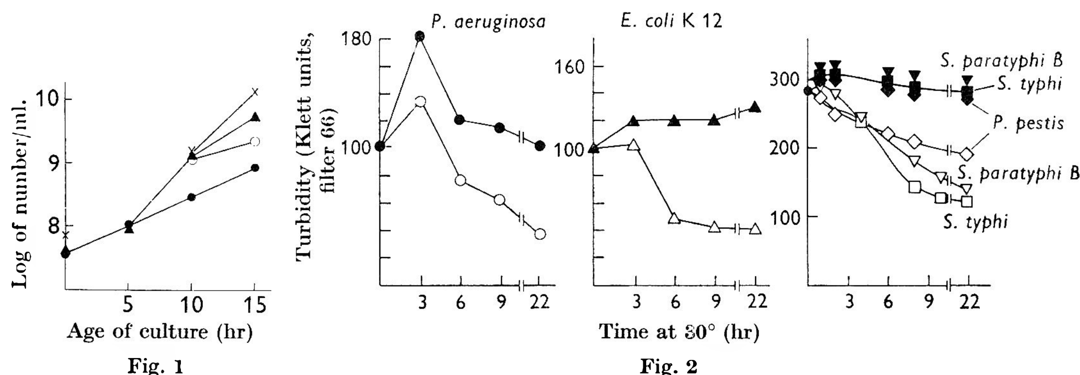

Fig. 1. Total and viable counts of host-independent *Bdellovibrio bacteriovorus* A3.12 isolate measured by different methods during exponential growth stage on nutrient broth. ● plaque-forming units; ▲ viable count, colonies; ○ viable count, dilution tube technique; × particle number, Coulter electronic counter.

Fig. 2. Lysis of suspension of different host species by host independent *B. bacteriovorus* A3.12 isolate. Conditions of lysis as described under Methods; the ratio of parasites to host organisms was 10:1 in all tests. Solid points are host controls; open points are host+parasite suspension. Key to host species: ○ ● *Pseudomonas aeruginosa*; △ ▲ *Escherichia coli* K12; ▽ ▼ *Salmonella paratyphi B*; □ ■ *S. typhi* O901; ◇ ◆ *Pasteurella pestis* EV76.

Table 2. *Ability of host-independent Bdellovibrio bacteriovirus A3.12 isolates, grown continuously in two-membered cultures, to develop in the absence of host organism*

	Viable bdellovibrios/ml. found by:	
Expt.	plaque count	dilution*
1	1×10^9	5×10^8
2	3×10^8	$3{\cdot}5 \times 10^8$

* Dilution medium; medium C containing 2–4 μg. thiamine HCl/ml.

dilution-tube technique. The results of two experiments (Table 2) show that practically every plaque forming unit was also able to grow in absence of host bacteria.

After prolonged subcultivation in host-free media, several host-independent isolates showed a progressive decrease in predacity as measured by the ratio of plaque-forming units to viable organisms in cultures. Such organisms, on microscopic examination, showed no loss of motility.

Specificity of attachment to host bacteria and then lysis by Bdellovibrio bactieriovorus A 3.12 isolates and B. bacteriovorus 109

All the bdellovibrio strains isolated by Stolp & Petzold (1962) and Stolp & Starr (1963) were separated into different groups according to their plaque-forming ability for specific hosts. In our work, the ability of the parasite to attach to and lyse host organisms in suspension showed that under such conditions the spectrum of host specificity was often markedly broader than that observed when plaque formation was the sole criterion. Thus, (wild-type) *Bdellovibrio bacteriovorus* A3.12 and its host-independent mutants were capable of lysing suspensions of many living Gram-negative organisms, including *Escherichia coli* B, *E. coli* K-12, *Pseudomonas aeruginosa*, *Salmonella typhimurium* LT2, *S. paratyphi B*, *S. typhi* O 901 and *Pasteurella pestis* EV76, all of which did not show plaque formation. Certain other Gram-negative organisms, such as *Caulobacter* sp., *Rhodopseudomonas spheroides*, *Rhodospirillum rubrum* and the Gram-positive bacterium *Bacillus megaterium* did not show attachment and lysis in suspension.

The kinetics of lysis in suspension of several host species with a host-independent *Bdellovibrio bacteriovorus* A3.12 isolate are shown in Fig. 2. The residual turbidity seen in these mixtures was due to ghosts of host bacteria and to the parasite organisms, which were visible on microscopic examination.

Figure 3 shows the effect of the degree of multiplicity (parasite: host ratio) on the rate of lysis of *Escherichia coli* B in suspension with a *Bdellovibrio bacteriovorus* A3.12 mutant. As can be seen from Fig. 3, the rate of lysis was dependent on the degree of multiplicity and even with a low ratio of two parasites one bacterium lysis of the host occurred.

To test whether the lysis of suspensions of host bacteria not suitable for plaque formation involved a selective enrichment of bdellovibrios having different and broader host specificities, the following experiment was done. The plaque-forming efficiency of a host-independent *Bdellovibrio bacteriovorus* A3.12 mutant on each host bacteria was tested before and after a series of three sequential transfers of the bdellovibrio on each of the following host suspensions: *Escherichia coli* B, *E. coli* K-12, *Aerobacter aeruginosa* and *Pseudomonas fluorescens* (the specific 'plaque-forming' host). Even after these repeated transfers and total lysis of the different hosts in suspension, no enriched suspension of any bdellovibrio mutant with new host plaque-forming specificity was found. The parasites of every one of the four transfer lines showed the same high plaque-forming capacity on *P. fluoroscens* (0·1–1·0 plaques/parasite) and very low plaque formation ($\geqslant 10^{-6}$ plaques/parasite) on any of the other host species tested. Similarly, tests of isolates from some of the rare plaques formed by the bdellovibrio strain on *E. coli* or *P. aeruginosa* showed that such isolates did not develop plaque-forming specificities differing from that of the initial strain.

Bdellovibrio bacteriovorus strain 109 lysed suspensions of several *Escherichia coli* mutants (including the UDP-galactose-4-epimeraseless strains CM7, *Gal*-16, *Gal*-22) which are deficient in ability to synthesize normal cell wall-lipopolysaccharides when grown on a galactose-free, glucose-containing mineral medium (Yarmolinsky, Wiesmeyer, Kalckar & Jordan 1959). Similarly, lysis was obtained in suspensions of the *E. coli Gal*-23 mutant (low in UDPG synthetase; Fukasawa, Jokura & Kurahashi, 1962; Sundarajan, Rapin & Kalckar, 1962) with the *B. bacteriovorus* 109 strain.

Protease formation by various bdellovibrio strains

Bdellovibrio bacteriovorus A 3.12 (grown in the presence of its specific host *Pseudomonas fluorescens*) or its host-independent mutants all grown in NB or NB/10 media released a potent protease into the medium. This exoenzyme lysed many different heat-killed bacteria (Fig. 4) dissolving most of the internal components of the bacteria and leaving empty hulls of cell walls. This exoenzyme also degraded typical protease substrates such as the coloured collagen preparation Azocoll (Calbiochem AG, Lucerne, Switzerland; Fig. 4). The spectrum of lytic activity against the heated bacteria extended beyond that observed with these parasites against living hosts in suspensions, and even included Gram-positive organisms such as *Bacillus megaterium*.

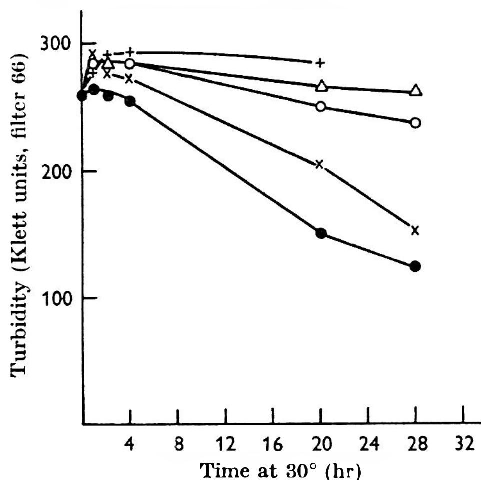

Fig. 3

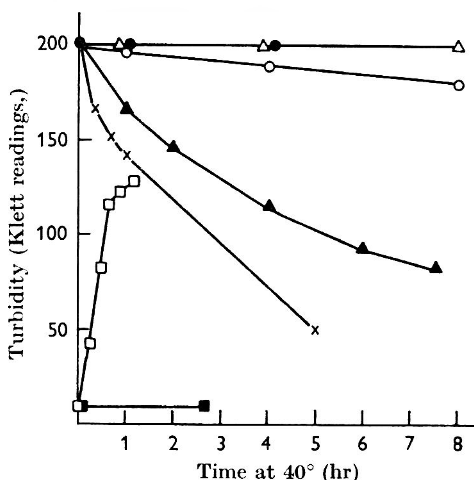

Fig. 4

Fig. 3. Effect of parasite-host ratio on rate of lysis of living *Escherichia coli* B in suspension with host independent *Bdellovibrio bacteriovorus* A3.12 isolate. Conditions of incubation as described under Methods for lysis of host suspensions. The parasite and host suspensions were mixed to give the desired parasite:host ratios. Ratio of parasite organisms to host organisms: 2:1 [△]; 5:1 [○]; 12:1 [×]; 24:1 [●]; host control [+].

Fig. 4. Digestion of different heat-killed bacteria and decomposition of Azocoll by culture supernatants of different bdellovibrio strains. Cell-free supernatants of 48 hr cultures of host independent strain A 3.12 or of two membered cultures of strain 109 or 321 on their specific hosts were obtained by centrifugation (20 min. at 15,000 r.p.m., Servall SS-1 at 3°). Heat killed (120°, 10 min.) *Pseudomonas fluorescens* or *B. megaterium* were suspended, respectively, in parasite culture supernatant and incubated at 40°. Proteolytic activity was measured by decrease in extinction (Klett–Summersen colorimeter, filter 66). 50 mg. Azocoll were suspended in 10 m. strain A3.12 culture supernatant. Mixture was incubated at 40°; at specified times, 2 ml. aliquots were removed, filtered and turbidity of 1 part of filtrate diluted in 4 parts distilled water was read (Klett–Summersen colorimeter, filter 54). ● *Pseudomonas fluorescens* and *Bacillus megaterium* controls. △ *Bdellovibrio bacteriovorus* 321 culture supernatant + *P. fluorescens*. ○ *B. bacteriovorus* 109 culture supernatant + *P. fluorescens*. ▲ *B. bacteriovorus* A 3.12 culture supernatant + *B. megaterium*. × *B. bacteriovorus* A 3.12 culture supernatant + *P. fluorescens*. □ *B. bacteriovorus* A 3.12 culture supernatant + Azocoll. ■ Azocoll control.

Figure 5 shows the formation of protease exoenzyme in a growing bdellovibrio culture as a function of time. Maximal exoenzyme activity in culture supernatant fluids was found at the time when the rapid decline in bdellovibrio numbers began

(24–48 hr). It can also be seen from Fig. 5 that the enzyme activity was rapidly lost.

The exoenzyme activity of the *Bdellovibrio bacteriovorus* A 3.12 culture supernatant fluids in dissolving heat-killed bacteria was markedly suppressed by phosphate at 10^{-1} to 10^{-2} M (Fig. 6). Similar phosphate inhibition of enzyme activity was observed in the decomposition of Azocoll.

Tests for exoenzyme formation by other bdellovibivrio strains showed that strains 321 and 109 did not form any protease, or only trace amounts (see Fig. 4), when grown on their specific hosts in NB medium. These two strains also differ from A 3.12 in the morphological sequence which leads to lysis of their host organisms. With strains 321 and 109, lysis involved the formation of cytoplasmic extrusions (strain 321, Pl. 2, figs. 1–4) or spheroplast formation (strain 109, Pl. 2, fig. 5).

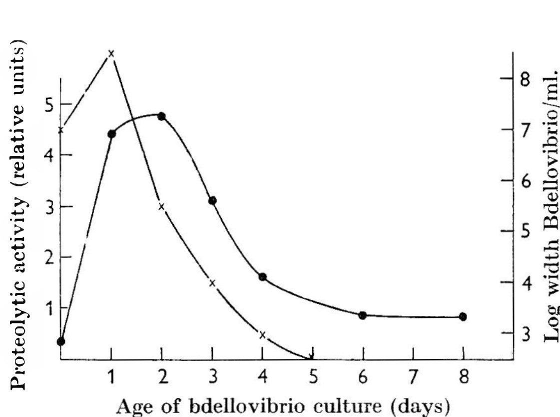

Fig. 5

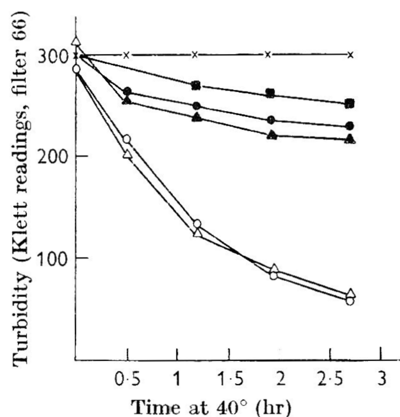

Fig. 6

Fig. 5. Formation of proteolytic exoenzyme in host-independent *Bdellovibrio bacteriovorus* A 3.12 isolate cultures (in NB medium). × Number of viable bdellovibrios measured by colony formation on solid medium C. ● Proteolytic activity of parasite culture supernatant fluid as measured by decrease in turbidity of standard suspension with heat-killed *Pseudomonas fluorescens* (120°, 10 min.) incubated for 50 min. at 37°.

Fig. 6. Inhibitory effect of phosphate on exoenzyme activity of host-independent A 3.12 culture supernatant fluid. Heat-killed *Pseudomonas fluorescens* (120°, 10 min.) was suspended in parasite culturesupernatant fluid (from 48-hr host-independent *Bdellovibrio bacterivorus* A 3.12 culture in NB medium) prepared as described for Fig. 4. Different buffers were added to give final pH values and molarities as indicated below. Control, nutrient broth only [×]; Sørensen buffer 0·1 M, pH 6 [■]; Sørensen buffer 0·1 M, pH 7 [●]; Sørensen buffer 0·1 M, pH 8 [▲]; Tris buffer 0·2 M, pH 8·4 [△]; pH 8. no buffer added [○]. Conditions of incubation and measurement of lysis were as for Fig. 4.

Table 3. *Analyses of the predacity of host-independent Bdellovibrio bacteriovorus A 3.12 mutants during successive transfers in pure culture*

Transfer	Viable count by: Dilution	Colony count	Plaque count	Plaque dilution
5th	$1·2 \times 10^9$	$3·5 \times 10^8$	$1·2 \times 10^9$	1·00
10th	$1·6 \times 10^9$	$1·4 \times 10^9$	$0·4 \times 10^9$	0·25
26th	$8·5 \times 10^9$	8×10^9	1×10^8	0·01

Sensitization of bacteria to bdellovibrio exoenzyme

Cell-free exoenzyme preparations of *Bdellovibrio bacteriovorus* A 3.12 did not affect intact living organisms of their hosts (*Pseudomonas fluorescens*, *Escherichia coli* B, *E. coli* K-12) in suspension. However, when such host bacteria were heated (70°, 10 min.) or treated with various agents such as 5 % butanol, acid or EDTA, which damage cell walls, in a manner similar to those described by Noller & Hartsell (1961*a*, *b*), the bacteria were rendered sensitive to lysis by the exoenzyme. When such cell-free culture supernatant fluids were added to pseudomonas host bacteria immediately after these hosts had attached a multiplicity of washed host-independent *B. bacteriovorus* A 3.12 mutant, an enhancement in the rate of lysis was observed over and above that observed when similar host + mutant mixtures were suspended in the growth medium alone.

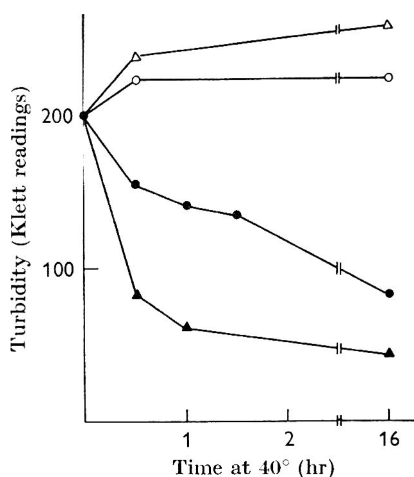

Fig. 7

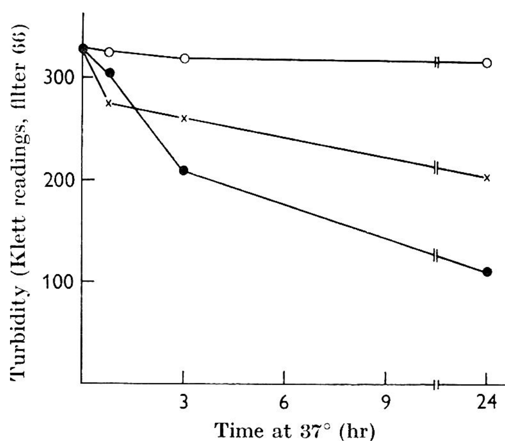

Fig. 8

Fig. 7. Effect of acid treatment of host bacteria on their sensitivity to lysis by bdellovibrio exoenzyme. Washed host bacteria were incubated for 50 min. at 40° at pH 2 (pH adjustment with dilute HCl). After neutralization, lysis was followed in suspension with host-independent *Bdellovibrio bacteriovorus* A 3.12 culture supernatant fluid (prepared as described under Fig. 4), incubated at 37°. ● *Escherichia coli* B + *B. bacteriovorus* A 3.12 culture supernatant fluid. ○ *E. coli* B control. ▲ *Pseudomonas fluorescens* + *B. bacteriovorus* A 3.12 culture supernatant fluid. △ *P. fluorescens* control.

Fig. 8. Effect of EDTA on sensitivity of *Pseudomonas fluorescens* to lysis by parasite exoenzyme. Living *Pseudomonas fluorescens* (from 24 hr cultures) were incubated with EDTA (di-sodium salt; Matheson, Coleman and Bell, Norwood, Ohio; final concentration, 133 μg. EDTA/ml.) in the presence and absence of the *B. bacteriovorus* A 3.12 culture supernatant fluid (prepared as for experiment of Fig. 4). All tests were made at pH 8·5, 37°. ○ *B. bacteriovorus* A 3.12 culture supernatant fluid only; ● EDTA + *B. bacteriovorus* A 3.12 culture supernatant fluid; × EDTA in nutrient broth.

Figure 7 shows the effect of acid treatment of bacteria on their sensitivity to lysis by exoenzyme; Fig. 8 summarizes the effect of EDTA on the lytic activity of the exoenzyme.

DISCUSSION

Bdellovibrio bacteriovorus at present occupies a unique position in the microbial world: it is the first example of a group of bacteria specifically able to attach to and to lyse certain other bacteria. In addition, the widespread occurrence of bdellovibrios as shown in soil and sewage samples in Germany and California (Stolp & Petzold, 1962; Stolp & Starr, 1963) and recently in soil samples in Israel (Miss M. Varon, private communication) suggests their possible ecological importance.

The isolation of predacious bdellovibrios in axenic cultures was a step towards a better understanding of the inter-relationship between parasite and host. With such cultures, an approach has been made in the present work towards the elucidation of the nutritional requirements of the parasite and the mechanisms of bacterial lysis induced by it.

Several earlier attempts by Stolp & Petzold (1962) and Stolp & Starr (1963) to isolate host-independent strains appeared to involve the loss of predacity and motility. In our experiments with *Bdellovibrio bacteriovorus* A3.12 isolates, however, even after repeated subcultivation, most of the parasite organisms retained their predacity. The media used in this work fully supplied the nutritional requirements of these mutants, although massive inocula (10^4 organisms/plate) were required to initiate colony formation on the NB agar or C agar media. The addition to the solid media of cell-free parasite culture fluid showed that the fluid had growth-initiating ability and abolished the growth-controlling effect of the inoculum. The growth-initiating factor was relatively heat stable; its activity was retained after heating to 120° for 15 min. A similar effect was observed by Mager (1964) for different strains of *Pasteurella tularensis*, where a growth-initiating factor present in culture supernatant fluid or cell extracts induced in recipient pasteurellas a capacity to synthesize new growth-initiating factor, thus insuring a continuous self-supply by the proliferating cultures.

The spectrum of host specificity of bdellovibrio strains was markedly dependent on the conditions prevailing during host/parasite interaction. The lytic spectrum of various host bacteria in suspension was broader than when plaque-formation was used as criterion for host specificity, and therefore appears to be a more useful way to assess the host specificity of the parasites. The much more restricted host range for plaque formation, as opposed to the ability of the parasite to attach to and to lyse different bacterial hosts in suspension, may be explained by the fact that plaque formation involves, besides attachment and lysis, the capacity of the parasite to multiply and spread in the presence of the multiplying host bacterium. Inhibition of growth by host metabolites could thus prevent plaque formation by parasites on solid media.

Lysis of host bacteria by certain predacious bdellovibrio strains, such as A3.12, seems to involve two stages, of which the first is the attachment of the bdellovibrio to its specific bacterial host; during this stage some damage of the outer layer of the host cell wall probably occurs. This damage may unmask or dissociate components of the inner layers of the cell wall, thus exposing the host bacterium to non-specific lysis by the bdellovibrio exoenzyme (second stage). The enhancement of lysis of living *Pseudomonas fluorescens* by multiple attachment of washed *Bdellovibrio bacteriovorus* A3.12 organisms after addition of exoenzyme-

containing culture supernatant fluid further supports the two-stage interpretation of lysis. The morphology of lysis by exoenzyme-producing bdellovibrio strains (Pl. 1, figs. 3–5) is markedly different from the sequence observed with strain 321 (Pl. 2, figs. 1–4), or strain 109 (Pl. 2, fig. 5), which did not form exoenzyme or only formed trace amounts. In these latter strains, the absence of exoenzyme production would then allow the formation of spheroplasts or cytoplasmic extrusions.

Sensitization of various Gram-negative bacteria to the action of proteolytic enzymes by dissociation of the outer lipoprotein layer of the cell wall has been demonstrated by many investigators who have used: heat treatment (Salton, 1958; Horikoshi & Shigeji, 1959; Becker & Hartsell, 1954; Bender, 1963); freezing and thawing (Kohn, 1960); extraction with lipid solvent or alkali (Bender, 1963; Becker & Hartsell, 1955) or with EDTA (Repaske, 1958). Treatment of host bacteria by heat, 5% (v/v) butanol, EDTA or acid in our experiments sensitized bacteria to the lytic activity of strain A3.12 exoenzyme.

In contrast to the work of Fukasawa & Nikaido (1960) on the loss of receptor site for phage P 22 in *Salmonella typhimurium*, *Bdellovibrio bacteriovorus* 109 did not lose its ability to attach to and to lyse hosts such as UDP-galactose-4-epimeraseless *Escherichia coli* mutants in the absence of galactose, and the UDPG synthetase defective mutant of *E. coli*. The polysaccharide composition of the cell walls of these host mutants does not seem, therefore, to be of critical importance for the attachment of the parasites.

We are grateful to Professor R. Y. Stanier for his hospitality in making it possible for one of us (M.S.) to spend a sabbatical leave in his laboratory, for suggestion of the subject and for his continued interest in this work. We thank Dr H. Stolp for kindly making his bdellovibrio strains available to us.

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EXPLANATION OF PLATES

All figures (except Figs. 6–8; Pl. 1) photographed under oil immersion in Reichert Zetopan Phase Contrast microscope, × 1200.

PLATE 1

Fig. 1. Degenerate form of a host-independent bdellovibrio isolate, 3 to 5-day-old culture in medium NB.

Fig. 2. Attachment of host-independent *Bdellovibrio bacteriovorus* A3.12 isolate on *Pseudomonas fluorescens* 2 min. after mixing with host cells. The 48-hr-old parasite culture was grown in NB medium.

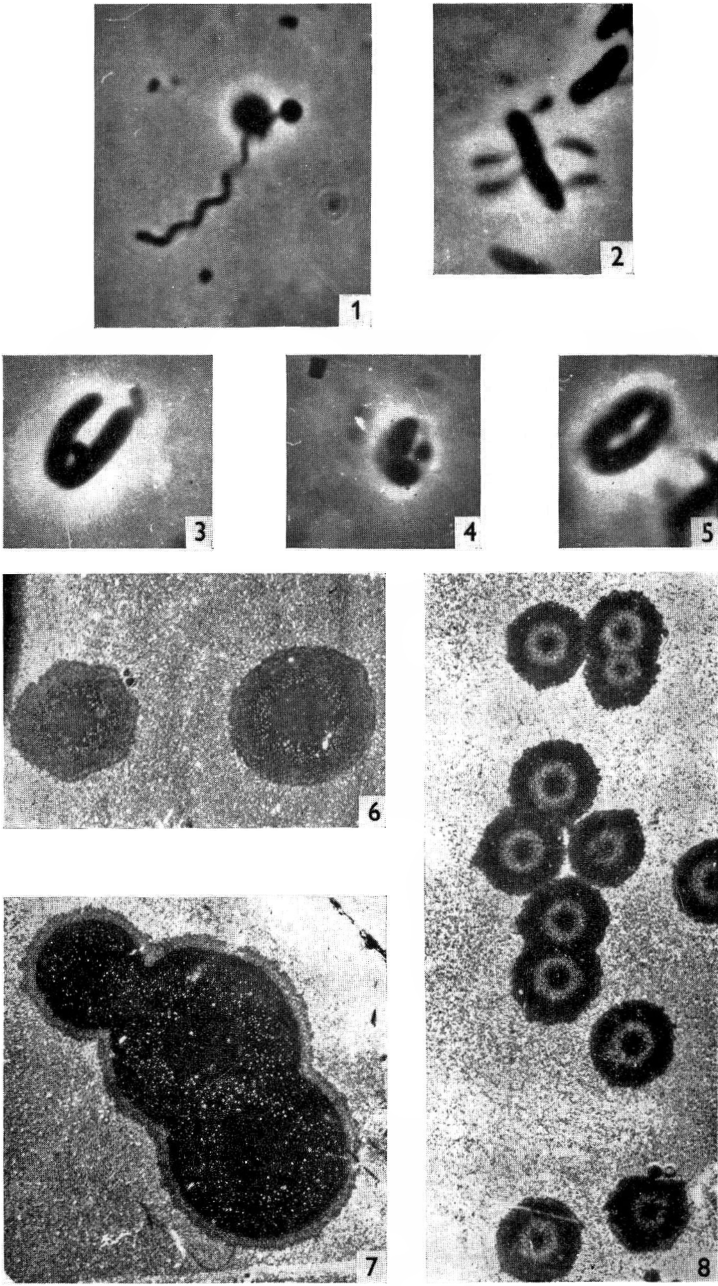
Figs. 3–5. Morphological changes in host organisms (see explanation Fig. 2 above) 30–60 min. after mixing cultures.

Figs. 6–8. Different plaque types of host-independent strain A3.12 isolates after 4 days incubation (30°, NB/10 medium as described in Methods) on *Pseudomonas fluorescens* lawns.

PLATE 2

Figs. 1–4. Formation of cytoplasmic extrusions of *Pseudomonas phaseolicola* induced by *Bdellovibrio bacteriovorus* 321 photographed 30–60 min. after mixing host organisms with parasites from two-membered culture in which hosts had been fully lysed.

Fig. 5. Spheroplast formation of *Pseudomonas fluorescens* induced by strain 109 photographed 30–60 min. after mixing host organisms with parasites from two-membered culture in which hosts had been fully lysed.



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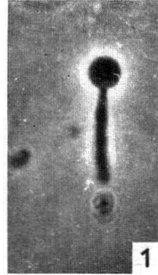

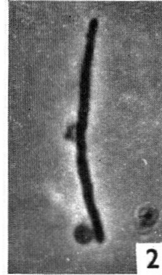

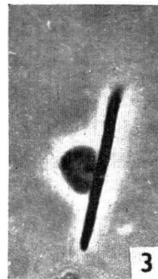

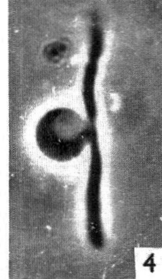

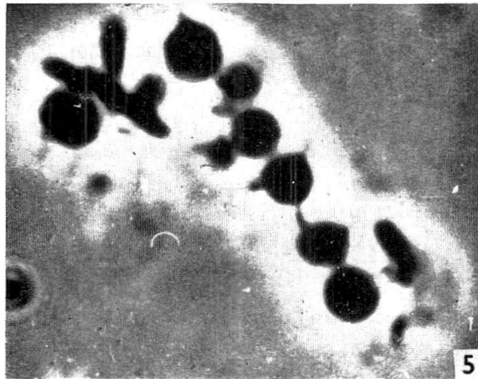

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Separation, Characteristics and Minimal Amino-Acid Requirements of Six Variants Derived from a Strain of *Bacillus cereus*

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SUMMARY

Maintenance of a stock culture of *Bacillus cereus* by subculture on nutrient agar plates resulted in dissociation of the culture manifest as the development of fimbriate outgrowths from the initially entire peripheries of confluent streaks and isolated colonies. Auxanographic determination of minimal amino acid requirements of the dissociated culture indicated that it was composed of at least two types of organism: one was sensitive to (i.e. probably lysed by) L-lysine and the other resistant. They were further separated on the basis of sensitivity to lysine and colonial morphology into six different types. The microscopic appearance of organisms and their arrangement within colonies indicated that the original culture and each of the variants was a rough (R) type. At 28°, four variants required only L-glutamate for growth, while the remaining two required L-cysteine or L-α-alanine in addition. At 35° a greater number of amino acids had to be supplied for growth to occur. At neither temperature did the variants show a requirement for growth factors of the vitamin B-complex. These nutritional requirements, together with infra-red spectra of whole organisms and biochemical characteristics determined by conventional methods, confirmed that each variant, though showing minor differences, did represent a culture of *B. cereus*. On auxanographic plates in the presence of L-lysine at 28°, three variants were lysed, one was unaffected, and the growth of two enhanced.

INTRODUCTION

The ease with which bacteria, and in particular the aerobic sporeformers, dissociate is well known and is one cause of the difficulties encountered in identification and classification of members of the genus *Bacillus*. Though there have been many studies of dissociation of Bacillus (Smith, Gordon & Clark, 1952; Braun, 1947), few have been concerned with detecting alterations in metabolic and nutritional patterns. The dissociation has usually been correlated with change of colonial morphology or capacity to form a particular metabolic product. The requirements for growth factors and amino acids have been separately reported in some detail for a few members of the genus, namely: *B. polymyxa* and *B. macerans* (Katznelson, 1944), *B. larvae* (Katznelson & Lochhead, 1948), *B. larvae* White (Lochhead, 1942), *B. alvei* and *B. para-alvei* (Katznelson & Lochhead, 1947), *B. anthracis* (Brewer *et al.* 1946), *B. popilliae* Dutky and *B. lentimorbus* Dutky (Dutky, 1947), *B. subtilis* (Teas 1950; Demain, 1958), and *B. coagulans* (Cleverdon, Pelczar & Doetsch, 1949*a*, *b*;

Campbell & Sniff, 1959). The B vitamin and amino acid requirements of almost al of the species within the genus were reported by the comprehensive nutritional surveys of Knight & Proom (1950) and Proom & Knight (1955). Although this latter work provided data for a nutritional classification the elucidation of minimal amino acid requirements was not then necessary. In the present work the dissociation, cultural characteristics and minimal amino acid requirements of one species, *B. cereus*, have been investigated in greater detail.

METHODS

Amino acids. These were obtained from British Drug Houses Ltd., or Light and Co. Ltd. Before use each was checked for purity by paper chromatography.

Sugars. Glucose was of A.R. quality. The maltose sample used was found to contain appreciable quantities of several amino acids upon examination in the Beckman/Spinco Amino Acid Analyser; it is subsequently referred to as impure maltose.

Inorganic salts. All were of A.R. quality.

Water. Glass-distilled water, passed through an Elgastat de-ionizer to yield an effluent having 2–4 MΩ resistance, was used for making all solutions.

Culture flasks. The specially designed flasks (Moore, 1963) were made from soda glass having the following composition (%, w/w): SiO_2, 71·5; Al_2O_3, 2·2; CaO, 5·7; MgO, 3·0; BaO, 1·7; Na_2O, 14·0; K_2O, 1·5. This analysis is given since, under the same growth conditions, equivalent flasks of Pyrex glass caused a marked lag in growth response.

Agar. The material used was of Korean origin.

Organism. Bacillus cereus strain CN 753 was selected from the Wellcome Research Laboratories Culture Collection.

Media. Nutrient broth was used for growing crops of organisms to be used subsequently as inocula. For nutritional investigations a chemically defined medium similar to that of Knight & Proom (1950) was used. The complete basal medium (BM) contained (g./l. final volume): glucose (or maltose) 5·0; KH_2PO_4, 1·5; $(NH_4)_2HPO_4$, 7·0; $MgSO_4.7H_2O$, 0·5; in mg.; $MnCl_2.4H_2O$, 37; $FeSO_4.7H_2O$, 2·5; $(NH_4)_6Mo_7O_{24}.4H_2O$, 2·0.

Maintenance of cultures. (*a*) During the early phase of the work a freeze-dried stock culture was reconstituted in 0·5 ml. nutrient broth, incubated for 2–3 hr at 28° and then plated on nutrient agar to check purity. The culture was kept at room temperature and further maintained by fortnightly or monthly subculture on to plates of fresh medium. (*b*) Following the change in growth characteristics and subsequent separation of variants, freeze-dried stocks of each were stored at 4°.

Preparation of inocula for growth experiments. For each experiment the appro priate freeze-dried culture was reconstituted and plated to check purity and colonial morphology. A liquid culture in broth inoculated from a single typical colony was then grown at 28° overnight. The organisms contained in a 6 ml. portion were then washed successively in three 6 ml. portions of sterile saline and finally suspended in a fourth 6 ml. portion. After determining the concentration of the suspension it was diluted to correspond to 85 μg. dry weight bacteria/ml. This suspension served as the inoculum, 0·4 ml. being added to about 20 ml. of final medium. Finally the suspension was plated to check viability, purity and colonial morphology.

Inocula for auxanography. Crops of organisms were grown and washed as described above and enough bacterial suspension added to cooled molten agar medium to produce a concentration of organisms in the final plates corresponding to 10 μg. dry wt. bacteria/ml.

Measurement of amount of organism in inocula. The extinction of a known dilution of a washed cell suspension of organism was measured in 1 cm. optical cells at 404 mμ, (E_{404}) by using a Unicam SP 1400 spectrophotometer. The amount of organism, in terms of μg. dry wt. bacteria/ml. suspension was then read from a calibration curve, and the value calculated for the undiluted suspension. The latter was then diluted before use to a concentration of organism equivalent to 85 μg. dry wt. bacteria/ml.

Measurement of growth. Extinctions of cultures in liquid chemically defined medium were read *in situ* by using specially designed culture flasks (Moore, 1963), the side-arms of which fitted into the optical cell carrier of the same spectrophotometer. The values are recorded in terms of μg. dry wt. bacteria/ml.

Preparation of chemically defined medium. Each of the inorganic salts was kept as a concentrated aqueous stock solution at room temperature. Glucose, or maltose, solution (25 %, w/v) in de-ionized water and separate solutions of amino acids in 0·25 M-phosphate buffer (pH 7·0) were freshly made for each experiment.

Complete basal (BM) medium. Appropriate volumes of stock inorganic salt solutions were added to about 800 ml. de-ionized water, the mixture adjusted to pH 7·5 and the volume made to 900 ml. The mixture was then heated to boiling, cooled quickly to about 15° and filtered (Whatman no. 1). The volume of the filtrate was readjusted to 900 ml. and to pH 7·5. Portions of 18 ml. were then distributed into culture vessels and sterilized. Glucose or maltose solution (0·4 ml.) was added aseptically and the completed medium left for 18–24 hr at room temperature before being inoculated.

For auxanography, agar (2 g.) and BM medium (75 ml., sugar omitted) were autoclaved in admixture, and glucose or maltose solution (4 ml.) added aseptically to the cooled mixture.

Complete chemically defined (CDM) medium. Solutions of amino acids were added asceptically to each of the portions of BM medium so that the final concentration of each compound (in μg./ml.) was DL-leucine, DL-isoleucine, DL-valine, DL-threonine, L-arginine, L-cystine, DL-methionine, DL-tryptophan, DL-serine, 50; L-glutamic acid, 200; L-lysine, 1000; glycine, 1000.

Sterilization. Solutions of methionine and serine were filtered through Ford SB pads. Sugar solutions, BM medium and solutions of the other amino acids were autoclaved separately at 15 lb./in.^{2} for 10 min. (121°).

Incubation. Culture flasks, maintained at 28° or 35° $\pm$ 0·1° as required, were shaken in a Gallenkamp Metabolic Shaker (reciprocal) at 200 strokes (2·5 cm. amplitude)/min. This effected some degree of aeration and also maintained the organisms in suspension.

Auxanographic tests. A technique similar to that described by Pontecorvo (1949) was used. Korean agar (2 g.) was washed by repeated suspension in fresh portions (1 l.) of de-ionized glass-distilled water during the day before use. After standing in water overnight the agar was washed once more, drained of excess water, mixed with BM medium (glucose omitted) and autoclaved (121°; 15 min.). Glucose (or

maltose) and amino acids as required were added aseptically to the cooled mixture (37–42°). This was then inoculated with the calculated volume of washed suspension of organism. After thoroughly mixing, portions (10 ml.) were quickly run into Petri dishes, allowed to set, and then dried in an inverted position for 1 hr at 35°. Each plate was then overlaid with 5 ml. of an aqueous solution of washed agar (2 %, w/v), and dried similarly. Compounds to be tested were applied to this top layer in very small quantities at well-separated marked positions. Not more than six compounds were applied to dishes of 8·5 cm. diameter.

Replica plating. The technique has been described in detail by Lederberg & Lederberg (1952).

RESULTS

Dissociated culture. The fimbriate edges of colonies which developed after repeated subculture of *Bacillus cereus* CN 753 on nutrient agar at 28° are shown in Pl. 1, fig. 2. Such a culture will be referred to as a dissociated culture; for comparison the appearance of colonies immediately on reconstitution from freeze-dried specimen is shown in Pl. 1, fig. 1.

Minimal amino acid requirements for growth at 28°. Auxanographic tests with BM medium and glucose as carbon energy source showed that at 28° the culture did not grow when ammonium ion was the sole source of nitrogen. Of sixteen common amino acids tested singly only L-glutamic acid or L-aspartic acid supported good growth (Pl. 1, fig. 3). Subsequent tests in liquid BM medium confirmed these results and indicated that a concentration of 1000 μg. L-glutamate/ml. was optimal. Growth response to L-aspartate under the same conditions was erratic. At best, only thin, clumped growth was obtained with L-aspartate at 250 μg./ml.

The effect of lysine + glycine mixture on growing organisms. The effect of these amino acids was first seen during an attempt to determine auxanographically whether or not minimal amino acid requirements varied with the carbon energy source supplied. A series of auxanographs containing pure glucose showed growth only around L-glutamic acid and L-aspartic acid; effects on growth due to the presence of L-lysine and glycine were not demonstrable. However, in a replicate series, the overall growth which resulted from the chance use of a sample of impure maltose (subsequently shown to be contaminated by several amino acids) made it possible to observe, in the particular plate bearing L-lysine and glycine in close proximity, the development of a clear zone between the points at which each had been applied. Within 48 hr after completion of the clearing, comparatively large colonies had developed in the clear zone. The final appearance of such a plate is shown in Pl. 1, fig. 4. Since overall growth was followed by clearing the effect is referred to as lysis rather than as inhibition, although proof of this is wanting. Growth of the dissociated culture, or of certain of the separated variants in repeat tests, did not consistently demonstrate the necessity for the presence of glycine, in addition to L-lysine, for lysis to occur. The shape assumed by a lysed area varied, therefore, between a biconvex area often situated exactly between the separate points of application of lysine and glycine, and a circle surrounding the lysine or lysine + glycine applications. Lysis was never detected when only glycine was present. The apparent necessity of both amino acids is shown in Pl. 1, fig. 5.

Prevention of the lytic effect. Plates of BM medium agar containing impure maltose (1 mg./ml.), L-lysine (1 mg./ml.), and glycine (1 mg./ml.), and seeded with the

dissociated culture, were poured, dried as usual, and then a variety of compounds touched on at separate points. Protection against the lytic effect was shown by the development of dense growth around the active compound. The remaining area of each plate was clear except for a general scatter of a few large colonies of the resistant variants (Pl 1, fig. 6). Compounds having this 'protective' property were the following: L-aspartic acid, L-cystine, L-cysteine, L-histidine, L-glutamine, L-glutamic acid, DL-leucine, DL-isoleucine, L-methionine, L-tyrosine, DL-threonine, DL-valine, DL-norvaline. Those which did not protect were: L-α-alanine, β-alanine, L-arginine, DL-α-amino-n-butyrate, DL-β-amino-n-butyrate, DL-γ-amino-n-butyrate, L-hydroxyproline, DL-serine, DL-β-phenylalanine, DL-tryptophan, L-proline, DL-penicillamine, adenosine, guanine, xanthine, orotic acid, hypoxanthine, thymine, uracil, thiamine, nicotinamide, Ca-D-pantothenate, p-aminobenzoic acid, pteroylglutamic acid, pyridoxamine, riboflavine.

Separation of variants sensitive and resistant to mixtures of lysine and glycine

Lysine-glycine resistant variant (LR 1)

An inoculum taken from a colony of *Bacillus cereus* CN 753 having fimbriate edges was grown in nutrient broth, the organisms washed and then inoculated into cooled molten medium consisting of BM medium (glucose omitted), impure maltose (1 %) and agar (2 %). Plates were poured, dried as usual, and crystals of glycine and L-lysine placed together at marked positions, and the plates then incubated at 28°. Six of the resistant colonies which developed within a lysed area were removed and macerated in 1 ml. sterile nutrient broth. This suspension was then inoculated into 20 ml. sterile broth and incubated at 28° on a shaker for 18 hr. The organisms were harvested and a washed suspension of just perceptible turbidity prepared. Tenfold-serial dilutions were made from this, 0·05 ml. of each dilution spread over the surface of a separate nutrient agar plate and the plates incubated at 28° overnight. A plate bearing 50–100 well-separated colonies was replica plated in duplicate on BM medium agar containing impure maltose (1 %) + L-lysine (1 mg./ml.) + glycine (1 mg./ml.), and also on nutrient agar. To obtain colonies of reasonable size it was necessary to incubate the BM medium plates for four days at 28°. Nutrient agar plates required only overnight incubation at 28° and were then retained at 4°. All the colonies on the nutrient agar replica appeared on the selective defined medium; some had an entire edge, while others had such a pronounced lobate edge that they assumed a satellite appearance (Pl. 2, fig. 7). Since the form of the lobate colony was retained on duplicate plates it seemed that it was not the result of a mechanical artifact. Probably the colonial form represented the resistant portions of a colony remaining after those portions sensitive to lysine + glycine had lysed. Isolates were obtained by picking from a single colony of each type. Organisms grown from these, washed and seeded into BM medium containing impure maltose + glycine + agar, showed no lysis around applied crystals of lysine. Similar plates seeded with the dissociated culture of *B. cereus* CN 753 showed the typical lytic effect. A freeze-dried stock was prepared from the smooth-edge type and designated LR 1.

Lysine-glycine sensitive variant (LS)

Using an inoculum of washed cells grown from a fimbriate colony of *B. cereus* CN 753, replica plates were again prepared as described above but with BM medium

agar + impure maltose. Prior to incubation, crystals of L-lysine and glycine were placed together on the plates at precisely located positions. The plates were then incubated and colonies sought which had lysed (in areas close to the lysine + glycine applications) and remained so upon extended incubation (1 week at 28°). Inocula taken from corresponding colonies on the nutrient agar replica were then replica plated repeatedly until many colonies present on the nutrient agar replica remained completely absent from the selective replica. When this was attained, a colony present on the nutrient agar plates but absent from the corresponding position on selective replicas was then picked and grown in nutrient broth to provide organisms from which a freeze-dried stock of the sensitive variant, LS, was prepared.

An auxanographic comparison of the purity of inocula grown from variants LS and LR1 is shown in Pl. 2, figs. 8, 9. Subsequent work indicated that variant LS remained as pure as on isolation but LR1 seemed to develop an increasing amount of the sensitive component. This was manifest as a definite thinning of the opacity of plates inoculated with LR1 in the vicinity of superimposed crystals of lysine and glycine. A suitable plate, inoculated with LR1 and bearing L-lysine and glycine at one site, was therefore selected and one matt colony and one smooth colony picked from the surface at a thinning area. Each colony was suspended in a small volume of saline and then streaked on to BM medium agar containing impure maltose (1 %) + L-lysine (1 mg./ml.) + glycine (1 mg./ml.). Growth was slow, but after 7 days at 28° single colonies were large enough to transfer to nutrient broth; after overnight growth at 28° the crops were freeze-dried to provide stocks designated LR2/R and LR2/S, respectively.

