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The Relationships of Bacteria Within the Family Bacteroidaceae as shown by Numerical Taxonomy

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SUMMARY

A comparison, by numerical taxonomy methods, was made of 72 named or freshly isolated strains of Gram-negative anaerobes which were considered to belong within the family Bacteroidaceae. In the first analysis 57 strains were compared. Representative strains were then selected and compared with additional named strains in a second analysis. Four phena were identified: (1) strains of *Sphaerophorus*, *Fusobacterium* and *Bacteroides melaninogenicus*, (2) *Bacteroides*, (3) unnamed poultry isolates, (4) a second group of unnamed poultry isolates. The most useful differential tests were found to be: (*a*) cell morphology, (*b*) the terminal pH value in glucose broth and the production of formic, acetic, propionic or butyric acids, (*c*) the production of propionic acid from threonine, (*d*) growth stimulation by bile, (*e*) the effect of various inhibitors.

INTRODUCTION

Difficulties in the identification and classification of the anaerobic non-sporing Gram-negative bacteria were first encountered in studies of the intestinal flora of chickens, when a group of organisms was isolated which bore no resemblance to any named strains (Barnes & Goldberg, 1962; Goldberg, Barnes & Charles, 1964). It was decided to use the methods of numerical taxonomy in an attempt to assign these organisms to a particular genus and at the same time to eliminate some of the confusion which exists within the family Bacteroidaceae. At present it is difficult even to attach particular characteristics to named organisms since several different classifications are in use (Prévot, 1938; *Bergey's Manual* 1957; Beerens, Castel & Fievez, 1962), and, owing to the difficulties of maintaining these organisms as freeze-dried cultures, there has been little interchange of isolates between workers of different countries.

The tests used in this investigation were based on the more important characters described in *Bergey's Manual* (7th ed. 1957) together with those tests which were considered important by Beerens, Schaffner, Guillaume & Castel (1963). It was hoped to obtain further information on the relationships of various named strains to each other, and also to select key tests for identification purposes, by using strains from as many sources as possible in a combination of tests.

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METHODS

Sources of organisms. The origin of the strains used in the two computer analyses is given in Table 1.

Media and cultural conditions. Details of the media used and conditions of culture have already been described by Goldberg *et al.* (1964) and Barnes, Impey & Goldberg (1966). The incubation temperature was 37° unless otherwise stated.

Table 1. *The origin of the strains used in the two computer analyses*

Source	Computer analysis
From the National Collection of Type Cultures (NCTC)	
Bacteroides necrophorus 7155	1
B. fragilis 9343	1 and 2
B. melaninogenicus 9337	2
From the American Type Culture Collection (ATCC)	
Bacteroides vulgatus 8482, *B. ovatus* 8483, *B. thetaiotaomicron* 8492, *B. distasonis* 8503, *B. symbiosis* 12829, *Sphaerophorus varius* 8501, *S. necrophorus* 12290, *Fusobacterium polymorphum* 10953	1 and 2
From Dr H. Beerens (Institut Pasteur, Lille)	
Fusiformis biacutus 25, *Eggerthella clostridiformis* 22, 61, 353, 354, *Ristella variabilis* 285	1
Ristella convexa 265, *R. incommunis* 308, *R. variabilis* 264, 266, 267, *Sphaerophorus necrophorus*, *S. funduliformis*, *S. freundii*	1 and 2
Sphaerophorus necrophorus Fievez N117, N167, N252, *Fusiformis fusiformis* 389	2
From Dr T. Mitsuoka (Animal Physiology Laboratory, The Institute of Physical and Chemical Research, Tokyo, Japan): isolated from chicken caeca	
Strains AIII-45, AU23-33, CH32-17, CH32-20 CH36-9, N5-43, N209-12, N210-25, N212-47	1
Strain AU21-27	
From Dr S. M. Finegold (Dept. of Medicine, Wadsworth Veterans Hospital, Los Angeles, California)	
Bacteroides melaninogenicus B477, B536, B537	2
From Dr R. J. Gibbons (Forsyth Dental Centre, Boston)	
Fusobacterium polymorphum, *Fusobacterium* JF5	2
Isolated by the authors	
From human faeces—strains 9/1 and 12/1	1
From chicken caeca—strains belonging to Groups 1, 2, 3 and 4 of Barnes & Goldberg (1965)	1 and 2
Group 1, strain EBF59/96P	1 and 2
Group 2, strains EBF59/91P, 95P	1
Group 3, strains EBF61/31B, 66, 67, 68 and 69	1
Group 3, strain EBF61/60B	1 and 2
Group 4, strains EBF59/78, 85, 92P, 100, EBF60/26, EBF61/30, 63	1
Group 4, strains EBF58/74, EBF59/72, EBF61/42, 56, 61	1 and 2
From duck caeca—strains EBD1/1B, 3B and 4A	2
From turkey carcass—strains EBT2/61 and 68	2

For *Bacteroides melaninogenicus* strains all the media were supplemented with menadione 0·5 μg./ml. and laked blood 5% (v/v) which was prepared by repeated freezing and thawing.

Morphology. Examinations for Gram reaction, size, shape and appearance, were carried out using 18–20 hr. cultures in Reinforced Clostridial Medium (RCM) of (Hirsch & Grinsted, 1954). Colonies on RCM agar (Hirsch & Grinsted, 1954) or blood agar (Beerens *et al.* 1963) were examined after 2 or 3 days incubation in a 90% hydrogen and 10% carbon dioxide atmosphere.

Lecithinase. Lecithinase production was determined using the method of Fievez (1963). The egg-yolk plates were examined daily up to 5 days.

Table 2. *Characters examined*

Test	Features scored
Morphology	
Gram reaction	Negative or variable
Shape of cells	Rods, beaded rods or filaments
Arrangement	Single rods, diplobacilli, or chains
Length	Short (0·2–0·59 μ), medium (0·6–3·0 μ), long (over 3 μ)
Diameter	Slender (0·2–0·9 μ), stout (1·0–2·0 μ)
Colonies on RCM agar	
Size	Small (2 mm or less), large (over 2 mm)
Edge	Entire or uneven
Surface	Raised or convex
Colour	White, grey or black
Appearance	Opaque or translucent
Colonies on blood agar	
Haemolysis	Negative or β haemolysis
Growth in RCM broth	
20 °C	Negative or positive
45 °C	Negative or positive
Turbidity	Flocculent or uniform
Gas production	Copious or negative
Glucose fermentation	
Terminal pH	pH 5·5 and below or above pH 5·5
Volatile fatty acids produced	
Formic, acetic, propionic or butyric acids	Positive when present at > 1 μM/ml.
Fermentation of:	
Sucrose, cellobiose, galactose, arabinose, xylose, mannitol, inositol, salicin, and glycerol	Negative or acid produced
Cysteine milk	No change, acid, or acid and clot
Lecithinase production	Negative or positive
Gelatin liquefaction	Negative or positive
Indole production	Negative or positive
Hydrogen sulphide production	Negative, weakly positive, strongly positive
Propionic acid from threonine	Negative or positive
Growth stimulation by 10% bile	Negative or positive
Growth in the presence of	
Brilliant green 1/100,000, penicillin 10 μg./ml., neomycin 25 μg./ml., polymyxin B 10 μg./ml., and chlortetracycline 10 μg./ml.	Negative or positive
Growth stimulation by menadione	Negative or positive

Biochemical tests. For carbohydrate fermentation, H_2S production, indole, etc., additions were made to the basal medium of Beerens (1953–54) as described by Goldberg *et al.* (1964).

Detection of volatile fatty acids. The volatile fatty acids produced from the fermentation of glucose were determined by the method of Guillaume, Beerens & Osteux (1956) as modified by Charles & Barrett (1963).

Bile Stimulation. Stimulation of growth was determined by adding 10% bile to the Basal Glucose Phosphate (BGP) medium of Barnes & Goldberg (1962).

Effect of inhibitors. Growth in the presence of brilliant green 1/100,000 (v/v),

penicillin 10 μg./ml., neomycin 25 μg./ml., polymyxin B 10 μg./ml., or chlortetracycline 10 μg./ml. was tested by adding the required concentration of each inhibitor to RCM broth.

Theonine test. The production of propionic acid from threonine was detected following the method of Beerens (personal communication). This test was later modified by Beerens & Tahon-Castel (1965). The strains were examined using both techniques.

Characters. The organisms were examined for the characters given in Table 2.

Computer analysis. Both the computer analyses were carried out by Mr J. C. Gower (Rothamstead Experimental Station, Harpenden, Hertfordshire) using the similarity coefficient defined by Gower (1967) and a single linkage sorting programme (Sneath, 1957). The coefficient of similarity used allows for the inclusion of quantitative and multivalue qualitative characters as well as dichotomies for which negative matches do not contribute to the similarity coefficient. A detailed discussion of the choice of coding has been given by Thornley (1967).

In both analyses four tests were recorded as dichotomies, these were the production of formic, acetic, propionic or butyric acids, whilst hydrogen sulphide production was scored quantitatively (Table 2). All of the remaining tests were recorded as alternatives where equal weighting was given to the positive or negative answer. In the first analysis 83 and in the second 86 features were analysed.

RESULTS

Preliminary computer analysis. In the original computer analysis the main aim was to try and relate the chicken isolates to named strains. As they were intestinal in origin it was considered probable that they would relate more closely to *Bacteroides* than to the fusobacteria. It was also evident that morphologically they bore little resemblance to *Fusobacterium* species. Hence most of the named strains used for comparative purposes were either *Bacteroides* (*Ristella* in some classifications) or strains designated *Eggerthella* (Beerens *et al.* 1963). Only two strains of fusobacteria were included.

The 57 strains tested are listed in Table 1, whilst the characters analysed are given in Table 2. All the characters were used with the exception of haemolysis, lecithinase production and growth stimulation by menadione (as *Bacteroides melaninogenicus* strains were omitted from the first analysis). All of the strains were obligate anaerobes and inhibited by 10 μg./ml. chlortetracycline, none fermented glycerol or hydrolysed gelatin. These properties were therefore omitted from the analysis.

The similarity matrix is shown in Fig. 1. Five phena were defined which contained 50 of the strains examined.

(1) *Eggerthella clostridiformis.* The four strains received from Beerens were identical with *Bacteroides necrophorus* strain NCTC 7155. Although at the time of analysis these strains were not considered to form spores, it was subsequently shown that all of these five strains produced spores after prolonged incubation on RCM agar (but not on the VL agar of Beerens). They were therefore excluded from further consideration.

(2) *Bacteroides.* This phenon included the ATCC strains, those of Beerens and *Bacteroides fragilis* strain NCTC 9343, together with 8 of the chicken isolates from Mitsuoka. *Sphaerophorus necrophorus* ATCC 12290 also had a high similarity to the *Bacteroides* strains.

(3) *Sphaerophorus*. The strain *S. varius* ATCC 8501 was closely related to three strains obtained from Beerens. One of the chicken isolates 59/96P also grouped with these strains although having a much lower similarity value.

(4) *Chicken isolates*. Group 4. Fourteen of the strains previously designated group 4 (Barnes & Goldberg, 1965) had a low similarity value to either *Bacteroides* or *Sphaerophorus*. A full description of these organisms was given by Goldberg, Barnes & Charles (1964) and the computer results justify their caution in not assigning the organisms to a particular genus.

(5) *Chicken isolates*. Group 3. A further group of 6 chicken isolates had the lowest similarity to all the other groups. They were originally designated group 3 by Barnes & Goldberg (1965). They have since been shown to be Gram-positive in very young cultures and their position within the family Bacteroidaceae is thus in doubt.

The only two fusobacteria, *Fusobacterium polymorphum* and *F. biacutus*, showed little similarity to each other. *Fusobacterium biacutus* has since been shown to produce spores (Dr H. Beerens, personal communication) and should therefore be eliminated from the family Bacteroidaceae.

Further analysis of representative strains

The results from the first analysis were sufficiently encouraging that the computer analysis was repeated to include additional named strains. In particular, three further strains of *Sphaerophorus necrophorus* were included as the strains originally obtained from Dr Beerens were atypical in certain characters. Two further strains of *Fusobacterium* were also tested but it was difficult to obtain representative strains of these organisms as they tend not to be maintained in stock culture collections. Four strains of *Bacteroides melaninogenicus* were added, the requirement for menadione being scored as a separate character. Representative chicken isolates were included, together with a few freshly isolated strains from ducks and turkeys.

Two further tests were used which had been considered important in the differentiation of the pathogenic strains of *Sphaerophorus* (Fievez, 1963). These were lecithinase production and haemolysis.

In all 40 strains were analysed; of these 25 strains had been used in the first analysis. The additional 15 strains are listed above. The similarity matrix is shown in Fig. 2. It is evident that the additional strains and the extra tests used widened the differences between the main groups of organisms shown in Fig. 1. Four phena were now evident:

(1) Containing *Sphaerophorus*, *Fusobacterium* and *Bacteroides melaninogenicus* together with 3 poultry isolates.

(2) *Bacteroides*, together with the representative chicken isolate of Mitsuoka.

(3) The chicken isolates belonging to Group 4 (Barnes & Goldberg, 1965).

(4) The most representative chicken isolate belonging to Group 3 (Barnes & Goldberg, 1965) together with freshly isolated turkey and duck strains.

The family relationships are shown in the dendrogram Fig. 3, whilst the characteristics of the organisms are given in Tables 3 and 4.

In considering these results the discussion will be confined mainly to representative strains obtained from the Type Culture Collections and other workers. The poultry isolates will be discussed in detail elsewhere (Barnes & Impey, to be published).

Phenon 1: Sphaerophorus, Fusobacterium and Bacteroides melaninogenicus. The main characters which were common to *Sphaerophorus* and *Fusobacterium* and which

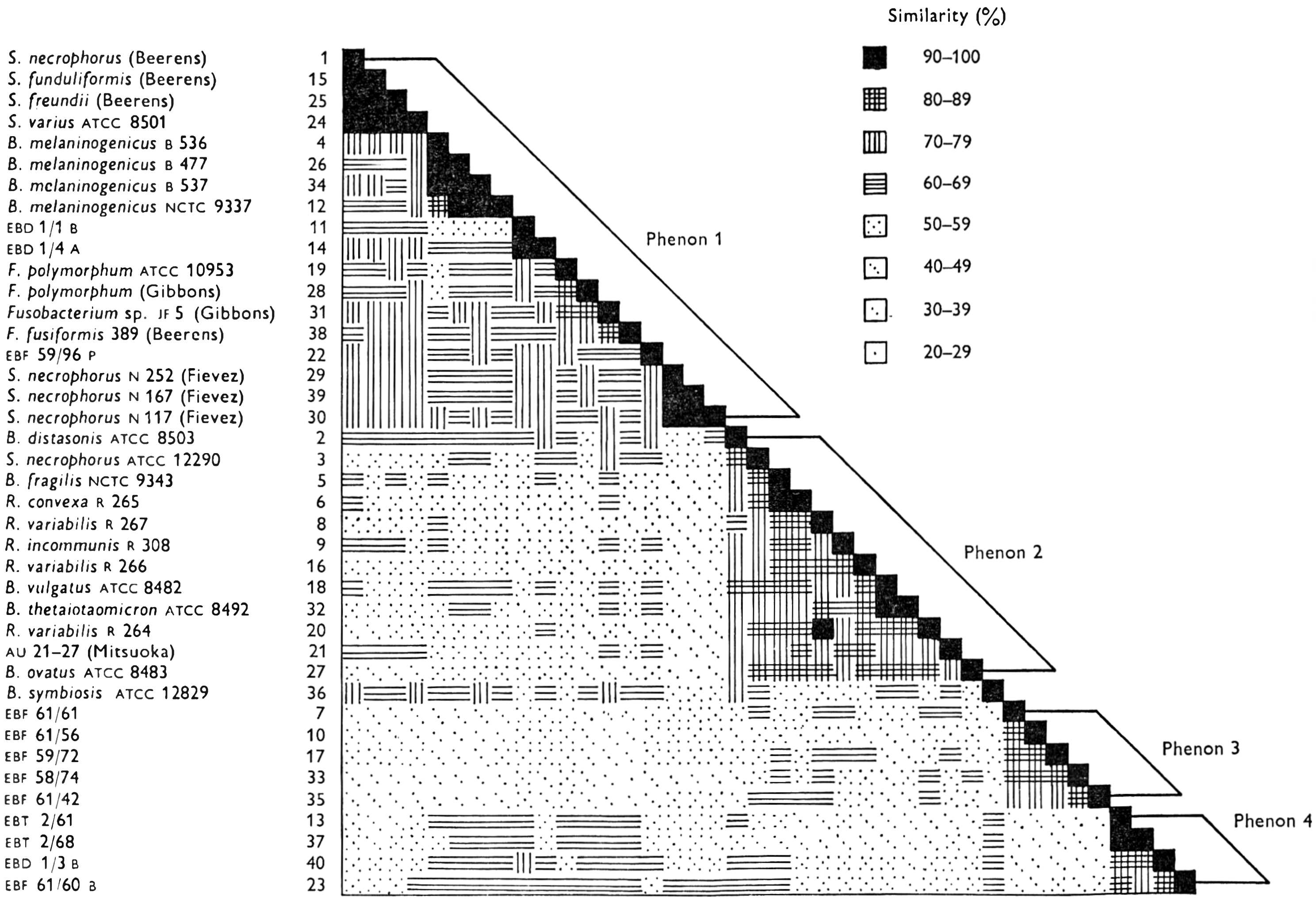

Fig. 2. Similarity matrix of 40 strains, 25 of which were also used in the first analysis.

differentiated these organisms from *Bacteroides* were the production of propionic acid from threonine, the high terminal pH in glucose broth, the production of butyric acid in glucose broth and the failure to produce acid from most of the other carbohydrates tested. All the strains tested grew in the presence of brilliant green 1/100,000.

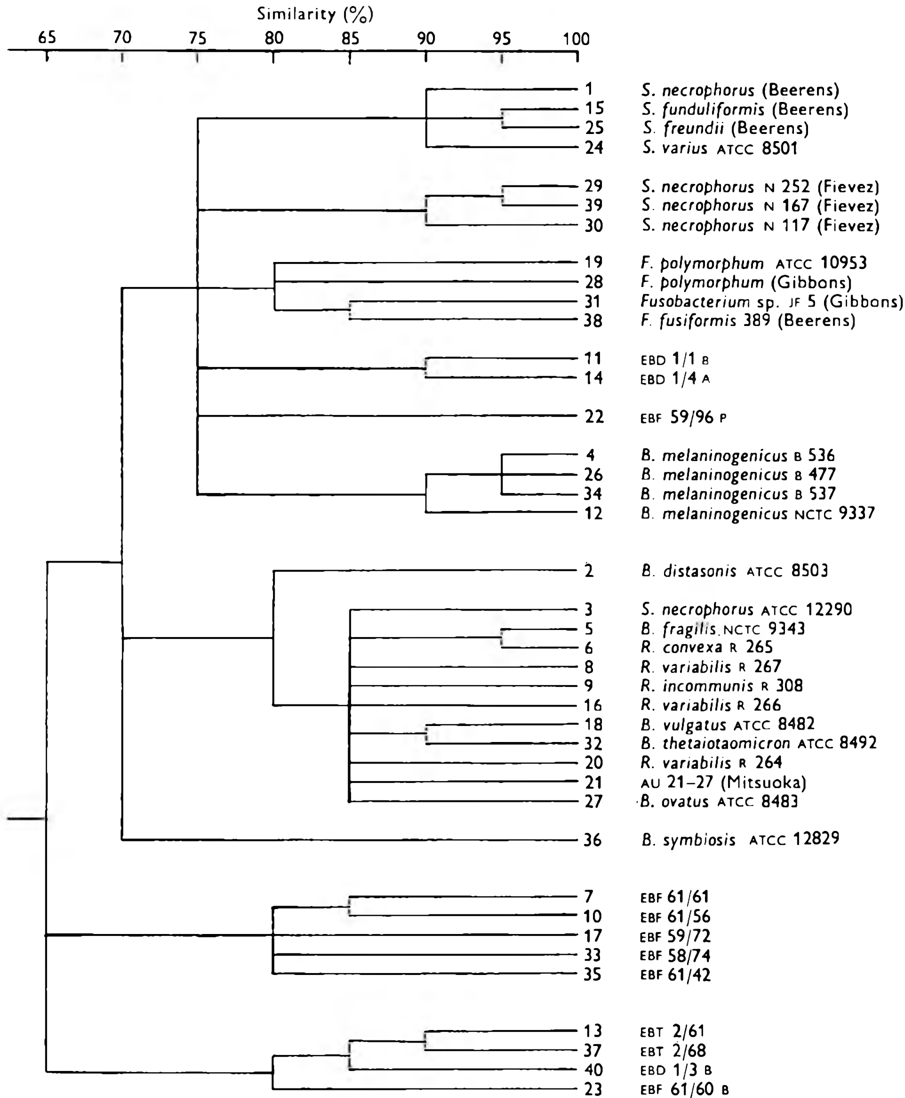

Fig. 3. Taxonomic hierarchy of strains of *Sphaerophorus*, *Fusobacterium*, *Bacteroides* and unnamed poultry isolates.

The strains of *Sphaerophorus* differed morphologically from those of *Fusobacterium*. They also produced copious gas, and generally produced propionic acid as well as acetic and butyric acids from glucose. They were inhibited by polymyxin 10 μg./ml. They produced lecithinase and grew at 20°.

The *Fusobacterium* spp. were characterized by forming long rods or filaments with

Table 3. *Some of the more important differential characters within the family Bacteroidaceae*

	No. of strains*	Description of cells	Glucose fermentation: Terminal pH 4·6 to 5·5	Terminal pH 5·6 to 6·2	Volatile fatty acids produced: Formic	Acetic	Propionic	Butyric	Propionic acid from threonine	Copious gas production	Stimulation by bile	Stimulation by menadione
Sphaerophorus spp.	4	Medium, slender rods, round ends, single, or filaments	−	+	−	+	+ (3)†	+	+	+	−	−
S. necrophorus, Fievez strains	3	Long slender rods, round ends, and filaments	−	+	+	+	+	+	+	+	−	−
Fusobacterium spp.	4	Long slender rods and filaments with pointed ends	−	+	+	+ (3)	−	+	+	−	−	−
B. melaninogenicus	4	Medium slender rods with rounded ends; single	−	+	+	+	− (3)	+	−	−	−	+
Bacteroides spp.	12	Single, short, medium or long rods, generally slender, rounded ends	+	−	− (7)	+	− (8)	−	−	−	+ (9)	−
Chicken isolates, group 4	5	Single, very large, stout rods, rounded ends	+	−	−	+	+	−	− (3)	−	−	−
Chicken isolates, group 3	4	Medium-to-long slender rods and beaded chains, pointed or rounded ends	+ (3)	− (3)	+	− (3)	−	+ (3)	−	−	−	−

* Strains EBD 1/1 B, 4 A, EBF 59/96 P and *B. symbiosis* ATCC 12829 have not been included in this Table.
† Parentheses denote number of strains with that particular reaction.

Table 4. *Other characters of the anaerobic non-sporing Gram-negative rods*

	No. of strains*		Production of:			Growth at:			Growth in presence of:				Acid from:							
		β-haemolysis	Lecithinase	Indole	H_2S†	20°	45°	Behaviour in cysteine milk‡	Brilliant Green 1/100,000	Polymyxin 10 μg./ml.	Neomycin 25 μg./ml.	Penicillin 10 μg./ml.	Sucrose	Cellobiose	Galactose	Arabinose	Xylose	Mannitol	Inositol	Salicin
Sphaerophorus spp.	4	–	+	+ (3)§	+ + + (3)	+	– (3)	NC	+	–	+	–	–	–	+	–	–	–	–	–
S. necrophorus Fievez strains	3	+	+	+	+ + +	+	+	NC	+	–	–	–	–	–	–	–	–	–	–	–
Fusobacterium spp.	4	–	–	+	+ + + (3)	–	– (3)	NC	+	+ (2)	+	–	– (3)	– (3)	+ (3)	–	–	–	–	–
B. melaninogenicus	4	N.T.‖	N.T.	+	+ + + (2) – (2)	+	+	NC	–	+	+	– (3)	–	–	–	–	–	–	–	–
Bacteroides spp.	12	+ (9)	–	– (8)	+ (8) + + + (4)	+ (7)	– (10)	A + C(4) A(5) NC(3)	– (9)	+	+	– (11)	+	– (9)	+	+ (9)	+ (11)	–	–	– (8)
Chicken isolates Group 4	5	+	+ (3)	–	– (1) + (4)	– (3)	+	A + C(4) A(1)	+ (3)	–	– (3)	+ (3)	– (3)	+ (4)	+	+	+	+	+ (3)	+ (3)
Group 3	4	+ (3)	– (3)	–	+ (3)	–	+	NC	+	+	+	– (3)	–	–	–	–	–	–	–	–

* Strains EBD 1/1B, 4A, EBF 59/96P and *B. symbiosis* ATCC 12829 have not been included in this Table.
† H_2S: + + +, strongly positive; +, positive; –, negative.
‡ NC, no change; A, acid; A + C, acid and clot.
§ Parentheses denote number of strains with that particular reaction.
‖ N.T., not tested.

pointed ends; they were non-haemolytic; did not produce lecithinase; did not produce propionic acid from glucose.

The strains of *Bacteroides melaninogenicus* were related more closely to the *Sphaerophorus* strains than to *Bacteroides*. Their resemblance was based partly on their morphology, the production of formic, acetic and butyric acids in glucose broths and their failure to ferment sucrose, cellobiose, arabinose, xylose, mannitol, inositol and salicin. They differed from *Sphaerophorus* and *Fusobacterium* in their failure to produce propionic acid from threonine, inhibition by brilliant green 1/100,000, and growth in the presence of polymyxin 10 μg./ml. In these latter three characters they resembled *Bacteroides*.

Phenon 2: Bacteroides. Morphologically the *Bacteroides* strains could not be differentiated from *Sphaerophorus*. The group was differentiated by failure to produce propionic acid from threonine, fermentation of glucose with a low terminal pH (4·6–5·5), the failure to produce butyric acid. The organisms grew in the presence of polymyxin 10 μg./ml. and generally fermented a wider range of carbohydrates. Many but not all of the strains were stimulated by bile.

Phenon 3: Chicken isolates (group 4). These organisms were characterised by their large size and shape, the production of acetic and propionic acids in glucose broth and the fermentation of a wide range of carbohydrates, in particular mannitol. They showed a low similarity both to *Sphaerophorus* and *Bacteroides*.

Phenon 4: Chicken isolates (group 3). These organisms showed the least similarity to all other groups. In 6 hr broth cultures the organisms had a Gram-positive reaction but tended to be negative in 24 hr cultures. The organisms were very pleomorphic, their shapes ranging from coccoidal forms to long rods. Of the carbohydrates tested only glucose was fermented. Formic acid was produced by all the strains together with trace amounts of acetic and butyric acids with some of the strains.

DISCUSSION

There have been several detailed studies recently on individual genera within the family Bacteroidaceae, e.g. *Fusobacterium* (Baird-Parker, 1960), *Sphaerophorus* (Fievez, 1963), *Bacteroides* (Beerens *et al.* 1963), and the tests used above were based on those found to be important for each of these genera. The results obtained justify the division of the family into a number of groups but these groups can only be assigned to specific genera after agreement has been reached on the correct nomenclature.

The properties of the *Fusobacterium* strains agreed closely with those described by Baird-Parker (1960), whilst the *Sphaerophorus necrophorus* strains (Fievez N 117, N 167, N 252) conformed with the description of the species given by Fievez (1963), but differed in several characteristics from the other four *Sphaerophorus* strains tested (Table 4). The close relationship between *Fusobacterium* and *Sphaerophorus* which was shown in this analysis is supported by Sebald (1962), who found that the DNA base ratios were similar for the organisms within the two genera and suggested that they should be included together in one genus.

The separation of the *Bacteroides* strains into a separate group from *Fusobacterium* and *Sphaerophorus* is also supported by the DNA base ratio analyses of Sebald (1962) who showed that the G+C ratio was > 41 % as compared with 27–34 % for the other two genera. Beerens *et al.* (1963) suggested that many of the species described within the

genus *Bacteroides* (or *Ristella*) should be grouped together within one species *Eggerthella convexa*. This analysis confirmed the difficulty of differentiating between the various species but certain tests such as those used by Reinhold (1964) to differentiate *B. convexus* from *B. thetaiotaomicron* were not included.

It is evident that a number of type-culture collection strains have been incorrectly named—in particular the strain *Bacteroides necrophorus* NCTC 7155 which is identical with *Eggerthella clostridiformis* (Beerens) and does not resemble the other *Sphaerophorus* strains. The strain *S. necrophorus* ATCC 12290 is almost certainly a *Bacteroides* strain. *B. symbiosis* ATCC 12829 shows little resemblance to the other *Bacteroides* strains and needs further investigation.

There was no difficulty in recognizing the strains of *Bacteroides melaninogenicus* because of their typical black colonies and requirements for menadione; however, the taxonomic position of this organism within the family needs careful consideration, It was assigned by Beerens *et al.* (1962) to a separate genus, and support for this suggestion comes from its low similarity to the other *Bacteroides* strains.

One of the main purposes of the analysis was to determine which tests were most useful for identification purposes. Perhaps the most difficult problem was initially to determine which organisms might come within the family Bacteroidaceae, which is defined as containing Gram-negative anaerobic non-sporing rods. Strains were included in the first analysis which were subsequently shown to produce spores on particular media. Included amongst these was *Fusiformis biacutus* which has always been considered a non-sporing organism. Similarly, the *Eggerthella clostridiformis* strains of Beerens formed spores. The whole relationship of the Gram-negative sporing organisms to the Bacteroidaceae needs further investigation. The problem of obtaining a reliable Gram reaction has again been demonstrated. In these analyses the group 3 chicken isolates were included initially as Gram-negative bacteria but were subsequently found to be Gram-positive. However, they had a low similarity to all the other organisms tested, indicating that they also differed in many other properties.

As can be seen from Table 3, the morphological differentiation of the *Sphaerophorus* strains from *Bacteroides* was difficult, but *Fusobacterium* and the two groups of chicken isolates all had a characteristic morphology. Most of the traditional tests (Table 4) such as indole production, hydrogen sulphide production and the fermentation of various carbohydrates were too variable within the groups to be used to separate them. Amongst the differential tests considered to be most useful are those shown in Table 3. These were: (1) the terminal pH in glucose broth and the types of volatile fatty acids produced, i.e. formic, acetic, propionic or butyric acids, (2) the production of propionic acid from threonine, (3) growth stimulation by bile. Other useful tests shown in Table 4 included the effect of various inhibitors, in particular polymyxin 10 μg./ml.

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The Influence of Certain Trace Metals on Bacterial Growth and Magnesium Utilization

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SUMMARY

The uptake of Mg^{2+} in Mg^{2+}-limited cultures of the Gram-negative *Escherichia coli* is rapid and is complete some time before the onset of the stationary phase. In similar cultures of the Gram-positive *Bacillus megaterium* and *B. subtilis* F3 growth and Mg^{2+} assimilation cease at the same time and when only part of the available Mg^{2+} has been utilized; thereafter efflux of the cation may occur. In these cultures, as in dilute suspensions of the bacilli in a Mg^{2+}-deficient medium, viability is maintained in a high percentage of the organisms, and growth occurs on the addition of Mg^{2+}, even if this is delayed for 20 hr. The minimum growth-requirement for Mg^{2+} varies for different Gram-positive bacilli and is particularly low for *Bacillus subtilis* var. *niger*. The response of this organism to Mg^{2+} is unaffected by Mn^{2+}. The Mg^{2+} requirements of *B. megaterium* and *B. subtilis* F3, however, are reduced by 25 μM Mn^{2+}. Although this concentration of Mn^{2+} is unable to support growth of these bacilli in the complete absence of Mg^{2+}, it appears to stimulate the uptake of the latter cation from dilute solutions. Mn^{2+} also is assimilated during growth by both Gram-positive and Gram-negative bacteria, although less efficiently than Mg^{2+}, and is incorporated into the ribosomes.

INTRODUCTION

In a recent paper Tempest, Dicks & Meers (1967) have reported that the magnesium contents of Mg^{2+}-limited *Bacillus subtilis* var. *niger* and *Aerobacter aerogenes*, when grown in continuous culture at corresponding rates, are not significantly different, and that, at low dilution rates, the uptake of the cation by both of these organisms is almost complete. Tempest *et al.* (1967) conclude from these findings and batch culture experiments with *B. subtilis* var. *niger* and *B. megaterium* that the interpretation of previous observations (Webb, 1966) on the differences in the abilities of certain Gram-positive bacilli and Gram-negative bacteria in batch culture to concentrate Mg^{2+} from simple chemically defined media is incorrect. The authors suggest that any variation in the response of these organisms to low concentrations of Mg^{2+} must be due to physiological factors other than differences in assimilation, and in this connexion infer the importance of a differential effect of Mg^{2+} deficiency on the death rates of Gram-positive and Gram-negative organisms in aqueous environments. This hypothesis, which presumably would require the more efficient retention of Mg^{2+} by the Gram-negative bacteria, is supported by the difference that has been observed in the relative binding affinities of *A. aerogenes* and *B. subtilis* var. *niger* for Mg^{2+} (Tempest *et al.* 1967).

Several other explanations for the discrepancies between the work of Webb (1966)

and of Tempest *et al.* (1967), however, are possible. First, the results that are described in the two papers refer to cultures in different phases of growth, i.e. the stationary and logarithmic phases respectively. The cellular content of Mg^{2+} is related to that of RNA (Dicks & Tempest, 1966; Tempest, Dicks & Hunter, 1966), which is known to decrease at the end of exponential growth (e.g. Malmgren & Hedén, 1947). It is possible therefore that the content per organism of bound Mg^{2+} is greater in exponentially growing than in stationary populations, and that in the Gram-positive bacilli this excess cation is returned to the medium at the end of the logarithmic phase.

Secondly, the Gram-positive bacilli differ amongst themselves with regard to the Mg^{2+} concentration that is required to initiate growth (Webb, 1966). It is possible, therefore, that *Bacillus subtilis* var. *niger* may assimilate Mg^{2+} more efficiently and thus exhibit a lower requirement for the cation than the organisms that have been studied hitherto.

Thirdly, the growth medium of Tempest *et al.* (1967) contains 'trace amounts' of other bivalent cations (Cu^{2+}, Mn^{2+}, Zn^{2+}) and of the molybdate anion. Any of these ions, which are not included in Webb's (1966) medium, may influence the utilization of Mg^{2+}.

The present paper is concerned with an investigation of these possibilities. It is shown that, in contrast to the Gram-negative *Escherichia coli*, efflux of cellular Mg^{2+} does occur when cultures of the Gram-positive bacilli enter the stationary phase, but in Webb's (1966) Mg^{2+}-limited medium the assimilation of the cation is incomplete even during the logarithmic growth phase. The Mg^{2+} requirements of these organisms, however, are reduced considerably by the presence of Mn^{2+} at the concentration (25 μM) that is included in the medium of Tempest *et al.* (1967). Under these conditions growth occurs at low concentrations of Mg^{2+} and the uptake of this cation is proportionately greater. In the absence of Mn^{2+} and other trace metals, the response of *Bacillus subtilis* var. *niger* to Mg^{2+} is similar to that of other Gram-positive bacilli, although the concentration of the cation that is necessary to initiate growth is much lower.

METHODS

Organisms. The sources and conditions of maintenance of *Bacillus megaterium* (KM), *B. subtilis* (F 3), *B. mesentericus* and *Escherichia coli* have been given previously (Webb, 1966). *B. subtilis* var. *niger* was obtained from Dr D. W. Tempest and was maintained by monthly subculture on Evans peptone-agar slopes containing 0·2 % glucose.

Growth conditions. Cultures were grown in shaken flasks at 37° in either P medium (Webb, 1966) or T medium (Tempest *et al.* 1967). These solutions were supplemented with Mg^{2+} and other ions as stated in the text and were sterilized by filtration through sintered glass filters. Inocula for the experimental series were taken from organisms that had been subcultured at least three times in the appropriate defined medium. Growth was measured turbidimetrically as described previously (Webb, 1966).

Viable counts were made by plating on to Evans peptone (2 %, w/v)-glucose (0·2 %, w/v)-agar (2 %, w/v).

Isolation of ribosomes from cells grown in the presence of $^{54}Mn^{2+}$. Cultures were grown in P medium (500 ml.) supplemented with $^{54}Mn^{2+}$ (10 μc; The Radiochemical Centre, Amersham, Bucks.) and Mg^{2+} as indicated in Table 2. After being harvested and washed 3 times with solution L (0·01 M-tris buffer, pH 7·4, 0·06 M-KCl and

0·014 M-$(CH_3COO)_2Mg$), the organisms were resuspended in the same solution (15 ml.) and treated at 0° with ultrasonic vibrations in an M.S.E. machine (4 × 30 sec. at 1·5 A with 30 sec. intervals of rest). This method, which yielded ribosomal preparations that were comparable with those obtained from organisms that were crushed in the French press, was used in preference to the more conventional procedures for the disintegration of the labelled organisms. The homogenates were treated with DNAse (1 μg./ml.; Sigma Chemical Co., London; RNAse-free) for 5 min. at room temperature and then centrifuged at 4° for 10 min. at 18,000 *g*. The supernatant fractions were centrifuged for 70–90 min. at 114,000 *g* (Spinco Model L centrifuge, no. 40 rotor) and 0°. The crude ribosomal pellets were dissolved in mixture L, the solutions centrifuged for 5 min. at 12,000 *g* to remove insoluble and aggregated material, and then diluted as necessary to contain 0·9–1·0 mg. RNA/ml. Portions (0·2 ml.) of these solutions were layered on to continuous gradients of 10%–25% (w/v) sucrose in mixture L, and centrifuged for 100 min. at 125,000 *g* and 4° in the no. 39 rotor of the Spinco ultracentrifuge. Three gradients were run with each crude preparation. After centrifugation the bottom of each tube was pierced with a no. 16 hypodermic needle, and a series of 20-drop fractions was collected. These fractions were diluted with solution L (1·0 ml.) and the main ribosomal components located by measurement of the *E* 260 values. The appropriate fractions from each series were combined and the ribosomes were recovered by centrifugation for 90 min. at 114,000 *g*. The particle dry weight was calculated from the decrease in the *E* 260 value on centrifugation of each solution according to Imsande & Caston (1966).

Measurement of radioactivity. The ribosomal or cell pellets were dissolved in 98% (w/v) formic acid (0·5 ml.) at 60° before assay. Radioactivity was measured as described by Daniel, Dingle, Webb & Heath (1963).

Analytical methods. Mg^{2+} and Mn^{2+} were determined in culture supernatants by atomic absorption, the former by direct analysis in the presence of La^{3+} (Webb, 1966) and the latter by scale expansion in conjunction with the Perkin Elmer Recorder Readout accessory.

RESULTS

Utilization of Mg^{2+} in Mg^{2+}-limited cultures of Escherichia coli, Bacillus megaterium and B. subtilis F3

Variation of the Mg^{2+} content of P medium over the range 0·25–2·0 μg Mg^{2+}/ml. influenced both the rate and duration of the logarithmic growth phase of *Escherichia coli*, but had little effect on the length of the initial lag (Table 1). In Mg^{2+}-limited cultures of this organism the uptake of Mg^{2+} occurred rapidly, and the concentration of the cation in the medium was reduced to zero, usually within 2½–3 hr of the end of the lag phase (Fig. 1). After the complete utilization of the available Mg^{2+}, which correlated approximately with the transition from logarithmic growth to the phase of decreasing multiplication rate, the cell density increased by at least 33% in the absence of the exogenous cation. In cultures that contained initially 0·2 and 0·5 μg. Mg^{2+}/ml. the cell densities reached maximum values and then decreased by about 32% and 21% respectively between 8 and 20 hr. Mg^{2+} ions were not liberated from the cells during this period. At higher levels of Mg^{2+} (1·5–4·0 μg. Mg^{2+}/ml.) some slight efflux of the cation occurred between 6 and 8 hr (Table 1).

In agreement with previous findings (Webb, 1966), the growth of *Bacillus mega-*

terium and *B. subtilis* F 3 in P medium was limited to a greater extent by low concentrations of Mg^{2+} than was that of *Escherichia coli* (Fig. 1). Throughout the period of incubation of cultures of the Gram-positive bacilli the utilization of Mg^{2+} from media with initial concentrations of 0·5, 0·9 and 1·2 μg. Mg^{2+}/ml. was not quantitative. In

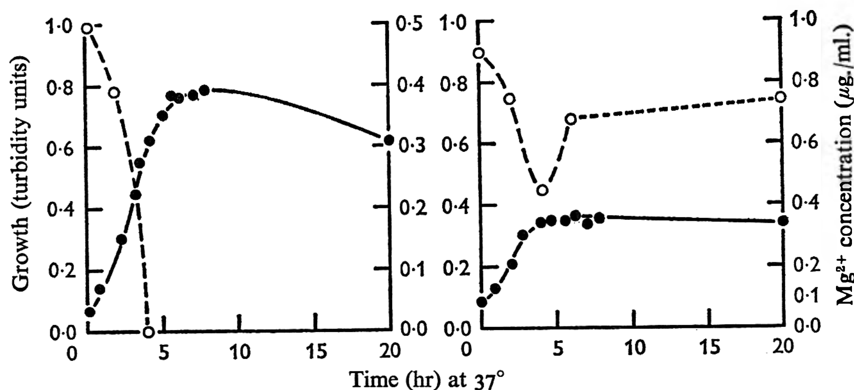

Fig. 1. Growth (●——●) and magnesium utilization (○ - - - ○) in cultures of *Escherichia coli* with 0·5 μg. Mg^{2+}/ml., and in cultures of *Bacillus megaterium* with 0·9 μg. Mg^{2+}/ml. in P medium.

Table 1. *Magnesium utilization in Mg^{2+}-limited cultures of Escherichia coli*

The experimental cultures were grown with shaking in P medium (15 ml.) supplemented with different amounts of Mg^{2+} in conical flasks (50 ml.) that were fitted with side arms for turbidimetric measurements. At the intervals shown, portions (3·0 ml.) of the cultures were removed aseptically, centrifuged and the supernatant fractions analysed for Mg^{2+}. The inocula (0·3 ml.) were taken from a 16 hr culture in P medium with 2 μg. Mg^{2-}/ml.

Time (hr) at 37°	Mg²⁺ concentration (µg./ml.)					
	0·2	0·5	1·0	1·5	2·0	4·0
	µg. dry wt organisms/ml.					
1·5	19	23	26	26	24	32
2·0	40	33	33	37	36	41
3·0	85	94	79	78	90	90
3·75	122	154	154	156	159	163
4·0	137	188	204	206	214	217
5·0	169	263	379	409	430	440
6·0	180	302	459	539	595	714
7·0	199	344	514	616	775	794
8·0	197	359	533	735	760	950
	Residual Mg²⁺ (µg./ml.)					
2·0	0·18	0·50	0·87	1·32	1·79	3·42
4·0	0·04	0·03	0·19	0·66	1·02	2·66
6·0	0·02	0·01	0·03	0·03	0·01	0·03
8·0	0·00	0·00	0·00	0·01	0·04	0·10

each culture, the cation content of the medium decreased to a minimum at the onset of the stationary phase and then increased. At the low medium concentrations of Mg^{2+} the liberation of the cation coincided with lysis, as shown by the decrease in turbidity of the cultures after 3 hr. At the higher levels of Mg^{2+}, however, turbidity remained essentially constant between 8 and 20 hr.

Mg^{2+} requirements of Bacillus subtilis var. niger

In cultures of *Bacillus subtilis* var. *niger* in P medium the lag in the growth response to increasing concentrations of Mg^{2+} was much shorter than with other Gram-positive bacilli that have been studied previously in batch culture (Webb, 1966). Growth began at about 0·15–0·2 µg. Mg^{2+}/ml., and then increased rapidly with the concentration of the cation, whilst in cultures that contained low levels of Mg^{2+} the utilization of the cation was almost complete.

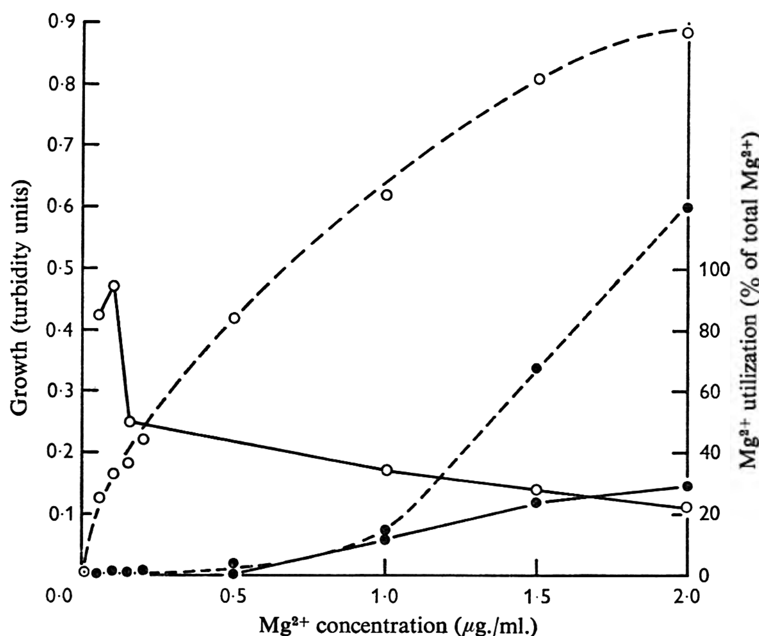

Fig. 2. Growth (– – –), and Mg^{2+} utilization (——) as functions of Mg^{2+} concentration in cultures of *Bacillus subtilis* F 3, in T medium (○) and P medium (●).

Influence of other ions on the utilization of Mg^{2+} by Gram-positive bacilli. In the medium of Tempest *et al.* (1967), both the growth response to Mg^{2+} and the utilization of the cation by *Bacillus subtilis* F 3, *B. megaterium* and *B. mesentericus* were different from those in P medium. Thus in T medium the growth lag at low levels of Mg^{2+} was eliminated and the utilization of the cation at concentrations below 0·1 µg. Mg^{2+}/ml. was almost complete (Fig. 2). Under these conditions there was little difference, for example, in the response of *B. subtilis* F 3 (Fig. 2) and of *B. subtilis* var. *niger*.

As the major components of P and T media were similar, these observations indicated that the Mg^{2+}-requirements of at least certain Gram-positive bacilli were modified by the additional trace metals (Mn^{2+}, Cu^{2+}, Zn^{2+} and MoO_4^{2-}) of T medium. In P medium the Mg^{2+}, requirements for growth of *Bacillus subtilis* F 3 and *B. megaterium* were reduced greatly by Mn^{2+}, but were increased by $Cu^{2+}Zn_4^{2+}$ and MoO_4^{2-} when these ions were added separately in the amounts present in T medium (Fig. 3). Although Mn^{2+} (25 µM) alone was unable to support the growth of these organisms, it stimulated the uptake of Mg^{2+} when this ion was present in low concentration. With

B. subtilis var. *niger* the utilization of Mg^{2+} was increased slightly by Mn^{2+}, and decreased by Cu^{2+}, Zn^{2+} and MoO_4^{2-}, although none of these ions had any significant effect on the growth response of this organism to increasing concentrations of Mg^{2+}. The latter finding was confirmed by a study of the response of *B. subtilis* var. *niger* to Mg^{2+} in T medium from which the various trace metal components were omitted separately. When Mn^{2+}, Cu^{2+} and MoO_4^{2-} were omitted together, however, growth did not occur at Mg^{2+} concentrations lower than 0·15 μg. Mg^{2+}/ml., and at 2 μg. Mg^{2+}/ml. the cell

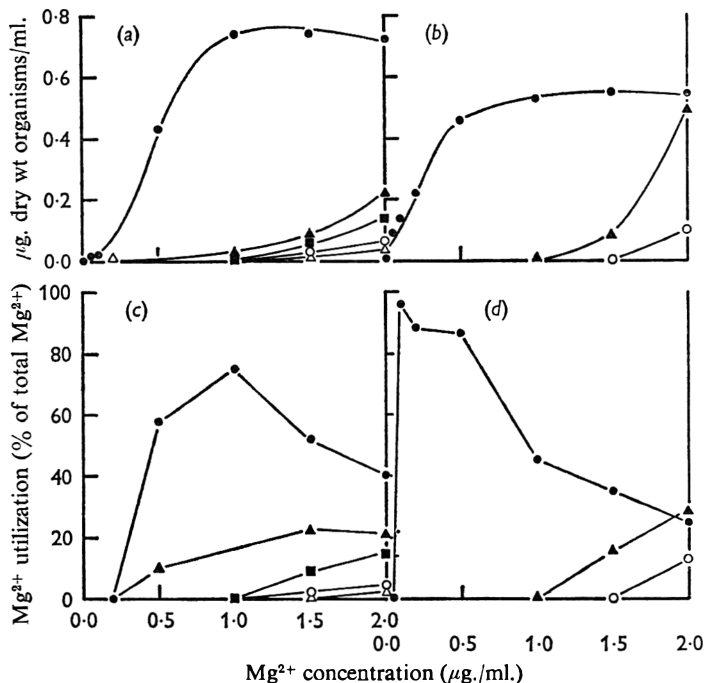

Fig. 3. Influence of other ions on growth (upper figures) and Mg^{2+} utilization (lower figures) in Mg^{2+}-limited cultures of *Bacillus megaterium* (*a*, *c*) and *Bacillus subtilis* F3 (*b*, *d*). Additions to P medium were as follows: none (▲), 25 μM-Mn^{2+} (●), 5 μM-MoO_4^{2-} (■), 10 μM-Zn^{2+} (●), 5 μM-Cu^{2+} (○).

density in fully grown cultures was 25 % less than in the complete medium. In some experiments with this organism in the absence of Cu^{2+}, Zn^{2+} and MoO_4^{2-}, Mn^{2+} (25 μM) antagonized the uptake of Mg^{2+} at concentrations of less than 0·5 μg./ml. and, at the lowest levels of Mg^{2+}, caused the liberation of this cation from the cells of the noculum. This antagonism, however, was not consistently reproducible. At higher concentrations (1·0–2·0 μg./ml.) of Mg^{2+}, Mn^{2+} acted synergistically, the increase in dry wt organisms/ml. under these conditions being 95–98 % of that in corresponding cultures in the complete medium, and the uptake of Mg^{2+} about 35 % greater.

Utilization of Mn^{2+} by Escherichia coli, Bacillus megaterium and B. subtilis var. niger. Analysis of cell-free supernatant fractions from the above experiments established that, irrespective of the Mg^{2+} concentration, only a small fraction of the available Mn^{2+} was assimilated by each of the three organisms. As the decrease in the content of this cation in the media was too low to be determined accurately by atomic absorption, uptake was measured with $^{54}Mn^{2+}$ as tracer.

In *Bacillus megaterium* and *B. subtilis* var. *niger*, as in *Escherichia coli*, Mn^{2+} was taken up during growth and incorporated into the ribosomes (Table 2). It is probable that in the three organisms the Mn^{2+} contents of the native, intracellular ribosomes were higher than those found for the isolated particles, since these were prepared in the presence of a relatively high concentration (14 mM) of Mg^{2+} to prevent dissociation to the 50 S and 30 S sub-units. Although the binding affinity of bacterial (*E. coli*) ribosomes for Mn^{2+} is about 3 times that of Mg^{2+} (Sheard *et al.* 1967) it is to be expected that some displacement of the former cation by the latter would occur under the conditions of the present experiments. In culture, Mg^{2+} antagonized the uptake of Mn^{2+}. Thus, as shown in Table 2, a 10-fold increase in the Mg^{2+} concentration of the medium depressed the Mn^{2+} content of the whole cells and isolated ribosomes of *E. coli* by 87 % and 80 % respectively. In this connexion it is interesting that the Mn^{2+}

Table 2. *Incorporation of* $^{54}Mn^{2+}$ *by Escherichia coli, Bacillus megaterium and Bacillus subtilis var. niger*

The cultures were grown with aeration at 37° in P medium (500 ml.) supplemented with 12·5 µmoles Mn^{2+} (10 µc $^{54}Mn^{2+}$) and Mg^{2+} as shown, from inocula (5 ml.) that contained medium concentrations of Mg^{2+} of 1 µg./ml. (*E. coli*), 2 µg./ml. (*B. subtilis* var. *niger*) and 5 µg./ml. (*B. megaterium*). Growth was followed turbidimetrically to enable the two cultures of *E. coli* and of the Gram-positive bacilli to be harvested in the logarithmic phase at approximately the same dry wt organisms/ml. The procedures for the isolation of ribosomes and the determination of Mn^{2+} were as described in the Materials and Methods section.

Organism	Mg^{2-} content of culture medium (µg./ml.)	Yield (mg. dry wt organisms)	Total Mn^{2+} incorporated by cells (µmoles)	Mn^{2+} content (mµ-moles/mg. dry wt)	
				Cells	Ribosomes
E. coli	0·5	94·6	0·123	1·30	1·32
E. coli	5·0	91·7	0·016	0·17	0·27
B. subtilis var. *niger*	1·0	134·7	0·122	0·81	1·04
B. megaterium	2·0	148·0	0·264	1·78	1·81

contents of *B. megaterium* cells and ribosomes were approximately double those of *B. subtilis* var. *niger*, although the Mg^{2+} concentration in the culture of the former was twice that of the latter. It seems therefore that *B. megaterium* may have a lower affinity for Mg^{2+} than *B. subtilis* var. *niger*, but a higher affinity for Mn^{2+}.

Loss of viability in Gram-positive bacilli in the absence of Mg^{2+}

Although *Bacillus megaterium*, *B. subtilis* F 3 and *B. subtilis* var. *niger* failed to grow in either P or T medium in the absence of Mg^{2+}, viability was maintained. With both *B. subtilis* F 3 and *B. megaterium*, the viable count fell by about 45 % during 20 hr at 37°, a 25 % decrease being observed within the first 3·5 hr of incubation. The death-rates of these organisms in the Mg^{2+}-deficient P medium were little affected by Mn^{2+}, Cu^{2+} and MoO_4^{2-}, but were increased by Zn^{2+} (10 µM), in some experiments by as much as 90–95 % in 6 hr. Even in the presence of Zn^{2+} some cells survived for 20 hr and were capable of growth when the medium was supplemented with Mg^{2+} (10 µg./ml.).

In cultures of *Bacillus subtilis* F 3 in P medium with 0·5 µg. Mg^{2+}/ml., as in those of *B. megaterium*, there was no stable stationary state, but a decrease in cell density after 6 hr (Fig. 4). This decrease in density was exaggerated by the turbidimetric measurements since once the growth maximum was attained, the cells tended to

agglutinate. Agglutination was correlated with a change in morphology from Gram-positive rods to Gram-negative filaments of uneven thickness, the majority of which were swollen at one or both ends. Sporulation did not occur. With most Gram-positive bacilli, the formation of filaments in response to Mg^{2+} deficiency occurs in complex media, but not in simple nutrient solutions unless these are supplemented with amino acids (Webb, 1951 *b*). This atypical behaviour of *B. subtilis* F 3, however, has been described previously by Grunau (1958).

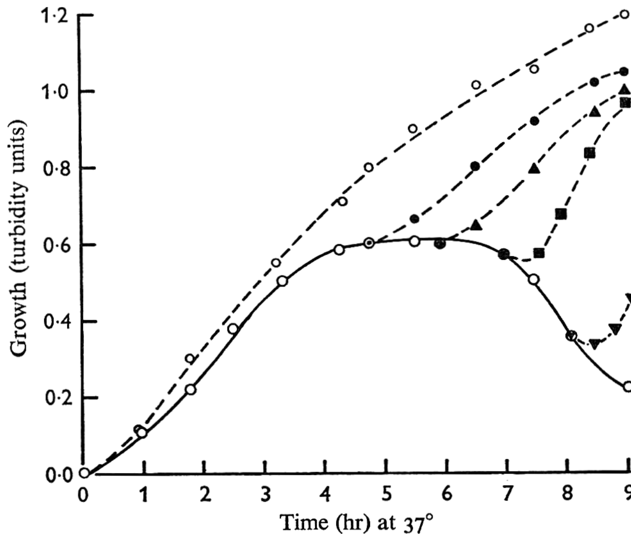

Fig. 4. Response of Mg^{2+}-limited cultures of *Bacillus subtilis* F3 to Mg^{2+}. The parent culture (○——○) was grown with shaking in P medium with limited Mg^{2+} (0·5 µg./ml.) at 37° under the conditions described in Table 1. Portions of this culture were supplemented with additional Mg^{2+} (10 µg./ml. at 0 hr (○ - - - ○), 4·75 hr(● - - - ●), 5·75 hr (▲- - - ▲), 7 hr (■ - - - ■), and 7·75 hr (▼ - - - ▼).

On the addition of Mg^{2+} (10 µg./ml.) to a Mg^{2+}-limited culture of *Bacillus subtilis* F 3 growth recommenced with little or no lag with the production of a new population of morphologically normal, Gram-positive cells. An interesting and unexpected feature of these results (Fig. 4) was that the rate of multiplication in the second growth phase increased the later the Mg^{2+} was added, and was greatest during the period of the decrease in turbidity of the parent culture. It appears therefore that the Mg^{2+} requirements of cells which survive during the period of degeneration in Mg^{2+}-deficient cultures of *B. subtilis* F 3, and which resume growth on the addition of Mg^{2+}, are reduced by the presence of products from other autolysed cells. Certain amino acids, for example, are known to reduce the Mg^{2+} requirements of various Gram-positive bacilli in simple media (Webb, 1951 *b*, 1966).

Effect of other ions on the leakage of Mg^{2+} from Gram-positive bacilli

Previously (Webb, 1966) it was reported that when Gram-positive bacilli are transferred to a Mg^{2+}-deficient medium leakage of Mg^{2+} occurs progressively with time and often precedes a decrease in cell density. A number of observations suggest that a bivalent cation, usually considered to be either Mg^{2+} or Ca^{2+}, is necessary to maintain

the integrity of the bacterial cell wall, cell membrane or permeability barriers (e.g. Strange, 1964: Gray & Wilkinson, 1965: Hamilton-Miller, 1966; Goldman, 1966). The trace amounts of Cu^{2+} and Mn^{2+} in the medium of Tempest *et al.* (1967) also appeared to stabilize Gram-positive cells and, in the absence of exogenous Mg^{2+}, prevented both the leakage of this cation and the decrease in cell density. The loss of Mg^{2+} which occurred, for example, when cells from the exponential phase of a culture of *Bacillus subtilis* in P medium with 2 μg. Mg^{2+}/ml. were transferred to fresh medium without Mg^{2+} was prevented completely by Cu^{2+} (5 μM) and reduced by 85 % in the presence of Mn^{2+} (25 μM).

DISCUSSION

The present results confirm and extend those reported previously (Webb, 1966) on the difference in the abilities of certain Gram-positive and Gram-negative bacteria in batch culture to concentrate Mg^{2+} from a simple chemically defined medium. In Mg^{2+}-limited cultures of the Gram-negative *Escherichia coli* for example, the utilization of the cation is rapid and is complete some time before the stationary phase is reached. In contrast, in similar cultures of the Gram-positive *Bacillus megaterium* and *B. subtilis* F 3 only part of the available Mg^{2+} is utilized; growth and Mg^{2+} assimilation cease at the same time, and thereafter efflux of the cation may occur. This efflux of Mg^{2+} may be either accompanied or followed by some cell lysis. In such stationary cultures of the Gram-positive bacilli, as in dilute cell suspensions in Mg^{2+}-deficient media, some cells remain viable and are able to grow on the addition of Mg^{2+}, even if this is delayed for 20 hr. Thus contrary to the suggestion of Tempest *et al.* (1967), loss of viability of the Gram-positive bacilli cannot account for the differences in the growth response of these and Gram-negative bacteria to low concentrations of Mg^{2+}.

The concentration of Mg^{2+} that is necessary to initiate growth of various Gram-positive bacilli varies with the species (Webb, 1949, 1966), and appears to be extremely small for *Bacillus subtilis* var. *niger*, the organism that has been used in most of the comparative studies of Tempest *et al.* (1967). This strain of *B. subtilis* is able to grow in the P medium, and to assimilate Mg^{2+}, at concentrations of the cation that are inadequate for a number of other bacilli.

In cultures of certain of the Gram-positive bacilli the Mg^{2+} requirements for growth are reduced by Mn^{2+} (25 μM). The synergistic action of Mn^{2+} is in agreement with previous observations (Webb, 1951 *a*) on the partial ability of this cation to substitute for Mg^{2+} in the nutrition of these organisms. The present results, however, show that in the complete absence of Mg^{2+} this limited concentration of Mn^{2+} is unable to support growth of either the Gram-negative *Escherichia coli* or a number of Gram-positive bacilli, and suggest that either there is some fundamental reaction that has absolute specificity for the former cation, or the transport of Mn^{2+} is energy-dependent and is activated by Mg^{2-}. Assimilation of Mn^{2+} is much less efficient than is that of Mg^{2+}, and, at least in *E. coli*, is decreased by increased concentrations of the latter cation. It seems therefore that the relationship between the utilization of these two ions may be synergistic or antagonistic according to their relative concentrations.

It is significant that in the presence of low levels of Mg^{2+}, Mn^{2+} is taken up by bacterial cells and incorporated into the ribosomes (Table 2), since, chemically, the inhibition of protein synthesis is the main result of Mg^{2+} deficiency, particularly in Gram-positive bacilli (Webb, 1953). Kennell & Kotoulas (1967) have shown that

Mn^{2+} partially protects the ribosomes of *Aerobacter aerogenes* against degradation due to Mg^{2+} deficiency, whilst the ability of Mn^{2+} to exchange rapidly with the bound Mg^{2+} of isolated ribosomes from *E. coli* has been described by Sheard *et al.* (1967). Earlier, Tissières, Schlessinger & Gros (1960) reported that Mn^{2+} was 50% as efficient as Mg^{2+} in the activation of protein synthesis by *Escherichia coli* ribosomes. It seems therefore that incorporation of Mn^{2+} into the ribosomes of Mg^{2+} deficient bacteria would maintain the structure and function of the particles, and also liberate some bound Mg^{2+}, which would thus become available for other processes.

The reduced Mg^{2+} requirements of a number of Gram-positive bacilli in the medium of Tempest *et al.* (1967) can be explained by the presence of Mn^{2+}. Individually, the other trace metal components of this medium have little effect on either growth or Mg^{2+} utilization, although under certain conditions Cu^{2+} may stabilize the cells and prevent both the efflux of Mg^{2+} and partial lysis that are liable to occur in the absence of the latter cation. The discrepancies between the results of Tempest *et al.* (1967) and of Webb (1966) thus seem to be due to the use by the former authors of (*a*) *Bacillus subtilis* var. *niger*, a Gram-positive bacillus that is atypical in its Mg^{2+} requirements, and (*b*) a medium that contains Mn^{2+}, which can substitute, at least in part, for Mg^{2+} in the nutrition of both Gram-positive and Gram-negative bacteria.

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Formation and Structure of Extracellular Glucans Produced by Claviceps Species

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SUMMARY

Submerged cultures of *Claviceps fusiformis* (Loveless) became progressively viscous during the incubation because of the production of an extracellular polysaccharide. The polysaccharide was shown to be a branched glucan with a $\beta 1 \rightarrow 3$ linked main chain having single glucopyranosyl units at intervals along it in the $\beta 1 \rightarrow 6$ configuration. For most of the media investigated growth at 27° and between pH 5 and 6 was accompanied by glucan production, which ceased when growth was arrested by lack of nutrient. The glucan was re-metabolized by the fungus only to a limited extent as the culture aged; the degree of branching varied during the culture period. The unit amount of glucan synthesized was not affected by numerous successive subculturings over a period of several months, but the product from the later cultures had a greater degree of branching. Branched glucans of similar structure to the above were also detected in low concentration in the natural sclerotia. The presence of $\beta 1 \rightarrow 3$ linked glucans in the cell walls of this and other fungal strains examined was also indicated.

INTRODUCTION

During studies on the production of clavine alkaloids in submerged culture by strains of *Claviceps fusiformis*, it was noted that the cultures became very viscous during incubation, to the extent that the fluid culture became practically immobile. This viscosity interfered with aeration (Chain, Gualandi & Morisi, 1966) and thus impeded the production of the alkaloids. It was therefore necessary to study the nature of the viscous metabolite in order to find conditions to prevent its formation; the present paper is concerned with a study of its production and of its structure. Preliminary studies showed that it was a polysaccharide of the glucan type.

Extracellular polysaccharides with various degrees of structural complexity frequently occur in fungal cultures and some of these have been shown to be glucans (for reviews see Clarke & Stone, 1963; Nordin & Kirkwood, 1965). Bouveng, Kiessling, Lindberg & McKay (1963) studied the structure and production of α- and β-linked glucans produced in shake cultures by *Pullularia pullulans* from a variety of sugar substrates. Davis, Rhodes & Shulke (1965) studied the production of glucans in shake culture by the Ascomycete fungi *Plectania occidentalis* and a Helotium species, Wallen, Rhodes & Shulke (1965) investigated the structure and physical properties of these glucans and found them to be branched polymers containing appreciable amounts of $\beta 1 \rightarrow 3$ linkages. Perlin & Taber (1963) and Vining & Nair (1966) have

found that cultures of Claviceps species give rise to polysaccharides. The former workers demonstrated that the polymer arising from *Claviceps purpurea* had a $\beta 1 \rightarrow 3$ linked primary chain with single glucose units attached in the 6 position ($\beta 1 \rightarrow 6$) at every fourth point, on average, along it.

METHODS

Organisms. Strain F 2047, used in most of these experiments, was derived by selection of a strain (no. B 37 received from Dr J. Renz of Sandoz Ltd., Basle, Switzerland), which had been isolated from a sclerotium on *Pennisetum typhoideum*, Rich. (millet) from the Tschad region of Africa. This organism has recently been described as *Claviceps fusiformis* (Loveless, 1967).

Other organisms used were: I.C. 49, related to Claviceps, isolated from sclerotia on Sorghum in Africa (received originally from M. C. Futrell, Zaria, Nigeria); F-114, isolated from sclerotia of *Claviceps litoralis* by Dr A. Tonolo, Istituto Superiore di Sanita, Rome; *Sclerotium rolfsii* (IMI 74210—Commonwealth Mycological Institute, London).

Sclerotia of *Claviceps fusiformis* were kindly supplied by the Director, Station Agricole de Sarid, Koudougou, Haute-Volta. The uninfected seeds of *Pennisetum typhoideum* (millet) were obtained from the same source.

Maintenance of the cultures. Stock cultures of F 2047 were prepared on potato glucose medium (see below) containing 2% agar and stored in the refrigerator.

Slope cultures for immediate use were prepared on the same medium at 24° and were transferred about once a month.

Submerged culture procedures

Media

The media used were as follows.

Medium I for seed production: a potato infusion was made by boiling 300 g. cut potatoes in 500 ml. water for 15 min.; after cooling the suspension was filtered through muslin and brought to pH 7 before addition of 30 g. glucose and making up to 1 l.

Medium II, a modification of the medium used by Stoll *et al.* (1954) for clavine alkaloid production: sucrose, 50 g.; ammonium succinate, 3·2 g.; KH_2PO_4, 1·0 g; $MgSO_4.7H_2O$, 0·3 g.; $FeSO_4.7H_2O$, 13 mg.; $ZnSO_4.7H_2O$, 4 mg.; adjusted to pH 5·2 with concentrated ammonia, water to 1 l.

Medium III, as medium II, but at pH 5·5. Distilled water was used for shake-flask media, and tap water for fermenters (see below).

Sterilization conditions were as follows: for 100 ml. to 500 ml. Erlenmeyer flasks, 113° for 30 min.; for 1 l. to 3 l. flasks, 113° for 50 min.; for stirred fermenters containing 60 l. medium, 100° for 20 min. and 121° for 20 min.

Seed cultures

A suspension of mycelium and conidia obtained by shaking an agar slope culture with sterile water was used to inoculate seed cultures. These were maintained in shake culture at 27° and transferred every 3–4 days. For the first subculturings the seed culture was homogenized at speed 80 for 30 sec. in an Omni mixer. A 10% (v/v)

inoculum was used for all media. Washed inoculum was used for experiments on carbon source.

Fermentations in shake flasks

For seed and polysaccharide production, cotton-plugged 500 ml. Erlenmeyer flasks were placed on a rotary shaker at 27°. Under the conditions of agitation (200 rev./min., eccentric throw 10 cm.) the aeration rate in the absence of mycelium, measured by the method of Cooper, Fernstrom & Miller (1944), was equivalent to 30 ml. O_2 absorbed/100 ml. sulphite solution/hr.

For sampling, duplicate flasks were removed at intervals, and the course of fermentation followed by microscopic examination and by measurement of the following parameters: pH value, sugar utilization, ammonia utilization and dry weight of mycelium and polysaccharide. All experiments were at least duplicated.

Fermentations in stirred fermenters

Jacketed stainless-steel fermenters of 90 l. total capacity (Paladino, Ugolini & Chain, 1954) containing 60 l. medium II at 27° were used. Aeration was effected through a ring sparger with a sterile air flow of 30 l./min. for the first 24 hr and at 60 l./min. thereafter at an over-pressure of 1 atmosphere.

Agitation was provided with a top-driven stirrer rotating at 183 rev./min. and fitted with an 8-bladed turbine propeller (ratio diameter of fermenter: diameter of propeller, 3·5:1). Antifoam silicon R.D. (Midlands Silicones Ltd.) was added.

The fermenters were inoculated with 2 l. mycelial suspension grown in 3 l. shake flasks for 4 days at 25°.

Analytical techniques

Mycelial dry weight. As a standard procedure, the following was done: a sample of the culture was filtered through a filter paper supported by a nylon mat on a Hartley 3-piece Büchner funnel. The mycelium was washed twice with distilled water on the filter and then dried at 70° to constant weight.

Polysaccharide. The filtrate from above was mixed with 1–2 volumes of absolute ethanol and the precipitated polysaccharide collected on a glass rod and dried at 70° to constant weight. For structural studies, the precipitated polysaccharide was not immediately dried but resuspended in water to form a gel and then reprecipitated; this procedure was repeated several times (up to 6) before the final purified product was freeze-dried from aqueous suspension and weighed.

Total carbohydrate. Total carbohydrate was estimated by the anthrone method (Morris, 1948) and by use of the phenol+sulphuric acid reagent (Dubois *et al.* 1951, 1956).

Reducing sugar. This was determined by the method of Somogyi (1952).

Sucrose in culture filtrates was estimated by the picric acid method of Thomas & Dutcher (1924) modified as follows. To the aqueous 5 ml. sample was added 1 ml. 2N-NaOH and 4 ml. of the picric acid reagent; the mixture was heated for exactly 10 min., cooled and diluted to 25 ml. with distilled water before reading at 560 mμ (see below).

Glucose was specifically determined by a modified glucose oxidase method (Fleming & Pegler, 1963).

Nitrogen. Ammoniacal nitrogen was determined after distillation in the micro-Kjeldahl apparatus or Conway unit (Conway, 1962) followed by Nesslerization (Koch & Hanke, 1948), and, similarly, total nitrogen after digestion of the material with concentrated sulphuric acid and selenium.

Coloured complexes obtained were read at their respective maximum absorbencies in a Unicam SP. 500 spectrophotometer using a 1 cm. light path.

Chromatographic procedures. Compounds were separated by chromatography on paper (Whatman no. 1 or 3 MM) and on thin-layer plates with cellulose (Whatman CC 41). The following solvents were used: (*a*) butan-1-ol+pyridine+water (10+3+3, by vol.); (*b*) benzene+butan-1-ol+pyridine+water (1+5+3+3, by vol.); (*c*) ethyl acetate+pyridine+water (2+5+7, by vol.); (*d*) butan-1-ol+ethanol+water (4+1+1·9, by vol.); (*e*) propan-1-ol+ethyl acetate+water (7+1+2, 7+1+3 and 6+1+3, by vol.); (*f*) butan-1-ol+acetic acid+water (100+22+50, by vol.).

Paper electrophoresis was done by using Whatman 3 MM paper, at approximately 40 V./cm. in 0·05 M-borate, (pH 9·5) and 0·05 M-germanate (pH 10·7) buffers.

Detection reagents. Reducing sugars were detected with aniline hydrogen phthalate (Partridge, 1949), *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950) and benzidine (Bacon & Edelman, 1951): sugar alcohols were detected by using silver nitrate and sodium hydroxide (Trevelyan, Proctor & Harrison, 1950).

Identification of compounds. The following methods were used for identifying compounds: (1) Infra-red spectroscopy of solid films or nujol mulls measured with a Unicam SP. 200 G spectrophotometer. (2) Optical rotation recorded in a 1 dm. tube on a Perkin Elmer Model 141 polarimeter. (3) Chromatographic mobility. (4) M.p. and mixed m.p. in the case of crystalline products.

Enzymic methods. The following enzymes were used: a β-glucuronidase preparation from *Helix pomatia* (L'Industrie Biologique Française, S.A., Gennevilliers, France), β-glucosidase and maltase (Koch–Light Laboratories Ltd., Colnbrook, Bucks.), α-amylase (British Drug Houses, Poole, Dorset) and cellulase (Meiji Seika Kaisha Ltd., Tokyo, Japan); the specific exo-$\beta 1 \rightarrow 3$-glucanase, derived from Basidiomycete QM806 (Reese & Mandels, 1959) was kindly given by Dr E. T. Reese (U.S. Army Natick Laboratories, Massachusetts).

Apart from the exo-$\beta 1 \rightarrow 3$-glucanase, all enzyme reactions were done at 25° in 0·03 M-phosphate buffer (pH 6·0). The exo-$\beta 1 \rightarrow 3$ glucanase digests were done at 50° in 0·05 M-acetate buffer (pH 4·6). The products of enzymic hydrolysis were separated by chromatography in solvents *e* and *f*. Normally 1 %, w/v (final), solution of substrate and 0·1 % w/v (final) solution of enzyme were used; enzyme activity was terminated by addition of 0·01 M-mercuric chloride or immersion of the vessel containing the digest in boiling water for 5 min.

Incubations were for periods up to 24 hr.

Determination of branching in the polysaccharide. For accurate determinations of branching by using the exo-$\beta 1 \rightarrow 3$-glucanase, the incubation time was decreased to 5 hr (see below: Table 8). A typical procedure was as follows. The products of incubation of 30 mg. glucan with the exo-$\beta 1 \rightarrow 3$-glucanase were separated by chromatography (solvent *e*) eluted from the chromatogram with water and the major components characterized as their β-acetates (see above) to give: β-D-gentiobose octaacetate (15·2 mg.), m.p. 194–195°, identical with an authentic specimen and β-D-glucose pentaacetate (25·2 mg.), m.p. 131–132°. Alternatively, the eluted compounds

were assayed by using the phenol+sulphuric acid reagent to determine the ratio of glucose to gentiobiose and hence the degree of branching.

Preparative and counting techniques using ^{14}C

Preparation of ^{14}C*-labelled polysaccharides.* Polysaccharide labelled with ^{14}C was prepared by growing the organism in the usual way on medium III but with 1 % (w/v) sucrose, instead of 5 %, containing 50 μc of U-^{14}C sucrose/flask. The polysaccharide was isolated and purified as above.

Counting techniques. Products derived by enzymic hydrolysis of the radioactive glucan were separated by paper chromatography (solvent *e*) and the dried papers exposed to 'Kodirex No-screen' X-ray film. The autoradiograph obtained was used to determine the radioactive areas, which were removed, but into small pieces (10 × 10 mm.) and immersed in liquid scintillant (Bray, 1960). The radioactivity in the samples was determined on the paper in a Packard counter (model 3003). Corrections for variable efficiencies were made.

Procedures for structural determination of carbohydrates

Fractionation of extracellular polysaccharides. A sample (116·1 mg.) of purified polysaccharide from a 17-day culture was stirred with successive amounts of 5 N-sodium hydroxide (5 ml.) for 10 min. each time until all the material had dissolved. Each fraction was neutralized with acetic acid and the glucan, precipitated by addition of excess ethanol, was washed with ethanol, centrifuged and freeze-dried.

Fractionation of sclerotial polysaccharides. The powdered sclerotia (1 g.) were suspended in 100 ml. of 5 % sodium hydroxide and stirred at 20° for 3 hr under a stream of nitrogen. After neutralization with 7·3 ml. glacial acetic acid, the extract was centrifuged for 1 hr at 12,000 *g*. The pale-brown opalescent supernatant fluid was brought with absolute ethanol to a final concentration of 70 % (v/v) ethanol. The off-white flocculent precipitate was collected by centrifugation at 8000 *g* for 15 min., washed with 70 % (v/v) ethanol in water and dried over $CaCl_2$ in vacuum. The dried residue (109 mg.) was free from sugars of low molecular weight as indicated by chromatography in solvents *e* and *f*.

Total hydrolysis of glucan. A typical procedure was as follows. A solution of glucan (70 mg.) in 1 ml. of 72 % (w/v) concentrated sulphuric acid in water was stirred at 5° for 1 hr, after which water (25 ml.) was added and the solution was boiled for 4 hr. The filtrate, neutralized with barium hydroxide, was examined by paper chromatography (solvents *a* and *b*) and by paper electrophoresis in both buffers. After concentration in vacuum, acetylation of the residue (dried over phosphorus pentoxide) was done with acetic anhydride (0·7 ml.) and sodium acetate (50 mg.) by the method of Fischer (1916) and gave β-D-glucose pentaacetate, m.p. 130·5–131·0° (70 mg.), which was identical with an authentic specimen.

Partial hydrolysis of glucan. As the glucan proved intractible to partial hydrolysis in N-H_2SO_4, it was first dissolved by stirring overnight at 50° in 80 % phosphoric acid (1 g./20 ml.). The dissolved glucan was precipitated at 5° by adding 10 ml. water followed by 90 ml. ethanol. The precipitate was collected by centrifugation at 8000 *g* for 15 min., washed with cold 75 % (v/v) ethanol in water until neutral, and dried over $CaCl_2$ in vacuum to yield 390 mg. of white product. The treated glucan (2 mg.) was

hydrolysed with 100 μl. of N-H_2SO_4 at 90°. Samples (10 μl.) of the reaction mixture were removed at intervals over a period of 5 hr, neutralized with barium hydroxide and chromatographed in solvents *e* and *f*. Some of the products obtained were partially identified, after spraying the papers, by co-chromatography with known sugars in the above solvents.

Hydrolysis of the methylated glucan and identification of hydrolysis products

Methylated glucan (5·6 mg.—see below) was dissolved in 72% (w/v) concentrated sulphuric acid in water (0·1 ml.) and stirred at 0° for 1 hr. Water (1 ml.) was added and the solution was boiled for 4 hr. Examination of the hydrolysate, neutralized with barium hydroxide, by paper chromatography (solvent *d*) revealed components with the mobilities of a tetra-*O*-methyl, a tri-*O*-methyl and a di-*O*-methyl-D-glucose. The ratio of these components was determined by hypoiodite oxidation (Chandra, Hirst, Jones & Percival, 1950) after separation by paper chromatography.

Methylated glucan (51·3 mg.) was hydrolysed essentially as above and the hydrolysis products isolated and identified after chromatography on Whatman 3 MM paper (solvent *d*). Three homogeneous fractions were obtained. Fraction A had the same mobility as 2,3,4,6-tetra-*O*-methyl-D-glucose and after isolation had a m.p. 91–94°. It proved identical to an authentic specimen. Fraction B had the same mobility as 2,4,6-tri-*O*-methyl-D-glucose. After isolation it had a m.p. 122–125°. It was identical with an authentic specimen, kindly given by Dr G. O. Aspinall (University of Edinburgh). Fraction C was chromatographically and electrophoretically homogeneous but could not be obtained crystalline. It was dissolved in water (0·5 ml.) and sodium borohydride (20 mg.) was added. After 18 hr the solution was neutralized to pH 6·5 with 2 N-acetic acid and sodium metaperiodate (50 mg.) added. After 3·75 hr at 3°, excess aqueous barium chloride was added. The filtered solution, which gave a positive test for formaldehyde with chromotropic acid reagent (MacFadyen, 1945), was extracted exhaustively with chloroform and the extract, dried with $MgSO_4$, was evaporated. The product had a mobility of a di-*O*-methylpentose (solvent *d*). A solution of this product in dry dichloromethane (1 ml.) was demethylated with boron trichloride by the standard procedure (Allen, Bonner, Bourne & Saville, 1958). Examination of the product by paper chromatography (solvent *e*) and electrophoresis in both buffers revealed the presence of xylose only.

Methylation of the glucan. The glucan proved remarkably refractory to methylation. Eventually the following procedure was adopted (Wallenfels *et al.* 1963, and references cited therein). Glucan (128·5 mg.) was stirred with a solution of sodium borohydride (86 mg.) in water (20 ml.) for 12 hr and the resultant gel dissolved in 40% (w/v) aqueous sodium hydroxide (10 ml.) and stirred with dimethyl sulphate (4 ml.) at 20° for 18 hr. After warming to decompose excess dimethyl sulphate and cooling, the solution was dialysed against distilled water and concentrated by evaporation in vacuum at 40°. The above methylation was repeated twice and the final solution freeze-dried.

A solution of the partially methylated glucan in dimethyl formamide (20 ml.) was shaken with barium hydroxide (1 g.) and methyl iodide (5 ml.) at room temperature for 24 hr. A further amount of barium hydroxide (1 g.) and methyl iodide (5 ml.) was added and the mixture shaken for 24 hr. Water (200 ml.) was added and the suspension extracted with chloroform (5 × 50 ml.). The extracts, dried over $MgSO_4$, were

evaporated to 1 ml. and addition of light petroleum (b.p. 60–80°) gave a precipitate (22·2 mg.), which was still incompletely methylated (ν_{max} 3500 cm.$^{-1}$).

A solution of the product in dimethyl sulphoxide (4 ml.) was shaken with barium oxide (1 g.) and methyl iodide (5 ml.) for 24 hr. This procedure was repeated and the product (20 mg.), isolated essentially as above, was free from absorption at 3500 cm.$^{-1}$ in its infra-red spectrum.

Periodate oxidations. The procedures were as follows.

(1) A sample of the glucan (12·5 mg.) was dispersed in water (5 ml.) and a solution of sodium metaperiodate (5 ml., 0·022 M) added. The suspension was stirred at 3° and samples (1 ml.) withdrawn at intervals for analysis for periodate uptake (Fleury & Lange, 1933) and formic acid liberation (Anderson, Greenwood & Hirst, 1955).

(2) Smith degradation (Goldstein, Hay, Lewis & Smith, 1959, 1965): a dispersion of the glucan (78·0 mg.) and sodium metaperiodate (144·9 mg.) in water (50 ml.) was stirred at 3° for 72 hr. Ethylene glycol (100 μl.) was added and after 0·5 hr the oxidized glucan was centrifuged down, washed with cold 50% (w/v) acetic acid and water and freeze-dried.

The resultant white solid (48·3 mg.) was suspended in water (10 ml.) and stirred overnight with sodium borohydride (100 mg.). Acetic acid (10 ml.) was added and the centrifuged precipitate was washed with 50% (v/v) aqueous ethanol. Sulphuric acid (0·2 N, 10 ml.) was added to the gel suspended in 10 ml. of water and the suspension stirred at room temperature for 24 hr. The precipitate was centrifuged down, washed well with water and freeze-dried to give 34 mg. of the degraded glucan.

Examination of the final supernatant fluid and washings, after neutralising with barium hydroxide, by paper chromatography (solvent *c*) showed the presence of glycerol only. After chromatography on Whatman 3 MM paper (solvent *c*) the glycerol was characterized as the tri-*p*-phenylazobenzoate m.p. 212–213°, prepared by the method of Baggett, Foster, Haines & Stacey (1960), which was identical with an authentic specimen prepared from glycerol (found on analysis: C, 71·1; H, 4·7; N, 11·8. $C_{42}H_{32}O_6N_6$ requires C, 70·4; H, 4·5: N, 11·7%).

Preparation of cell wall from mycelium

Suspensions of washed mycelium were shaken at about half the maximum amplitude in a Mickle disintegrator (Mickle Lab. Engineering Co., Surrey); each container held 0·75 g. wet wt mycelium, 5 ml. water and 5 ml. dry ballotini (no. 10). After 40 min. shaking, the hyphae were broken into short lengths and the cell contents had exuded. To free the cell walls from adhering cytoplasm, the process was repeated after the ballotini had been replaced by 4 ml. of 4% (w/v) sodium lauryl sulphate (pH 6·8), containing 10 mM-magnesium chloride. The large particles still remaining were allowed to sediment and the supernatant fluid decanted off. The cell walls were separated by centrifugation at 2000 *g* for 5 min. and washed by centrifugation 4 or 5 times with water. The residue was freeze-dried, yielding a white fluffy solid weighing about 50 mg. Microscopic observations, after staining with methylene blue and lactophenol cotton blue, indicated that the product was about 90% free from cell inclusions.

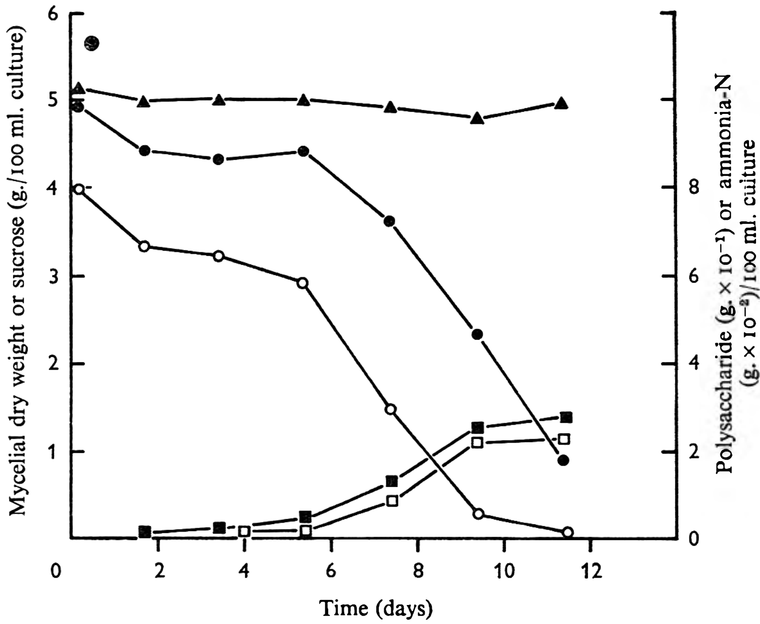

Fig. 1. *Claviceps fusiformis* strain F2047. Course of metabolism in stirred fermenters. Culture in medium II at 27° stirred at 183 rev./min. in a 60 l. fermenter with sterile air blown through at 30 l./min. for the first 24 hr and at 60 l./min. thereafter at an over-pressure of 1 atm. ▲, pH; ●, sucrose; ○, ammonia nitrogen; ■, mycelium; □, polysaccharide.

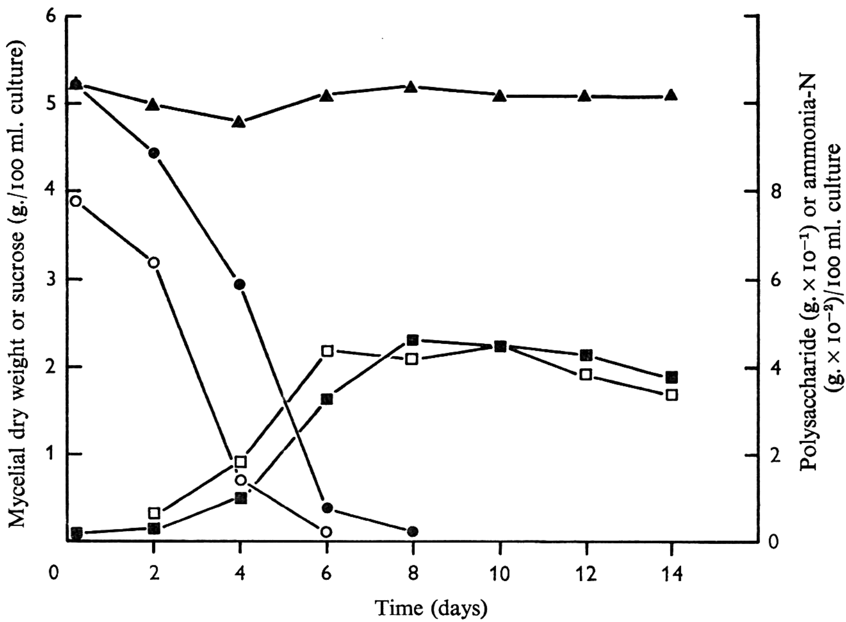

Fig. 2. *C. fusiformis* F2047. Course of metabolism in shake-flasks. Culture (100 ml. in 500 ml. Erlenmeyer flasks) shaken at 200 rev./min. in medium II at 27°. ▲, pH; ●, sucrose; ○, ammonia nitrogen; ■, mycelium; □, polysaccharide.

RESULTS

Physiological conditions controlling formation of the polysaccharide

In this work polysaccharide formation occurred between 24° and 27° and yields up to 4 g. dry wt mycelium/l. culture fluid were obtained.

The course of fermentation using medium II is given in Figs. 1 and 2. The polysaccharide was produced in shaken flasks and in the fermenters and reached maximum yields when growth ceased. In the fermenters growth was preceded by a long lag phase and the course of metabolism was slower. After the growing period, little or no decline

Table 1. *Claviceps fusiformis strain* F2047: *effect of pH value of medium on polysaccharide production*

Shake-flask culture at 27° in medium II at various pH values; harvested at 8 days.

pH value of culture		Yield of mycelium (g. dry wt/100 ml. culture)	Polysaccharide (mg./100 ml. culture)	Sporulation*
Initial	Final			
4·2	3·0	0·48	50	—
4·7	3·0	0·61	76	—
5·0	4·4	1·06	145	+
5·2	5·0	1·48	194	++
5·5	5·0	1·49	411	++++
5·7	5·0	1·67	353	++++
6·2	5·0	1·70	178	++++

*Arbitrary scale: no sporulation; + → ++++ degrees of sporulation.

Table 2. *Claviceps fusiformis strain* F2047: *effect of ammonium succinate concentration on polysaccharide production*

Shake-flask culture at 27° with various ammonium succinate concentrations (salts and sucrose concentration as in medium II); harvested at 8 days

Initial conc. of ammonium succinate in medium (%)	N (mg./ml.)	Mycelium (g./100 ml. culture)	Polysaccharide (mg./100 ml. culture)	Relative degrees of sporulation
0·13	0·24	1·191	85	—
0·21	0·39	1·191	86	—
0·32	0·59	1·489	209	++
0·41	0·76	1·408	210	++
0·54	1·00	1·196	263	++
0·64	1·18	1·196	266	++++

in the quantity of polysaccharide present in the culture was observed for at least 14 days (Fig. 2; Table 6) which indicated that it was not further metabolized by the fungus under the conditions used. This contrasts with the work of Davis *et al.* (1965), who found marked decreases in the polysaccharides produced by *Plectania occidentalis* and a Helotium species after 14 and about 2·5 days, respectively.

The effect of pH value on polysaccharide production in shake flasks is shown in Table 1; maximum production occurred between pH 5 and 6. The cultures were white below pH 5·2; with higher pH values a red pigment developed in increasing amounts.

Increasing the nitrogen concentration of the medium above a minimum value of

0·4 mg. N/ml. (Table 2) had little effect on polysaccharide production. Polysaccharide production was also independent, within fairly wide limits, of the sucrose concentration (Table 3). Sporulation occurred under most conditions towards the end of the incubation.

In addition to sucrose, the fungus utilized glucose, fructose, cellobiose and mannitol for growth, but not galactose, xylose, maltose, lactose, sorbitol, glycerol, starch or

Table 3. *Claviceps fusiformis strain F2047: effect of sucrose concentration on polysaccharide production*

Shake-flask culture at 27° with various sucrose concentrations (salts and ammonium succinate concentration as in medium II); harvested at 7 days.

Initial sucrose conc. in medium (%)	Mycelium (g./100 ml. culture)	Polysaccharide (mg./100 ml. culture)	Relative degree of sporulation
1	0·436	69	+ + + +
2·5	1·272	105	+ + +
5	1·982	205	+ +
10	2·401	205	+

Table 4. *Claviceps fusiformis strain F2047: effect of carbon source on mycelial growth and polysaccharide production*

Shake-flask culture at 27° with various carbon sources (salts and ammonium succinate concentration as in medium II); harvested at 8 days.