Further resolution of variants LS and LR2/S

During subsequent work it was noticed that on plating the crops obtained by growing these variants in chemically defined (CDM) medium some of the colonies maintained an entire edge, whereas others quickly developed a fimbriate edge. Microscopically each type of colony consisted of large Gram-positive rods; macroscopically the colonial appearances were not sufficiently dissimilar to suggest contamination. An extreme example is shown in Pl. 2, fig. 10: all colonies from one culture vessel are entire-edged while almost all of those from another are fimbriate though both culture flasks were inoculated with variant LS.

Subcultures on nutrient agar were made from the centre of an entire-edge colony and the periphery of a fimbriate type, and by repeated subculture of young colonies (18 hr at 28°) cultures which retained their entire and fimbriate colonial characteristics were obtained (Pl. 3, figs. 11–14). Freeze-dried stocks were prepared from crops grown in nutrient broth and designated variants LS/S, LS/R, LR2/SS, LR2/SR.

Derivation of variant LR/VR

Routine plating of variant LR1 revealed the existence of a minute proportion of colonies which developed exaggerated fimbriate characteristics. One was picked, repeatedly subcultured on nutrient agar and a crop then freeze-dried for stock. The relationship of the variants to each other is shown in Fig. 1, and their colonial appearances in Pl. 3, figs. 11–14.

Investigations with the separated variants

Re-typing. The use of infra-red spectrophotometry as an aid in the identification of bacteria has been suggested by many workers (Stevenson & Bolduan, 1952; Levine, Stevenson, Chambers & Kenner, 1953; Riddle *et al.* 1956), though several consider it of limited application. The work of Blackwood & Epp (1957), and Haynes

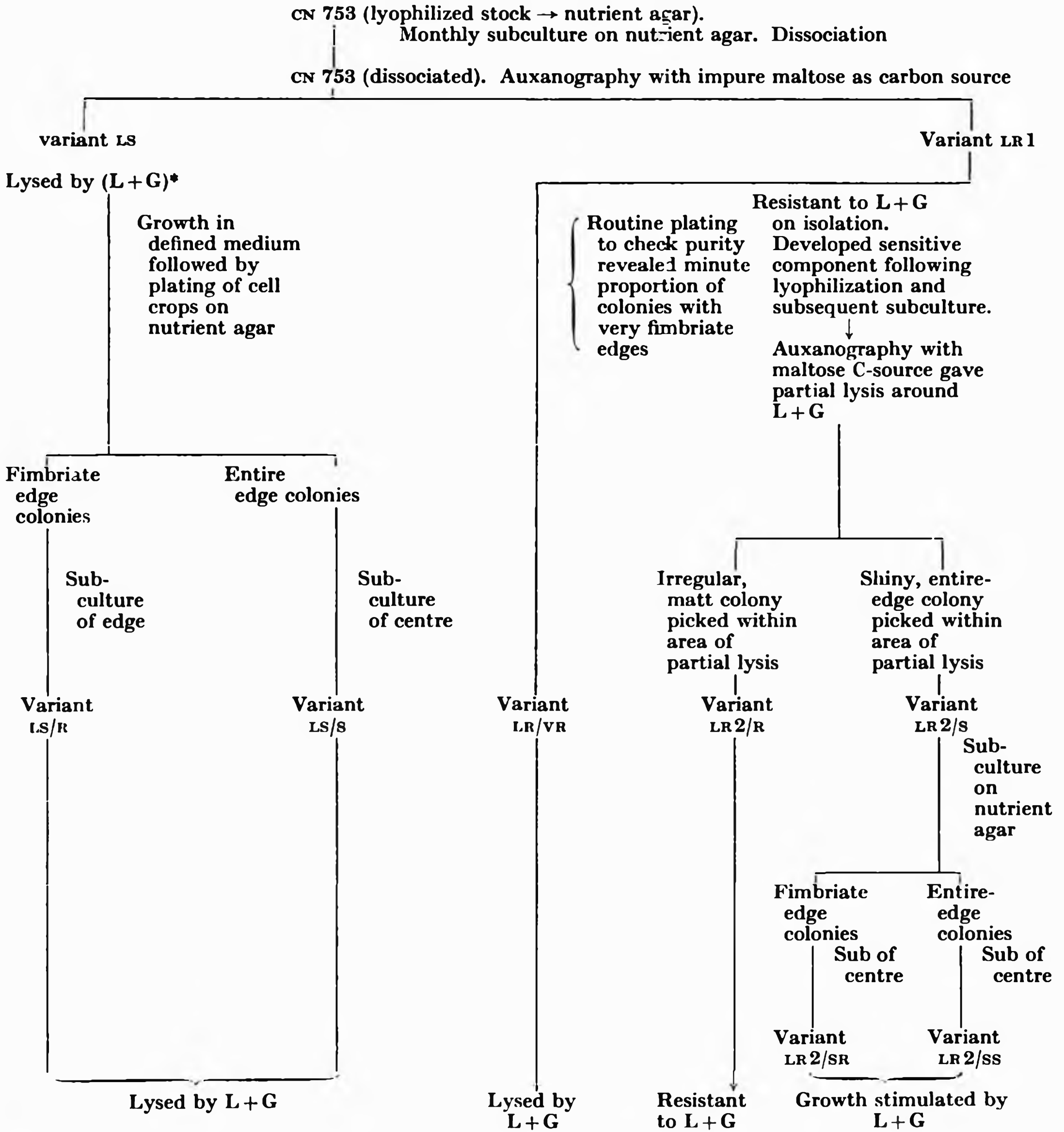

Fig. 1. The relationship and derivation of variants isolated from the dissociated culture of *Bacillus cereus* CN 753.

et al. (1958), however, indicated that the technique was useful for characterizing members of the genus *Bacillus*. By virtue of their content of poly-β-hydroxybutyric acid, which absorbs strongly at 5·7 μ, whole organisms of members of this genus are endowed with a characteristic '5·7 μ-type' absorption spectrum (Haynes *et al.* 1958) when grown under appropriate conditions. Whole organisms of the variants and of the dissociated culture, grown in nutrient broth (0·2 % glucose) at 28°, had identical infra-red spectra, each with strong absorption band at 5·7 μ.

Tests based upon those suggested by Smith *et al.* (1952) showed that each isolate exhibited biochemical characteristics typical of *Bacillus cereus*, viz.: gelatin liquefaction +; casein hydrolysis +; starch hydrolysis +; anaerobic growth (Robertson's broth) +; aerobic growth (pH 6·0) +; acetylmethylcarbinol formation +; methylene-blue reduction −; lecithinase (egg-yolk reaction) +; methyl red test +; indole formation −; formation of nitrite from nitrate +; utilization of citrate −; urease formation −; bromocresol purple milk, alkaline with peptonization; utilization of carbohydrates. (*a*) Inorganic medium: glucose, growth with formation of acid; D-xylose and L-arabinose, no growth, no acid formation. (*b*) Organic medium: glucose, growth with acid formation; growth and acid formation in the presence of L-arabinose or D-xylose was atypical.

Nutritionally each isolate required a supply of preformed amino acids for growth in defined medium but a supply of growth factors of the vitamin B complex was not necessary. These requirements are in agreement with those found for *Bacillus cereus*, by Knight & Proom (1950).

S-R classification. Impression preparations of young (9 hr) colonies were made according to the method of Klieneberger & Smiles (1942), but stained with tannic acid crystal violet according to Robinow (1947) to reveal the cell-walls. On the basis of the microscopic appearance of the organisms and their arrangement within the colonies each variant appeared to represent a rough (R) type (Bisset, 1938, 1948) (Pl. 3, figs. 11–15).

At 28° on nutrient agar entire-edge types, namely, variants of LS/S and LR2/SS produced colonies having only a suggestion of medusa-head structure; this became a little more pronounced at 35°. The remainder of the variants produced typical medusa-head colonies at both temperatures.

Minimal amino acid requirements. These were determined auxanographically with glucose as carbon energy source and for ease of comparison are shown in Table 1. The different auxanographic responses to lysine, glycine, and lysine + glycine are compared in Pl. 4, figs. 16–21. Lysine was not replaceable by cadaverine, putrescine, histamine, benzylamine or ethanolamine, when tests were carried out with variant LS/S, though partial lysis occurred when lysine was replaced by 11-aminoundecanoic acid, ω-aminocaprylic acid, or α-aminopimelic acid.

The growth response of the dissociated culture and of each of the variants at 28°, in BM medium containing 500 μg. L-glutamate/ml., is shown in Fig. 2.

The effect of temperature on growth and amino acid requirements. In a defined medium consisting of BM, glucose and L-glutamate (1 mg./ml.) washed bacteria of the dissociated culture grew heavily at 28° but not at all at 35°. Growth of unwashed bacteria in the same medium at 35° showed that proliferation at this temperature was, in fact, possible, and that one or more factors present in broth were essential for growth. The latter could not be replaced by a second amino acid, or purines,

pyrimidines, nucleotides and factors of the vitamin B-complex, singly or in admixture. The compounds concerned were in fact a group of amino acids, namely: DL-leucine, DL-threonine, L-arginine, DL-isoleucine and DL-valine. Incorporation of each of these, at 50 μg./ml. into BM containing glucose and L-glutamic acid, together with L-lysine, glycine and L-cysteine, present in the medium of Johnson (1961), resulted in rapid heavy growth of washed bacteria of the dissociated culture and of each variant at 35°. Omission of the amino acids singly showed that for

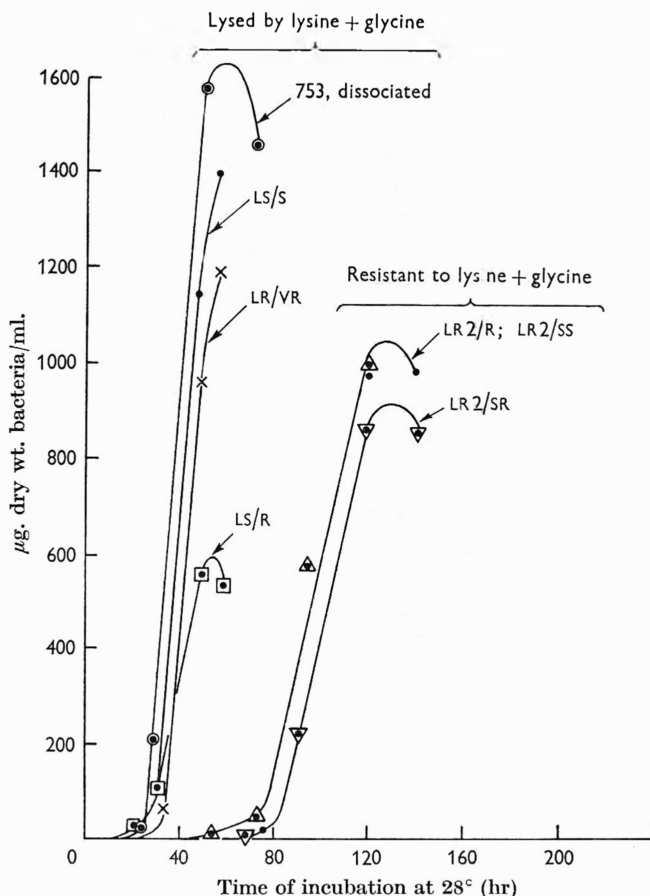

Fig. 2. Growth at 28° of the dissociated culture of *Bacillus cereus* CN 753 and each of the variants in defined medium (salts + glucose) containing L-glutamic acid (500 μg./ml.) as sole nitrogen source.

growth of variant LS/S (the only variant investigated) at 35°, DL-isoleucine and L-cysteine were not essential, DL-serine and glycine were stimulatory, L-lysine inhibitory, and the remaining amino acids, namely, L-glutamic acid, DL-leucine, DL-valine, DL-threonine and L-arginine, essential.

individual differences in amino acid requirements are shown. Variant LS/S probably represents the major proportion of the population of the original dissociated culture since it grows most rapidly and is least exacting, i.e. it will grow in the presence of a single amino acid of the group, glutamic acid, aspartic acid, proline, histidine, threonine or methionine. The other variants are progressively more exacting in that either the number of amino acids which can support growth is fewer (one only for variant LS/R, three for LR/VR) or that at least two amino acids together are essential (variant LR2/R). These facts presumably reflect differences in the equilibrium positions of metabolic reactions amounting, in some instances, to deficient formation of certain essential compounds. Such variants thus become less well equipped to establish themselves in a mixed population containing variant LS/S organisms. The fact that only one or two colonies of variant LR/VR can ever be seen when the undissociated culture of *B. cereus* CN 753 is plated possibly bears this out. The number of colonies representing the more exacting (lysine resistant) variants, namely, LR2/R, LR2/SS, LR2/SR, in lytic areas of auxanographic plates is also so much smaller than the total number of bacteria originally present per unit volume, that their combined numbers can represent only a fraction of the total population of the inoculum.

That the ability of the dissociated culture to grow at 35° in the presence of additional amino acids is an expression of the proliferation of a variant favoured by the higher incubation temperature seems unlikely since all the variants isolated show the same effect and are recoverable from cultures grown at 35°. Since growth occurs at 28° in the presence of glutamate only, and at 35° only when additional amino acids are supplied, it appears that at the higher temperature the ability of the bacteria to derive necessary amino acids from glutamate is impaired.

From a consideration of colonial morphology and amino acid requirements it seems that while the pairs of variants LS/S, LR2/SS, and LR2/R, LR/VR represent extremes (the former being least exacting and barely maintaining rough (R) colonial characteristics, and the latter more exacting and distinctly R-types), variants LR2/SR and LS/R might well represent intermediate types, or populations of incompletely separated variants. Thus nutritionally these retain less exacting characteristics, due perhaps to the presence of an LS/S-type, but morphologically the irregular edges of their colonies may represent the proliferation of an LR2/R-type.

The variants fall into two groups on the basis of growth rate and lysine sensitivity, the faster growing (LS/S, LS/R, LR/VR) being sensitive to L-lysine and the slower (LR2/R, LR2/SS, LR2/SR) resistant. Although elaboration of lytic factors by members of the genus *Bacillus* is very well known (Waksman, 1945) the nature of the factors is often less well established. Smith *et al.* (1952) considered the lytic activity present in their cultures of *B. cereus* due to bacteriophage, whilst Nomura & Hosoda (1956) and Ivanovics & Alföldi (1957) described, respectively, the formation of an autolytic enzyme by *B. subtilis*, and a proteinaceous antibacterial lytic substance (megacin) by *B. megaterium*. Reports of the lytic effect of cysteamine and its close derivatives for members of the genus *Bacillus* have been published by Weinberg, Saz & Pilgren (1958) and Judith & Weinberg (1959). In the former paper several mechanisms were suggested which would provide an explanation of the lytic effects observed and equally of the lytic effect obtained with L-lysine in the present work. The fact that 11-aminoundecanoic acid, ω-aminocaprylic acid, or α-aminopimelic acid can replace L-lysine to some extent suggests, on the basis of similarity of chemical

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Appearance of colonies of undissociated *Bacillus cereus* CN 753 after reconstitution of freeze-dried stock and cultivation on nutrient agar at 28° for 18 hr.

Fig. 2. As Fig. 1, but after repeated subculture on nutrient agar.

Fig. 3. Auxanographic response of dissociated *B. cereus* CN 753 to L-aspartate (17) and L-glutamate (21), basal salts medium with glucose as carbon energy source.

Fig. 4. As fig. 3, but with impure maltose present instead of glucose, showing overall growth with lysed area (containing resistant colonies) between L-lysine (19) and glycine (20).

Fig. 5. As fig. 3, but inoculated with variant LS/S and supplemented with L-methionine (1 μg./ml.); showing lysed areas between lysine and glycine, and around lysine + glycine.

Fig. 6. Plate containing basal salts medium, impure maltose, L-lysine (1 mg./ml.), glycine (1 mg./ml.) and seeded with dissociated *B. cereus* CN 753, showing protection against lysis afforded by glycyl-DL-asparagine (10).

Plate 2

Fig. 7. 'Satellite' appearance of replica plated colonies of *Bacillus cereus* CN 753 variant LR 1, possibly indicating lysis of contaminant LS-type bacteria. A few colonies, virtually pure LR-types, show no satellites. Solidified basal salts medium + maltose (impure) + L-lysine (1 mg./ml.) + glycine (1 mg./ml.).

Figs. 8, 9. Auxanographic comparison of the purity of isolates LS (fig. 2) and LR 1 (fig. 3) by their responses to lysine + glycine mixture.

Fig. 10. Plate to check purity of variant LS after growth in defined medium. Crops represented pure cultures but a high proportion of colonies developing from bacteria taken from one culture flask (10) show fimbriate edges, while all those from replicate flask (12) show entire edges.

Plate 3

Appearance of organisms and colonies of variants of *Bacillus cereus* CN 753 grown on nutrient agar at 28°, for 9 hr (bacteria), 18 hr (colonies). Preparations by the impression technique. Tannic acid crystal violet stain. Magnification: colonies × 3½; organism × 1600.

Fig. 11. Variants LS/S or LR2/SS, showing colonies with entire peripheries.

Fig. 12. Variant LS/R, showing colonies with irregular peripheries which later become fimbriate.

Fig. 13. Variant LR2/SR, showing colonies with irregular peripheries which later become fimbriate.

Fig. 14. Variant LR/VR, showing pronounced fimbriate peripheries of young colonies.

Fig. 15. Variant LR2/R, showing small colonies with edges which remain irregular.

PLATE 4

Auxanographic response of variants of *Bacillus cereus* CN 753 to L-lysine, glycine and L-lysine + glycine.

Fig. 16. Variant LS/S showing lysis around lysine, and lysine + glycine, but not around glycine.

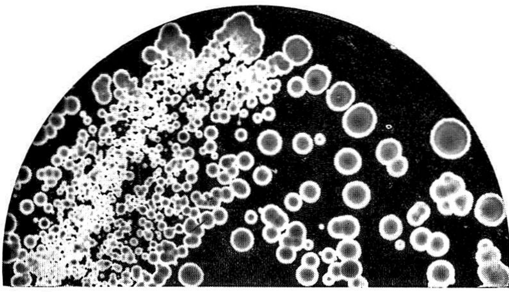
Fig. 17. Variant LS/R (and LR/VR), Growth is poor but lysis just discernible around lysine and lysine + glycine.

Fig. 18. Variant LR2/SS; growth increased by lysine.

Fig. 19. Variant LR2/SR; growth increased by lysine. The crescent-shaped areas of thinning growth between L, G, and L, L + G applications indicate the presence of a small percentage of sensitive cells.

Fig. 20. Variant LR2/R. Growth unaffected by lysine, glycine, or lysine + glycine.

Fig. 21. Dissociated *Bacillus cereus* CN 753. Growth lysed by L-lysine and L-lysine + glycine. Resistant colonies developing within the lytic area.



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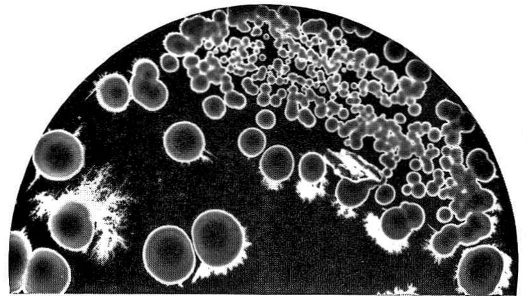

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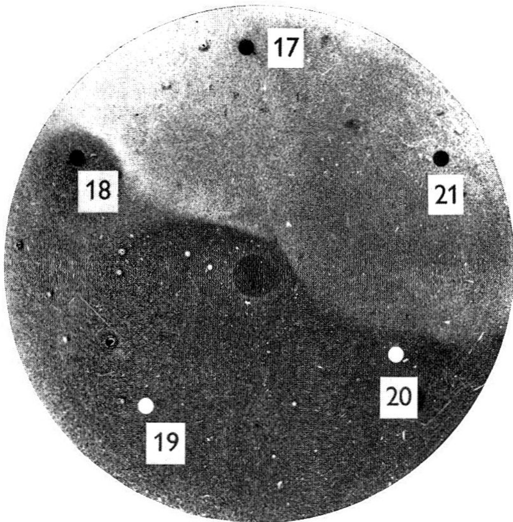

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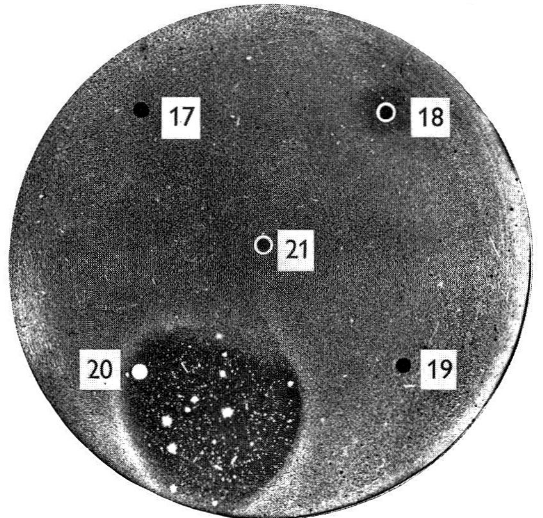

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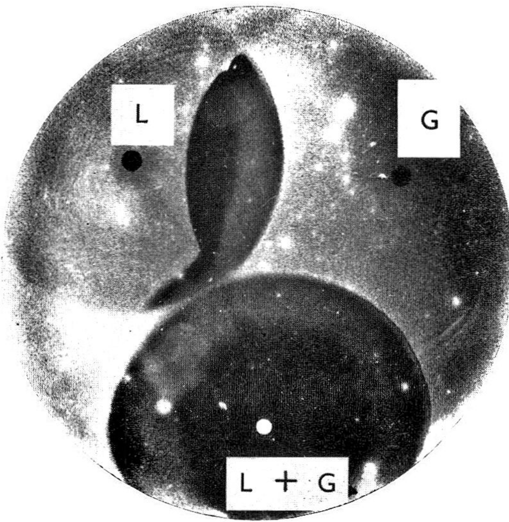

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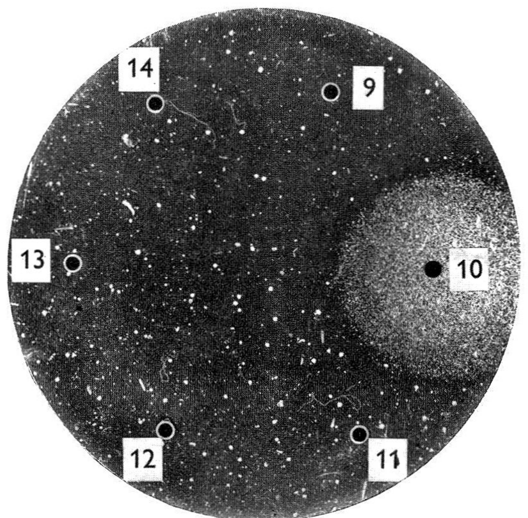

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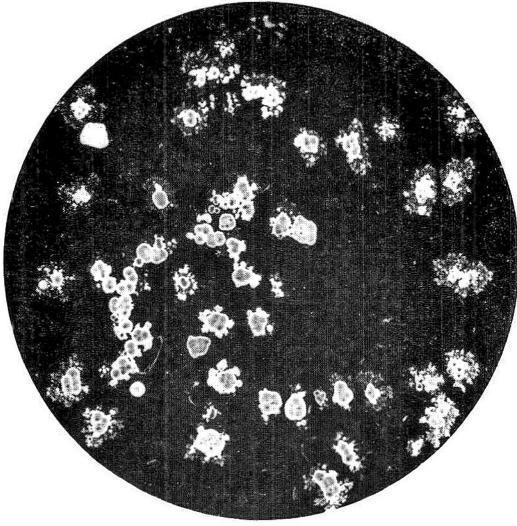

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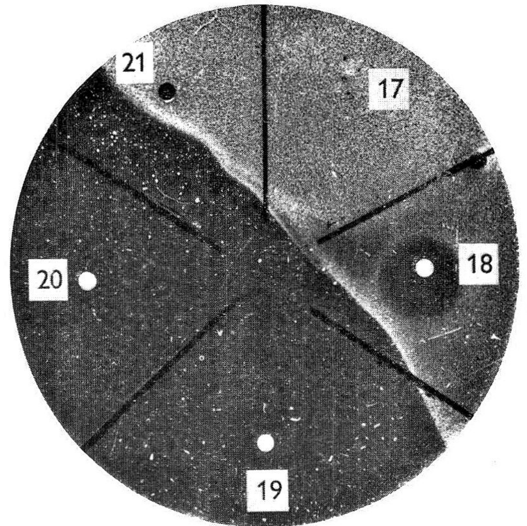

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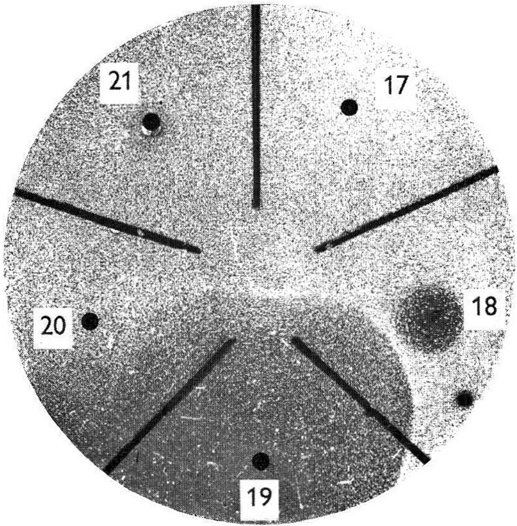

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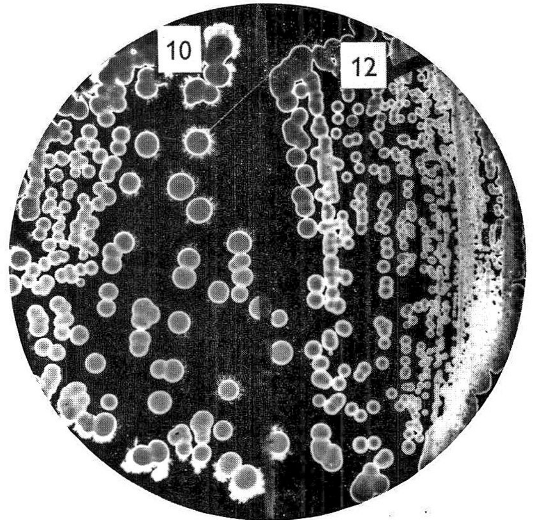

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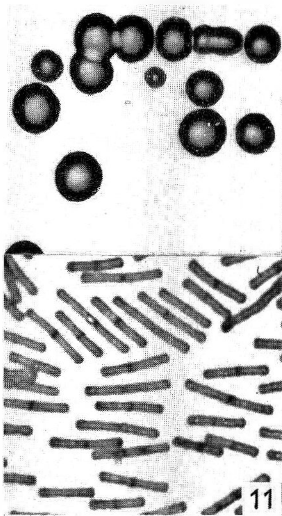

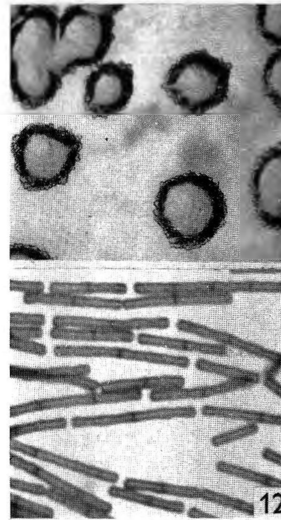

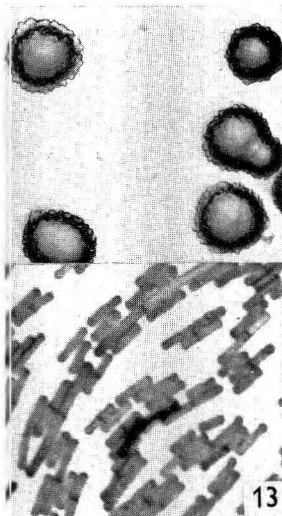

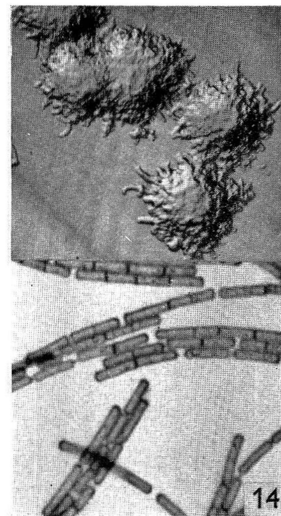

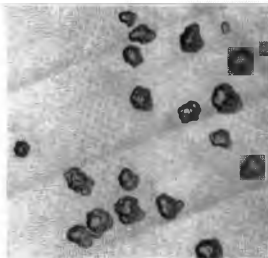

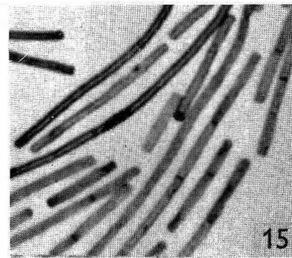

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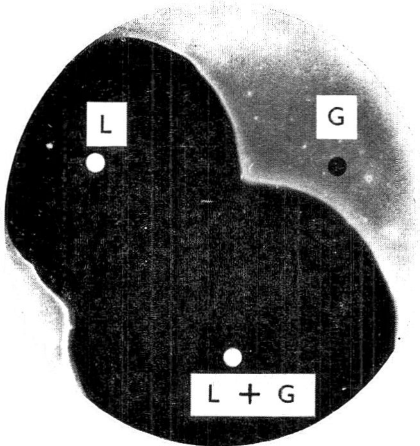

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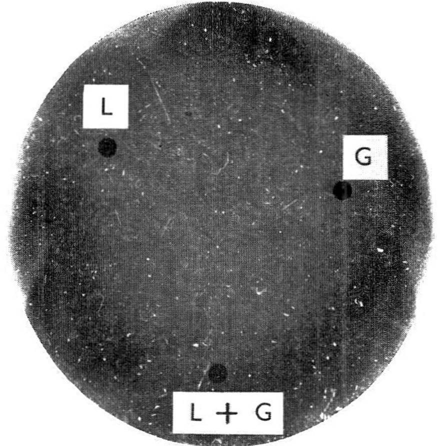

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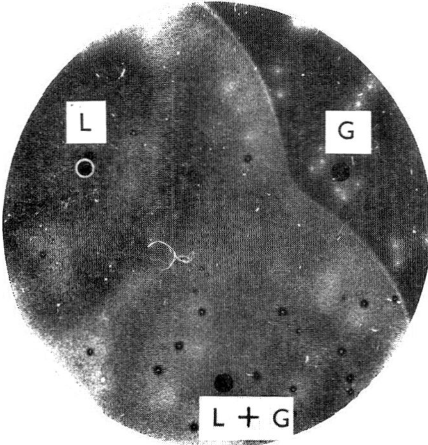

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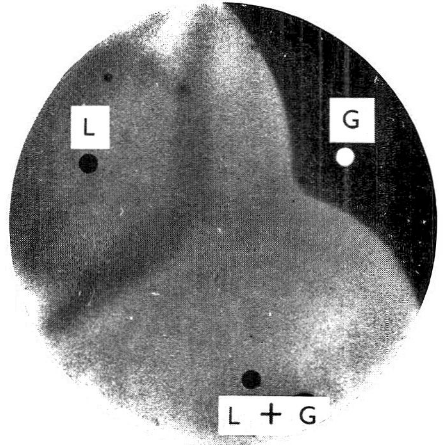

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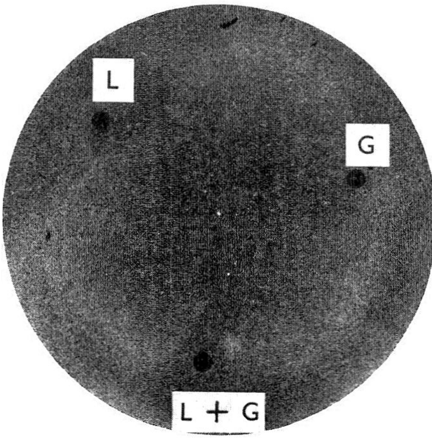

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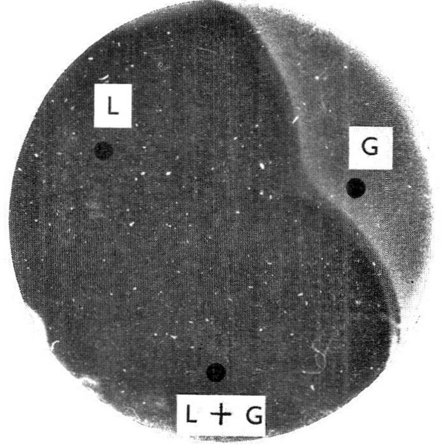

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An Alcaligenes Species with Distinctive Properties Isolated from Human Sources

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SUMMARY

The distinctive properties of an Alcaligenes-like organism isolated from human pathological material are described. The organism is readily recognizable by its characteristic colonial appearances, fruity smell and greening of blood agar. It resembles to a considerable extent *Alcaligenes faecalis*, 'Bacterium alcali-aromaticum', and *A. odorans*; the name *A. odorans* var. *viridans* is proposed for the organism. It appears to be non-pathogenic, but may be confused with *Pseudomonas aeruginosa.*

INTRODUCTION

This communication describes a member of the genus *Alcaligenes*, occasionally found in mixed bacterial cultures from human pathological material, which does not appear to be identical with any organism previously described. It has very distinctive properties which should make for instant recognition in medical bacteriology.

METHODS

An investigation of this organism was first undertaken in this laboratory in 1952 based on a study of 20 strains (17 from urine, 3 from wound infections). Similar strains were isolated subsequently, but were not fully examined until the present work was undertaken with a collection of 27 strains isolated from routine specimens in 1963 and 1964. These 27 organisms came from different patients, and were found in 22 urine specimens, 3 ear swabs and 2 wound swabs. Except where indicated, the results of tests given below are based on an investigation of 12 of these strains, usually incubated at 37°.

Burdon's (1946) method was used to stain for *sudanophilic inclusions* after cultivation on 2 % glucose peptone agar for 3 days (Hayward & Hodgkiss, 1961).

Motility was determined by direct microscopy of overnight broth cultures, and by observation of stab cultures in tubes of 0·3 % nutrient agar.

Colonial appearances were examined on nutrient agar and horse blood (5 %) agar (28°, 37°); ox blood, sheep blood and rabbit blood agar; heated blood agar, MacConkey's medium, and deoxycholate citrate agar (4 strains); 0·03 % cetrimide agar (Lowbury & Collins, 1955). Fluorescin production was tested with ultraviolet radiation on the medium B of King, Ward & Raney (1954). Growth at 42° was tested on nutrient agar slopes immersed in a water bath.

Final pH value in fluid culture was determined in Difco 'Bacto' nutrient broth incubated for 7 days, using 'Lyphan' multi-strip pH papers.

Carbohydrate reactions. Standard peptone water media (glucose, lactose, maltose, sucrose, salicin, mannitol and dulcitol) were incubated for 21 days. Acid production from glucose, mannose, maltose, arabinose, xylose and glycerol was also tested in the medium of Hayward & Hodgkiss (1961) incubated for 12 days (6 strains).

Lipase. Sierra's (1957) medium, incubated at 28° for 6 days.

Hydrolysis of casein and starch. The media of Hayward & Hodgkiss (1961).

Indole production was tested with Ehrlich's reagent in peptone water cultures incubated for 6 days.

H_2S production was tested in peptone water containing 0·01 % L-cysteine hydrochloride (lead acetate papers) incubated for 6 days.

Urease. Christensen's (1946) method; incubation for 21 days.

Oxidase. Kovacs's (1956) method.

Catalase. A loopful of culture from nutrient agar was held in a drop of '10 vol.' H_2O_2 on a slide and examined macroscopically for effervescence.

Voges-Proskauer (Barritt's method; Mackie & MacCartney, 1960) and *methyl red* reactions. Glucose phosphate broth cultures incubated for 6 days.

Nitrate reduction. Nutrient broth containing 0·1 % (w/v) KNO_3, incubated for 5 days, then tested with Griess–Ilosvay reagents, and with zinc dust for residual nitrate.

Gelatin liquefaction. Kohn's (1953) method, incubation for 14 days.

Citrate utilization. Koser's (1923) medium, incubation for 2 days.

Gluconate oxidation. Shaw & Clarke's (1955) method.

Malonate utilization and phenylalanine deaminase activity. Combined medium of Shaw & Clarke (1955), incubation for 6 days.

Growth in KCN. Moeller's (1954) method.

Decarboxylases. Moeller's (1955) method.

Two isolates were examined by electron microscopy on nitrocellulose-coated grids, shadowed with gold + palladium at an angle of 15°; magnification × 20,000.

Antibiotic sensitivities were determined by the agar gel diffusion method, with 'Mast' antibiotic discs.

RESULTS

The organism was a Gram-negative rod, average size $1{\cdot}5\,\mu \times 0{\cdot}6\,\mu$ with coccoid and elongated forms (18 hr cultures on blood agar); non-sporing, non-capsulated and not acid-fast. Scattered sudanophilic inclusions were found in 5 of 9 strains examined. It was actively motile after overnight growth in broth at 37°. Electron micrographs clearly showed a peritrichous arrangement of flagella, varying in numbers up to about 12 per organism.

After incubation on blood agar at 37° for 24 hr, two distinct types of colony were recognizable, with a range of intermediate forms. The usual and more characteristic colony was umbonate, with a central plateau raised to a button in the middle, and a thin matt spreading edge. There was a surrounding zone of bright green discoloration of the medium, visible on blood (horse, sheep, ox, rabbit) agars. Colonial size varied considerably up to 6 mm. in diameter. The elevation of the colonies varied from flat-topped to almost conical. Frequently there was no central button. In areas of confluent growth, adjacent colonies were often demarcated by straight indentations, resulting in a 'paving-stone' effect, the area of growth being outlined

by a skin-like fringe. The other type of colony was high-convex, greyish white, with a smooth glistening surface and a circular outline devoid of fringe. It appeared to correspond to the central button of the 'rough' type. Usually a particular culture showed a preponderance of one type, but both types might be present in the same culture. Several strains incubated simultaneously under identical conditions might show a preponderance of different types. Neither type bred true on subculture.

After incubation at 37° for 48 hr the characteristic colonial features were no longer evident, and blood plates showed complete lysis. After incubation at 28° for 24 hr the diameter of the colonies was similar, but the fringe was developed at

Table 1. *Antibiotic sensitivities of 17 of the strains of Alcaligenes odorans var. viridans examined*

	Reactions of the 17 strains		
Antibiotic	Sensitive	Moderately sensitive	Resistant
Penicillin	—	—	17
Methicillin	—	—	17
Ampicillin	9	2	6
Streptomycin	7	2	8
Chloramphenicol	16	1	—
Tetracycline	14	1	2
Erythromycin	—	11	6
Neomycin	4	8	5
Kanamycin	5	—	12
Polymixin B	16	—	1
Polymixin E	17	—	—
Sulphonamide	14	—	3
Novobiocin	—	1	16
Ristocetin	—	—	17
Nitrofurantoin	17	—	—
Bacitracin	—	—	17

the expense of the convex portion, so that the colonial mass was diminished. Cultures on blood agar nearly always had a pleasant strongly aromatic odour, resembling that of apples. This odour was very characteristic and could often be detected even when the organism was outnumbered in mixed cultures. Colonies on nutrient agar were smaller, and had a less distinctive, somewhat cheesy smell. Poor growth occurred on MacConkey's medium with an alkaline reaction and on deoxycholate citrate agar after incubation at 37° for 24 hr. Growth did not occur at 42° nor on solid media incubated anaerobically at 37° for 7 days. Fluorescin was not produced on the medium B of King *et al.* (1954). Discoloration was not produced on heated blood agar. Appreciable growth occurred on 0·03 % cetrimide agar, although the size of colonies was then much decreased as compared with a control strain of *Pseudomonas aeruginosa* which grew freely. Cultures in nutrient broth showed a uniform turbidity with an occasional surface pellicle, and reached pH 8·1 after 7 days. Litmus milk cultures turned blue after 4 days, and were bleached subsequently.

The following tests were always positive: catalase, oxidase, H_2S production, citrate and malonate utilization.

There was no visible growth in presence of KCN (Moeller, 1954) at 24 hr, but there was at 48 hr.

The following tests were negative: lipase, casein and starch hydrolysis, gelatinase, V.P. and M.R., indole, urease, nitrate reduction, gluconate oxidation, phenylalanine deaminase; arginine, lysine and ornithine decarboxylases. No carbohydrates were attacked in the medium of Hayward & Hodgkiss (1961).

DISCUSSION

Records of the previous collection of 20 strains examined 12 years ago showed that, although several recently introduced tests were not then performed, the organisms were almost certainly the same as are now reported upon, with the same characteristic aromatic smell, colonial appearances and discoloration of blood.

This organism belongs to the genus *Alcaligenes* as defined in *Bergey's Manual* (1957), being a Gram-negative rod, motile by peritrichous flagella, producing an alkaline reaction in litmus milk and not attacking carbohydrates. The genus has aroused controversy since Petruschky's (1896) original description of 'Bacterium alcaligenes faecalis', which was stated to possess peritrichous flagella. The existence of such strains was doubted for a time, but several subsequent workers, notably Leifson (1960), Thibault (1961), and Hugh & Ryschenkow (1961) have maintained that the genus *Alcaligenes* should include only organisms motile by peritrichous flagella; we have followed them in this. The present organism differs from the type species *A. faecalis* Castellani & Chambers (1919) in possessing a strong fruity odour and producing greening of blood agar. A search of the literature has not revealed a description of any organism which exactly corresponds to our own, but three papers have described similar or possibly related organisms.

Stutzer (1924) described an organism found in the stools of patients with cholera and dysentery which he called 'Bacterium faecalis aromaticum'. This was a small Gram-negative rod, $1\ \mu \times 0{\cdot}3$–$0{\cdot}5\ \mu$, with similar colonial appearances to those of our strains. One type of colony was matt flat-conical in elevation with radial furrows and a polyhedral outline, reaching 3–4 mm. in diameter after incubation for 24 hr. The other type had a moist glistening surface, and had an intense agreeable aromatic odour. Unlike our strains, it was non-motile and liquefied coagulated serum and gelatin; greening of blood agar was not mentioned.

Berlin (1927) isolated from human faeces an organism which he called 'Bacterium alcali-aromaticum'. This was a Gram-negative rod, motile by peritrichous flagella. Colonies showed a raised greyish white centre and an irregular plateau, which later became cone-shaped, with regular peripheral radial furrows and a transparent edge. At 16–18° the colonies had an intense fruity odour which disappeared after a week, to be replaced by a cheesy odour; the fruity odour was not apparent at 37°. On sheep blood agar, weak haemolysis was evident after 3–4 days. Growth in broth at 37° was described as granular, reaching pH 8·0 after 7–10 days. It was indole negative, H_2S negative, did not liquefy serum or gelatin or reduce nitrate, and did not attack a wide range of carbohydrates. This organism showed only minor differences from our strains but lacked the intense fruity odour at 37° and did not produce greening of blood agar after overnight incubation.

Málek, Radochová & Lysenko (1963) studied 4 strains of an organism previously

described as *Pseudomonas odorans* (Málek & Kazdová-Koží̌ková, 1946) and reallocated them to the genus *Alcaligenes*. They were Gram-negative bacilli, motile by peritrichous flagella as shown by electron microscopy. On meat peptone agar after incubation for 2 days at 28°, colonies were either flat and spreading with irregular edges, or convex, and round with entire edges. No haemolysis occurred on blood agar. Young cultures had an odour of jasmine or strawberries, replaced by that of ammonia in older cultures. Unlike our strains, their organisms were non-haemolytic on blood agar, and differed in their antibiotic sensitivities, being resistant to tetracycline and polymixin, and only slightly sensitive to streptomycin and chloramphenicol.

Our strains resemble *A. odorans* too closely to be regarded as a separate species, and it is proposed that they be distinguished as *A. odorans* var. *viridans*. The type strain has been deposited in the National Collection of Type Cultures as NCTC 10388.

The resemblances to *Pseudomonas aeruginosa* are only superficial; but confusion may arise in clinical bacteriology because of the colonial appearances, obligate aerobic growth, aromatic odour, green discoloration of medium, positive oxidase reaction, appreciable growth on 0·03% cetrimide agar, and marked sensitivity to polymixins. Our strains have been isolated only in mixed cultures, with other Gram-negative bacilli. They have not been shown to have a pathogenic role. The widely differing antibiotic sensitivity patterns of the strains are strong evidence that the organism was not recurring as a laboratory contaminant.