Carbon source (5%, w/v, medium)	Mycelium (g./100 ml. culture)	Polysaccharide (mg./100 ml. culture)
Sucrose	1·60	224
Glucose	1·54	209
Fructose	1·39	215
Galactose	0·032	—
Xylose	0·032	—
Maltose	0·38	45
Lactose	0·047	—
Cellobiose	1·69	309
Mannitol	1·61	255
Sorbitol	0·12	—
Glycerol	0·13	27
Starch	0·21	16
Succinic acid	0·034	—

Table 5. *Claviceps fusiformis strain F2047: effect of nitrogen-source on polysaccharide production*

Shake-flask culture at 27° with various nitrogen sources (salts and sucrose concentration as in medium II); harvested at 8 days.

N source in medium (N = 0·75 g./l.)	Final pH value of medium	Mycelium (g./100 ml. culture)	Polysaccharide (mg./100 ml. culture)
Ammonium succinate	5·0	1·486	200
KNO 3	6·4	0·904	—
Urea	6·2	1·792	Trace
Peptone	5·6	1·527	149
NH_4NO_3	5·8	1·715	85

succinic acid (Table 4). As expected, polysaccharide production took place only when mycelial growth occurred. However, correlation between mycelial growth and polysaccharide production was not observed when the nitrogen source was varied while the carbon source (5 % sucrose) was kept constant (Table 5). All nitrogen sources tested were utilized for growth but with nitrate or urea no polysaccharide production took place.

Structure of polysaccharide

A single specimen of the polysaccharide obtained from a 7-day shake-flask culture was used for the following structural studies. The purified freeze-dried product (sample A) was a white fibrous solid which had a total carbohydrate content of 97 %, a total nitrogen content of 0·5 % and, although it had been soluble in the original culture medium, was largely insoluble in water and only partially soluble in fresh culture medium and in 20 % sucrose solution. Acid hydrolysis yielded only glucose as shown by paper chromatography and electrophoresis; the reducing power of the hydrolysate was equivalent to 88·5 % anhydroglucose. D-glucose was isolated in good yield as the pentaacetate. Partial acid hydrolysis of this glucan at 90° yielded a series of oligosaccharides, two of which were tentatively identified by chromatography as laminaribose and gentiobiose, which suggested the presence of $\beta 1 \rightarrow 3$ and $1 \rightarrow 6$ links. The optical rotation of the original material ($[\alpha]_D^{20}$ $+6{\cdot}8° \pm 0{\cdot}3$ in 5 N-sodium hydroxide) and its infra-red spectrum (ν_{max} 896 cm.$^{-1}$) were further indications of β- linkages. Acid hydrolysis of the methylated glucan gave 2,3,4,6-tetra-*O*-methyl-D-glucose; 2,4,6-tri-*O*-methyl-D-glucose and 2,4-di-*O*-methyl-D-glucose in the ratio of 1·15:2·7:1·0; this indicated the presence of non-reducing glucopyranosyl end-groups, $1 \rightarrow 3$ glucopyranosyl linkages and 1,3,6 branch points, respectively, in the same ratio.

Periodate oxidation studies were in agreement with the methylation analysis. After 95·5 hr at 3° the glucan had consumed a limiting value of 0·51 mole/periodate/anhydrohexose unit and had liberated 0·20 mole formic acid. Only trace amounts of formaldehyde were detected. This is consistent with a structure containing either one non-reducing end-group or a $1 \rightarrow 6$ linkage for every five units: the 70 % periodate resistant units obtained indicate $1 \rightarrow 3$ linkages or branch points with no vicinal hydroxyl groups. The slightly high value for the periodate consumption (0·5 mole) over that expected from the formic acid production (0·2 mole) remains to be explained.

Evidence for the fine structure of the glucan was obtained from a Smith degradation from which was isolated glycerol and a residual polymer only. The latter was not attacked further by periodate and its structure was confirmed enzymically (see below) as an unbranched linear $\beta 1 \rightarrow 3$ linked glucan.

The above results indicate that the original glucan consisted of chains of $1 \rightarrow 3$ linked β-D-glucopyranosyl units to which single β-D-glucopyranosyl units are attached by $6 \rightarrow 1$ linkages on every fourth unit, on average, along the chain. Supporting proof for the structure of the glucan was obtained from a concurrent series of enzymic investigations. The presence of β linkages was indicated by hydrolysis of the polymer to glucose by an enzyme preparation from *Helix pomatia* known to contain a specific β-glucosidase. Similarly, the polymer, and oligosaccharides obtained by its partial acid hydrolysis, were hydrolysed to glucose by a β-glucosidase from almonds but were unaffected by a fungal maltase and bacterial α-amylase. A partially purified fungal cellulase had only a slight hydrolytic action on this glucan which suggested that the linkages were not $\beta 1 \rightarrow 4$. Enzymic proof for the $\beta 1 \rightarrow 3$ nature of the linkages was

obtained by use of the specific exo-$\beta 1 \rightarrow 3$-glucanase, which hydrolysed the polymer to a mixture of glucose and gentiobiose in a molecular 3:1. The polymer ratio of derived by the Smith degradation (see above) gave only glucose on hydrolysis by this enzyme.

Glucan from stirred fermenters

The infra-red spectrum, optical rotation and products from complete acid hydrolysis and hydrolysis with the exo-$\beta 1 \rightarrow 3$-glucanase obtained with the product from the stirred fermenter were identical with those found for the shake flask polysaccharide. The average glucose to gentiobiose ratio for the fermenter polysaccharide was 2·2:1.

Variations in glucan structure

Claviceps fusiformis strain F2047 did not lose its capacity for polysaccharide synthesis in submerged culture after many vegetative transfers (> 30) in contrast with the findings of Davis *et al.* (1965) with *Plectania occidentalis* and an Helotium species. However, variations in the structure of the *C. fusiformis* glucan were observed. Table

Table 6. *Claviceps fusiformis strain F2047: variation in the degree of branching of the glucan during 20 days of incubation*

Samples of the glucan, obtained from a shake-flask culture at 27° on medium II, were hydrolysed at 1% (w/v) in 100 μl. of 0·05 M-acetate buffer (pH 4·6) with 0·1% (w/v) of exo-$\beta 1 \rightarrow 3$-glucanase for 3 hr at 50°.

Time of growth (days)	Yield of glucan mg./100 ml. of culture	Molecular ratio of glucose to gentiobiose*
3	44	3·6:1
7	102	3·2:1
10	133	2·4:1
14	208	1·8:1
17	137	2·1:1
20	88	2·3:1

*Mean value obtained on products from enzyme hydrolysis. Ratio of units in main chain with branch to those without = 1:glucose/gentibiose ratio.

6 shows that the degree of branching of the glucan, as measured by the ratio of the glucose to gentiobiose produced on enzymic hydrolysis with the specific exo-$\beta 1 \rightarrow 3$-glucanase, gradually increased to a maximum of one branch on every third residue after 14 days, then slowly declined. Furthermore, chemical and enzymic evidence suggested that over a period of months the overall degree of branching of polysaccharide produced after 10 days submerged culture had slowly increased from about one branch per four units to one per three units. The highest degree of branching corresponded to the highest degree of water solubility and lowest viscosity.

The variation in the degree of branching observed amongst these samples and between them and specimen A suggested the possibility that the glucan was not homogeneous but consisted of several components differing in their degrees of branching. However, fractionation experiments in N-sodium hydroxide using a single purified sample from a 17-day culture did not support this hypothesis. All fractions obtained were indistinguishable by infra-red spectroscopy and had approximately the same degree of branching, as indicated by hydrolysis with the exo-$\beta 1 \rightarrow 3$-glucanase and measurement of the glucose and gentiobiose liberated (Table 7).

Tests on the polysaccharide derived from mycelium grown on media with various

carbon and nitrogen sources showed that neither had any effect on the degree of branching.

A sample of ^{14}C-glucan obtained from an ammonium succinate [U-^{14}C]sucrose (1 %) medium enabled an accurate check to be made on the degree of branching, as measured by the enzyme technique. The high value obtained at 24 hr (Table 8) was probably due to the fact that the exo-$\beta 1 \rightarrow 3$-glucanase had a slight activity towards the $\beta 1 \rightarrow 6$ link, (already noted by Dr E. T. Reese, personal communication, 1966).

Table 7. *Claviceps fusiformis strain F2047: degree of branching of glucan fractionated in 5 N-sodium hydroxide*

Sample of the glucan (116 mg.) obtained from a shake-flask culture at 27° on medium II, harvested at 17 days, fractionated as under 'Methods'; fractions hydrolysed at 1 % (w/v) in 100 μl. of 0·05 M-acetate buffer (pH 4·6) with 0·1 % w/v. of exo-$\beta 1 \rightarrow 3$-glucanase for 3 hr at 50°.

Fraction	Weight of extracted polysaccharide in fraction (mg.)	Molecular ratio of glucose to gentiobiose
1	31·5	1·75:1
2	31·5	1·74:1
3	14·1	2·06:1
4	8·15	1·85:1
5	7·95	1·98:1
6	1·70	1·86:1

Table 8. *Claviceps fusiformis strain F2047: molecular ratio of the glucose to the gentiobiose liberated from [U-^{14}C]glucan during hydrolysis by exo-$\beta 1 \rightarrow 3$-glucanase*

Sample of the glucan, obtained from a shake-flask culture at 27° with 1 % (w/v) [U-^{14}C]sucrose containing 50 μc./flask (salts and ammonium succinate concentration as in medium III), hydrolysed at 5 % (w/v) in 500 μl. of 0·05 M-acetate buffer (pH 4·6) with 0·1 % (w/v) of exo-$\beta 1 \rightarrow 3$-glucanase at 50°.

Incubation period	Hydrolysis of glucan (%)	Molecular ratio of glucose to gentibiose
0	0·90	—
3 min.	13·3	2·02:1
10 min.	35·7	2·03:1
40 min.	81·9	2·02:1
2 hr	98·5	2·09:1
5 hr	98·9	2·19:1
24 hr	99·3	2·44:1

Cell-wall glucans

Loosely filtered mycelium to which glucan still adhered was incubated with the exo-$\beta 1 \rightarrow 3$ glucanase to ascertain whether the glucan could be removed in this way from the hyphal walls. However, the dry weight of the mycelium after this treatment was lower than could be accounted for by removal of the glucan alone; moreover, microscopic observation clearly indicated that the cell walls had undergone some decomposition. Furthermore, the molecular ratio of glucose to gentiobiose in the enzyme digest was over 6:1, i.e. higher than in the glucan alone. Accordingly, cell-free samples of cell walls were prepared and subjected to enzymic hydrolysis by the $\beta 1 \rightarrow 3$ glucanase; they yielded glucose 26 %. Attempts to record the infra-red spectrum of the cell-wall material in nujol were not completely successful due to the highly particulate nature of the material; however, a small peak at 898 cm.$^{-1}$ and the absence of peaks

at 850 and 935 cm.$^{-1}$ indicated β linkage, which supports the evidence for the presence of chains of $\beta 1 \rightarrow 3$ linked glucose units in the walls.

The indication of $\beta 1 \rightarrow 3$ linked glucans in the walls of *Claviceps fusiformis* strain F2047 suggested that the natural sclerotium might contain similar polysaccharides. Sclerotia were ground and the resulting powder subjected to the action of the exo-$\beta 1 \rightarrow 3$ glucanase. No hydrolysis was observed. It was considered that this negative result might be due to the fact that potentially hydrolysable structures were not available to the enzyme because of the complex structure of the sclerotia. However, extraction of the sclerotial powder by alkali under nitrogen, followed by ethanol precipitation, yielded a product which represented 10·9% of the original and contained 58% total carbohydrate and 2·9% total-nitrogen. This extract was susceptible to prolonged enzymic hydrolysis and yielded glucose and gentiobiose in a ratio of approximately 2:1 (3·03 mg. were incubated with the exo-$\beta 1 \rightarrow 3$ glucanase for 20 hr and yielded after hydrolysis 0·94 mg. anhydroglucose). The glucose yield after hydrolysis indicated that the carbohydrate in the extract consisted of about 53% glucan containing $\beta 1 \rightarrow 3$ linkages and $\beta 1 \rightarrow 6$ branch points, in all representing 3·4% of the original dry sclerotia. Uninfected millet, used as a control, gave no indication of $\beta 1 \rightarrow 3$ glucans after extraction.

Allied organisms producing $\beta 1 \rightarrow 3$ glucans

During this work two other organisms related to Claviceps and a basidiomycete *Sclerotium rolfsii*, all of which produce polysaccharides, were tested for the presence of $\beta 1 \rightarrow 3$ linked glucans. Strain I.C.49 was isolated from sclerotia on Sorghum in Africa; strain F-114 was isolated from sclerotia of *Claviceps litoralis*; the basidiomycete was kindly given by Dr B. E. J. Wheeler (Imperial College, London). All three fungi were found to produce $\beta 1 \rightarrow 3$-linked glucans with $\beta 1 \rightarrow 6$ branches. Strain I.C.49 gave a molar ratio of glucose to gentiobiose of about 4:1. The glucan produced by F-114 gave the highest degree of branching observed in the work (glucose:gentiobiose ratio about 1·5) and was the most water-soluble glucan. The presence of $\beta 1 \rightarrow 3$-linked glucans from *S. rolfsii* has already been noted (Johnson *et al.* 1963); the culture used (IMI74210—Commonwealth Mycological Institute, London) produced a glucan in large quantities after addition of 2% (v/v) ethanol to the culture medium (Dr B. E. J. Wheeler, personal communication, 1966); this glucan had a molar ratio of glucose to gentiobiose of about 3:1 and was very sparingly water-soluble.

Microscopic examination of the mycelia of the above organisms after treatment with the exo-$\beta 1 \rightarrow 3$ glucanase showed that the cell walls of all three organisms were attacked, thus suggesting that glucans with $\beta 1 \rightarrow 3$ linkages were present. The presence of $\beta 1 \rightarrow 3$ linked glucans in the cell walls of other fungi appears to be well established (Clarke & Stone, 1963; Horikoshi & Iida, 1964; Manners & Patterson, 1966; Wessels, 1966). Perlin & Taber (1963) noted that in some cases the glucan produced by *Claviceps purpurea* was attached to the mycelium, as has been found here for the glucan produced by the above strain of *Sclerotium rolfsii* and to a lesser extent the various strains of Claviceps tested. Evidence is accumulating to suggest that there may be a structural connexion between the cell walls and the glucan produced extracellularly.

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A Microthread Technique for Studying the Viability of Microbes in a Simulated Airborne State

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SUMMARY

A technique is described in which airborne micro-organisms are captured on ultra-fine spider threads. In this state the organisms may be subjected to any environment of interest for extended periods of time, during which their loss of viability may be assayed. Experiments are described which show that the loss of viability is quantitatively similar to that in the true airborne state, provided that the microthread and aerosol environments are identical and that certain other precautions are observed.

INTRODUCTION

For some diseases it is well established that infection can be acquired by inhalation of airborne particles which consist of, or contain, viable bacteria or viruses. In the study of these infections it is obviously desirable to be able to investigate such factors as: (a) the length of time in which micro-organisms can remain viable in the airborne environment; (b) the extent to which loss of viability is affected by the bulk or physical nature of the particle; (c) the effect of sterilizing agents in the air, such as ultraviolet radiation and vapours toxic to the microbes. These aspects can be studied to some extent in small rooms or specially constructed chambers, but drift, fall-out, loss on walls, etc., is usually such as to limit severely the time period of such experiments. The effect of radiation from a sterilizing source on airborne particles in rooms, chambers, etc., is particularly difficult to measure quantitively because random air movement continually changes the distance of the particles from the radiation source. The rotating-drum apparatus (Goldberg *et al.* 1958) can be used to hold small particles of artificially generated aerosols in the airborne state for very long periods, but the survival of micro-organisms in these tightly enclosed conditions may not be the same as in inhabited environments. Therefore the results are of uncertain value in public health applications.

The extent to which fungal spores and pollens remain viable is also difficult to study because, when captured from the open air, their age is usually unknown and their size is such that in any enclosure they rapidly fall out from the aerosol.

In an effort to overcome these difficulties we have studied the survival of micro-organisms when they are supported on ultra-fine threads stretched across a small metal frame. This system gives a form of 'captive aerosol' with which, for example, it is easy to subject particles to accurately measured dosages of sterilizing vapours or radiation for any length of time and in any condition of humidity and temperature. By combining the viable particles with an inert tracer such as a stable bacterial spore or a

radioactive substance the loss of viability of the particles with time can be studied in detail, though their infectivity as airborne particles cannot be tested.

For our purposes we consider the basic requirements for microthreads to be:

(i) Their diameter should be as small as possible, and in any case smaller than that of the smallest viable particle. Access to the particle of vapours, radiation, humidity change, etc., should then not be seriously impeded by the presence of the supporting threads.

(ii) They should be biologically inactive so that there is no toxic or protective action at the point of contact of particle with thread.

(iii) They should be sterile and free from surface contamination before use.

(iv) They should be easy to handle and to wind round their holding frames in a standardized manner.

(v) When wound on the frames they should be resistant to shock, shaking and to the forces of air movement.

(vi) They should be in plentiful supply.

(vii) A standardized method of winding should give a constant length of thread on every frame so that the number of particles caught shall be predictable.

Commercially or experimentally made fibres fail to meet one or more of these requirements, particularly (i) and (iv). Kordyum & Bobchenko (1959) studied the growth of micro-organisms on fine glass threads (50 μ) diameter but we do not know of any means of obtaining uniform and regular supplies of glass threads to our requirement for fineness (< 1 μ diameter) in a form convenient for handling. Dessens (1949) used spider threads to collect and study atmospheric haze particles. He allowed spiders to crawl over metal frames and pointed out that some of the threads were as fine as 0·01 μ.

We now routinely use the 'safety-line' threads released by recently hatched specimens of those spiders which spin orb webs. The safety-line threads are produced by the spider in its normal escape activity when it falls from a vigorously tapped rod or frame. These threads are about 0·5 μ in diameter; but the spider also produces, in a random and unpredictable manner, side threads which are too fine to be seen under the high-power microscope, so that they can only be detected when particles adhere to them. Presumably in nature these side threads act as wind-blown anchors; in the present work they caused the number of particles caught from a flowing aerosol of known concentration to be unpredictable, except to within a factor of 2 or 3. In all other respects the fine spider threads appear to fulfil requirements (i)–(vi) perfectly. Also, the weight of the spider, which is exactly right for the thread it produces, gives a constant winding tension for all turns.

APPARATUS AND METHOD

Frames for microthreads. The frames used for supporting the microthreads were designed so that they are convenient (*a*) to handle when winding on the threads, (*b*) to expose in any desired situation, (*c*) to immerse in sampling fluid when removing the organisms for assay. A frame is shown in Fig. 1A. The handle, 7·5 cm. (3 inch) long, is made from 13-gauge stainless steel, as are the two cross-pieces. These cross-pieces are 22 mm. ($\frac{7}{8}$ inch) long and are joined by the two side members made from 5·5 cm. ($2\frac{1}{8}$ inch) lengths of 19-gauge stainless steel. The outer edges of these side

members are 1·9 cm. ($\frac{3}{4}$ inch) apart. All joints are silver soldered and all frames are assembled on a jig so that all are identical. A useful degree of protection to the microthreads when wound round the side members is afforded by the cross-pieces, which are thicker than the side members and project beyond the latter at the corners (see Fig. 1 A).

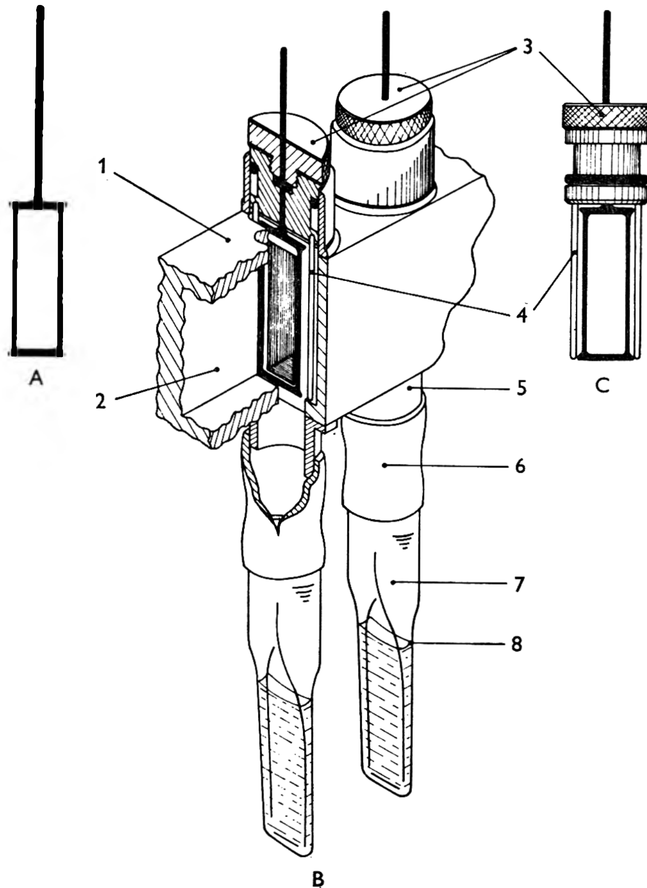

Fig. 1. A, Stainless steel frame. B, Isometric cut-away sketch of 'sow' tube showing positioning of frames and sampling cells. C, Frame mounted in removable, O-ring sealed cap.

Spiders. As a source of microthreads we usually employ newly hatched or young specimens (bodies not greater than 3 mm.; $\frac{1}{8}$ inch) of *Araneus diademata* or of *Zygiella-x-notata* which are the two commonest orb spinners. The latter can be artifically reared by feeding on Drosophila, to obtain a continuous supply for most of the year. There is no doubt that many other species of web-spinners would be equally satisfactory, but spiders which do not spin web snares are of no value.

Method of winding spider threads on the frames. To wind a frame, a spider is placed on it (a wire loop is a useful transferring tool) and the frame, held horizontally, is given a sharp tap. The spider drops on its safety line which is then wound round the frame by spinning the handle the required number of turns. Normally we use 50 complete turns (100 lateral threads) spacing the threads equally along the frames but only to within $\frac{1}{4}$ inch of the cross-pieces (Pl. 1, fig. 2). The winding process may also be done

by holding the handle in a chuck which is mounted on a lead screw with a pitch of 1 mm. so that, on hand rotation, the frame advances 1 mm. per turn, with each turn indicated on a counter. When a spider of about 3 mm. ($\frac{1}{8}$ inch) body is used for this process and the room air is still, very perfectly wound frames can readily be produced. The threads are too fine to be visible in transmitted light but may be readily seen in Tyndall illumination at certain angles. Very close inspection makes possible the rejection of frames which carry an excessive number of the ultra-fine cross-threads, should this be desired. These frames would capture more particles than their neighbours, which may have fewer or no cross-threads. Plate 1, fig. 1, shows a photomicrograph of two main threads and a single cross-thread, the existence of which is only revealed in transmitted light by the presence of the micro-organisms on it. While a frame is being wound, the spider must not be allowed to climb back onto it or the spider will at once tangle up the threads which have been already wound. A single spider in good health will provide enough thread before it tires to wind from two to eight frames, according to the size of the spider. Spiders larger than about 3 mm. ($\frac{1}{8}$ inch) in body size move too quickly and actively to be readily handled and the thread diameter increases in proportion.

Spider threads wound in this way will withstand air velocities well in excess of 20 miles/hr (32 km./hr) without breaking, their strength relative to their size being extremely high. The silk threads are not ejected by the spider, they are drawn out from the spinnarets and thread formation from the viscous fluid is said to be a process of molecular rearrangement of the amino acids therein and not of drying, oxidation or other chemical process (Savory, 1952).

Frames are washed and sterilized before winding and the threads as produced by the spider are sterile. Provided that wound frames are stored in a closed container, stocks of them may be stored for many weeks without deterioration before use. Contaminated wound frames may be sterilized by ultraviolet radiation or by heating at 60° for 1 hr.

Equipment for capturing and sampling aerosol particles on the microthreads. To deposit aerosol particles on the microthreads a tube called a 'sow' is employed. A cutaway isometric sketch of a small section of a sow is shown in Fig. 1B. The rectangular body of the sow tube (1) is built from brass and the internal rectangular channel (2) through which the aerosol passes is $2 \times \frac{1}{2}$ inch ($5 \times 1{\cdot}3$ cm.). Slots, cut right through the sow body (1) from the top to bottom allow just enough room for a frame to pass right through. To load the sow with frames, the handle of each frame is first pushed through the holding cap (3), a side elevation of which is shown in Fig. 1C and a section in Fig. 1B. This holding cap has an internal O-ring which seals and grips the handle of the frame. On the external shaft of the holding cap is an O-ring, as shown, which provides a rolling seal when the cap is pushed into its socket on top of the sow.

When the holding cap is pushed home in the sow it holds the frame in the position shown in Fig. 1B. The metalwork of the frame is completely recessed within the slot in the walls of the sow so that the aerosol, when passing through the rectangular channel (2), only encounters the microthreads in its direct passage. In fact, owing to some eddies within the slot recesses, about 1–2% of the number of particles intercepted by the microthreads may lodge on the frames (see Pl. 1, fig. 2). The holding caps have two side-prongs (4) which serve as protectors to the frames and as guides when the cap is pushed home in the sow.

Below each frame station is the tube (5). A short length of thin-walled rubber tubing (6) connects tube (5) to the glass (or plastic) sampling cell (7). These sampling cells are attached as shown in Fig. 1 B when it is desired to perform an experiment on the survival of the micro-organisms within the enclosure of the sow tube. In such an experiment a screw-clip (not shown in Fig. 1 B) seals off the tube (6). At suitable time intervals frames can be withdrawn from the sow without opening it to the air by fully opening the screw clip and pushing the handle of the frame down through the holding cap (3) by means of a ramrod of the same diameter as the handle. The frame drops into the cell (7) and at the same time the ramrod maintains the integrity of the seal within (3) as it passes through the small internal O-ring. The screw clip is re-tightened and the cell (7) is withdrawn.

The sampling cells (7) are formed over a mandrel to the shape shown and are normally filled with 4 ml. of sampling fluid. The meniscus height (8) is such that when a frame is dropped home its shoulder or upper cross-piece is above the meniscus. A vigorous up-and-down agitation of the frame handle moves the lower cross-piece up and down in the narrow channel, so giving a violent stirring action within the sampling fluid. This removes micro-organisms from the microthreads and thoroughly mixes the suspension for assay. There is no splashing as only the vertical side members of the frame penetrate the liquid surface.

Sows of the Fig. 1 B pattern have been made with six frame stations. The ends of the rectangular body have flanges so that coned end-tubes may be bolted on. These cones taper to a 1-inch (2·54 cm.) diameter tube, a convenient size for the induction of aerosols. With coned pieces removed, two sows may be bolted together in series. In another form of sow, used solely for depositing particles on the microthreads before removal of the frames to exposure sites, the lower tubes (5) are dispensed with, and a second row of sockets for the caps (3) is built into the intermediate positions. In this way, 13 frames may be charged within the same length as needed for 6 frames in the Fig. 1 B form.

Methods of generating aerosols. In the laboratory artificial aerosols for passage through the sow may be generated by any convenient device, from liquid suspensions or powdered material. For generating aerosols of very small particle size we routinely use a Collison spray system similar to that described by Henderson (1952) but with the addition of humidity and temperature control. For aerosols of large uniformly sized particles the wind-tunnel system of Druett & May (1952), which embodies a spinning-top sprayer, is employed. The vibrating-reed apparatus of Wolf (1961) is also useful for depositing perfectly uniform particles on the threads. With Wolf's device the generated droplets may be allowed to fall directly onto the microthreads before evaporation. This avoids the use of the sow but the number of droplets caught is only very roughly predictable. 'Natural' aerosols may be drawn through the sow for capture on the threads, provided that their concentration is high enough to give an adequate number of viable organisms for assay. A sneeze aerosol could be captured in this way. Particle size selection may be incorporated when desired by fitting a simple impaction system of the required performance to the sow intake hole. Details of suitable impactor design parameters are given for example by May (1967).

Capture efficiency of threads

Small particles generated from a liquid suspension readily adhere to a microthread after touching it. The well-known van der Waals forces between small particles no doubt play a part, as do surface tension forces at the points of contact, where a minute liquid film condenses in the zone of high curvature (Cross & Picknett, 1964). When an unevaporated droplet is intercepted by the threads, its residue after drying will be bonded firmly round the thread. Thus, fine aerosol particles, when intercepted by the threads will stay in place in spite of subsequent wind forces or mechanical shock, yet are readily removed and dispersed by the standard procedure in the sampling cells, as already described. However, liberties may not be taken with particles dangerous to health. An initial shake off or blow off of a very small proportion of deposited particles can be demonstrated in certain circumstances and in free air there is always the possibility that a fly, floating feather, etc., might carry off some of the threads.

In very dry conditions and with larger particles adherence to the spider threads is much less satisfactory. Pollens and fungal spores, for example, with their hard dry rough surfaces are very easily removed from the threads by wind or shock. To study the viability of these in the 'captive aerosol' state it may be necessary to use, for example, Terylene threads, which can be obtained down to 15 μ diameter, a size which is still quite small as compared to some pollens and fungal spores. Such threads could be dipped, after winding on the frames, into a solution which would leave a thin adhesive film on the threads. If it were acceptable to spray the pollens or spores of interest from a liquid suspension, a small proportion of inert adhesive could be incorporated in the liquid, and the particles would then adhere firmly to any type of thread. The very fine spider threads we have described do not have sufficient absolute strength to withstand dipping into an adhesive solution. Electrostatic forces of attraction might also be effective with pollens and spores.

When particles adhere to a thread on contact, the effective 'capture cross-section' of a frame is the product of the total length of thread exposed to the aerosol and the sum of the diameters of the particle and thread. Hence the fraction of particles of a given size removed from the passing aerosol by any one frame is $nl(D+d)/hl$, where, in consistent units, n = number of threads across the frame, D and d = diameters of aerosol particle and thread, h and l = height and width of sow channel. The number of organisms caught on the frame is $N = n(D+d)\ CVt/h$, where C = total number of organisms per unit volume of air, in the particles of diameter D, V = volumetric flow rate and t = time of exposure.

As $n(D+d)/h$, the fraction of a passing aerosol caught, is very small successive frames are exposed to only slightly decreasing dosages. If the required parameters are known the above calculation is sufficiently accurate for assay dilution values to be estimated, but the variable number of ultra-fine cross-threads, together with certain anomalies of flow within the sow, cause variations in the catch from frame to frame which in extreme cases can exceed 4:1. A tracer incorporated with the decaying particles nullifies these variations and should therefore be used when possible. When a tracer cannot be used it is necessary to pool the samples from several frames at one time, having rejected before use any frames seen to have excessive fine cross-threads.

Methods of exposure of frames

After depositing an adequate number of organisms (usually at least 2000) on the microthreads they may be exposed to the environment of interest in any convenient way. For example, the effect of toxic vapours may be studied by flowing the gas mixture slowly through a sow, with a clean-air sow as a control. To do this two complete sows are set up in series with clean filtered air drawn through at, say, 10 l./min. and the toxic vapour is introduced at the mid-point junction of the sows. Any increased decay rate in the downstream sow as compared to that in the upstream sow is then due to the introduced vapour. The effect of flow velocity will be discussed later.

For exposure in rooms, holding-caps ((3) in Fig. 1) with their frames still in place may be inverted and the cap stood on a level surface with the frame handle pushed half-way through the cap. Alternatively a low table-like framework, with holes bored through the level surface, may be used. The holding caps rest on the holes with the microthread frame projecting beneath. This arrangement allows full air circulation but gives some physical protection to the threads.

To study the effects of sunlight on micro-organisms a number of small lightweight counterbalanced wind-vanes were constructed. Each holds a single frame, inverted in respect to Fig. 1A. A small polythene windscreen is mounted on the horizontal shaft of the vane just in front of the frame to give the threads protection from the direct force of the wind, dust particles, insects, etc. The polythene is partially transparent to all solar wavelengths in the visible and near ultraviolet.

Whatever the exposure situation, the standard sampling cells have been used for assay.

Measurement of very low degrees of viability

As already mentioned, the number of small particles lodging on the metal sides of a frame is about 1–2 % of those caught on 100 threads across the frame (Pl. 1, fig. 2). It has been found that micro-organisms in contact with the stainless steel will, in general, survive better than those fully exposed on the threads. Therefore measurements of viability have low significance when the viability has decreased to about 1 % of its original level. To overcome this we have used two-pronged forks made from thin stainless-steel wire. The prongs will just pass down inside the side members of the metal frame and when coated with an inert adhesive remove only the microthreads with their attached organisms. These may then be dropped into the standard sampling cells. Reasonably consistent estimates of viability can be obtained in this way at levels below 1 % of the initial viability.

Validity of the technique

Before the microthread technique could be established as a useful tool it had to be shown that survival of micro-organisms supported on the threads resembled that of the particles in the true airborne state in the same environment. The range of experiments in which such a comparison can be made is limited because the main feature of the microthread technique is that it can be used to study viability where other methods cannot. We were able to make comparison tests in the following three situations where the airborne micro-organisms could be assayed by the liquid impinger technique, which is well-established and considered to be reliable. (1) Survival in small enclosures.

Survival of true aerosols in the Goldberg rotating drum was compared to survival on the microthreads held both in a closed sow and in a drum. (2) Survival of an aerosol sprayed into a room compared to survival on microthreads in a similar room. (3) Survival of a range of particle sizes under sterilizing radiation (sunlight). The experiments were designed to ensure that the aerosol particles in the airborne state and on the threads came from the same source and were identical in all respects.

The test organism was a strain of *Escherichia coli* known to be robust in the aerosol state. Into the stock suspension was mixed a proportion of the tracer, spores of *Bacillus subtilis* var. *niger*, which are highly resistant to the airborne state and which grow into colonies at about the same rate as *E. coli* on the same media. Sampling cells and impingers were filled with phosphate buffer (pH 7) containing Manucol in M-sucrose solution; serial dilutions of the samplers were made in the same fluid. Plating was on tryptone agar. After incubation, differential counts of the two contrasting types of colony were made on each plate.

(1) *Survival in small enclosures.* We considered that if attachment to a microthread were to cause the survival of a micro-organism to be different from that in the true airborne state, then the difference would be more obvious for single-bacterium particles than for particles containing many bacteria, where mutual protection between the organisms might be expected. Single bacteria are nearer to the diameter of the threads and have a large fraction of their surface area in contact with the thread, so that they would be more subject to any adverse influence of the thread. Therefore these first comparisons were made with single-bacterium aerosols from the modified Henderson apparatus. This type of aerosol is in any case the most suitable for holding in the Goldberg rotating drum.

Aerosol was led from the spray mixing tube through the drum and sow in parallel. Drum and sow were then sealed off and held in a constant-temperature environment Samples from the drum were taken at intervals with the 'raised impinger' (May & Harper 1957), and frames were dropped from the sow at the same intervals.

Comparisons at 65° F (17·2° C) and 95% RH are shown in Fig. 2 and at 60° F (15·5° C) and 22% RH in Fig. 3. Both show a close agreement between the two methods. In Fig. 3 the triangles show results obtained without a tracer by finding the initial *Escherichia coli* recovery from 3 pooled frames and expressing successive *E. coli* recoveries from similar pooled samples as percentages of the initial number. The curve shows that it is possible to dispense with a tracer, given an adequate number of frames to assess and provided that the organisms survive the stress of aerosolization to give an initial viability close to 100%.

A typical comparison made at 68° F (20 °C), 50% RH is shown in Fig. 4. In this series of comparisons a persistently higher viability was obtained from the threads, the reason for which is unknown. The difference between the curves is, however, not important in comparison with the much higher rates of die-away which are usual when micro-organisms are being studied in larger enclosures such as a room where the air is in movement and contains sterilizing components (see Fig. 5). One difficulty encountered in long-term holding experiments is that very slight traces of a toxic vapour in a holding vessel will show a very pronounced unfavourable effect on the maintenance of viability in that vessel. Therefore it is essential to ensure that holding vessels used for control experiments are scrupulously clean inside.

In other tests, survival in the drum against survival in the sow was compared as

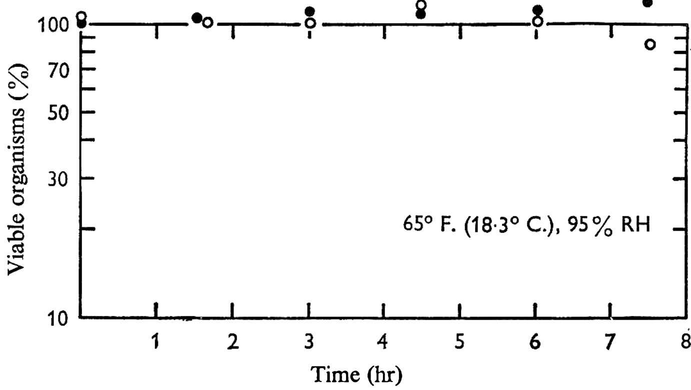

Fig. 2

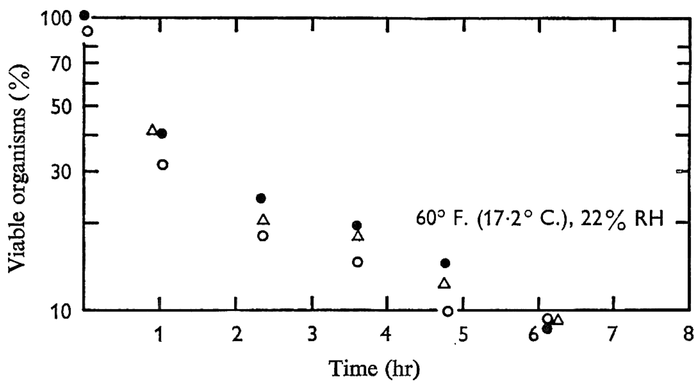

Fig. 3

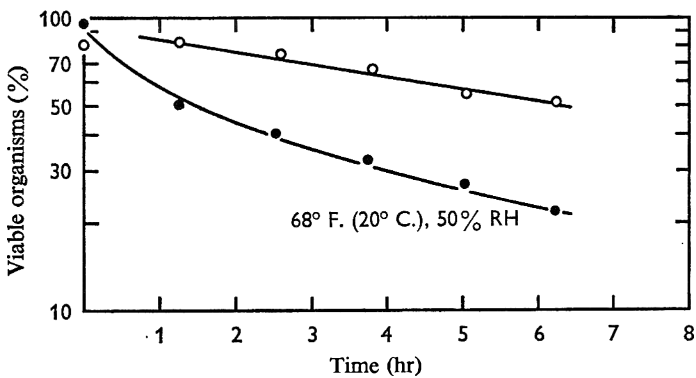

Fig. 4

Fig. 2. Comparison of survival of *Escherichia coli* 162 single-bacterium particles at high humidity; ○, on microthreads; ●, aerosol particles in rotating drum. Each point is the mean from three replicate samples.

Fig. 3. As Fig. 2 but at low humidity. △, *Escherichia coli* viabilities estimated without use of tracer.

Fig. 4. As Fig. 2 but at intermediate humidity.

above, but in addition survival on threads held in an otherwise empty aerosol drum was compared to that in the adjacent sow. After 21 hr at 69° F (20·5° C) 63% RH the measured viabilities in all three systems were in the range 4–10%. It was concluded that, provided that full precautions were taken in cleanliness and in establishing identical environmental conditions, there is no serious systematic difference between survival of particles in the airborne state in the drum and the same particles held on the microthreads.

(2) *Survival in a room.* A sealed chamber, 16 × 8 × 12 ft., was filled by an aerosol from the standard suspension by a remotely controlled pneumatic spray. The chamber was fanned for a short period to allow thorough mixing and evaporation of the spray droplets. The final particle diameters ranged from 6 μ downwards. Twenty litres of the aerosol was then drawn through a sow via an access port in 1 min. and an impinger

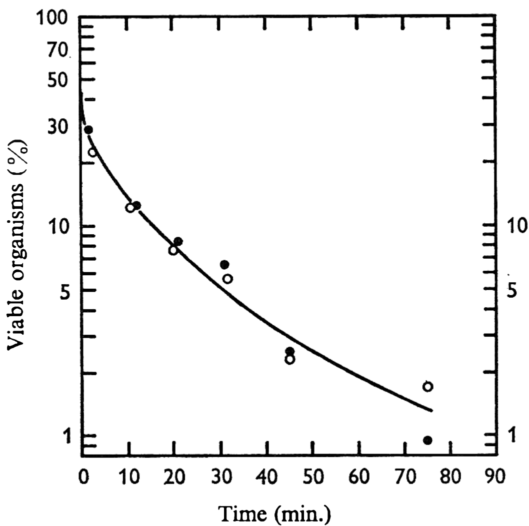

Fig. 5

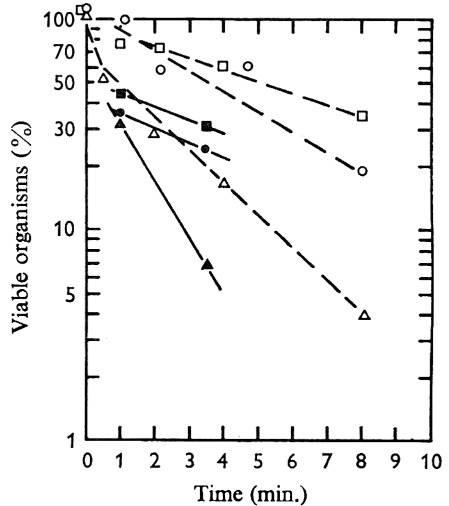

Fig. 6

Fig. 5. Comparison of survival of *Escherichia coli* 162 particles in a room; ○, on microthreads; ●, aerosol particles sampled by impinger.

Fig. 6. Comparison of survival of sprayed particles of *Escherichia coli* 162 in sunlight, in three particle-size ranges. On microthreads; △, particles < 3 μ.; ○, particles 3–6 μ; □, particles > 6 μ. Airborne particles; ▲, particles < 3 μ; ●, particles 3–6 μ; ■, particles > 6 μ.

sample was taken at the same time. The sow was then removed to an adjoining chamber, which was a replica of the first, the frames were removed and exposed on a table. Both chambers were fanned at frequent intervals, and impinger and frame samples were taken simultaneously from each chamber during the experiment. Three experiments gave very similar results,one of which is shown in Fig. 5. The agreement between the two methods was very close.

The rate of loss of viability in Fig. 5 is very high compared to the result of Fig. 4, which was obtained in tightly enclosed conditions in rather similar temperature and humidity. Some toxic component in the air of the chambers may have been present on this occasion, as it is possible to obtain prolonged survival of this strain of *Escherichia coli* in a room (cf. Fig. 7).

(3) *Survival in sterilizing radiation.* Tests on a three-stage glass impinger were described by May (1966). This device separates an aerosol into particle-size fractions of > 6 μ, 6–3 μ, and < 3 μ, and permits viability determinations to be made with each fraction. In these tests a pneumatic spray of a suspension of *Escherichia coli* and *Bacillus subtilis* var. *niger* was released into the air and it was possible to sample some of the aerosol after it had travelled downwind for 3–4 min. To compare the microthread technique in these conditions, frames in three sows were charged with particles of 9 μ, 4·5 μ and 1–2 μ, respectively, to represent the mid-points of the ranges in the 3-stage impinger. The frames were then taken to a flat roof on a fine summer day

Table 1. *Percentage viability of airborne Escherichia coli*

	Time airborne	Particle size range		
		> 6 μ	6–3 μ	< 3 μ
Sampler 1	1 min.	55	38	35
Sampler 2	1 min.	35	36	31
Average	1 min.	45	37	33
Sampler 1	3½ min.	30	28	10
Sampler 2	3½ min.	32	21	4
Average	3½ min.	31	24·5	7

Table 2. *Decay rates (%/min.) of airborne and threadborne Escherichia coli*

	Average particle size		
	9 μ	4·5 μ	1–2 μ
Impinger samples	14	16	63
Thread samples	13	22	36

with about 5/10 cloud and were mounted in the 'Vane' holding and exposure device at about the same time as the pneumatic spray cloud was released nearby. The suspensions for the airborne cloud and for the threadborne particles came from the same stock. The 1–2 μ particles were generated in a Henderson apparatus at the ambient temperature and humidity and the larger particles were obtained from the Druett & May wind-tunnel apparatus. Because of the practical difficulties of the tests it was possible to obtain no more than duplicate 3-stage impinger samples at two time-intervals in the airborne state, namely after 1 min. and 3·5 min. average travel. The percentage of *E. coli* viable in the impinger samples is given in Table 1 (taken from May, 1966, table 2, p. 567, runs 4 and 5). The averages from Table 1 are plotted in Fig. 6 for comparison with the microthread experiment done at the same time. The impinger data are obviously scanty but there is general agreement with other figures given by May for similar atmospheric conditions. Straight lines drawn through the plotted points in Fig. 6. appear to start from different origins, perhaps because the pneumatic spraying into the air gave an initial kill of some of the *E. coli*. Slopes of these lines are given in Table 2, in terms of percentage loss of viability among the *E. coli* cells per minute. Table 2 indicates agreement between the 'captive' and true aerosol viability measurements in that both show rapid and rather similar decay under the powerful sterilizing action of the diffuse or direct insolation and that both show the smallest particles dying off more rapidly than the larger ones. Col. 3 suggests that the smallest

particles survived rather better on the threads. Thread and particle were of comparable dimension here, and there may well have been some radiation shielding. Further experiments were made, but are not reported because comparisons were not made simultaneously. There was no suggestion from the latter that the microthread results had any gross systematic difference from the airborne cloud results.

Precautions required by the technique

Air velocity over the threads. An airborne particle falls through air at its terminal velocity, which is, for example, 0·00351 cm./sec. for a 1 μ particle and 0·309 cm./sec. for a 10 μ particle. These velocities are extremely small compared to normal air movements past stationary microthreads. To study the effect of air velocity, an 18-inch radius arm was set up inside an open-topped drum and could be electrically rotated to give a tip speed of 20 miles/hr. (32 km./hr). Frames were mounted in a special holder on the tip of the arm and a similar holder half-way along the arm gave 10 miles/hr (16 km./hr). Further frames could be held very close to the axis in almost stagnant air. Frames were mounted edge-on to the air flow to minimise collection of the room dust by the microthreads.

The result of an experiment in a room containing clean, stable, almost still, air favourable to the survival of the *Escherichia coli* (in single-bacterium particles) is shown in Fig. 7. There is a very clear relation between death rate and air velocity, the death rates at 20, 10 and 1 mile/hr (32, 16, 1·6 km./hr) being approximately 3, 2 and 1 %/min. Figure 8 shows the result of another experiment, which was carried out in an identical fashion to that in Fig. 7 except that the air contained a sterilizing component and was itself in fairly rapid movement above the drum. In these conditions there was little difference in death rate. After the first 30 min. the viability decreased at about 17 %/min. at the 20 miles/hr (32 km./hr) velocity, but the three sets of points (i) at 10 miles/hr (16 km./hr), (ii) in the nearly still air at the centre of the shielding drum, and (iii) from the stationary frames outside the drum, all gave about 10 %/min.

The reason for this difference in velocity dependence in favourable and hostile atmospheres is not understood. The effect has been well established by several experiments replicating those of Figs. 7 and 8. In another series of experiments air was drawn at a steady rate through a tube whose cross-section area decreased in steps. Frames could be exposed at different points down this tube so that all were in the same air but experienced air velocities ranging from 0·2 to 23 miles/hr (0·32–36 km./hr) according to their site of exposure. Comparisons between favourable (filtered) air and hostile (containing sterilizing components) air closely matched those of Figs. 7 and 8 in all respects. This dependence upon air velocity when rates of decay are low is clearly a difficulty with the microthread technique. To overcome or minimize this we are careful to expose microthreads to air which is in the same state of movement as would obtain in the airborne cloud which is being simulated. This was the situation in Figs. 2–6, where satisfactory agreement with the comparison situations was obtained.

Frame-to-frame variations. Our experience is that replicate assays (using a tracer) from a number of identically exposed frames usually show wider scatter in viability determination than replicate impinger samples from an aerosol. Single or duplicate frame assays at single points in time may therefore give less smooth decay curves than is desirable. The simplicity of the technique will usually allow several frames to be assayed per point to obtain greater precision if required. Sampling and assay

procedures must always be carried out in an identical, standardized, manner. Studies with radioactively labelled organisms have shown that the proportions of test and tracer organisms caught on the threads is the same as in the passing aerosol (provided that the particles are similar in size and in surface characteristics) so that assay variations beyond statistical expectations arise between dropping the frames into the sampling fluid and counting the incubated colonies.

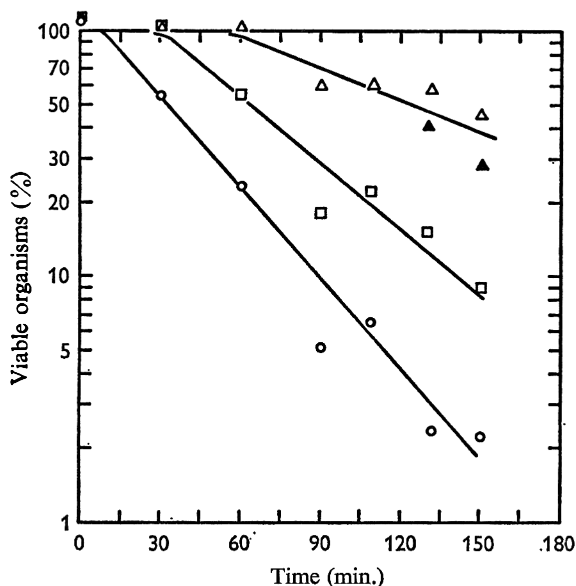

Fig. 7

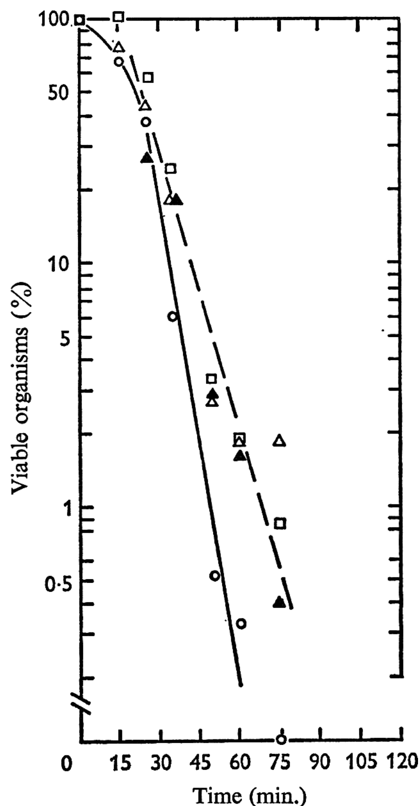

Fig. 8

Fig. 7. Effect of air velocity on survival of *Escherichia coli* 162 single-bacterium particles in whirling-arm experiment in a room containing clean air; ○, 20 miles/hr (32 km./hr); □, 10 miles/hr (16 km./hr); △, ~ 0 miles/hr at axis of arm; ▲ ~ 0 miles/hr on bench in room.

Fig. 8. Effect of velocity of air containing a sterilizing component. Whirling-arm experiment. Symbols as in Fig. 7.

Conclusions

The comparisons described in this paper together with 4 years experience with the microthread technique lead us to conclude that it is a useful tool in the study of air borne microbes, provided that the proper precautions are observed. Of particular value is its versatility, in that there is, within reason, no limit to the length of time, ranges of atmospheric conditions, radiation intensity, etc., in which the airborne state can be simulated. Moreover, organisms such as pathogens can be studied in actual living spaces instead of having to be confined, in the aerosol state, to unrealistic sealed vessels. Extensive studies have been made of the sterilizing effect of various vapours and atmospheres; these will be published later.

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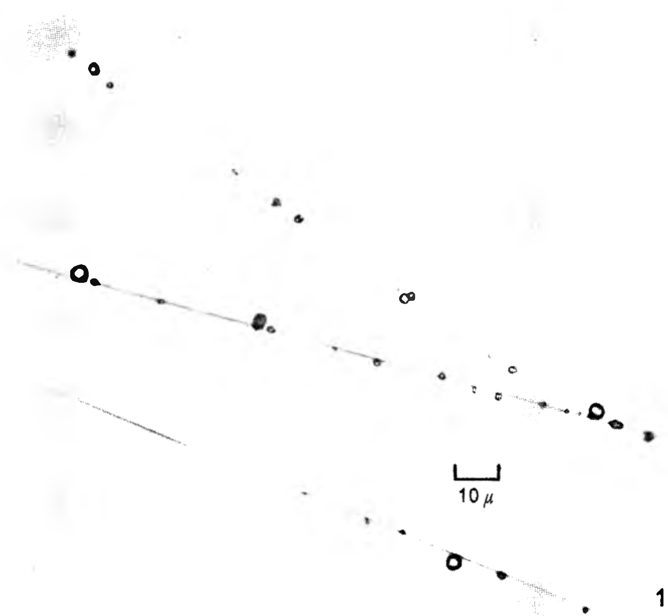
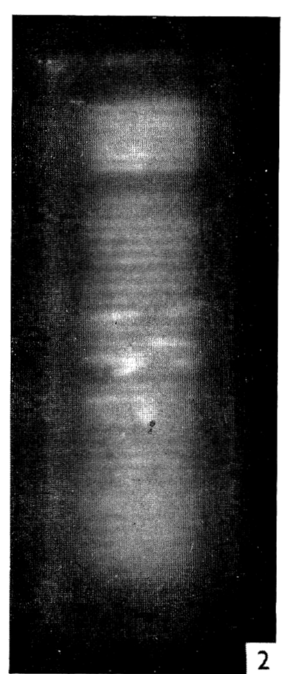
EXPLANATION OF PLATE

Fig. 1. Photomicrograph of dried particles, from a sprayed bacterial suspension, adhering to microthreads.

Fig. 2. Autoradiograph of frame after exposure in a sow to an aerosol of particles containing ^{131}I. Note faint deposition on metal work and heavy deposition on the microthreads.


10 μ
1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Cell Wall Composition of the Mycelial and Blastospore Forms of *Candida albicans*

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SUMMARY

Cell walls were obtained from the mycelial and blastospore forms of *Candida albicans*; these were delipidized and separated into alkali-soluble and alkali-insoluble fractions. The detailed composition of these fractions was determined in organisms grown on different media at 37° and on the same medium at different temperatures (blastospores 30°, mycelium 40°). The composition of the wall of each form was found to be constant, irrespective of growth conditions, except for some variation in the amounts of mannose and glucose in wall hydrolysates. The alkali-insoluble fraction from the mycelial form contained 3 times as much chitin as that from blastospores and only about one third as much protein. The protein from these two fractions showed marked differences in amino acid composition. Differences between the two morphological forms in the amounts of carbohydrate and protein in the alkali-soluble fractions were also found. The results are discussed in relation to other studies of cell-wall composition in the dimorphic fungi.

INTRODUCTION

An increased incidence of candidiasis in certain conditions, e.g. diabetes mellitus and hypoparathyroidism, has long been recognized; more recently it has been recorded as a complication of treatment with corticosteroids and with broad-spectrum antibiotics (for review see Seelig, 1966). *Candida albicans* has been shown to occur as a saprophyte in blastospore form in a high percentage of normal healthy individuals; figures varying from 15–40% have been given (Van Uden, 1960; Dubos & Hirsch, 1965). With tissue invasion, a change to a predominantly mycelial form occurs, and it has been suggested that this determines its pathogenicity (Hill & Gebhardt, 1956; Taschdjian & Kozinn, 1957; Rogers, 1957).

Several factors have been shown to affect the blastospore-mycelium balance in *in vitro* culture. Thus Nickerson (1954, 1963) and Nickerson & Falcone (1956) postulated that the mycelial form develops in cultures generating insufficient H^+ or thiol groups to maintain reduction of cell-wall protein disulphide linkages or in the presence of chelating agents which inactivate a protein disulphide reductase. Widra (1964) also postulated that blastospore formation is dependent upon an adequate supply of reducing groups and has stressed the importance of adequate concentrations of available magnesium. Thus the development of the mycelial form is seen by these workers to be a result of the inhibition of normal cell division. To further understanding of the role of the mycelial form in susceptibility to Candida infection it seems

important to compare the composition and biochemical activities of the two forms. The present paper reports a comparative analysis of the cell-wall material of mycelial and blastospore forms of a single strain of *C. albicans*. In order that the material analysed should be as near as possible to the pathogenic type of growth all analyses were effected on organisms grown from fresh isolates obtained from a child with chronic widespread Candida infection.

METHODS

Organism. A strain of *Candida albicans* was used which was freshly isolated from mouth swabs of an 8-year-old child with a widespread infection of several years' duration, except that a few experiments were made, following the death of the patient, from freeze-dried cultures of initial isolates. Following isolation subculture was effected on to Sabouraud agar slopes and the growth harvested by suspension in sterile water for inoculation into media for large-scale growth.

Media. Media were devised based on those of Nickerson & Mankowski (1953), Roth & Goldstein (1961) and Widra (1964); for blastospore production medium A containing glucose (0·2 %), sodium chloride (0·3 %), Difco yeast extract (0·1 %) and Difco Bactopeptone (5·0 %) with incubation at 37°, and medium B as medium A but substituting Difco Neopeptone for the Bactopeptone and starch for glucose with incubation at 30°. Mycelial phase was grown on medium B with incubation at 40° and on 100 % ox serum (medium C) with incubation at 37°. Ox blood was collected aseptically, allowed to clot overnight and the serum used after centrifugation.

All media were dispensed in a volume of 500 ml. in 1 l. flasks, inoculated heavily with a blastospore suspension and incubated for 18 hr in a New Brunswick Environmental Incubator Shaker (100 rev./min.).

Preparation of cell-wall fractions. Organisms were centrifuged down and washed 3 times by suspension in 0·05 M-tris (pH 6·8). Mycelial growth on the starch–Neopeptone medium at 40° was accompanied by some production of blastospores; these were removed by homogenizing a suspension of organisms in the tris buffer in an MSE glass homogenizer, followed by repeated filtration through a sintered-glass disc. After about 10 such filtrations the mycelial residue was free from blastospores. A heavy suspension of washed mycelium or blastospores in the tris buffer was put through a French press at 20,000 lb./in.^{2} pressure. The product was examined by light microscopy for the extent of cell breakage and, when necessary, the process was repeated until at least 90 % cell breakage had been achieved. The final suspension was centrifuged at 1400 *g* for 10 min. and then successively washed 3 times each at 0° with M-sucrose, 0·1 N-sodium chloride and distilled water, increasing the time and speed of centrifugation to a maximum of 2000 *g* for 20 min. This procedure gave clean wall material as judged by light microscopy and the absence of staining with methylene blue. The final product was freeze-dried and stored at −20°.

Lipid readily extractable was separated from the dried wall preparation (400 mg.) by shaking for 1 hr at 4° 3 times with 40 ml. lots of chloroform + methanol mixture (1 + 1, v/v). Chemically bound lipid was extracted by the method of Kessler & Nickerson (1959).

Experience showed that material reacting in the orcinol test for pentose could be extracted with tris buffer from the de-lipidized wall and thus all preparations were

extracted for 2·5 hr with 0·2 M-tris buffer (pH 7·8; 5 mg. de-lipidized wall/3 ml. buffer), conditions which were found to effect complete removal of this material which was probably due to traces of sucrose from the washing procedure. The wall material was then separated by the procedure of Kessler & Nickerson (1959) into an alkaline-insoluble fraction and alkali-soluble fractions 1 and 2.

Acid hydrolysis of cell-wall fractions. All tubes containing solutions for hydrolysis were evacuated to remove dissolved air and then sealed under nitrogen. All acid hydrolyses for determination of sugar components were effected in duplicate on 10 mg. samples together with a third hydrolysate containing a 5 mg. sample + 5 mg. of component to be estimated. All results were corrected for recovery of added sugar, since losses during hydrolysis were found to be considerable and variable. Acid hydrolysis conditions were as follows. (*a*) *Amino sugars*: 4 N-HCl (5 ml.) heated at 110° for 16 hr; HCl was removed in vacuum in a rotary evaporator, the residue dissolved in water, passed through Norit NK (Hopkin and Williams Ltd) on a sintered glass disc, the Norit washed with water, the combined filtrates adjusted to pH 7·0 and made to 25 ml. with water. (*b*) *Total reducing sugars* by the method of Johnston (1965). (*c*) *Amino acids*: 6N-HCl (2 ml.) heated with wall fraction (10 mg.) for 18 hr at 105° and the hydrolysate treated as for amino sugars. (*d*) *Total phosphorus*: wall material (5 mg.) digested for 60 min. with 10N-H_2SO_4 (0·4 ml.) at 130°, cooled and 30% H_2O_2 (0·1 ml.) added and then heated at 130° for 10 min. After cooling, distilled water (1·0 ml.) was added and after heating at 100° for 30 min. the orthophosphate formed was estimated by the method of Fiske & SubbaRow (1925).

Enzymic digestion of wall material. β-Glucanase was separated from a crude preparation of snail digestive juice (suc digestif d'*Helix pomatia* obtained from Industrie Biologique Française, Gennevilliers, Seine, France) by the method of Anderson & Millbank (1966). Fraction A was used and shown to be devoid of chitinase activity. Pronase and chitinase were obtained from Calbiochem Ltd. Alkali-insoluble wall fraction (100 mg.) was suspended in McIlvaine citrate phosphate buffer (pH 5·8; 50 ml.) and incubated with the β-glucanase (25·0 mg.) at 30° for 8 hr. The washed residue was suspended in 0·2 M-tris (pH 7·1; 50 ml.) and incubated with pronase (25 mg.) at 25° for 72 hr. A sample (5·0 mg.) of the residue was suspended in 0·05 M-phosphate buffer (pH 6·3, 5 ml.) and incubated with chitinase (10 mg.) at 36° for 22 hr. All incubation mixtures were shaken.

Analytical methods. Total reducing sugars were estimated by the method of Nelson (1944), glucose according to Marks (1959) except that 4·0 ml. of the 'working' solution and 0·4 ml. of the glucose-containing sample were used. The reaction was stopped after 12 min. by the addition of 8 N-HCl (0·25 ml.), giving a yellow colour which was read at 440 mμ (Dr M. Green, personal communication). Mannose was calculated from the difference between the values from the above two methods, also making allowance for hexosamine when present. Hexosamine was estimated according to Tracey (1955), total-N by the micro Kjeldahl method and α-NH_2 nitrogen according to Moore & Stein (1954). RNA was estimated by digestion of wall material (5 mg.) with RNAse (1·5 mg.) in 0·2 M-tris (pH 7·8; 3 ml.) at room temperature for 40 min., followed by addition of 5% TCA (7 ml.) and heating at 90° for 15 min. After centrifugation, ribose was estimated on the supernatant solution by the method of Mejbaum (1939). Amino acids were determined in a single-column spherical-bead apparatus with Amberlite CG 110 resin and elution with citrate buffers at pH 3·5, 4·6 and 6·5.

Chromatography. Chromatography of sugars was effected on Whatman no. 1 paper with the following solvents: (*a*) *n*-propanol+acetic acid+water (7+1+2, by vol.); (*b*) *iso*-propanol+*n*-butanol+water (7+1+2, by vol.). Detection was with the alkaline silver nitrate reagent of Trevelyan, Procter & Harrison (1950). Ninhydrin-reacting materials were separated on Whatman no. 4 paper on 2-way chromatograms with the following solvents: (*a*) *n*-butanol+acetic acid+water (4+1+5, by vol.); (*b*) the phenol+ammonia mixture of Smith (1960).

RESULTS

Two factors which affect the yeast/mycelial morphology of dimorphic fungi are temperature of growth and medium composition (for review see Mariat, 1964). Both these factors also affect the morphology of *Candida albicans* and therefore the cell-wall composition of the two forms was studied when produced (*a*) under constant incubation temperature (37°) with different media (media A and C) and (*b*) with a common medium (medium B) but different incubation temperatures, 30° for blastospores and 40° for mycelium. Growth at the latter temperature gave a greater proportion of mycelium than at 37°.

Table 1. *Lipid fractions extracted from cell walls of blastospore and mycelial phases of Candida albicans as percentage of lyophilized whole wall*

	Blastospore growth medium		Mycelial growth medium	
Lipid fraction	A, 37°	B, 30°	B, 40°	C, 37°
Chloroform+methanol extract	0·55	3·32	1·9	5·5
Acidified ethanol+ether extract	—	7·3	3·14	—

Composition of cell wall from yeast and mycelial phases

Lipid. Typical results for the content of lipid extracted by chloroform+methanol from freeze-dried wall fractions and subsequent extraction with acidified ethanol+ether are shown in Table 1. The former lipid is seen to be variable in amount, being affected by medium composition and temperature. The tenfold increase in this fraction when the organism was grown in serum at 37° as compared with medium A at the same temperature may be a reflexion of the fact that the serum medium was the only medium with a significant lipid content. This result may be of interest, since *in vivo* growth is in the presence of such materials. Chemically bound lipid was only extracted from wall material grown on medium B; the amount found in blastospores grown at 30° was approximately twice that of mycelium grown at 40°. Since the reagents used in the extraction of this fraction may affect linkages between wall components (Phaff, 1963), this extract has been omitted from the described fractionation of wall material.

Fractionation of chloroform–methanol extracted wall. The percentage distribution between the alkali-soluble and alkali-insoluble fractions and the recovery of material during fractionation is shown in Table 2. The composition of these fractions as obtained from blastospores and mycelium is shown in Table 3 for growth on different media at 37° and in Table 4 for growth on medium B at different temperatures. Chromatographic study showed glucose and mannose to be present in the hydrolysates of all fractions; glucosamine only in those indicated in Tables 3 and 4, and

Table 2. *Alkali fractionation of cell wall of blastospore and mycelial phases of Candida albicans*

	Blastospore growth medium		Mycelium growth medium	
	A, 37°	B, 30°	B, 40°	C, 37°
	Recovery as % of lyophilized wall taken			
	87	100	92·5	99
	Composition of fractions as % of total wall recovered			
Alkali-insoluble	63·2	52	61·5	55·7
Alkali-soluble	36·8	48	38·5	44·5
Alkali-soluble 1	16·1	18·5	7·0	14·5
Alkali-soluble 2	20·7	29·5	31·5	31·0

Table 3. *Percentage composition of cell-wall fractions of Candida albicans grown in different media at 37°*

Blastospores were grown on medium A; mycelium was grown on medium C

	Cell-wall Fraction					
	Alkali-insoluble		Alkali-soluble 1		Alkali-soluble 2	
Component	Blastospores	Mycelium	Blastospores	Mycelium	Blastospores	Mycelium
Total reducing sugar	59·0	61·8	8·6	15·6	29·3	14·4
Glucosamine	2·7	9·9	Nil	0·9	Nil	2·6
Glucose	45·0	36·0	4·2	13·3	1·9	3·8
Mannose	14·0	25·8	4·4	2·3	27·4	10·6
Total-N	6·0	2·2	9·2	8·7	2·2	3·8
Protein*	36·8	9·0	57·5	53·8	13·8	22·5
Phosphorus	2·2	1·3	2·6	1·8	2·6	1·4
RNA	1·5	0·7	—	—	—	0·38

* Total N × 6·25 after allowing for glucosamine-N.

Table 4. *Percentage composition of cell-wall fractions of Candida albicans grown on medium B at different temperatures*

Blastospores were grown at 30°, mycelium at 40°

	Cell-wall fraction					
	Alkali-insoluble		Alkali-soluble 1		Alkali-soluble 2	
	Blastospores	Mycelium	Blastospores	Mycelium	Blastospores	Mycelium
Total reducing sugar	63·6	64·3	10·0	21·3	26·7	20·3
Glucosamine	2·9	10·6	Nil	Nil	Nil	Nil
Glucose	47·0	45·3	3·3	12·3	1·0	5·3
Mannose	16·6	19·0	6·7	9·0	25·7	15·0
Total N	3·8	2·2	9·6	7·3	1·5	3·6
Protein*	22·5	8·8	60·0	45·6	9·4	22·5
Phosphorus	0·6	0·6	0·5	1·4	0·6	0·7
RNA	0·54	0·17	—	—	0·53	0·35

* Total N × 6·25 after allowing for glucosamine-N.

no other sugars were demonstrated. Total phosphorus values were consistently higher in wall fractions grown on media A and C than in those grown on medium B. This difference was not simply due to a higher medium concentration of phosphorus, since medium A contained 19·1 mg. P/100 ml. and medium B 14·5 mg. P/100 ml., while ox serum has a value of about 3–8 mg. P/100 ml.

Composition of alkali-insoluble fraction

The composition of this fraction from blastospore walls was virtually constant under the two sets of growth conditions; that of mycelium was also constant except for a change in the glucose:mannose ratio from 1·4:1 on medium C to 2·4:1 on medium B. The most striking differences between the two forms was in the glucosamine content of the hydrolysates, which in the mycelial fraction was several times greater than that from the blastospores, irrespective of growth conditions, and the appreciably higher level of protein in the latter material.

Nature of amino sugar binding. Chitin has been shown to be present in the cell wall of a number of yeast species, including *Candida albicans*, on the basis of chemical tests or X-ray diffraction studies (Roelofsen & Hoette, 1951; Houwink, Kreger & Roelofsen, 1951; Bishop, Blank & Gardner, 1960) and its presence in blastospores of *Candida albicans* inferred from glucosamine estimations by Kessler & Nickerson (1959). Korn & Northcote (1960) and Eddy (1958) showed that only a small proportion of the glucosamine present in baker's yeast cell-wall hydrolysates was derived from chitin. The form in which glucosamine was present in the alkali-insoluble fractions has been examined by extraction with 2% HCl and 3% NaOH as described by Korn & Northcote (1960) and by X-ray diffraction studies of the fractions as initially prepared and after digestion with β-glucanase and pronase. The extraction procedure showed that 96% of the glucosamine of both mycelial and blastospore fractions was resistant to extraction and thus had the solubility properties of chitin.

The results of enzymic digestion of the fractions is shown in Table 5. The fact that 75% of the glucan in the blastospore fraction was broken down and only 44% of that in the mycelial fraction may indicate a difference in wall structure between the two forms. Pronase digested 55% of the protein of the blastospore and 35% of the mycelial fraction. The final residue contained from 90 to 95% of the glucosamine of the original material. Chitinase treatment of this residue released as N-acetyl glucosamine 62% of the hexosamine from the mycelial and 43% from the blastospore fraction. X-ray diffraction studies of the original fractions and the residue after glucanase and pronase treatment showed the presence of chitin in material from both forms. These results indicate that the glucosamine of the alkali-insoluble fraction from both growth forms is present almost entirely as chitin, the level in the mycelial form being significantly greater than that of the blastospores.

Protein. The amino acid composition of acid hydrolysates of alkali-insoluble wall fractions from both growth forms is shown in Table 6. The most striking difference between the two materials is the occurrence of histidine as the principal basic amino acid in the blastospore fraction and its absence from the mycelial fraction, with arginine showing the opposite distribution. In addition, glutamic acid, proline, valine, methionine and lysine show major differences in their distribution between the two forms, and minor components, mainly unidentified, also show differences. Glucosamine appeared to be present in appreciably larger amounts in the mycelial fraction,

but the interpretation is difficult owing to incomplete separation from phenylalanine. These results indicate that the protein in this fraction is different in the two growth forms, although the insolubility of the material has made it impossible to determine whether a single protein or a mixture is present.

Table 5. *Digestion of the alkali-insoluble fraction of Candida albicans cell wall with β-glucanase, pronase and chitinase*

The wall fractions were treated successively with β-glucanase, pronase and chitinase under conditions described in the text. Values of components estimated are given as % of original fraction taken. The α-amino-N values have been corrected for glucosamine content.

	Material released by:					
	β-glucanase		Pronase		Chitinase: N-acetyl glucosamine	Acid hydrolysis of chitinase substrate: Glucosamine
Morphological form of culture	Glucose	Glucosamine	α-Amino-N	Glucosamine		
Mycelium	18·8	0·12	0·6	0·05	6·7	10·8
Blastospore	39·2	0·4	1·74	0·16	1·8	4·26

Table 6. *Amino-acid composition of acid hydrolysates of the alkali-insoluble fraction of cell walls of the blastospore and mycelial forms of Candida albicans*

Values are the mean of two determinations and are expressed as μmole amino acid/1000 μmole. The material analysed was obtained from growth on medium B under conditions as described in the text.

Amino acid	Mycelium	Blastospores	Amino acid	Mycelium	Blastospores
3-hydroxy proline*	2·2	—	Leucine	75·1	63·3
4-hydroxy proline*	29·0	—	Tyrosine	36·1	50·9
Aspartic acid	65·4	65·3	Glucosamine + phenylalanine†	140·5	47·1
Threonine	74·3	61·0	O‡	42·6	—
Serine	56·5	55·5	P‡	—	4·3
Glutamic acid	90·7	65·5	S‡	—	15·0
Proline	36·9	63·1	U‡	—	8·8
Glycine	73·6	69·3	Lysine	7·7	25·8
Alanine	107·0	88·8	Histidine	—	112·2
Valine	93·8	50·6	Arginine	53·4	—
Methionine	21·6	79·3	Recovery	89·9%	98·4%
Isoleucine	58·7	53·2			

* Identified only by absorption maximum at 440 mμ.
† Complete separation of glucosamine and phenylalanine was not achieved.
‡ Unidentified components.

Composition of alkali-soluble fractions

Fraction 1 (Tables 3 and 4). This fraction from both growth forms showed a high protein content. The composition of blastospores was constant irrespective of growth conditions; that of mycelium was also constant except for variation in the glucose: mannose ratio from 5·8:1 on medium C to 1·4:1 on medium B. The principal difference shown between the two forms was in the concentration of glucose, this being approximately 3 times greater in the mycelial form. Glucosamine was absent from this fraction in both forms.

Fraction 2 (Tables 3 and 4). The material from blastospores was of constant composition irrespective of growth conditions; that from mycelium was also relatively constant

except for variation in the mannose values, this being lower on medium C. Comparison of the two forms showed a considerably higher proportion of mannose in the blastospore fraction and of protein in the mycelial fraction. Glucosamine was absent or at low value in both forms. The most notable feature of these analyses is the low recovery (i.e. some 40 %) of material as sugar, amino acid derivatives and RNA.

DISCUSSION

The composition of the cell wall of blastospores of *Candida albicans* as revealed by analysis of hydrolysates of wall fractions was constant when grown on different media and at different temperatures. The cell-wall composition of the mycelial form showed a similar constancy, except for variation in the values for glucose and mannose units present, according to the growth conditions used. Comparison of the composition of the cell walls of the two growth forms showed a qualitative similarity but considerable quantitative variations, particularly in terms of glucosamine, glucose, mannose and protein content. Such qualitative similarity, accompanied by quantitative variations in wall composition of dimorphic forms, is also seen in *Mucor rouxii* (Bartnicki-Garcia & Nickerson, 1962) and *Histoplasma capsulatum* (Kobayashi & Guilacci, 1967). This suggests that the blastospore ↔ mycelium change is directed by variation in the activity of enzymes which produce wall components rather than in the elaboration of new components. Comparative study of metabolic activities in the two forms of *C. albicans* is the subject of further work.

The presence of a higher value for chitin in the mycelial form of *Candida albicans* suggests a similarity in wall structure to many filamentous organisms. A higher value for chitin in the mycelial form of growth has not previously been recorded in the dimorphic fungi. Bartnicki-Garcia & Nickerson (1962) found similar amounts in the two forms of *Mucor rouxii*, and whilst Kobayashi & Guiliacci (1967) demonstrated a greater hexosamine content in the yeast form of *Histoplasma capsulatum*, they did not show the presence of chitin in this organism. This difference in chitin values between the two growth forms of *C. albicans*, together with the differences in susceptibility to digestion by β-glucanase and in the protein composition of the alkali-insoluble wall fraction, indicate that considerable differences are likely to be present in the tertiary structure of the polysaccharide–protein complexes of the cell wall. These may result in differences in permeability, with changes in resistance to host defence mechanisms, and may be in part responsible for the greater persistence of the mycelial form *in vivo*. The differences in amino acid composition of the proteins analysed are striking, in particular the presence of histidine, but no arginine in the blastospore fraction and the reversal of this distribution in the mycelial fraction. Kessler & Nickerson (1959) reported a predominance of acidic amino acids in a corresponding wall fraction from baker's yeast and suggested that the acidic groups may be of importance in the cell-wall structure through ester linkages between polysaccharide and amino acid residues. No such preponderance of acidic amino acids was found in the *C. albicans* fractions studied here.

The only other recorded comparative analysis of the two forms of this organism is that by Kessler & Nickerson (1959) in which the wall material from blastospores and a divisionless mutant derived from the same strain were studied. Both forms were grown on the same medium and at the same temperature (28°) for 48 hr. The analyses

showed that the wall fractions had virtually the same composition, irrespective of the growth form. The mycelium produced by this mutant was non-septate 'pseudo'-mycelium, whereas that produced by the organism and under the growth conditions used here was normal septate mycelium; this difference and the different environmental conditions required to induce mycelium production may account for the differences in composition between the two sorts of mycelium. Alternatively, the cell wall of a freshly isolated pathogenic strain of the organism may differ markedly from that of a laboratory-developed mutant strain. Differences are also apparent between the composition of the blastospore wall described here and that recorded by Kessler & Nickerson. The principal differences are the presence of markedly higher protein contents in all our fractions and of appreciable quantities of mannose in the alkali-resistant fraction. Growth temperature did not affect blastospore wall composition and is unlikely to explain these differences. The inclusion by Kessler & Nickerson of the extraction with acidified ethanol+ether may have affected the stability of the macromolecular complexes of the wall, otherwise the different growth media used or strain differences are the most likely cause of these differences. Nickerson (1963) suggested that the development of the mycelial form results from impaired cell division arising from lack of intracellular sulphhydryl groups which normally result from protein disulphide reductase activity. Where this is decreased or lacking the elasticity of the mannan-protein complex is considered to allow extension of the cell without division. The results recorded above indicate that a more complex mechanism may be involved in the blastospore ↔ mycelium change, since recorded differences between the two forms indicate a requirement for increased chitin synthesis and changes in the type of protein produced in the development of mycelium.

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Moraxella kingii sp.nov., a Haemolytic, Saccharolytic Species of the Genus *Moraxella*

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SUMMARY

A new Moraxella species, *Moraxella kingii*, is described. It is characterized by β-haemolysis, acid production from glucose and maltose, and lack of catalase activity. The strain 4177/66, isolated from a nose swab, is designated as the type strain.

INTRODUCTION

In 1960 the late Miss Elizabeth O. King of Atlanta, Georgia, U.S.A., sent a few strains to one of us with a request for an opinion as to whether these were strains of the genus *Moraxella*, and, if so, whether they belonged to any well-defined species. Two of the strains were β-haemolytic and saccharolytic. Morphologically and in growth characteristics they showed considerable likeness to Moraxella species, but the conclusion was that they represented a new species, and that *Moraxella* appeared to be the most suitable genus to allocate it to. Later we obtained Miss King's permission to include these strains in studies on the transformation reactions of Moraxella and related organisms which were then in progress, but it was our understanding that Miss King would describe this new species as soon as she found time to do so. Unfortunately this was prevented by her death. When we isolated an additional strain of this species from a nose culture in 1966, we felt that publication of a description of the new species was desirable. Inquiries to the Communicable Disease Center in Atlanta revealed that there was no objection to the inclusion of Miss King's strains in our study.

METHODS

Organisms. One of the strains (4177/66) was isolated in Oslo in 1966. Two other strains of the material had previously been received from the late Miss Elizabeth O. King, and 6 strains, also from her collection, were received from Dr R. E. Weaver (Atlanta). The strains were designated as follows (with source in parentheses): 2941 (blood), 5530 (blood), 5963 (1) (blood), 8623 (blood), 9571 (throat), A 1702 (knee joint), A 2471 (bone lesion), A 6042 (throat) and 4177/66 (nose).

Morphological, cultural and biochemical tests. With some modifications these were identical with the procedures previously described for the examination of *Moraxella phenylpyrouvica* (Bøvre & Henriksen, 1967*b*). Haemolysis was studied on plates with ox, sheep, horse and rabbit blood in addition to the commonly used human blood agar medium. Tests for growth in citrate were made only in Koser's fluid medium.

Examination for acid production from carbohydrates was extended to the use of ascites agar slants with 1 % glucose, maltose, fructose, galactose, lactose, saccharose, arabinose, xylose, rhamnose, mannitol, dulcitol, sorbitol and glycerol, respectively.

DNA base determinations. Guanine + cytosine (G + C) contents of DNA were determined by Dr W. Szybalski (Professor of Oncology, University of Wisconsin) with the CsCl buoyant density method on extracts prepared in the authors' laboratory.

Transformation methods. A quantitative procedure with short-term DNA exposure and a more sensitive, but less accurate approach, with continuous DNA exposure, were employed. The principles of mutant selection, transformation techniques, transformant assay and control measures, have been extensively described previously (Bøvre, 1964, 1967*a*, *c*). The genetic marker used was exclusively one-step mutation to high streptomycin resistance.

RESULTS

Morphological, cultural and biochemical characteristics

Microscopical examination revealed coccoid to medium long diplo-bacteria with square ends, in pairs or chains. The bacteria were generally of rather small diameter as compared with previously studied Moraxella species. Pleomorphism was not pronounced in any of the strains. Microphotographs of two of the strains are given in Plate 1, fig. 1, 2. The organisms were Gram-negative, but in many instances some resistance to decolorization was observed. There were no distinct capsules. Motility was not observed.

All strains grew with small colonies, reaching diameters in the range 0·1–0·6 mm. in culture on human blood agar plates after 20 hr of incubation at 33° and 37°. Single colonies of some strains increased to 1–2 mm. in size on prolonged incubation. The colonies were generally of the low hemispherical type. In some instances there was a tendency towards colony growth down into the medium, with resulting pitting of the surface. In these cases the colonies were flat, extending very little from the surface of the surrounding medium, and the growth mass appeared adherent to the medium. The colonies were non-pigmented, translucent or slightly opaque, with a smooth and sometimes mucoid appearance. Their consistency varied from soft, giving easy emulsification in physiological saline (strain 5530), to coherent, like gonococci, with resulting spontaneous agglutination (the other 8 strains). The latter phenomenon was only temporary, since complete emulsification occurred after a while in saline.

In all strains a distinct, but most often very narrow zone of β-haemolysis was observed around colonies on human blood agar. The haemolysis was more pronounced on plates with ox, sheep, horse or rabbit blood.

There was no anaerobic growth. In semifluid nutrient agar stab cultures growth could be observed down to about 5 mm. below the surface. Very slight growth was observable with some of the strains at room temperature. There was no difference in growth capacity at 33° and 37°. Most strains survived exposure to 45° for 10 min., but not for 30 min. None of the strains resisted heating to 49° for 10 min.

Growth in peptone media such as the indole and nitrate media used was absent or very scarce, as it was on Triple Sugar Iron Agar (Difco) and in the MR–VP medium. Only very little improvement of growth was observed when 10 % (v/v) horse serum was added to fluid peptone media or when heated blood was substituted for ordinary blood in agar plates. This phenomenon seems to distinguish our strains from other

Table 1. *Some cultural and biochemical characteristics of Moraxella kingii, as compared with other known species of the genus Moraxella*

Species	Catalase production	Growth on Hugh and Leifson's medium	Growth in Koser's citrate medium	Nitrite production from nitrate	Urease activity	Oxidative deamination of phenyl-alanine and tryptophan	Serum liquefaction	Beta-haemolysis	Acid production from glucose
*M. lacunata**	+	− or +	−	+	−	−	+	−	−
M. nonliquefaciens	+	−	−	+	−	−	−	−	−
M. bovis	+	+	−	−	−	−	+	+	−
M. osloensis	+	+	+†	+ or −	−	−	−	−	−
M. phenylpyrouvica	+	+	− or (+)	+, (+) or −	+ or −	+	−	−	−
M. kingii	−	−	−	− or (+)	−	−	−	+	+‡

+ = positive reaction, (+) = doubtful reaction, − = negative reaction. † Weakly positive. ‡ Acid also from maltose.
* Including *M. liquefaciens*, as proposed by Henriksen & Bøvre (1968).

fastidious moraxellas, according to our experience. There was no satellite phenomenon with staphylococci. All strains were able to grow almost as well on high-quality nutrient agar as on the same medium with blood added.

The strains all had a remarkable inability to survive on blood agar plates at room temperature; cultures always became sterile in 6–12 days.

The catalase reaction was uniformly negative. This is in accordance with notes made by Miss Elizabeth O. King, placed at our disposal. The negative catalase reaction distinguishes our strains from all other known moraxellas, which have been extensively examined for this character in our laboratory (unpublished results). With the tetramethyl-*p*-phenylenediamine reagent all strains were strongly oxidase positive. With the dimethyl compound the reaction was also distinct with all strains, but perhaps weaker than with other known Moraxella species.

Strong acid production in 1 day was observed from glucose and maltose on ascites agar media. There was no apparent attack on the other carbohydrates and alcohols tested, except for very weak acid reactions in the galactose medium. No growth and no glucose attack was observed on Hugh and Leifson's medium. In 1 % peptone liquid media with glucose growth was either absent or very weak, and only very late and irregular acid reactions were seen under these conditions.

No hydrogen sulphide or indole production was detected. Some results of cultural and biochemical tests are presented in Table 1, together with a comparison with other moraxellas recently studied in this laboratory. The results of antibiotic sensitivity tests are listed in Table 2.

DNA base composition

The guanine+cytosine (G+C) content of all DNAs examined (from strains 2941, 5530, A1702, 4177/66) was 44·5 moles %.

Table 2. *Sensitivity to antibiotics of the 9 strains of Moraxella kingii*

Antibiotic	Range of inhibition zones diam. (mm.)	Approximate range of minimum inhibitory concentrations (m.i.c.)*
Penicillin	37–54	0·02–< 0·005
Streptomycin	27–34	0·2–< 0·01
Chloramphenicol	36–45	0·1–< 0·03
Oxytetracycline	25–30	0·4–0·08
Eythromycin	31–45	0·3–< 0·008

* Method of Ericsson, Høgman & Wickman (1954). Minimal inhibitory concentration (m.i.c.) values calculated from zone diameters by means of regression equations for each antibiotic (Ericsson, 1960). M.i.c. given as i.u./ml. for penicillin, as μg./ml. for the other antibiotics.

Transformation reactions

The strain 4177/66 was simultaneously exposed to marker DNAs of the same strain and of the strains 2941, 5530 and A1702. There was a uniform distribution of interstrain transformation frequencies close to the frequency of intrastrain (autologous) transformation, as presented in Table 3. The resulting ratios of inter- to intra-strain transformation can be seen to range from $4{\cdot}6\times 10^{-1}$ to $9{\cdot}2\times 10^{-1}$. Values of this order are consistent with a close taxonomic relationship and similar to those previously found between strains of one and the same species (see Bøvre, 1967*b*).

In Table 4 are presented results of five quantitative transformation experiments designed to elucidate the transformation compatibility of *Moraxella kingii* with other Moraxella species. No such compatibility was observed. Depending on the sensitivity of the individual experiments (i.e. the transformability or state of competence of the

Table 3. *Quantitative streptomycin resistance transformation between strains of Moraxella kingii. Recipient strain 4177/66. Recipient count 7·6 × 10⁷/ml.*

Duration of DNA exposure 15 min. Donor mutants selected at 500 μg. streptomycin/ml.; transformants selected at 50 μg./ml.

Donor strain	Transformants per ml.	Ratio of inter- to intrastrain transformation
2941	$2·9 \times 10^5$	$9·2 \times 10^{-1}$
5530	$1·5 \times 10^5$	$4·6 \times 10^{-1}$
A1702	$2·2 \times 10^5$	$6·9 \times 10^{-1}$
4177/66	$3·2 \times 10^5$	—

Table 4. *Quantitative streptomycin resistance transformation in Moraxella, with Moraxella kingii as donor or recipient in interspecies experiments*

Recipient strain (recipient count/ml. in parentheses)	Donor strain	Transformants per ml.	Ratio of inter- to intrastrain transformation
*M. nonliq** 7784	*M. kingii* 5530, A1702, 4177/66	< 10	$< 1·4 \times 10^{-5}$
($4·2 \times 10^8$)	*M. nonliq.* 7784	$7·0 \times 10^5$	
M. osloensis 5873	*M. kingii* 2941, 5530, A1702, 4177/66	< 10	$< 8·3 \times 10^{-6}$
($9·0 \times 10^8$)	*M. osloensis* 5873	$1·2 \times 10^6$	
M. bovis ATCC 10900	*M. kingii* 2941, 5530, 4177/66	< 10	$< 2·3 \times 10^{-4}$
($8·0 \times 10^7$)	*M. bovis* ATCC 10900	$4·4 \times 10^4$	
M. lacunata ATCC 11748	*M. kingii* 4177/66	< 10	$< 3·7 \times 10^{-3}$
($3·2 \times 10^7$)	*M. lacunata* ATCC 11748	$2·7 \times 10^3$	
M. kingii 4177/66	*M. bovis* ATCC 10900	< 10	$< 1·0 \times 10^{-3}$
($6·0 \times 10^7$)	*M. phen.*† A390	< 10	$< 1·0 \times 10^{-3}$
	M. kingii 4177/66	$1·0 \times 10^4$	

Duration of DNA exposure 15 min. Donor mutants selected at 500 μg. of streptomycin per ml., transformants selected at 50 μg./ml.

* *nonliq.* = *nonliquefaciens*; † *phen.* = *phenylpyrouvica.*

recipients in question) ratios of inter- to intra-strain transformation between *M. kingii* and the other species were below $1·4 \times 10^{-5}$ for *M. liquefaciens*, below $8·3 \times 10^{-6}$ for *M. osloensis*, below $2·3 \times 10^{-4}$ for *M. bovis*, below $3·7 \times 10^{-3}$ for *M. lacunata* and below $1·0 \times 10^{-3}$ for *M. phenylpyrouvica*. In all the experiments of Table 4, except for that with *M. lacunata* as recipient, the occurrence of transformants was also studied in continuous DNA exposure (without termination of DNA activity by DNase), which was performed in parallel. These experiments (not tabulated) were also negative, indicating that the transformation compatibility between *M. kingii* and the other species was considerably lower than the limits for these interactions listed above. Previous experiments with other organisms have shown that continuous DNA exposure was usually more than 100 times more sensitive for the detection of low transformation compatibility, as compared with the 15 min. DNA exposure technique (Bøvre, 1965).

In Table 5 are listed a few of the results obtained when highly competent Moraxella and Neisseria recipients were exposed continuously to DNAs of streptomycin-

resistant *Moraxella kingii* on the one hand, and simultaneously to other distantly related donors on the other. It can be seen that when the conditions of transformation permitted the *M. phenylpyrouvica* 2863 donor to give 246 transformants in the *M. osloensis* 5873 recipient, several donors of *M. kingii* were still completely unable to elicit such transformants. Since the compatibility between these strains of *M. phenylpyrouvica* and *M. osloensis* is known to be of the order 6×10^{-6}, as expressed by ratio of inter- to intra-strain transformation (Bøvre & Henriksen, 1967*b*), ratios of transformation between *M. kingii* and *M. osloensis* would be lower than 3×10^{-8}. Whereas *M. osloensis* shows some very distant relations to all other moraxellas (Bøvre, 1967*a*; Bøvre & Henriksen, 1967*a*, *b*), there was no indication of any transformation compatibility between the former species and *M. kingii.*

Table 5. *Semiquantitative comparison of low transformation frequencies (without termination of DNA exposure by DNase), with streptomycin resistance as the genetic marker*

Recipient strain	Donor strain	Transformants per plate	Approximate ratio of inter- to intrastrain transformation
Moraxella osloensis 5873	*M. phen.** 2863	246	6×10^{-6}‡
	M. kingii 2941, 5530, A 1702, 4177/66	0	$< 3 \times 10^{-8}$
Neisseria catarrhalis NE 11	*M. nonliq.*† 7784	3500	3×10^{-5}‡
	M. osloensis 5873	276	2×10^{-6}‡
	M. phen. 2863	613	5×10^{-6}‡
	M. kingii A 1702	0	$< 9 \times 10^{-9}$
Neisseria ovis 199/55	*M. osloensis* 5873	1300	3×10^{-5}‡
	M. phen. 2863	561	1×10^{-5}‡
	M. kingii A 1702	0	$< 3 \times 10^{-8}$

Donor mutants selected at 500 μg. of streptomycin per ml., transformants selected at 50 μg. per ml. For methodology, see Bøvre (1967*a*).