Our thanks are due to Professor W. A. Gillespie, under whose direction this work was done, for his invaluable advice and encouragement. We are greatly indebted to the late Dr K. J. Steel for considerable help in revising the manuscript, and to C. A. Bassett of the Department of Physics, University of Bristol, for making electron micrographs of the organism.

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A Comparative Study on the Biochemical Bases of the Maximum Temperatures for Growth of Three Psychrophilic Micro-Organisms

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SUMMARY

Three psychrophilic micro-organisms (strains of Arthrobacter, Candida and *Corynebacterium erythrogenes*) were capable of growth for a period when exponential-phase cultures in chemically defined media were transferred from temperatures at or near the optima for growth (20, 10 and 15°, respectively), to 37, 25 and 30°, respectively. The latter temperatures were 3–5° above the maxima for the growth of the organisms in freshly inoculated cultures. Growth at the higher temperatures was greatest with the Candida and least with the Arthrobacter. Cultures of the Arthrobacter and Candida grew when transferred back to the optimum temperatures for growth, after a lag which increased with the length of time that the cultures had spent at the higher temperatures. *C. erythrogenes* cultures grew almost immediately after they were transferred back to the optimum growth temperature. Growth of the organisms at the higher temperatures was not affected by supplementing cultures with bacteriological peptone and/or yeast extract. There was a rapid decline in the viability and in the rates of respiration of endogenous reserves and of exogenous glucose and pyruvate when Arthrobacter and Candida cultures were transferred to the higher temperatures. But with *C. erythrogenes* the respiratory activities were not so markedly affected by the change in incubation temperature, while the viability of this bacterium increased slightly after the transfer of cultures to the higher temperature. The activities of many of the tricarboxylic acid cycle enzymes in Arthrobacter and Candida were diminished after the transfer of organisms from the optimum temperature to one above the maximum for growth; but the activities of these enzymes in *C. erythrogenes* were less affected by the change in incubation temperature. There was no marked intracellular accumulation or excretion of ultraviolet-absorbing compounds by the organisms after the transfer of cultures to the higher temperatures. The results are discussed in relation to the biochemical factors which may determine the maximum temperatures for growth of these organisms.

INTRODUCTION

Psychrophilic micro-organisms differ from mesophils in having lower minimum temperatures for growth. But the maximum temperatures for growth of psychrophils can vary from around 18°, as with a strain of *Serratia marcescens* (Kates & Hagen, 1964) to between 40 and 50° which is in the range of maximum temperatures for growth of many mesophils. Very little is known about the biochemical bases of the maximum temperatures for growth of micro-organisms, although several factors are thought to be involved including the denaturation of enzymes (Chick,

1910; Edwards & Rettger, 1937), denaturation and possible degradation of DNA (Marmur & Doty, 1959) and RNA (Califano, 1952; Strange & Shon, 1964) and changes in the properties of membrane lipids (Luzzati & Husson, 1962; Byrne & Chapman, 1964; Hagen, Kushner & Gibbons, 1964). Enzyme denaturation is usually assumed to be a major factor, and excellent agreement between the maximum temperatures for growth of several bacteria and the minimum temperatures at which certain of their respiratory enzymes were inactivated was reported by Edwards & Rettger (1937). By using a technique in which exponentially growing organisms were transferred from the optimum temperature for growth to a temperature some 3–5° above the maximum for growth, Hagen & Rose (1962) showed that the low maximum temperature for growth of a psychrophilic Cryptococcus (about 28°) was determined at least in part by the heat-sensitive nature of one or more of the tricarboxylic acid (TCA) cycle enzymes. Upadhyay & Stokes (1963) reported the presence of a heat-sensitive formate hydrogenlyase in a psychrophilic bacterium and Burton & Morita (1963) showed that the malate dehydrogenase in a psychrophilic marine bacterium was also abnormally sensitive to heat denaturation, although in neither of these reports was there evidence that the heat sensitivity of the enzyme determined the maximum temperature for growth of the given organism. The present paper records the results of a comparative study on the biochemical bases of the maximum temperatures for growth in three psychrophilic microorganisms which have maximum temperatures within the range 22–33°.

METHODS

Organisms. The origin and maintenance of the strains of Arthrobacter (No. R22/3A), Candida (No. A3E-2) and *Corynebacterium erythrogenes* (NCMB 5) examined here were described by Rose & Evison (1965).

Experimental cultures. The strains of Arthrobacter and *Corynebacterium erythrogenes* were grown in the defined medium (pH 6·7) described by Rose & Evison (1965) and the strain of Candida in the glucose + salts + vitamins medium (pH 4·5) of Rose & Nickerson (1956) supplemented with D-biotin (2·0 μg./l.). Portions (100 ml.) of bacterial or yeast medium in 350 ml. conical flasks were prepared as described by Rose & Evison (1965). In certain experiments, cultures (6 ml.) were grown in Samco tubes covered with anodized aluminium caps (Oxo Ltd., Queen Street Place, London, E.C. 4; Northam & Norris, 1951). Solutions of substances were occasionally added to these 6 ml. cultures as described later. These solutions were adjusted to pH 4·5 when added to yeast cultures, or to pH 6·7 when added to bacterial cultures, and were sterilized separately by autoclaving momentarily at 115°. Portions of sterile medium were inoculated as described by Rose & Evison (1965). Cultures were incubated statically at the temperatures stated. Growth was measured turbidimetrically in Samco tubes with the Hilger 'Spekker' absorptiometer (Model H760) with neutral green-grey H 508 filters and a water blank. Turbidity readings were related to dry weight by using a calibration curve for each organism.

Viable counts of organisms in cultures were made by spreading samples (0·1 ml.) from successive ten-fold dilutions in water on well-dried plates of malt wort-agar (10 %, w/v, spray-dried malt extract, 'Muntona', Munton and Fison, Ltd., Stow-

market, Suffolk, +2 %, w/v, agar, +0·5 %, w/v, NaCl) for the yeast, or on plates of nutrient agar for the bacteria. Triplicate plates were used with each dilution. Plate cultures of the Candida were incubated at 10° for 144 hr, those of the Arthrobacter at 20° for 48 hr and of *C. erythrogenes* at 20° for 72 hr. The colonies on plates which had received suitably diluted portions were counted with an electric colony counter (Sintacell, Ltd., London, E.C. 4). The contents of viable organisms in cultures are expressed as the number/mg. dry wt. organism.

Respirometry. The respiratory activities of organisms were determined as described by Umbreit, Burris & Stauffer (1964) with the constant volume Warburg respirometer (B. Braun, Melsungen, West Germany; Model S 85) fitted with cooling coils through which was circulated cold water from a low temperature water bath (model LB 405; Grant Instruments Ltd., Barrington, Cambridge) when required. Organisms were harvested from cultures at the times indicated in a refrigerated centrifuge at 0°. The yeast was washed three times with phosphate buffer (M/15 KH_2PO_4; pH 4·5) and the bacteria with 0·85 % (w/v) NaCl. The washed organisms were suspended in a volume of phosphate buffer (pH 4·5 for the yeast; pH 7·0, Gomori, 1955, for bacteria) to a concentration suitable for use in the Warburg respirometer. The centre well of each Warburg flask contained 0·2 ml. 10 % (w/v) KOH and a small filter-paper wick. A portion of suspension containing a suitable quantity of organisms was added to the flask and the total volume adjusted to 2·5 ml. with buffer. The side arm contained 0·3 ml. of a solution (2·5 %, w/v; pH 4·5 or 7·0) of oxidizable substrate, or 0·3 ml. water when measuring the respiration of endogenous reserves. After the Warburg flasks had been attached to the manometers, they were equilibrated in the water bath for 10–30 min., depending upon the temperature of the bath. After the manometer taps had been closed and the substrate tipped from the side arm, the uptake of oxygen by the organisms was followed over a period of 1 hr. The respiratory activities are quoted as the Q_{O_2} values (μl. oxygen consumed/mg. dry wt. organism/hr) for the respiration of endogenous reserves, and of exogenous substrate after subtracting the value for the respiration of endogenous reserves.

Preparation of cell extracts. Extracts of organisms for use in the measurement of enzyme activities were prepared by ballistic disintegration. Organisms were harvested from cultures by centrifugation at 0° in a refrigerated centrifuge. Bacteria were washed twice with 0·85 % (w/v) NaCl and the yeast with phosphate buffer (pH 4·5). The equivalent of 50–200 mg. dry wt. organisms was washed once with ice-cold water, suspended in 5·0 ml. ice-cold water and shaken with 3 g. Ballotini beads (Grade 12) in a Mickle tissue disintegrator (Mickle, 1948) as described by Ahmad & Rose (1962). Cell-free extracts were obtained by centrifuging the suspension of disrupted organisms at 1300*g* for 20 min. at 0°. Extracts were either used immediately or stored at −20° until required. The protein contents of the extracts were determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine plasma albumin (L. Light and Co., Ltd., Colnbrook, Bucks.) as a standard. Acid-soluble ultraviolet (u.v.)-absorbing compounds were extracted from portions of washed organisms (containing equiv. 5 mg. dry wt. organism) by using 5 % (w/v) trichloroacetic acid as described by Ahmad, Rose & Garg (1961).

Enzyme assays. The enzyme nomenclature used is that recommended in the Report

of the Commission on Enzymes of the International Union of Biochemistry, 1961, although only the suggested trivial names are used for the dehydrogenases studied since the experimental results do not permit precise identification of the enzymes concerned. The activities of all of the enzymes studied were calculated from initial reaction velocities determined over a period during which plots of the amount of substrate changed against time were linear. All activities are expressed as specific activities (μmole-substrate consumed/mg. extract protein/hr).

The activities of aconitate hydratase, isocitrate dehydrogenase, fumarate hydratase and L-malate dehydrogenase were measured spectrophotometrically by using the S.P. 500 quartz spectrophotometer fitted with a constant temperature cuvette housing (Unicam Ltd., Cambridge) through which was circulated water at an appropriate temperature. Reactions were carried out in 1 cm. quartz cuvettes and all reaction constituents except cell extracts, which were kept at 0°, were equilibrated in the cuvettes at the test temperature before starting the reaction. The temperature of the reaction mixture in the cuvettes was measured by using either a mercury thermometer or a Rustrak miniature temperature recorder coupled to a hypodermic thermistor which fitted down the inside of the cuvette (Grant Instruments Ltd., Toft, Cambridge).

Aconitate hydratase (citrate (isocitrate) hydrolyase; EC 4.2.1.3) was assayed by a method based on that of Racker (1950) which depends on measuring the increase in extinction at 240 mμ attendant upon the conversion of citrate to *cis*-aconitate. Each cuvette was charged with sodium citrate (87 μmoles; pH 7·4) and sodium phosphate buffer (145 μmoles; pH 7·4) in 2·9 ml. water. The reaction was started by adding 0·1 ml. of a suitably diluted portion of cell extract containing approximately 100 μg. protein; 0·1 ml. water was added to the control cuvette. The increase in extinction at 240 mμ, caused by the formation of *cis*-aconitate, was followed at 30 sec. intervals for a period of 5 min. Specific activities were calculated using the value for the extinction in 3·0 ml. water of 1 μmole *cis*-aconitate at 240 mμ quoted by Williams & Rainbow, (1964).

Isocitrate dehydrogenase (NADP-linked) was assayed by following at 340 mμ the increase in extinction on reduction of NADP (Ochoa, 1948; Kornberg & Pricer, 1951). Each cuvette contained sodium DL-isocitrate (0·5 μmole; pH 7·0), potassium phosphate buffer (100 μmoles; pH 7·0), NADP (0·5 μmole), $MgCl_2$ (10 μmoles) in 2·9 ml. water. The reaction was started by adding 0·1 ml. of diluted cell extract containing about 100 μg. protein, and the increase in extinction at 340 mμ was followed at 30 sec. intervals over a period of 3 min., with a blank reaction mixture lacking cell extract. The extinction of a second blank reaction mixture containing all of the constituents except DL-isocitrate was measured at the beginning and at the end of the period of observation. Specific activities were calculated from the change in extinction using the molar extinction coefficient for $NADPH_2$ quoted by Horecker & Kornberg (1948).

Fumarate hydratase (L-malate hydro-lyase; EC 4.2.1.2) activity was assayed using a method based on that of Racker (1950) which depends on measuring the decrease in the extinction at 300 mμ attendant on the conversion of fumarate to L-malate. Each cuvette contained sodium fumarate (49 μmoles; pH 7·3) and sodium phosphate buffer (95 μmoles; pH 7·3) in 2·9 ml. water. The reaction was started by adding 0·1 ml. of cell extract containing about 400 μg. protein; 0·1 ml. water was

added to the control reaction mixture. The decrease in extinction at 300 mμ, caused by the decrease in the concentration of fumarate, was followed at 30 sec. intervals over a period of 5 min. Specific activities were calculated using a value for the extinction of 1 μmole fumarate in 3·0 ml. at 300 mμ of 0·015.

Two methods were used for assaying L-malate dehydrogenase (NAD-linked). The activity of this enzyme in cell extracts of the Candida was assayed by a modification of the method of Beaufay, Bendall, Baudhuin & de Duve (1959) with L-malate as substrate and following at 340 mμ the increase in the extinction of the reaction mixture on the reduction of NAD. Each cuvette contained tris buffer (58 μmoles; pH 8·5), potassium-L-malate (600 μmoles; pH 8·5), NAD (0·7 μmole; pH 8·5), NaCN (30 μmoles; pH 8·5) and ethylenediaminetetra-acetic acid (EDTA) (3 μmoles; pH 8·5) in 2·9 ml. water. The constituents were incubated at room temperature (18–22°) for 2 hr to allow the NAD and NaCN to equilibrate. The reaction was started by adding 0·1 ml. of diluted cell extract containing about 100 μg. protein and the increase in extinction at 340 mμ was followed at 30 sec. intervals for a period of 4 min. Two blank reaction mixtures were used with each experiment, one lacking NAD and cell extract and the other lacking L-malate. Specific activities of the Candida extracts were calculated by using the molar extinction coefficient for $NADH_2$ reported by Horecker & Kornberg (1948). Only very slight malate dehydrogenase activity was detected in extracts of the Arthrobacter or of *Corynebacterium erythrogenes* by this assay method. Malate dehydrogenase activity could, however, be assayed in extracts of these bacteria using oxaloacetate as substrate and following the decrease in extinction caused by the oxidation of $NADH_2$ (Ochoa, 1955*a*). Each cuvette contained sodium phosphate buffer (60 μmoles; pH 7·4), potassium oxaloacetate (0·76 μmole; pH 7·4) and $NADH_2$ (0·15 μmole; pH 7·4) in 2·9 ml. water. The reaction was started by adding 0·1 ml. of a suitably diluted portion of cell extract containing about 100 μg. protein and the decrease in extinction at 340 mμ was followed at 30 sec. intervals over a period of 3 min. Specific activities were calculated by using the molar extinction coefficient for $NADH_2$ quoted by Horecker & Kornberg (1948). Extracts of the Candida did not show malate dehydrogenase activity when assayed using this method even when the pH value of the reaction mixture was raised to 8·4 or lowered to 5·8.

The activities of pyruvate, 2-oxoglutarate and succinate dehydrogenases in cell extracts were assayed manometrically by the conventional constant volume respirometer technique (Umbreit *et al.* 1964). Pyruvate dehydrogenase activity was assayed by measuring carbon dioxide evolution with lithium pyruvate as substrate and potassium ferricyanide as electron acceptor (Jaganathan & Schweet, 1952). Each Warburg flask was charged with lithium pyruvate (100 μmoles), sodium bicarbonate (50 μmoles), $MgCl_2$ (20 μmoles) and thiamine pyrophosphate (200 μg.; adjusted to pH 6·0 just before use). These constituents were added as a solution which had been flushed with carbon dioxide gas for 2–3 min. A portion of cell extract containing 3–8 mg. protein and water to a volume of 3 ml. were also added to the flask, the side arm of which contained potassium ferricyanide (100 μmoles). The flasks were attached to the manometers, and flushed with carbon dioxide gas for 10 min., with a glass manifold to ensure even gassing. The manometer units were then quickly transferred to the water bath and equilibrated for 10 min. After the potassium ferricyanide had been tipped in from the side arm, the evolution of

carbon dioxide was followed during a period of 10 min. Control flasks contained all of the constituents of the reaction mixture except cell extract. Specific activities were calculated from the amount of carbon dioxide evolved.

2-Oxoglutarate dehydrogenase activity in cell extracts was assayed by measuring carbon dioxide evolution in a system which contained 2-oxoglutarate as substrate and potassium ferricyanide as electron acceptor (Sanadi, Littlefield & Bock, 1952). The main compartment of each Warburg flask was charged with sodium 2-oxoglutarate (50 μmoles), sodium bicarbonate (400 μmoles), thiamine pyrophosphate (200 μg.; adjusted to pH 6·9 just before use) and $MgCl_2$ (20 μmoles). These constituents were added as a solution that had been flushed for 2–3 min. with carbon dioxide gas. The solution in the Warburg flasks was supplemented with bovine plasma albumin (L. Light and Co. Ltd., Colnbrook, Bucks; 30 mg.), a portion of cell extract containing 3–8 mg. protein and water to a volume of 3 ml. The side arm of the flask contained potassium ferricyanide (50 μmoles). The flasks were attached to the manometers, flushed with carbon dioxide gas for 10 min., quickly transferred to the Warburg bath and equilibrated for 10 min. After the potassium ferricyanide had been added from the side arm, the evolution of carbon dioxide was recorded during 10 min. Control flasks contained all of the constituents of the reaction mixture except cell extract. Specific activities were calculated from the amount of carbon dioxide evolved.

Succinate dehydrogenase activity in cell extracts was assayed by measuring the amount of oxygen uptake with succinate as a substrate in the presence of phenazine methosulphate as electron carrier (Bernath & Singer, 1962). Each Warburg flask was charged with sodium phosphate buffer (150 μmoles; pH 7·6), KCN (30 μmoles; pH 7·6), cell extract containing 3–4 mg. protein and water to 3·0 ml. Sodium succinate (60 μmoles; pH 7·6) and phenazine methosulphate (0·2, 0·1, 0·07, 0·05 or 0·04 ml. of a 1 %, w/v, solution) were added to the side arm. KCN was added last, the flasks immediately attached to the manometers and the stopcocks closed. The excess pressure was released momentarily after the units had been placed in the Warburg bath. After equilibrating for 10 min. the contents of the side arms were tipped into the flasks and the uptake of oxygen was recorded during 20 min. Control flasks contained all of the constituents of the reaction mixture except cell extract. The reciprocal of the Q_{O_2} value (calculated over a period of 2–12 min.) was then plotted against the reciprocal of the concentration of phenazine methosulphate and the oxygen uptake extrapolated to infinite phenazine methosulphate concentration. This value for the oxygen uptake was used to calculate the specific activities of succinate dehydrogenase in the extracts (Bernath & Singer, 1962).

The method of Ramakrishnan & Martin (1954) was used for assaying the citrate synthase (citrate oxaloacetate-lyase (Co A-acetylating); EC 4.1.3.7) activities of cell extracts. This involved using acetyl phosphate, coenzyme A and transacetylase to generate acetyl coenzyme A which was then allowed to react with oxaloacetate to give citrate in a reaction catalysed by citrate synthase. In this system, the amount of acetyl phosphate used is proportional to the amount of citrate synthase present (Ochoa, 1955*b*). The reaction was carried out in Warburg flasks which were placed in a tray of ice and charged with potassium phosphate buffer (25 μmoles; pH 7·4), potassium oxaloacetate (20 μmoles; pH 7·4), acetyl phosphate (10μmoles) coenzyme A (0·05 μmole), L-cysteine (10 μmoles; pH 7·4), $MgCl_2$ (4 μmoles), trans-

acetylase preparation (0·04 ml. containing approximately 0·8 mg. protein; Ramakrishnan & Martin, 1954), a portion of cell extract containing 0·5–4·0 mg. protein and water to 1 ml. The transacetylase preparation was obtained from *Escherichia coli* strain NRC 482 as described by Ramakrishnan & Martin (1954), except that the extract was not fractionated with ammonium sulphate. Control flasks lacking cell extract were set up. The Warburg flasks were placed on the manometers and immediately incubated at the test temperature for 20 min. The flasks were then removed from the manometers and placed in the tray of ice for 5 min., and the acetyl phosphate remaining in the reaction mixture was determined by the hydroxamate method of Lipmann & Tuttle (1945). Specific activities were calculated from the amount of acetyl phosphate used.

RESULTS

Effect of change of incubation temperature on growth

Exponential-phase cultures of a psychrophilic strain of Cryptococcus were shown by Hagen & Rose (1961) to grow rapidly for a period, at a temperature about 3° above the maximum for growth in freshly inoculated culture, when they were previously incubated at or near the optimum temperature for growth (16°).

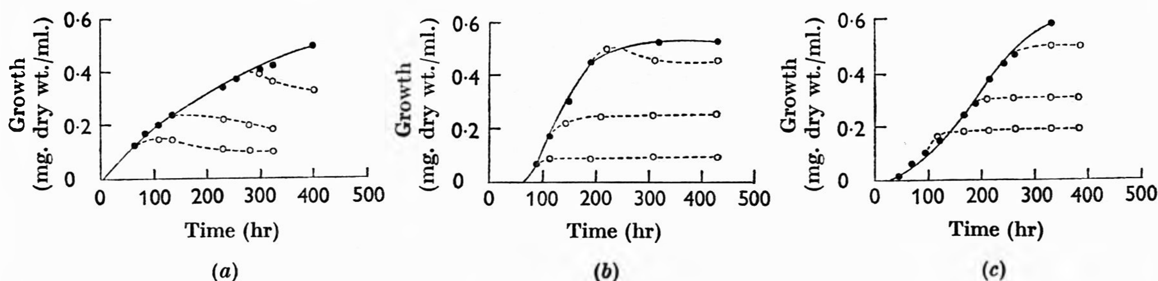

Fig. 1. Growth of cultures (6 ml.) of Arthrobacter (*a*), Candida (*b*), *Corynebacterium erythrogenes* (*c*) after transfer from a temperature at or near the optimum for growth (●——●) to one 3–5° above the maximum for growth (○ - - ○). Cultures of Arthrobaeter were transferred to 37° after 62, 135 and 255 hr incubation at 20°; cultures of Candida to 25° after 90, 114 and 191 hr incubation at 10°; *C. erythrogenes* cultures to 30° after 96, 192 and 264 hr at 15°.

The effects of transferring cultures of each of the organisms used in the present work from a temperature at or near the optimum for growth to one 3–5° above the maximum for growth in freshly inoculated cultures are shown in Fig. 1. All three organisms grew to some extent after transfer to the higher temperatures. The amount of growth at the higher temperatures was greatest with the Candida and least with the Arthrobacter, but none of the organisms grew to the same extent as did the psychrophilic Cryptococcus (Hagen & Rose, 1961) after transfer to the higher temperatures. The turbidity of some Arthrobacter cultures decreased after prolonged incubation at 37°. Hagen & Rose (1961) also reported that cultures of the Cryptococcus which had stopped growing after they had been transferred to the higher temperature began to grow when they were transferred back to the optimum temperature for growth. There was usually a lag period before growth occurred at the optimum temperature, and this lag was proportional to the

duration of the incubation at the higher temperature. The results of subjecting each of the organisms used in the present work to a similar regimen of changes in incubation temperature are shown in Fig. 2. Cultures of each of the organisms grew after being transferred back to the optimum temperature for growth. The lag period for growth of the Arthrobacter and Candida increased with the length of time that the cultures had spent at the higher temperatures. But *Corynebacterium erythrogenes* cultures grew almost immediately after they were transferred back to the optimum temperature for growth.

It was possible that the inability, or limited ability, of the organisms to grow following the transfer of cultures to the higher temperatures was due to an additional nutritional demand that could not be met by the media (Brown, 1957). Several reports have appeared showing that, at temperatures above the maxima for growth in minimal medium, micro-organisms may become auxotrophic for growth factors that are not required at the optimum temperatures for growth (see review

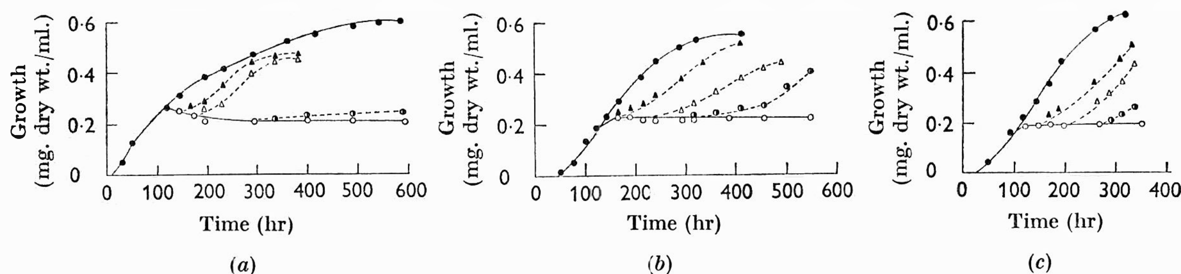

Fig. 2. Growth of cultures (6 ml.) of Arthrobacter (*a*), Candida (*b*), *Corynebacterium erythrogenes* (*c*) after transfer from a temperature at or near the optimum for growth (●——●) to one 3–5° above the maximum for growth (○——○) followed by a return to the optimum temperature (- - - -). Cultures of Arthrobacter were transferred to 37° after 120 hr at 20° and were returned to 20° after 24 (▲), 48 (△) and 175 (◑) hr at 37°; cultures of Candida were transferred to 25° after 120 hr at 10°, and were returned to 10° after 12 (▲), 36 (△) and 60 (◑) hr at 25°; *C. erythrogenes* cultures were transferred from 15° to 30° after 92 hr and were returned to 15° after 52 (▲), 100 (△) and 172 (◑) hr at 30°.

by Langridge, 1963). Little attention has been given to the reasons for this increase in nutritional requirements with temperature, although it is assumed that they are caused in part at least by the thermal denaturation of one or more enzymes concerned in the synthesis of some cell constituent. To test for an increase in nutritional requirements at the higher temperatures. cultures of each of the three organisms were incubated at or near the optimum temperature for growth and, when the cultures had reached the mid-exponential phase, they were transferred to a higher temperature. At the time of transfer, duplicate cultures of each organism were supplemented with 0·5 ml. of a solution of bacteriological peptone ('Oxoid', Oxo Ltd., London, E.C. 4) to concentrations 1·0, 0·1 or 0·01 % (w/v), or of yeast extract ('Yeastrel', Brewers' Food Supply Co. Ltd., Edinburgh) to final concentrations 0·1 or 0·01 % (w/v). Other cultures were supplemented with peptone (to 1 %, w/v) + yeast extract (to 0·1 %, w/v). Control cultures were supplemented with water. However, none of these supplements had any detectable effect on the growth of the organisms at the higher temperatures. Although these results do not exclude the

possibility that transfer to the higher temperatures caused additional nutritional demands by the organisms, they show that such demands, if created, were not satisfied by the constituents of the bacteriological peptone and yeast extract. Neither of the bacteria grew in nutrient broth at the higher temperatures.

Effect of change in incubation temperature on viability and respiratory activity

From the report by Hagen & Rose (1962) it seemed likely that further information on the biochemical bases of the maximum temperatures for growth of the organisms might be obtained by examining the behaviour of organisms transferred to temperatures above the maxima for growth. The data in Fig. 3 show the effect

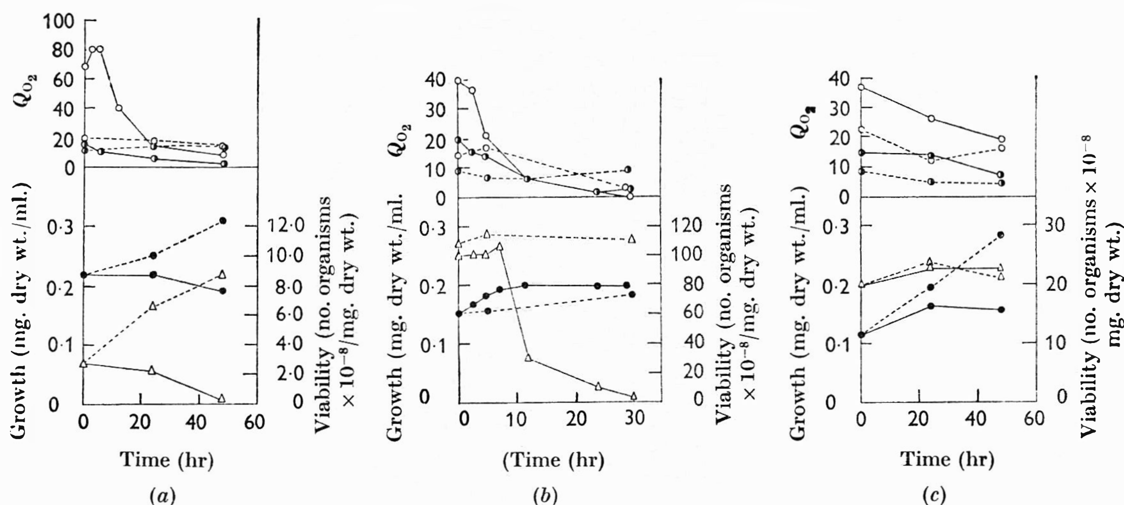

Fig. 3. Effect of change in incubation temperature on growth (●), viability (△) and the rate of respiration of endogenous reserves (◑) and of exogenous glucose (○) by Arthrobacter (*a*), Candida (*b*), *Corynebacterium erythrogenes* (*c*). Cultures (100 ml.) of Arthrobacter were transferred from 20° to 37° after 72 hr incubation; cultures of Candida from 10° to 25° after 120 hr; and *C. erythrogenes* cultures from 15° to 30° after 120 hr. ——— indicates the activities of organisms at the higher temperatures, and — — — the activities of organisms at the lower temperatures. Respiratory activities were measured at the temperatures at which the organisms had been incubated. Plate cultures for the estimation of viability were incubated at the lower temperature for each organism.

of such a change in incubation temperature on the viability and respiratory activity of each organism. The imposition of these thermal stresses caused a rapid decline in the rates of respiration of exogenous glucose and endogenous reserves by the Arthrobacter and the Candida and this was accompanied after a brief lag period by a marked decrease in the content of viable organisms in the cultures. *Corynebacterium erythrogenes*, on the other hand, was much less sensitive to the thermal stress. The rates of respiration of this bacterium were not so markedly affected after the transfer of cultures to 30°, while the content of viable organisms in these cultures actually increased slightly.

An attempt to locate the heat-sensitive lesions in the respiratory metabolism of the organisms was made by examining the ability of each organism to respire intermediates of the TCA cycle before and after transfer to the higher temperature.

Organisms were tested for the ability to respire pyruvate, citrate, isocitrate, 2-oxo-glutarate, succinate, fumarate, malate and oxaloacetate. Only a few of these substrates were respired to any appreciable extent by organisms grown at the optimum temperatures for growth; presumably those compounds not respired were unable to penetrate the organisms. Tucker (1960) examined the ability of a strain of *Corynebacterium erythrogenes* to respire TCA cycle intermediates after growth at

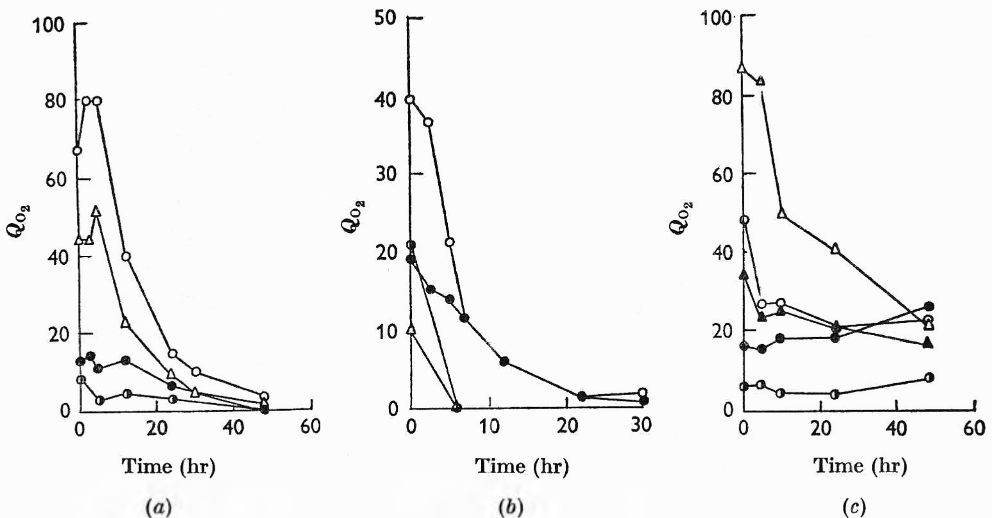

Fig. 4. Effect of change in incubation temperature on the ability of Arthrobacter (*a*), Candida (*b*), *Corynebacterium erythrogenes* (*c*) to respire endogenous reserves (●), glucose (○), pyruvate (△), succinate (◑) and oxaloacetate (▲). Cultures (100 ml.) of Arthrobacter were transferred from 20° to 37° after 120 hr; cultures of Candida from 10° to 25° after 120 hr; and *C. erythrogenes* cultures from 15° to 30° after 120 hr. All respiratory activities were measured at the higher temperatures. Data are given only for those substrates that the organisms were able to oxidize before transfer to the higher temperatures.

25° in a medium containing glucose as carbon source, and obtained results similar to those reported here. The data in Fig. 4 show that the effects of the thermal stresses on the ability of the organisms to respire pyruvate were similar to the effects on glucose respiration. This suggested that one or more of the heat-sensitive lesions in each of the organisms was among the reactions of the TCA cycle, although it did not exclude the possibility that the ability of the organisms to transport the substrates had been affected by the change in incubation temperature. The abilities of Arthrobacter and Candida to respire succinate were similarly affected, which suggested that in these organisms there were heat-sensitive lesions among those reactions of the TCA cycle concerned with the oxidation of succinate. The effects of the increase in incubation temperature on the ability of *Corynebacterium erythrogenes* to respire pyruvate and oxaloacetate were also similar to the effect on glucose respiration. But the ability of this Corynebacterium to respire succinate was not adversely affected by the change in incubation temperature, which suggested that the enzymes concerned in the oxidation of succinate by it are not particularly heat-labile.

Activities of tricarboxylic acid cycle enzymes in organisms after changes in incubation temperature

The data in Table 1 show the effect of a change in incubation temperature on the activities of eight TCA cycle enzymes in each of the three organisms. The enzyme activities in extracts of the organisms that had been transferred to the higher temperatures were assayed at those temperatures and also at the temperatures at which the organisms had been grown before transfer. Control cultures were retained at the lower temperatures, and enzyme activities in extracts of organisms from those cultures were assayed only at those temperatures. Frequently there was some variation between experiments in the activities of enzymes in extracts of organisms. Nevertheless, it can be seen (Table 1) that the transfer of organisms from the optimum temperatures for growth to higher temperatures caused a marked decrease in the activities of many TCA cycle enzymes, particularly in Arthrobacter and Candida. With the exception of succinate dehydrogenase, the activities of each of the TCA cycle enzymes in Arthrobacter were diminished after cultures of the bacterium had been transferred to 37°. The diminution in activity was greatest with isocitrate dehydrogenase which was completely inactivated in bacteria that had been incubated at 37° for 48 hr. The activities of several of the enzymes in extracts of Arthrobacter that had been transferred to 37° were lower, when assayed at 20°, than the activities in organisms before they were transferred to 37°, which suggested that the loss of activity of these enzymes caused by the thermal stress was not completely reversible. However, the decrease in activity of malate dehydrogenase and to some extent of aconitate hydratase was reversed when the extracts were assayed at 20°. In Candida, the pyruvate dehydrogenase was most sensitive to the thermal stress although other enzymes (isocitrate dehydrogenase, fumarate hydratase) in this organism were not affected. The loss in activity of certain of the Candida enzymes was not completely reversible. The inactivation of malate dehydrogenase and aconitate hydratase was, however, reversed when the extracts were assayed at 10°. With *Corynebacterium erythrogenes*, the thermal stress had little if any effect on the activities of several of the enzymes, including pyruvate, 2-oxoglutarate and succinate dehydrogenases, and fumarate hydratase. Nevertheless, the activity of the isocitrate dehydrogenase in this bacterium was markedly decreased by the thermal stress.

Release of ultraviolet-absorbing compounds from organisms after changes in incubation temperature

That the lethal effect of high temperatures on micro-organisms may be caused partly by the breakdown of nucleic acids was first suggested by Califano (1952), Strange & Shon (1964) reported that heat-accelerated death of *Aerobacter aerogenes* in non-growing suspensions at 47° was accompanied by the degradation of endogenous RNA which led to an increase in the ultraviolet (u.v.)-absorption of cold acid extracts of the bacterium and of the suspending fluid. It seemed of interest, therefore, to examine the u.v.-absorption of cold acid extracts and culture filtrates of the organisms used in the present work, after the transfer of cultures to high temperatures, to assess the extent to which RNA degradation occurred. The results (Table 2) showed that the transfer of Candida cultures from 10° to 25° and of *Coryne-*

Table 1. *Effect of change in incubation temperature on the activities of tricarboxylic acid cycle enzymes in three micro-organisms*

Arthrobacter cultures were transferred to 37° after 120 hr at 20°; Candida cultures to 25° after 144 hr at 10°; *Corynebacterium erythrogenes* cultures to 30° after 96 hr at 15°. Control cultures were maintained at the lower temperatures. After the times indicated, cultures (100 ml.) of each organism were removed, and the organisms harvested and washed by centrifugation at 0°. The methods used for preparing cell extracts and assaying enzyme activities in the extracts are as described under Methods.

Enzyme	Arthrobacter					Candida					*Corynebacterium erythrogenes*				
	Temperature of		Specific activity: Incubation (hr)			Temperature of		Specific activity: Incubation (hr)			Temperature of		Specific activity: Incubation (hr)		
	I*	A†	0	24	48	I*	A†	0	5	24	I*	A†	0	24	48
Pyruvate dehydrogenase	20°	20°	0·5	0·4	0·7	10°	10°	0·3	—	0·6	15°	15°	0·4	—	0·4
	37°	20°	0·5	0·6	0·2	25°	10°	0·5	0·1	0·0	30°	15°	0·4	0·5	0·2
	37°	37°	0·9	1·2	0·3	25°	25°	1·3	0·3	0·0	30°	30°	0·5	0·6	0·5
Citrate synthase	20°	20°	1·3	0·9	0·6	10°	10°	4·2	—	4·1	15°	15°	2·6	5·7	4·3
	37°	20°	1·3	0·6	0·3	25°	10°	5·6	7·1	4·9	30°	15°	2·6	2·8	3·4
	37°	37°	1·7	0·7	0·3	25°	25°	10·7	10·8	8·8	30°	30°	2·4	1·8	2·0
Aconitate hydratase	20°	20°	4·3	4·9	6·3	10°	10°	0·3	—	1·8	15°	15°	2·2	2·4	3·9
	37°	20°	7·2	5·1	4·7	25°	10°	0·2	0·2	0·2	30°	15°	5·0	3·6	3·4
	37°	37°	21·5	15·0	7·4	25°	25°	0·8	0·5	0·3	30°	30°	11·8	13·2	8·9
Isocitrate dehydrogenase	20°	20°	3·7	2·7	3·7	10°	10°	0·5	—	0·5	15°	15°	2·4	4·8	4·3
	37°	20°	4·3	0·5	0·5	25°	10°	0·5	0·7	0·4	30°	15°	2·1	0·7	0·2
	37°	37°	3·7	1·3	0·0	25°	25°	1·2	1·2	1·1	30°	30°	7·0	3·1	1·8
2-Oxoglutarate dehydrogenase	20°	20°	0·7	0·3	0·3	10°	10°	0·7	—	0·8	15°	15°	0·6	0·3	0·5
	37°	20°	0·6	0·4	0·1	25°	10°	0·4	0·4	0·4	30°	15°	0·5	0·6	0·3
	37°	37°	1·1	0·5	0·2	25°	25°	0·5	0·5	0·1	30°	30°	0·6	1·0	0·6
Succinate dehydrogenase	20°	20°	0·4	0·6	0·4	10°	10°	0·7	—	0·8	15°	15°	0·9	0·9	0·6
	37°	20°	0·9	0·9	0·8	25°	10°	0·5	0·5	0·4	30°	15°	0·6	0·6	0·0
	37°	37°	1·8	2·8	2·6	25°	25°	0·5	0·9	0·4	30°	30°	1·5	1·3	1·4
Fumarate hydratase	20°	20°	25·2	22·8	41·6	10°	10°	18·0	—	16·8	15°	15°	14·4	13·2	0·6
	37°	20°	29·2	32·0	20·0	25°	10°	8·8	0·6	8·0	30°	15°	0·6	10·0	8·4
	37°	37°	61·2	50·4	45·2	25°	25°	20·4	18·4	45·4	30°	30°	5·6	7·6	9·6
Malate dehydrogenase	20°	20°	4·5	4·7	7·8	10°	10°	2·1	—	2·8	15°	15°	2·1	2·7	4·7
	37°	20°	2·9	2·1	3·0	25°	10°	2·2	3·8	2·3	30°	15°	6·9	4·2	3·3
	37°	37°	5·3	3·0	4·9	25°	25°	6·1	2·9	3·9	30°	30°	12·4	10·2	9·7

* I = incubation. † A = assay.

bacterium erythrogenes cultures from 15 to 30° did not lead to any additional intracellular accumulation or excretion of u.v.-absorbing compounds. There was, however, an increase in the u.v. absorption of filtrates from the cultures of Arthrobacter which had been transferred to 37°, although this was not accompanied by an increased intracellular accumulation of these compounds.

Table 2. *Effect of change in incubation temperature on the contents of ultraviolet-absorbing compounds in culture filtrates and cell extracts of the three organisms*

Arthrobacter cultures were transferred to 37° after 144 hr at 20°; Candida cultures to 25° after 168 hr at 10°; *Corynebacterium erythrogenes* cultures to 30° after 120 hr at 15°; control cultures were maintained at the lower temperatures. After the times indicated, duplicate cultures (100 ml.) of each organism were removed and the organisms harvested by centrifugation at the temperature at which they had been incubated. Portions of each culture filtrate were filtered through a Millipore filter with 25 mm. HA filters. The extinctions of filtrates from Candida cultures were measured at 260 mμ in quartz cuvettes with 1 cm. light path, but because of interference from amino acids filtrates from Arthrobacter and *C. erythrogenes* cultures were measured at 300 mμ. The crops of organisms were washed twice with phosphate buffer (pH 7·0 for bacteria; pH 4·5 for yeast), a portion of suspension containing equiv. 5 mg. dry wt. organism taken and the organisms extracted with 3 ml. 5 % (w/v) trichloroacetic acid at room temperature. The supernatant fluids were quickly removed by centrifugation and made to 5·0 ml. with 5 % trichloroacetic acid. The extinctions of these extracts were measured at 260 mμ in 1 cm. quartz cuvettes.

		Cultures maintained at optimum temperature			Cultures transferred to temperature above maximum for growth		
		Ultraviolet absorption of			Ultraviolet absorption of		
Organism	Period of incubation after transfer (hr)	Cell extract ($E^{1\,cm.}_{260\,m\mu}$)	Filtrate ($E^{1\,cm.}_{300\,m\mu}$)	Growth (mg. dry wt./ml.)	Cell extract ($E^{1\,cm.}_{260\,m\mu}$)	Filtrate ($E^{1\,cm.}_{260\,m\mu}$)	Growth (mg. dry wt./ml.)
Arthrobacter	0	0·31	0·08	0·26	—	—	—
	24	0·31	0·10	0·31	0·04	0·19	0·26
	48	0·22	0·08	0·42	0·31	0·48	0·25
	72	0·24	0·12	0·42	0·25	0·63	0·24
Candida	0	0·07	0·08*	0·20	—	—	—
	5	0·09	0·07*	0·21	0·11	0·11*	0·23
	24	0·07	0·12*	0·23	0·03	0·17*	0·25
	48	0·07	0·17*	0·27	0·04	0·16*	0·25
Corynebacterium erythrogenes	0	0·20	0·06	0·11	—	—	—
	24	0·23	0·0	0·13	0·26	0·0	0·13
	48	0·10	0·0	0·18	0·22	0·01	0·18

* Extinction measured at 260 mμ

DISCUSSION

In the work described in the present paper, we were concerned only with two of the biochemical factors that seemed likely to be involved in determining the maximum temperatures for growth of the organisms, namely, enzyme denaturation and nucleic acid breakdown; no attempt was made to examine the effect of thermal stress on the properties of membrane lipids in the micro-organisms. Moreover, since we set out to study the biochemical bases of the maximum temperatures for growth

of these organisms, all experiments in which the organisms were subjected to a thermal stress were done with cultures of organisms rather than with washed suspensions or cell extracts, the conditions in which are known to alter the susceptibility of certain microbial cell constituents to heat inactivation (Militzer & Burns, 1954; Strange & Shon, 1964).