* *phen.* = *phenylpyrouvica*; † *nonliq.* = *nonliquefaciens*.

‡ Previously estimated (Bøvre, 1967*a*; Bøvre & Henriksen, 1967*b*).

Similar comparisons of low transformation frequencies in continuous DNA exposure were performed with *Neisseria catarrhalis* and *N. ovis* recipients (Table 5). These experiments revealed no transformants with *Moraxella kingii* donors under conditions where *M. nonliquefaciens*, *M. osloensis* and *M. phenylpyrouvica* donors elicited hundreds or thousands of transformants in the same recipients. The activities of the latter three donors had been estimated previously (Bøvre, 1967*a*; Bøvre & Henriksen, 1967*b*), and by means of these values the ratio of inter- to intra-strain transformation between *M. kingii* and *N. catarrhalis* would be situated below 9×10^{-9}, and between *M. kingii* and *N. ovis* the ratio would be below 3×10^{-8}. So far there is no indication of any transformation compatibility between *M. kingii* and *N. catarrhalis* or *N. ovis*, in contrast to the distinct, but often very distant relations in these terms previously found between all other moraxellas and these two Neisseria species.

Continuous DNA exposure experiments were also performed to detect compatibility between *Moraxella kingii* and *Neisseria flavescens* or *N. meningitidis*, with negative results. However, the recipients of these experiments were not of particularly high competence, so that a final decision cannot be made as regards absence of affinity between *M. kingii* and the latter Neisseria species.

DISCUSSION

The results indicate that the strains included in this study are a homogeneous group which is sufficiently distinct from all bacterial species known to us, to deserve the status of a species. This new species shares many of the characteristics of the genus *Moraxella* in morphology, cultural behaviour, oxidase reaction and antibiotic sensitivity, and it has a DNA base composition close to the range of the other Moraxella species. Like the majority of the latter it is transformable. On the other hand, the group shows marked differences from all the other known Moraxella species, the most conspicuous of which are the ability to produce acid from glucose and maltose, and the lack of catalase.

Flamm (1956) described a new species, *Moraxella saccharolytica*, which, like our organism, produced acid from glucose, maltose, fructose, saccharose, glycerol, starch; but not from arabinose, dulcitol, galactose, inositol, lactose, mannitol, melizitose, raffinose, rhamnose, salicin, sorbitol, xylose. It was non-haemolytic and it further differed from our strains in the following characters: good growth at 22°, viable on blood agar for 4 weeks, gelatin liquefaction, catalase production, and resistance to streptomycin, chloramphenicol and tetracyclines. As far as we know strains of this organism have not been maintained, at least no strain has been available to us; but it seems quite obvious that *M. saccharolytica* differs from our strains in too many characters to belong to the same species. The study of new isolates of *M. saccharolytica* would be necessary to determine the taxonomic position of this species and its relationships to other Moraxella species.

Whereas other Moraxella species show some, even if very slight, signs of mutual compatibility in transformation experiments, no sign of compatibility of the new species with other Moraxella species or with Neisseria could be detected. This suggests that the new species is more distantly related to the other Moraxella species than the latter are to one another. Nevertheless, we feel that the likeness to other species of the genus *Moraxella* is sufficient to admit the new species to this genus; we find at present no reasonable alternative.

The habitat of the new species is uncertain in view of the fact that so few strains are known; 3 of the 9 strains were isolated from throat or nose of patients. It may be pointed out that this organism could very easily be mistaken for α- or β-haemolytic streptococci or for haemolytic Haemophilus species, and it is possible that a systematic search for this organism might reveal that it is less rare than it may appear.

We name this species *Moraxella kingii* in honour of the late Miss Elizabeth O. King, who did the pioneer work on this species and who collected most of the existing strains. The strain 4177/66 is designated as type strain. It is being deposited in the National Collection of Type Cultures (Great Britain) and in the American Type Culture Collection. A condensed description of the species follows.

Description of Moraxella kingii sp.nov.

Micromorphology. Organisms coccoid to medium-sized rods, somewhat smaller than those of other Moraxella species, with square ends, in pairs and chains. Gram-negative, with some tendency to resist decolorization. Not capsulated. Non-motile. No endospores.

Colonies. Delicate, translucent or slightly opaque, 0·1–0·6 mm. in diameter after

20 hr on blood agar. Usually low hemispherical, smooth, but occasionally flat with some pitting of the agar surface, or of a mucoid appearance. No pigment. Consistency soft or coherent, in the latter case showing transient spontaneous agglutination in saline. Colonies surrounded by distinct zones of beta-haemolysis.

Relation to oxygen. Aerobe.

Temperature. Slight growth at room temperature (about 20°). Optimal growth at 33–37°. Usually killed by exposure to 45° for 10–30 min.

Growth requirements. Comparatively fastidious. Little or no growth in peptone media. No growth on Hugh & Leifson's medium. Practically no improvement of growth by addition of 10 % (v/v) horse serum to liquid peptone media. Growth on high quality nutrient agar almost as good as on blood agar. No apparent need for X or V factors.

Viability. Blood agar cultures become sterile within 6–12 days at room temperature.

Biochemical reactions. Catalase reaction negative. Oxidase reaction strongly positive with tetramethyl-, less strongly positive with dimethyl-*p*-phenylenediamine. Nitrate not reduced or slight reduction. Acid rapidly produced on ascites fluid agar slopes from glucose and maltose. Trace of acid from galactose. No acid from fructose, lactose, saccharose, arabinose, xylose, rhamnose, mannitol, dulcitol, sorbitol, glycerol. Gelatin or serum not liquefied. Indole or hydrogen sulphide not produced. Urease negative. No oxidative deamination of phenylalanine or tryptophan.

Antibiotic sensitivity. Highly sensitive to penicillin, streptomycin, chloramphenicol, oxytetracycline, erythromycin.

Habitat. Strains have been isolated from throat, nose, blood, bone lesion and joint.

Pathogenicity. Uncertain, probably low.

DNA base composition. 44·5 mole % guanine+cytosine (CsCl buoyant density method).

Genetic compatibilities. Strains are mutually compatible with ratios of interstrain to intrastrain transformant numbers close to 1. No compatibility detected with other Moraxella or with Neisseria species.

Our thanks are due to Drs W. B. Cherry and R. E. Weaver, both of Atlanta, Georgia, U.S.A., for allowing us to use Miss King's strains in the study, and for kindly sending us additional strains from her collection. We are most grateful to Professor W. Szybalski for performing the DNA base determinations.

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Fig. 1 Fig. 2

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EXPLANATION OF PLATE

Fig. 1. *Moraxella kingii*, strain 4177/66 (type strain). Stained by Gram's method. × 1600.

Fig. 2. *Moraxella kingii*, strain A 1702. Stained by Gram's method. × 1600.

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The Taxonomy of the Genera *Moraxella* and *Neisseria*

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SUMMARY

A reclassification of the Neisseriaceae is proposed, involving: (1) Inclusion of *Moraxella* as a genus of Neisseriaceae. (2) Transfer of *Neisseria catarrhalis*, *N. ovis* and *N. caviae* to *Moraxella*, or, alternatively, creation of a new genus for these species. (3) Unification of the species *Neisseria flava*, *N. perflava* and *N. subflava* under the name *N. subflava*. (4) Unification of the species *Moraxella lacunata* and *M. liquefaciens* under the name *M. lacunata*. (5) Exclusion of the genera *Veillonella* and *Gemella* from Neisseriaceae. The relationships of the organisms *Acinetobacter anitratus* and *A. lwoffi* to the Neisseriaceae, if any, have not yet been sufficiently clarified to suggest the inclusion of these organisms in Neisseriaceae. Until this has been studied in more detail, the genus *Acinetobacter* appears to be suitable.

INTRODUCTION

The results of recent studies on the relationships of organisms belonging to the genera *Moraxella* and *Neisseria* (Bøvre, 1967; Catlin & Cunningham, 1961, 1964*a*, *b*) indicate that a revision of the taxonomy of these organisms is desirable. Transformation experiments and determination of DNA base composition have given particularly suggestive results. The significance of such criteria in taxonomy has been discussed elsewhere (Bøvre, 1967; Bøvre & Henriksen, 1967*a*). Here it may suffice to state that the following discussion is based upon the hypothesis that genetic homologies as demonstrated by genetic recombination between organisms have a greater significance in the taxonomy of bacteria than many phenotypic expressions which are commonly utilized in taxonomy. We also feel that the DNA base composition represents valuable supporting evidence, in the sense that wide differences between the over-all base composition of two organisms speak against close taxonomic relationship, whereas similar base compositions are compatible with, but do not prove, close relationship. At the present stage of our work we find it permissable and useful to suggest a new classification of these organisms, mainly based on the available transformation results and DNA composition data. Some of the taxonomic problems have been only tentatively solved, however. Future nucleic acid hybridization experiments may prove supporting or modifying on these points.

Relationships between Moraxella and Neisseria

Some years ago it was suggested (Henriksen, 1952) that the best choice of a family for the genus *Moraxella* might be Neisseriaceae. This suggestion was based upon similarities between the genera *Moraxella* and *Neisseria* in morphology, biochemical reactions, growth characteristics and requirements, antibiotic sensitivity and habitat. The results of transformation experiments give support to this idea. Thus transfor-

mation of relatively high efficiency, as high as or higher than between some species within each of the two genera, has been shown between *Neisseria catarrhalis*, *N. ovis* and *N. caviae* on one hand, and *Moraxella lacunata*, *M. liquefaciens*, *M. nonliquefaciens* and *M. bovis* on the other (Bøvre, 1967). Particularly high ratios between interspecific and intrastrain transformant numbers (in the following such ratios are called transformation ratios for simplicity) were found in experiments with *N. ovis* and *M. bovis*. We feel that it is reasonable to assume that such high transformation ratios (up to about 0·1 %), as occurs in these cases, are indications of fairly close relationships. When this is considered in the light of the many other similarities which have been pointed out before (Henriksen, 1952), we feel that it is a sound idea to include the genus *Moraxella* in Neisseriaceae as one of its genera.

Relationships within Neisseria

Results of transformation experiments and DNA base determinations made elsewhere (Catlin, 1960, 1961; Catlin & Cunningham, 1961; Marmur, Falkow & Mandel, 1963) and on our own strain material (summarized by Bøvre, 1967) indicate that Neisseria species can be divided in two subgroups. One subgroup consisting of *Neisseria gonorrhoeae*, *N. meningitidis*, *N. sicca*, *N. flava*, *N. perflava*, *N. subflava*, *N. flavescens* and *N. cinerea* is characterized by guanine+cytosine (G+C) content of the DNA in the vicinity of 50 mole %. The species which have been studied show high mutual compatibility in transformation, with transformation ratios above 1 %. In the case of *N. gonorrhoeae*, this species is highly compatible with *N. meningitidis* in DNA hybridization experiments (Kingsbury & Duncan, 1967). All these organisms clearly are closely related and belong to the same genus. It might even be discussed whether they are too closely related to be separated in different species. In the case of the species *N. flava*, *N. perflava* and *N. subflava* the relationships are particularly close, with transformation ratios above 10 % in all combinations. These organisms only differ from one another in comparatively minor details, and it has always been difficult to distinguish clearly between them. It has been previously suggested that they be united in one species. We feel that the transformation data give strong support to this view, and that these species should be united. According to the data given in *Index Bergeyana* (Buchanan, Holt & Lessel, 1966) the name would have to be *N. subflava*, which appears to be the oldest legitimate name.

Neisseria gonorrhoeae, *N. meningitidis*, *N. sicca*, *N. flavescens*, and *N. cinerea* are also apparently closely related to *N. subflava* and to one another, and it might be discussed whether they should be considered as varieties of the same species rather than as separate species. However, this might cause unnecessary confusion. In particular it seems desirable to keep organisms such as *N. gonorrhoeae* and *N. meningitidis* as separate species. We suggest that also *N. sicca*, *N. flavescens* and *N. cinerea* be maintained as separate species for the time being.

The second subgroup, consisting of *Neisseria catarrhalis*, *N. ovis* and *N. caviae* have G+C contents of the DNAs in the same range as the 'classical' Moraxella species (*N. catarrhalis* 41–42·5 moles %) or slightly above this range (*N. ovis* and *N. caviae* 44·5–45 moles %). They show mutual compatibility in transformation, although this is low between the former and the two latter species, and also comparatively high compatibility with some Moraxella species. They do not attack sugars, but reduce nitrate like the majority of Moraxella species. In other words,

they appear to be more closely related to some Moraxella species than to the other subgroup of the genus *Neisseria*. For these reasons it seems sensible to separate these organisms from *Neisseria*. This might be done by transferring them to the genus *Moraxella* under the names *Moraxella catarrhalis* (with two biotypes, possibly deserving species distinction), *M. ovis* and *M. caviae*; or alternatively by creating a new genus for these species. This may be a controversial question. If genetic compatibility is to be considered more significant than phenotypic characters such as morphology, the natural choice would be to transfer these species to *Moraxella*. This also would be the simplest solution, which would avoid the creation of a new genus. On the other hand, some microbiologists may object to the inclusion of both rods and cocci in the same genus. We are in favour of the first alternative and suggest that the three species be transferred to *Moraxella*, but we feel that the second alternative might also be acceptable.

Relationships within Moraxella

Strains named *Moraxella lacunata* and *M. liquefaciens*, and most probably corresponding to these entities, were found to be highly compatible (Bøvre, 1965), with transformation ratios between 10 and 100 % in all tested combinations. The difference in conventional characters between these organisms is very slight, probably consisting in a slightly different sensitivity to certain inhibitory substances contained in some batches of peptone (Lwoff, 1947); with most of the media now in use the difference is even more difficult to demonstrate (Bøvre, 1965). Furthermore, the DNAs contain approximately the same percentages of G+C, 41·5–43 moles %. There seems to be very little reason to keep these organisms in separate species, and we propose that they be united in the species *M. lacunata*.

The species *Moraxella nonliquefaciens* and *M. bovis* appear to be very closely related to *M. lacunata* and to one another, with transformation ratios ranging from 0·1 to 1·8 %, and their DNAs show G+C values in the same range, from 40 to 43 moles %. Again it can be discussed whether these organisms should be considered as varieties of the same species or as separate species. It is true that the difference between them, both in genetic compatibility and in conventional characters, is small. But the difference is clear enough to allow easy identification. The use of varieties is slightly more cumbersome than of separate species and is rather uncommon in bacteriology. We feel that one may have to accept that the degree of relationship between the species within the same genus varies, and we can see no advantage in changing the present usage on this point. For these reasons we suggest that these three entities should remain separate species.

Two other Moraxella species, *Moraxella osloensis* (Bøvre & Henriksen, 1967*a*) and *M. phenylpyrouvica* (or *M. polymorpha*; Bøvre & Henriksen, 1967*b*) show slight transformation compatibility with one another. Both species are slightly compatible also with the Neisseria species which we have proposed to transfer to the genus *Moraxella*. *Moraxella osloensis* has revealed slight compatibility with the species *M. lacunata*, *M. nonliquefaciens* and *M. bovis*.

Moraxella osloensis and *M. phenylpyrouvica* have DNA base compositions in the same range as the former Moraxella species (G+C 43–43·5 moles %). They both show great likeness to *M. nonliquefaciens*, with which species they have been confused until very recently, and we feel at present that it is most sensible to keep the two species in *Moraxella*.

Moraxella kingii (Henriksen & Bøvre, 1967) differs more clearly from the other Moraxella species in biochemical characters, transformation and DNA base composition. This species is able to attack some carbohydrates. It appears to be completely incompatible with all other Moraxella species, as well as with *Neisseria catarrhalis* and *N. ovis*. It also has a slightly higher G+C content than the other oxidase positive rods (44·5 moles %). Nevertheless, for the time being it seems most reasonable to consider it as a member of the genus *Moraxella*.

Relationships to Veillonella

The genus *Veillonella* has been included in Neisseriaceae on the basis of rather superficial morphological resemblance; otherwise there is little or no similarity to *Neisseria*. The Neisseria and Moraxella species are aerobes, whereas Veillonella species are anaerobes. We feel that there is a fundamental difference between these two kinds of metabolism. Furthermore, there is little or no other evidence apart from the coccal shape and the, perhaps questionable, Gram-negativity of *Veillonella* (Burnett & Scherp, 1962) to indicate any relationship. We feel that there is no proof that *Veillonella* is sufficiently related to *Neisseria* to belong to the same family, and propose that it be excluded from the Neisseriaceae. It might, perhaps form a separate family, at least until its relationships have been better clarified.

Relationships to Gemella

Berger (1960*a*, *b*) showed clear differences and little resemblance between the genera *Neisseria* and *Gemella*, to which genus he removed the former *N. haemolysans*. We are of opinion that it would be better to keep *Gemella* out of the Neisseriaceae, since this would leave a more homogeneous family.

Relationships to Acinetobacter

Opinions about the relationships of the organisms variously called Acinetobacter, Achromobacter, Mimeae, Moraxella, etc., to the 'classical' Moraxella species are divided, some workers wishing to place these organisms in the genus *Moraxella* or at least in the same family, whereas others wish to remove these organisms to a different family, e.g. Achromobacteriaceae.

The results of transformation experiments do not indicate close relationship between these organisms and oxidase positive bacteria. The very slight transformation reactions obtained with DNA from Acinetobacter strains on *Neisseria catarrhalis*, *N. ovis* and *Moraxella osloensis* are very difficult to evaluate, and absolutely no compatibility with *Moraxella lacunata*, *M. nonliquefaciens* and *M. bovis* has been demonstrated. The relationship, therefore, if any, would appear to be distant. In view of the many other differences in conventional characters such as growth characteristics, mode of attack on sugars, oxidase reaction, antibiotic sensitivity and habitat we feel that the time is not yet ripe for the admission of these organisms to the genus *Moraxella* or to the family Neisseriaceae.

The results of the studies on these organisms which are now in progress in many laboratories may be expected to clarify the situation and should be awaited before a decision is made in this controversial matter. We conclude that these organisms should wait for admission into the Neisseriaceae until better evidence in favour of

this becomes available. In the meantime the classification of these organisms in the genus *Acinetobacter* seems to be sound, at least as a temporary measure.

A revised classification of the Neisseriaceae, with a description of the family and the genera

Family Neisseriaceae Prévot, 1933

Organisms spherical in pairs with adjacent sides flattened, or rod-shaped in pairs and short chains. Organisms usually plump with diameters of 1 μ or more. Non-flagellated and often non-motile. Some species have been reported to show gliding motility. Gram-negative with some tendency to resist decolorization. Some species form yellow pigments. Aerobic. Some species prefer or require a humid atmosphere, with or without added CO_2. Some species grow poorly or not at all in some usual media without mammalian body fluids due to sensitivity to certain inhibitory substances in peptone, but may grow in simple defined media. Optimum temperatures about 32–37°. All known species are parasitic. Give positive oxidase reaction with both dimethyl- and tetramethyl-*p*-phenylenediamine. Usually sensitive to penicillin and most other antibiotics. The type genus is *Neisseria.*

Genus I. *Neisseria* Trevisan, 1885

Organisms coccal. Some species produce yellow pigment. Grcwth on standard media may be poor or fail. Biochemical activities limited. A few carbohydrates may be utilized. Indole is not produced. Nitrate not reduced. Catalase is produced. G+C content of DNA in the range about 50 moles%. Parasites of mammals. The type species is *Neisseria gonorrhoeae* Trevisan.

Species 1. *N. gonorrhoeae.* Species 3. *N. sicca.* Species 5. *N. flavescens.*
Species 2. *N. meningitidis.* Species 4. *N. subflava.* Species 6. *N. cinerea.*
(Other species which have been described, may be included.)

Genus II. *Moraxella* Lwoff, 1939

Organisms coccal or rod-shaped. Tendency to pleomorphism. No pigment. Growth on standard media may be poor or fail. Biochemical activities limited. Most species do not attack carbohydrates. Indole is not produced. Nitrate may or may not be reduced. Catalase may or may not be produced. G+C content of DNA in the range 40–45 moles%. Parasites of mammals. The type species is *Moraxella lacunata* (Eyre 1900).

Species 1. *M. lacunata* (includes the biotype *M. liquefaciens*).
Species 2. *M. nonliquefaciens* (Scarlett, 1915), Murray, 1948.
Species 3. *M. bovis* (Hauduroy *et al.* 1937), Murray, 1948.
Species 4. *M. osloensis* (Bøvre & Henriksen, 1967).
Species 5. *M. phenylpyrouvica* (Bøvre & Henriksen, 1967).
Species 6. *M. kingii* (Henriksen & Bøvre, 1967).
Species 7. *M. catarrhalis* (Frosch & Kolle, 1896), Henriksen & Bøvre, 1967.
Species 8. *M. ovis* (Lindqvist, 1960), Henriksen & Bøvre, 1967.
Species 9. *M. caviae* (Pelczar, 1953), Henriksen & Bøvre, 1967.

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Evolution of Developmental Stages in Cultures of *Bacillus cereus*

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SUMMARY

Cultures of *Bacillus cereus* grown in a chemically defined medium with glucose under standardized conditions followed a reproducible pattern of development giving good spore yields in less than 24 hr. Samples were withdrawn at intervals and individual organisms were classified into developmental states by four methods: (1) Gram reaction; (2) growth potential as revealed by the microcultures derived from single organisms or chains of organisms isolated into microdrops; (3) ability of individual organisms to take up neutral red; (4) electrophoretic distribution of organisms through a pH gradient. From the results we conclude that at most times the population was heterogeneous with respect to state of the organisms and that at least some of the shifts in state were quantal, in the sense that each state was relatively stable and that the transition from one state to the next rapid. There was a cyclical appearance and disappearance of organisms stainable with neutral red. Waves of stainable organisms were correlated with the break-up of chains into smaller units and with the lysis of a proportion of the organisms.

INTRODUCTION

In developing bacterial cultures there are progressive changes in the properties of the organisms and the culture medium produced by mutual interactions. We were interested to study the role of these interactions in determining morphogenesis of the organisms. The ideal approach to this problem would be to start from a complete description of the organism + medium system in every biochemical detail. At present a more realistic approach is to describe the states of individual organisms at various stages, to study the kinetics and mode of transition from state to state, and to identify the morphogenesis-determining interactions between the organisms and the medium without necessarily knowing their nature. We used this second approach in the model case of a culture of *Bacillus cereus* from spore germination through vegetative growth to sporulation. In work described in this paper, we describe changes in properties of the cultures and organisms in terms of a time scale.

METHODS

Organism and medium

The bacterial strain used was *Bacillus cereus* 569 R from the collection of Dr C. B. Thorne. The growth medium (SMCA) contained (%, w/v): 0·025 K_2HPO_4, 0·025 KH_2PO_4, 0·01 $MgSO_4$, 0·0005 $FeSO_4 \cdot 7H_2O$, 0·0005 $MnSO_4 \cdot 4H_2O$, 0·5 vitamin-free

Casamino acids (Difco), 0·2 glucose, and (μg./ml.): 0·025 biotin, 0·25 niacin, 0·27 Ca pantothenate. This medium was selected because it permits almost complete sporulation of *B. cereus* 569 R within 24 hr. The germination medium consisted of 0·04 M-tris buffer (pH 8·0) containing 0·001 M-KH_2PO_4 and 2·5 mg. L-alanine/ml.

Culture conditions

A washed heat-shocked suspension of spores in water (0·1 ml. containing 2×10^8 spores as determined by microscopic counts and assays of viable heat-resistant spores) was heated at 65° for 15 min. One ml. of L-alanine germination medium was added, and the tube shaken at 37° for 30 min. The contents were added at $t = 0$ hr to 50 ml. growth medium in a 250 ml. Erlenmeyer flask. Flasks were shaken at 37° on a reciprocal shaker, and samples removed at desired intervals. When large samples were to be removed, duplicate cultures were used. Identifiable events occurred at reproducible times within 0·5 hr for $t = 0$ to $t = 7$ hr, and within 1 hr for $t = 7{\cdot}5$ to $t = 13$ hr.

Miscellaneous

The extinction at 440 mμ (E_{440}) was measured with a Zeiss PMQII spectrophotometer.

A Petroff–Hauser chamber was used for counts of organisms and chains. To count the individual organisms in chains, saturating sodium chloride was added to shrink the organisms (Powell, 1958). Counts were not accurate after 5 hr because of clumping.

Identification of states of organisms

Organisms in different physiological or developmental states were scored on the basis of four properties.

(1) *Type of development of individual organisms.* A small drop of mineral oil was placed on a microscope slide. A sample of the culture was diluted when necessary into fresh SMCA medium to about 5×10^6 organisms or chains/ml. With a pipette drawn from capillary glass tubing a pattern of 100 droplets (volume about 10^{-7} ml. each) was placed on the glass under the oil by the use of a dissecting microscope; a coverslip was then placed on the oil drop. Most droplets contained either one organism or a chain or none. The slides were studied microscopically to locate organisms or chains, incubated for 2 days at 37° in a moist chamber, and the droplets scored for the following types of development: (*a*) no growth, (*b*) division without spore formation, (*c*) division with spore formation, (*d*) spore formation without division. The results served to calculate the frequency of organisms which gave each type of development, by using the Poisson distribution.

(2) *Staining with neutral red.* An aqueous solution of neutral red (0·85 %, w/v) was used to stain living organisms in samples of the developing culture with 1 vol. dye solution to 4 vol culture. The organisms were examined by phase-contrast microscopy and photographed under oil immersion with 35 mm. Kodak Plus-X or Panatomic-X film. Individual organisms were scored as bright (stained), dark (unstained), or bright with dark nuclear structures.

(3) *Gram reaction.* A heated-fixed smear was stained with 0·5 % (w/v) crystal violet for 1 min., drained, treated with Gram iodine solution for 1 min., washed with 95 % (v/v) ethanol in water, rinsed with water and dried.

(4) *Distribution through a pH gradient.* The method of electrophoretic separation of bacteria on the basis of their isoelectric points was adapted from Kolin (1958). The apparatus was constructed of glass tubing of the same dimensions as in the Kolin apparatus.

The gradient pH 7·5 to 3·5 was established with 0·1 M-tris HCl buffers, with 2 M-sorbitol to make the dense acidic buffer. Sorbitol was used to stabilize the gradient. The procedure for filling the U tube and forming the 'M layer' (the region containing the sample) was as described by Kolin (1958). A millimetre scale was attached to one arm of the U tube and the 'M layer' was formed in 1 cm. in the centre of this scale. The samples were either concentrated by centrifugation followed by resuspension with supernatant fluid or diluted with SMCA medium to give an extinction E_{440} of about 0·6 after an equal volume of 2 M-sorbitol had been added. All solutions were chilled in ice before use to decrease the effect of heat on the organisms during electrophoresis. The glass tubes containing the platinum electrodes were connected to the U tube chamber by filter-paper wicks. With a Beckman Duostat power supply, current was applied for 1 min. at 2 mA. and increased to 10 mA., 150 V. for a total of 20 min. Bands of organisms formed in the 'M layer' did not change position after this time. Organisms were scored by band position in the pH gradient.

RESULTS

Once a set of parameters (initial medium, volume, temperature, aeration, inoculum) is chosen, the course of development of a culture is completely determined. The changes in state of the organisms are not directly dependent on time, but are determined by a number of other variables which are altered by the growth of the organisms (population density, pH value of the medium, nutrient supply and other changes of the medium). With a single set of parameters, all these dependent variables follow a reproducible pattern, and the time of incubation may be used to define the state of the culture at any instant.

Description of the culture properties

Figure 1 gives data obtained by sampling from a standard culture (see Methods) for the period from $t = 2$ to $t = 13$ hr of incubation. The extinction of samples removed at intervals from the growing culture indicated progressively decreasing growth rates. During the stationary phase a reproducible decrease in E_{440} occurred at $t = 9·5$ hr. The increases in counts of organisms corresponded closely to increases of extinction. The apparent breaks in the continuous curve of the count coincided with periods of lysis (see below), but may have been due to inaccuracies of counting. *Bacillus cereus* characteristically grows in chains. The first one or two divisions resulted in organisms which then separated; chain formation began after incubation for about 3 hr. The chains broke up into smaller units mainly at two times, $t = 5$ hr and $t = 7$ hr. After 7 hr, growth was mainly in chains of 2 to 4 organisms. The pH value of the standard culture medium dropped to a low point at about $t = 5$ hr, gradually rose until about $t = 10$ hr, and then continued near a maximum of pH 7·4. The decrease in pH value followed by a rise has been observed by other workers with different sporulation media (Halvorson, 1957).

Classification of states of organisms

To study the developmental changes in the organisms during growth it was necessary to use tests by which individual organisms (rather than the culture as a whole) could be classified according to their developmental or physiological states. The use of such tests permitted us to determine whether organisms changed state in a gradual or in a sudden, quantal manner, and whether the population consisted of organisms which were all in one state of development at any given time, or whether it was heterogeneous. Four such techniques were used.

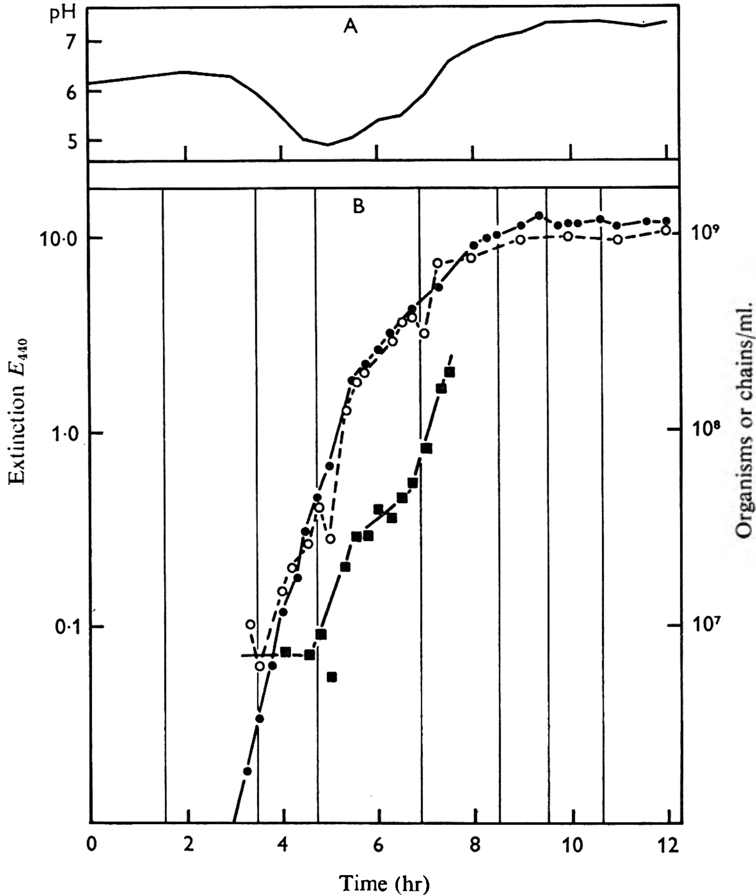

Fig. 1. *Bacillus cereus* 569 R. A. Changing pH during culture development. B. Growth of standard culture. (●) extinction, (○) organism counts, (■) chain counts.

Gram reaction

Samples of the standard culture were removed at 5 min. intervals and stained by the Gram method. All the organisms in samples taken before $t = 3{\cdot}5$ hr were Gram-negative. During the period from $t = 3{\cdot}5$ to $3{\cdot}75$ hr, all the organisms uniformly became more darkly stained; after $t = 3{\cdot}75$ hr. all the organisms were Gram-positive (Fig. 2, top).

Staining with neutral red

Samples taken at intervals from the culture were stained with aqueous neutral red, examined microscopically (phase contrast), and representative fields photographed. Stained organisms appeared refractile (bright) while unstained organisms or parts of organisms were dark. In some organisms dark bodies or complex structures, probably nuclear material, were seen in bright cytoplasm.

(*a*) *Germination and outgrowth.* Even after pre-treatment with heat and L-alanine the spores did not germinate simultaneously in the standard culture. The events occurring during the first 2 hr are complex and will be dealt with in a separate paper. Plate 1, fig. 3 and 4, show stages in this process. At 1·5 hr (Pl. 1, fig. 3*b*) the outgrowing organisms were stainable with neutral red and appear bright under phase contrast. At 2–2·5 hr some organisms were seen to disintegrate or lose their contents (Pl. 1, fig. 4).

(*b*) *Periodicity in the frequency of bright organisms.* During the period from $t = 1$ to $t = 12$ hr, neutral red staining of samples taken at intervals showed that bright organisms appeared in 7 successive waves, reaching their peaks at the following times: $t = 1{\cdot}5, 3{\cdot}5, 4{\cdot}75, 6{\cdot}75, 8{\cdot}5, 9{\cdot}5, 10{\cdot}75$ hr, respectively. These peaks are indicated by vertical lines and numbered at the top of Fig. 2. Except for the first peak, at 1·5 hr, the maximum proportion of bright organisms did not exceed 30% of the total number of organisms, the rest remaining dark. Counts of stained organisms were made in detail for peak no. 4 and are plotted on Fig. 2.

The bright organisms appeared either within dark chains (Pl. 1, fig. 7; Pl. 2, fig. 10), or in chains consisting entirely of bright organisms, or in chains composed of bright organisms and organisms with bright cytoplasm and dark nuclei (Pl. 2, fig. 10). In the rising phase of each wave, fully bright organisms in the dark chains appeared in groups of one, two, or three with about equal frequency. This is a non-clonal and non-random distribution. Very few intermediately bright organisms were observed. In the falling phase of each wave, the brightness of the organisms slowly faded (Pl. 1, fig. 5; Pl. 2, fig. 11*b*) and internal structure could sometimes be observed (Pl. 2, fig. 9). Between waves the organisms appeared uniformly dark (Pl. 1, fig. 6).

(*c*) *Lytic phenomena.* Soon after the number of bright organisms in any given wave reached a maximum, microscopic observation showed that some organisms were disintegrating, or cell material was being extruded through the cell wall at one or more places (Pl. 1, figs. 4, 5; Pl. 2, figs. 8, 11*a*). The times at which this apparent lysis was observed are marked by arrows on Fig. 2. Lysis was observed also in unstained organisms (Pl. 2, fig. 8).

Growth potential.

Single organisms or chains isolated in microdrops under oil were allowed to develop at 37° for 2 days (see Methods). Among organisms taken from the standard culture at times from 2 to 13 hr after inoculation, four clearly distinct types of development were observed. Five cell 'states' (α–ϵ) were inferred from these results as indicated in Table 1.

Organisms in state α were usually single and divided only once if at all; chains in state β grew into clones of about 100 organisms and no spores were formed by the progeny. Chains in state γ (2–14 organisms) also formed clones of about 100 organisms and usually all of these progeny formed spores. Occasionally, a single very long chain of unsporulated organisms was present among hundreds of free spores in the

same microdrop. Organisms in state δ were a minority group of very short chains (about 2 organisms) which did not develop. Organisms or short chains in state ϵ (1–4 organisms) did not divide but formed spores in the original organisms. Samples of 100 microdrops containing organisms taken from the developing culture at any given time showed that the organisms were not usually all in the same state. This heterogeneity is shown by the overlapping curves α–ϵ in Fig. 2.

It is likely that oxygen was limiting in the microdrops, and this may account in part for the fact that organisms in different states show different types of development. Halvorson (1957) showed that a sharp peak in oxygen demand followed the low pH point in sporulation cultures of *Bacillus cereus*. In our system this peak would correspond to the time at which the organisms shifted from state β to state γ. Therefore, if ample oxygen were not available in the microdrops, the organisms may not have been able to make the shift.

Table 1. *Bacillus cereus 569* R. *Definition of developmental states of organisms in culture*

Single organisms or chains were isolated from a standard culture into microdrops. Types of growth were observed after incubation for 48 hr.

State of organism	Time of appearance in standard culture (hr)	Growth observed in microcultures	
		Clone formation	Spore formation
α	2–4	−	−
β	3–6	+	−
γ	4·5–13	+	+
δ	7·5–13	−	−
ϵ	9·5–13	−	+

The interpretation of these findings would be clearer if individual organisms were used instead of chains. We were unable to break the chains successfully by ultrasonic treatment. Nevertheless, the sharp distinctions between the types of growth resulting from individual chains isolated into microdrops means that the states were sharply differentiated and did not grade into each other.

Correlation of observed events with a time-scale

Figure 2 shows the data, described above, in terms of a time-scale. In this figure, as well as in Fig. 1, vertical lines have been drawn through the times at which peaks of bright organisms were observed, as numbered at the top of Fig. 2. Some lysis was noted microscopically after each peak of bright organisms, at the times indicated by arrows in Fig. 2. The curve of visible organisms counts (Fig. 1) shows decreases in numbers following bright organism peaks 2, 3 and 4, and a decrease in extinction occurred after peak 6. A break-up of the chains into smaller units followed peaks 3 and 4 (Fig. 1). These correlated events seem to show in the development of the culture a periodicity not directly related to the division cycle. The shift from Gram-negativity to Gram-positivity occurred at the time of peak 2 (Fig. 2) and the low pH point of the medium at the time of peak 3 (Fig. 1). The numbers of organisms in each development state, as determined by their growth potential in microdroplets, is plotted against time in Fig. 2. The overlapping curves α–ϵ illustrate the heterogeneity observed in the culture at each time. The shifts in state of organisms do not appear to occur at times correlated in any simple way with the periodic phenomena listed above, except possibly for the small group of non-viable δ organisms.

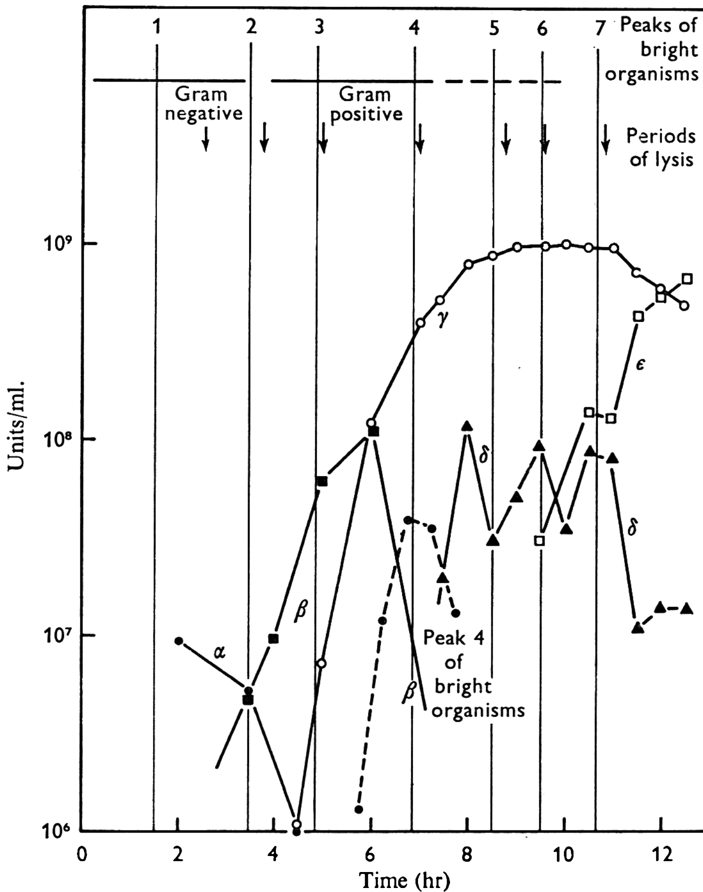

Fig. 2. *Bacillus cereus* 569 R. Population changes in organism state during development. Upper line, numbers indicate peaks of organisms which appeared bright when stained with neutral red; second line, Gram reaction; third line, arrows indicate periods of lysis. Solid curves (●) organisms in state α; (■) in state β; (○) in state γ; (▲) in state δ; (□) in state ϵ. Dashed curve, organisms staining with neutral red in peak 4.

Classification of types of organisms by isoelectric point

Samples of the culture taken at different times were subjected to electrophoresis through a pH gradient stabilized with sorbitol (see Methods). This technique determines the range of isoelectric points of the organisms in a suspension and is not a measure of their electrophoretic mobility (Kolin, 1958). The purpose of the experiment was to determine whether the population at any one time was heterogeneous in isoelectric point, whether there were discrete groups of points or whether the isoelectric points varied over a continuous range. The results are shown in Fig. 3. It is clear that the organisms were grouped into 4 to 6 distinct bands. These bands are either sharp (groups of organisms with similar isoelectric points) or wide (groups of organisms or chains with isoelectric points continuously varying within a range). Repeated experiments gave results in general agreement but somewhat variable in detail. At certain times, bluish bands containing cellular debris but no intact organisms

were observed at the top of the gradient. These probably represented the products of the disintegration of organisms as seen microscopically.

Organisms were recovered with a micropipette from the major bands and tested for staining with neutral red. Each band consisted of organisms which stained alike, but the staining property was not correlated with the position of the band in the gradient and did not correspond to the staining ability of the organisms in the growing culture, since no mixed chains were observed.

The results permit the clear conclusion that at any one time there existed within the culture 4 to 6 groups of organisms which differed discontinuously in isoelectric point. The groups with a range of isoelectric points may have been due to mixed chains, such as those of the bright and dark organisms observed by neutral red staining. The isoelectric range of each sample indicated a greater complexity of the system than was obvious from the distribution experiments or the neutral red staining.

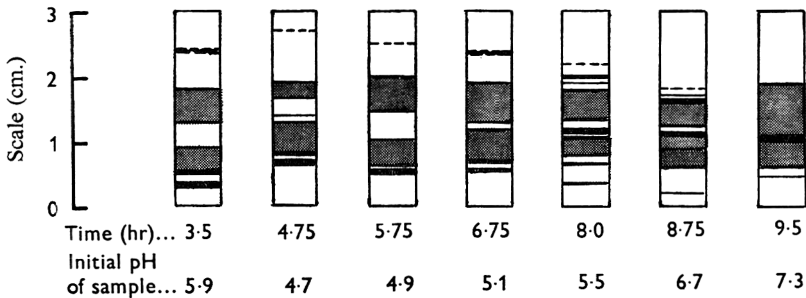

Fig. 3. Isoelectric ranges of samples from developing culture of *Bacillus cereus* 569 R. Diagram of location of bands after 20 min. electrophoresis at 10 mA, 150 V. Sample initially occupied area between 1 and 2 cm. pH of sample was determined after addition of sorbitol. Initial pH at 0 cm., 3·3–3·5; initial pH at 3 cm., 7·5. Bands indicated by dashes did not contain whole organisms.

DISCUSSION

A developing culture goes through a series of phases each of which is conditioned by the previous phases and in turn conditions the following ones. The process probably involves both intracellular changes, including cell division, and extracellular (medium) changes produced by the previous growth history of the culture. In this paper evidence is given that in these cultures individual organisms underwent quantal changes in shifting from one state to the next. In the case of the Gram reaction, all organisms shifted simultaneously and relatively suddenly from Gram-negative to Gram-positive. Since the organisms were not growing synchronously, this shift must have occurred in response to an environmental change. The rapidity of the shift suggests that this was a threshold reaction of the organisms to a more gradually changing environmental variable.

The growth potential tests show two distinct phenomena. First, a number of individuals taken from the culture at any given time and isolated into similar microdrops differ from each other with respect to their subsequent growth. This means that the sampled population was heterogeneous, and that the future behaviour of each organism when placed in the uniform environment of the microdrop had already been determined by its previous interactions in a spatially uniform but temporally

changing environment. Secondly, each state of the organisms was sharply defined by the type of growth resulting from single organisms or chains. Organisms in transition from state to state would be expected to produce intermediate types of growth. These were not observed and must be rare. Therefore, the shifts in state must occur rapidly in individual organisms. The isoelectric range experiments also showed that distinct groups of organisms existed within the culture at any given time. The separation of bands confirms that organisms in intermediate stages were rare and indicates that the shifts in isoelectric point must be rapid.

With neutral red staining during the appearance of bright organisms, transition stages from dark to bright were rare, again indicating that changes in state occurred rapidly. After the peak frequencies of bright organisms had been reached, however, the changes from bright to dark occurred gradually.

The bright organisms appeared in cyclical waves during the growth of the culture. This oscillatory behaviour presumably means that the first individuals which gave a certain response caused a new change of environment which altered the reaction of other individuals. Since after each wave a fraction of the organisms lysed, the substances so released may play a role in controlling the oscillation in frequency of bright organisms.

With many transformable strains of bacteria, competent organisms appear towards the end of logarithmic growth (Schaeffer, 1964), at the time at which wave 4 of bright organisms was observed in our cultures of *Bacillus cereus*. Occasionally, successive waves of competency have been observed (Thomas, 1955). Jensen & Haas (1963) reported correlation between the capacity for uptake of methylene blue and the presence of competent bacteria in a culture. Even through *B. cereus* 569 R has not been shown to be transformable it seems possible that in transformable strains competency may be coupled with changes in stainability like those revealed by the present experiments with neutral red.

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EXPLANATION OF PLATES

Figs. 3–11. Successive changes in morphology of organisms in the developing culture of *Bacillus cereus* 569 R. The organisms were stained with aqueous neutral red except for Pl. 2, fig. 6. The organisms were photographed under phase contrast at a magnification of × 1450. Stained organisms appeared bright; unstained organisms appeared dark.

PLATE 1

Fig. 3. Outgrowth. The organisms were concentrated by centrifugation. (*a*) 1·0 hr; (*b*) 1·5 hr; (*c*) 2·7 hr.

Fig. 4. Early growth and lysis, (*a*) Swollen pair, 2·75 hr, (*b*) lysis of bright organisms, 2·5 hr; (*c*) growth and lysis of dark organisms, 2·5 hr.

Fig. 5. Lysis, (*a*) leakage from fading bright chain, 4·0 hr; (*b*) lysis, 4·0 hr; (*c*) lysis, 4·8 hr.

Fig. 6. Dark organisms, 4·5 hr.

Fig. 7. Mixed chains, one intermediate organism, 4·8 hr.