The rapid decrease in the respiratory activities of the Arthrobacter and Candida, caused by transferring exponentially growing cultures to a temperature 3–5° above the maximum for growth in freshly inoculated culture, suggested that inactivation of the respiratory metabolism is a major factor in determining the maximum temperature for growth of these organisms. Such an inactivation might lead quickly to a shortage of metabolic energy in the organisms, and might explain the increased death rate which accompanied the decline in respiratory activity. The shortage of metabolic energy might also explain in part the inability of organisms to grow at the higher temperatures in media supplemented with bacteriological peptone and/or yeast extract.

The activities of several TCA cycle enzymes in the Arthrobacter and Candida were diminished after these organisms had been transferred to the higher temperatures. The isocitrate dehydrogenase in the Arthrobacter and the pyruvate dehydrogenase in the Candida were almost completely inactivated in organisms so transferred and this might explain the almost complete loss of respiratory activity by these organisms at the higher temperatures. However, the inactivation of other respiratory enzymes, such as those concerned in oxidative phosphorylation, might also contribute to this effect. Other workers have reported exceptionally heat-labile respiratory enzymes in psychrophilic micro-organisms. Upadhyay & Stokes (1963) showed that the formate hydrogenlyase in a psychrophilic bacterium was inactivated at a much lower temperature than was the corresponding enzyme in a mesophilic strain of *Escherichia coli*; and Burton & Morita (1963) and Morita & Burton (1963) reported that the malate dehydrogenase in another psychrophilic bacterium was inactivated at 30°, which is the maximum temperature for growth of this bacterium. However there has been no previous report of a comparison of the heat lability of several respiratory enzymes in any one psychrophilic organism. Burton & Morita (1963) reported that the denaturation of the malate dehydrogenase in extracts of their bacterium was to some extent reversible, and suggested that the bacterium might contain more than one malate dehydrogenase, each with a different heat lability. Certain of our results may also be explained by postulating the existence of isoenzymes with different degrees of heat lability. There is also the possibility that the thermal stresses affected not only the activities of certain of the TCA cycle enzymes but also the ability of the organisms to synthesize these enzymes.

The transfer of cultures of *Corynebacterium erythrogenes* from the optimum temperature for growth to the higher temperature also caused a decline in the respiratory activity of the organism, but this was much less marked than with Arthrobacter and Candida and was not accompanied by a decrease in viability. The lack of an effect on the viability explains why *C. erythrogenes*, which had been grown at 15° and then transferred to 30°, grew rapidly on being returned to 15°. The comparatively small effect of the thermal stress on the respiratory activity of *C. erythrogenes* was supported by the finding that the activities of several of the TCA cycle enzymes in extracts of this bacterium were not diminished after the transfer

of the bacteria from 15° to 30°. The most heat-sensitive enzyme in this corynebacterium was isocitrate dehydrogenase and the decline in activity of this enzyme was of the same order as the decrease in respiratory activity. Possibly therefore the bacterium can produce sufficient energy at the higher temperature to maintain viability but insufficient to enable the organisms to divide.

Breakdown of RNA, as detected by an increase in the u.v.-absorption of cold acid extracts of the organisms and culture filtrates, did not appear to be important in determining the maximum temperatures for growth of any of the organisms tested. There was some excretion of u.v.-absorbing compounds by Arthrobacter after transfer to 37°, but the amounts released were small and probably did not represent an appreciable loss of RNA. Strange & Shon (1964) showed that magnesium ion protected RNA in *Aerobacter aerogenes* against thermal denaturation; it is possible that the thermal decomposition of RNA in the organisms used in the present study was protected by Mg ions present in the medium.

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Growth abnormalities in Hfr Derivatives of *Escherichia coli* Strain C

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SUMMARY

From *Escherichia coli* strain C, made F$^+$ by infection with the sex factor F normally carried by *E. coli* strain K-12, several Hfr ('high frequency of recombination') strains were derived. Among these, four were found which exhibited a defective growth pattern on minimal media at 37°. Reversion to the F$^+$ state was accompanied by re-establishment of normal growth habit. In the case best studied (strain C-132) the Hfr bacteria form colonies smaller than normal, acquire a rough surface upon prolonged incubation, and are unable to grow at 42°. Growth is normal at room temperature and on rich media; it can be improved by the addition of methionine to minimal media. The rate of reversion from the Hfr to the F$^+$ state (i.e. from defective to normal growth) is of the order of 1/20,000 per generation. Defective growth is not due to a genetic peculiarity of the F factor, nor is it dependent on the map location of the 'origin' or leading end of the particular Hfr strain, or on its direction of chromosome transfer; possibly it results from the manner in which the F factor is integrated at any given chromosomal site.

INTRODUCTION

It is known (see reviews by Jacob & Wollman, 1961, and by Hayes, 1964) that strain K-12 of *Escherichia coli* carries a genetic element, the sexual fertility factor F which is necessary for the occurrence of genetic recombination by conjugation, and is itself transmissible by cell contact to cells which lack it. The F factor can be carried by cells in two different states, corresponding to the two types of strains, called F$^+$ and Hfr, the latter being recognized by its much higher capacity to give genetic recombination (fertility level) with unidirectional transfer of chromosomal markers, and by low efficiency of transmission of F itself. Little is known about the stability of the two states. Hfr cells originate from F$^+$ cells and can revert to the F$^+$ state. In practice, most F$^+$ cultures contain a small minority of Hfr cells, which might be responsible for all the recombination obtained with an F$^+$ culture and probably most Hfr cultures contain a fraction of F$^+$ cells. The presence of F affects also other properties of the cell that carries it, for example, surface antigens, susceptibility to specific bacteriophages, etc. In general however such properties are more or less equally expressed in both F$^+$ and Hfr cells.

We report here cases in which the presence of the F factor in the Hfr state (but

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not in the F^+ state) produces certain side effects on the carrier cell. These effects seem to depend on the manner in which the Hfr state is established, and disappear when the Hfr cell reverts to the F^+ state.

Table 1. *List of bacterial strains used*

Collection number	Genetic structure	Origin and special properties
c-1	*Escherichia coli strain* c. *F⁻ prototrophic*	Bertani & Weigle (1953); Lieb, Weigle & Kellenberger (1955)
c-1a	*F⁻, prototrophic*	'Adapted' c-1, i.e. maintained for several years on Davis minimal medium. Grows much more rapidly than c-1
c-2	*F⁺*	Bertani & Six (1958)
c-6	*F⁻, arg-d-1, T1,5-r-1*	From c-3 (Bertani & Six, 1958) by spontaneous mutation to T1 resistance
c-20	*F⁻, arg-d-1, T1,5-r-1, gal-n-2*	From c-6, by mutation following irradiation with ultraviolet light
c-64	*F⁻, ura-d-1*	Bertani & Six (1958)
c-66	*F⁺, ura-d-1*	Bertani & Six (1958)
c-129	*Hfr-4, ura-d-1*	From c-66, following ultraviolet irradiation (low dose: about 20% survival; see under Methods)
c-130	*Hfr-5, ura-d-1*	From c-66, following ultraviolet irradiation heavy dose: about 10^{-6} survival; see under Methods)
c-131	*Hfr-6, ura-d-1*	From c-66, as above
c-132	*Hfr-7, ura-d-1*	From c-66, as above
c-167	*Hfr-7, ura-d-1, str-r-5*	From c-132, by spontaneous mutation
c-436	*F⁻, arg-d-1, T1,try-d-1, str-r-1, his-d-1*	From c-8 (Bertani & Six, 1958). Isolated by B. Kelly following irradiation with ultraviolet light
c-463	*F⁻, arg-d-1, T1,try-d-1, str-r-1, thr-d-1, P2-r-27*	From c-8. Isolated by B. Kelly in two steps: *thr* after ultraviolet irradiation, *P2-r* spontaneous
c-512	*Hfr-2*, $(P2)_{III}$	From c-77 (Kelly, 1963). Isolated by B. Kelly following ultraviolet irradiation
c-1057	*F⁻, arg-d-1, T1,5-r-1, gal-n-2, T6-r-1, azi-r-2, str-r-7*	From c-20, by spontaneous mutation (three steps)
c-1069	*F⁻, ura-d-1, gal-n-2, str-r-7, azi-r-2, T1,5-r-1*	Recombinant from a cross betweeen c-1057 and c-132. *T6* resistance not tested
c-1072	*Hfr-8*, $(P2)_{III}$	From c-512 (see under Results)
c-1073	*Hfr-9*, $(P2)_{III}$	As above
c-1075	*Hfr-10, ura-d-1*	From an n-type clone of c-132 (see under Results)
c-1077	*Hfr-11, ura-d-1*	As above
c-1091	*Hfr-12, ura-d-1*	As above

Explanation of symbols:

F⁻, F⁺, Hfr, fertility types. $(P2)_{III}$, lysogenic for phage P2 in location III.

T1, try-d, resistant to phage T1 and requiring tryptophan for growth (also abbreviated as *try*).

T1,5-r, resistant to phages T1 and T5; *azi-r*, to azide; *str-r*, to streptomycin; *T6-r*, to phage T6; *P2-r*, to phage P2 (unable to adsorb it).

arg-d, requiring arginine (also abbreviated as *arg*); *his-d*, requiring histidine; *met-d*, methionine; *thr-d*, threonine; *ura-d*, uracil; *xan-d*, xanthine (or guanine).

gal-n, galactose non-fermenting.

r, resistant; *d*, dependent; *n*, non-fermenting. Wild type alleles symbolized as *ura⁺*, *try⁺*, etc.

The number following the symbol refers to the isolation of the mutant and distinguishes mutants independently isolated which might have identical phenotypes.

METHODS

Organisms. The bacterial strains used (Table 1) are all derivatives of *Escherichia coli* strain C. The F factor, however, originated from *E. coli* strain K-12. The Hfr strains C-129, C-130, C-131, and C-132 were all isolated from a culture of strain C-66, following irradiation with 'germicidal' ultraviolet light, by replica-testing (Lederberg & Lederberg, 1952) some 10,000 surviving colonies for their ability to recombine efficiently with strain C-436, both when selecting for ura^+ and arg^+, and for ura^- and try^+. The isolation of other Hfr strains will be described under Results.

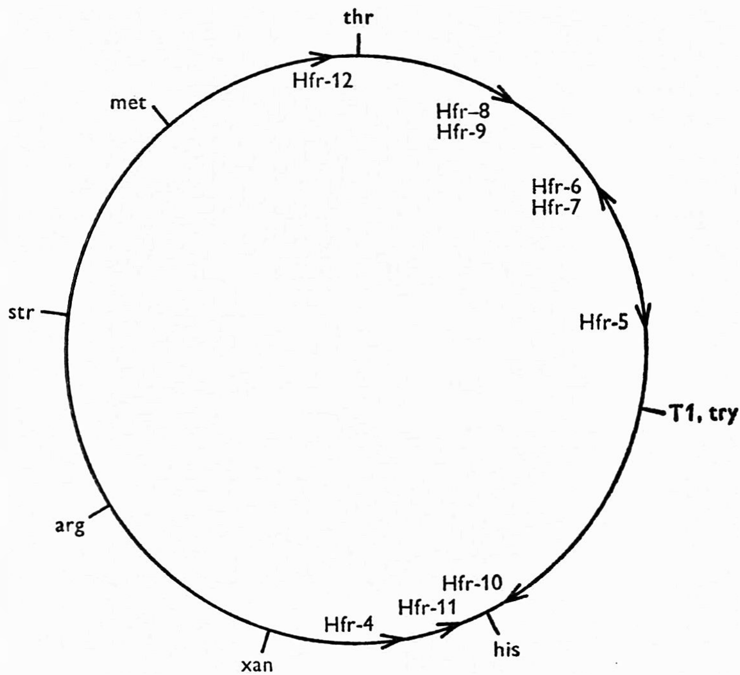

Fig. 1. Preliminary genetic map of *Escherichia coli* strain C constructed from results of interrupted conjugation experiments following the criteria of Jacob & Wollman (1961). The arrows indicate the *approximate* locations of the points of origin of the corresponding Hfr strains and their directions of transfer. The total length of the map is approximately 110 min. Symbols are explained in the legend to Table 1.

In collaboration with Dr Beatrice Kelly and Miss Marianne Wiman, we have obtained information (by means of interrupted conjugation experiments) concerning the times of entry of various markers for the Hfr strains mentioned here. These data (to be presented on a later occasion) permit the construction of a preliminary genetic map for strain C (Fig. 1), and the determination of the directions of transfer and of the points of origin of the Hfr strains used, with sufficient precision for the purposes of this report. All strains classified here as Hfr (with the exception of C-512) give under optimal conditions no less than 1 % recombination frequency (as defined below) when selecting for the leading marker and contra-selecting for a marker sufficiently distant from it.

Media

The compositions of the media used are given below in g./l. distilled water.

Tryptone broth: 10, Bacto-Tryptone (Difco); 5, NaCl; pH 7·0–7·2. For strains which require uracil, this medium was supplemented with 0·005, uracil (although the addition may not be strictly necessary).

Tryptone agar: tryptone broth with 10, agar.

Tryptone soft agar: tryptone broth with 5, agar.

Crossing buffer: 0·2, sodium aspartate; 2·5, glucose; in 0·05 M-sodium phosphate buffer (pH 6·4; Fisher, 1957).

Davis (minimal) agar: 7, K_2HPO_4; 2, KH_2PO_4; 0·5, Na citrate.$5H_2O$; 1, $(NH_4)_2SO_4$; 0·1, $MgSO_4.7H_2O$; 1, L-asparagine; 5, glucose; 14, Difco agar.

UTA agar: Davis agar supplemented with 0·02, uracil; 0·04, DL-tryptophan; 0·02, L-arginine.

Gal.EMS agar: 5, Na succinate; 1, NaCl; 5, $(NH_4)_2SO_4$; 1, $MgSO_4.7H_2O$; 2, K_2HPO_4; 0·4, eosin Y; 0·065, methylene blue; 10, Ionagar No. 2 (Oxoid); 5, galactose.

Double strength agar: 28, Difco agar.

Amino acid supplements were added as needed in amounts of 0·02 or 0·04 (for L and DL preparations, respectively,) uracil, 0·02, streptomycin (sulphate, Glaxo) 0·2.

Crossing technique

With the exception of some preliminary work, all bacterial crosses were made as follows. The F^- bacteria were prepared from a non-aerated overnight culture in tryptone broth, centrifuged once, and resuspended in crossing buffer at a final concentration about 5×10^8 bacteria/ml. The F^+ or Hfr bacteria were prepared from actively growing (at about 10^8 bacteria/ml.) static cultures in tryptone broth inoculated on the day of the experiment either with a 1/50 volume of a similar culture incubated overnight, or with a suspension made from a whole colony grown on UTA agar. These suspensions were centrifuged once and resuspended in crossing buffer at about 10^8 bacteria/ml. The suspensions of recipient and donor bacteria thus obtained were mixed in a ratio (v/v) of 3:1 and incubated for 2 hr. They were then plated, after appropriate dilution in crossing buffer, on the appropriate selective media. As a rule this plating was done by the soft agar layer method (as used for bacteriophage assays). The soft agar (2·5 ml./plate) was prepared by mixing 2 vol. crossing buffer + 1 vol. melted double-strength agar, and was kept at 45° until used. One drop of a 1/5 dilution of tryptone broth in water was added to each soft agar tube. The plates were inspected after incubation for 48 hr. Control platings were done in an identical manner for the recipient alone, the donor alone, and also for the two together, immediately after mixing. The concentrations of recipient and donor bacteria in the crossing mixture were estimated by viable counts made on tryptone agar (soft agar layer method) of the two suspensions before mixing. The frequency of recombination is expressed as the ratio of the number of recombinants obtained on a given selective medium to the number of donor bacteria in the mixture plated.

For interrupted conjugation experiments (Jacob & Wollman, 1961) to estimate

the time of entry of chromosomal Hfr markers, 1/10 dilutions of the crossing mixture were made at various times in 10 ml. chilled crossing buffer, immediately stirred for 5 sec. with an Ultraturrax high speed mixer (Janke & Kunkel KG, Staufen i. Br.) at full speed, and plated.

Unless otherwise stated, all incubation was at 37°.

RESULTS

Defective growth of Escherichia coli Hfr strain C-132

In the course of recombination experiments with Hfr strain C-132, during several months following its isolation, it was noted that its fertility level tended to decrease with time. It was also accidentally observed that cultures of such a strain, plated by spreading on UTA agar and incubated for 48 hr at 37° gave a heterogeneous

Table 2. *Measurement of the rate of transition from s-type to n-type in Escherichia coli strain* C-132

A diluted suspension of one s-type colony in tryptone broth was distributed into many small tubes (0·2 ml./tube) and simultaneously assayed on UTA agar. The tubes were incubated for 5–5·5 hr. Then for each tube the whole content was spread on a UTA plate (rinsing off the inside of the tube with two drops of added fluid). The plates were scored after 2 days, and more thoroughly after 7 days of incubation, for n-colonies present. Sectored colonies were neglected (although some ambiguity remained in two cases as to the scoring of single n-type colonies in crowded plates). The plates from the assays of the original suspension were also scored for n-type colonies, which were present with a frequency of about 0·2 %. The same suspension was used as inoculum in the two experiments.

	Experiment	
	a	*b*
Total number of tubes, T	60	59
Average number of bacteria/tube at inoculation, N_0	22	21
Average number of bacteria/tube after 5–5·5 hr of incubation, N_t	1780	1810
Number of plates with s-type colonies conly	51	49
Number of plates with numerous* n-type colonies	1	4
Same, expected from observed frequency of n-type bacteria in inoculum	2·6	2·4
Number of plates with a few n-type colonies, T_n distributed as follows:	8	6
with 1 n-colony	6	4
with 2 n-colonies	1	1
with 3 n-colonies	0	1
with 6 n-eolonies	1	0
Rate of transition from s- to n-type/bacterial generation†	$5{\cdot}2 \times 10^{-5}$	$3{\cdot}9 \times 10^{-5}$

* The n-type clones classified in this group had sizes of 42 (for *a*) and 17, 20, 32, and 24 (for *b*). We assume they represent the progeny of n-type bacteria present in the inoculum, even though their sizes were smaller than one would expect from the fact that n- and s-bacteria multiply equally well in tryptone broth. The possibility of a difference in the length of the lag period was not investigated. See also note below.

† Approximated as $T_n \ln 2/T\,(N_t - N_0)$. This estimate is subject to large errors on at least two counts. (*a*) The estimates of N_t had to be obtained from a small sample of the very crowded plates. (*b*) The bacteria in the inoculum (a suspension made from an s-colony grown on UTA agar) and those (actively multiplying in tryptone broth) at the end of the incubation period were obviously in different physiological states: hence the possibility of systematic differences in efficiency of plating affecting unequally the estimates of N_0 and N_t.

population of colonies (Pl. 1, fig. 1). Some of the colonies (n-type) were of normal size (i.e. as large as those of the parent strains C-66 and C-64); when picked and restreaked on the same medium they usually gave homogeneous populations of n-type colonies. Other colonies (s-type) were smaller; when picked and restreaked on UTA agar they usually gave a mixed population composed of a majority of s-colonies and a minority of n-colonies. The actual proportion of the latter type fluctuated very much from s-clone to s-clone, as one would expect if the variation were caused by a genetic change taking place randomly in time during the growth of the mother colony. The frequency of occurrence of such a change (transition from s-type to n-type) was measured by the method of Luria & Delbrück (1943) and is of the order of 1 in 20,000/bacterial generation (Table 2). The difference in colony size is clearly due to a difference in growth rate between s- and n-bacteria: the average doubling times for s- and n-bacteria were measured by means of viable counts in liquid cultures, in a medium identical to UTA agar but without agar, at 37°, in the exponential phase of growth, and found to be 100–120, and 60–80 min., respectively. An examination with the microscope revealed no obvious difference between bacteria from s- or n-colonies.

In platings as described above often a small proportion of colonies even smaller than the s-type was seen. This is to be attributed to uncontrolled variations (environmental or physiological as opposed to genetic): when picked and restreaked on UTA agar such very small colonies behaved in general like the most common type that was present on the plate from which they had originated. They will thus be neglected in what follows.

The use of UTA agar for the early observations was quite accidental. When the phenomenon was first observed, histidine was also present in the medium: as it does not appear to be necessary for the expression of the phenomenon, plain UTA agar was used in later experiments. When tryptophan or arginine or both together were omitted, the efficiency of plating of cultures of strain C-132 (grown in tryptone broth) was decreased and the fraction of very small colonies increased to a point which made the classification of s- and n-colonies very difficult.

Even on UTA agar it was sometimes difficult to recognize immediately upon inspection the two types of colonies. However, when such plates were incubated for 5 or 6 days at 37°, the difference between the two types became clear-cut: the n-type colonies were smooth in appearance (like those of strains C-66 or C-64 under similar conditions) when observed under a dissecting microscope, whereas the s-type colonies had a rough surface (Pl. 1, fig. 2, 3). Often the s-type colonies showed smooth papillae growing from them.

When mixtures of s- and n-bacteria were plated on tryptone agar, homogeneous populations of colonies were obtained (Pl. 1, fig. 4), indistinguishable from those of strains C-66 or C-64. Similarly, the differences between n- and s-types were almost undetectable on Davis medium supplemented with casein hydrolysate. L-methionine (20 μg./ml.) added to UTA agar stimulated the growth of s-type colonies, but not to the extent of obliterating the difference between the two types.

No difference between s- and n-colonies was detected on UTA agar when the plates were incubated at room temperature (18° to 24°; Pl. 1, fig. 5). Conversely, when incubation was at 42°, the n-colonies grew more or less normally, whereas the s-colonies as a rule did not appear at all. The addition of L-methionine permitted

Table 3. *Fertility levels typical of the two colony types obtained from Escherichia coli strain c-132*

Colonies of n- or s-type obtained on UTA agar from strain c-132 were picked and streaked on the same medium. From each streak a daughter colony of the same type as the parent was picked and used as inoculum for a culture. For each such culture, a sample was used in a standard cross to the F^- strain c-436, and another sample was plated on UTA agar to estimate the composition of the culture in terms of n and s types (progeny test).

	Type of colony	
	n	s
Number of colonies tested	8	8
Frequency of colonies clearly classifiable by inspection as n-type in progeny tests		
Average	97 %*	11 %
Range	83–100 %*	0–28 %
Recombination frequency:		
(A) Selecting for *str-r* and *T*1, try^+		
Average	$6{\cdot}4 \times 10^{-5}$	$1{\cdot}0 \times 10^{-1}$
Range	$2{\cdot}9$–$12{\cdot}0 \times 10^{-5}$	$0{\cdot}71$–$1{\cdot}5 \times 10^{-1}$
(B) Selecting for arg^+ and ura^+		
Average	$5{\cdot}9 \times 10^{-5}$	$4{\cdot}4 \times 10^{-3}$
Range	$1{\cdot}9$–$9{\cdot}0 \times 10^{-5}$	$1{\cdot}8$–$11{\cdot}0 \times 10^{-3}$

* That not all colonies could be here classified as n-type by mere inspection must be attributed to uncontrolled non-genetic sources of variation in colony size.

Table 4. *Transfer of the F factor by bacteria of n-type colonies of Escherichia coli strain c-132*

The ' nfection tubes' (5 ml. tryptone broth + uracil) were inoculated with small amounts of fully-grown cultures of various strains in the combinations given in the Table. After overnight incubation (during which the F-factor was expected to infect the majority of the F^- bacteria present, here represented by strain c-1a or strain c-64), fresh cultures in tryptone broth were started from each infection tube and used as donor cultures in a standard cross to the F^- strain c-1069, selecting on Gal.EMS agar for *str-r* gal^+ and ura^+ ('recombination test'). Selection for ura^+ excluded the possibility of a direct participation of the uracil-requiring F-donors in the formation of the recombinants scored.

Infection tubes and controls	Inocula used for infection tubes		Recombination test: Bacteria from donor culture/ml. mixture in crossing buffer ($\times 10^{-7}$)	Recombinants obtained by plating 0·1 ml. of crossing mixture on selective medium	
	0·1 ml. of	0·002 ml. of		Immediately after mixing	After 2 hr incubation
A*	c-1a	c-132 pure n-type	2·2	0	39
B	c-1a	c-66	2·1	0	71
C	c-1a	c-64	1·5	0	0
D	c-2	c-64	2·2	2	136
E	c-66	—	2·4	0	0
F	c-1a	—	3·4	0	0

* In a similar experiment five other n-type isolates (each originating from a different s-type colony of strain c-132) were tested and found to give results similar to those for tube A.

s-bacteria to form colonies at 42°. Also, s-colonies were helped to grow at the higher temperature by the presence of an n-colony on the same plate: as a consequence, the test for temperature sensitivity was often difficult to interpret. No difference was noted between s- and n-colonies at 42° on tryptone agar.

The s- and n-type colonies behaved quite differently in recombination experiments (Table 3). The s-colonies showed fertility values typical of the Hfr strain as originally isolated. The n-colonies behaved like F^+ strains, both in fertility level and in their ability to transfer the F factor to F^- bacteria as efficiently as other F^+ strains (Table 4). A sample of a C-132 culture which had been freeze-dried very soon after isolation of the strain was shown to contain, after reconstitution and plating on UTA agar, a majority of s-type bacteria. There must therefore have been representative of the original Hfr isolate.

As one might expect, the mutant derivative C-167 of strain C-132, behaved just like C-132 in respect to the properties described above. Such properties are by no means common to all Hfr derivatives of *Escherichia coli* C, as shown by the study of the following three Hfr strains which were obtained at the same time and from the same C-66 culture as C-132 was.

Strain C-129 produced on UTA agar homogeneous populations of colonies, indistinguishable from the n-type of C-132. Strain C-130 showed great colony size variability ion UTA agar (the largest colonies being like the n-type of C-132): it was not possible, however, to demonstrate convincingly the inheritance of colony size or a correlation between it and fluctuations in fertility level. Strain C-131 gave homogeneous populations of colonies resembling the n-type. These three Hfr strains grew at 42° as well as did the parental strains C-66 and C-64.

Re-isolation of Escherichia coli Hfr strains from low fertility clones of strain C-132, and their properties

Next was examined whether the n-type segregants of strain C-132 could revert to the highly fertile s-type with a measurable probability. In one experiment 10 clones originating from a repeatedly purified n-type segregant of C-132 were analysed by plating on UTA agar (10 plates/clone; average 350 colonies/plate) and looking for small colony variants. The more minute types of colony (perhaps a hundred in the whole sample) were neglected. A total of 87 colonies resembling in size the s-type were picked and streaked on UTA agar. In none of these streaks was found a clearly recognizable proportion of s-type colonies. All 87 colonies were also tested for fertility level by spotting a loopful of each suspension on four different selective agar media coated with a thick suspension of bacteria of the F^- strain C-463. One colony gave a significant amount of recombinant growth on such plates and was purified and studied further (strain C-1091). In another experiment, 10 more clones obtained from another, independently obtained n-type segregant of C-132, were analysed (10 plates/clone; average 210 colonies/plate), looking for rough colonies or rough-looking sectors after 6 days of incubation. One rough colony and several sectors were found, picked and streaked on UTA agar. Two such streaks were clearly heterogeneous as to colony sizes: one small size colony from each was further purified and shown to be highly fertile in crosses to F^- bacteria (strains C-1075 and C-1077, the former originating from the whole rough colony).

The three new Hfr strains thus obtained differ from C-132 in the map positions of

their origins (Fig. 1). Two have the direction of chromosomal transfer opposite to that of C-132. Their other properties are as follows. Strain C-1091, although isolated by selecting a small colony, gives on UTA agar n-type colonies which show, however, a greater size variability than, for example, strain C-66. As in the case of strain C-130, it was impossible to demonstrate genetic heterogeneity. The strain grows reasonably well at 42°. Strain C-1075 behaves like C-132 in respect of colony size variation (Pl. 1, fig. 6) and correlated fertility changes. It differs from C-132, however, in the following properties. The s-type colonies after 3 days of incubation on UTA agar are partially overgrown by peripheral papillae of n-type bacteria, in consequence of which the colony acquires a characteristic morphology (Pl. 1, fig. 7). To obtain reasonably pure populations of s-bacteria it was therefore necessary to pick very young colonies. Although, as with C-132, s- and n-colonies of C-1075 cannot be recognized at room temperature on UTA agar or at 37° or 42° on tryptone agar, unlike strain C-132, the addition of L-methionine to UTA agar did not improve the growth of s-colonies of C-1075. Several n-colonies of C-1075 were tested for fertility level: all were at least 10–100 times less efficient in recombination than the s-colonies.

Strain C-1077 gives both s- (Pl. 1, fig. 8) and n-colonies, and only the latter breed true. The one n-colony tested showed a fertility value typical of F^+ strains. The s-colonies did not grow or grew very poorly at 42° on UTA agar, and were helped by the addition of methionine. Unlike strain C-132, no evident roughness of the s-colonies of C-1077 appeared on prolonged incubation, and the two types of colonies could be recognized even when grown at room temperature.

Defective growth of an Hfr strain not descended from strain C-132

The *ura* marker present in C-132 is probably not involved in the phenomena described here; this was shown as follows. The prototrophic strain C-512, originally isolated as a typical Hfr, shows now, after several passages on nutrient agar, a decreased fertility level (about ten-fold higher than that typical of an F^+). Several colonies obtained from a subclone of C-512 were tested for fertility level by spotting on F^- bacteria, as described above. Two of these showed a high fertility level and were purified and studied further (strains C-1072, C-1073). Strain C-1072 (Pl. 1, fig. 9) showed colony size variation correlated with changes in fertility level, was temperature sensitive and was affected by methionine like strain C-132. The s-colonies of C-1072, however, did not show after long incubation on UTA agar the surface changes typical of strain C-132. The colony size variation was observable when uracil was not added to the medium. With strain C-1073 we did not succeed in showing on UTA agar any inheritable heterogeneity in colony size.

A word of caution may be added about the low recombination values of n-type clones isolated from Hfr strains which show defective growth patterns. Although in some cases the results obtained in recombination tests were sufficiently precise and involved a sufficient number of markers for concluding that the recombination frequency was of the same order as with F^+ strains, this conclusion should not be over-generalized. We made no special effort to see whether the frequency of the so-called F' types or of other types which give frequencies of recombination intermediate between those typical of Hfr and F^+ strains was particularly high among such normal-growing revertant colonies.

DISCUSSION

We have shown that under certain conditions some, but not all, Hfr derivatives of *Escherichia coli* strain C exhibit defective growth patterns. Such patterns are not identical, but have as a common feature their dependence on substrate composition and incubation temperature. This property is apparently contingent upon the Hfr state; normal-growing clones are found in such strains, but they no longer recombine with high frequency and may behave like typical F+. From such revertant clones both Hfr strains showing defective growth pattern and normal-growing Hfr strains can be isolated. These facts suggest that defective growth pattern in the Hfr state is not the consequence of a genetic peculiarity of the F factor involved. Defective growth pattern seems also to be independent of the map position of the origin (i.e. the leading end in unidirectional transfer at conjugation) of the Hfr chromosome or of the direction of transfer. It would seem therefore that defective growth pattern results from the manner in which the bacterium and the F factor interact to establish a given Hfr line.

More specific suggestions can be made starting from Jacob & Wollman's theory of the formation of Hfr strains, and Campbell's (1962) model for the integration of the F factor (and of episomes in general). According to Jacob & Wollman (1961 and earlier), an Hfr clone is formed when the F factor, believed to be present in several freely floating copies in the F+ bacteria, becomes 'integrated' at one of several points on the circular bacterial chromosome, determining thereby the origin and direction of the new Hfr strain. According to Campbell (1962), the F factor is a ring of genetic material which can insert itself into the bacterial chromosome by a single reciprocal cross-over. Such cross-over would require the existence of genetically identical or homologous segments (attachment sites) on the chromosome (chromosite) and on the F factor or episome (episite). The facts clearly indicate the existence of several chromosites at different locations on the chromosome. If all chromosites and the episite were identical, it would obviously be difficult to conceive how the manner of attachment of the F factor can differ for different Hfr strains. It is possible however that: (*a*) the various chromosites are neither identical nor homologous and therefore several corresponding episites exist; or (*b*) the homology within a matching episite chromosite pair is not perfect. In such cases, a variety of modes of insertion of the F factor might ensue, depending on which episite is used for the insertion or at which point within the matching sites the reciprocal cross-over has taken place. The F factor is known to control several properties of the cell that carries it, most likely by possessing some genes which are not usually present in an F− cell. If the insertion point should happen to be within one such gene or block of genes, it is conceivable that detrimental effects may follow for the growth or multiplication of the Hfr cell. There is some evidence supporting the idea that duplication of an Hfr chromosome begins as a rule at the point of attachment of the F factor (Nagata, 1963). If this be the case, one could also imagine that certain modes of attachment of the F factor interfere under certain conditions with the inception of the duplication process, thereby increasing the generation time of such cells. We are unable to propose at this time a more precise interpretation.

Most of the work on Hfr behaviour has been done with *Escherichia coli* strain K-12. Although Hfr derivatives of K-12 are also known to revert to the F+ type, there

is to our knowledge no precise estimate of such rate of reversion. It should be noted that our estimate for strain C-132 is of the same order of magnitude as the rate of spontaneous phage liberation in certain lysogenic bacteria (e.g., Bertani, 1951; Six, 1959).

To our knowledge there is no mention in the literature of similar defective growth patterns for Hfr derivatives of strain K-12, although the F factor used in our work does originate from such a strain. It is possible that the phenomenon observed with derivatives of strain C is to some extent dependent on a lack of reciprocal adaptation between F factor and host genotype. Possibly significant might also be the fact that strain C already grows rather slowly as compared with strain K-12 and most other *E. coli* strains.

We are indebted to Dr Beatrice Kelly and Miss Marianne Wiman for the isolation of some of the strains used by us. This work was aided by United States Public Health Service Research Grant AI-04390 from the National Institute of Allergy and Infectious Diseases, and by a joint grant from the Swedish Medical and Natural Sciences Research Councils and the Swedish Cancer Society.

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EXPLANATION OF PLATE

(Magnification: ×2 throughout)

Fig. 1. Mixture of s- and n-type colonies from *Escherichia coli* strain c-132 after 2 days incubation at 37° on UTA agar.

Fig. 2. Same mixture as in Fig. 1 after 7 days of incubation.

Fig. 3. Colonies of strain c-66 after 7 days of incubation at 37° on UTA agar.

Fig. 4. Same mixture as in Fig. 1, after incubation for 1 day at 37° on tryptone agar.

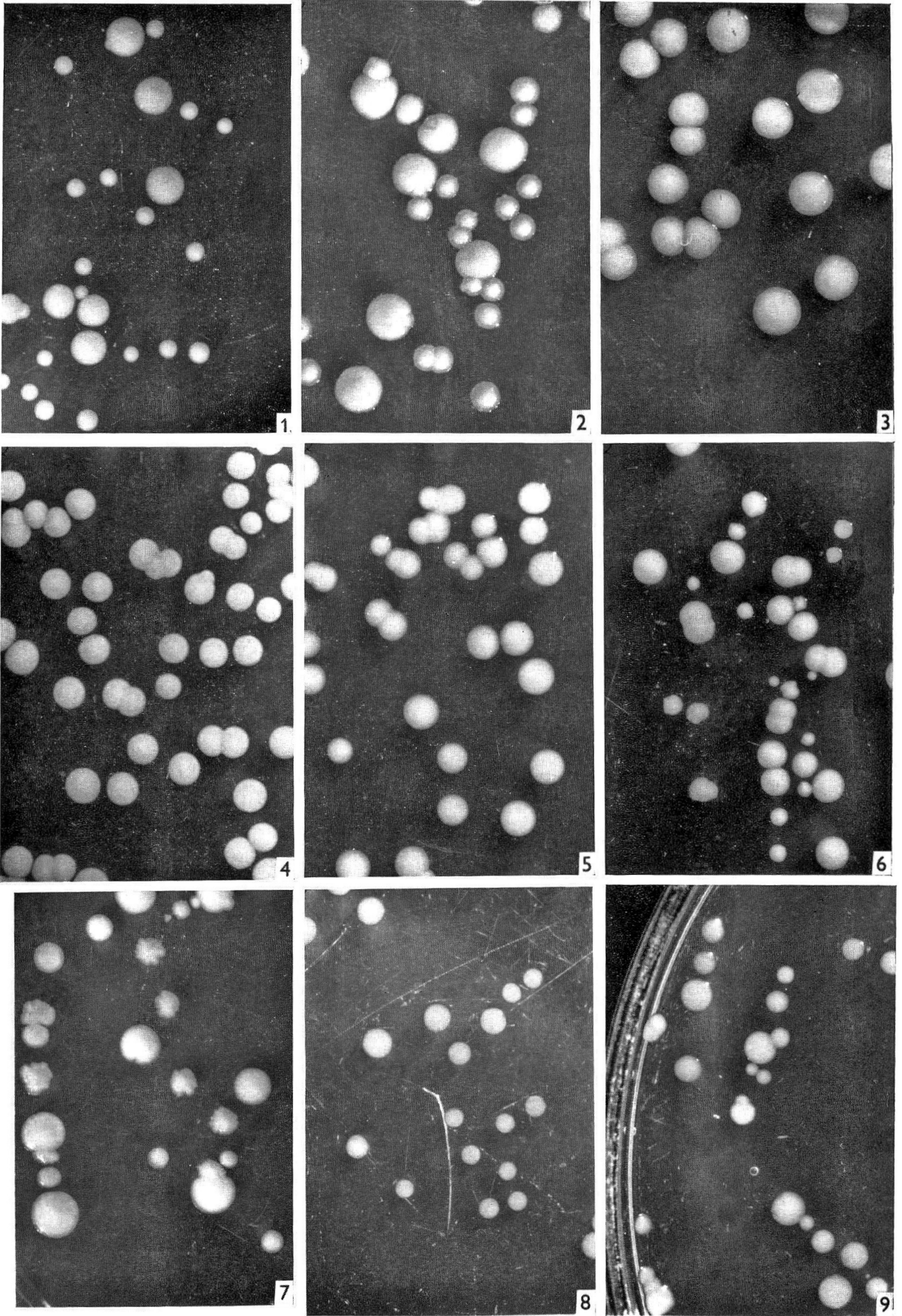
Fig. 5. Same mixture as in Fig. 1, after 3 days of incubation at room temperature on UTA agar.

Fig. 6. Progeny of an s-type colony of strain c-1075 after 2 days of incubation at 37° on UTA agar.

Fig. 7. Same as in Fig. 6 after 7 days of incubation.

Fig. 8. Progeny of an s-type colony of strain c-1077 after 2 days of incubation at 37° on UTA agar (only s-type colonies are present in the picture).

Fig. 9. Progeny of an s-type colony of strain c-1072 after 2 days of incubation at 37° on UTA agar.



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Mutations in Symbiotic Effectiveness in *Rhizobium trifolii* Caused by Transforming DNA and Other Agents

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SUMMARY

A strain of *Rhizobium trifolii* able to fix nitrogen in root nodules of clover plants lost its effectiveness when treated with deoxyribonucleic acid (DNA) from an ineffective strain. Attempts to transform two ineffective strains to effectiveness failed, even when the donor of DNA and the recipient strains were genetically related and apparently differed only in symbiotic property. The efficiency of transformation by DNA to ineffectiveness was compared with mutagenic and selective treatments. The results support the idea that symbiotic effectiveness involves compatability between several plant and bacterial factors, changes in any one of which makes the bacterium ineffective.

INTRODUCTION

Individual strains of nodule Rhizobium are usually stable, maintaining their morphological, serological, symbiotic and other features unaltered during many years of cultivation on artificial media. New properties appear only rarely and irregularly. Work done on the conditions which affect the frequency and regularity of appearance of variants has often given conflicting results (see review, Kleczkowska, 1950). The treatments used by different workers do not give consistent results, and the main reason for these may be that different bacterial strains behave differently. The work described in the present paper was done to see whether deoxyribonucleic acid (DNA) of the bacteria acts as a transforming factor and can change symbiotic properties. When DNA was found to do so its efficiency was compared with the results of previous work (Kleczkowska, 1950; Gupta & Kleczkowska, 1961) where, due to mutagenic (u.v. irradiation) and selective agents (streptomycin or bacteriophage), similar genetic mutants were obtained.

METHODS

Three strains of *Rhizobium trifolii* Dung. were used: strain A121111, which is effective in nitrogen fixation, and strains f12 and HKC which are ineffective on the host plant, *Trifolium pratense* L. These strains had been maintained over a long period on yeast water agar medium and had often been passed through clover plants without showing any genetic changes. Strain f12 is a stable ineffective variant of strain A121111 (Nutman, 1946), from which it cannot be distinguished culturally or serologically. Strain HKC differs serologically from A121111; strains A121111 and f12 are both susceptible to bacteriophage A121111, to which strain HKC is resistant. The proportion of phage-resistant bacteria in cultures of these organisms never exceeded 0·01% and was usually less than 0·001%.

The methods of obtaining phage-resistant and streptomycin-resistant mutants, and also the technique of isolating surviving bacteria after exposure to ultraviolet (u.v.) radiation were described previously (Kleczkowska, 1950; Gupta & Kleczkowska, 1961). The proportion of streptomycin-resistant bacteria in a culture of strain A121111 is about 0·00001%. Strain A121111 was exposed to u.v.-radiation so that the proportion of surviving bacteria was about 0·5%. The ineffective strains f12 and HKC were not tested with streptomycin or u.v.-radiation. Preparations of DNA were obtained from large quantities of strains A121111 and HKC grown either on agar medium or in a chemostat. For continuous culture (apparatus described by Skinner & Walker, 1962) a liquid medium of the following composition was used: 0·5 g. KH_2PO_4; 0·1 g. $MgSO_4.7H_2O$; 0·5 g. $CaCl_2.6H_2O$; 10 g. sucrose; 100 ml. yeast water extract; 1 ml. 0·1 N-HCl; 900 ml. water; adjusted to pH 6·8–6·9 with 5% (w/v) Na_2CO_3. Growth was at 27° and the steady-state numbers of bacteria were 13×10^8/ml. The bacteria were separated in a Sharples centrifuge.

The agar medium (Kleczkowska, 1950) was distributed in 150 ml. portions in 16 oz. medicine bottles and allowed to set horizontally. Each bottle was inoculated with 3 ml. of a 48 hr liquid culture and incubated for 7 days at 28°; the bacteria were then washed from the surface of the agar with sterile saline solution and centrifuged down. Although both methods were satisfactory, strain A121111 grew better on the agar medium.

DNA was prepared from the bacteria by the method described by Balassa (1960). It was finally precipitated with ethanol, centrifuged down, dissolved in sterile saline and kept at −20° until needed. The u.v.-absorption spectrum of the preparations differed from that of thymus DNA by showing more absorption at wavelengths shorter than 260 mμ, indicating that they were probably contaminated with protein. The concentration of DNA was estimated colorimetrically by using diphenylamine in acetic acid (Dische, 1930), against a standard commercial preparation of thymus DNA.

For transformation tests the DNA preparations were added to sterile liquid media to a concentration of 0·025–0·1 mg./ml. and the mixture then inoculated with the bacteria. The cultures were incubated for times (4, 14, 21 days) at 28°; single colonies were then isolated by the plate dilution technique. Since the time of incubation did not affect the results, the results of the different times have been treated together. Each colony isolate after treatment was tested for effectiveness in nitrogen fixation on four sterile seedlings of red clover grown singly in tubes on a nitrogen-deficient agar medium (Nutman, 1946). The plants were allowed to grow for about 3 months until control plants inoculated with untreated strain A121111 had 8 true leaves. At the end of a test plants were classified as to effectiveness. The effective class included a small proportion of plants that were intermediate in size, but these were clearly distinguished from ineffective plants which made no better growth than uninoculated controls.

RESULTS

Table 1 shows the effect on strain A121111 of the selective and mutagenic treatments in terms of the distribution of plant response in sets of four test plants. The results with the control untreated bacteria of strain A121111 confirmed Nutman's (1954) observations that a proportion of clover plants did not fix nitro-

gen with this strain. That this reflects heterogeneity in the plant population and not the bacterial colonies is concluded from the fact that plants which did not fix nitrogen were distributed randomly among the sets of four plants inoculated with different bacterial colonies. This is shown by the following statistical examination. A total of 680 plants in 170 sets of four were inoculated with untreated control bacteria of which 48 plants did not respond, so that the probability of a plant not responding was $P = 48/680 = 0{\cdot}07$. With a random distribution the expected numbers of sets of four plants in which 0, 1, 2, 3 and 4 plants did not respond are

Table 1. *Effect of various mutagenic and selective treatments on symbiotic effectiveness on red clover in Rhizobium trifolii strain* A121111

Treatment	Total numbers of colonies tested	Response in four test plants: All four effective	Three effective	Two effective	One effective	All four ineffective
		Number of colonies in each response				
Control (untreated)	170	131	35	5	1	0
Streptomycin	28	21	5	1	0	1
U.v. irradiation	23	14	6	1	0	2
Phage A 121111	68	22	6	10	8	22
DNA from strain HKC	170	104	44	5	3	14
DNA from strain HKC + phage A 121111	20	3	0	1	4	12

Table 2. *A comparison of observed results of inoculating 170 sets of four red clover plants with different colonies isolated from untreated strain* A121111 *of Rhizobium trifolii (control) with values expected on the assumption of random distribution of plants which responded ineffectively*

Number of ineffective responses in a set of four plants	Expected number of sets		Observed number of sets		χ^2
0	127·2		131		0·1102
1	38·3		33		0·7392
2	4·323	4·54	5	6	0·4695
3	0·217		1		
4	0·004		0		
				Total	1·3189
				D.F. 1*; $P = 0{\cdot}25$	

* There is a loss of a degree of freedom for estimation of p.

given by the successive terms of the expanded binomial $(0{\cdot}93+0{\cdot}07)^4$ multiplied by 170. Table 2 compares the expected numbers with those obtained. The χ^2 test shows that the probability of deviations from expectation exceeding by chance those observed was about 0·25. The agreement between the observed and expected number is therefore good. The bacterial colonies can be assumed to be identical and the population of plants heterogeneous, consisting of two genotypes of plants which give different symbiotic response with the same bacterial strain. The proportion

of plants with ineffective response is 0·07. This will be the basis for assessing the results by subjecting *Rhizobium trifolii* strain A121111 to the treatments indicated in Table 1.

If strain A121111 remained unchanged the probability of a given set of four plants all failing to respond is $P_1 = 0{\cdot}07^4 = 0{\cdot}000024$, and consequently the probability of a set of four plants not all responding ineffectively (i.e. some or all responding effectively) is $q_1 = 1-0{\cdot}07^4 = 0{\cdot}999976$.

Of 28 sets of plants inoculated with colonies obtained after treating strain A121111 with streptomycin (Table 1), the probability of at least one set of 4 plants all failing to respond effectively would therefore be $1-q_1^{28}$ which is about 0·0015, assuming no change in the bacterial strain. Similarly, the probability of at least two sets of 4 plants not responding out of the 23 inoculated after u.v.-irradiation, would be $1-q_1^{23}-23p_1q_1^{22}$, which is about 0·0006. These probabilities are very small and those relating to treatment with phage, DNA, or phage + DNA are infinitesimal. It can therefore be concluded that these bacterial colonies ineffective on all four test plants must almost certainly be genetically changed.

All the treatments shown in Table 1 gave ineffective mutants of strain A121111, either by selection (bacteriophage and possibly streptomycin) or by mutagenesis (DNA from ineffective strain HKC and probably u.v.-irradiation), the highest incidence being produced by the combined treatment with DNA and bacteriophage.

Table 3. *A comparison of observed results on sets of four red clover plants, some of which responded effectively, with results to be expected on the assumption that the rhizobia remained unchanged and that ineffectively responding plants were distributed at random*

Treatment	Treated colonies (no.)		Numbers of ineffectively responding plants in sets of 4 test plants			
			0	1	2	3
Streptomycin	27	o	21	5	1	0
		e	20·2	6·1	0·67	0·03
U.v.-irradiation	21	o	14	6	1	0
		e	15·7	4·7	0·5	0·027
Phage A121111	46	o	22	6	10	8
		e	34·4	10·4	1·2	0·059
DNA from strain HKC	156	o	104	44	5	3
		e	116·7	35·1	4·0	0·2
DNA from strain HKC + phage A121111	8	o	3	0	1	4
		e	6·0	1·8	0·2	0·01

A similar procedure can be used to determine whether the bacterial colonies that gave less than four ineffectives in each set of four plants were also mutants. Table 3 compares the observed numbers of sets containing different numbers of ineffectives with those to be expected had the bacteria not been affected by the treatments. Agreement is quite good for exposure to streptomycin and u.v. irradiation. The bacterial colonies, obtained after these treatments, which were not all effective on four test plants, were therefore probably unaltered in nitrogen-fixing effectiveness. In contrast, with phage, DNA, or phage + DNA, the observed and expected results

disagree, indicating that the colonies that were ineffective on only a proportion of test plants had also suffered change as a result of the treatment.

The smallest difference between observed and expected numbers was with DNA, with which three sets out of 156 were ineffective in three out of four test plants. If the bacteria remained unaltered, the probability of at least three ineffectives occurring in a set of four plants would be $p_2 = p^4 + 4p^3q$, where $p = 0{\cdot}07$ and $q = 0{\cdot}93$. This gives a value of 0·0013, so that the probability of this not occurring in a set of four plants is $q_2 = 0{\cdot}9987$. Therefore the probability of the occurrence of at least three sets each with at least three ineffective plants in a total of 156 sets, would be

$$1 - q_2^{156} - 156 q^{155}{}_2 p_2 - \tfrac{1}{2}(156 \times 155) q_2^{154} p_2^2,$$

which equals 0·0012. This excludes the possibility that the bacteria used were identical with the original strain A 121111 of *Rhizobium trifolii*.

Table 4. *Loss of susceptibility to phage, as a result of treatment of the rhizobium strain A 121111 with DNA from rhizobial strain HKC*

Colonies tested (no.)	Nitrogen fixation			Susceptibility of strain A 121111 to phage	
	Effective*	Intermediate†	Ineffective‡	Susceptible	Resistant
54	21	.	.	0	21
	.	25	.	14	11
	.	.	8	5	3

* Effective response on all 4 test plants.
† intermediate: mixed effective and ineffective responses among 4 test plants.
‡ Ineffective response on all 4 test plants.

Ineffective and effective bacterial isolates obtained after treatments of strain A 121111 with DNA from strain HKC all agglutinated with strain A 121111 antiserum to the same end point as did unheated strain A 121111 itself. Ineffectiveness was therefore transmitted independently of serological characteristics. All the isolates that retained their nitrogen-fixing effectiveness also remained susceptible to phage A 121111; but among ineffective mutants some isolates were also resistant to this phage. Table 4 shows the reaction to phage A 121111 of bacterial isolates after DNA treatment. These results suggest a connexion between the characteristics, possibly through the juxtaposition of corresponding genetic loci. This is also suggested by the fact that 60% of phage-resistant mutants of strain A 121111 are also ineffective. DNA and phage treatment together gave the very large proportion of 85% of ineffective mutants.

After phage treatment, all the surviving bacteria are at first phage-resistant, but after several subcultures on phage-free medium, most regained susceptibility to phage. Ineffective strains which regained susceptibility to phage remained ineffective. So mutation to ineffectiveness seems more stable than mutation to phage-resistance.

In contrast to strain A 121111, the ineffective strains f12 and HKC showed very little or no tendency to change their symbiotic response, although strain f12 was derived from strain A 121111 and does not differ from it in any known feature other

than in being ineffective. Like strain A121111, strains f12 and HKC have been shown (Kleczkowska, 1950) to form mutants resistant to their respective phages, but none of the resistant forms of strain HKC was effective and strain f12 formed only a few effective variants after exposure to phage.

DISCUSSION

These results show great frequency and stability of the induced mutations in certain *Rhizobium trifolii* strains to ineffectiveness in symbiotic nitrogen fixation. Difficulties were experienced in obtaining the reverse mutation. In speculating on the significance of these relationships it should be remembered that symbiotic effectiveness is not simply a property of a bacterial strain for, as the behaviour of individual plants of the control series emphasized (Table 1), a bacterial strain effective on one host can be ineffective on another. Nutman's (1954*a*) studies on the inheritance of the effective response in the host and on the relationship between host genes and bacterial strain characteristics, suggest that effectiveness is an expression of compatibility between plant and bacteria. This compatibility can be broken by mutation in either host or bacterial genes. In some symbioses, changes in one of the bacterial genes suffice to interfere with the process which leads to nitrogen fixation, in others more than one gene may be involved. For example, Kleczkowska (1957, 1958) showed that the partially effective rhizobium strain 'Coryn' gave a mixed response in the late-flowering Montgomeryshire variety of red clover, but when tested in selected lines of the same host plant gave different responses. Some host lines did not respond at all; in some the rhizobial strain was effective; and in other host lines gave mixed responses, as in the original variety. Other effective and ineffective rhizobial strains behaved in these selected lines of clover as in unselected material, showing that the source of variation lay with the host.

The apparent irreversible nature of the change to ineffectiveness at first suggests simply a gene loss, but in view of the complex compatibility relationships between plant and bacteria there is reason to suspect other explanations. If the change from effectiveness to ineffectiveness can result from mutation in any one of several genes it will happen correspondingly more often than mutation to effectiveness in one particular gene.

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Carbohydrate Metabolism of Iron-Rich and Iron-Poor *Staphylococcus aureus*

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SUMMARY

Comparative studies were made of the metabolic activities of a strain of *Staphylococcus aureus* grown in iron-poor and iron-rich trypticase medium, with and without glucose. Four nutritionally distinctive types of organisms were produced: iron-rich without glucose (Fe+ G−); iron-poor without glucose (Fe− G−); iron-rich with glucose (Fe+ G+); iron-poor with glucose (Fe− G+). Fe+ G− cocci oxidized glucose, L- and D-lactate, pyruvate, acetate, formate, and Krebs cycle intermediates to completion. Of these substrates, only glucose and the lactates were oxidized by the Fe− G− organisms. These latter oxidations proceeded at decreased rates and incompletely to acetate and acetoin. Fe+ G+ cocci in comparison with the Fe+ G− cocci showed glucose inhibition of oxidative capacity by metabolizing only glucose, L-lactate and pyruvate to acetate and failing to oxidize D-lactate, acetate and Krebs cycle intermediates. The Fe− G+ cocci showed the most severe restriction of oxidative capacity by oxidizing only glucose with the accumulation of much lactate and minor amounts of pyruvate, acetate and acetoin. The Fe− G−, Fe− G+, and Fe+ G+ cocci glycolysed glucose at comparable rates, while the highly oxidative form Fe+ G− showed a markedly decreased glycolytic activity. The catalase activity of both types of iron-poor cocci was very much lower than that of the iron-rich organisms. Of the latter, the Fe+ G+ cocci showed a catalase activity only 20% that of Fe+ G− cocci.

INTRODUCTION

The growth characteristics of *Staphylococcus aureus* in complex media of various iron availabilities have recently been reported (Theodore & Schade, 1965). Not only the rate of growth but also the final size of the population were dependent upon the concentration of free iron in the medium. Preliminary investigations of the metabolic effects of nutritional iron deficiency showed that iron restriction of growing *S. aureus* resulted in qualitatively and quantitatively altered aerobic metabolism of common carbon sources (Schade, 1963). Since iron is required for the formation of haemoproteins essential for many enzymically catalysed biological oxidation-reduction reactions and thus must be provided in the growth media of practically all micro-organisms, the possible metabolic alterations produced in *S. aureus* by iron-deficiency have been examined. Such a study is of significance since

the nutritional conditions provided by the circulating animal body fluids approximate an iron-restricted state for *S. aureus* affected by the iron-chelating activity of their contained siderophilin [transferrin], (Schade, 1960). A more satisfactory understanding of the pathogenicity of *S. aureus* can be anticipated from the knowledge of its metabolic characteristics as they are determined by iron restriction, rather than from that derived from previous studies of organisms grown in media containing essentially excess iron. In the present work, comparative studies of the oxidative and glycolytic activities of suspensions of iron-rich and iron-poor *S. aureus* were made. In an attempt to determine the site of action of iron in the metabolism of these organisms, qualitative and quantitative analyses of the end products formed from various carbohydrate intermediates have been made.

METHODS

Organism. A penicillin resistant strain of *Staphylococcus aureus*, phage type 80/81, was used throughout. Stock cultures on nutrient agar slopes were kept in the cold and subcultured every 2 months.

Media. The basal medium used for the production of iron-rich and iron-deficient organisms contained: 2% (w/v) iron-depleted trypticase medium (0·01–0·02 μg. Fe/ml.); 1% (v/v) egg white (1·0 ml. egg white through its contained conalbumin binds about 20 μg. iron, Schade & Caroline, 1944); growth factor amounts of biotin (0·11 μg./ml.), nicotinic acid (0·05 μg./ml.), thiamine HCl (0·05 μg./ml.). When desired, glucose was added to the medium to a final concentration 0·2% (w/v). All media were initially at pH 7·6. To obtain conditions of restricted and unrestricted iron availability, iron as ferrous ammonium sulphate ($Fe(NH_4)_2(SO_4)_2.6H_2O$; Fisher C. P.) was added to the media at 0·01 and 0·228 μg./ml. The resulting percentages iron saturation of the egg-white conalbumin were 10–15 and 115–120 respectively. Procedures for cleaning glassware, removal of iron from the trypticase medium (Baltimore Biological Laboratories, 0·6–0·8 μg. Fe/ml.), and the preparation of various medium components were described in a previous paper (Theodore & Schade, 1965). Media were dispensed in 500 ml. batches in 2 l. screw-capped Erlenmeyer flasks. Seed cultures were grown at 37° for 12 hr in iron-depleted trypticase (2%) medium without egg white or glucose.

Preparation of washed coccal suspensions. For manometric studies and for determining products of glycolysis and oxidation of substrates, suspensions of *Staphylococcus aureus* were prepared from log-phase cocci grown in iron-deficient and iron-enriched media, in absence or presence of glucose. Organisms grown in these media will be referred to herein as: Fe– G– cocci, Fe+ G– cocci, Fe– G+ cocci and Fe+ G+ cocci, respectively. Five hundred ml. of the appropriate media were inoculated with 0·3 ml. of a 1/100 dilution of the seed culture which had an extinction $E^{1\cdot0\,cm}_{600\,m\mu}$ of approximately 0·3, and incubated at 37° on a rotary shaker. The incubation times and the final pH values of the cultures grown in the four media and harvested while still in their logarithmic phase of growth were: (Fe– G–) 13 hr, pH 7·5; (Fe+ G–) 10 hr, pH 7·7; (Fe– G+) 12 hr, pH 7·5; (Fe+ G+) 9 hr, pH 6·5; respectively. At harvest the cultures were passed through Pyrex glass wool filtering fibre and centrifuged at 4° at 4000g for 20 min. Analyses for glucose in the supernatant fluids of all cultures were positive only in those from the high- and

low-Fe + glucose media. For oxygen uptake studies, cocci were washed twice with 0·1 M-phosphate buffer (pH 7·4) and resuspended in the same buffer. Organisms used for the measurement of glycolytic activity were washed twice and resuspended in 0·03 M-$NaHCO_3$ buffer (pH 7·4). For the manometric assay of catalase activity, the cocci were washed and resuspended in 0·85 % (w/v) NaCl. All suspensions were diluted to an extinction at 600 mμ appropriate to their intended use. Preliminary analytical studies established that a $E^{1\cdot 0\,cm}_{600\,m\mu}$ of 1·0 was equivalent to approximately 320 μg. dry wt. organism/ml. and 2×10^9 cocci/ml. of the suspension measured. These values were applicable to the organisms grown in all four media.

Analytical procedures. Standard Warburg manometric techniques were used for measuring O_2 uptake and CO_2 production (Umbreit, Burris, & Stauffer, 1957). The main chamber contained 2·0 ml. washed coccal suspension (equiv. 3·2 mg. dry wt.) and the substrates in the sidearms were added in 0·1 and 0·2 ml. amounts. The temperature was maintained at 37°. For analysis of the products of glycolysis and oxidation of the substrates used, duplicate Warburg vessels were shaken in the bath with the same proportion of cocci to substrate and were set up in conjunction with the manometric experiment. Following complete utilization of the substrate, as measured manometrically, the vessels were chilled and samples removed, centrifuged in the cold, and the supernatant fluids frozen and kept for analysis.

Catalase activity was assayed for manometrically (Schade, 1963). Iron analysis of the medium and the iron-binding capacity of the egg white were determined by the bound-iron and unsaturated iron-binding capacity methods of Schade, Oyama, Reinhart & Miller (1954). Glucose was determined by glucostat (Worthington Biochemical Corp., Freehold, New Jersey). Pyruvate remaining in the supernatant fluids was assayed as the 2,4-dinitrophenylhydrazone, with benzene extraction (Friedemann & Haugen, 1943); lactate was determined by the method of Barker & Summerson (1941); acetoin was measured by the method of Westerfeld (1945); acetate was determined enzymically by the method of Soodak (1957).

RESULTS

Oxidative activities of iron-rich and iron-poor Staphylococcus aureus

The oxidative capabilities of iron-rich and iron-poor staphylococci, when grown in the presence or absence of glucose, were measured with common glycolytic and Krebs cycle intermediates at non-limiting substrate concentrations (Table 1). Fe+ G – suspensions oxidized glucose, L-lactate, malate, D-lactate pyruvate, acetate and formate. In contrast, Fe – G – suspensions oxidized glucose, L-lactate and D-lactate at approximately one-half to one-third the rate of that of the Fe + G – cocci. Malate oxidation was greatly suppressed while pyruvate, acetate and formate were not utilized. Thus, it is clear that the growth of *Stavhylocossus aureus* in an iron-restricted medium resulted in organisms with impaired respiratory activity. When Fe+ G+ suspensions were similarly examined (Table 1), they oxidized glucose, L-lactate, D-lactate, pyruvate and formate at more or less severely decreased rates as compared with those found with Fe+ G – cocci; for example, the Q_{O_2} on pyruvate showed an eightfold difference. Fe – G+ suspensions were completely devoid of any activity on these same substates except for a very small rate of oxidation of glucose.

End product analysis studies on iron-rich and iron-poor suspensions grown in the absence of glucose

Since differences in oxidative rates were found as between suspensions of iron-rich and iron-poor staphylococci on various selected substrates, the effects of iron deficiency on the qualitative and quantitative results of such oxidations were studied. With limiting amounts of various substrates, total O_2 uptake and CO_2 production were determined and analyses for the accumulated end products were made. Figure 1 summarizes the manometric measurements of O_2 consumption by Fe+ G− and Fe− G− suspensions. Formate, acetate, succinate, malate, and to a

Table 1. *Rate of oxidation of various substrates by suspensions of iron-rich and iron-poor Staphylococcus aureus grown in the absence or presence of glucose*

Warburg vessels contained equiv. 3·2 mg. dry wt. cocci in 2·0 ml. 0·1 M-potassium phosphate buffer (pH 7·4); 25 μmole substrate were added from the side-arm; total volume 2·4 ml. The centre well contained 0·2 ml. 10 % (w/v) KOH; the gas phase was air. The Q_{O_2} values represent μl. O_2 consumed/hr./mg. dry wt. cocci, calculated on the maximum rate of oxygen consumption following a 10 min. initial equilibration period and corrected for the endogenous rate.

	Coccal suspension			
	Fe− G−	Fe+ G−	Fe− G+	Fe+ G+*
	Q_{O_2} values			
Substrate				
Endogenous	6	23	2	13
Glucose	101	316	23	104
L-Lactate	143	306	0	87
D-Lactate	33	105	0	22
Pyruvate	0	202	0	26
Acetate	0	124	0	0
Formate	0	124	0	63
Malate	7	58	0	0

* Fe− G−, Fe+ G−, Fe− G+, Fe+ G+ mean respectively, Fe poor without glucose; Fe rich, without glucose; Fe poor, with glucose; Fe rich, with glucose.

lesser extent citrate, were oxidized by Fe+ G− cocci. End-product analysis showed that formate and acetate were oxidized completely with 92 and 96 % of the carbon accounted for as CO_2, respectively (Table 2). Fe− G− coccal suspensions, as shown in Fig. 1 and Table 2, were unable to use any of these substrates. It appeared possible that the Fe− G− cocci contained all the enzymes necessary for the oxidation of the above substrates, but that one or more were present as apoenzymes which required free ionic iron for the completion of activation. To test this, suspensions of Fe− G− cocci were pre-incubated in Warburg vessels with 3·0 μg. iron for 30 min. at 37°; then formate and acetate were tipped into the suspensions. No O_2 uptake was observed.

Suspensions of Fe+ G− and Fe− G− cocci were examined for their ability to oxidize glucose and various intermediates of glycolysis (Fig. 2). Fe+ G− cocci oxidized glucose, L-lactate, D-lactate and pyruvate essentially to completion (Table 2). In all cases the only major end-product was CO_2. The difference in the amount of carbon initially provided and that accounted for as CO_2 produced

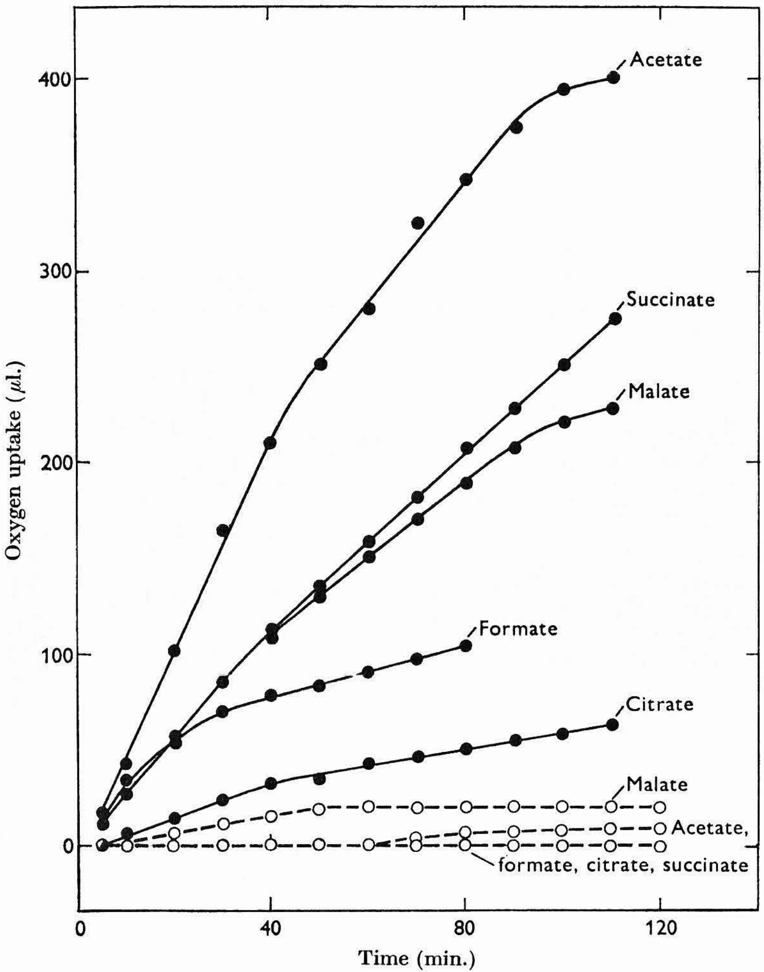

Fig. 1. Oxidation of various substrates by suspensions of iron-poor and iron-rich *Staphylococcus aureus* grown in the absence of glucose. -○--○--, Fe− G− cocci; —●—●—, Fe+ G− cocci. Experimental details as in Table 1; substrates used expressed as μmoles: acetate, 10; formate, 10; succinate, 5; malate, 5; and citrate, 5. Endogenous values subtracted.

Fig. 2. Same as Fig. 1; substrates employed expressed as μmoles; glucose, 5; L-lactate, 5; D-lactate, 5; pyruvate, 5. Endogenous values subtracted.

probably indicated assimilated carbon. Fe − G − cocci, on the other hand, oxidized glucose, L-lactate and D-lactate at a decreased rate and effected a much smaller total O_2 consumption by the time the utilization of added substrate had practically ceased (Fig. 2). Pyruvate, in contrast to the results obtained with Fe + G − cocci, was not oxidized. As calculated from the data in Table 2, the products of glucose oxidation by Fe − G − cocci as percentages of the initial carbon available were: CO_2, 46 %; acetate, 28 %; acetoin, 9 %. L-Lactate was oxidized to CO_2, 49 %, acetate, 18 %; and acetoin, 10 %. From D-lactate no acetoin was found, only CO_2, 45 % and acetate, 27 %. When acetoin was offered to high-Fe and to low-Fe cocci, none was utilized. Attempts to recover some part of the 17, 23, and 29 % of the unaccounted-for carbon from the glucose, L-lactate and D-lactate oxidations respectively, as acetaldehyde or pentose sugars, were unsuccessful. It is assumed that

Table 2. *Substrate dissimilation by suspensions of iron-rich and iron-poor Staphylococcus aureus grown in the absence of glucose**

Experimental details as for Figs. 1 and 2

Substrate	Organisms	Lactate	Pyruvate	Acetate	Acetoin	CO_2	O_2	R.Q.	% carbon recovered
Glucose†	Fe + G −	0	0	0	0	4·31	4·42	0·98	72
	Fe − G −	0	0	0·851	0·141	2·74	2·52	1·09	83
L-Lactate	Fe + G −	0	0	0	0	2·04	2·02	1·01	68
	Fe − G −	0	0	0·273	0·077	1·46	1·46	1·00	77
D-Lactate	Fe + G −	0	0	0	0	2·52	2·50	1·01	84
	Fe − G −	0	0	0·399	0	1·34	1·26	1·06	71
Pyruvate	Fe + G −	0·05	0	0	0	2·18	1·64	1·33	78
	Fe − G −	0	0·97	0	0	0	0	—	97
Acetate	Fe + G −	0	0	0·052	0	1·81	1·75	1·03	96
	Fe − G −	0	0	1·01	0	0	0	—	101
Formate	Fe + G −	0	0	0	0	0·92	0·56	1·70	92
	Fe − G −	0	0	0	0	0	0	—	—

* Results expressed as μmole/μmole substrate consumed. O_2 and CO_2 corrected for endogenous.
† No glucose detected in supernatant fluids.

the unrecovered carbon had been assimilated. The observed respiratory quotients of both iron-rich and iron-poor cocci (Table 2), when considered in conjunction with the oxidation state of the accumulated end-products, are in reasonable agreement with the calculated theoretical values based on complete oxidation of the substrate to CO_2 and water.

End-product analysis studies on iron-rich and iron-poor Staphylococcus aureus grown in the presence of glucose

Figure 3 records the O_2 uptake by Fe + G + and Fe − G + cocci on selected substrates. In contrast to Fe + G − cocci (Figs. 1, 2), Fe + G + cocci were unable to use acetate, malate, succinate or citrate. Glucose, L-lactate, D-lactate and pyruvate were oxidized at decreased rates and incompletely, while formate was oxidized to the same extent by Fe + G − cocci, but at a slower rate. With Fe − G + cocci only glucose was oxidized. The total O_2 consumed by these cocci was one-quarter that of the Fe − G − cocci.

Table 3 summarizes the end-product analyses of the substrates oxidized by Fe+ G+ and Fe− G+ cocci. The products of glucose oxidation by the Fe+ G+ cocci in percentages of initial carbon available were: CO_2, 38 %; acetate, 24 %; lactate, 8 %. L-Lactate was oxidized to CO_2, 49 %, acetate, 18 %; and pyruvate, 11 %. Only 10 % of the D-lactate was used, with 40 % of this amount being converted to acetate and 40 % to CO_2. It is possible that D-lactate was not used at all but was contaminated with some L-lactate that was readily used. At the time of analysis, 46 % of the initially available pyruvate had been metabolized and was accounted for as CO_2, 40 %; acetate, 51 %; and lactate, 10 %. All the formate was utilized, with 88 % accounted for as CO_2. The end products of glucose oxidation by Fe− G+ cocci were: CO_2, 12 %; acetate, 12 %; lactate, 64 %; pyruvate, 10 %; acetoin, 11 %; at the time of analysis, no glucose remained.

Table 3. *Substrate dissimilation by suspensions of iron-rich and iron-poor Staphylococcus aureus grown in the presence of glucose**

Experimental details as for Fig. 3

Substrate	Organisms	Lactate	Pyruvate	Acetate	Acetoin	CO_2	O_2	R.Q.	% carbon recovered
Glucose	Fe+ G+	0·15	0	0·704	0	2·3	2·34	0·98	70
	Fe− G+	1·28	0·19	0·35	0·17	0·70	0·61	1·15	108
L-Lactate	Fe+ G+	0	0·112	0·274	0	1·48	1·52	0·97	79
	Fe− G+	1·01	0	0	0	0	0	—	101
D-Lactate	Fe+ G+	0·9	0	0·058	0	0·116	0·104	1·11	98
	Fe− G+	1·06	0	0	0	0	0		106
Pyruvate	Fe+ G+	0·05	0·537	0·351	0	0·56	0·46	1·37	101
	Fe− G+	0	1·00	0	0	0	0		100
Formate	Fe+ G+	0	0	0	0	0·88	0·58	1·52	88
	Fe− G+	0	0	0	0	0	0		

* Results expressed as μmole/μmole substrate consumed. O_2 and CO_2 corrected for endogenous.
† No glucose detected in supernatant fluids.

Glycolytic activity of iron-rich and iron-poor staphylococci

To determine the glycolytic activity of iron-rich and iron-poor *Staphylococcus aureus* when grown aerobically in the absence or presence of glucose, glucose fermentation studies were made (Fig. 4; Table 4). Fe+ G− cocci fermented glucose anaerobically at approximately one-third to one-quarter the rate of the Fe− G− cocci. When grown in the presence of glucose, iron-rich and iron-poor cocci both fermented this substrate at about the same rate and to the same extent. In all cases (Table 4), the products of glycolysis were mostly lactate (73–94 %), acetate (4–7 %) and traces of pyruvate. These results show that growth on glucose enhanced the glycolytic activity of iron-rich cocci while the activity of iron-poor cocci was high and essentially the same, irrespective of the presence or absence of glucose in the growth medium. It appears that the iron-poor cocci deficient in normal respiratory activity relied on anaerobic fermentation and thus showed high activities of glycolyzing enzymes even in the absence of glucose. The acetate found probably resulted from the dismutation of pyruvate. Studies on the pyruvate dismutation activity of these organisms grown in absence of glucose showed that iron-rich cocci were

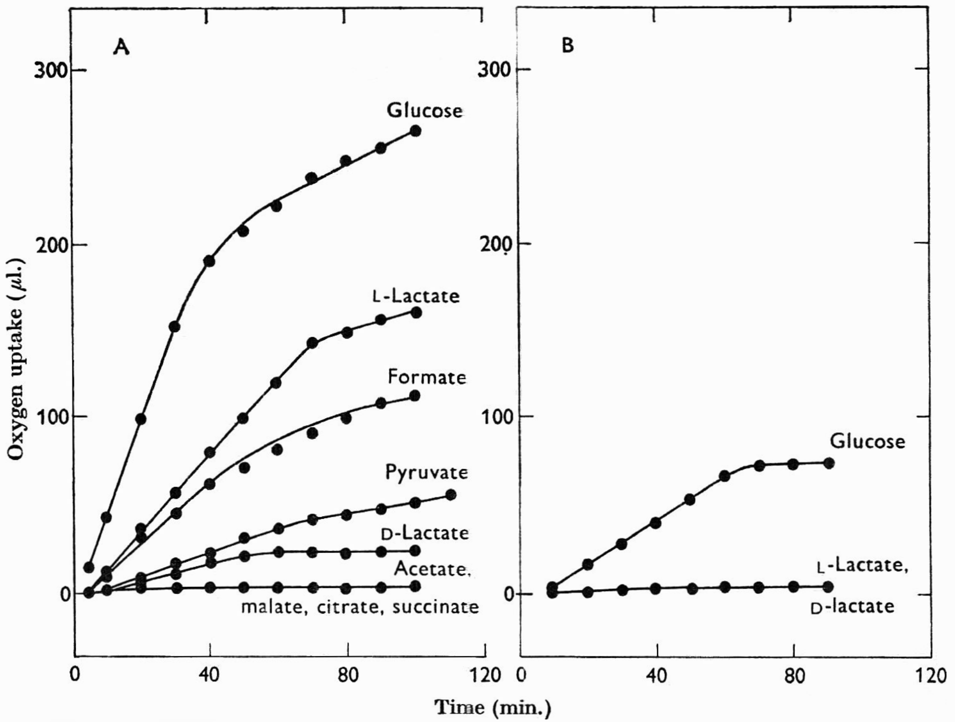

Fig. 3. Oxidation of various substrates by iron-rich and iron-poor *Staphylococcus aureus* grown in the presence of glucose. A, Fe+G+ cocci; B, Fe−G+ cocci. Experimental details as in Table 1; substrates used expressed as μmoles: glucose, 5; L-lactate, 5; D-lactate, 5; pyruvate, 5; succinate, 5; malate, 5; citrate, 5; acetate, 10; formate, 10. Endogenous values subtracted.

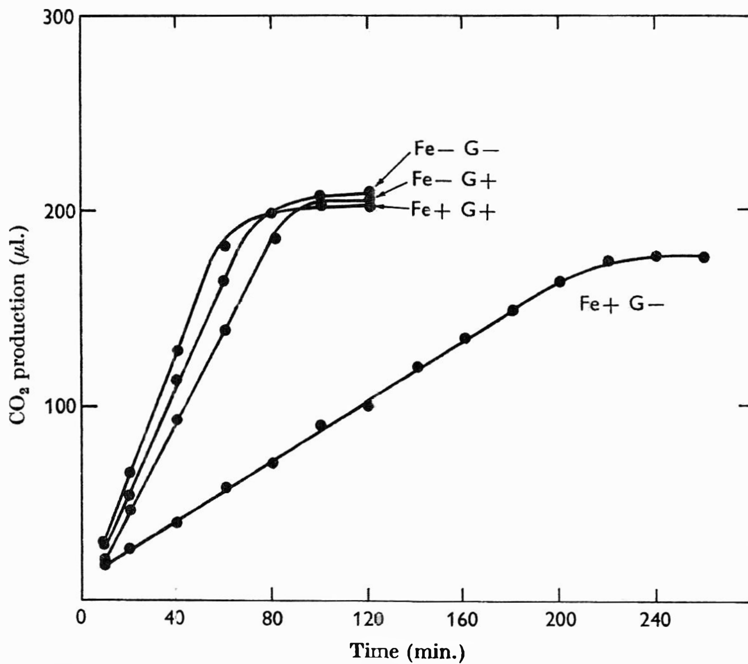

Fig. 4. Glycolytic activity by suspensions of iron-poor and iron-rich *Staphylococcus aureus* grown in the absence or presence of glucose. The experimental details are as described in Table 4.

three times as active as the iron-poor cocci (Q of high-Fe = 99 *versus* Q of low-Fe = 31, where Q = μl. CO_2/hr/mg. dry wt. organism).

Catalase activity of iron-rich and iron-poor cocci

When iron-rich and iron-poor staphylococci grown in the absence of glucose were compared for their relative catalase activities, Fe− G− cocci (Q = 1·3) were practically devoid of this iron-containing enzyme while Fe+ G− cocci (Q = 43) as expected were relatively rich. Of particular interest was the apparent inhibitory effect that glucose had on the catalase activity of organisms grown in the medium containing high-Fe+glucose. Fe+ G+ cocci (Q = 8·6) possessed only 20 % of the activity of cocci grown in the absence of glucose. Fe− G+ cocci (Q = 2·5), like the Fe− G− cocci, were practically devoid of any catalase activity (Q values = μ-moles O_2 produced/min./mg. dry wt. cocci).

Table 4. *Rate of glycolysis and products of glucose dissimilation by suspensions of iron-poor and iron-rich Staphylococcus aureus grown aerobically in the absence or presence of glucose**

Warburg vessels contained equiv. 3·2 mg. dry wt. cocci in 2·0 ml. 0·03 M-$NaHCO_3$, (pH 7·4); 5 μmole of glucose were added from the sidearm; total volume 2·1 ml. The gas phase was 90 % (v/v) N_2+10 % (v/v) CO_2. Temp. 37°. Q_{O_2} values represent μl. CO_2 produced/hr/mg. dry wt. cocci.

Organisms	Q_{O_2} values	Lactate	Pyruvate	Acetate	Acetoin	% carbon recovered
Fe− G−	58	1·78	0·02	0·13	0	94
Fe+ G−	16	1·45	0·02	0·21	0	81
Fe− G+	44	1·88	0·01	0·15	0	102
Fe+ G+	63	1·65	0·02	0·18	0	90

* Results expressed as μmole/μmole glucose fermented.

DISCUSSION

Theodore & Schade (1965) showed that the growth rate of a strain of *Staphylococcus aureus* 80/81 in iron-restricted trypticase media was dependent on the amount of free ionic iron available to the organism. Addition of glucose to such media at all concentrations of iron investigated increased further the logarithmic growth rate. The responses of these bacteria to iron and glucose additions made investigation of their metabolic characteristics of especial interest since their growth-rate patterns followed those of the same strain grown in human serum at various concentrations of siderophilin-iron saturation (Schade, 1963). Comparison of the metabolic activities of iron-rich and iron-poor staphylococci grown in the absence of glucose showed the high oxidative capacity of Fe+ G− cocci and the decreased and impaired oxidative capacity of the Fe− G− cocci. In some respects, these results are similar to findings of Waring & Werkman (1944) and Webley, Duff & Anderson (1962) who studied the metabolic activities of iron-deficient glucose-grown *Aerobacter aerogenes* and *Nocardia opaca*, respectively.

Suspensions of Fe+ G− *Staphylococcus aureus* oxidized glucose, L-lactate, D-lactate, pyruvate, acetate and formate completely to CO_2 and water at rapid rates. Krebs cycle intermediates (malate, succinate) were also rapidly oxidized, apparently to completion. Citrate was oxidized at a decreased rate in comparison to other

Krebs cycle intermediates, probably because of the impermeability of the cocci to this substrate (Stedman & Kravitz, 1955). Fe – G – staphylococci, however, oxidized only glucose, L-lactate and D-lactate, at decreased rates, and incompletely, in contrast to Fe + G – staphylococci. Pyruvate, acetate, formate and Krebs cycle intermediates were not used. The inability of these organisms to use pyruvate suggests that the pyruvic oxidase of this *S. aureus* may be iron dependent. O'Kane (1954) showed that the pyruvic oxidase of *Clostridium butyricum* required ferrous iron. The end-products from glucose and L-lactate metabolism by Fe – G – staphylococci were CO_2, acetate and acetoin, while only CO_2 and acetate resulted from D-lactate utilization. That pyruvate was not found as an end-product of these oxidations and was itself not used as a substrate suggested that either glucose and lactate are converted to acetate and CO_2 by a lactate oxidase system similar to that found in mycobacteria (Sutton, 1954) and not involving pyruvate; or that Fe – G – cocci are impermeable to exogenous pyruvate. To account, however, for the production of acetoin from glucose and L-lactate without pyruvate as an intermediary is not possible on the available evidence. Further, since the CO_2 produced from the oxidation of glucose and lactate was in considerable excess of that expected from the quantity of acetate found as end product, assuming that the path of such oxidations is via lactate to acetate and CO_2, and since acetate is demonstrably not oxidized, some other metabolic oxidative system must be operative in these organisms. The percentages of carbon recovered indicate some assimilation of these substances. The possibility exists that the excess CO_2 was derived from oxidation of an assimilated material.

The results obtained with iron-rich staphylococci when grown with and without glucose were similar to those found by Collins & Lascelles (1962), Gershanovitch, Palkina & Burd (1963), and Strasters & Winkler (1963) who showed that growth on glucose repressed the formation of enzymes required for the oxidation of acetate and tricarboxylic acid cycle intermediates. In the present work, Fe + G + staphylococci not only did not oxidize these substrates, but also produced acetate as an end product from glucose, L-lactate and pyruvate. Fe – G – staphylococci, with their already impaired respiratory activity, were so inhibited by the presence of glucose that they did not oxidize L- or D-lactate and only slowly attacked glucose itself. The accumulation of large amounts of lactate from glucose oxidation suggested that there was increased reliance on anaerobic fermentation for energy production necessary to growth and maintenance of the Fe – G + staphylococci. From these results, it can be seen that the metabolic activity of *Staphylococcus aureus* was affected not only by the amount of available iron in the medium but also by the presence or absence of glucose. Since there are many examples in the literature of repression of enzyme synthesis by glucose, any studies of the metabolic capacities of organisms as affected by a particular nutrient, e.g. iron, must consider the possibility of misinterpretation of data when glucose has been used as the common carbon + energy source. For instance, Fe – G + staphylococci did not use L- or D-lactate and only slowly oxidized glucose with the accumulation of large amounts of lactate. These findings, considered by themselves, might have been ascribed to iron deficiency, however, they were actually due to glucose inhibition since Fe – G – staphylococci grown in the absence of glucose were able to oxidize L- and D-lactate and to oxidize glucose without accumulation of lactate.

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On the Function and Structure of the Septal Pore of *Polyporus rugulosus*

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SUMMARY

Micrographs of serial sections of *Polyporus rugulosus* Lev. show minute and regularly spaced pore-cap perforations which are of similar dimensions to nuclear membrane pores. The presence of two types of pore apparatus in Basidiomycetes is established, and suggestions are made about the function and evolutionary development of the septal pores of Basidiomycetes.

INTRODUCTION

The septal pores of Basidiomycetes have been studied by several authors; although the general structures reported are similar there are differences in detail and different interpretations for function of this apparatus have been given. Moore & McAlear (1962) studied five Basidiomycetes and suggested that the pore apparatus has an occluding function, allowing only humoral continuity; Gibardt (1961) seemed to be in agreement, calling the pore cap a 'verschlussband'. Bracker & Butler (1963) showed micrographs of mitochondria passing through the pore of *Rhizoctonia solani* Kühn, and tentatively concluded that even nuclei could pass through this pore. In the present work the pore structure of *Polyporus rugulosus* has been examined in serial sections, longitudinal and transverse. The results indicate a variation in the structure and function of the pore apparatus in Basidiomycetes.

METHODS

Polyporus rugulosus Lev. was isolated from decaying timber and cultured on sterile cellophan discs on corn meal agar. Material was fixed in an aqueous unbuffered 2%(w/v) $KMnO_4$ solution at room temperature for 30 min., dehydrated in a graded series of ethanol + water mixtures and embedded in Epon 812 (Luft, 1961). Sections were cut on a Reichert Ultra-microtome and stained with lead hydroxide (Feldman, 1962). The grids used were carbon coated, and the specimens were viewed with a Siemens Elmiskop I, operating at 80 kV.

RESULTS

The terminology used by Bracker & Butler (1963) is used in the present descriptions.

In Pl. 1, fig. 1, a nearly median section of a septal pore is seen; it seems clear that the pore cap is continuous with the endoplasmic reticulum (ER) which lies parallel to the cross-wall. Small interruptions can be seen in the cap, suggesting the presence of openings.

Plate 1, fig. 2, shows the layered cross-wall extending into the septal swelling, being separated from the actual pore only by the ectoplast which is resolved as a double layer. Bracker & Butler (1964) interpreted such a pore as a fully open one in which the septal swelling has been depressed during active cytoplasmic streaming. In Pl. 1, fig. 2, some membranous matter can be seen in the subcap matrix, and is most probably in the process of passing through the pore.

Serial sections of pore, sections parallel to hyphal axis. Only six of nine sections are shown here. Pl. 1, fig. 3, represents the peripheral region of the pore apparatus and interrupted strands of endoplasmic reticulum (ER) can be seen lying parallel on both sides of the cross-wall. In Pl. 1, fig. 4, the perforated pore cap begins to appear. The swelling on the cross-wall appears in Pl. 1, fig. 5, and a large area of the pore cap can now be seen on both sides.

In Pl. 2, fig. 6, the very regular arrangement of the pore-cap perforations can be seen, the openings are very regular in shape and size, being ±800 Å in diameter, and ±500–700 Å apart. In Pl. 2, fig. 7, the actual pore opening is beginning to appear; Pl. 2, fig. 8, represents a median section, where it can be seen that the pore cap is continuous with the endoplasmic reticulum (ER). The pore-cap perforations now appear as lighter regions alternating with darker ones.

Sections of pore apparatus, sections transverse to hyphal axis. The apical part of the dome-shaped pore cap is seen in transverse section in Pl. 3, fig. 9; the regular spacing and size of the pore-cap perforations is again evident. An interesting comparison can be made between the dimension of the pore-cap perforation and mitochondrion size in Pl. 3, fig. 10, which is a lower magnification of an adjacent serial section.

The interrupted nature of the endoplasmic reticulum which is continuous with the pore cap could not be understood in longitudinal sections. Plate 3, fig. 11, is a cross-section very near the cross-wall, and it includes the endoplasmic reticulum (ER) referred to above. This is clearly reticulated, consisting of narrow tubules. Lamellations can be seen in the pore swelling, and the double ectoplast lines the pore.

Nuclear membrane. An almost tangential section of the nuclear membrane (Pl. 3, fig. 12) reveals pores, the dimensions of which are almost identical with the pore-cap perforations.