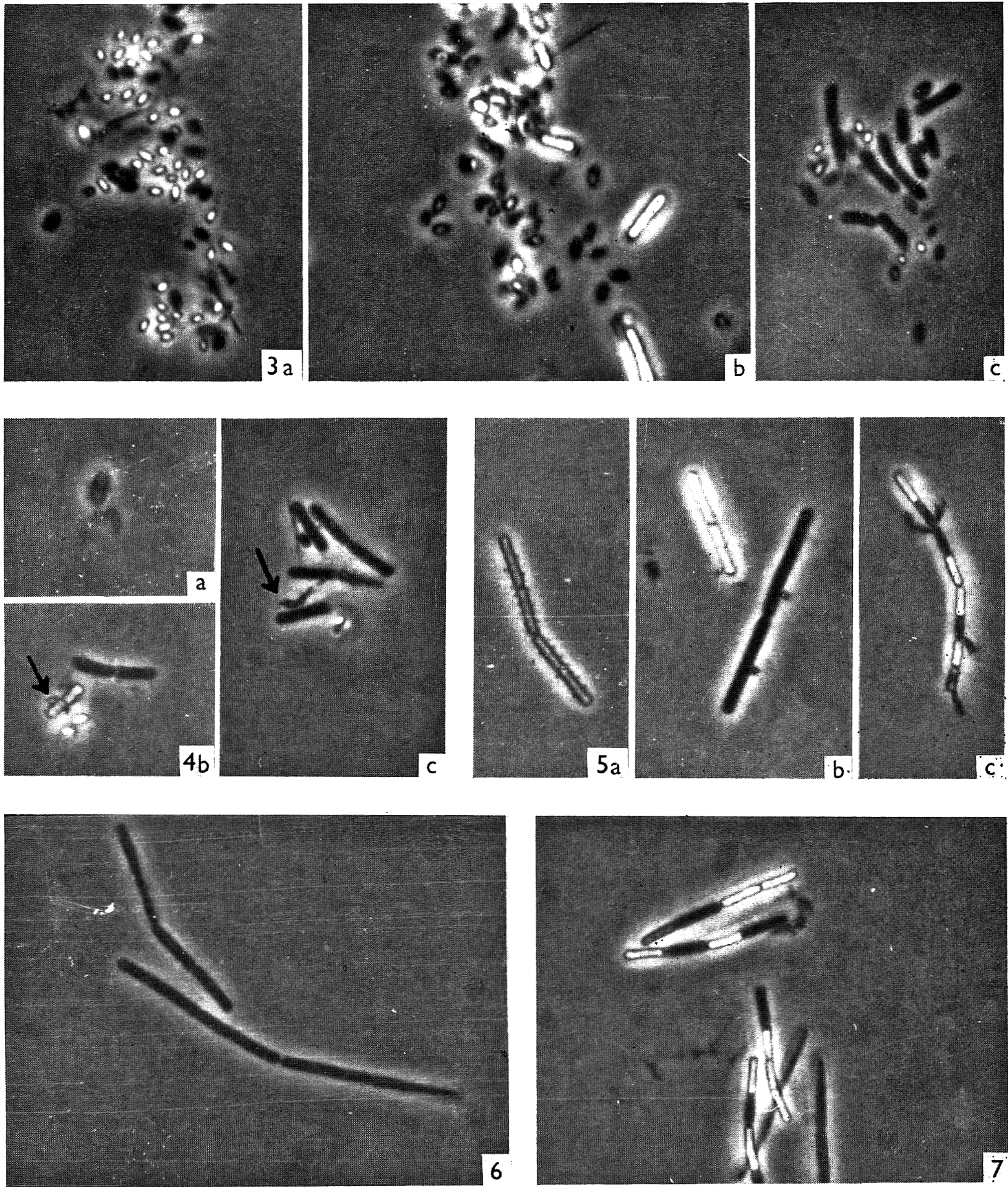
PLATE 2

Fig. 8. Lysis, 5·0 hr. Unstained.

Fig. 9. Complex structures in fading organisms, 5·75 hr.

Fig. 10. Bright organism distribution. (*a*) and (*b*) 6 hr; (*c*) 6·25 hr.

Fig. 11. (*a*) Lysis of bright organism, 7 hr; (*b*) fading of bright organisms, 7 hr.



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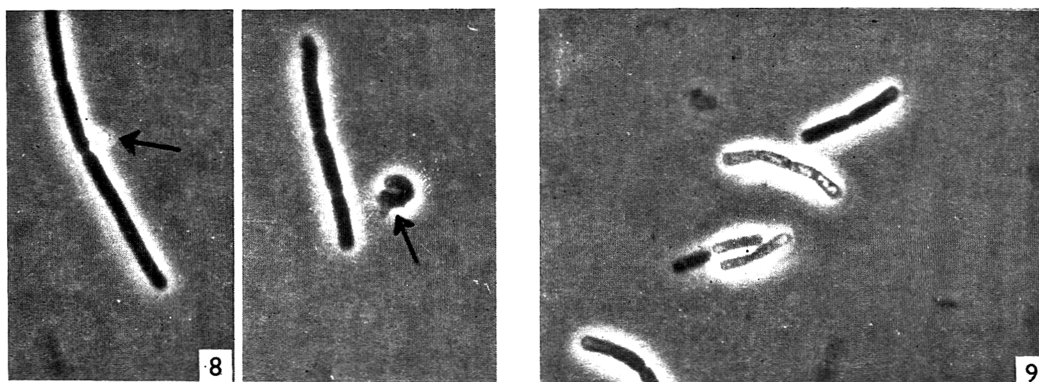

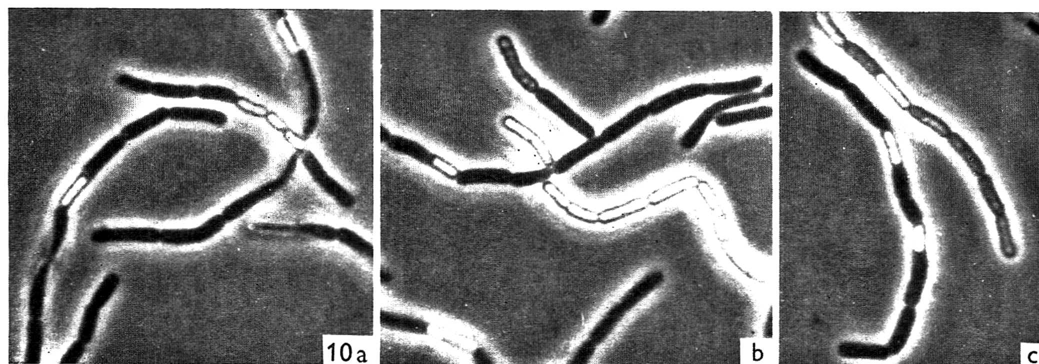

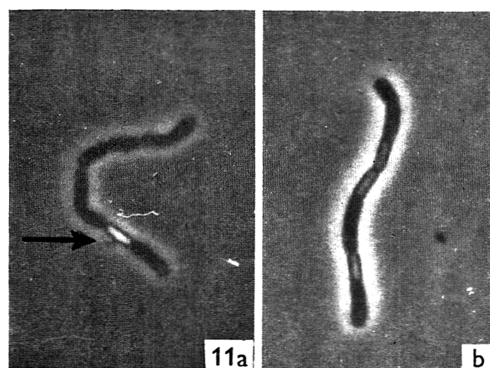

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Ultrastructural Changes During Germination of Ascospores of *Neurospora tetrasperma*

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SUMMARY

A technique is described for fixing and embedding dormant ascospores of *Neurospora tetrasperma*, whose impermeability has made them difficult subjects for electron microscopy. The ultrastructure of dormant and germinating spores has been compared. Endoplasmic reticulum occurs relatively infrequently in dormant ascospores whereas germinating ones have large amounts. An unusual structure, consisting of concentric membranes of varying degrees of complexity, appears after activation and disappears after extension of the germ tube. This membrane complex is continuous with the endoplasmic reticulum and may be its origin during germination. Mitochondria in dormant ascospores are larger and less numerous than in germinating ones. Two new elements of the ascospore wall are described, one of which may contribute to the relative impermeability of these forms. Nuclei increase in number during germination, but the exact number of divisions is not known.

INTRODUCTION

The ascospore of *Neurospora tetrasperma* is a resistant organism which can sustain itself during a long dormant period, as well as for several hours after germination, through the oxidation of endogenous substrates (Sussman, 1961). Its protoplast is enclosed in a complex wall consisting of at least three layers (Lowry & Sussman, 1958), with a germ pore at each end of the elliptical cell. This pore appears under the light microscope to be a perforation in the outer two major wall layers which is closed by the innermost layer. Perhaps because of the very elaborate wall and associated structures, the dormant ascospore is remarkably resistant to a variety of environmental factors including extremes of temperature (Lingappa, Y. & Sussman, 1959) and toxic chemicals. For example, these spores survive after being exposed for days to the fixatives commonly used for electron microscopy (R. J. Lowry, unpublished). Upon the disruption of dormancy by heat, the metabolic capacity of ascospores is enhanced 20- to 30-fold (Goddard, 1939) and there is a shift from the oxidation of lipids alone to the rapid utilization of trehalose as well (Lingappa, B. T. & Sussman, 1959). Several observations led us to believe that important changes at the ultrastructural level must accompany the dramatic shifts in metabolism. Thus, Holton (1960) showed that the endogenous content of cytochrome *c* in the mitochondria of dormant ascospores was much lower than that in mitochondria of germinating spores. One possibility to explain these data is that the mitochondria change in structure during germination. Moreover, after protrusion of the germ

tube, sensitivity to toxic substances increases markedly as does permeability (Sussman, Holton & von Böventer-Heidenhain, 1958). These observations lead to the surmise that the ascospore wall may undergo changes which help to account for some of the events described above. Therefore, we have studied the changes in the ultrastructure of dormant and germinating ascospores in order to check these possibilities and to describe the events that occur during germination.

METHODS

Activation and germination of ascospores. Ascospores of *Neurospora tetrasperma* were obtained by the means described by Goddard (1935). In addition, they were incubated at 24° in 0·1 % (w/v) EDTA at pH 7·2 for 7 days on a reciprocal shaking machine and then washed in several changes of distilled water to ensure good reproducibility in germination. Activation was done by exposing dormant ascospores to 60° for 30 min., after which they were brought to 24° and incubated on a reciprocal shaker. Germination began at about 3 hr after activation under these circumstances.

Fixation and embedding. Germinating spores were fixed in 2 % (w/v) potassium permanganate at 4° for 1–2 hr. They were then washed briefly in distilled water and embedded as a concentrated suspension in 0·5 % agar. The agar containing the embedded spores, cut into suitably small pieces, was dehydrated through a graded series of acetone or ethanol solutions, passed through three changes of propylene oxide and placed in a mixture of 1/3 part Epon (Luft, 1961)+2/3 parts propylene oxide. The propylene oxide was allowed to evaporate slowly overnight, whereupon the agar blocks were transferred to pure Epon in gelatin capsules. Polymerization was done at 60°.

Dormant spores presented special problems because of their extreme impermeability to fixing solutions and to Epon. The procedure finally evolved was as follows. Dormant spores, suspensions in distilled water at 4°, were centrifuged at 3000*g* and the pellet resuspended in a small volume of 2 % potassium permanganate. A drop of the suspension was placed between a pair of 2 in. square pieces of plate glass and pressure applied with parallel-jaw glass pliers until microscopic examination showed that most of the spores had been cracked. Squeezing was repeated until enough spores were broken to form a convenient sample for embedding. The cracked spores were allowed to remain in the 2 % permanganate solution at 4° for 2 hr, after which they were dehydrated and embedded by the same methods as used for germinating spores. A similar technique was described by Ekundayo (1966) except that sporangiospores of *Rhizopus arrhizus* were broken in fixative in a Mickle disintegrator.

Sections were cut on an LKB Ultratome with either a glass or a diamond knife, mounted upon formvar-coated copper grids and sections examined in an RCA-EMU 3G electron microscope at 50,000 V.

RESULTS

The spore wall

Our previous light-microscope study of the ascospore wall indicated that it is composed of three major layers including an inner layer, the endosporium, a middle layer, the episporium, and an outer layer, the perisporium (C, B and A, respectively

in Pl. 2, fig. 4). Observations with the electron microscope revealed the presence of two additional layers. One of these is relatively electron-transparent and covers the ridges of the perisporium and fills in between them (arrow, Pl. 2, fig. 3, and upper arrow, Pl. 2, fig. 4). It is lamellate with a finely fibrous surface and can be sharply distinguished from the perisporium itself, which is electron-dense and, presumably, chemically distinct. The presence of this outermost layer, at least in wet spores, results in an organism with a relatively smooth surface. The outermost fibrous portion of this layer can be seen to be continuous with the fibrous outer layer of the germ tube (arrow, Pl. 3, fig. 5).

The second additional wall layer revealed by the electron microscope is composed of a very electron-dense material lying between the episporium and the endosporium (lower arrow, Pl. 2, fig. 4). The inner boundary of this layer is not sharply defined and penetrates the episporium for a short distance in the form of isolated dense granules (arrow, Pl. 4, fig. 8). There appears to be an accumulation of this material at the germ pore which seems pushed out of the way by the emerging germ tube (Pl. 3, fig. 6). That this layer is associated with the endosporium is indicated by the fact that when the endosporium pulls away from the episporium, because of damage in cutting, the dense layer always remains associated with the endosporium (Pl. 2, figs. 3, 4).

The protoplast of the dormant spore

Numerous swollen mitochondria are present in dormant ascospores (Pl. 1, fig. 1). Attempts were made to prevent the swelling of these mitochondria by increasing the osmotic concentration of the fixing solution by adding up to 5% (w/v) NaCl, but to no avail. Otherwise the mitochondria of dormant spores appeared not to differ greatly from those of other forms (Pl. 2, fig. 3).

Dormant ascospores possess vacuoles of two types, including those of type 1, which appear empty following permanganate fixation (V1, Pl. 1, fig. 1), and those of type 2, which contain a coarsely precipitated material (V2, Pl. 1, fig. 1). The vacuoles are bounded by a single membrane. Only an occasional fragment of what appears to be endoplasmic reticulum is found in dormant spores (arrow, Pl. 1, fig. 1). The background cytoplasm appears coarse and somewhat like a flocculent precipitate (Pl. 1, fig. 1).

Inasmuch as the dormant spores were subjected to violent mechanical forces as a result of the methods used in fixation, it was necessary to assess the damage that may have been caused by this method of preparation. Accordingly, ascospores were activated and allowed to begin germination and were then broken in the fixative in the same manner as were dormant spores. The results are presented in Pl. 1, fig. 2, and reveals that the mitochondria do not appear to be swollen. Therefore, it seems reasonable, assuming that the spores were not fixed in the few seconds that they were in the fixative before breaking, that the swollen mitochondria found in the dormant ascospore are not merely the result of mechanical damage. Germinating ascospores prepared by being broken in fixative have endoplasmic reticulum (arrows, Pl. 1, fig. 2) of a type found in such spores that have not been broken (Pl. 2, fig. 3). Consequently, the lack of such membranes in the dormant cracked spores seems to be a normal feature of the dormant condition and not an artifact. Moreover, the vacuoles in broken germinating and dormant ascospores appear to be alike, but the coarsely precipitated cytoplasm of the dormant ascospores does not occur to the same degree as in the cracked germinating spores.

The protoplast of the germinating spore

The germinating ascospore contains numerous mitochondria of various shapes which are unlike the swollen ones found in the dormant spore (Pl. 2, fig. 3). Vacuoles of the two types described in the dormant ascospore also are found in germinating spores (V 1, V 2, Pl. 2, fig. 3). Endoplasmic reticular membranes of a type found in material fixed in permanganate are present in the ascospore (Pl. 2, fig. 3), as well as in the germ tube (Pl. 3, fig. 6). Nuclei are present and are more numerous in sections of germinating ascospores than in those of dormant ones (Pl. 2, fig. 3). The emergence of a germ tube through a germ pore can be seen in Pl. 3, figs. 5 and 6, where the continuity of the germ tube wall and the endosporium can be seen.

In germinating ascospores from 3 to 6 hr after activation a complex of concentric membranes that is continuous with the endoplasmic reticulum at one or more points is found. Such bodies are illustrated in sections from different spores in Pl. 4, figs. 7 and 8. The connexion between the outer membrane of these bodies and the endoplasmic reticulum of the spore is particularly well shown at the arrow in Pl. 4, fig. 7. In one instance, two such bodies were noted in one section, indicating that more than one can occur in a spore. However, the frequency of their occurrence must be low, because only an occasional section is found in which they occur.

DISCUSSION

Activation of dormant ascospores of *Neurospora tetrasperma* triggers a series of ultrastructural as well as metabolic and physiological changes. Among the most striking is in the endoplasmic reticulum, which, although very sparse in dormant ascospores, appears in larger amounts in germinating spores. Synthesis of endoplasmic reticulum seems to be a common feature of the germination process in fungus spores, for it has been observed in most of the cases studied, including sporangiospores of Rhizopus (Hawker & Abbott, 1963; Buckley, Sjaholm & Sommer, 1966), conidia of *Botrytis cinerea* (Hawker & Hendy, 1963) and uredospores of *Puccinia graminis tritici* (Williams & Ledingham, 1964). Moreover, spores of *Blastocladiella emersonii* also are devoid of endoplasmic reticulum, so that synthesis must occur upon their germination (Cantino, Lovett, Leak & Lythgoe, 1963).

A membrane complex, often connected to elements of the endoplasmic reticulum (Pl. 4, figs. 7, 8) is formed after activation but was not seen in germinating spores after 8 hr. Its appearance during the time of active synthesis of the endoplasmic reticulum, and its continuity with these membranes, suggests a function in the generation of the reticulum. A similar organelle may be the site of unit membrane synthesis in some animals, and consists of convolutions of endoplasmic reticulum, according to Robertson (1961). That these membrane complexes may be induced in higher plants under the stress of treatments like high doses of radiation, and anaerobiosis, has been reported by Whaley, Kephart & Mollenhauer (1964). Moreover, anaerobically growing *Torulopsis utilis* forms membranous organelles (Linnane, Vitols & Nowland, 1962) which may be involved in the formation of mitochondria, because they show dehydrogenase activity. Membrane complexes also have been found in actinomycetes (Overman & Pine, 1963), hyphae of *Armillaria mellae* (Berliner & Duff, 1965) and in somatic cells of Sporobolomyces (Prusso & Wells, 1967). Therefore, membrane complexes are widely distributed and may have diverse roles.

Another change which occurs during germination is in the size of the mitochondria in that they are very large in dormant ascospores of *Neurospora tetrasperma* but much smaller and more numerous in germinating spores. Whether this change in size is associated with biochemical differences like that described by Holton (1960) is not known for Neurospora but Biggs & Linnane (1963) indicated that the cytochrome content of yeast cells and the size and organization of mitochondria were closely related. A decrease in the size of mitochondria during spore germination has been noted in a Rhizopus species (Hawker & Abbott, 1963) and the latter authors also reported increased numbers after germination, as did Hawker & Hendy (1963) in *Botrytis cinerea* conidia. This must also be the case for *Blastocladiella emersonii*, in whose spores only a single mitochondrion exists (Cantino *et al.* 1963). More cristae have been reported to be present in the mitochondria of the germ tube of the wheat stem rust than in its uredospores and their orientation is more regular as well (Williams & Ledingham, 1964). Therefore, changes in mitochondria during the germination of fungus spores are common, at least in the relatively few cases reported. However, as Hawker & Abbott (1963) pointed out, care must be taken to ascertain that artifacts do not arise because of the difficulties encountered in fixing and embedding fungus spores, difficulties to which we can readily attest.

That nuclear divisions occur during germination is suggested by the fact that more nuclei are seen after activation than before. It is likely that these occur before protrusion of the germ tube but their number remains to be determined.

The studies have shown that the wall of *Neurospora tetrasperma* ascospores consists of at least 5 distinct elements: endosporium, episporium, perisporium, a layer on the outside of the latter and one which is between the two innermost walls. The dense layer associated with the endosporium may be responsible for the relative impermeability of dormant ascospores, for it is the only layer that appears to be ruptured upon germination (arrow, Pl. 3, fig. 6). Moreover, the perisporium and episporium, being perforate at the germ pore (Lowry & Sussman, 1958), cannot act as barriers; nor does it seem likely that the endosporium acts in this way for it is continuous with the wall of the germ tube, which is readily permeable to many substances.

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EXPLANATION OF PLATES

Electron micrographs of ascospores of *Neurospora tetrasperma*

Abbreviations used: A, perisporium; B, episporium; C, endosporium; ER, endoplasmic reticulum; M, mitochondrion; N, nucleus; V1, vacuole lacking precipitate; V2, vacuole having precipitate.

PLATE 1

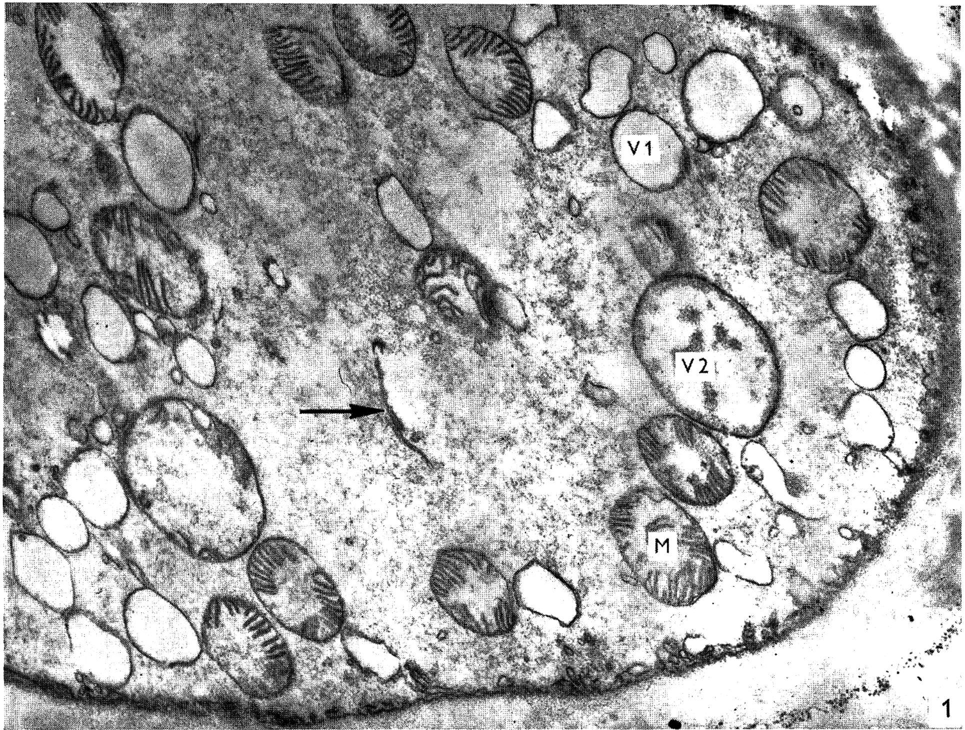
Fig. 1. Dormant ascospore showing two types of vacuoles, V 1 and V 2, and endoplasmic reticulum-like membrane at arrow. Note also the swollen mitochondria (M). (× 18,000.)

Fig. 2. A cracked, germinating ascospore showing typical endoplasmic reticulum (arrows), and a nucleus (N). Vacuoles and mitochondria also are present. (× 14,000.)

PLATE 2

Fig. 3. Germinating ascospore showing wall layers, nuclei (N), mitochondria (M) and vacuoles. The arrow indicates the less dense component of the outer wall layer. (× 8000.)

Fig. 4. A portion of a cross-section of a germinating ascospore showing the wall layers: perisporium (A), consisting of electron-dense ridges and a less dense component (arrow) covering the ridges and filling the space between them; episporium (B), showing characteristic features due to cutting; endosporium (C), which has an extremely dense layer indicated by the arrow. (× 31,000.)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V2
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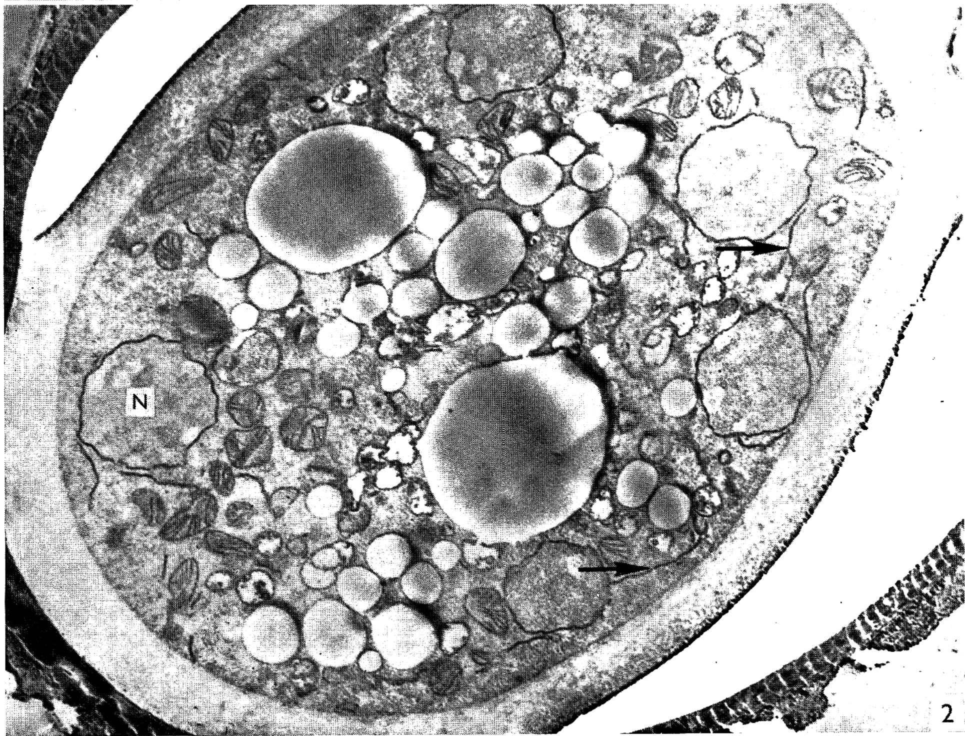
1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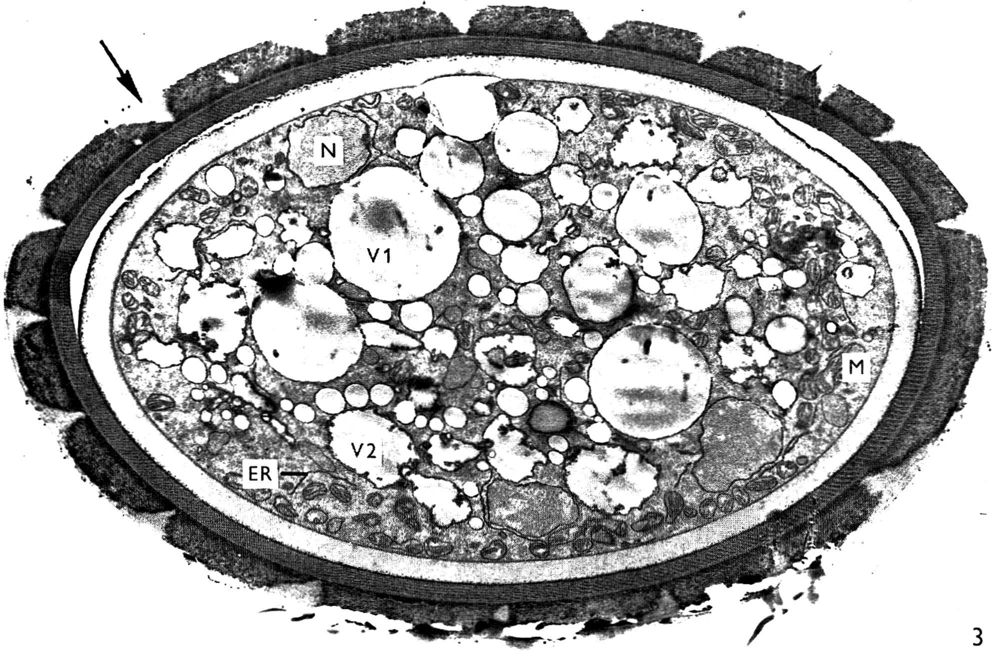

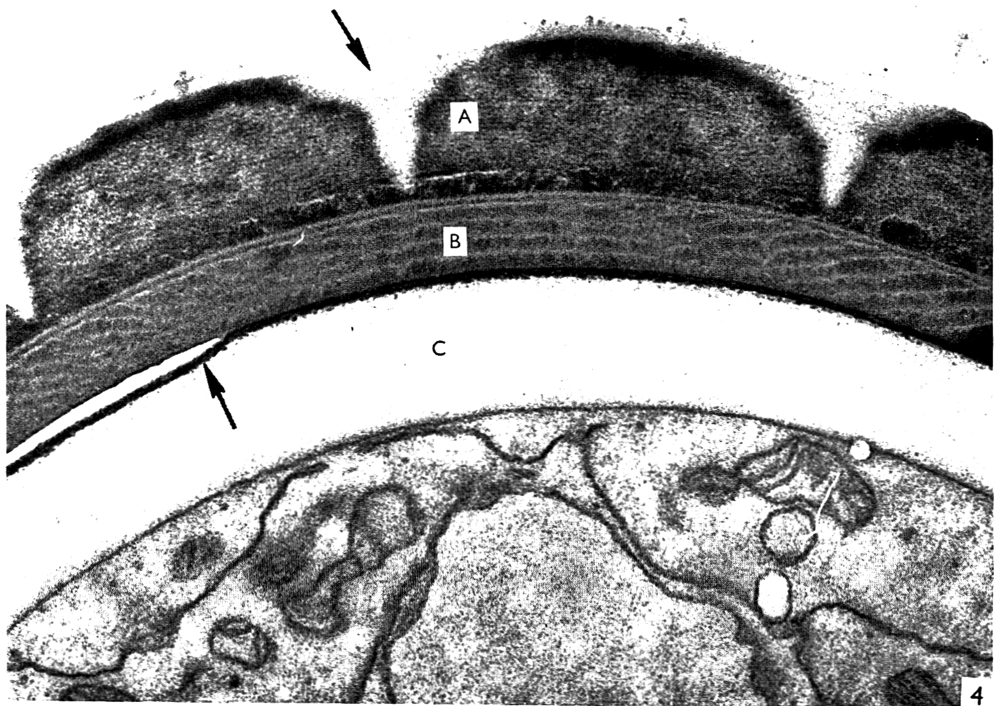

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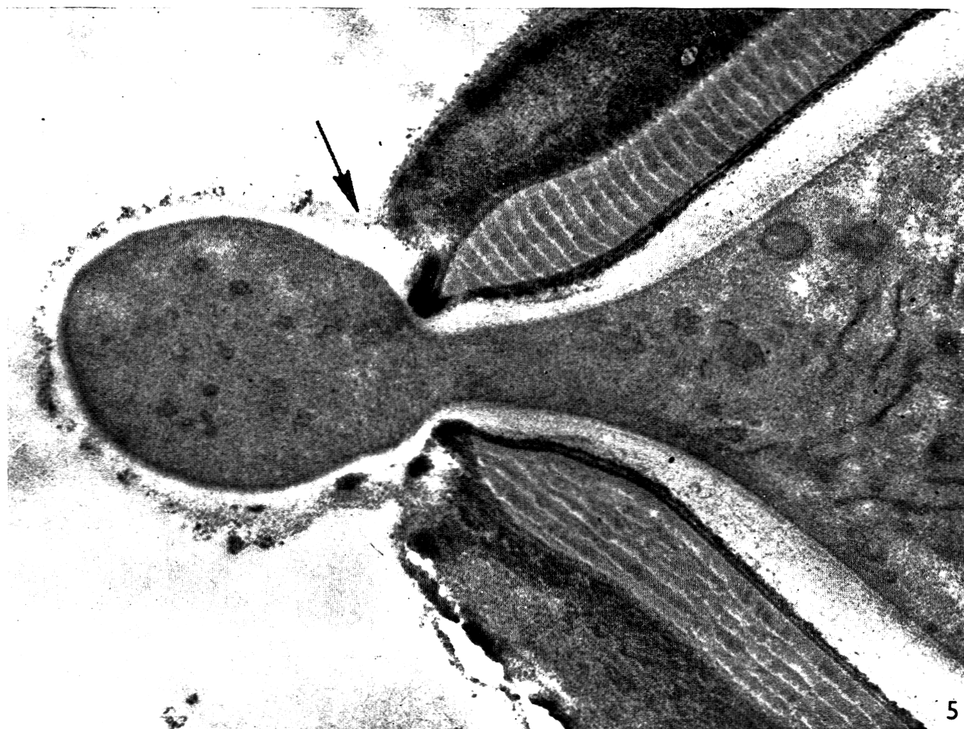

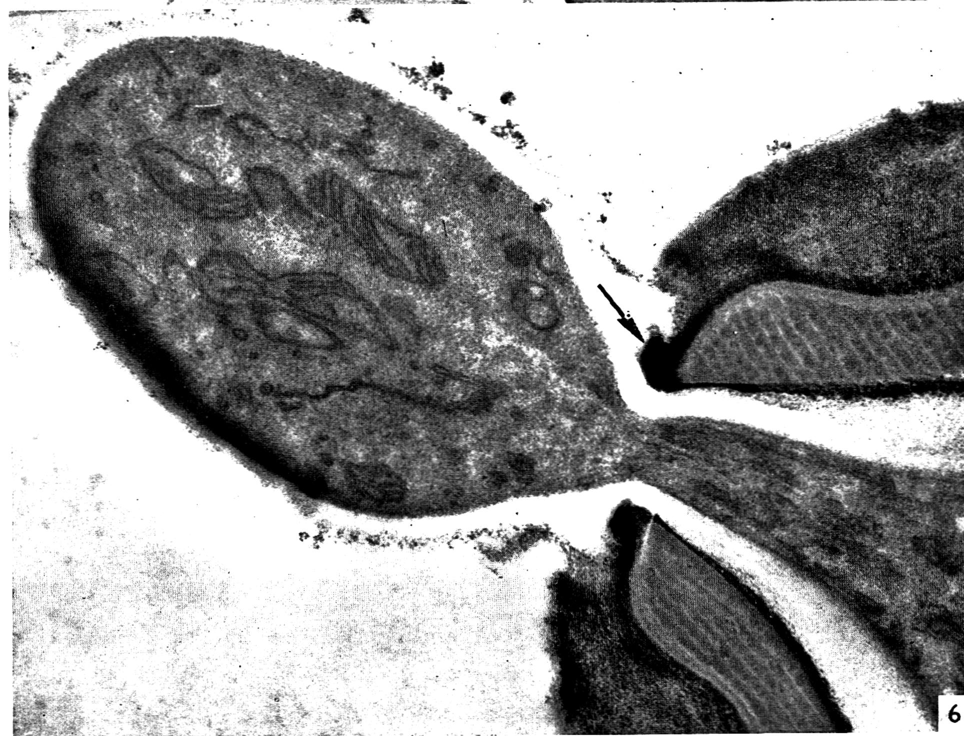

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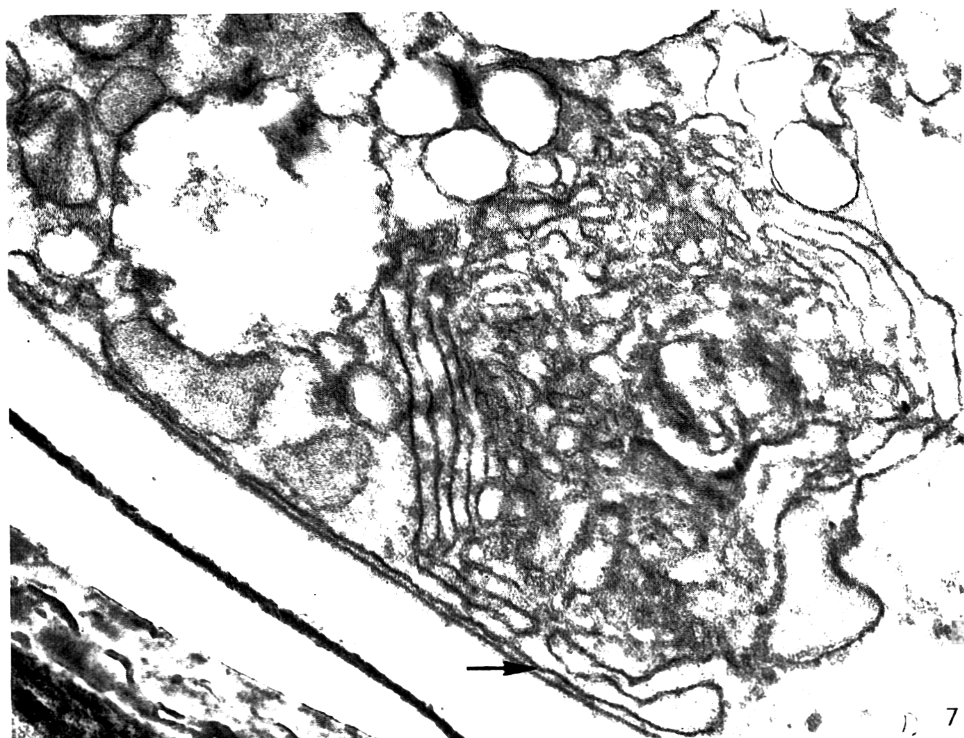

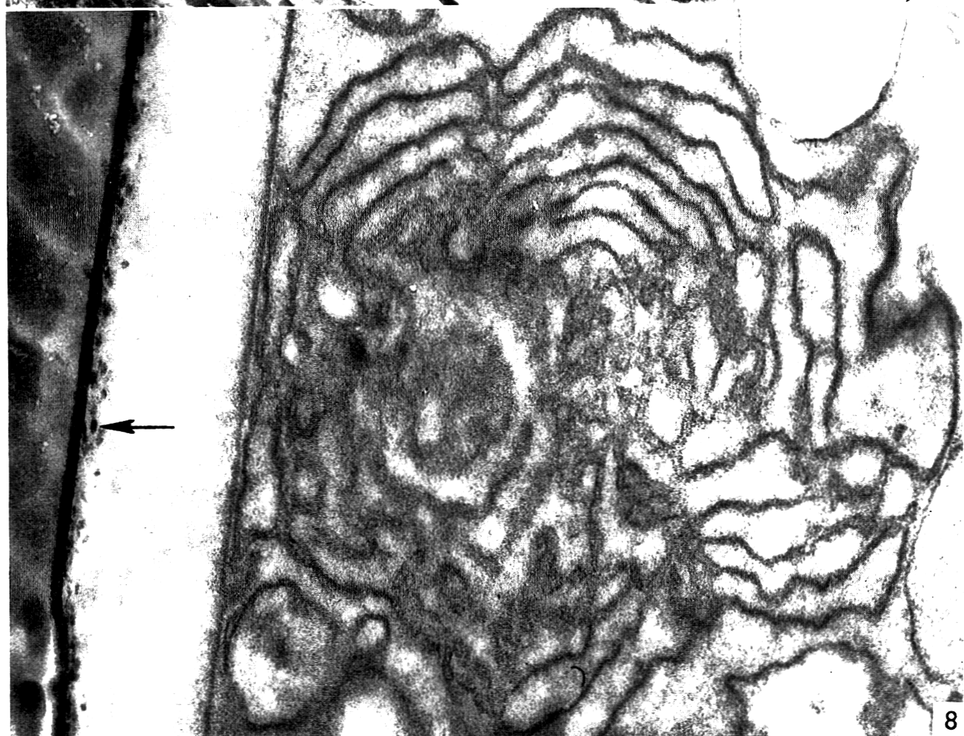

R. J. LOWRY AND A. S. SUSSMAN

PLATE 3

Fig. 5. A germinating ascospore showing the emerging germ tube and the continuity of its wall with the endosporium. The continuity of the outermost layer of the spore wall and the fibrous layer of the germ tube wall also can be seen at the arrow. (× 32,000.)

Fig. 6. An older germ tube showing mitochondria and endoplasmic reticulum. Material which may have formed a plug in the germ pore before germination began is shown at the arrow. (× 32,000.)

PLATE 4

Fig. 7. Ascospore in an early stage of germination, before protrusion of the germ tube. The continuity of the membrane complex and the endoplasmic reticulum is indicated at the arrow. (× 32,000.)

Fig. 8. Ascospore in an early stage of germination, before protrusion of the germ tube. Arrow indicates the very electron-dense wall layer which occurs between the episporium and endosporium. (× 35,000.)

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Levels of Resistance in ribosomes from Genetically Linked, Streptomycin-resistant Mutants of Pneumococcus

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SUMMARY

A streptomycin-sensitive strain and six streptomycin-resistant mutants of pneumococcus have been studied. These strains differed in resistance to streptomycin over a 5000-fold range, and the resistance mutations occurred at recombinationally distinct sites of a single genetic locus (the *str* locus). The effects of streptomycin on cell-free amino acid incorporating systems prepared from each of the strains were studied. Both polyuridylic acid (poly U)- and endogenous mRNA-directed systems were employed. As in *Escherichia coli*, sensitivity to streptomycin was found to reside in the ribosomal fraction of the amino acid incorporating system.

Streptomycin caused both inhibition of ^{14}C-phenylalanine incorporation and stimulation of ^{3}H-isoleucine incorporation in poly U-directed experiments. The resistance of the amino acid incorporating systems to these effects of streptomycin paralleled the streptomycin resistance of the strain from which the system was derived. In endogenous mRNA-directed systems the incorporation of ^{14}C-valine and ^{3}H-isoleucine was followed simultaneously. Streptomycin caused an inhibition of valine incorporation and a lesser inhibition of isoleucine incorporation. At a higher magnesium concentration streptomycin caused a stimulation of isoleucine incorporation while still inhibiting valine incorporation. The disparate behaviour of isoleucine and valine incorporation in the presence of streptomycin may be due to streptomycin-induced misreading of endogenous mRNA. As in the poly U-directed experiments, the magnitude of the effects of streptomycin on endogenous mRNA directed amino acid incorporation correlated in an inverse fashion with the resistance of the strain from which the system was prepared.

These results lead us to conclude that the ribosome is the primary target for streptomycin in pneumococcus. We propose that the affinity of the ribosome for streptomycin at a critical site or sites determines the level of resistance of the bacterium, and that this affinity is affected by mutations in the *str* locus.

INTRODUCTION

In *Escherichia coli* mutations to streptomycin (SM) resistance and dependence are closely linked (Newcombe & Nyholm, 1950). Rotheim & Ravin (1961, 1964) and Mishra & Ravin (1966) showed that this is true as well in pneumococcus. By means of transformation analysis a large number of streptomycin resistance mutations was mapped in this organism. Although these mutations varied greatly in phenotype, raising the level of resistance of the pneumococcus by as little as tenfold to as much as several thousand fold, they were all found to occupy sites within a single genetic

locus. Mutations conferring relatively low levels of resistance were usually inter-recombinable, while mutations conferring high levels of resistance replaced the low-level resistance mutations rather than combining with them.

Several workers (Flaks, Cox, Witting & White, 1962; Speyer, Lengyel & Basilio, 1962; Davies, 1964; van Knippenberg *et al.* 1965) have shown that a mutation to streptomycin resistance in *Escherichia coli* is associated with an alteration in the ribosomes, so that ribosomes isolated from resistant cells are comparatively resistant to the stimulatory, inhibitory, and misreading effects of streptomycin *in vitro*. Streptomycin resistance in *E. coli* is not always associated, however, with resistant ribosomes. Rosenkranz (1964) has described a streptomycin-resistant *E. coli* strain in which the resistance was due to the presence of a multiple-drug-resistance episome. In this case the cell apparently became impermeable to, or destroyed the antibiotic while the ribosomes remained sensitive.

The purpose of the present investigation was to determine whether the ribosomes of the previously mapped pneumococcal mutants were resistant to streptomycin, and if they were to compare the level of resistance of the ribosomes to the level of resistance of the cells from which the ribosomes had been isolated. Ribosomal resistance was studied *in vitro* by determining the levels of streptomycin which were necessary to cause inhibition of amino acid incorporation and misreading in cell-free systems prepared from each of the mutants. Both poly U- and endogenous mRNA-directed cell-free systems were studied.

METHODS

Materials. L-valine-1-^{14}C and DL-phenylalanine-1-^{14}C were obtained from New England Nuclear Corp., Boston, Mass. In addition, some of the valine used was synthesized by Dr Leon Miller of the University of Rochester. L-isoleucine-^{3}H was obtained from Schwarz BioResearch, Inc., Orangeburg, N.Y. ATP and phosphocreatine were purchased from Sigma, St Louis, Mo. Mercaptoethanol and GTP were obtained from Calbiochem, Los Angeles, Cal.; *Escherichia coli* B sRNA (stripped) came from General Biochemicals, Chagrin Falls, Ohio; and poly U was purchased from Miles Chemical Co., Elkhart, Ind. Streptomycin sulphate was purchased from Eli Lilly and Co. Erythromycin lactobionate was obtained from Abbott Laboratories and chlortetracycline from Lederle Laboratories.

Strains. The strains used and the maximum amounts of streptomycin which they can withstand *in vivo* without significant loss of colony-forming ability are as follows: *SIII-1* (sensitive, about 2 μg./ml.); *str-r 42* (30 μg./ml.); *str-r 3* (150 μg./ml.); *str-r 48* (150 μg./ml.); *str-r 2* (300 μg./ml.); *str-r 1* (5000 μg./ml.); and *str-r 51* (between 10,000 and 15,000 μg./ml.). Streptomycin kills at concentrations exceeding those listed for the respective strains, the rate of killing being dependent upon the concentration of antibiotic. The origins and genetic relationships among these strains have previously been reported (Rotheim & Ravin, 1961, 1964); the most recent genetic map is contained in Mishra & Ravin (1966).

Growth of organisms. The media and procedure for growing pneumococcus have previously been described (Ephrussi-Taylor, 1951; Rotheim & Ravin, 1961). The cocci were harvested after 75 ml. of N-NaOH (necessary for the neutralization of the acid produced by the growing cells) had been added to a 3 l. volume of a growing culture. At this point the cell density, determined by viability count, was about 1×10^{9}

cocci/ml. Six litres of culture yielded 9–11 g. of wet cocci. The harvested cocci could be frozen or used immediately for the preparation of a cell-free extract.

Cell-free extract (S-30). Cell-free extracts were prepared essentially according to the method of Matthaei & Nirenberg (1961). When it was appropriate, the S-30 extract was further separated into ribosome and S-100 fractions as described by these authors.

Preincubated S-30 (S-30 pre.). When poly U was the messenger, the S-30 was preincubated (Nirenberg & Matthaei, 1961) in order to minimize incorporation due to the endogenous messenger. S-30 and preincubated S-30 were stored at -65° until use.

Amino acid incorporation in cell-free extracts. Endogenous mRNA-directed incorporation was carried out in a volume of 0·5 ml. The following were present (μmoles/ml.): 100 tris(hydroxymethyl)-aminomethane, pH 7·8 at 37°; 100 ammonium acetate, 11 or 16 magnesium acetate; 6·0 mercaptoethanol; 0·03 each of 20 L-amino acids minus valine; 0·03 ^{14}C-valine (10 μc/μmole); 0·03 GTP, 6·0 phosphocreatine; 1·0 ATP; 40 μg. creatine kinase; from 1 to 2 mg./ml. cell-free extract protein. In most experiments the incorporation of ^{3}H-isoleucine and ^{14}C-valine was followed simultaneously, in which case isoleucine was omitted from the amino acid mixture and 0·03 μmole ^{3}H-L-isoleucine (100 μc/μmole) was added in its place. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1956) using a standard solution of bovine serum albumin (Standard Scientific Supply Corp., New York, N.Y.) as a standard. Antibiotics were added where indicated.

The same components were present when poly U was the messenger except that the cold amino acid mixture was omitted and the labelled amino acids present were ^{14}C-DL-phenylalanine (5 μc/μmole) and ^{3}H-L-isoleucine (100 μc/μmole). 300 μg./ml. of *Escherichia coli* B sRNA (stripped) was added as a supplement. Poly U was present at a concentration of 20 μg./ml. All assays were performed in duplicate or triplicate.

Poly U-directed phenylalanine incorporation did not occur at 37°, but took place when the incubation temperature was lowered to 27°. Our investigations of this temperature effect show that a component of the pneumococcal ribosome can destroy poly U at 37° but not at 27°. Because of this effect, poly U-directed reactions were incubated for 2 hr at 27°, at which time they were virtually completed. Amino acid incorporation directed by endogenous mRNA was found to take place at either 27° or 37°, and since only the rate of incorporation is affected by temperature, the reactions were incubated at the higher temperature. The reactions were completed in 45 min. Experiments were terminated by adding 0·5 ml. of 10% trichloroacetic acid. The protein precipitates were digested at 90–100° for 15 min., filtered on to discs of Whatman 542 filter paper, and washed with 4% trichloroacetic acid containing the appropriate cold amino acids at 1% concentration to reduce the adsorption of the labelled amino acids. The filter discs were counted in a Nuclear Chicago thin-window gas-flow counter, or when it was necessary to count ^{3}H and ^{14}C simultaneously, they were counted in a Packard Tri-Carb Liquid Scintillation Spectrometer. In the latter case NCS Reagent (Nuclear Chicago, Des Plaines, Ill.) (Hansen & Bush, 1967) was used to dissolve the protein precipitates in a toluene-based scintillation fluid. The efficiency of gas-flow counting for ^{14}C was about 25%. The efficiency of scintillation counting was 11% for ^{3}H and 50% for ^{14}C. No quenching correction was necessary since the protein precipitates themselves did not quench and NCS Reagent, which did quench, was present in the same concentration in samples and standards.

RESULTS

Effect of magnesium ion concentration on poly U-directed phenylalanine and isoleucine incorporation

Many workers have shown in the *Escherichia coli* system that the concentration of magnesium ion exercises an important influence on the kind and amount of amino acid incorporation (Davies, Gilbert & Gorini, 1964; So, Bodley & Davie, 1964; Szer & Ochoa, 1964; van Knippenberg *et al.* 1965). Therefore we studied the effect of magnesium concentration on amino acid incorporation into the endogenous mRNA- and poly U-directed cell-free system from *SIII-1*, the sensitive strain.

Figure 1 shows the incorporation of ^{14}C-phenylalanine and ^{3}H-isoleucine directed by poly U as a function of the magnesium concentration. The experiment was done both in the absence and in the presence of $1{\cdot}0 \times 10^{-5}$ M-streptomycin. It is evident that in pneumococcus as in *Escherichia coli* (Davies *et al.* 1964) streptomycin causes an inhibition of phenylalanine incorporation and a stimulation of isoleucine incorporation. The former appears to be maximal at 11 mM-magnesium, while the latter is greatest at 16–21 mM-magnesium. The explanation commonly given for the isoleucine incorporation, which occurs in the presence of streptomycin despite the fact that only the triplet UUU coding for phenylalanine is present, is that streptomycin causes misreading: in its presence the triplet UUU is sometimes read as AUU, which is a codon for isoleucine (Davies, Gorini & Davis, 1965). In this experiment no isoleucine was incorporated in the absence of streptomycin. In later experiments variable results were obtained so that sometimes a small incorporation of isoleucine was noted even though no antibiotic was present. Davies *et al.* (1964) have reported similar results with the *E. coli* system.

Effect of magnesium ion concentration on amino acid incorporation directed by endogenous mRNA

Figure 2 shows the incorporation of ^{14}C-valine and ^{3}H-isoleucine directed by endogenous mRNA as a function of magnesium concentration. About three times as much valine as isoleucine was incorporated at 10 mM-magnesium ion concentration in the absence of streptomycin. When $1{\cdot}0 \times 10^{-5}$ M-streptomycin was added, the incorporation of both amino acids was inhibited, but not to the same extent, the incorporation of valine being more greatly suppressed than that of isoleucine. In fact, at magnesium concentrations greater than 14 mM streptomycin caused an actual stimulation of isoleucine incorporation.

The magnesium concentration dictated, therefore, whether the incorporation of isoleucine in the endogenous system would be stimulated by streptomycin or partially inhibited, but at all magnesium concentrations the addition of streptomycin resulted in an increase in the ratio of isoleucine to valine incorporated. The increase did not occur, however, if the cell-free system was derived from a highly resistant strain (see Fig. 7A, B). In a cell-free system derived from a sensitive strain the ratio of isoleucine to valine incorporated was usually about 0·5 and rose to 1·0 or more in the presence of streptomycin. The ratio was not significantly influenced by the time of incubation (Fig. 3) or by the presence of the antibiotics erythromycin (EM) or chlortetracycline (CTC) which, like streptomycin, caused inhibition of phenylalanine incorporation in

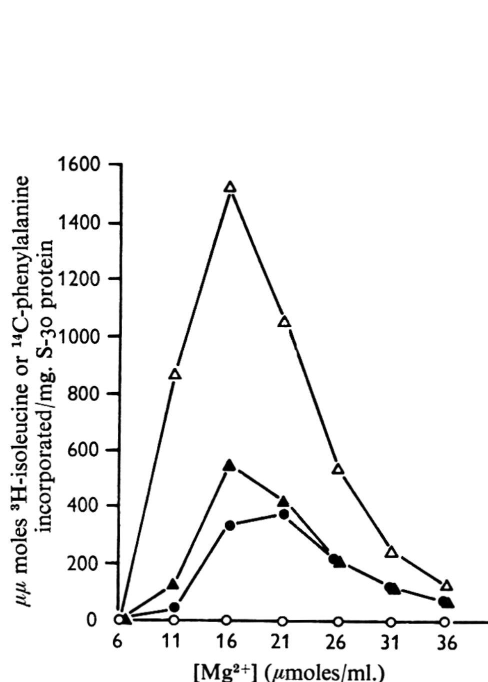

Fig. 1

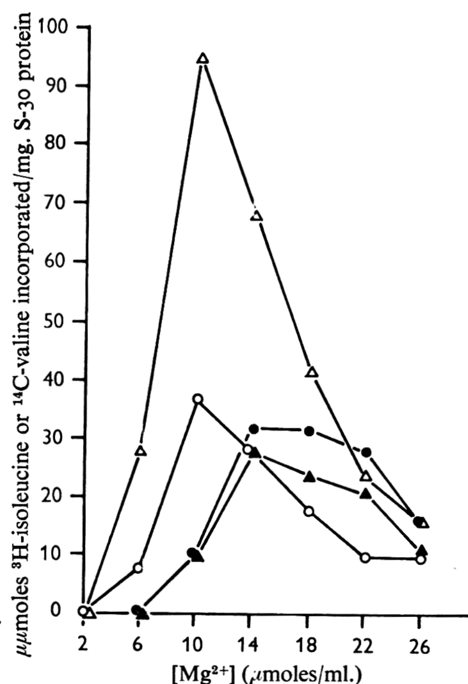

Fig. 2

Fig. 1. Incorporation of ^{14}C-phenylalanine and ^{3}H-isoleucine into *SIII-1* S-30 stimulated by poly U, in the absence and in the presence of SM ($1{\cdot}0 \times 10^{-6}$ M) as a function of Mg^{2+} concentration. Symbols: △, phenylalanine in the absence of SM; ▲, phenylalanine in the presence of SM; ○, isoleucine in the absence of SM; ●, isoleucine in the presence of SM.

Fig. 2. Incorporation of ^{14}C-valine and ^{3}H-isoleucine into *SIII-1* S-30, stimulated by endogenous mRNA in the absence and in the presence of SM ($1{\cdot}0 \times 10^{-5}$ M) as a function of Mg^{2+} concentration. Symbols: △, valine in the absence of SM; ▲, valine in the presence of SM; ○, isoleucine in the absence of SM; ●, isoleucine in the presence of SM.

Table 1. *Influence of chlortetracycline (CTC), erythromycin (EM), and streptomycin (SM) on the incorporation of ^{14}C-phenylalanine and ^{3}H-isoleucine into SIII-1 S-30 pre. under poly U direction, and on the incorporation of ^{14}C-valine and ^{3}H-isoleucine into SIII-1 S-30 under the direction of endogenous mRNA.*

The Mg^{2+} concentration was 11 mM in the endogenous mRNA-directed experiments and 16 mM in the poly U-directed experiments.

Expt.	Addition	Messenger*	Isoleu. inc. ($\mu\mu$moles/ mg. prot.)	Valine inc. ($\mu\mu$moles/ mg. prot.)	Phe. inc. ($\mu\mu$moles/ mg. prot.)	Ratio isoleu.:valine or isoleu.:phe.
1	None	Poly U	0	—	414	0·00
2	1×10^{-4} M SM	Poly U	155	—	231	0·67
3	1×10^{-6} M CTC	Poly U	0	—	45	0·00
4	1×10^{-4} M EM	Poly U	4	—	257	0·02
5	None	End.	72	132	—	0·55
6	1×10^{-5} M SM	End.	28	26	—	1·08
7	1×10^{-6} M CTC	End.	3	12	—	0·25
8	1×10^{-4} M EM	End.	38	64	—	0·59

* End. = endogenous mRNA.

poly-U-directed systems, but did not cause the phenylalanine–isoleucine ambiguity (Table 1). The increase in the isoleucine-to-valine ratio appeared therefore to be streptomycin-specific.

Demonstration of ribosomal basis of streptomycin resistance

In order to determine whether the mutants in our possession, like streptomycin-resistant *Escherichia coli* cells and the streptomycin-resistant pneumococcal mutant reported by Sawada & Suzuki (1964), have acquired streptomycin-resistant ribosomes,

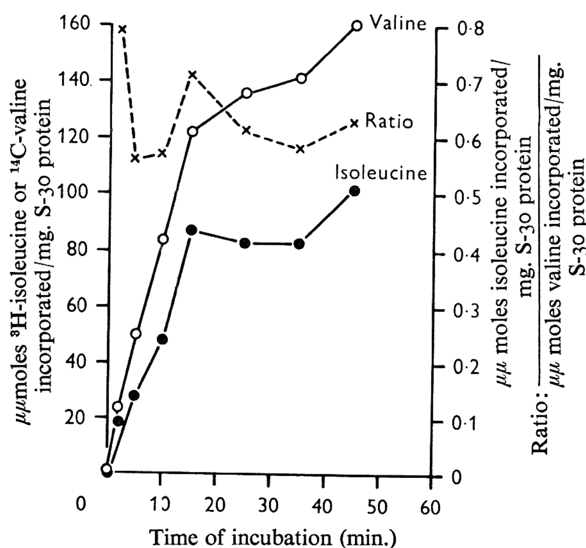

Fig. 3

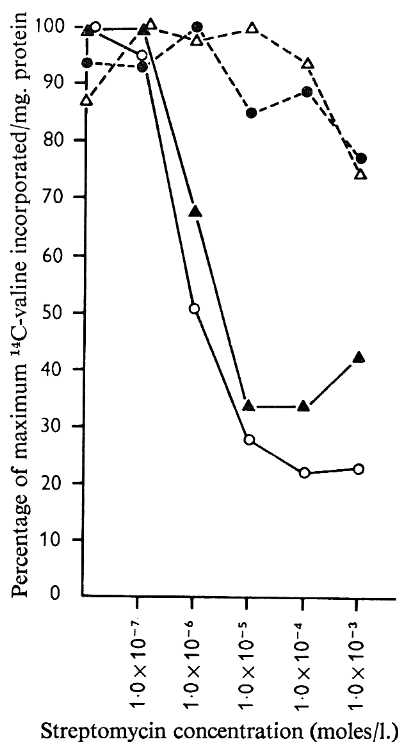

Fig. 4

Fig. 3. The endogenous mRNA-directed incorporation of ^{14}C-valine and ^{3}H-isoleucine into *SIII-1* S-30 *v.* time. The ratio of μμmoles isoleucine to μμmoles valine incorporated is also plotted. The Mg^{2+} concentration was 11 mM.

Fig. 4. The effect of streptomycin on ^{14}C-valine incorporation into ribosomes-plus-S-100 from *SIII-1* (sensitive), *str-r 51* (resistant), and interchanged ribosomes and supernatant from the two organisms. Symbols: ○, *SIII-1* ribosomes plus *SIII-1*, S-100; △, *str-r 51* ribosomes plus *str-r 51* S-100; ●, *str-r 51* ribosomes plus *SIII-1* S-100; ▲, *SIII-1* ribosomes plus *str-r 51* S-100. The Mg^{2+} concentration in these experiments was 12 mM. The incorporation of valine in the absence of SM varied from 90 to 180 μμmoles/mg. protein.

a cross experiment was performed using cell-free extracts from the sensitive *SIII-1* and resistant *str-r51* strains. The *str-r51* strain was chosen because the *str-r51* mutation genetically replaces (i.e. is allelic with) all of the other *str-r* mutations used in this study, and hence any finding concerning the biochemical locus affected by the *str-r51* mutation would be applicable to the other mutations as well. Figure 4 shows that the incorporation of ^{14}C-valine directed by endogenous mRNA is inhibited by strepto-

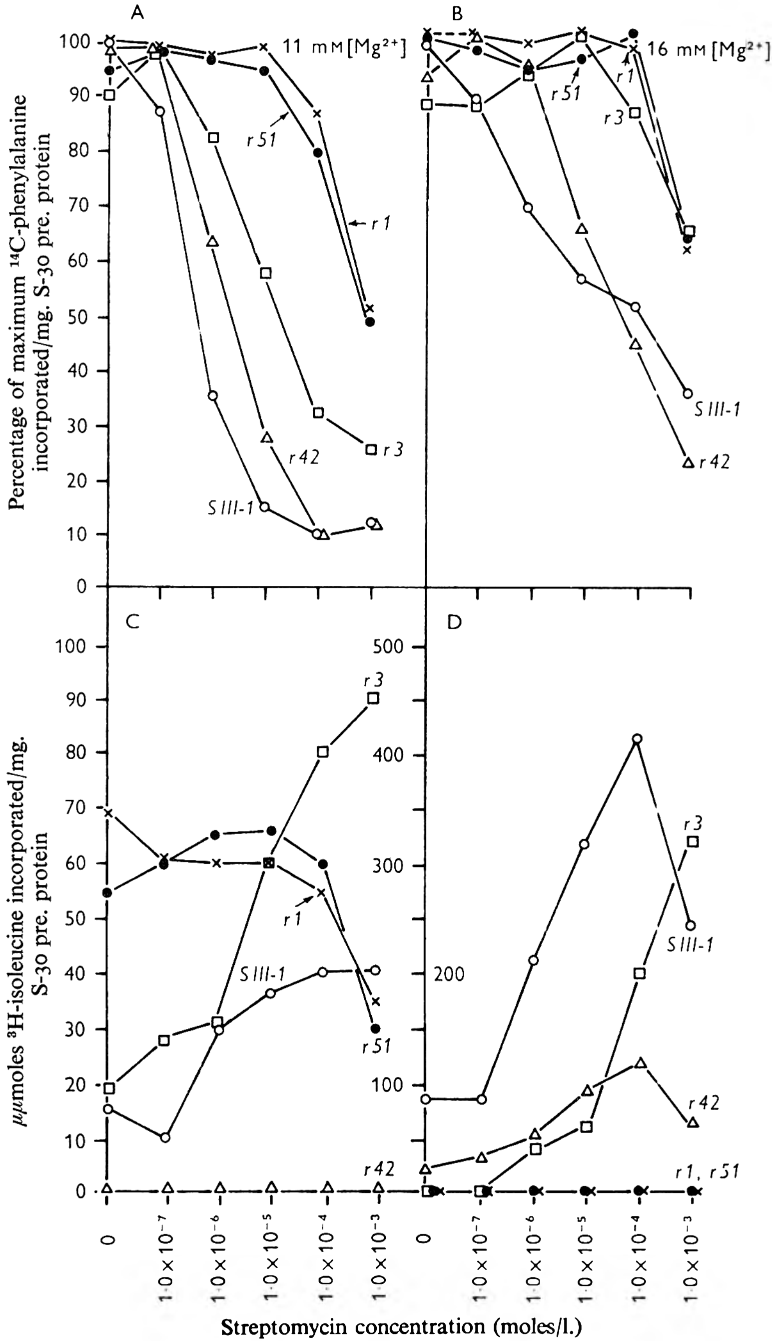

Fig. 5. The effect of SM on the poly U-directed incorporation of ^{14}C-phenylalanine and ^{3}H-isoleucine into S-30 pre. from a sensitive and four SM-resistant strains of pneumococcus: A and B, incorporation of ^{14}C-phenylalanine at 11 mM and 16 mM-Mg^{2+}; C and D, incorporation of ^{3}H-isoleucine at 11 mM and 16mM-Mg^{2+}. The maximal incorporation of ^{14}C-phenylalanine at 11 mM-Mg^{2+} varied from 2000 to 760 μμmole/mg., depending on the preparation used. At 16 mM-Mg^{2+} the maximum incorporation of phenylalanine varied from 1860 to 760 μμ-mole/mg.

mycin only when sensitive ribosomes from *SIII-1* are present in the incubation mixture. The source of the supernatant has no influence on the results.

Parallelism of in vivo bacterial resistance and in vitro resistance of ribosomes

It being ascertained that resistant ribosomes were associated with mutations to resistance *in vivo*, further experiments were performed using mutants making up a spectrum of resistance varying from 30 μg./ml. to slightly over 10,000 μg./ml. of streptomycin. The question to be answered was whether the resistance of the ribosomes of the different mutants to the inhibitory and misreading actions of streptomycin *in vitro* paralleled the *in vivo* levels of resistance of the organisms. Both endogenous mRNA- and poly U-directed systems were studied, and experiments were performed at each of two magnesium concentrations, 11 mM and 16 mM, which are near optimal for streptomycin-induced inhibition and misreading respectively. Figure 5 shows the poly U-directed incorporation of phenylalanine and isoleucine into preincubated S-30 (S-30 pre.) prepared from five of the seven pneumococcal strains which are listed in the Methods section. No data are shown for the other two strains, *str-r48* and *str-r2*. It was found that the ribosomes of *str-r48* could not be distinguished from those of *str-r3*. Both mutants have the same *in vivo* streptomycin resistance. Although the amino acid incorporating system from *str-r2* was clearly more resistant to streptomycin than that obtained from the sensitive strain, repeated preparations failed to give as active a system as that obtained from all of the other strains we have studied. We suspect that there may be something unusual about the ribosomes of this mutant. Perhaps they are more fragile than those of the other mutants.

At 11 mM-magnesium in poly U-directed systems there was a good correlation between the *in vivo* resistance of the mutant which supplied the ribosomes and the level of resistance of the ribosomes themselves to the inhibition of phenylalanine incorporation by streptomycin (Fig. 5A). *SIII-1*, *str-r42*, and *str-r3* could be distinguished from one another and from *str-r1* and *str-r51*. The latter two strains could not, however, be distinguished from one another. These two strains are closer to each other in *in vivo* level of resistance than are the others.

At 11 mM magnesium there was no obvious correlation between the quantities of isoleucine incorporated because of misreading and the levels of resistance of the mutants (Fig. 5C). In these experiments there was sometimes isoleucine incorporation at a low level in the absence of streptomycin.

When the magnesium ion concentration was raised to 16 mM (Fig. 5B, D) the differences among the ribosomes became less distinguishable as far as resistance to streptomycin-caused inhibition of phenylalanine incorporation was concerned, but at the higher magnesium concentration a correlation could be seen between the level of streptomycin needed to stimulate isoleucine incorporation and the level of resistance of the strain from which the ribosomes were taken.

In summary, in poly U-directed systems, at a magnesium concentration favouring inhibition of amino acid incorporation, ribosomal resistance to such inhibition correlated well with the *in vivo* resistance of the strains. Similarly, at a magnesium concentration favouring misreading (isoleucine incorporation), ribosomal resistance to such misreading was again well correlated with the *in vivo* levels of resistance.

The findings with endogenous mRNA directed systems at 11 and 16 mM-magnesium were in many ways similar to those obtained with the poly U system. Figure 6 shows

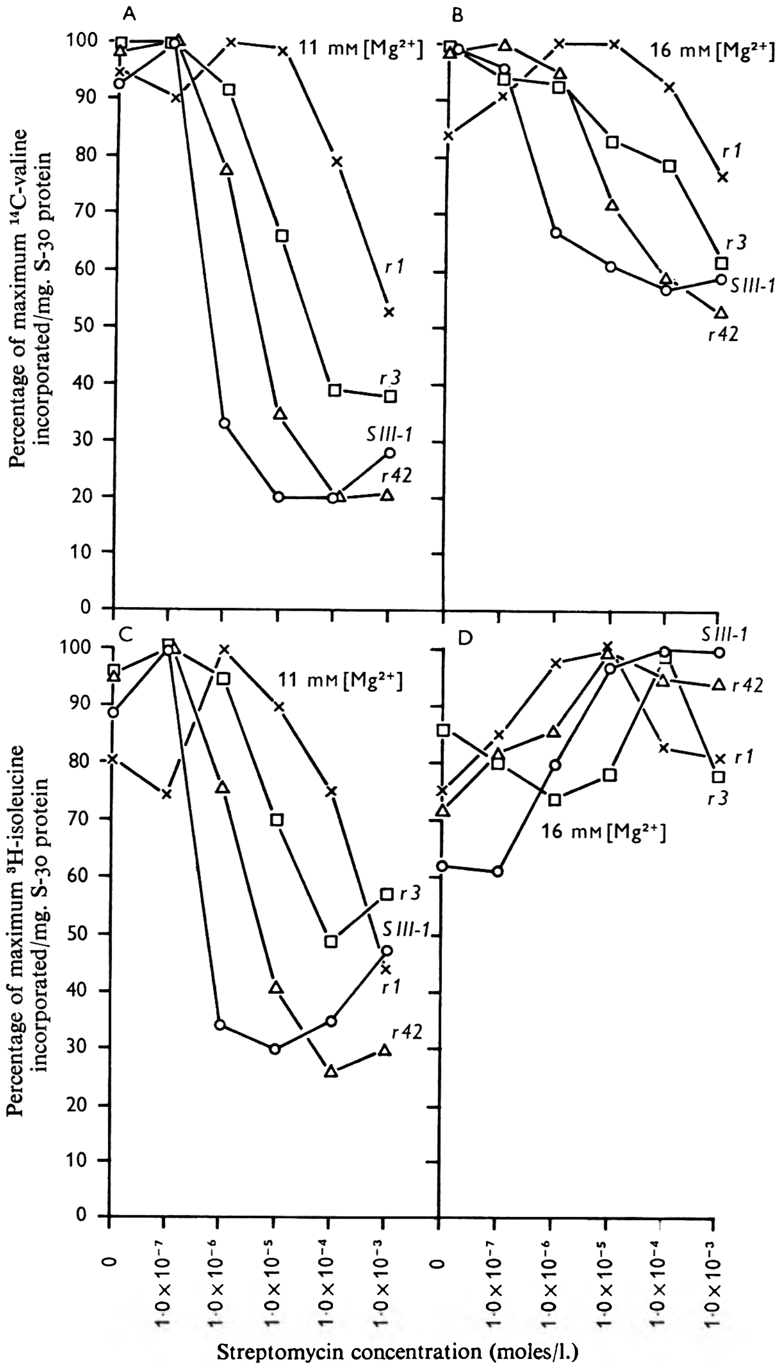

Fig. 6. The effect of SM on the endogenous mRNA-directed incorporation of ^{14}C-valine and ^{3}H-isoleucine into S-30 from a sensitive and three SM-resistant strains of pneumococcus: A and B, incorporation of ^{14}C-valine at 11 mM and 16 mM-Mg^{2+}; C and D, incorporation of ^{3}H-isoleucine at 11 mM and 16 mM-Mg^{2+}. The maximal incorporation of ^{14}C-valine at 11 mM-Mg^{2+} varied from 280 to 120 $\mu\mu$moles/mg., depending on the preparation used. At 16 mM-Mg^{2+} the maximum incorporation of valine varied from 160 to 85 $\mu\mu$mole/mg.

these results. The same strains were studied as in the poly U experiments except that *str-r51* was not included because it was not expected that it could be distinguished in any way from *str-r1*.

As in the poly U experiments, a correlation was observed between the *in vivo* resistance of the pneumococcal mutants and the level of resistance of their ribosomes to the inhibitory action of streptomycin on ^{14}C-valine incorporation. In the endogenous system this correlation was found at both 11 mM (Fig. 6A) and 16 mM-magnesium (Fig. 6B), but the inhibitory action of the antibiotic was less at the higher magnesium concentration. Table 2 shows a detailed comparison between the *in vitro* resistances of the mutant ribosomes to streptomycin-caused inhibition of valine incorporation at 11 mM magnesium and the *in vivo* resistance of the organisms which supplied the ribosomes. The streptomycin concentration at which half of the maximal inhibition of valine incorporation was achieved was taken as a measure of the *in vitro* resistance of the ribosomes. It can be seen that between 10 and 100 times as much streptomycin was needed to prevent a cell from forming a colony as to inhibit amino acid incorporation in a cell-free system derived from the cell.

Table 2. *Comparison between the in vitro resistance of ribosomes to inhibition of valine incorporation by SM at 11 mM-Mg^{2+} and the in vivo resistance of the cells which supplied the ribosomes*

Strain	Streptomycin concentration at which half max. inhibition achieved (moles/l.)	Resistance of ribosomes *in vitro* (μg. SM/ml.)	Resistance of cells *in vivo* (μg. SM/ml.)
SIII-1	4×10^{-7}	0·3	2
str-r 42	3×10^{-6}	2	30
str-r 3	8×10^{-6}	6	150
str-r 1	$1{\cdot}3 \times 10^{-4}$	95	5000

Unlike poly U, endogenous mRNA can be expected to code naturally for isoleucine. Indeed, the incorporation of isoleucine at 11 mM-magnesium could be inhibited by streptomycin in much the same fashion as valine in the endogenous system (Fig. 6C). However, the incorporation of isoleucine was not inhibitable to quite the same extent as valine, as noted earlier. This can be seen from Fig. 7A and B; here the isoleucine–valine ratio is plotted *v.* streptomycin concentration for the four pneumococcal strains. It is the increase of this ratio with increasing streptomycin concentration which indicates that misreading may be occurring in the translation of the endogenous messenger. The amount of ratio increase which can be caused by the addition of streptomycin is inversely proportional to the *in vivo* resistance of the strain from which the cell-free system was derived.

At 16 mM-magnesium streptomycin causes an actual stimulation of isoleucine incorporation in all strains (Fig. 6D). In the case of the highly resistant mutant *str-r1* it is to be noted however, that the stimulation of isoleucine incorporation is accompanied by an equivalent stimulation of valine incorporation (Fig. 6B). Thus the isoleucine–valine ratio remains constant in *str-r1* with increasing streptomycin concentration (Fig. 7B). A similar stimulatory effect at low streptomycin concentrations on the incorporation of phenylalanine was observed consistently in poly U-directed experiments employing the *str-r3* cell-free preparation. We do not know the significance of this effect.

DISCUSSION

The discovery by Gorini & Kataja (1964) of *Escherichia coli* mutants which have a genetic defect suppressible by streptomycin provided the first evidence that the antibiotic can cause the misreading of mRNA. Such misreading has clearly been shown to occur in artificial polynucleotide-directed cell-free systems (Davies *et al.* 1964; Davies, Jones & Khorana, 1966). Schwartz (1965) has reported that streptomycin causes misreading of the asparagine codon when the RNA of f2 bacteriophage is employed as a messenger in an *E. coli* cell-free system. No evidence has appeared,

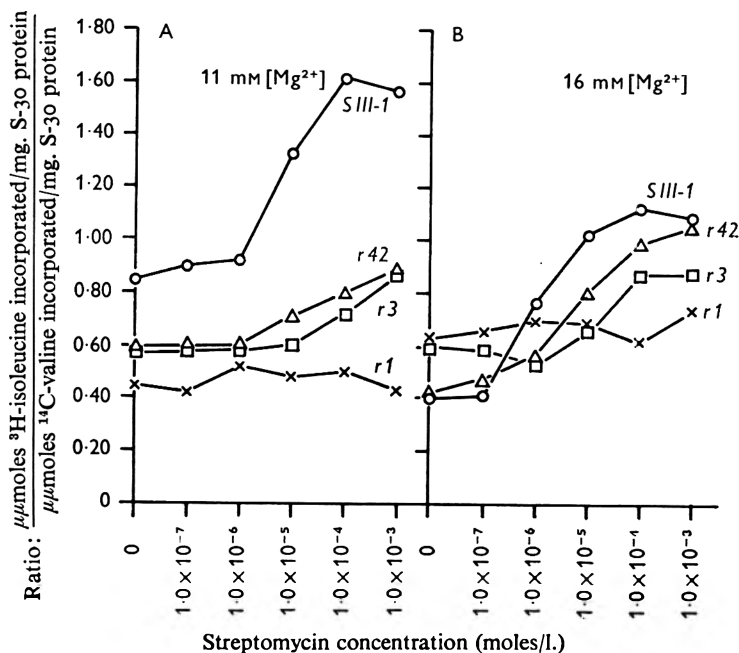

Fig. 7. The effect of SM on the ratio of the amounts of ^{3}H-isoleucine and ^{14}C-valine incorporated under the direction of endogenous messenger into S-30 from a sensitive and three SM-resistant strains of pneumococcus. Experiments at two different Mg^{2+} concentrations are shown.

however, which documents the misreading of endogenous mRNA *in vitro* under the influence of streptomycin. In this regard, our finding that streptomycin causes an increase in the ratio of isoleucine to valine incorporated into cell-free systems prepared from sensitive and low-resistance strains is of interest. The increase in the ratio can be interpreted as the result of the streptomycin-induced misreading of endogenous mRNA. There are two lines of evidence to support this idea. In the first place, the antibiotics erythromycin and chlortetracycline, which inhibit amino acid incorporation without causing misreading of poly U, do not bring about any alteration in the ratio of isoleucine to valine incorporated under the direction of endogenous mRNA. Secondly, the isoleucine–valine ratio in endogenous mRNA-directed experiments behaves much like the isoleucine-phenylalanine ratio in poly U-directed experiments. In both cases streptomycin causes an increase in isoleucine incorporation relative to

that of valine or phenylalanine, and the concentration of streptomycin needed to cause the increase is greater, the greater the resistance of the strain from which the amino acid incorporating system was prepared. In the poly U-directed system the streptomycin-induced increase in isoleucine incorporation can be clearly attributed to the misreading of UUU triplets. While it is not possible to determine which codons are being read in endogenous mRNA, one expects that streptomycin would change the relative amounts of some of the amino acids incorporated in a system directed by endogenous messenger. Interpreted in this light, therefore, the increase in the isoleucine–valine ratio which we observe in the presence of streptomycin could be the result of misreading of the endogenous messenger.

In considering other possible explanations for the ratio increase, the possibility was entertained that a fraction of the isoleucine incorporation was due to the activity of a soluble amino acid incorporating system like that reported by Kaji, Kaji & Novelli (1965) and that it was resistant to inhibition by streptomycin. This possibility was discarded, however, when we were unable to find evidence that such a system was contributing to the isoleucine incorporation in our experiments.

Spotts & Stanier (1961) were the first to propose that the ribosome is the primary site of the action of streptomycin. The evidence for this was, and still is, chiefly genetic: mutations to streptomycin resistance or dependence are allelic and result in demonstrable changes in the ribosome. According to the Spotts & Stanier hypothesis, the development of resistance to streptomycin is explained by the loss of affinity of the ribosomes in the resistant cells for the streptomycin molecule. Our results support the above ideas. Since genetic mapping of the *str* locus in pneumococcus has shown that mutations to different levels of streptomycin resistance in this organism arise within a single locus, and since the ribosomes are demonstrably changed when such mutations occur, it seems clear that the *str* locus of pneumococcus must carry the information for the synthesis of a ribosomal component. Whether this component is RNA or protein is not known with certainty. However, Leboy, Cox & Flaks (1964) have reported that the locus governing the synthesis of a certain ribosomal protein in *Escherichia coli* K 12 maps close to or is identical with the streptomycin locus. Their finding offers some support for the idea that the ribosomal component involved in streptomycin resistance is a protein.

Our experiments show, moreover, that there is a good correspondence between the *in vivo* resistance of ribosomes to streptomycin-caused inhibition and misreading and the *in vivo* resistance of the pneumococcal cells from which the ribosomes were derived. The widely different levels of resistance which occur in our strains can be explained by the idea that mutation-caused changes in the ribosome result in changes in the magnitude of the constant for ribosome-to-streptomycin binding. This idea implies that it is ribosomally bound streptomycin which causes interference with cell multiplication and cell death. Just how ribosomally bound streptomycin kills the cell is not clear. The exciting discovery of Gorini & Kataja (1964) and of Davies *et al.* (1964) that streptomycin causes misreading of mRNA *in vivo* and *in vitro* led for a time to the idea that streptomycin kills the cell by causing the formation of non-functional proteins. Later work (Freda & Cohen, 1966*a*, *b*; Stern, Barner & Cohen, 1966) has indicated that the misreading effect of streptomycin alone may not be able to account for cell death. The present state of knowledge about misreading has been reviewed by Gorini (1967).

There is evidence from Cohen's laboratory (Freda & Cohen, 1966*b*) that streptomycin killing is associated with an increase in ribosomal RNA synthesis. Cohen *et al.* (1967) have recently proposed that polycationic streptomycin may kill the cell by cross-linking the ribosome–DNA–RNA complex involved in co-ordinate transcription–translation. Our results are not inconsistent with this suggestion provided one postulates that it is the ribosome which dictates whether or not streptomycin can bind to the ribosome–RNA–DNA complex.

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The Effects of Various Anions and Cations on the Lysis of Yeast Protoplasts by Osmotic Shock

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SUMMARY

The lysis of yeast protoplasts as a function of the tonicity and composition of the suspension medium was examined. The resistance of the protoplasts to lysis by osmotic shock was lowered by certain chelating agents and at low pH values. The effects of chelating agents on the lysis of the protoplasts were inhibited by K^+, Na^+, Mg^{2+} and spermidine. Chelating agents increased lysis only when the protoplasts were undergoing osmotic stress, and the cations did not influence lysis unless chelating agents were also present. The presence on the protoplast membrane of a cation-binding site which is involved in maintaining the structure of the membrane is inferred.

INTRODUCTION

The lysis of bacterial protoplasts is influenced not only by the relative permeability of the protoplast membrane to various solutes, but also by the ability of certain compounds to interact with the membrane itself (McQuillen, 1960; Mager, 1959; Harold, 1964; Marquis, 1965). Apart from the usefulness of such studies in providing a basis for a systematic approach to the problems of obtaining stable protoplast preparations, they may lead to a better understanding of the properties of the protoplast membrane. A variety of stabilizing media have been used by different workers to preserve yeast protoplasts during their preparation and subsequent storage (Gascon & Villanueva, 1965), but in this case few attempts have been made to study the response of the protoplasts to the various solutes used. In the present work the effects of certain anions and cations on the lysis of yeast protoplasts by osmotic shock was examined.

METHODS

Preparation of yeast protoplasts. The yeast used was a strain of *Saccharomyces carlsbergensis* NCYC 74. The protoplasts were isolated by a method based on that of Eddy & Williamson (1957, 1959). Dried snail-gut juice (2·5 mg./ml. of Helicase. Industrie Biologique Française, 49 Quai Du Moulin De Cage, Gennevilliers, Seine, France) was used to dissolve the cell wall, the digestion medium being buffered with 0·01 M-imidazole HCl at pH 6·4. Less lysis occurred during protoplast formation under these conditions than in the citrate+phosphate buffer used by Eddy & Williamson. The protoplasts were washed at 0° in 10% (w/v) mannitol solution containing 0·01 M-imidazole HCl buffer (pH 6·4), suspended in this solution at a concentration of about 10^9 protoplasts/ml. and stored at 0° for up to 3 days.

Osmotic lysis measurements. A series of tubes containing 5 ml. samples of an appropriate buffer and from 2 to 10% (w/v) mannitol in 0·5% (w/v) steps were prepared and brought to 25°. Protoplasts (0·05–0·10 ml. of stock suspension) were added to the tubes of the series, the contents of which were then intimately mixed by shaking. The interval between the addition of protoplasts and the mixing of the solutions was about 1 min. The extinction of each suspension was measured at 5 min. intervals for about 20 min. with a Hilger Spekker with 1 cm. cells and Ilford H. 508 (neutral) filters. Extinction changes were correlated with lysis of the protoplasts by counting the intact cells in a Neubauer counting chamber.

In experiments where the effects of selected anions on the stability of the protoplasts were investigated the solutions were prepared from the free acids and brought to the required pH value with tris; other cations were added as the chlorides. Generally the contribution of the substance tested to the net osmotic pressure of the medium was not significant, but when necessary iso-osmotic conditions between the control series (no additions) and the test series were maintained by reducing the mannitol concentration in the test series appropriately.

RESULTS

The relationship between extinction changes and lysis

When samples of a stock suspension of protoplasts in 10% (w/v) mannitol solution were added to hypotonic solutions of mannitol the extinction values of the mixtures decreased rapidly during the next 5–10 min. and were then almost constant. It will be seen from Fig. 1 that the initial decrease of extinction was greater the lower the concentration of mannitol in the suspension medium.

Extinction changes observed in suspensions of protoplasts or subcellular particles may result from both swelling and lysis. To estimate the relative contributions of swelling and lysis to the extinction changes recorded in these experiments samples of the protoplasts were suspended in various hypotonic media and the numbers of unlysed protoplasts counted after 15–20 min. A linear relationship between the number of intact protoplasts and the extinction value of the lysate was observed for a range of experimental conditions (Fig. 2).

In further tests the effects of swelling on the extinction readings were estimated by restoring the concentration of mannitol in the medium to 10% (w/v) after the lysis period. Generally this treatment produced only a small increase in the extinctions of the test mixtures, indicating that the observed changes resulted mainly from lysis of the protoplasts. Under conditions where most of the protoplasts resisted lysis, swelling contributed more significantly to the observed extinction changes; but the effects were not sufficiently large to be revealed above the scatter of experimental points of Fig. 2.

In view of these findings extinction measurements were taken to provide an approximate measure of protoplast lysis in subsequent work, and the possible differential effects of swelling under the various test conditions were ignored.

Measurement of lysis resistance

The above observations implied that in a protoplast population there existed a distribution of resistances to lysis by osmotic stress, since not all the protoplasts lysed in a given hypotonic solution. This suggested that the response of the protoplasts to

osmotic stress might be better represented in the form of resistance curves than in the form of progress curves as in Fig. 1. To do this the extinction observed in a given suspension 15–20 min. after the protoplasts were added to the various hypotonic media was plotted against the respective concentration of mannitol (see Fig. 3). Plots of the experimental data on arithmetical probability paper (Papart *et al.* 1947) showed that in general the resistance curves determined from osmotic lysis tests made in a number of different media approximately followed a normal distribution. Therefore many of the results were condensed by reporting only the tonicity of the medium (in %, w/v, mannitol) at the median of the resistance curve (the median tonicity). On this basis the influence of a certain compound on the resistance of the protoplasts to lysis was expressed quantitatively (in %, w/v, mannitol) as the difference between the median tonicities in the presence and absence, respectively, of that compound. For convenience this quantity is called the osmotic shift, C_m.

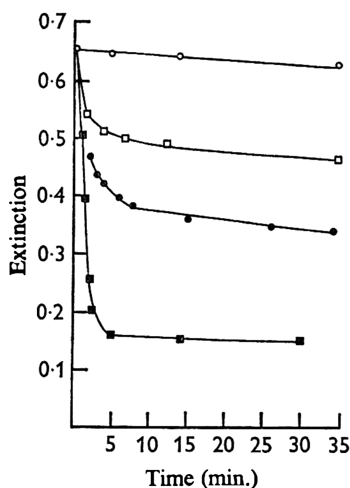

Fig. 1

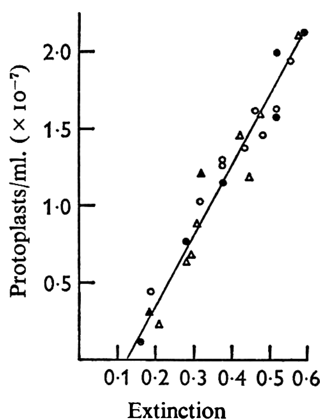

Fig. 2

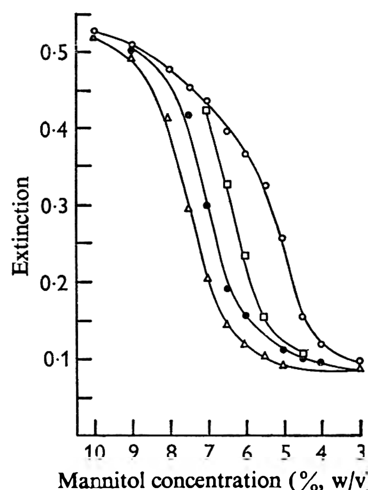

Fig. 3

Fig. 1. Extinction changes with time during osmotic lysis of yeast protoplasts. The protoplasts were suspended at 25° in 10 % (○), 6 % (□) 5 % (●) and 4 % (■), w/v, mannitol solution buffered with 0·01 M-imidazole HCl (pH 6·4).

Fig. 2. The relationship between the extinction and the number of unlysed protoplasts after the subjection of protoplast populations to osmotic stress. Protoplast populations containing about 2×10^7 protoplasts/ml. were partially lysed by osmotic shock in 0·01 M-imidazole HCl buffer (pH 6·4) (○); 0·01 M-imidazole HCl (pH 6·4)+0·01 M-citrate tris (●); 0·01 M-imidazole HCl buffer (pH 6·4)+0·01 M-citrate tris+0·01 M-KCl (△); 0·01 M-succinate tris buffer (pH 5·3) (▲).

Fig. 3. Osmotic lysis resistance curves showing the effects of citrate, K^+ and pH value on lysis. Protoplasts were lysed by osmotic shock in media containing graded amounts of mannitol. Lysis in 0·01 M-imidazole HCl buffer (pH 6·4) (○); imidazole buffer + 0·01 M-citrate tris (△); imidazole buffer+0·01 M-citrate tris+0·01 M-KCl (□); succinate tris buffer (pH 5·3) (●).

The influence of selected anions on lysis

When osmotic lysis tests were carried out in the presence of citrate (0·01 M), progress curves similar to those shown in Fig. 1 were observed, except that complete lysis of the protoplast population occurred when the mannitol concentration was 6 % (w/v).

This finding is illustrated by the resistance curves shown in Fig. 3. Table 1 shows that of the anions tested only those which are strong chelating agents, namely citrate, EDTA, ATP and to a lesser extent oxalate, pyrophosphate and isocitrate, decreased the resistance of the protoplasts to osmotic stress.

Protoplasts which were suspended in mannitol solution (10%, w/v) together with citrate (0·01 M) or EDTA (0·01 M) did not lyse during 30 min. When these protoplasts were then tested in the absence of these anions they exhibited resistance curves which were identical with those of untreated, control protoplasts. This finding indicated that certain anions were able to influence lysis only when the protoplasts were osmotically stressed. To examine this possibility further samples of a stock suspension of protoplasts were suspended in 6·5% (w/v) mannitol solution and citrate (0·01 M) was then added at various times. The results of this experiment (Fig. 4) supported the idea that citrate increased lysis only when the protoplasts were undergoing osmotic stress. Similar results were obtained with ATP and EDTA.

Table 1. *The effects of some anions on the osmotic lysis of yeast protoplasts*

Tests were made in 0·01 M-imidazole HCl buffer (pH 6·4) and 25°. Anions (0·01 M) were added as the tris salts. C_m values were calculated as the difference between the median tonicities of the lysis resistance curves in the presence and absence, respectively, of anion. Deviations are the standard deviations and figures in parentheses the number of experiments.

Anion (0·01 M)	C_m % (w/v) mannitol	Anion (0·01 M)	C_m % (w/v) mannitol
Citrate	2·2 ± 0·2 (20)	Succinate	0 (5)
EDTA	2·5 ± 0·2 (10)	Phosphate	0 (5)
ATP	2·5 ± 0·25 (6)	Sulphate	0·1 (3)
Pyrophosphate	1·8 ± 0·2 (5)	Malonate	0 (3)
Isocitrate	1·1 ± 0·1 (6)	Glutamate	0 (3)
Oxalate	0·9 ± 0·2 (5)	Aconitate	0 (3)
Acetate	0 (3)	Malate	0 (3)

Lysis and anion concentration

The relationship between the observed osmotic shift, C_m, and the concentration of chelating anion in the lysis medium is shown in Fig. 5. It can be seen that each anion tested produced a sharp increase in the lysis of the protoplasts over a fairly narrow range of concentration.

If it be assumed that: (1) these anions influence lysis by binding with receptor sites on the protoplast membranes, (2) the C_m value is a measure of the extent of binding, (3) the amount of anion bound is a negligible fraction of the amount added, then these curves (Fig. 5) represent the dissociation functions of the complexes. On the basis of these assumptions the data were analysed by using a Scatchard plot (Sanui & Pace, 1962). The results showed that the data did not follow simple mass law relationships; they suggested that co-operative interactions might be involved.