DISCUSSION

The general structure of the pore apparatus of Basidiomycetes, as suggested by Gibardt (1961), Moore & McAlear (1962), and Bracker & Butler (1963), is confirmed in the present work for *Polyporus rugulosus*: the actual pore opening is lined by the ectoplast, a barrel-shaped septal swelling is present, the pore is covered by a dome-shaped pore cap on both sides of the cross-wall, and the pore cap is regarded as a specialized region of the endoplasmic reticulum.

The Polyporus type of pore cap (with its regular small perforations) will not allow the passage of organelles which are much bigger than 800 Å. In *Polyporus rugulosus* the mitochondria are remarkably large (Pl. 3, fig. 10) and one cannot visualize the movement of mitochondria through the pore. This was confirmed by studying vitally stained mycelia of this fungus and by phase microscopy. Rapid streaming was observed, mitochondria were recognized, but no migration of particles through the pore was seen. The pore cap of *Polyporus rugulosus* therefore acts

as a sieve, allowing only the migration of particles smaller than 800 Å or particles that can shrink or be compressed to this dimension. The pore-cap perforations are of almost the same diameter as the nuclear membrane pores. The pore cap therefore will maintain the same physical boundary between adjacent cells as that present between the nuclear sap and the cytoplasm. These minute pore-cap perforations can also be seen in the pore cap of *Polystictus versicolor* in the micrographs of Gibardt (1961). Some preliminary work has shown similar perforations in *Poria monticola*. These genera all belong to the Polyporaceae. Nuclear migration is obviously impossible through an intact Polyporus-type pore. It is suggested that in such Basidiomycetes functional diploidy is maintained, while humoral continuity exists between different cells. These results are in agreement with those of Moore & McAlear (1962) and Gibardt (1961).

In comparison, the situation in Rhizoctonia (Bracker & Butler, 1963) is very different: the perforations in the pore cap are irregular and much larger, while the mitochondria are smaller. The migration of mitochondria shown in the micrographs of Bracker & Butler (1963) can be confirmed by phase-contrast microscopy. In Rhizoctonia even the actual pore opening is larger, and in this Basidiomycete the pore cap does not have an occluding function; if nuclear migration occurs the fungus is not even a functional diploid. The Rhizoctonia-type of pore cap has recently also been shown to occur in *Exidia nucleata* (Wells, 1964).

There are therefore two types of pore apparatus in Basidiomycetes and although the present data are very inadequate, it would appear that modifications of septal pore structure within this group of fungi may have phylogenetic significance. At this stage it is impossible to say which type preceded the other; more information is necessary, including more about the possible function of the Rhizoctonia-type pore cap. If the function of the Rhizoctonia-type pore cap is indeed protection of a 'delicate' pore swelling (and we do not know whether the pore swelling is delicate), one might regard this as an initial evolutionary development. The Polyporus-type pore cap could then be interpreted as a further differentiation of the pore apparatus. It is tempting to believe this hypothesis because the Polyporaceae are considered by mycologists to be a much more advanced group than fungi like Rhizoctonia and Exidia.

Bracker & Butler (1963) suggested that the actual pore opening can be increased during rapid cytoplasmic streaming as a result of the depression of the septal pore swelling. One can understand such a depression of the pore swelling when mitochondria (or nuclei) pass through a pore and in doing so exert lateral pressure on the swelling, but in Polyporus the pore cap acts as a sieve which allows only the migration of particles much smaller than the actual pore opening. In Polyporus pores can be seen which would be interpreted by Bracker & Butler (1963) as widened pores, but we cannot visualize hydrodynamic forces which will exert unilateral forces to cause the lateral depression of the pore swelling. From some observations Bracker & Butler (1963) concluded that the septal swelling was of different chemical nature than the cross-wall, but nothing more is known about the substance of the pore swelling. We consider that septal pore swelling variation as due to rapid streaming to be unlikely. It may be due to structural variation, but it must be appreciated that the swelling will appear different in median and near-median sections.

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EXPLANATION OF PLATES

E, ectoplast; ER, endoplasmic reticulum; G, compound lipid granule; Mi, mitochondrion; N, nucleus; NM, nuclear membrene; NP, nuclear pore; PC, pore cap; PCP, pore cap pore; S, septal pore swelling; SP, septal pore; V, vacuole; XW, cross-wall.

PLATE 1

Fig. 1. Hypha with septum, section not median thus actual pore opening not showing. Small pores are apparent in the dark pore cap which is continuous with the endoplasmic reticulum. Compound granules (G), thought to be of lipid nature, are present in the cytoplasm. × 12,000.

Fig. 2. Median section of 'fully open' pore. Lines and numbers refer to figs. 3–8. × 60,000.

Figs. 3–5. These are the first three sections through the septal pore apparatus. Position of the sections are indicated in fig. 2. × 60,000.

PLATE 2

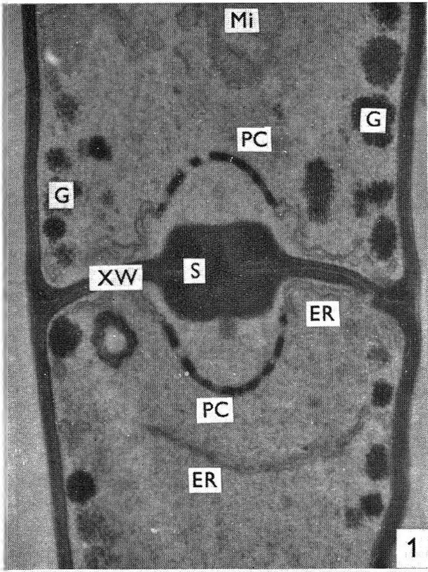
Figs. 6–8. These are the following three serial sections, omitting a section between figs 6 and 7. Approximate position of sections indicated in Pl. 1, fig. 2. × 60,000. The pore cap is thus shown to be a dome-shaped cap extending over the pore swelling, and having perforations of very regular size and distribution.

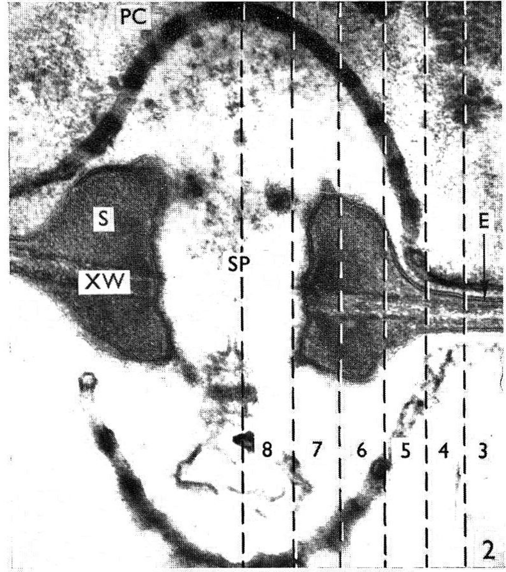
PLATE 3

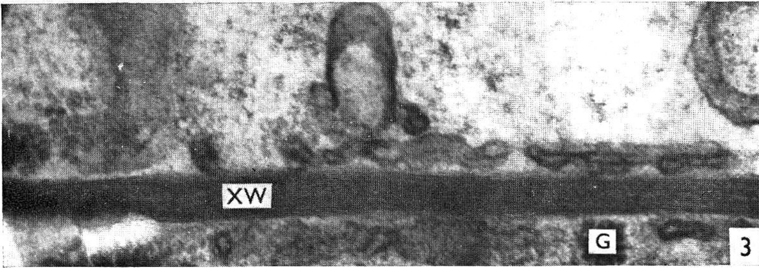
Figs. 9, 10. Serial sections at different magnifications of the pore cap, transverse to hyphal axis. Note difference in size between pore cap perforations and mitochondria. Fig. 9, × 60,000; fig. 10, × 36,000.

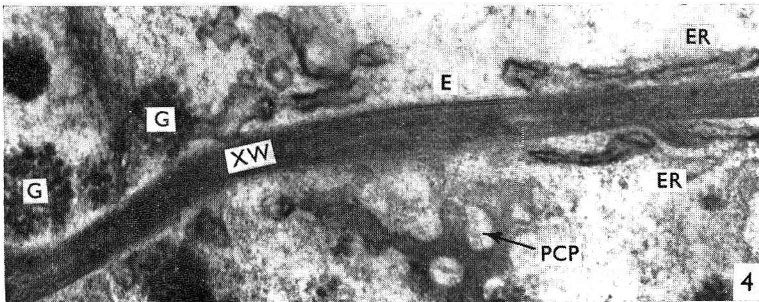
Fig. 11. A transverse section very close to the cross-wall including the endoplasmic reticulum which is continuous with the pore cap. × 60,000.

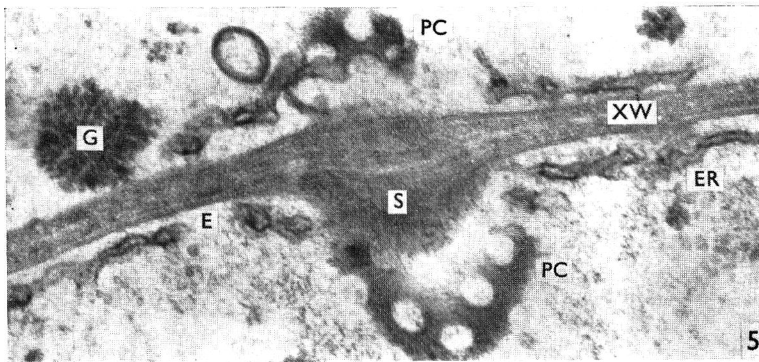
Fig. 12. An almost tangential section of the nuclear membrane. Compare size of nuclear pores with pore-cap pores in fig. 9. × 60,000.











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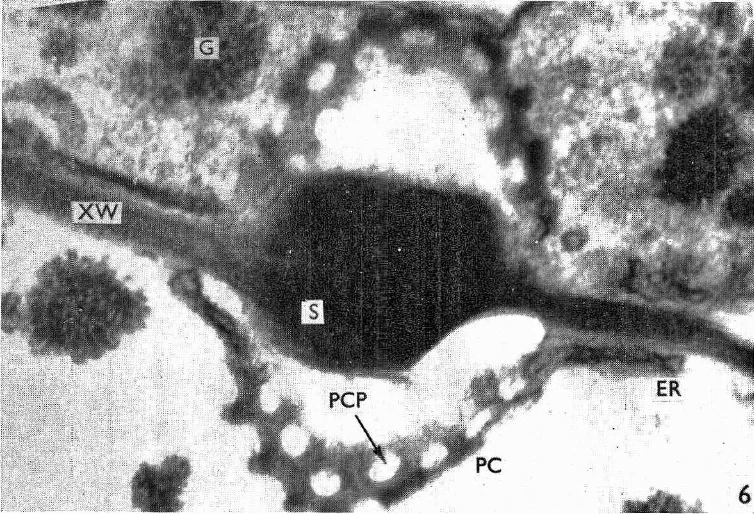

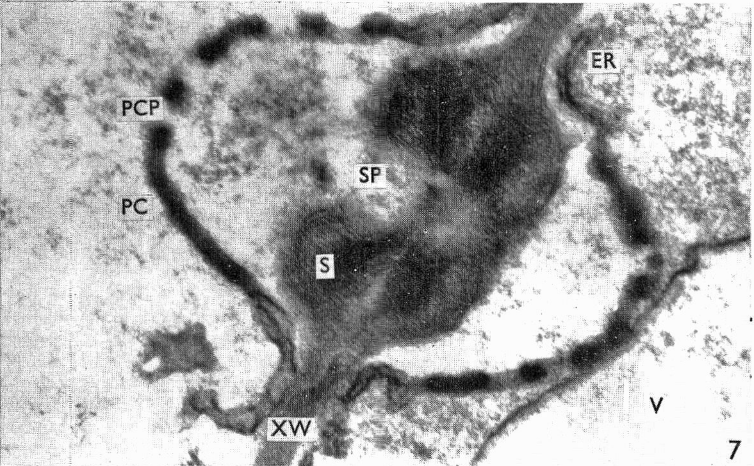

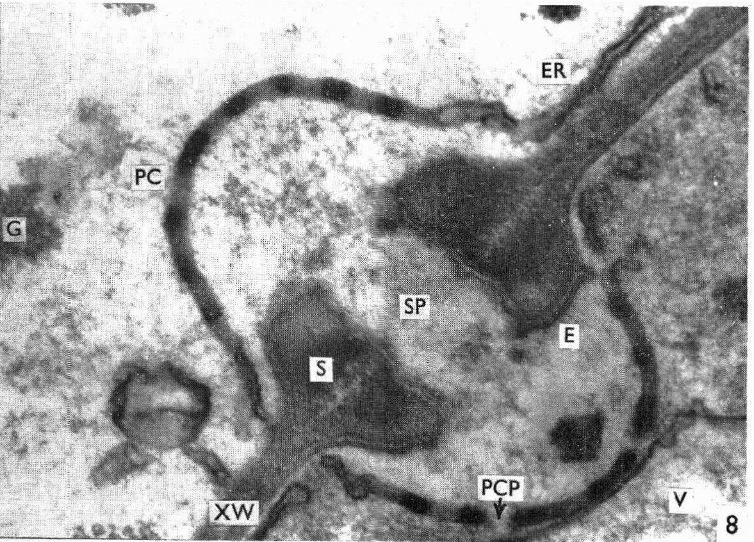

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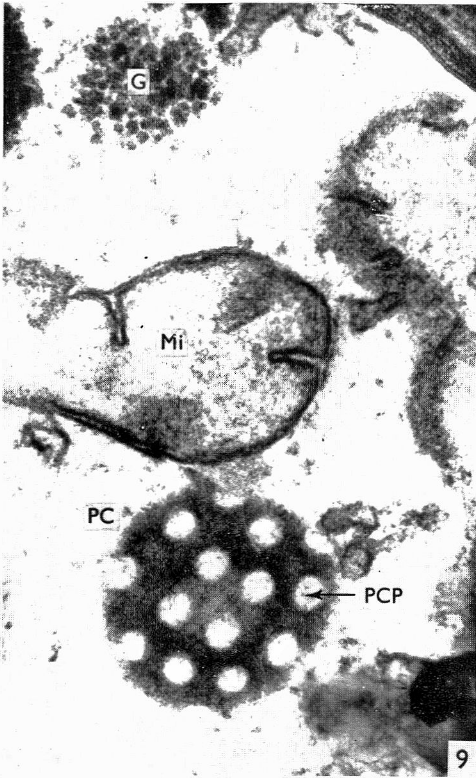

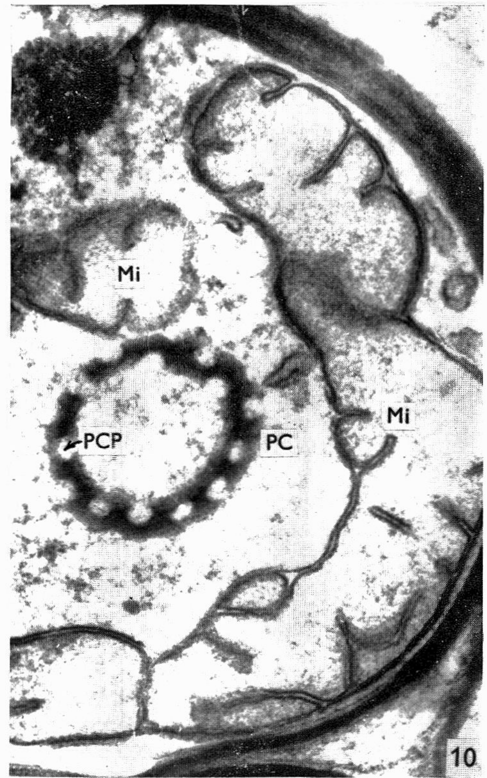

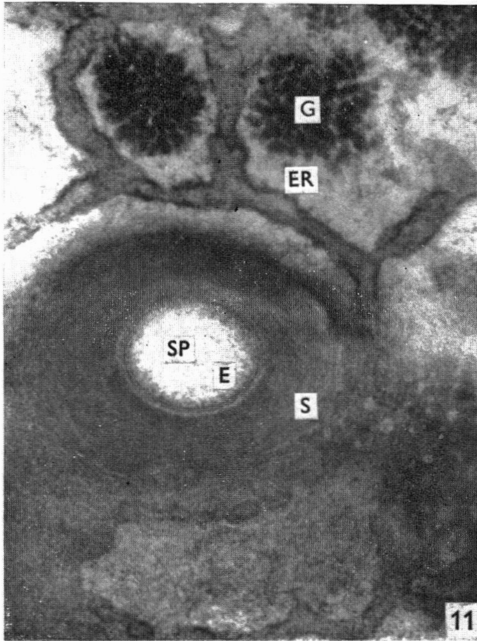

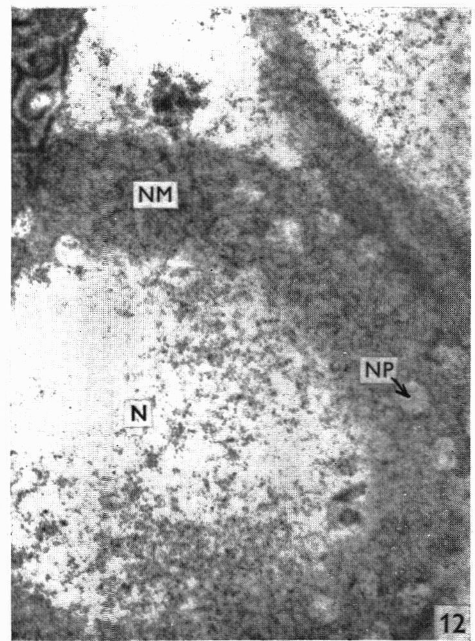

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The Role of Lomasomes in Wall Formation in *Penicillium vermiculatum*

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SUMMARY

When ascospores are formed within the ascus each nucleus and associated cytoplasm becomes enclosed by a cell wall. The origin of this wall is traced in a soft cleistothecial species, *Penicillium vermiculatum* Dangeard. Asci in this fungus arise by budding, and some aspects of bud-wall formation are recorded here.

INTRODUCTION

Very little is known about the formation of the ascospore wall and its subsequent thickening. Moore & McAlear (1962) reported the origin of the wall as the appearance of a thin electron-transparent shell which subsequently becomes thickened. Moore (1963) gave a more detailed account of ascospore wall thickening, associating the process with the presence of a 'peripheral net' of endoplasmic reticulum against the ascus wall, but gave no account of the initial stages. Because lomasomes have always been observed next to cell walls, we considered the possibility that they might play a role in wall formation. Moore & McAlear (1961), however, remarked on the absence of lomasomes from ascospores, and in the micrographs of Moore (1963) no lomasomes can be seen. If this be true then the role of the lomasomes in wall formation must be excluded. The present study is a re-investigation of the developmental stages of ascospore walls. New wall development during budding was also followed.

METHOD

Penicillium vermiculatum Dangeard, isolated from soil near Johannesburg, was grown on sterile cellophan discs on corn meal agar in a Petri dish. Maturing yellow cleistothecia were fixed and prepared for electron microscopy as described elsewhere (Wilsenach & Kessel, 1965).

RESULTS

Asci at different stages of development and a number of mature liberated ascospores can be seen with associated nutritive hyphae enclosed by a cleistothecial wall formed by densely packed hyphae in Pl. 1, fig. 1. The existence of all the different stages within a single cleistothecium facilitated the study of developmental changes.

Early bud formation can be seen as an infolding of the wall (Pl. 1, fig. 2). There is evidence that final cytokinesis is not accomplished by this infolding, but a new cell-wall (NBW) is laid down spanning the gap between the infolding walls (IBW) (Pl. 1, fig. 3). A lomasome can be seen on both sides of the newly formed bud wall (Pl. 1, fig. 4).

Within the young ascus numerous dark granules (G) which appear to have a compound structure can be seen (Pl. 2, figs. 5, 6). These seem to represent a recently formed metabolic product since their appearance is considerably less in earlier stages (see Pl. 1, fig. 2). The dense appearance of these granules after $KMnO_4$ fixation suggests that they were of lipid nature; and this was confirmed by Sudan IV staining of living mycelium. Their relative abundance decreased sharply during the final stages of ascospore differentiation, during significant increase in ascospore wall thickness (see Pl. 2, figs. 7, 8). It is therefore suggested that this lipid material is incorporated in the ascospore wall.

Ascospore wall formation

Before wall formation takes place the primary ascus nucleus undergoes three divisions, resulting in 8 nuclei. The first evidence of future wall formation is a sheath consisting of a double membrane which becomes orientated around each nucleus and adjacent cytoplasm (Pl. 2, fig. 6). At earlier stages only strands of endoplasmic reticulum (ER) are present in the cytoplasm (Pl. 2, fig. 5). Before this membrane system (interpreted as endoplasmic reticulum ER) forms a complete sheath, portions of it become swollen (Pl. 3, fig. 9). Very soon after this small vesicles accumulate in the swollen part, which now appears like a lomasome (Pl. 3, fig. 10).

Cell-wall material then begins to accumulate between the two membranes which become pushed apart, the wall at this stage consists of three layers, a thin central electron-dense layer bordered on either side by lighter regions. Lomasomes are now very prominent and they appear between the two membranes, inside the dark wall layer (Pl. 3, fig. 12). In Pl. 3, fig. 11, the relationship between lomasome and endoplasmic reticulum (ER) can clearly be seen. Both the dark layer and the inner lighter region increase in thickness (Pl. 2, fig. 7; Pl. 4, fig. 13). The ectoplast (or plasmalemma) of the ascospore protoplast is therefore derived from the endoplasmic reticulum ER, and so is the spore membrane. Further thickening of the wall is almost entirely limited to the lighter region, but the spines are extensions of the dark layer (Pl. 2, fig. 8; Pl. 4, fig. 14).

DISCUSSION

Lomasomes were first recorded by Moore & McAlear (1962) in nine different fungi from all the major groups, and were described as aggregates of vesicles which always appeared against the cell wall. No function was proposed for these organelles, and it was stated that they had not been observed in conidia or ascospores. Since this initial report, lomasomes have been described in other papers on fungal fine structure (Bracker & Butler, 1963; Gibardt, 1961), but a possible function is not known. These bodies were presumed to be exclusive to the fungi, although Manocha & Shaw (1964) claimed to have seen similar structures in the mesophyll cells of wheat.

The appearance of the lomasomes between the two membranes of the endoplasmic reticulum (reported for the first time in this paper) immediately precedes the formation of cell-wall material, which is also produced between these same two membranes. The suggestion is therefore made that lomasomes are associated with wall formation. This accounts for the initial appearance of the ascospore wall, which was not explained by Moore & McAlear (1962) or Moore (1963). The process of cell-wall formation described here differs from that described by Moore (1963) who

reported that a peripheral net of endoplasmic reticulum lies internally to the ectoplast and somehow contributed to the ascospore wall thickening. Moore (1963) could not explain the origin of the spore membrane and suggested that this membrane had a protective function. In the present study the origin of this membrane is explained and is not linked with the function of protection, but is seen as the remains of the endoplasmic reticulum which was involved in cell-wall formation.

Crawley (1965) and Barton (1965) published accounts of a cytoplasmic organelle associated with cell walls in *Chara* and *Nitella*, suggesting that its function is involved in wall formation. The structure of these organelles differs from lomasomes in that tubules are present (and not vesicles), and are continuous with the plasmalemma. Although structural differences are present, this organelle has the same topography and the same suggested function as lomasomes. These bodies appear to be homologous with the lomasomes of fungi. In higher plants it has been suggested that the Golgi bodies are involved in cell-plate formation; cell-wall material is said to be contained in the vesicles which subsequently coalesce to form the cell plate (Frey-Wyssling, Lopez-Saez & Mühlethaler, 1964). It is suggested here that the vesicles of the fungal lomasomes behave in a similar way and it is perhaps significant that Golgi bodies have not been observed in the fungi in which lomasomes have been found.

It is difficult to account for the observation by Moore & McAlear (1962) that lomasomes were not found in ascospores, unless they had disappeared or become less conspicuous when cell-wall thickening had ceased. Although the initial appearance of the lomasome in the endoplasmic reticulum seems contrary to earlier observations, its final position in the ascospore has the same topography as that described by previous authors, namely between the ectoplast and the cell wall. During active bud-wall formation the lomasome is seen in the typical position (Pl. 1, fig. 4). An interesting confirmation of the role of lomasomes in wall formation was provided by an anomalous chain of buds in which, in a particular ascus, mature ascospores were strangely lobed and multinucleate. In a younger adjacent ascus several nuclei are surrounded by a similarly shaped sheath of endoplasmic reticulum with associated conspicuous lomasomes (Pl. 4, fig. 15).

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EXPLANATION OF PLATES

Ascospore wall development and the associated role of lomasomes in *Penicillium vermiculatum* Dangeard. A, ascus; AS, ascopore; ASW, ascospore wall; AW, ascus wall; BW, bud wall; E, ectoplast; EP, epiplasm; ER,endoplasmic reticulum; G, compound lipid granules; IBW, infolding bud wall; Lo, lomasome; Mi, mitochondrion; N, nucleus; NBW, new bud wall; NM, nuclear membrane; P, initial ascospore envelope; SM, spore membrane; W, wall.

PLATE 1

Fig. 1. Segment through a maturing cleistothecium. The soft cleistothecial wall consists of dark hyphae. Mature and maturing asci can be seen. × 3900.

Fig. 2. Budding of ascogenous hypha. Notice infolding of the bud wall. Relatively few electron-dense granules are evident in the cytoplasm. × 12,000.

Fig. 3. Infolding of wall as seen at a later stage, with the new cell wall accomplishing final cytokinesis. × 60,000.

Fig. 4. Lomasome associated with the new bud wall. × 60,000.

PLATE 2

Fig. 5. Dividing nucleus in young ascus. Apparently the nuclear membrane does not disappear during cell division. Endoplasmic reticulum can be seen in the cytoplasm, and the dark granules have increased in abundance. × 13,000.

Fig. 6. Young 8-nucleated ascus. The young ascospore is delimited by endoplasmic reticulum, enclosing cytoplasm and nucleus. The nuclear membrane is not clear at this stage. × 12,000.

Fig. 7. Maturing ascus. The ascospore wall has increased in thickness, the nuclear membrane is clearer and mitochondria can be seen in the ascospore cytoplasm. A large lomasome can be seen associated with the wall of one ascospore. × 12,000.

Fig. 8. Mature ascus. The ascospore wall has become considerably thickened and shows the echinulations characteristic for this species. Notice the disappearance of the dark granules during maturation of the ascus. × 12,000.

PLATE 3

Details of different stages of ascospore wall formation.

Fig. 9. The initial ascospore envelope consists of endoplasmic reticulum (ER) which becomes swollen at places. Approx. × 60,000.

Fig. 10. At a later stage small vesicles appear in the swollen part of the ascospore envelope. This is interpreted as the origin of the lomasomes subsequently seen. × 60,000.

Fig. 11. Conspicuous lomasomes appearing between the two layers of the ER which is the ascospore envelope (P). × 60,000.

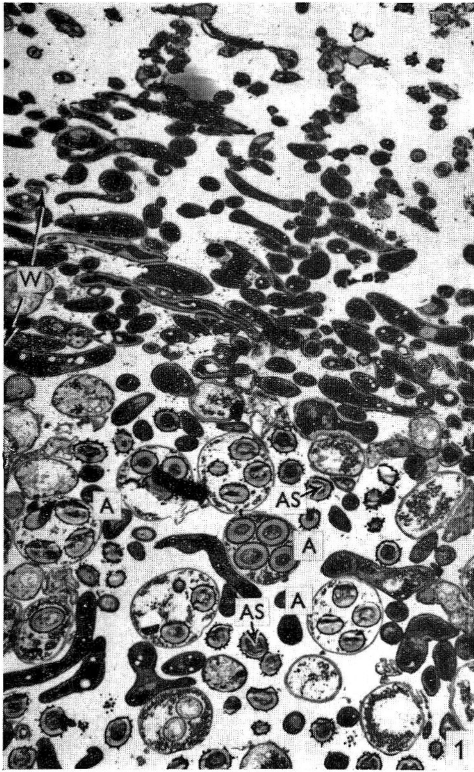
Fig. 12. Wall material is laid down between the two layers of ER. × 60,000.

PLATE 4

Fig. 13. The wall now consists of a thin outer light layer, a thick central dark layer and a thick inner light layer. These three layers can be seen in Fig. 12 and it is clear that the thickening occurs mainly in the dark and inner light layers. The outer membrane of the original ER is now the spore membrane (SM) and the inner layer, the ectoplast. The lomasome now occupies the typical position between the ectoplast and the cell wall. × 60,000.

Fig. 14. Mature ascospore wall showing the considerably thickened light layer. The echinulation appears to arise from the dark layer, and is bounded by the spore membrane (SM). × 60,000.

Fig. 15. Adjacent asci with the one showing a mature lobed ascospore and the other a similar shaped spore in an early stage of development with lomasomes conspicuous in the young ascospore envelope. × 12,000.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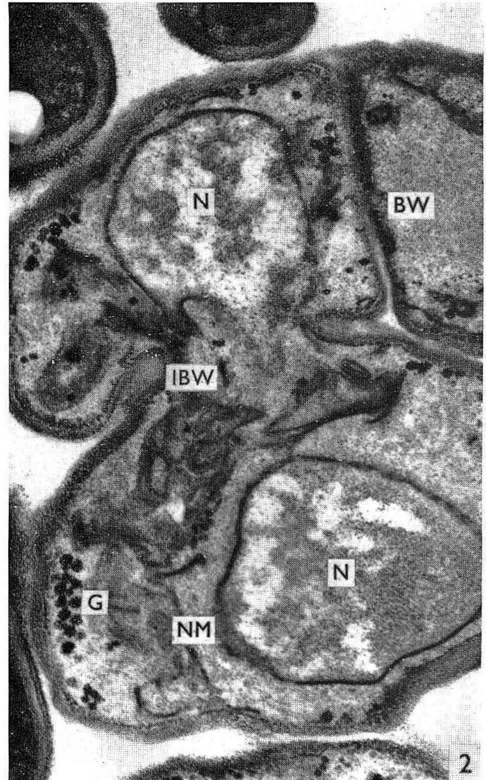
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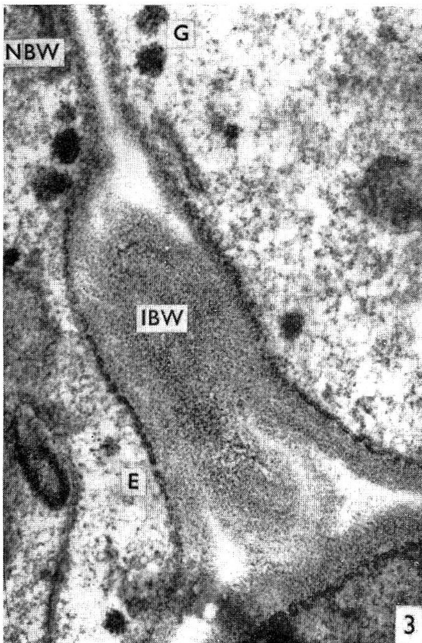
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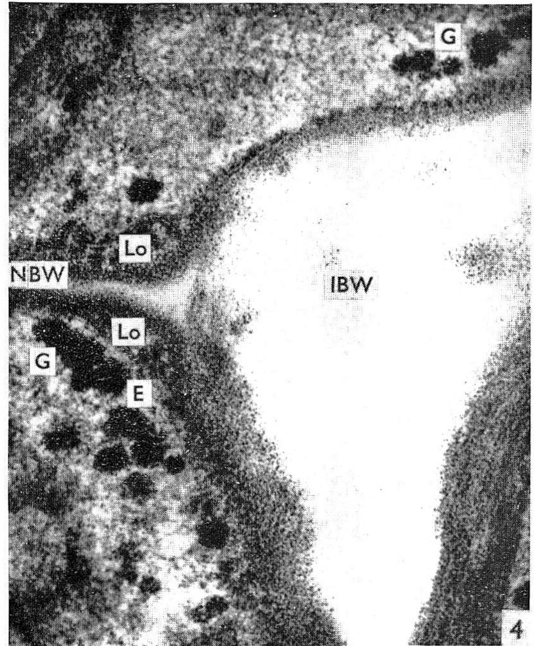
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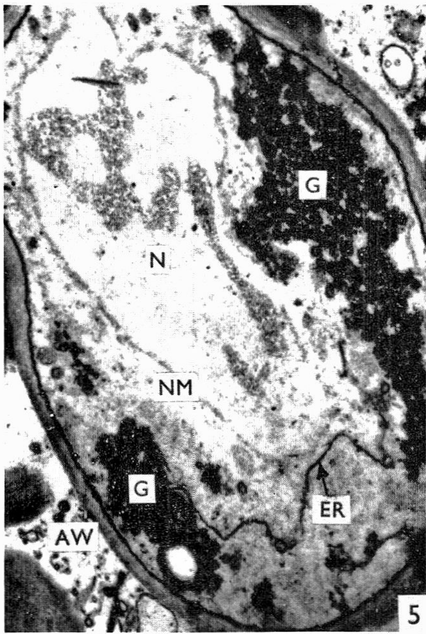

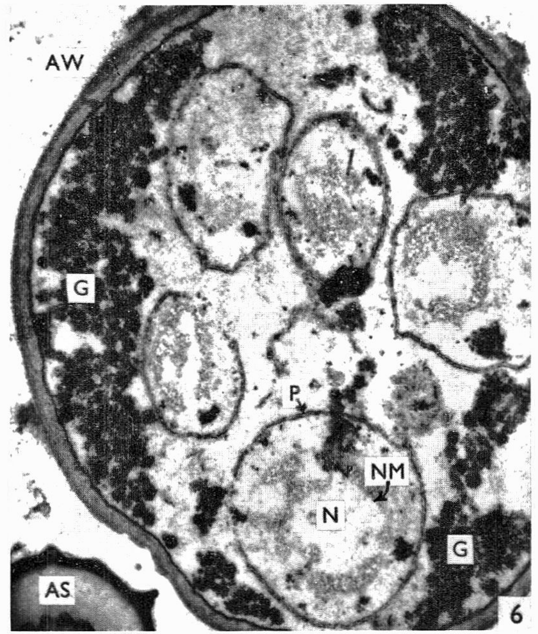

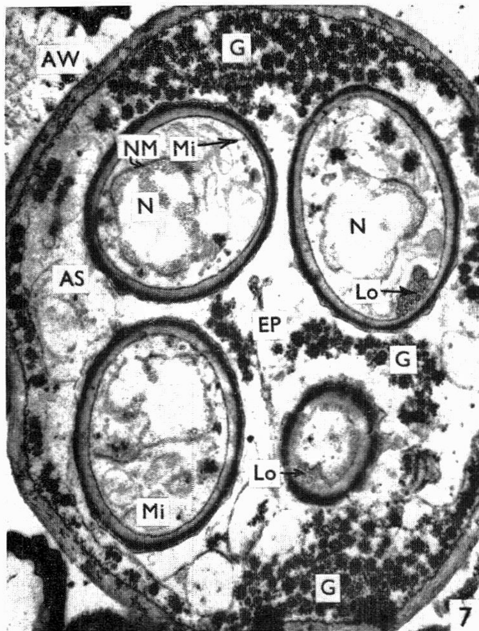

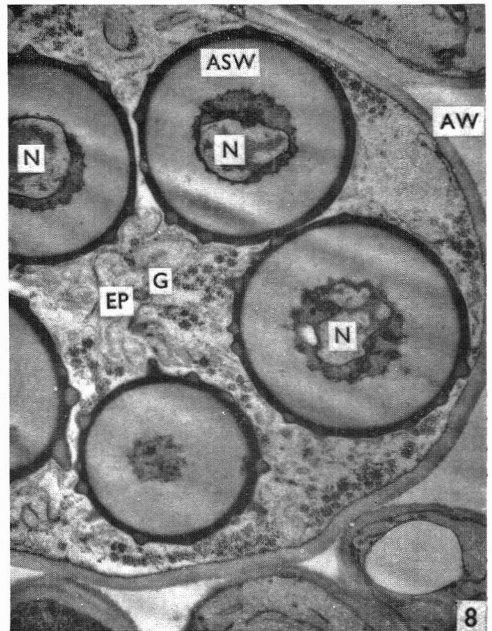

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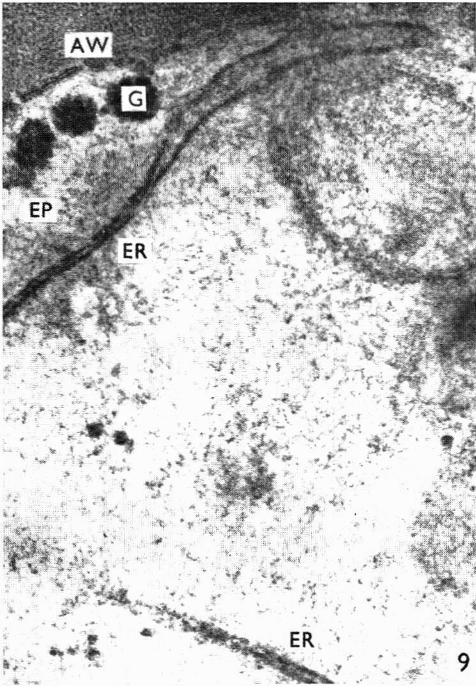

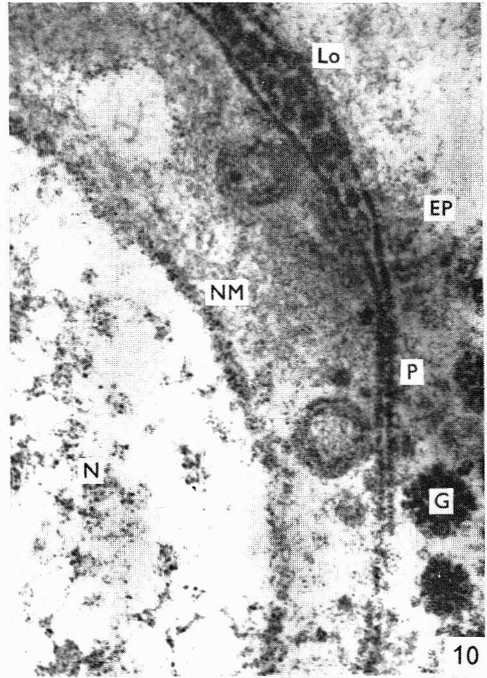

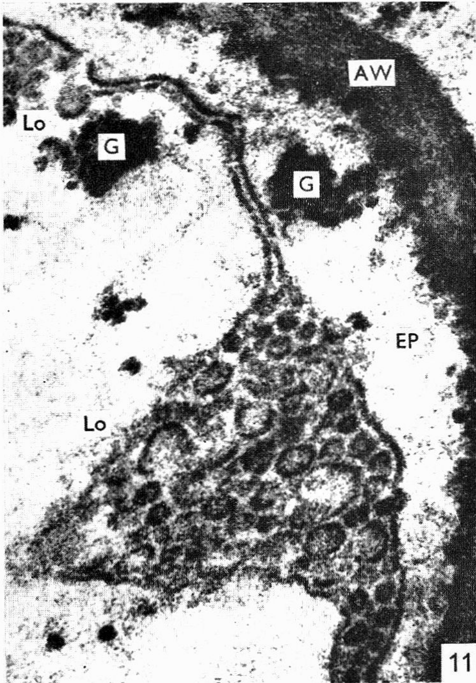

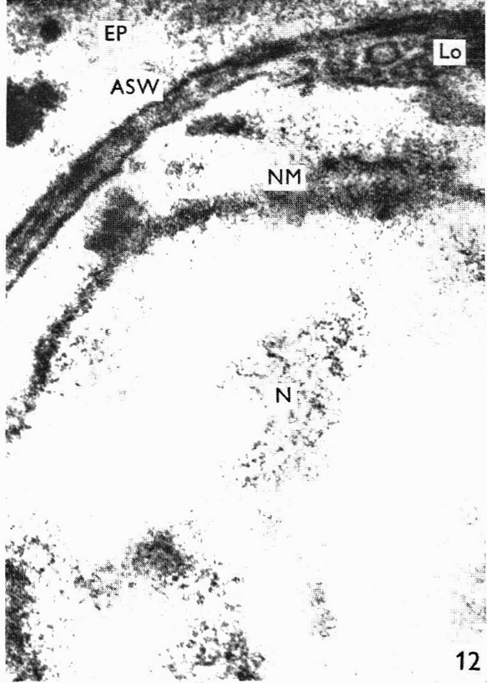

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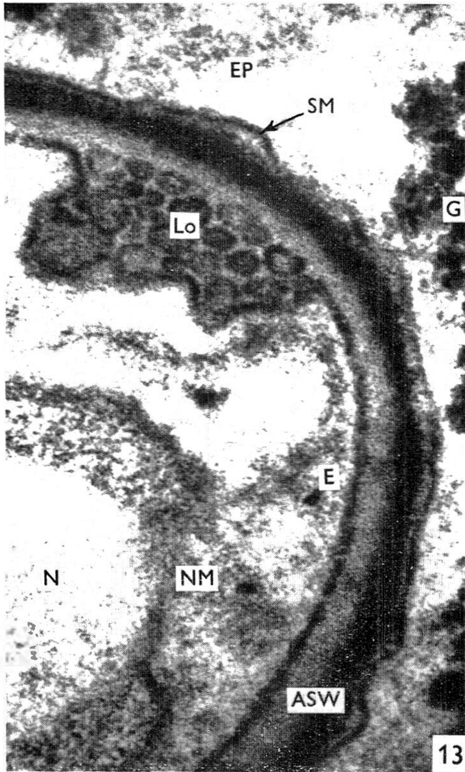

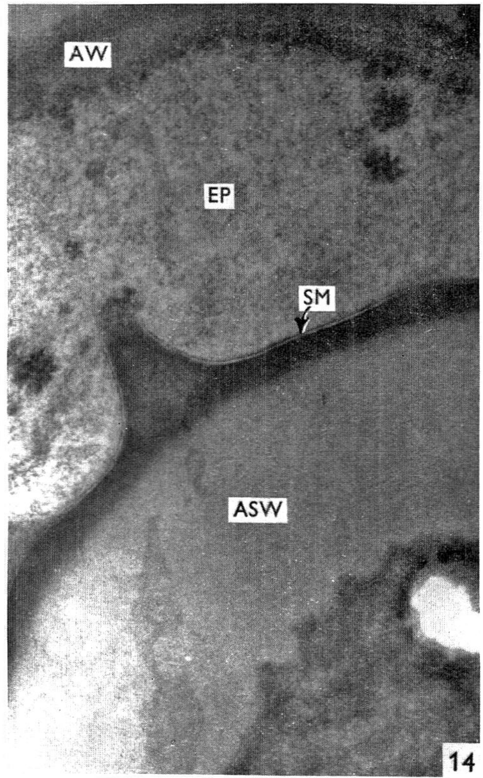

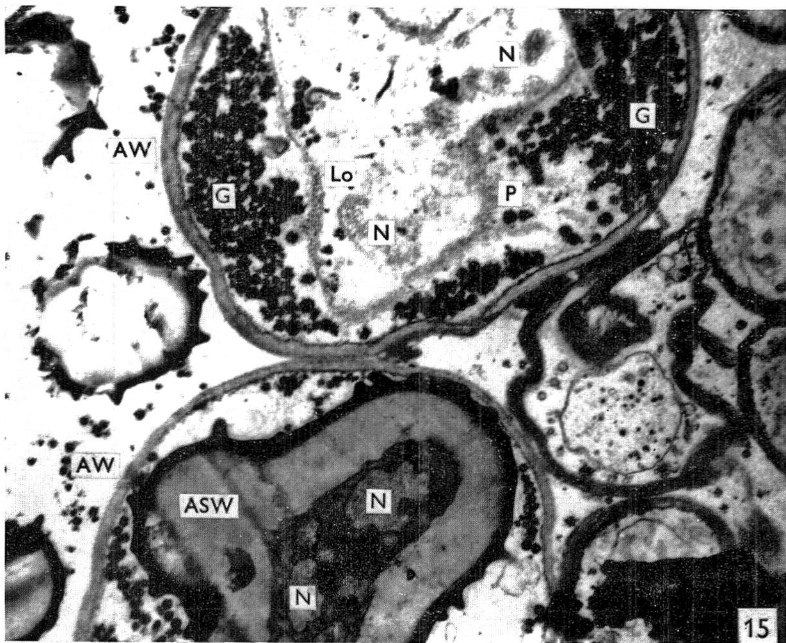

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The Chemical Composition of the Nucleic Acids and Other Macromolecular Constituents of *Mycoplasma mycoides* var. *capri*

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SUMMARY

Samples of deoxyribonucleic acid (DNA), microsomal ribonucleic acid (RNA) and a neutral polysaccharide from *Mycoplasma mycoides* var. *capri* were isolated and analysed. The DNA was found to have an adenine + thymine/guanine + cytosine value of 3·04, and the RNA to have an adenine + uracil/guanine + cytosine value of 1·21.

The amino acid composition of the protein of the organism was also determined and found to be in close agreement with the values expected by extrapolating the curves obtained from the amino acid contents of the protein of several bacteria having DNA's with a wide range of adenine + thymine/guanine + cytosine values (Sueoka, 1961). The values were also in close agreement with those obtained for the protein of *Tetrahymena pyriformis*, which has a DNA of similar base composition (A + T/G + C = 3·0). Two significant exceptions were noted: the value for proline was almost twice as large as would have been expected and a large amount of cysteine was found.

The mycoplasma organisms contained a very small amount of a neutral polysaccharide in which only glucose was detected.

INTRODUCTION

The information available about the chemical composition of the Mycoplasmatales shows that there was little difference between the strains tested and bacteria except for a lower nucleic acid content (Lynn & Smith, 1957), the presence of cholesterol (Lynn & Smith, 1960; Morowitz *et al.* 1962) and the evidence presented in a preliminary communication (Jones & Walker, 1963*a*) that the deoxyribonucleic acid (DNA) of *Mycoplasma mycoides* var. *capri* had an adenine + thymine/guanine + cytosine ratio of 3·00. This is, as far as we are aware, the highest value found for the DNA from any micro-organism. The present communication gives more details about the isolation and analysis of the DNA and describes the isolation and analysis of the RNA and a neutral polysaccharide. The amino acid composition of the total protein of the mycoplasma organism was also determined and compared with the values obtained by Sueoka (1961) for the amino acid content of the protein from several bacteria and a protozoon.

METHODS

Organism. The strain *Mycoplasma mycoides* var. *capri* P.G. 3 was obtained from Dr D. G. ff. Edward (Wellcome Research Laboratories, Beckenham, Kent).

Medium. The organism was grown in either of the following media: (1) 'normal

medium' containing Oxoid brain heart infusion (3·7 %, w/v), Burroughs Wellcome horse serum (1 %, w/v), penicillin (100 units/ml.), thallous acetate (0·01 %, w/v); (2) 'dilute medium' containing Oxoid brain heart infusion (0·45 %, w/v), Oxoid peptone (1·0 %, w/v), NaCl (0·5 %, w/v), sodium dihydrogen phosphate (0·25 %, w/v), Burroughs Wellcome horse serum (1·0 %, w/v), glucose (0·2 %, w/v), with penicillin and thallous acetate as before.

Total phosphorus was estimated by the method of Jones, Lee & Peacocke (1951).

Nitrogen was estimated by the method described by Jones & Walker (1963*b*).

Pentose was determined by the method of Euler & Hahn (1946) with a purified yeast nucleic acid preparation as a standard.

Deoxypentose was determined by the method of Dische (1955) with a purified calf thymus DNA preparation as a standard.

Purine and pyrimidine contents of nucleic acids. DNA samples were hydrolysed as described by Wyatt & Cohen (1953) and the bases determined as described by Wyatt (1951). RNA samples were hydrolysed and the bases determined as described by Markham & Smith (1951) after separating the bases in the solvent system described by Kirby (1955).

Amino acid composition of the proteins. Protein samples were hydrolysed by the method of Spackmann, Stein & Moore (1958), and the amino acid composition was analysed by a Technicon Automatic Amino Acid Analyser.

Hydrolysis of the polysaccharide. The polysaccharide was hydrolysed for 3 hr at 100° in M-sulphuric acid, and neutralized with barium hydroxide. The monosaccharide components were detected and identified chromatographically.

RESULTS

Growth and harvesting of organisms

Growth curves were obtained for the organisms when they were grown in the 'normal' and in the 'dilute' medium. The results showed that maximum opacity of the solution was obtained in the 'normal' medium after incubation for 18 hr, but about 36 hr were needed in the case of the 'dilute' medium. The maximum opacities reached in the two media were similar and the same yield of organisms was obtained from the same quantity of the two media. The organism was grown in 300 ml. medium in a 1 l. bottle which was inoculated with a 48 hr culture (2 ml.) and incubated at 37° for 18 hr in normal medium, or 42 hr in dilute medium. The organisms were harvested at 11,600 rev./min. in a continuous-flow refrigerated centrifuge (Measuring & Scientific Equipment Ltd.). When harvesting the organisms from the dilute medium it was found necessary to cool the suspension to 0° before centrifugation to prevent enzymic degradation of the nucleic acid. This was achieved by passing the suspension through polythene tubing (2000 cm. × 1 cm.) immersed in an ice + salt cooling mixture. No pre-cooling was necessary when the organism was grown in the normal medium.

Isolation of the RNA, DNA and a neutral polysaccharide

The organisms from 20 l. medium were added to a phenol solution saturated with water (100 ml.) and sodium *p*-aminosalicylate solution (6 %, w/v; 100 ml.) and the mixture shaken for 1 hr at 4°. The nucleic acids and polysaccharide were precipi-

tated from the aqueous layer by the addition of ethanol (3 vol.) and the phenol layer and cell debris were re-extracted with salicylate solution until no more material precipitable with 3 vol. of ethanol was obtained; usually five extractions were necessary. The combined ethanol precipitates were dissolved in water (100 ml.) and centrifuged at 105,000*g* (Spinco ultracentrifuge) for 30 min. to remove cell debris; the supernatant fluid was then made M with respect to NaCl and allowed to stand at 0° for 18 hr. The precipitate was removed by centrifugation at 105,000*g* for 30 min., washed with M-NaCl, dissolved in water, dialysed and freeze-dried to give RNA (20 mg., probably microsomal).

To the supernatant fluid from the RNA precipitate, ethanol (3 vol.) was added and the resulting precipitate dissolved in 0·1 M-NaCl. Cetyltrimethylammonium bromide (CTAB, British Drug Houses Ltd., 5 %, w/v) was added until precipitation of acidic material was complete and the precipitate centrifuged down. Ethanol (3 vol.) was added to the supernatant fluid to precipitate the neutral polysaccharide, which was centrifuged down, separated and dissolved in water. Contaminating CTAB was removed by shaking with chloroform (3 times), and the aqueous solution dialysed and freeze-dried to give the polysaccharide (3 mg.).

The material precipitated with CTAB was dissolved in M-NaCl (20 ml.), CTAB added to the solution so that its final concentration was 1 % (w/v), and the concentration of the NaCl decreased to 0·6 M by adding water (13·3 ml.), the temperature being kept at about 20°. The cetyltrimethylammonium salt of DNA which precipitated was removed and dissolved in M-NaCl, ethanol (3 vol.) was added and the resulting precipitate was dissolved in water, extracted with chloroform to remove cetyltrimethylammonium salts and the aqueous solution dialysed and freeze-dried to give DNA (16·5 mg.).

Composition of the DNA

The DNA was isolated in three separate experiments. The base contents of the samples are shown in Table 1. The RNA and protein contents of all samples were less than 1 %. Phosphorus content was 8·9 % (w/w), the nitrogen to phosphorus ratio 1·69 (w/w) and the extinction of a solution containing 1 g. atom phosphorus/l. at pH 7·0 was 7500 at 260 mμ.

Table 1. *Base composition of the DNA of Mycoplasma mycoides var. capri,* P.G. *3*

I and II DNA isolated from organisms grown on 'normal' medium. III DNA isolated from organisms grown on 'dilute' medium. Other bases, particularly 5-methylcytosine and 6-methylaminopurine, were not detected. The amounts were determined from the ultraviolet absorption of the eluate from paper chromatograms after hydrolysis of the DNA in formic acid. The amounts are given in moles base/100 g. atoms P (corrected to 100 % recovery of phosphorus; recovery was 90–95 %).

Isolation	Guanine (G)	Adenine (A)	Cytosine (C)	Thymine (T)	$\frac{A+T}{G+C}$
I	11·3	37·8	13·0	37·8	3·11
II	12·5	38·0	11·6	37·9	3·14
III	12·5	38·0	13·3	36·1	2·88
Average	12·1 ± 0·6	37·9 ± 0·1	12·6 ± 0·7	37·3 ± 0·8	3·04 ± 0·11

Composition of the RNA

The RNA was isolated in three separate experiments. The base contents of the samples are shown in Table 2.

Table 2. *Base composition of the RNA of Mycoplasma mycoides var. capri*, P.G. 3

All samples were from organisms grown in the dilute medium. The amounts were determined from the ultraviolet absorption of the eluate from paper chromatograms after hydrolysis of the RNA in hydrochloric acid. The amounts are given in moles base/100 g. atoms P (see Table 1).

Isolation	Guanine (G)	Adenine (A)	Cytosine (C)	Uracil (U)	$\frac{A+U}{G+C}$
I	25·7	29·8	19·8	24·7	1·20
II	25·5	31·0	19·4	24·1	1·23
III	25·9	30·3	19·2	24·5	1·21
Average	25·7 ± 0·2	30·4 ± 0·5	19·5 ± 0·2	24·5 ± 0·3	1·21 ± 0·1

G + U/A + C = 0·99; purine/pyrimidine = 1·27.

Table 3. *Amino acid composition of the total protein of Mycoplasma mycoides var. capri*, P.G. 3

The protein was hydrolysed with 5N-HCl *in vacuo* at 110° for 22 hr. The amino acid composition was analysed by a Technicon Automatic Amino Acid Analyser. The results are expressed in the same form as those quoted by Sueoka (1961) and his values for the amino acid composition of the protein of *Tetrahymena pyriformis* are given for comparison. Molar amounts of amino acids lysine, histidine, arginine, aspartic acid and asparagine, glutamic acid and glutamine, proline, alanine, valine, leucine, tyrosine and phenylalanine, which are known to be stable and to be well recoverable in the analysis, were summed. The other amino acids are classified as unstable amino acids. The amount of each amino acid both stable and unstable is expressed by its proportion to the sum of the stable amino acids.

Organism ...	*Mycoplasma mycoides* var. *capri.*	*Tetrahymena pyriformis*
G + C content of DNA (%) ...	24·7	25·0
Stable amino acids (average of 3 determinations)		
Lysine	14·0 ± 0·2	11·9
Histidine	3·0 ± 0·1	2·8
Arginine	4·3 ± 0·2	6·1
Aspartic acid	13·4 ± 0·1	16·5
Glutamic acid	16·2 ± 0·2	17·6
Proline	11·0 ± 0·2	5·1
Alanine	10·0 ± 0·2	10·3
Valine	7·2 ± 0·2	8·0
Leucine	11·8 ± 0·1	11·7
Tyrosine	3·6 ± 0·1	4·3
Phenylalanine	5·5 ± 0·1	6·0
Total	100·0	100·0
Unstable amino acids		
Glycine	6·0 ± 0·1	11·0
Threonine	6·8 ± 0·2	7·7
Serine	7·3 ± 0·2	8·1
Isoleucine	5·6 ± 0·2	8·8
Methionine	1·3 ± 0·3	1·6
Cysteine	2·7 ± 0·3	—
Total	29·9	37·2

Composition of the polysaccharide

The neutralized hydrolysate of the polysaccharide was added to chromatography papers, which were developed in three solvents. Apart from a trace of D-ribose from contaminating RNA only one spot was detected with the aniline hydrogen phthalate spray for reducing sugars (Partridge, 1949), and with the silver nitrate spray, which is a non-specific detecting reagent (Trevelyan, Procter & Harrison, 1950). The R_F values of the compound in the solvent systems were: in *n*-butanol + ethanol + water (4+1+5, by vol.) R_F 0·14 (R_F glucose 0·14); in *n*-butanol + acetic acid + water (4+1+5, by vol.) R_F 0·18 (R_F glucose 0·18); in pyridine + amyl alcohol + water (1+1+1, by vol.) R_F 0·35 (R_F glucose 0·35). The compound and the glucose marker gave identically coloured spots with the two detecting reagents, and the three solvent systems used ensured that all the possible hexoses would be separated.

Isolation of and determination of the amino acid content of the protein of Mycoplasma mycoides var. capri

Mycoplasma organisms which had been grown in 1 l. dilute medium were removed by centrifugation at 5000*g*, washed with physiological saline solution (3 times) and added to tris magnesium buffer (10^{-2} M-tris, 10^{-3} M-magnesium; pH 7·3; 10 ml.) and phenol saturated with water (10 ml.). The mixture was shaken for 1 hr, the phenol extracted with ether and the remaining aqueous suspension exhaustively dialysed. The protein suspension (0·5 ml.) was then heated in a sealed tube *in vacuo* with HCl (10 N; 0·5 ml.) at 110° for 22 hr. The resulting solution was filtered free from carbon, evaporated to dryness, dissolved in HCl (0·1 N; 2·5 ml.) and samples (0·25 ml.) taken for amino acid analysis. The results are given in Table 3; they are expressed in the same forms as those quoted by Sueoka (1961) and his values for the amino acid composition of the protein of *Tetrahymena pyriformis* are given for comparison.

DISCUSSION

In previous cases when base analyses have been quoted for the nucleic acids of the Mycoplasmatales (Lynn & Smith, 1957; Morowitz *et al.* 1962), the nucleic acids have either been analysed in the presence of other cellular constituents or degraded samples have been used. It has now been found possible to isolate samples of pure highly polymerized nucleic acids from *Mycoplasma mycoides* var. *capri*.

The expense of the normal medium was too great to allow the organism to be grown in the large volumes required to isolate sufficient nucleic acid to analyse (20 l.), so another (dilute) medium was found which gave the same yield of organism/l. medium but which contained only 12 % of the normal brain heart infusion content. With large volumes (20 l.) it was necessary to cool the suspension of organisms when grown in the dilute medium before centrifugation in order to prevent enzymic degradation during the time taken for the centrifugation. When this precaution was not taken degradation of the DNA occurred, so preventing precipitation of its cetyltrimethylammonium salt in 0·6 M-NaCl and hence preventing the separation of the DNA from the RNA.

The isolation and separation of the nucleic acids and neutral polysaccharide followed closely the method reported previously for *Serratia marcescens* (Jones & Walker, 1963*b*). As the Mycoplasmatales have no rigid cell wall the phenol was

sufficient to rupture the cells and to liberate the nucleic acids and polysaccharide into the aqueous layer and also to prevent enzymic degradation.

The base analysis of the DNA shows that it contained more adenine and thymine than any bacterial DNA so far analysed. The only DNA to have a comparable base analysis is that from the protozoon, *Tetrahymena pyriformis*. The A + T/G + C ratio of 3·04 for *Mycoplasma mycoides* var. *capri* P.G. 3 has recently been confirmed by caesium sulphate density centrifugation technique (Dr W. Szybalski, personal communication). As previously remarked (Jones & Walker, 1963*a*) it is very unlikely that *M. mycoides* var. *capri* is an L form of a bacterium the composition of whose DNA is known; the present analysis lends support to the idea that the Mycoplasmatales are a distinct group.

The RNA base analysis shows that the RNA also contained more adenine and uracil than do most RNA's, although the moles of 6-amino bases equal the number of moles of 6-keto bases. *Tetrahymena pyriformis* has an RNA with a similar composition (Scherbaum, 1957).

Sueoka (1961) found a correlation between the amino acid content of the proteins and the guanine + cytosine (G + C) content of the bacterial DNA's examined. The values obtained here for the amino acid composition of the protein of *Mycoplasma mycoides* var. *capri* agree with the values expected from the curves obtained by Sueoka (1961) and also for those obtained for *Tetrahymena pyriformis*, which has a DNA of almost identical base composition. In the list of stable amino acids, all fit the curves as well as or better than the values for *T. pyriformis*, with the exception of proline. Sueoka (1961) found that the amount of proline decreased with decreasing G + C content. The value obtained in the present work is almost twice the expected proline content of an organism with an extreme G + C-containing DNA. This difference might be attributed to the fact that in the present case the amino acid content of the whole organism was determined, whereas Sueoka used purified fractions and rejected cell wall and possibly cytoplasmic membrane as well. Although *M. mycoides* var. *capri* has no cell wall, the membrane constituents would be present, and these might have a high proline content. It is also possible that structures of this type may be produced in a way that does not depend directly on a DNA template (e.g. the capsule of *Bacillus anthracis*; Salton 1960).

It is difficult to show correlations between the values obtained for the unstable amino acids since the amounts of these which are present depend to a large extent on the nature of any material contaminating the protein and on the exact conditions of hydrolysis. The amount of glycine observed was rather low, but, since it is known that this amino acid can arise from the degradation of the bases in any contaminating nucleic acids (Fraser, 1957), no significance can be attached to this. The only other amino acid content to differ significantly from that in bacteria was that of cysteine, which is usually not detectable but was present in quite large amounts in the protein of *M. mycoides* var. *capri*.

The polysaccharide of a bovine pleuropneumonia-like organism was investigated by Plackett & Buttery (1958), who found a galactan comprising about 10 % of the dry weight of the organism. No evidence for the presence of galactose or any other sugar apart from glucose was found in the neutral polysaccharide fraction of *M. mycoides* var. *capri* examined in the present work and this polysaccharide only comprised about 0·5 % of the dry weight of the organisms.

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A Comparison by the Use of Gel Electrophoresis of Soluble Protein Components and Esterase Enzymes of some Group D Streptococci

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SUMMARY

Soluble cell constituents of strains of *Streptococcus faecalis*, *S. faecium* and *S. durans* were studied by electrophoresis in polyacrylamide gels followed by staining the gel to reveal the patterns of separated proteins and esterase enzymes. The patterns of proteins and of esterases found in *S. faecalis* differed from those in *S. faecium* and *S. durans*. Strains of *S. faecalis* differing in serotype and variety showed a very similar series of proteins, some differences in patterns of esterases were found, but similar patterns occurred in strains differing in type and variety. The major protein bands of *S. faecium* and *S. durans* differed in mobility from the major proteins of *S. faecalis*; different strains of *S. faecium* and *S. durans* showed some differences in other protein bands. Esterase activity of *S. faecium* and *S. durans* was weaker than that of *S. faecalis*, and in the case of most strains only faint esterase bands were detected. A strain of *S. faecium* which gave protein and esterase patterns different from those of the remaining strains, differed also in showing motility.

INTRODUCTION

Previous studies of the serology and biochemical reactions of Lancefield group D streptococci have provided a basis for differentiating *Streptococcus faecalis* and its varieties *zymogenes* and *liquefaciens* from *S. faecium* and *S. durans*, and there is evidence of a marked difference between the metabolism of *S. faecalis* and *S. faecium* (Deibel, 1964). To obtain better understanding of the relationships between these organisms, it was thought to be of interest to compare protein macromolecules present in the organisms. Electrophoresis in polyacrylamide gels enables a separation of macromolecules according to their charge and molecular size (Ornstein, 1964; Raymond, 1964). During initial work this technique was used to compare the patterns of separated soluble protein components of cell extracts. The use of starch gel electrophoresis to study multiple forms of esterase enzyme in extracts of *Bacillus thuringiensis* was reported by Norris (1964) to provide a method of characterizing strains; classification according to 'esterase pattern' showed close correlation with a serological classification. In the experiments reported here the study of esterases has been extended to the group D streptococci.

METHODS

Organisms. Table 1 shows the organisms used. Strains of *Streptococcus faecalis* and its varieties 'liquefaciens' and 'zymogenes' were studied; strains 30/39 and 30/82 were isolated by Dr E. M. Barnes from chicken caeca; the remainder were originally obtained from Dr M. E. Sharpe and were discussed in a recent publication (Sharpe, 1964). The *S. faecium* and *S. durans* strains were described by Barnes (1964) except for B631 which was obtained from Dr M. E. Sharpe. Definitive biochemical tests were confirmed by the methods of Barnes, Ingram & Ingram (1956).

Table 1. *Strains of group D Streptococcus studied*

Streptococcus faecalis			**Streptococcus faecium* and *S. durans*			
Serological type	Strain		Serological type	Strain	Serological type	Strain
1	D76	Var. liquefaciens	16	NH53	31	NH2
1	30/39	Var. liquefaciens	18	S98	32	NH4
1	GB112	Var. zymogenes	24	H24	33	P3
3	30/82	—	25	S748	34	P7
3	H69D5	Var. zymogenes	26	CH1	35	N/R/64
4	GB122	Var. liquefaciens	26	C3†	36	P1/12
4	C1	Var. zymogenes	27	G3	37	P6/4
5	N83	—	28	HGH511	38	P17/8‡
6	B65	—	29	P14/6	39	P16/5‡
8	1AS	—	30	GE4B	42	B631
40	D10	Var. liquefaciens	—	—	Not typed	5422
41	D15	Var. liquefaciens	—	—	—	—

* Most strains showed the characters of *S. faecium*.
† This strain was typical of *S. durans* and was β-haemolytic.
‡ These strains could be classed as *S. durans* because of their failure to ferment L(+)-arabinose and mannitol, but were not β-haemolytic.

Preparation of cell extracts. Cultures were grown for 16–20 hr at 37° in brain heart infusion broth (Difco Laboratories) with 800 ml. medium in 1 l. flasks, without agitation or aeration. The bacteria were harvested, washed and finally suspended in water and disrupted by one of two methods.

Method 1. Six g. of a thick suspension + 6 g. ballotini beads (grade 12) per tube were shaken in a Mickle disintegrator for 45 min. giving 50–80% disruption as judged by Gram-stained smears. Ballotini and some cell debris was separated by low-speed centrifugation. *Method* 2. Bacterial suspensions were frozen at $-20°$, then disrupted in an X-press (AB Biox. Box 235, Nacka 2, Sweden) previously cooled to between -25 and $-30°$; with 3–4 ml. of suspension containing 1·5–2 g. wet weight of bacteria, three pressings gave 70–90% disruption as judged by Gram-stained smears. The extracts remained frozen and were stored at $-20°$ until required.

All extracts were clarified by centrifugation at about 60,000g for 1 hr and concentrated by vacuum dialysis in collodion shells (Membranfiltergesellschaft Göttingen, W. Germany) against tris citrate gel buffer (see below). The final extracts contained a protein concentration of the order of 40–60 mg./ml.; this was estimated

by the method of Lowry, Rosebrough, Farr & Randall (1957), with bovine albumin (British Drug Houses Ltd., B.D.H.) as standard. These extracts were stored at −20° until required.

Preparation of polyacrylamide gel

Monomer solution. Cyanogum 41 (B.D.H.), 8% (w/v) in tris citrate buffer (described below).

Catalyst (Raymond & Wang, 1960). Separate solutions (*a*) and (*b*) of the two components of the catalyst system were prepared: (*a*) 10% (w/v) 2-dimethylamino-ethylcyanide in water; (*b*) 10% (w/v) ammonium persulphate in water.

Gel mould. This consisted of a rectangular Perspex tray (internal dimensions, length 15·6 cm., width 14·1 cm., depth 0·8 cm.) with a well-fitting lid bearing a removable slot-former of the type described by Smithies (1955). A suitable slot-former carried ten projections, producing insert slots 0·8 cm. wide, 0·7 cm. deep, 0·05 cm. thick, spaced at 0·5 cm. intervals. To prepare the gel 1% of each of solutions (*a*) and (*b*) was added to the monomer solution, the mixture was poured into the Perspex tray and the lid lowered into position. Polymerization occurred within 2 hr at room temperature.

Electrophoresis

Buffer system. A discontinuous system based on that described by Poulik (1957) was used. The gel buffer (half the concentration used by Poulik) contained tris 38 mM. and citric acid 2·5 mM. (pH 8·7 at 25°). The buffer for the electrode vessels contained boric acid 7·22 g./l. and borax 15·75 g./l. (0·28 M in terms of borate; pH 8·8 at 25°).

Electrophoresis took place in a room maintained at +1° to +5°, at constant voltage (7–8 V./cm. gel), initial current 35–40 mA. decreasing to 10–15 mA. at the end of the experiment. When the marker dye (bromophenol blue) had travelled 8 cm. towards the anode (6–7 hr) the gel was removed, sliced, and stained to demonstrate proteins or esterases.

Protein stain. Naphthalene black 10B (1%; G. T. Gurr, Ltd.,) in 20% (v/v) acetic acid in water; the solution was applied for 30 min. and background stain then removed by successive rinses with 7·5% acetic acid in water.

Esterase stain (Lawrence, Melnick & Weimer, 1960). The gel slice was flooded with a freshly prepared solution containing tris maleate buffer (0·1 M; pH 6·4) 50 ml.; a solution of α-naphthyl acetate (1%) in 50% (v/v) acetone in water, 2 ml., Fast blue BB salt (Sigma Chemical Co.), 50 mg. Esterase activity, was shown by the appearance of dark red bands which developed in 1 hr at room temperature.

Photography. Stained gels were photographed by transmitted light on Pan F film (Ilford Ltd.) and (for protein patterns) with a yellow filter (Ilford Ltd., filter no. 110).

RESULTS

Effect of method of disintegration

Extracts prepared by the two methods of disintegration of the bacteria gave similar patterns of proteins and esterase bands. It is probable that for subsequent study of more labile enzymes the use of the X-press, in which the extract remains frozen, will be more satisfactory than the use of the Mickle shaker, in which it is difficult to prevent an increase of temperature during disintegration.

Protein bands

All of the strains of *Streptococcus faecalis* and its varieties *liquefaciens* and *zymogenes* gave a similar pattern of protein bands ('protein pattern'), with major protein components of the different extracts showing similar mobility. Typical results are shown in Pl. 1, fig. 1. Patterns shown by strains of *S. faecium* and *S. durans* are illustrated in Pl. 2, fig. 3. Major protein components of the different extracts showed similar mobility; with the less-marked bands there were some differences between different strains of *S. faecium* and *S. durans*, but of 20 strains initially studied (representing 19 known serotypes) all but one gave patterns resembling those of Pl. 2, fig. 3, in position of the major bands. The exception was strain P14/6, serotype 29.

The protein patterns of strains of *Streptococcus faecalis*, *S. faecium* and *S. durans* are compared in Pl. 2, fig. 4. When protein bands at a distance of 3–5 cm. from the inserts were examined a difference was apparent in the position of the most marked bands in extracts of *S. faecalis* (a), typical *S. faecium* (b, d) and a β-haemolytic *S. durans* (c). Plate 2, fig. 4, also shows the protein pattern given by *S. faecium* strain P14/6, which differed quite markedly from those of the remaining strains of *S. faecium*, *S. durans* and of *S. faecalis*.

Esterase bands

Streptococcus faecalis strains possessed active esterases, and well-defined bands were detected after electrophoresis; typical results are shown in Pl. 1, fig. 2. Esterase 1 produced a wide zone of marked enzyme activity, except in the case of strain C1 (fig. 2, extract f); in 1 strain (N83; fig. 2, extract g) out of 12 studied this band was replaced by a similar one with slightly greater mobility (esterase 1a). Esterase 2 was detected in 9 of the 12 strains but was either very weak or not detected in the other 3 strains. Esterase 3 was a faint band shown by 3 strains in Pl. 1, fig. 2 (extracts g, h, j). Other very faint bands were sometimes detected and can be seen in the photograph. *S. faecium* strains generally showed weak rather ill-defined esterase bands; *S. durans* strain C3 showed distinct bands but lacked the strong esterase-1 band generally characteristic of *S. faecalis*. In view of the faintness of the esterase bands of *S. faecium* these have not been described in detail; a striking difference was shown by strain P14/6 which, in contrast with the remaining strains of *S. faecium*, gave strong bands of esterase activity.

Esterase patterns of *Streptococcus faecalis* strain 30/82, *S. faecium* P3, *S. durans* C3 and *S. faecium* 5422 and P14/6 are illustrated in Pl. 2, fig. 5.

Streptococcus faecium strain P14/6, *serotype 29*

This strain, distinguished by its unusual pattern of proteins and esterases, differed from all the other strains in showing motility, readily observed in microscopic examination of 8–24 hr broth cultures at 30°, and demonstrated by diffuse growth in brain heat infusion broth containing 0·25% agar (Davis). This strain also produced variants which gave red colonies on the thallous acetate + tetrazolium agar medium of Barnes (1956). These characters were also noted for this strain by Sharpe & Fewins (1960) and by Barnes (1964).

DISCUSSION

Protein patterns

In this study of soluble proteins, strains of *Streptococcus faecalis* and its varieties 'zymogenes' and 'liquefaciens', of several serotypes, showed very similar protein patterns, which contrasted with the pattern shown by the strain of *S. faecium*. The patterns for *S. durans* strains (C3, β-haemolytic; P16/5 and P17/8, α-haemolytic) appear more like that of *S. faecium* than that of *S. faecalis*. These results substantiate the previous differentiation between *S. faecalis* and *S. faecium/S. durans*, made on the basis of serology and biochemical reactions. The different protein patterns of *S. faecalis* and *S. faecium/S. durans* may be due to differences between the metabolism of these organisms, and hence a quantitative and qualitative difference between the major enzymes present; an objective of future experiments will be to identify some of the major bands in order to understand the significance of this difference.

Esterase patterns

A probable difference between the metabolism of *Streptococcus faecalis* and that of *S. faecium/S. durans* is illustrated by the difference in esterase activity of these organisms. The ability to hydrolyse α-naphthyl acetate is shown by various types of esterase; the physiological significance of the enzymes observed here requires further study.

Plate 1, fig. 2, shows that similar esterase patterns were given by *Streptococcus faecalis* strains of different serotype (e.g. extracts c, d, e, k) and by varieties faecalis (d), liquefaciens (a, b, e, k) and zymogenes (c). In some cases a difference in esterase pattern was found between two strains of the same serotype, e.g. GB122, var. liquefaciens (e) and C1, var. zymogenes (f). Only one strain of serotype 5 (N83) was examined; it remains to be seen whether band 1a is a general feature of strains of this serotype.

In the case of *Bacillus thuringiensis* (Norris, 1964) esterase patterns were found to be type-specific and to provide a means of identifying new isolates. The major esterases of several of the *B. thuringiensis* serotypes showed similar mobilities, and a comparison of other fainter bands was necessary to make a distinction between the serotypes. It is possible that such faint esterase bands are better detected on starch gels, as used by Norris (1964), than on polyacrylamide gels. The latter technique was used in the present work with streptococci because it enabled a much better separation of components in the extracts prepared by Mickle disintegration at the outset of this work, and the transparency of the gel readily enabled a more sensitive detection of protein bands than did the use of starch gels.

Streptococcus faecium strain P14/6. The comparison of protein and esterase pattern distinguished this motile strain from the remaining non-motile strains of *S. faecium*. Preliminary experiments have shown that two other motile strains of this serotype (isolated from different sources) gave protein and esterase patterns similar but not identical to those of strain P14/6; a further study of the significance of these results is in progress.

Streptococcus faecium strain 5422 was labelled in our collection as belonging to serotype 29 (of which P14/6 is the type strain) and was non-motile. The finding that

protein and esterase patterns of strain 5422 differed from those of P14/6, caused a check to be made of the history of strain 5422. It was found that a mistake had occurred in the correlation of typing systems: 5422 did not belong to the same serotype as P14/6; this was confirmed experimentally.

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EXPLANATION OF PLATES

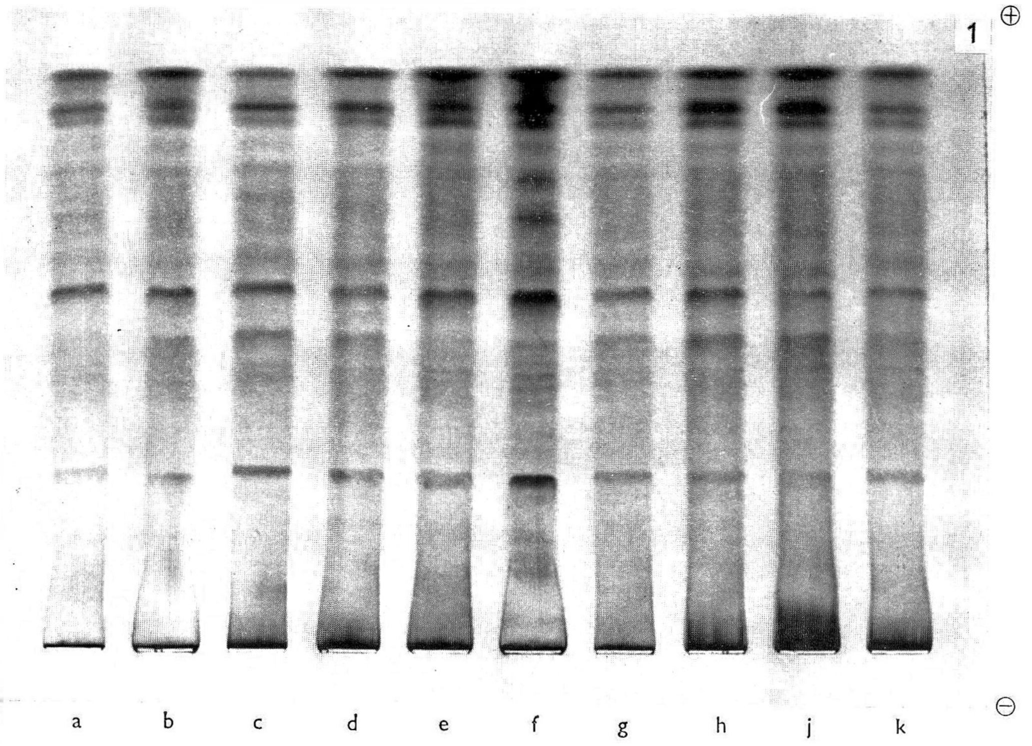
PLATE 1

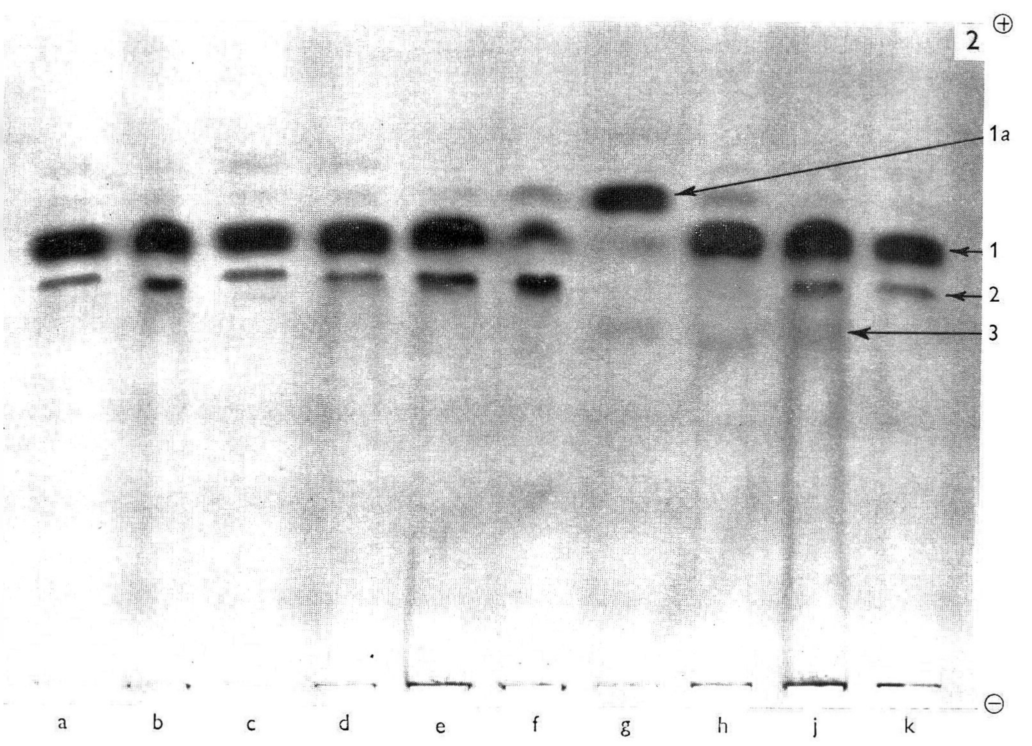
Study of soluble components of extracts of strains of *Streptococcus faecalis* by electrophoresis on polyacrylamide gel. The two different gels shown here contained the same series of extracts.

Fig. 1. Gel stained with naphthalene black 10B to detect protein bands.

Fig. 2. Gel incubated in the presence of α-naphthyl acetate and Fast blue BB to detect esterase enzymes.

(*a*) = strain D76, type 1 (var. liquefaciens); (*b*) = strain 30/39, type 1 (var. liquefaciens); (*c*) = strain GB112, type 1 (var. zymogenes); (*d*) = strain 30/82, type 3; (*e*) = strain GB122, type 4 (var. liquefaciens); (*f*) = C1, type 4 (var. zymogenes); (*g*) = strain N83, type 5; (*h*) = strain B65, type 6; (*j*) = strain D10, type 40 (var. liquefaciens); (*k*) = strain D15, type 41 (var. liquefaciens).





B. M. LUND

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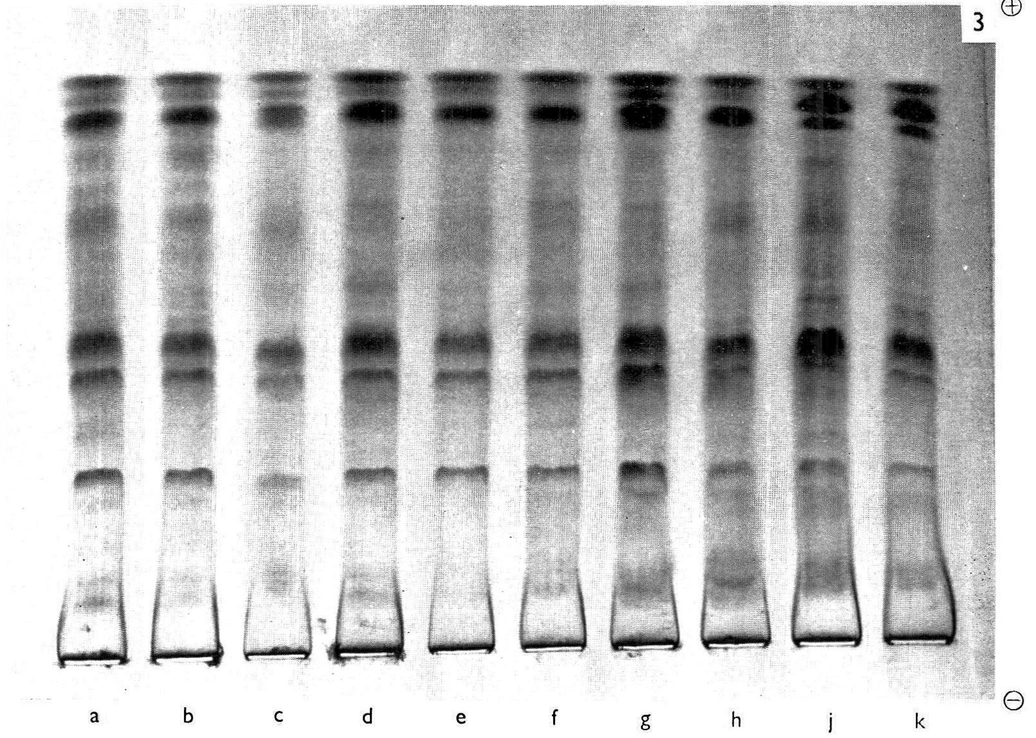

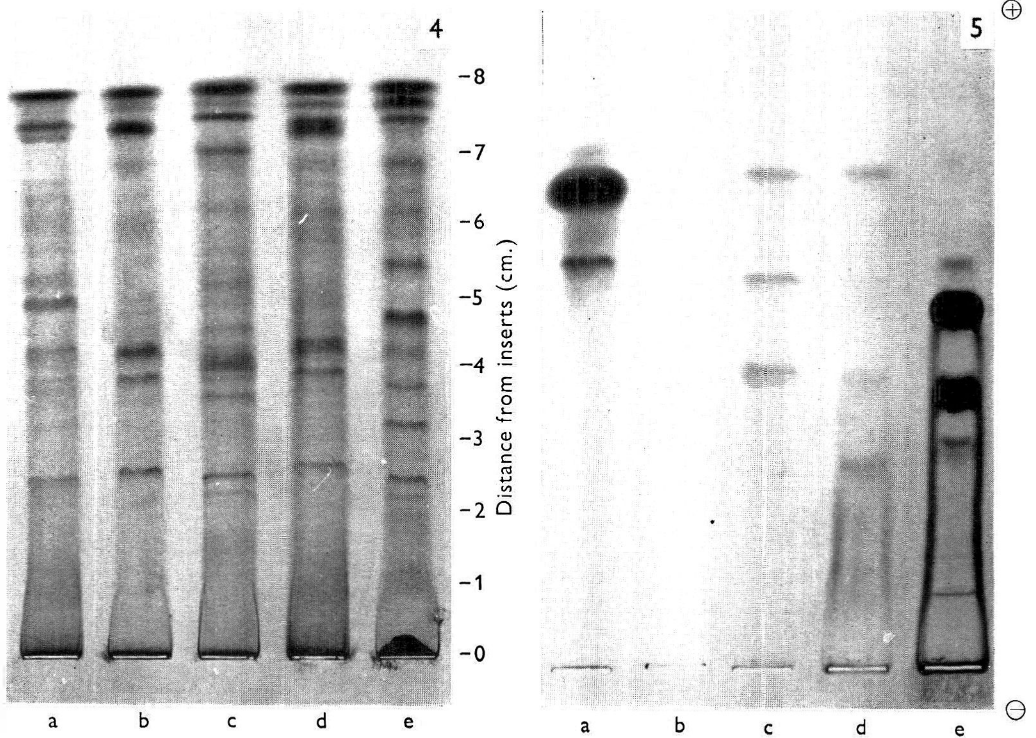

B. M. LUND

PLATE 2

Studies of soluble components of cell extracts by electrophoresis on polyacrylamide gel.

Fig. 3. Extracts of *Streptococcus faecium* and *S. durans*; gel stained with naphthalene black 10B to detect protein bands.

(*a*) = strain S98, type 18, *S. faecium*; (*b*) = strain H24, type 24, *S. faecium*; (*c*) = strain HGH511, type 28, *S. faecium*; (*d*) = strain GE4B, type 30, *S. faecium*; (*e*) = strain P3, type 33, *S. faecium*; (*f*) = strain P7, type 34, *S. faecium*; (*g*) = strain N/R/64, type 35, *S. faecium*; (*h*) = strain P1/12, type 36, *S. faecium*; (*j*) = strain P16/5, type 39, *S. durans*; (*k*) = strain P17/8, type 38, *S. durans*.

Figs. 4, 5. Comparison of extracts of strains of *S. faecalis*, *S. faecium*, and *S. durans*. The two different gels contained the same series of extracts.

Fig. 4. Gel stained to detect protein bands.

Fig. 5. Gel incubated with α-naphthyl acetate and Fast Blue BB to detect esterase enzymes.

(*a*) = *S. faecalis*, 30/82; (*b*) = *S. faecium*, P3, type 33; (c) = *S. durans*, C3, type 26 (β-haemolytic); (*d*) = *S. faecium*, 5422, not typed; (*e*) = *S. faecium*, P14/6, type 29.

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The Role of Phage in the Transduction of the Toxinogenic Factor in *Corynebacterium diphtheriae*

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SUMMARY

Strains of *Corynebacterium diphtheriae* which produced large amounts of toxin (e.g. PW8) were found to be non-lysogenic, but they contained a cytoplasmic toxinogenic factor designated as T^+. After infection with phage they produced less toxin. It is possible to remove the phage from the lysogenic strains without affecting the T^+ factor by treatment with small amounts of acriflavine. When treated with larger amounts of acriflavine the T^+ factor was also removed, rendering the strain non-toxinogenic. The toxin-inducing phages can be converted *in vitro* into non-toxinogenic phages by treatment with small amounts of acriflavine. After passage through the PW8T^+ strains these phages revert to toxin-inducing ability. Similarly, the naturally occurring, non-toxinogenic γ phage can be made to induce toxin production. This indicates that the role of phage in diphtheria toxin production is to transduce the T^+ factor.

INTRODUCTION

The conversion of non-toxinogenic *Corynebacterium diphtheriae* into toxinogenic strains after lysogenization with certain bacteriophages was shown by Freeman (1951); this observation has been confirmed by many workers. Freeman's work explained the possible mode of change to virulence of non-toxinogenic strains. Groman (1953*a*, 1955) ruled out the possibility that mutation, transformation or transduction might bring about the change to toxin production. He postulated that the B phage possessed converting ability which was an inherent and inseparable property of every plaque-forming particle. Groman & Eaton (1955) reported that there were certain phages, like the γ phage, which were unable to induce toxin production.

Barksdale & Pappenheimer (1954) reported that the PW8 strain of *Corynebacterium diphtheriae*, which produces large amounts of toxin, harboured a prophage which they claimed they were able to induce. However, while working with the SM-1 strain of Yoneda (a variant of the PW8 strain) Barksdale, Garmise & Horibata (1960) observed that no mature phage particle could be induced from this PW8 strain. They thought therefore, that the PW8 strain harboured a defective prophage, a mutant of the phage P which they designated as Pd. Our work has now clarified certain contradictions and wrong assumptions made by previous workers. We give evidence which indicates that the ability of *C. diphtheriae* to produce toxin depends on the presence of a cytoplasmic factor which we designate as T^+, which is merely

transduced by phage. Further, we have been able to convert toxinogenic phages into non-toxinogenic phages by treatment of the phages with small amounts of acriflavine *in vitro*.

METHODS

Corynebacterial strains. Corynebacterium diphtheriae strain PW8 (Weissensee-G). This is a variant of the PW8 Weissensee strain received from the Biogena Institute, Prague, Czechoslovakia. It has been adapted for growth and toxin production in the protein-free Holt medium (W.H.O., 1953) in our laboratory. This strain was used in most of our experiments; it gives 80–100 Lf toxin/ml.

C. diphtheriae PW8(P). A lysogenic variant of the PW8 strain was received from Dr L. Barksdale (New York). It was first isolated by Barksdale *et al.* (1960) from the SM-1 strain of Yoneda and shown to be lysogenic for the phage P; it yields 10 Lf toxin/ml.

C. diphtheriae non-toxinogenic strains C4 and C7 were received from Dr N. B. Groman (University of Washington, Seattle, Wash., U.S.A.). These were unable to grow on the Holt medium and therefore were grown on papain digest (PD) medium of Linggood & Fenton (1947).

Bacteriophages. The phage B was received as a broth lysate from Dr L. Barksdale. This phage can infect and lysogenize *Corynebacterium diphtheriae* strain C4 but not the PW8 strain. The phage γ was induced from the C4 (γ) culture received from Dr N. B. Groman. Phage P was induced from the PW8 (P) strain mentioned above, and can infect the *C. diphtheriae* C7 strain but not strain C4.

Method of phage induction. An 18 hr bacterial culture in liquid medium was poured into a sterile Petri plate so as to get a layer not more than 2 mm. thick. This plate was kept in a sterile cabinet in front of a 15 W. Philips u.v. 'Sterilamp' for 15 min. at a distance of 48 cm. from the centre of the lamp. The lysate obtained was filtered through L3 Chamberland candles and the phage activity was assayed by the over-layer technique of Adams (1950).

Detection of toxin. Toxin-producing strains were distinguished from non-toxinogenic strains by plating on solidified medium containing 3 i.u./ml. horse diphtheria antitoxin as suggested by Groman (1953*b*). The plates were incubated at 37° for 48 hr and then kept in a refrigerator for 2 days. Strains which produce large amounts of toxin showed a ring round the colonies (Pl. 1, fig. 1). Those which produced small amounts of toxin showed a small halo and the non-toxinogenic strains did not show a halo or ring (Pl. 1, fig. 2). Quantitative estimation of the toxin produced by a culture was done by (i) Ramon's flocculation test; (ii) by the sensitive intracutaneous injection test in rabbits. A culture was considered to be non-toxinogenic only when it did not show toxin production when tested by both methods.

RESULTS

Testing the lysogeny of Corynebacterium diphtheriae PW8 strains

According to Adams (1959) phage production and immunity to superinfection constitute the two criteria by which the presence of a prophage may be detected.

The PW8 (P) strain (obtained from Dr Barksdale) was readily induced and yielded phage P when ultraviolet (u.v.) irradiated. When tested by the overlayer technique this strain was found to resist infection by phage P.

The high toxin-yielding PW8 (Weissensee-G) strain yielded no mature phage particles when u.v.-irradiated. Induction of phage was also tried by the 'heat shock' treatment as used by Asheshov & Jevons (1963) to induce staphylococcal phages. An 18–24 hr growth of the culture in liquid medium was held at 56° for 2 min., cooled immediately and centrifuged; the supernatant fluid did not show the presence of any phage. It was then decided to test the immunity of PW8 (Weissensee-G) to phage P. When tested by the overlayer technique, the PW8 (Weissensee-G) culture was lysed by phage P and clear plaques were observed (Pl. 1, fig. 3). After lysogenization with P phage this strain resisted superinfection with phage P. Moreover, the yield of toxin obtained after lysogenization was only 10 Lf/ml. (Table 1). Since the PW8 culture was non-lysogenic, it was felt that a toxinogenic factor (T^+) and not the phage P was responsible for inducing toxin production.

Effect of acriflavine treatment on toxinogenic strains of Corynebacterium diphtheriae

Acriflavine is known to disinfect bacteria from cytoplasmic episomal factors (Hirota & Iijima, 1957) but it is ineffective when the episomal factors are integrated into the bacterial nucleus (Hirota, 1960). Pappenheimer, Howland & Miller (1962) reported the isolation of a non-toxinogenic strain of *Corynebacterium diphtheriae* by incubating the lysogenic strain C7 (β) in the presence of acridine orange 15 μg./ml. A loopful of 18–24 hr culture of *C. diphtheriae* PW8T^+ or PW8(P)T^+ in liquid medium was inoculated into 5 ml. Holt liquid medium containing acriflavine 5 μg./ml. The cultures were incubated at 37° for 18 hr. A homogeneous suspension was prepared of the scanty growth which appeared, by thorough shaking on a magnetic shaker. This suspension was plated on agar medium containing diphtheria antitoxin 3 i.u./ml. The plates were incubated at 37° for 48 hr and then refrigerated for 48 hr. In each case over 90 % of the surviving bacteria had stopped producing toxin, as indicated by the absence of halo or ring round the colonies on the antitoxin agar plates. Subcultures of colonies which did not show haloes or rings were further tested by the intracutaneous test in rabbits, to confirm their non-toxinogenic character. The non-toxinogenic bacteria thus isolated were labelled PW8T^-.

PW8T^- isolates exposed to phage P infection (about 10^4 bacteria to 1×10^6 phage particles) developed resistant (i.e. lysogenic) bacteria which were found to have reverted to toxin production. However, the yield of toxin given by these reverted cultures was never more than 10 Lf/ml. (Table 1). PW8T^- cultures exposed to phage B infection neither developed lysogenic bacteria nor reverted to toxin production. This was probably because PW8 strains (both PW8T^+ and PW8T^-) were found to be resistant to phage B. Incubation of strains C7(P), C4(B) and C4(γ) in a medium containing acriflavine 5 μg./ml. also converted them into non-toxinogenic and non-lysogenic (phage-free) strains.

Effect of acriflavine in vitro on toxin-inducing phages P and B

To test the effect of acriflavine on toxin-inducing phages, the phages P and B were purified by precipitation with ammonium sulphate. To 50 ml. of each phage lysate was added solid ammonium sulphate 0·3 g./ml. and the solutions were then centrifuged at 20,000g for 15 min. The pellet obtained was washed with ammonium

sulphate solution (28 g./100 ml. distilled water) and then suspended in 20 ml. sterile distilled water and centrifuged at 20,000*g* for 15 min. The supernatant fluid obtained was used for assaying phage; it contained about 1×10^6 phage particles/ml.

To 5 ml. samples of phage P suspension (purified as above), acriflavine was added to concentrations from 5 μg./ml. down to 0·1 μg./ml. and then stored in the refrigerator for 18 hr. Concentrations of acriflavine 2 μg./ml. was completely lethal to phage P, 1 μg./ml. decreased the titre to a negligible value; and 0·5 μg./ml. decreased the titre from $1{\cdot}0 \times 10^6$ to $1{\cdot}0 \times 10^2$ particles/ml. However, the surviving particles produced plaques on the indicator strain C7. The C7 culture made lysogenic to the acriflavine-treated phage P (Paf) was non-toxinogenic (Table 1).

Table 1. *Results of experiments in transduction in Corynebacterium diphtheriae*

Strain	Treatment	Result	Toxin production in Holt's medium (Lf/ml.)	U.v.-irradiated plaques on strain C7	Toxin production induced in strain C7
PW8T+	—	—	80–100	None	None
PW8T+	P phage	PW8(P)T+	10	+	–
PW8T+	Af* 5 μg./ml.	PW8T−	None	None	None
PW8(P)T+	Af 5 μg./ml.	PW8T−	None	None	None
PW8T−	P phage	PW8(P)T+	10	+	+
Phage P	Af 0·5 μg./ml.	Paf	–	+	None
PW8T+	Paf phage	PW8(Paf)T+	10	+	+
PW8T−	Paf phage	PW8(Paf)T−	None	+	None
PW8(P)T+	Af 0·2 μg./ml.	PW8T+	40–60	None	None

* Acriflavine.

When the phage B was similarly treated with 0·5 μg./ml. acriflavine, it led to the formation of phage (Baf) which produced plaques on the indicator strain C4 but did not induce toxin production. In this respect phage Baf resembled the γ phage, which also produces plaques on strain C4 but does not induce toxin production (Groman & Eaton, 1955).

To eliminate the possibility that the disinfecting action of acriflavine might have eliminated the T+ factor after rather than before lysogenization, the experiment was repeated after removing possible traces of acriflavine from the treated phage suspensions by dialysis at refrigeration temperature against 2% ammonium sulphate solution. The indicator strains made lysogenic to the acriflavine-treated phages remained non-toxinogenic.

To exclude the possibility of phage mutation to non-toxinogenic type, phage particles from forty-two plaques produced by phage Paf on strain C7 were individually isolated and, after infecting C7 cultures with them, the effect on toxin induction was observed by the intracutaneous injection test on rabbits: thirty-nine of the forty-two plaques thus tested indicated that the plaque-forming particles had lost the ability to induce toxin production in strain C7.

Role of phage as a carrier of the toxinogenic factor

Since acriflavine disinfected the toxinogenic PW8 cultures of their toxin-producing ability and also deleted the factor for toxin production from the toxin-inducing phages, it was felt that the genetic information for toxin production was due to the

cytoplasmic toxinogenic factor (T^+) and that the toxin-inducing phage was only a carrier of that factor. To test this possibility the following experiment was done Phage Paf was allowed to infect two cultures; PW8T^+ and its non-toxinogenic derivative PW8T^- obtained by acriflavine treatment. The proportion of phage particles to bacteria was approximately 2:1. On lysogenization with phage Paf, the yield of toxin given by PW8T^+ decreased from 80 Lf/ml. to about 10 Lf/ml. Strain PW8T^- remained non-toxinogenic on lysogenization with phage Paf. These lysogenic cultures PW8(Paf)T^+ and PW8(Paf)T^- were then subjected to u.v.-irradiation and the phages which were induced were allowed to infect the indicator strain C7. Strain C7 culture made lysogenic to the phage induced from strain PW8(Paf)T^+ became toxinogenic, as shown by the production of haloes on antitoxin agar plates and the intracutaneous injection test, while the strain C7 made lysogenic to the phage obtained from PW8(Paf)T^- remained non-toxinogenic. The phage had therefore acted as a carrier of the toxinogenic factor (Table 1).

Removal of phage from a toxinogenic and lysogenic culture of Corynebacterium diphtheriae to increase the yield of toxin

Since our results indicated that lysogenization decreased the yield of toxin produced by *Corynebacterium diphtheriae* strain PW8T^+, it was decided to eliminate the phage from this strain. Prolonged exposure to u.v. radiation for the purpose of 'curing' has been used by Lederberg & Lederberg (1953) to isolate non-lysogenic bacteria from a lysogenic culture of *Escherichia coli*. A 24 hr culture of *C. diphtheriae* strain PW8(P)T^+ was therefore u.v.-irradiated for 1, 1·5 and 2 hr, and the surviving bacteria then tested for the presence of the phage. The u.v.-irradiation did not 'cure' the diphtheria culture of the prophage. It was then decided to use acriflavine for this purpose. When *C. diphtheriae* strain PW8(P)T^+ was treated with acriflavine 5 μg./ml. it lost the phage and the T^+ factor. Concentrations of acriflavine ranging from 0·1 to 1·0 μg./ml. were added to Holt medium agar containing antitoxin 3 i.u./ml. and poured into Petri dishes. A 24 hr culture after shaking on the magnetic shaker was diluted 1/100 in 0·5 % saline; 0·5 ml. of this dilution was smeared on each plate, which was then incubated at 37° for 48 hr. Less than 10 colonies/plate were seen on the plates containing acriflavine 0·1–1·0 μg./ml. The same culture plated on antitoxin agar but not containing acriflavine showed over 120–150 colonies/plate. The plates were then refrigerated for 2 days.

Colonies grown on antitoxin + acriflavine agar plates which showed clear rings round them were individually subcultured and tested for phage and yield of toxin. Six out of eight colonies isolated from plates containing acriflavine 0·2 μg./ml. indicated that they had become non-lysogenic; they produced no mature phage particles when u.v.-irradiated. Moreover, in the overlayer technique the phage P produced plaques on the cultures. On an average these non-lysogenic cultures produced 40–60 Lf toxin/ml., while the parent culture produced only about 10 Lf/ml.

Conversion of the γ phage into a toxin-inducing phage

The γ phage was first induced from the *Corynebacterium diphtheriae* strain 124 of Freeman & Morse (1952) by Groman & Eaton (1955), who found that this phage, after infecting the indicator strain C4, did not induce toxin production, unlike phage B. They also reported that the phage γ was related to phage β, the temperate variant

of phage B. It was therefore felt possible to convert the γ phage into a toxin-inducing phage. The c4 strain of *Corynebacterium diphtheriae* was first made lysogenic to phage B. A sample (0·1 ml.) of an 18 hr culture of strain c4 grown in PD medium (Linggood & Fenton, 1947) was inoculated into 50 ml. of the same medium with about 10^8 particles of phage B. Lysis of bacteria was followed by lysogenization. Lysogenic bacteria of strain c4(B) were then isolated and were found to produce toxin (intracutaneous test). A sample (0·5 ml.) of 1/100 dilution of a 24 hr culture of strain c4(B) in PD medium was then plated on antitoxin agar plates containing acriflavine 0·2 μg./ml. Four to five colonies per plate appeared after incubation at 37° for nearly 36 hr. These colonies were subcultured and tested for toxin and phage production. Haloes round the colonies appeared when the plates were refrigerated for 48 hr, indicating that they were toxinogenic. U.v.-irradiation and the test for immunity to superinfection by the overlayer technique showed that these organisms were non-lysogenic. These cultures were termed c4T^+. An 18 hr culture of c4T^+ bacteria in PD medium was then exposed to infection with the non-toxinogenic phage γ, the proportion of phage particles to bacteria being 2:1. A culture which was lysogenic to phage γ was then isolated and u.v.-irradiated, and the induced phage particles (γT^+) were used to infect the indicator strain c4. Strain c4 made lysogenic to phage γT^+ when plated on antitoxin agar plates did not give prominent haloes. Nevertheless, when subcultures of ten of these colonies were tested by the intracutaneous test, two of them were toxinogenic. This indicated that phage γ was capable of transducing the T^+ factor, though not as efficiently as the other well-known toxin-inducing phages P and B.

DISCUSSION

The results of this work are summarized in Table 1. The data presented clarify some of the apparently contradictory observations made by earlier workers. The bacteriophage of the diphtheria bacillus certainly plays a vital role in the conversion of non-toxinogenic strains to toxinogenic strains as Freeman (1951) observed. However, it now appears that the toxin-inducing ability of the phage is not its inherent property, but that it acts as a transducing agent and the factor for toxin production can be removed from the phage by acriflavine treatment *in vitro*. So strong has been the conviction that the phage is indispensable to toxin production that Barksdale, Garmise & Rivera (1961) postulated that diphtheria toxin production is a post-lysis event, lysis being brought about by phage maturation. However, Pappenheimer, Miller & Yoneda (1962) proved this hypothesis to be wrong. Our results show that the high toxin-yielding PW8 strain of *Corynebacterium diphtheriae* is non-lysogenic but contains the T^+ factor. Lysogenization of this strain considerably decreased toxin production. Since this conclusion is based on the fact that strain PW8T^+ cultures succumb to infection with phage P it may be argued that this strain may harbour some other phage if not phage P. However, the well-known methods of phage induction have not shown the presence of a phage of this strain and, after treatment with acriflavine 5 μg./ml. non-toxinogenic strain PW8T^- cultures were obtained which reverted to toxin production only when phage P infected them. This indicates a certain compatibility between the phage P and strain PW8. Other phages of *C. diphtheriae* such as B and γ did not infect strain PW8 and it is not lysogenized by them.

It may be asked how Barksdale *et al.* (1960) could isolate the lysogenic strain PW8(P) from the SM-1 culture if the latter were non-lysogenic. We recently observed that a culture of strain PW8 (Weissensee-G) which showed a sudden decrease in the yield of toxin was in reality contaminated with a phage. It is quite likely, therefore, that the two types of micro-organisms observed by Barksdale *et al.* (1960) were the non-lysogenic PW8 bacteria and their lysogenic derivative PW8(P).

Support for our assumption that the phage acts as a carrier of T^+ factor can be had from the results of an experiment reported by Groman & Eaton (1955). They infected the non-toxinogenic c4(γ) strain of *Corynebacterium diphtheriae* with the toxin-inducing phage β and isolated five different cultures: c4(γ), 64(β), c4(γ'), c4(β'), c4(γ)(β). In the doubly lysogenic c4(γ)(β) culture toxinogenicity was dominant; c4(γ') was toxinogenic; c4(β') was non-toxinogenic. Groman & Eaton (1955) interpreted these results in terms of possible phage recombinations. It seems likely, however, that the γ' phage was actually phage γ carrying the T^+ factor and hence capable of inducing toxin production. Similarly, phage β' might have become non-toxinogenic by having lost T^+ factor.

It is generally believed that the disinfective action of acriflavine and other acridines is by dilution of an episome from a growing culture (Sneath, 1962). This perhaps explains in a general way the conversion of toxinogenic strains of *Corynebacterium diphtheriae* into non-toxinogenic strains on treatment with acriflavine 5 μg./ml. Recent investigations on the interactions of acridines with DNA, by means of X-ray diffraction analysis and other physical methods, have supported a hypothesis of Brenner, Barnett, Crick & Orgel (1961) that acridine mutations are caused by the deletion or addition of a base pair. To account for the elimination of T^+ factor from toxinogenic diphtheria phages after treatment with small amounts of acriflavine *in vitro*, we have to assume that, when acriflavine attaches itself either to phage DNA or to phage DNA and the T^+ factor in it, its action is lethal. This is evident from the large decrease in the number of phage particles after treatment with acriflavine 0·5 μg./ml. *in vitro*. When, however, an acriflavine molecule attaches itself only to T^+ factor and not to phage DNA, the phage can replicate in a susceptible host but the T^+ factor cannot and is lost. The specific action of acriflavine on the lysogenic cultures in rendering them non-lysogenic and at the same time increasing the yield of toxin has support from the work of Barksdale *et al.* (1960). They reported that proflavine, which prevented phage maturation, led to increase in toxin yield. In the light of our results it can be explained by the ability of small concentrations of acriflavine to disinfect prophage but not T^+ factor from a lysogenic and toxinogenic strain of *C. diphtheriae*.

Wohlhieter *et al.* (1964) showed that synthesis of β-galactosidase in a *lac*$^+$ strain of *Proteus mirabilis* was directed by a cytoplasmic episomal factor termed P. lac. Our conclusions, therefore, lead us to believe that synthesis of diphtheria toxin protein is similarly controlled by the T^+ factor. Although the experiments reported here point towards a cytoplasmic existence for this factor, it is likely that this factor is an episome and may be capable of alternating between autonomous cytoplasmic and an integrated chromosomal state, like other episomes such as the F factor and the colicinogenic factors of *Escherichia coli*. At present we know very little about the genetics of *Corynebacterium diphtheriae* and therefore it is worth while investigating this problem further.

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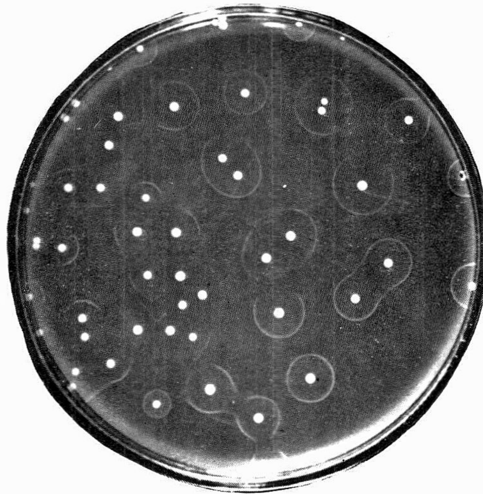

Fig. 1

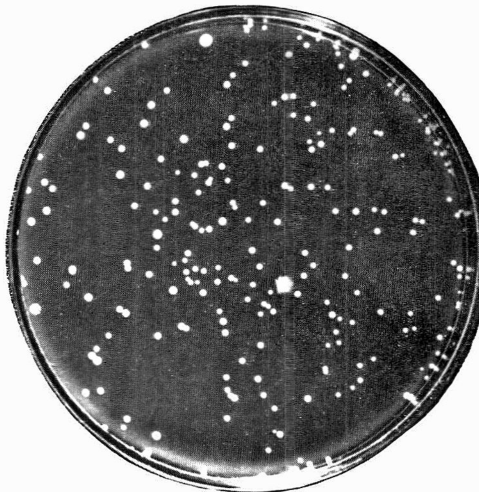

Fig. 2

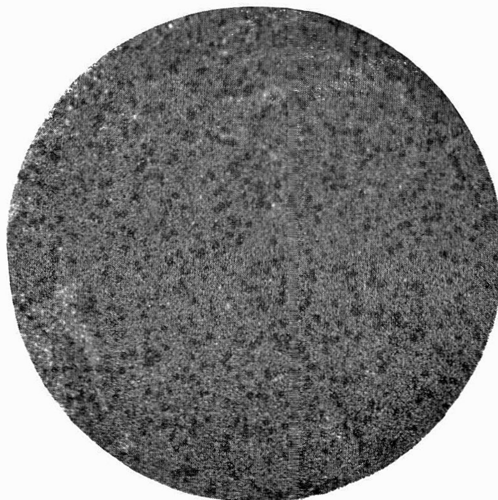

Fig. 3

A. B. RAJADHYAKSHA AND S. SRINIVASA RAO

(*Facing p.* 429)

EXPLANATION OF PLATE

Fig. 1. Detection of toxinogenic bacteria. An 18–24 hr culture of *Corynebacterium diphtheriae* PW 8T+ was plated on Holt's medium agar containing antitoxin 3 i.u./ml., incubated for 48 hr and then refrigerated for 48 hr. Production of toxin is known by the appearance of rings round colonies.

Fig. 2. Acriflavine treatment to convert a toxinogenic strain to non-toxinogenic strain. Acriflavine 5 μg./ml. was added to Holt's medium which was then inoculated with a 24 hr culture of the toxinogenic strain PW8T+ of *Corynebacterium diphtheriae.* After 18 hr the culture was plated on antitoxin agar, incubated for 48 hr and refrigerated for 48 hr. Absence of rings or haloes round colonies indicated conversion to non-toxinogenic strain.

Fig. 3. Plaques produced by phage P on *C. diphtheriae* strain PW8T+. Test done by Adams's overlayer technique.

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Interaction between *Phytophthora infestans* and Tissue Cultures of *Solanum tuberosum*

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SUMMARY

It has been shown that tissue cultures of *Solanum tuberosum* var. Majestic (susceptible) stimulated the growth of *Phytophthora infestans.* A similar stimulus was produced by cell suspensions of the *S. tuberosum* var. Orion (resistant), but was not manifested by larger tissue aggregates of this variety. The failure of Orion tissue aggregates to stimulate was independent of the size of the fungal inoculum and was annulled by boiling the Orion tissue. Freezing of both Majestic and Orion tissue aggregates destroyed the stimulus, but freezing followed by boiling or boiling followed by freezing did not. It is suggested that the failure of living Orion tissue aggregates to stimulate the growth of *P. infestans* was a direct effect of their excluding the fungus.

INTRODUCTION

It was suggested by Muller & Behr (1949) and by Muller (1958) that the resistance of the potato to *Phytophthora infestans* is the result of the action of 'phytoalexins' against the fungus. Further evidence for phytoalexins as antifungal substances was given by Spencer, Topps & Wain (1957) and Cruickshank & Perrin (1961). A nutritional basis for pathogenicity was suggested by Johnson (1953). Attempts have been made to study the interaction between host and fungus by using tissue cultures, notably by Morel (1944) and Coyier (1961). In the present paper the interactions between *P. infestans* and free cell cultures and tissue aggregates of *Solanum tuberosum* var. Majestic (susceptible), and var. Orion (resistant to races 0, 4 and others), are reported.

METHODS

Organisms. Phytophthora infestans race 4 was isolated from a leaf of the potato var. Duke of York during July 1964. At the time of the experiments it was pathogenic to var. Majestic and not pathogenic to var. Orion, when sporangial suspensions were placed on half tubers. Resistance to certain races of *P. infestans* is known to be expressed in the tubers of Orion (Lapwood & McKee, 1961).

Solanum tuberosum vars. Majestic and Orion. Both these potatoes were obtained from a commercial source and originally virus *X* infected; they were grown as tissue cultures.

Media. Stock cultures of *Phytophthora infestans* were normally grown on slopes of French bean agar, at 22°. This medium was made by homogenizing 128g. 'Birds Eye' frozen 'Sliced green beans', with 800 ml. distilled water, filtering the mush through muslin, adding 2% (w/v) Davis agar to the filtrate and autoclaving at 116° for 10 min.

The tissue cultures of *Solanum tuberosum* were grown in a liquid medium of the following composition (made up in 1 l. glass-distilled water): *mineral salts*: Na_2SO_4, 800 mg.; Na_2HPO_4, 33 mg.; KNO_3, 80 mg.; KCl, 65 mg.; $MgSO_4.7H_2O$, 180 mg.; $Ca(NO_3)_2$, 400 mg. *trace elements*: KI, 1·5 mg.; $ZnSO_4$, 0·3 mg.; H_3BO_3, 1·5 mg.; $MnCl_2$, 0·2 mg. *other additions*: glycine, 3·0 mg.; thiamine, 0·1 mg.; nicotinic acid, 0·5 mg.; pyridoxine, 0·8 mg.; Ca pantothenate, 2·5 mg.; EDTA (Na/Fe complex; Na_2EDTA, 0·0143 g. + $FeSO_4.7H_2O$, 0·01069 g.) 25 mg.; 2,4-dichlorophenoxyacetic acid, 6·0 mg.; α-naphthylacetic acid, 0·1 mg.; sucrose, 20 g.; Difco yeast extract, 1 g.; coconut milk, 130 ml.

Establishment and maintenance of tissue cultures. Tubers of *Solanum tuberosum* were washed with detergent and distilled water, followed by surface sterilization for 10 min. in a saturated solution of calcium hypochlorite. A plug of tissue was then removed from a tuber with a sterile no. 5 cork borer and surface sterilized in 95% (v/v) ethanol in water (1 min.) followed by 10 min. in saturated calcium hypochlorite. It was then washed twice in sterile glass-distilled water, cut into 0·5 cm. discs and placed on the tissue culture medium solidified with 1% (w/v) Davis agar.

The culture vessels were Pyrex boiling tubes, fitted with cotton-wool plugs and foil caps. Incubation was at 25°. Callus formation was normally visible after 1–4 weeks. Once established in this manner, the calluses were transferred to 25 ml. of the liquid medium in 100 ml. Pyrex conical flasks, plugged and fitted with foil caps. These were agitated on a rotary shaker (80 rotations/min.) at 25°. Under these conditions growth was continued, while the tissue mass soon broke down to give free cells and small tissue aggregates up to 0·5 cm. diameter.

Stock callus cultures were maintained on the solid medium. The liquid cultures were topped up with fresh medium monthly. New liquid cultures were periodically initiated by using inocula from established clones.

Cell Petri plate experiments. In these experiments the medium used for liquid tissue culture was solidified with 1% (w/v) Davis agar. Petri plates were seeded with 1·5 ml. of potato free cell suspensions of equal turbidity, measured by eye. Greater accuracy could not be obtained (and in the context, was not necessary), because of the lack of homogeneity of these suspensions and the difficulty in controlling contamination. Control plates were inoculated with 1·5 ml. of the liquid tissue-culture medium, in which no potato cells had been grown. All plates were further seeded with 100 sporangia of *Phytophthora infestans* (obtained from 7-day cultures of the fungus) and suspended in 1 ml. of the liquid medium. Incubation was at 22°.

Potato tissue aggregate experiments. In these experiments pieces of tissue aggregate of about 40 mm. diameter were placed near the edge, on opposite sides of a Petri plate. Each tissue aggregate was in contact with a separate streak of *Phytophthora infestans* sporangia (from 7-day cultures) suspended in liquid tissue-culture medium. The streaks were made with a glass loop of about 50 mm. diameter. The object of these experiments was to observe the influence of the tissue aggregate on sporangia, along a possible diffusion path. It was originally intended that tissue pieces were to be placed on pre-formed streaks of sporangia, but in practice it was found more convenient to begin the streaks from tissue aggregates already placed in position on the agar. At the same time, a control streak of sporangial suspension was made

in the centre of the plate. In later experiments the streaking of sporangia was dispensed with, and drops of tissue-culture liquid containing a known number of sporangia were placed on top of the tissue or directly on to the agar as controls.

RESULTS

Interaction of free potato cell suspensions and sporangia

Solanum tuberosum var. Majestic. When plates were simultaneously inoculated with Majestic cell suspensions and with 100 sporangia of *Phytophthora infestans*, growth of the *P. infestans* was stimulated, and after some 10 days well developed colonies were present on many of the plates. Control plates, inoculated with fresh tissue-culture medium, showed no development of macroscopic colonies (see Plate 1, fig. 1).

Table 1. *Colony formation on free-cell suspensions of Solanum tuberosum var. Majestic and var. Orion*

Figures for ten Petri plates each inoculated with 100 sporangia of *Phytophthora infestans.*

	Cells of potato variety		
	Majestic	Orion	Control
	Colonies of *P. infestans* (no.)		
Colony sizes (cm. diam.)			
< 1·0	8	7	1
> 1·0	24	8	1
> 0·5	9	9	0
Total sporangia germinated	41/1000	24/1000	30/1000

Solanum tuberosum var. Orion. Similar inoculations of var. Orion potato cell suspensions showed some stimulation and colony formation by *P. infestans*. Normally, in the cell Petri plates, all the *P. infestans* sporangia which germinated produced colonies. The germination of 2–4% of the added sporangia was the normal for 7-day sporangia of this isolate.

It might be argued that the transfer of potato free cell suspensions to the environment of the agar plate would lead to the death of these cells. This was investigated by using 2,3,5-triphenyltetrazolium chloride. Estimates were made of the reduction of this compound to the red triphenylformazan by fresh cells and by cells removed at daily intervals from the surface of the plate. The results showed that the activity of the cells, as measured by colour production, was at a maximum immediately after transfer, fell to one-third of the maximum in 120 hr and returned to two-thirds of the maximum activity within the next 24 hr. The activity remained at this value until the conclusion of the experiment at 12 days. After this time, the potato cells were seen to be growing on the agar plate. Considerable callus growth was observed after 6 weeks on plates inoculated with potato cell suspensions.

Interaction of tissue aggregates and sporangia

Normal tissues. Considerable development of the fungus took place within 3 days in that part of the streak of *Phytophthora infestans* sporangia in contact with the

Majestic tissue aggregate. On the same plate the control streak and the streak in contact with Orion tissue aggregate showed only slight growth, even after 8 days (Pl. 1, fig. 2).

Treated tissues; Solanum tuberosum var. Majestic. Living, frozen (–40°, 30 min.), and boiled (1 min.) tissue aggregates of var. Majestic were compared under the conditions outlined above. The results showed that the stimulus of *Phytophthora infestans* was decreased on boiling, and destroyed completely on freezing.

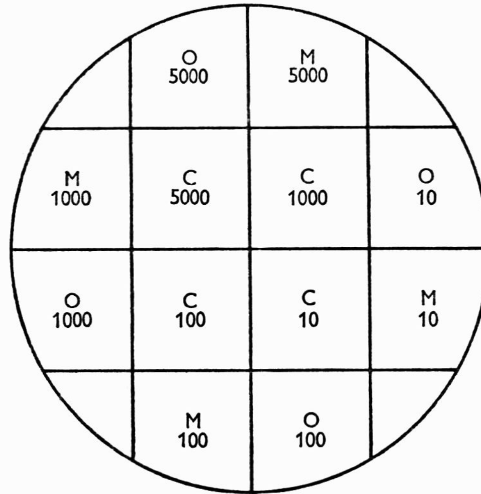

Fig. 1. Layout of experiment on relation of number of sporangia in inocula and resultant infection. M = *Solanum tuberosum* var. Majestic, O = var. Orion, C = control. 5000, 1000, 100 and 10 = no. sporangia of *Phytophthora infestans* in inoculum drop.

Table 2. *Stimulation of Phytophthora infestans by variously treated tissue aggregates of Solanum tuberosum var. Orion and var. Majestic*

	Treatment of potato tissue aggregates				
	Living	Boiled	Frozen	Boiled and frozen	Frozen and boiled
Var. Majestic	Strong stimulus	Decreased stimulus	No stimulus	Decreased stimulus	Decreased stimulus
Var. Orion	No stimulus	Some stimulus	No stimulus	n.t.	n.t.

n.t. = not tested.

When previously frozen tissue was boiled, or boiled tissue was frozen, their stimulatory effect on *Phytophthora infestans* was the same as that obtained with tissue that had only been boiled (Pl. 2, fig. 3).

Treated tissues; Solanum tuberosum var. Orion. Living tissue aggregates of var. Orion were compared with boiled and frozen tissues. The living tissues and the frozen tissues did not stimulate *Phytophthora infestans* but growth was stimulated by the boiled tissue after 8 days (Pl. 2, fig. 4).

Effect of size of inoculum. It was of interest to discover whether the lack of stimulation by var. Orion, which might be interpreted as resistance to infection,

could be overcome by increasing the size of the inoculum in the experiments. Petri plates, prepared as above, were marked off with lines into 12 squares, and the Orion and Majestic tissue aggregates laid out alternately as shown in Fig. 1. Inocula at 5000, 1000, 100 and 10 sporangia, suspended in 0·02 ml. liquid medium, were applied to the tissues, and as control drops directly on the agar, in the order shown in the figure. When read at 7 days the distinction between Majestic and Orion tissues was maintained at all sizes of inoculum, except 5000 sporangia where a slight fungal activity appeared round the Orion tissue. This was no greater than the fungal growth obtained from the control on the agar alone. With the inoculum of 10 sporangia, no growth was seen either on the Majestic or on Orion tissue.

DISCUSSION

Although *Phytophthora infestans* is clearly dependent on its host, it can be grown alone on artificial media. The medium which supports the growth of potato tissue cultures also supports the growth of *P. infestans*, and the growth of the fungus is relatively rapid when large inocula are used. However, when smaller inocula, e.g. suspensions containing about 100 sporangia, are added to this medium, germination takes place and usually a few colonies slowly become established. The addition of living Majestic potato cells to the surface of the medium stimulated the growth of the *P. infestans* in a spectacular fashion. This is perhaps not unexpected. When Orion cells were inoculated in the same manner they also stimulated the growth of the fungus, relative to the control, but were not as potent as the Majestic cells. It is surprising that Orion cells not only supported growth, but stimulated it, for with the intact plant var. Orion the *P. infestans* is rapidly excluded. Tomiyama, Takakuwa & Takase (1958) found that R genes were only expressed in slices of potato tissue which were more than ten cells in thickness. This may be analogous to the situation where Orion cell suspensions stimulated *P. infestans*, while tissue aggregates did not.

Tissue aggregates of Majestic stimulated the growth of *Phytophthora infestans*, while tissue aggregates of Orion did not. We saw no sign of any toxic inhibition of the limited fungal growth in the vicinity of the Orion tissue. Since both living cell suspensions and dead (boiled) tissue aggregates of Orion stimulated, we must adduce that living tissue aggregates of Orion prevent the fungus from responding to the stimulus. When the fungus, at the point of infection, is excluded by the Orion cells, the stimulus can never be transported to the rest of the fungal mycelium.

Our observations of the sporangial streaks on the agar plate lead us to suppose that the tissues have little or no effect on the germination of the sporangia or on germ-tube development. It is only when the fungus reaches and infects the susceptible potato tissue that the fungal growth is stimulated. This stimulus is prolonged and such that the fungus luxuriates eventually to fill the Petri plate.

It is of interest that killing the Majestic and Orion tissue by boiling left the stimulus intact although diminished. In the Orion tissue, killing by boiling inactivated the resistance mechanism either by killing the cells which generated it or by destroying any antifungal substances present in the tissue. This suggests that the resistance is a property of living and not of dead tissues. Tissues killed by freezing to -40°, at which temperature not all enzymes would be destroyed, behaved

differently from the boiled cells, and both Majestic and Orion tissues so treated did not stimulate the fungus. It is possible that the resistance of Orion is left intact by this treatment and that resistance is developed in Majestic. However, a simpler explanation is that enzymic destruction of the stimulating substance takes place in both tissues after they have thawed. This view is further strengthened by the demonstration that when freezing was followed by boiling, or boiling was followed by freezing, the stimulus was preserved. The fact that a stimulus was demonstrated in dead (boiled) tissue of Majestic suggests that it is a normal tissue metabolite, and not formed in response to infection.

This work has shown very clearly that tissue aggregates of *Solanum tuberosum* var. Orion can support and stimulate the growth of *Phytophthora infestans*, when the aggregates are dead, so that there is no basic nutritional reason for the failure of *P. infestans* to invade the tissue. Further it has shown that the living Orion cells act only when they are infected and that there is as yet no evidence of a massive diffusion of toxic material from the resistant tissue, either before or after fungal invasion or attempted invasion. Finally the possible effect of tissue mass in determining the expression of the resistance (see Tomiyama *et al.* 1958) has been demonstrated under conditions where the complicating effect of an extensive wound surface is absent.

Our thanks are due to Dr A. Harrison for advice on methods of tissue culture and to the Agricultural Research Council for the support of one of us (D. S. I.).

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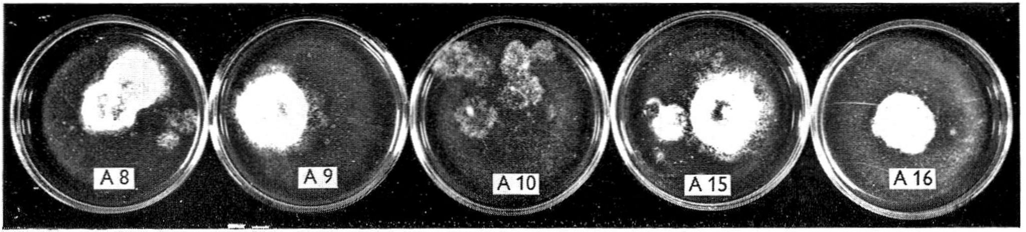

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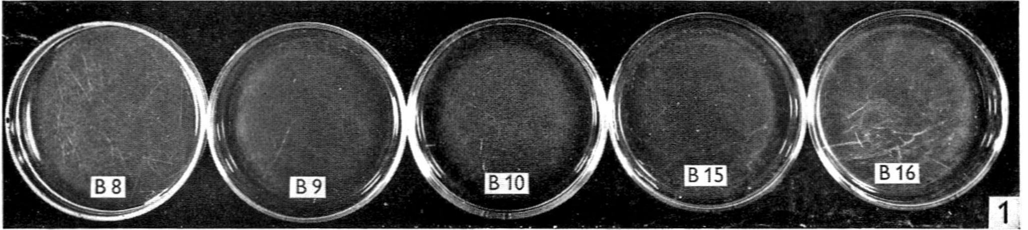

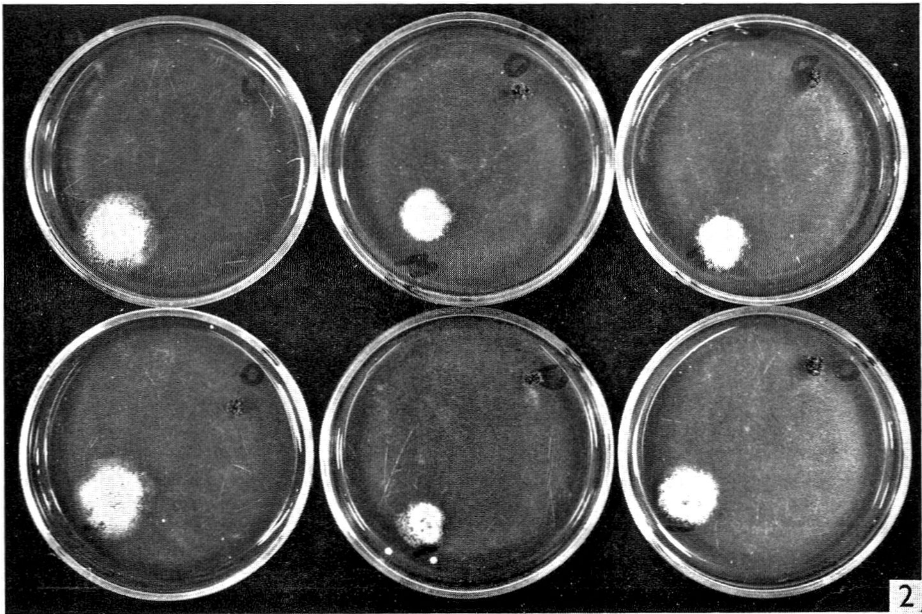

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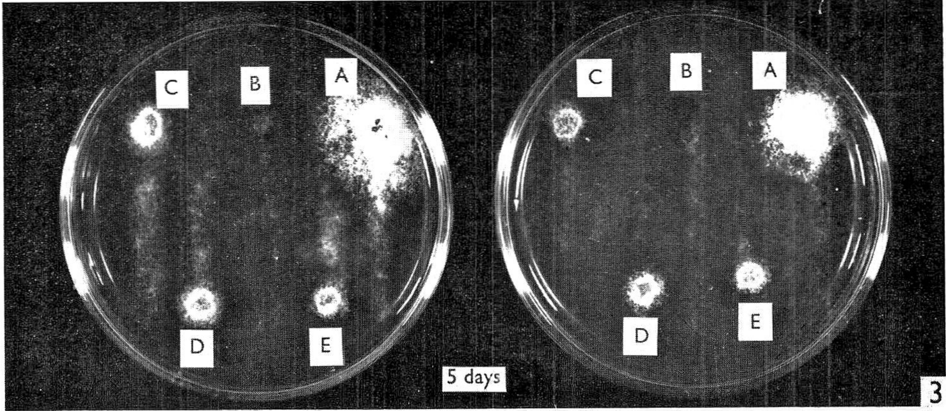

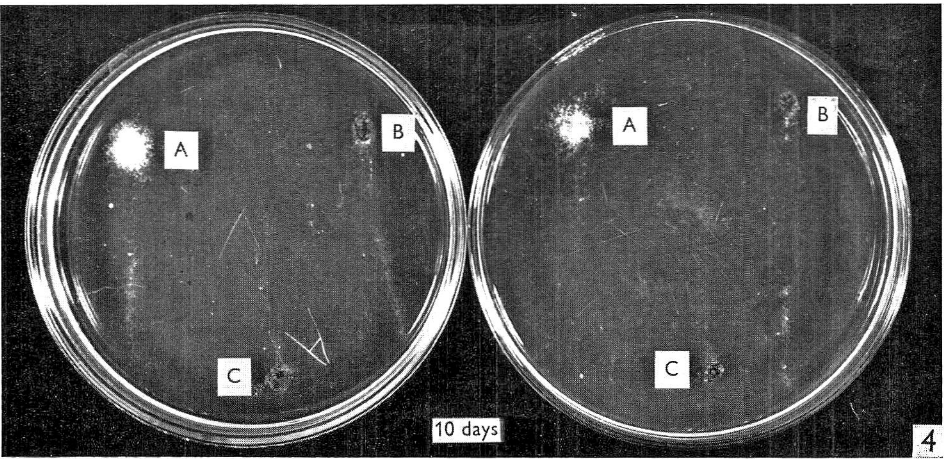

D. S. INGRAM AND N. F. ROBERTSON

EXPLANATION OF PLATES

Plate 1

Fig. 1. Five examples of the interaction between 100 sporangia of *Phytophthora infestans* and cell suspensions of *Solanum tuberosum* var. Majestic tubers and five controls. 14 days.

Fig. 2. Six examples of the interaction between streaks of sporangia of *Phytophthora infestans* and tissue aggregates of tubers of *Solanum tuberosum* var. Majestic and var. Orion. 5 days.

Plate 2

Fig. 3. Two examples of the interaction between streaks of sporangia of *Phytophthora infestans* and pre-treated tuber tissue aggregates of *Solanum tuberosum* var. Majestic. 5 days. A, living; B, frozen, C, boiled; D, boiled then frozen; E, frozen then boiled.

Fig. 4. Two examples of the interaction between streaks of sporangia of *Phytophthora infestans* and pre-treated tuber tissue aggregates of *S. tuberosum* var. Orion. 10 days. A, boiled; B, living; C, frozen.

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