The influence of cations

In the absence of chelating anions neither sodium chloride (0·01 M) nor potassium chloride (0·01 M) affected the osmotic lysis of the protoplasts at pH 6·4. However, these cations opposed the effects of those anions which decreased the resistance of the protoplasts to osmotic stress (Fig. 3). In a series of experiments the inhibition of the effects of ATP, EDTA and citrate on osmotic lysis by K^+ or Na^+ was investigated as a

function of the Na^+ or K^+ concentration in the medium. Adopting analogous assumptions to those detailed above the data were analysed by using a Scatchard plot (Sanui & Pace, 1962). Plots of the osmotic shift, C_m, against C_m/cation concentration for a range of concentrations of Na^+ or K^+ yielded straight lines (Fig. 6). The results were therefore consistent with a situation in which the formation of a reversible complex between Na^+ or K^+ and a binding site influenced the stability of the protoplasts. The validity of the interpretation is supported by the circumstance that the extrapolated

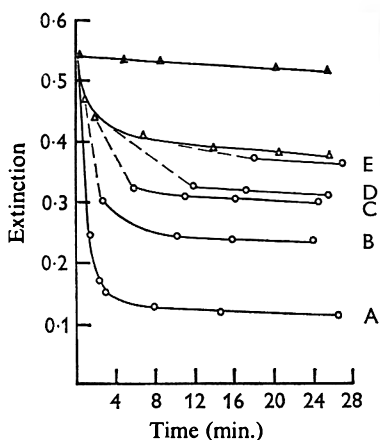

Fig. 4

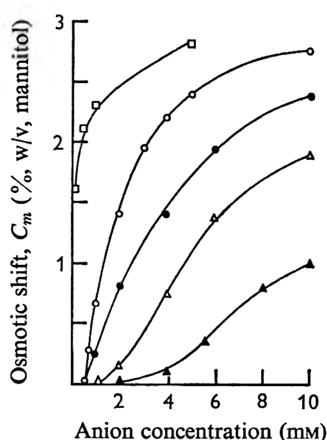

Fig. 5

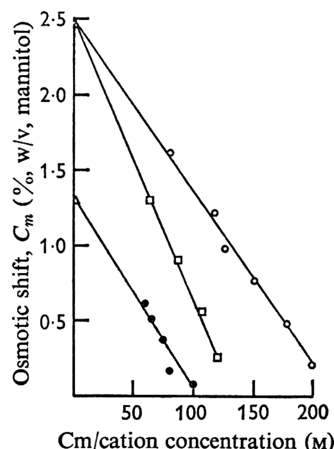

Fig. 6

Fig. 4. The effect of adding citrate ion during osmotic lysis of yeast protoplasts. Protoplasts were suspended in 0·01 M-imidazole HCl buffer (pH 6·4) containing 6·5% (w/v) mannitol, and 0·01 M-citrate tris was added at time zero (curve A), after 0·5 min. (curve B), after 1 min. (curve C), after 1·5 min. (curve D) and 5 min. (curve E). Control tests in 10 % (w/v) mannitol solution (▲) and 6·5% (w/v) mannitol solution without citrate (△).

Fig. 5. Osmotic lysis of yeast protoplasts as a function of the concentration of chelating anion. Osmotic shifts (C_m) plotted against the concentrations of EDTA (□), ATP (○), citrate (●), pyrophosphate (△) and oxalate (▲). C_m values were measured with respect to control tests in 0·01 M-imidazole HCl buffer (pH 6·4) which contained no chelating anions.

Fig. 6. Scatchard plots showing the inhibition of lysis in the presence of citrate ions as a function of Na^+ or K^+ concentration. Experiments in 0·01 M-imidazole HCl buffer (pH 6·4). Osmotic shifts (C_m) were calculated as the difference between the median tonicities of the lysis resistance curves without and with, respectively, added Na^+ or K^+. Lysis in 0·01 M-citrate tris+KCl (○) and NaCl (□). Lysis in 2·5 mM-citrate tris+KCl (●). Points on the ordinate axis marked △ were determined from control tests without added citrate and represent the osmotic shifts induced by citrate.

C_m values for Na^+ and K^+ (Fig. 6), corresponding to saturation of the hypothetical binding site, were numerically equal to the osmotic shifts caused by the anions initially, i.e. it appeared that saturating concentrations of K^+ or Na^+ would exactly annul the effects of the chelating anions.

The apparent dissociation constants of the presumed Na^+ and K^+ complexes as deduced from the slopes of the Scatchard plots shown in Fig. 6 were 0·02 M and 0·01 M, respectively. Similar values were obtained from experiments in which EDTA, ATP or citrate was used to decrease the resistance of the protoplasts to osmotic stress. The apparent dissociation constant of the K^+ complex was unchanged as the con-

centration of citrate (Fig. 6) or EDTA in the medium was varied. Thus K^+ behaved as a non-competitive inhibitor of the effects of these anions on the protoplasts.

Neither spermidine (10^{-4} M) nor magnesium chloride (10^{-3} M) affected the extinction changes during the osmotic lysis of protoplasts at pH 6·4 when chelating agents were absent. However, whereas lysis usually resulted in complete disruption of the protoplasts, the presence of these cations led to their gross structure being preserved. In the presence of either spermidine or Mg^{2+}, citrate or EDTA had less effect on the resistance of the protoplasts to osmotic stress (Table 2). Concentrations of spermidine or Mg^{2+} greater than about 10^{-4} and 3×10^{-3} M, respectively, caused precipitation of protoplast lysates accompanied by spurious extinction changes. For this reason a systematic study of their effects was not attempted. The addition of imidazole (0·01 M), tris (0·01 M) or triethanolamine (0·01 M) to tests made in media which were lightly buffered with 0·001 M-imidazole HCl (pH 6·4) either with or without EDTA (10^{-4} M) did not change the response of the protoplasts to osmotic stress.

Table 2. *Inhibition of osmotic lysis of yeast protoplasts by spermidine and Mg^{2+}*

Experimental conditions as for Table 1.

Addition	Median tonicity of resistance curve % (w/v) mannitol	
	EDTA (5 mM)	Citrate (4 mM)
None	7·8	7·0
MgCl 10^{-4} M	7·8	6·8
MgCl 10^{-3} M	7·2	6·0
MgCl 2×10^{-3} M	6·2	5·6
MgCl 4×10^{-3} M	—	5·4
No citrate or EDTA	5·3	5·3
None	7·4	6·4
Spermidine 10^{-6} M	7·2	6·3
Spermidine 10^{-5} M	6·7	5·9
Spermidine 10^{-4} M	6·3	5·8
No citrate or EDTA	5·0	5·0

The effect of pH value

The resistance of the protoplasts to osmotic stress decreased as the acidity of the medium was varied from pH 7·4 to 5·3 (Table 3; Fig. 3). This change was accompanied by a parallel decrease in the magnitude of the osmotic shift induced by citrate or by EDTA, a result which suggested that the effects of H^+ and chelating anions were related. The possibility that the succinate present in the buffer used for the tests at pH 5·3 was responsible for the decreased stability of the protoplasts at this pH value was considered, but seems unlikely since unbuffered suspensions brought to pH 5 with HCl lysed in a similar fashion.

A complication in the tests at pH 5·3 arose from the observation that the extent of lysis at this pH value appeared to vary with the rate of mixing of protoplasts with test medium. Large osmotic shifts resulted only when mixing was delayed for 1–2 min. after the addition of the protoplasts to the medium (see Methods). The explanation of this phenomenon is not known. At higher pH values the rate of mixing of the suspensions was not important.

K^+ and Na^+ were also effective in inhibiting the effects of low pH values on the response of the protoplasts to osmotic stress. The concentration dependence of the inhibition of lysis by these ions at pH 5·3 followed a simple adsorption isotherm with an apparent dissociation constant of roughly 10^{-2} M for both K^+ and Na^+; this figure is an approximate estimate and does not rule out the possibility of competition between Na^+ or K^+ and H^+.

Table 3. *The influence of pH value on the osmotic lysis of yeast protoplasts*

Tests at pH 7·5 in 0·01 M-tris HCl buffer; at pH 6·4 in 0·01 M-imidazole HCl buffer; at pH 5·3 in 0·01 M-succinate tris buffer. Temperature 25°.

	Median tonicity of resistance curve % (w/v) mannitol			
pH	No additions	Citrate (0·01 M)	EDTA (5 mM)	Succinate (0·01 M)
7·5	4·7	7·2	7·4	4·7
6·4	5·1	7·2	7·4	5·2
5·3	6·8	7·3	7·4	6·8

DISCUSSION

The experimental findings show that only those anions which are strong chelating agents lowered the resistance of the yeast protoplasts to osmotic stress. Considering the different chemical natures of these anions, it seems reasonable to conclude that their effects arise from their common ability to form stable chelate complexes. There is considerable evidence in the literature to indicate that calcium and magnesium are involved in natural membrane structures (Sampson & Karler, 1963; Carvalho, Sanui & Pace, 1963; Mikulecky & Tobias, 1964; Asbell & Eagon, 1966). Various papers, in which the association of membrane particles in the presence of magnesium ions has been reported, are particularly interesting from this viewpoint. (Blount, Rayner, Schmidt-Nielson & Tosteton, 1963; Brown, 1965; Razin, Morowitz & Terry, 1965). It is suggested therefore that the integrity of the yeast protoplast membrane depends on the presence of bound magnesium or calcium which can be replaced by Na^+ or K^+. (Preliminary work has shown that appreciable amounts of magnesium are present in cell membrane preparations of the protoplasts.)

The lysis reaction may then be formulated as follows. In the absence of chelating agents osmotic stress leads to the swelling of the protoplasts to a point where the membrane is no longer impermeable to mannitol; lysis then ensues. When chelating agents are present magnesium is withdrawn from the membrane, leading either to a direct breakdown of its structure or to a change in its permeability properties; in either case mannitol penetrates into the protoplast and lysis results. Robinson (1966) suggested that a similar situation exists in brain microsome preparations. Since K^+, Na^+ and spermidine influenced osmotic lysis only when chelating agents were added, it is suggested that these cations can substitute for magnesium in the membrane structure without changing its stability. Similarly, H^+ may compete with magnesium for binding sites on the membrane; but in this case leading to a structure with a decreased stability. It is relevant to note that competition between Na^+, H^+, K^+ and Ca^{2+} for binding sites of natural and model membrane systems has been reported (Rojas & Tobias, 1965; Carvalho, Sanui & Pace, 1963; Sanui & Pace, 1965).

On the basis of this model the results indicate that the magnesium of the membrane is not readily accessible to chelating agents in the medium since these anions were effective in decreasing the stability of the protoplasts only when these were undergoing osmotic stress. Two possibilities exist; either magnesium is exposed owing to the membrane becoming stretched, or the influx of water into the protoplasts enables the anions to penetrate the membrane structure. The former possibility requires that after the swelling phase the membranes of surviving protoplasts return to their original anion-insensitive state, presumably by loss of intracellular solutes and contraction. A similar synergistic action of chelating agents and hypotonicity on the stability of *Escherichia coli* protoplasts has been reported by Mager (1959).

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Metabolic Lysis of Yeast Protoplasts

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SUMMARY

Yeast protoplasts which were in osmotic equilibrium with a 0·55 M-mannitol solution underwent lysis when glucose was added to the suspension. Lysis was dependent on glucose metabolism and was accelerated when chelating agents were added to the medium. Lysis in the presence of glucose and chelating agents was inhibitied by alkali metal ions, magnesium, spermidine and by increasing the tonicity of the test medium. The inhibition of lysis by K^+ and Na^+ when chelating agents were present conformed to a Michaelis–Menten type relationship with apparent dissociation constants of $2–5 \times 10^{-4}$ M and $3–6 \times 10^{-3}$ M for K^+ and Na^+, respectively.

INTRODUCTION

While the growth and metabolism of bacterial and yeast protoplasts has been examined by various workers (McQuillen, 1960; Jeynes, 1961; Shockman & Lampen, 1962; Eddy & Williamson, 1959; Svoboda & Necas, 1966; Gascon, Ochoa & Villanueva, 1965), less attention has been paid to the circumstances in which the protoplasts may lyse as a result of their own metabolic activities (Abrams, 1959, 1960; Wachsman & Storck, 1960). In the present paper the influence of the composition of the test medium on the osmotic properties of metabolizing yeast protoplasts has been examined. The results show that in the presence of glucose the protoplasts tend to undergo metabolic lysis (Abrams, 1959) and that in short-term experiments the extent of lysis of the population is largely determined by the ionic composition of the test medium.

METHODS

Organism. Stock suspensions of protoplasts of the yeast *Saccharomyces carlsbergensis* NCYC 74 were prepared as described in the previous paper (Indge, 1968).

Experimental techniques. Samples (0·05–0·10 ml.) of the stock suspension of protoplasts were added to 5 ml. of a basal test medium containing mannitol (10%, w/v) and 0·01 M-imidazole HCl buffer (pH 6·4). The test solutions were equilibrated at room temperature (25°) during 10 min. and 0·25 ml. of glucose solution (10%, w/v) were then added to start the experiment. Extinction changes with time in the test samples were followed in a Hilger Spekker using neutral filters (Ilford, H. 508) and 1 cm. cells.

Compounds which were added to the basal test medium were made up in 10% (w/v) mannitol solution containing 0·01 M-imidazole HCl buffer (pH 6·4). Cations were added as the chlorides and anions as the tris salts.

Carbon dioxide evolution and oxygen consumption were measured by standard manometric techniques. When the respective rates of glucose metabolism and lysis were to be correlated the measurements were done in parallel. To estimate the initial rates of glucose metabolism in tests in which rapid rates of lysis were obtained, the suspension density of the protoplasts in the manometer flasks was increased threefold. Q values are reported in terms of the equivalent dry weight of whole yeast.

RESULTS

The influence of glucose

It can be seen from Fig. 1 that the extinction values of protoplast suspensions decreased only slowly with time during incubations in the absence of glucose. The addition of glucose (0·5 %, w/v) produced marked decreases in the extinctions of the suspensions, but when KCl (0·01 M) was also included in the medium the extinctions subsequently increased. This latter change was accompanied by aberrant cell-wall synthesis, swelling and growth of the protoplasts as described by Eddy & Williamson (1959). Sodium chloride (0·01 M) as opposed to KCl was found to decrease only slightly the extinction changes induced by glucose. When glucose alone was added all the protoplasts were lysed.

The effects of chelating agents

When glucose was added to osmotically stabilized protoplast suspensions containing citrate, ATP, EDTA or pyrophosphate (each 0·01 M) a rapid decrease in the extinctions of the mixtures ensued. This finding is illustrated by the results of an experiment in which citrate was added as the chelating agent (Fig. 2). Experience showed that a rapid decrease in the extinctions of the protoplast suspensions resulted only when both glucose and a chelating agent were added (Fig. 2). When the protoplasts were incubated with glucose for up to 30 min. before the addition of citrate, results similar to those of Fig. 2 were obtained.

Previous observations (Indge, 1968) had shown that in protoplast suspensions subjected to osmotic shock there was a linear relationship between the extinction change and the number of protoplasts lysed. A similar correlation between extinction changes and lysis of the protoplasts was found in the present experiments. When a suspension of metabolizing protoplasts was cooled to 0° (see later) and the number of intact protoplasts counted by using a Neubauer counting chamber, a linear relationship was found between the number of protoplasts lysed and the observed extinction change for a range of experimental conditions. Thus the extinction changes recorded in these experiments are a measure of protoplast lysis.

The relation between the rate of extinction decrease after the addition of glucose to the protoplast suspensions and the concentration of citrate, EDTA, ATP or pyrophosphate in the medium was investigated in a series of ten experiments. Representative data are given in Table 1. Analyses of the results of these experiments showed that in no instance did the kinetics conform to a Michaelis–Menten type relationship.

The role of glucose

The rate of lysis of protoplasts which were suspended in 10% (w/v) mannitol solution + citrate was influenced by the amount of glucose added. An analysis of the effects of glucose concentration on lysis showed that the initial rate of lysis of proto-

plast populations was proportional to the initial rate of glucose metabolism (Table 2). Parallel experiments done under nitrogen, or with Antimycin A (15 μg./ml.) added to the medium to suppress oxidative metabolism of the protoplasts, showed that while glucose respiration appeared to contribute significantly to the net rate of lysis, lysis did not depend absolutely on respiration. A point of interest emerging from these results

Table 1. *The stimulation of metabolic lysis of yeast protoplasts by chelating agents*

Protoplasts were suspended in 0·01 M-imidazole HCl buffer (pH 6·4) containing mannitol (10%, w/v), glucose (0·5, w/v) and certain chelating anions. The rate of protoplast lysis is expressed as the extinction decrease/5 min. observed after the addition of glucose.

	Chelator anion			
	Citrate	ATP	EDTA	Pyro-phosphate
Anion concentration (M)	Rate of lysis			
0	0·020	0·020	0·030	0·018
5×10^{-4}	0·056	0·040	0·120	0·018
2×10^{-3}	0·130	0·090	0·240	0·030
6×10^{-3}	0·275	0·220	0·370	0·102
1×10^{-2}	0·290	0·375	0·370	0·160

Table 2. *The relationship between glucose metabolism and yeast protoplast lysis*

The suspension medium contained 0·01 M-imidazole HCl buffer (pH 6·4), mannitol (10 %, w/v) and citrate (6 mM). The results are from parallel experiments in which extinction changes and gas exchanges were measured as a function of glucose concentration either with (+) or without (−) Antimycin A. Lysis rates expressed as extinction decrease/5 min. and Q values as μl./hr./mg. dry wt yeast.

Glucose added (%, w/v)	Lysis rate	$Q_{CO_2}^{Air}$	Q_{O_2}	Antimycin A (15 μg./ml.)
0	0·01	0	10	−
0·005	0·054	25	30	−
0·01	0·083	43	32	−
0·025	0·139	50	32	−
0·10	0·270	68	32	−
1·0	0·270	70	30	−
0	0·001	0	0	+
0·005	0·022	40	0	+
0·01	0·050	75	0	+
0·025	0·068	90	0	+
0·10	0·115	125	0	+
1·0	0·120	128	0	+

was that Antimycin A or anaerobiosis inhibited the slow extinction change usually observed in endogenous tests (see Figs. 1, 2). Several carbohydrates other than glucose were tested for ability to promote lysis in the presence of chelating agents. Of sixteen compounds tested only those which were metabolized by the protoplasts, namely sucrose, fructose and mannose, induced lysis when added to the medium at a concentration of 1 % (w/v).

Inhibition of metabolism

Concentrations of iodoacetate or iodoacetamide (0·005–0·01 M) which were sufficient to inhibit glucose metabolism completely also inhibited lysis of the protoplasts in the presence of chelating agents + glucose. The detailed effects of these inhibitors were however complex. For instance, when glucose and iodoacetamide ($1–5 \times 10^{-3}$ M) were added to the protoplasts together, the rate of lysis was increased twofold. Incubation of the protoplasts with iodoacetamide ($1–5 \times 10^{-3}$ M) during 20 min. before the addition of glucose resulted in either stimulation or inhibition of lysis, depending on the concentration of inhibitor. Sodium azide (5×10^{-4} M) and 2,4-dinitrophenol (2×10^{-3} M) completely inhibited the lysis reaction induced by the addition of glucose and citrate or EDTA to the protoplast suspensions.

The initial rates of lysis (extinction decrease per 5 min.) at 0°, 10°, 20° and 28° were 0·00, 0·03, 0·12 and 0·30, respectively, in one test done with glucose (0·5% w/v) + citrate (6 mM).

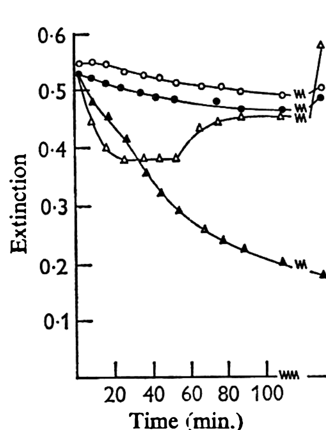

Fig. 1

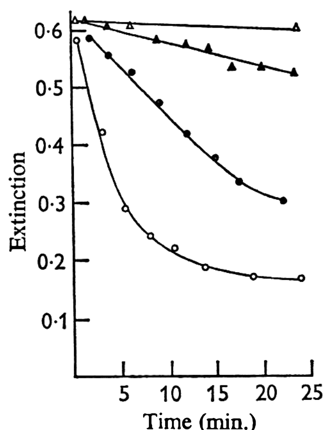

Fig. 2

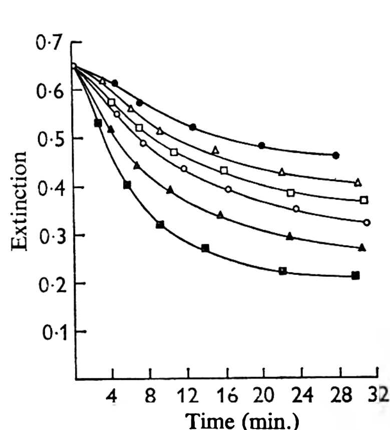

Fig. 3

Fig. 1. The effects of glucose and K^+ on yeast protoplasts. Protoplasts were incubated in 0·01 M-imidazole HCl buffer (pH 6·4), mannitol (10%, w/v) and glucose (0·5%, w/v) either with (△) or without (▲) KCl (0·01 M). Tests with (●) and without (○) KCl (0·01 M) in the absence of glucose. The broken lines signify further incubation for 16 h.

Fig. 2. Lysis of yeast protoplasts by the addition of glucose and citrate ions. Tests made in 0·01 M-imidazole HCl buffer (pH 6·4) with 10% (w/v) mannitol + glucose (0·5%, w/v) added. Citrate concentration 0·006 M (○); 0·001 M (●); zero (▲). Control test with citrate (0·006 M) but no glucose (△).

Fig. 3. Inhibition of lysis of yeast protoplasts by monovalent cations. The medium contained 0·01 M-imidazole HCl buffer (pH 6·4), mannitol (10%, w/v), glucose (0·5%, w/v), citrate (0·004 M) and the chloride of the cation under test (0·01 M). NH_4^+ ●; K^+ △; Rb^+ □; Cs^+ ○; Na^+ and Li^+ ▲ (these cations yielded coincident curves); control test ■.

Effects of cations

As can be seen from Fig. 3 monovalent cations (0·01 M) exhibited graded abilities to inhibit the rapid lysis of the protoplasts induced by citrate and glucose, NH_4^+ and K^+ being the most effective inhibitors. Of the cations tested Na^+ and K^+ were selected for more detailed studies. The relation between the rate of lysis and the concentration of K^+ or Na^+ in the medium was investigated. The results from a typical

experiment are shown in Fig. 4. It can be readily shown that when a cation binding site which controls the rate of lysis is inactivated by the formation of a reversible complex with monovalent cations, then the decrease in the rate of lysis in the presence of cations and the cation concentration would be related by a Michaelis–Menten type equation. When the kinetic data were plotted according to the procedure of Hofstee (1952) they yielded straight lines (Fig. 5). Thus the results conformed to the type of relationship suggested.

The apparent dissociation constants of the presumed Na^+ and K^+ complexes determined from the results of twelve experiments varied between $3–6 \times 10^{-3}$ M and $2–5 \times 10^{-4}$ M, respectively. These values were apparently independent of the nature or concentration of the chelating agent employed to stimulate the lysis reaction (Fig. 5). The results presented in Fig. 5 were obtained from measurements of the rates of extinction change in short-term experiments where the average rate of lysis was large (see Fig. 4). When the rate of lysis was small, for example in tests carried out with 0–2 mM-citrate, the addition of K^+ to the medium tended to increase the rate of lysis (see Fig. 1).

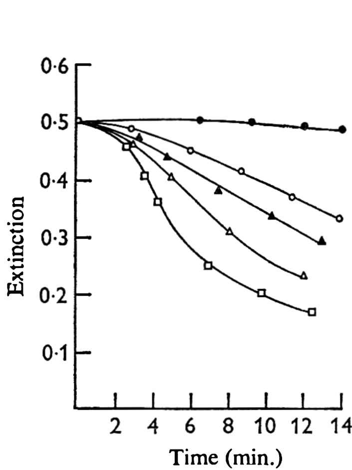

Fig. 4

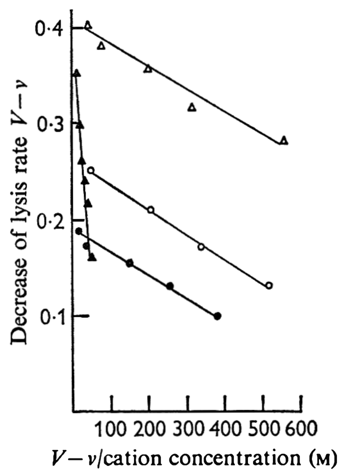

Fig. 5

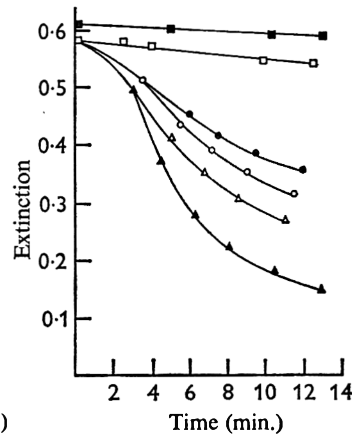

Fig. 6

Fig. 4. Kinetics of inhibition of yeast protoplast lysis by K^+. Test conditions as for Fig. 3. KCl 0·001 M, ○; 0·0005 M, ▲; 0·00025 M, △; zero, □. Control test with no citrate or K^+, ●.

Fig. 5. Inhibition of yeast protoplast lysis by Na^+ and K^+. The rate of lysis (V) in tests without Na^+ or K^+ minus the rate of lysis (v) in tests with Na^+ or K^+ is plotted against $V - v$/cation concentration. Lysis rates were estimated as the extinction decrease/5 min. The rates of lysis observed with no K^+ or Na^+ added are given below; note that in all cases the plots for K^+ cut the ordinate axis at values less than V (see text). 0·004 M-EDTA and K^+, △ ($V = 0·50$); 0·006 M-citrate and K^+, ○ ($V = 0·44$); 0·004 M-citrate and K^+, ● ($V = 0·25$); 0·006 M-citrate and Na^+, ▲ ($V = 0·40$).

Fig. 6. The effect of the concentration of mannitol in the medium on lysis of yeast protoplasts. The media contained 0·01 M-imidazole HCl buffer (pH 6·4), glucose (0·5 %, w/v), citrate (0·01 M) and 14 (●), 13 (○), 12 (△) and 10 (▲) % (w/v) mannitol. Control tests without citrate added in 14 (■) and 10 (□) % (w/v) mannitol.

Na^+ did not stimulate lysis appreciably under such conditions. Another aspect of the complex behaviour exhibited towards K^+ was that it did not annul completely the rapid lysis reaction induced by chelating agents and glucose (Fig. 4). Comparison of the intercepts on the ordinate axis of the plots of Fig. 5 with the rates of lysis of the

respective control tests also demonstrates this point. A possible explanation is that the protoplasts transport and accumulate K^+ during the experiments thereby increasing their osmotic pressure relative to that of the medium and thus increasing the rate of lysis.

Spermidine (10^{-5} to 10^{-4} M) and Mg^{2+} (10^{-4} to 10^{-3} M) decreased the rate of lysis of the protoplasts in the presence of citrate (6 mM) and glucose by up to 50%. Their effects were not investigated in detail, but even when excess citrate (6 mM) was present Mg^{2+} (10^{-4} M) decreased the rate of lysis by about 40%.

Osmotic pressure of the medium

In the osmotic lysis tests described in a previous report (Indge, 1968) chelating agents were shown to lower the resistance of yeast protoplasts to lysis by osmotic shock by an amount equivalent to a 2·5% (w/v) decrease in the concentration of mannitol in the suspending fluid. As shown in Fig. 6 the stimulation of the lysis of metabolizing protoplasts observed when citrate was included in the medium was not prevented even in 14% (w/v) mannitol solution. Increasing the concentration of mannitol in the medium to 14% (w/v) did not decrease the rate at which the protoplasts metabolized glucose.

The rate of lysis

Although the effects of chelating agents described were qualitatively highly reproducible, the rate of lysis varied somewhat from one batch of protoplasts to another. For example, the initial rates of lysis recorded in citrate solution (6 mM) and glucose varied from 0·15 to 0·45 (extinction decrease/5 min.), the average value being about 0·30, in a set of 30 measurements. A lag period of 5–10 min. after the addition of glucose was sometimes observed before lysis commenced.

Table 3. *The influence of anions on yeast protoplast lysis*

Tests were made in media containing 0·01 M-imidazole HCl buffer (pH 6·4), mannitol (10%, w/v) and glucose (0·5%, w/v). Lysis rates, which are expressed as the extinction decrease/5 min., were measured over a period of linear extinction change with time after the lag periods (see text).

Anion concentration (mM)	Anion: Phosphate	Succinate	Acetate
	lysis rate		
0	0·036	0·046	0·040
2	0·056	0·086	0·065
4	0·068	0·110	0·100
6	0·097	0·184	·
8	0·136	0·184	0·125
10	0·136	0·184	0·140

Preparations of protoplasts which lysed rapidly in the presence of glucose and strong chelating agents were also susceptible to lysis when weaker complexing agents such as sulphate, phosphate, acetate, succinate, malate or aconitate were added. The concentrations of these anions resulting in rapid rates of lysis were in each case between 10^{-3} M and 10^{-2} M (Table 3). Their effects were characterized by lag periods of up to 20 min. before any appreciable stimulation of lysis took place. In some instances it

was necessary to decrease the concentration of mannitol to 8·5% (w/v) in order to observe the effects of these anions on lysis conveniently. The actions of these anions on lysis were inhibited by K^+, Na^+ and Mg^{2+}.

DISCUSSION

The lysis of yeast protoplasts induced by glucose resembles the phenomenon of metabolic lysis observed by Abrams (1959, 1960) with *Streptococcus faecalis* protoplasts, in that the reaction depends on glucose metabolism. Certain properties of the yeast system, however, differ from those of the bacterial system studied by Abrams. In the yeast protoplast experiments reversible metabolic swelling did not appear to take place, and monovalent cations inhibited rather than stimulated lysis. The evidence indicated that K^+ tended to promote lysis of the yeast protoplasts, but this action was generally masked by the net inhibition of lysis produced by this cation.

The present results support the view proposed by Abrams that the addition of an energy source to protoplasts causes a change in the permeability properties of the protoplast membrane so that the osmotic stabilizer enters the cells. In yeast protoplasts this change appears to be accentuated by chelating agents, so that the rate of metabolic lysis is greatly increased. Lehninger (1962) discussed the similarities which exist between the swelling and contraction phenomena observed in mitochondria and in the protoplasts of micro-organisms.

It is of interest to compare the present results with those of the previous osmotic lysis experiments (Indge, 1968). Metabolizing yeast protoplasts were susceptible to the action of citrate ion up to 30 min. after the addition of glucose, which contrasts with the transient susceptibility of resting protoplasts to chelating agents during osmotic shock tests. Anions such as phosphate and succinate, which bind Mg^{2+} less strongly than do citrate or EDTA (Sillén & Martell, 1964), increased the rate of lysis in metabolic tests but did not influence the osmotic lysis of the protoplasts. These observations, and also the apparently larger effects of citrate ion on the stability of the protoplasts in the metabolic as opposed to the osmotic lysis tests (Fig. 6), can be explained in terms of the hypothesis put forward in the preceding paper (Indge, 1968). There it was suggested that bound Mg^{2+} or other cations in some way controlled the permeability properties of the protoplast membrane. Therefore it is proposed that in metabolizing protoplasts the Mg^{2+} of the membrane is accessible to the medium, the conformation and properties of the membrane being those which are exhibited during osmotic swelling of the protoplasts.

A further difference between the properties of the protoplasts in the two test systems is that the affinity of the membrane cation-binding site postulated to account for the inhibition of metabolic and osmotic lysis by Na^+ and K^+ was increased when the protoplasts were metabolizing glucose. The similarity between the apparent dissociation constants of the presumed Na^+ and K^+ complexes in metabolically active protoplasts and those of the cation transport system in yeast (Armstrong & Rothstein, 1964, 1967) leads one to suspect that these phenomena may be related. One interesting possibility is that the non-transporting modifier site postulated by Armstrong & Rothstein (1967) is involved in the permeability changes described in the present investigation. The phenomenon of metabolic lysis may be of practical importance in preparing yeast protoplasts. The results of the present work indicate that buffer

solutions containing chelating agents such as citrate should be avoided, and that the yeasts which exhibit high values of endogenous respiration incubations with snail-gut juice should be done anaerobically.

The author is indebted to Professor A. A. Eddy for valuable critical discussions and to Mr N. B. Roberts for his technical assistance.

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The Isolation and Properties of the Yeast Cell Vacuole

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SUMMARY

When glucose and a chelating agent such as EDTA were added to yeast protoplasts suspended in 8·5% (w/v) mannitol solution at pH 6·4 almost all the protoplasts lysed and the main vacuole was released as a discrete structure from about 75% of the protoplasts. The isolated vacuoles were themselves sensitive to osmotic shock and exhibited vital-staining reactions. They are therefore considered to be relatively undamaged. Some of the properties of the isolated vacuoles are described.

INTRODUCTION

Since the development of a method for isolating protoplasts from yeast cells (Eddy & Williamson, 1957) these bodies have become a favoured starting material for the isolation of various subcellular components. Eddy (1959) observed that ultrasonic vibration in certain circumstances released the main vacuole from the protoplast in a relatively intact form, but he did not develop a reliable method for preparing these bodies. Other methods of fragmenting the protoplasts include lysis in hypotonic media (Duell, Inoue & Utter, 1964; Boulton, 1965), ultraviolet irradiation (Svihla, Schlenk & Dainko, 1961) and treatment with detergent (Rozijn & Tonino, 1964). Such procedures have been used to prepare mitochondria, cytoplasmic membranes, vacuoles and nuclei respectively. Following the investigation into the stability of resting and metabolizing yeast protoplasts (preceding papers) it became apparent that the phenomenon of metabolic lysis (Abrams, 1959, 1960) might provide the basis of an alternative method of preparing subcellular structures. This is illustrated in the present work in which apparently intact vacuoles have been prepared from yeast protoplasts lysed metabolically in the presence of EDTA and glucose.

METHODS

Yeast protoplasts. Stock suspensions of protoplasts (about 10^9/ml.) of the yeast *Saccharomyces carlsbergensis* NCYC 74 were prepared as described previously (Indge, 1968). Protoplasts stored at 0° for 3 days and then washed exhibited the same behaviour during metabolic lysis as freshly prepared protoplasts.

Metabolic lysis. Samples (1 vol.) of the stock protoplast suspension were added to 50–100 vol. of 0·01 M-imidazole HCl buffer (pH 6·4) containing glucose (0·5%, w/v) and selected concentrations of mannitol, citrate ions or EDTA. Citrate ion and EDTA were added as the tris salts. The mixture was incubated at 30° for up to 20 min. and the progress of the metabolic lysis reaction was followed by measurements of extinction in the Hilger Spekker using neutral filters (Ilford H. 508) and 1 cm. cells.

Application of vital stains. Since contact with neutral red and toluidine blue was found to lyse both the protoplasts and the isolated vacuoles the behaviour of these bodies with the dyes was observed by irrigating slide preparations with isotonic solutions of the dyes. The dye concentrations given are those used in the irrigation fluid. By following the movement of the dye front across the slide it was possible to obtain photographs of the stained bodies before lysis occurred.

RESULTS

Metabolic lysis

It was shown in the preceding paper (Indge, 1968) that when yeast protoplasts (2×10^7/ml.) were incubated at 30° in a solution containing mannitol (10%, w/v), citrate ions (6 mM) or EDTA (5 mM) and glucose (0·5%, w/v) at pH 6·4 rapid metabolic lysis ensued. Examination by phase-contrast microscope of suspensions of protoplasts which were undergoing metabolic lysis revealed that the protoplasts swelled progressively and finally burst. As they lysed the protoplasts released spherical bodies 2–3 μ in diameter, each protoplast giving one such body, on average. These bodies have been identified with the cell vacuole. When the various particles released were collected by centrifugation at 2000 *g* for 4 min., the pellet consisted of a gelatinous membraneous material, vacuoles and unlysed protoplasts.

Conditions of metabolic lysis

By using the conditions described above, up to 25% of the protoplasts remained intact after incubation for 30 min. The composition of the lysis medium was therefore varied with the aim of decreasing the amount of this fraction. Figure 1 shows the effect of varying the concentration of mannitol in the lysis medium on the extinction changes taking place during lysis. Previous work had shown that the extinction as determined in these circumstances was a linear function of the number of unburst protoplasts (Indge, 1968). It can be seen that both the initial rate of change and the overall change in extinction increased as the concentration of mannitol in the medium approached 8% (w/v). Some of the protoplasts in the 8% (w/v) mannitol solution burst even in the absence of glucose (Fig. 1) and released intact vacuoles. On the basis of further similar experiments it was established that high yields of vacuoles, associated with extensive lysis of the protoplast population in the presence of glucose, occurred when the mannitol concentration was 8·5% (w/v).

A second factor which influenced lysis was the suspension density of the protoplasts in the test medium. Metabolic lysis was done with suspension densities of 1, 5 and 10 times that used in the above experiments (2×10^7/ml.), the suspensions being diluted appropriately for the purpose of measuring their extinctions. As shown in Fig. 2, although the initial rates of lysis in the three tests were similar, the number of protoplasts which lysed was smaller at the higher suspension densities. Moreover, the vacuoles and other membraneous elements of the protoplasts which lysed in the high density suspensions tended to remain attached to one another, forming structures which retained the gross morphology of the parent protoplasts. These results suggested that during lysis the protoplasts liberated some factor into the medium, possibly K^+ or Mg^{2+}, which stabilized the unlysed protoplasts. On the basis of the above results a solution containing mannitol (8·5%, w/v), glucose 0·5% (w/v), 0·01 M-imida-

zole HCl buffer (pH 6·4) and citrate ions (6 mM) or EDTA (5 mM) was expected to give the best yield of vacuoles. The initial suspension density of the protoplasts was about 2×10^7/ml. corresponding to an extinction of 0·5–0·6 (1 cm. cells). By using these conditions, between 95 and 100% of the protoplasts lysed during 20 min. at 30°; at least 75% of the lysed protoplasts gave vacuoles.

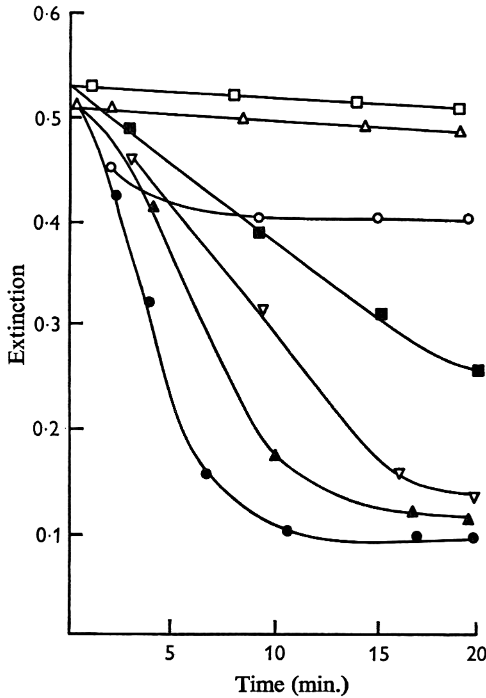

Fig. 1

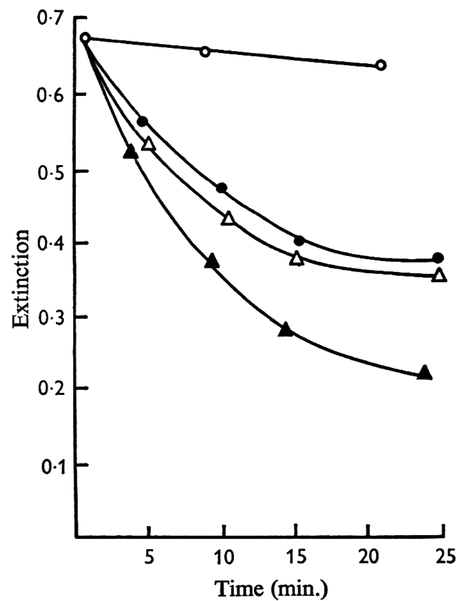

Fig. 2

Fig. 1. The effect of the concentration of mannitol in the medium on metabolic lysis of yeast protoplasts. Protoplasts were incubated at 30° in solutions containing: 0·01 M-imidazole HCl buffer (pH 6·4); glucose (0·5 %, w/v); citrate (6 mM); mannitol (%, w/v) 15 (■——■), 12 (▽——▽), 10 (▲——▲) or 8 (●——●). Control tests containing no glucose with mannitol (%, w/v) 15 (□——□), 10 (△——△), 8 (○——○).

Fig. 2. The influence of the suspension concentration of yeast protoplasts on metabolic lysis. Protoplasts suspended in: 0·01 M-imidazole HCl buffer (pH 6·4); glucose (0·5 %, w/v); citrate (4 mM); mannitol (10%, w/v) to give about 2×10^7 protoplasts/ml. (▲——▲), 10^8/ml. (△——△), 2×10^8/ml. (●——●). Tests containing 10^8 and 2×10^9 protoplasts/ml. were diluted before reading their extinctions. Control tests without glucose and 2×10^7 protoplasts/ml. (○——○).

The liberated vacuoles were concentrated by centrifugation at 2000 *g* for 4 min., when the vacuoles tended to remain dispersed in the pellet which formed at the bottom of the centrifuge tube, whereas unlysed protoplasts and other particles collected as a gelatinous, macroscopic aggregate. When the pellet was resuspended in a solution containing mannitol (10%, w/v) and 0·01 M-imidazole HCl buffer (pH 6·4) the gelatinous residue did not disperse and readily sedimented under gravity, leaving a supernatant solution rich in vacuoles which could be decanted from the other constituents. The appearance in the light microscope (Pl. 1, fig. 1) and the electron microscope (Pl. 3, fig. 7) of the material so obtained is shown.

Identification of the vacuole

A property common to many vacuoles as they occur *in vivo* is their ability to accumulate certain dyes (Zirkle, 1937). This so-called vital-staining reaction has been studied in the yeast cell by Guilliermond (1941). It seemed important to know whether the vacuole bodies released from the protoplasts during metabolic lysis would stain in a similar fashion.

The uptake of neutral red (0·01 %, w/v) at pH 7·0 by resting yeast cells followed the pattern described by Guilliermond (1941). Initially many stained granules exhibiting Brownian movement were observed in the vacuole; these became deposited against the vacuole membrane (Pl. 1, fig. 2) and coalesced to form large red globules which slowly dissolved in the vacuolar sap (Pl. 1, fig. 3). At pH 6·4 yeast protoplasts were stained similarly by neutral red (Pl. 2, fig. 5). A preparation of the bodies liberated by metabolic lysis of the protoplasts and stained with 0·005 % (w/v) neutral red is shown in Pl. 2, fig. 4. The dye caused the bodies to clump to some extent, and as staining continued, lysis ensued, apparently with the ejection of 'fingers' of precipitated dye which then rapidly dissolved. A few strongly stained particles were also released during lysis of the vacuoles.

The similarities between the interaction of these bodies with neutral red and the *in vivo* staining of the cell vacuole indicates that they are derived from the cell vacuole and are relatively undamaged. A similar parallelism existed between the reactions of the vacuoles *in vivo* and *in vitro* with toluidine blue (0·05 %, w/v). Isolated vacuoles were also lysed by this dye, with the liberation of large numbers of stained particles and a few rod-like structures (Townsend & Lindegren, 1953).

Physical properties of the isolated vacuoles

Fresh preparations of vacuoles adhered strongly to glass slides; this property was lost when the vacuoles were stored for 4–8 hr at 0°. Once the vacuoles were attached to a slide they could be drawn out to form beaded threads or long filaments, often without loss of optical contrast, by flooding isotonic buffer solution across the slide. Solutions flowing at low shear rates distorted the vacuoles, which reverted to their original shape, however, when the stress was removed. These circumstances made it possible to investigate the effects of certain compounds on selected groups of vacuoles by irrigating test solutions across the slide.

The isolated vacuoles were osmotically active in that they reversibly swelled and contracted as the concentration of mannitol in the medium was varied between 7·5 and 11 % (w/v). At higher concentrations of mannitol excessive contraction occurred with the formation of membrane 'tails' around the vacuoles. This process was not reversed when the osmotic pressure of the medium was decreased and the vacuoles lysed. The vacuoles lysed in 5–6 % (w/v) mannitol solution. During this lysis the optical contrast of the vacuole rapidly faded, leaving a spherical membrane ghost with no visible fracture in its surface. The ghosts remained adsorbed to the slide.

Stability of vacuoles

The stability of isolated vacuoles was investigated with the phase-contrast microscope. Isolated vacuoles survived 1–2 days at 0° when stored as a thick suspension in

mannitol solution (10%, w/v) at pH 6·4; at 30° considerable lysis took place within 30 min.

In an attempt to stabilize preparations of isolated vacuoles certain compounds were added to the buffered mannitol (10%, w/v) solution in which they were routinely suspended. The compounds tested included the chlorides, phosphates and sulphates of Na^+, K^+, Mg^{2+} and Ca^{2+} (0·005–0·05 M) which are known to stabilize plant cell vacuoles (Eichberger, 1933), and bovine serum albumin (1%, w/v), dextran 500 (Pharmacia, 2%, w/v), dextran 40 (Pharmacia, 2%, w/v) and Ficoll (Pharmacia, 1–5%, w/v). None of these compounds appeared to inhibit the spontaneous lysis of the vacuoles at 30°. Magnesium chloride (0·01 M) and calcium chloride (0·01 M) precipitated the contents of the vacuoles, giving spheres which exhibited Brownian movement (Pl. 2, fig. 6). The presence of these ions also caused the vacuoles to aggregate.

DISCUSSION

The success of the present technique in liberating apparently intact vacuoles from yeast protoplasts results from the change in the permeability properties of the protoplast membrane induced by glucose and chelating agents. Under the conditions of the lysis experiments the osmotic stabilizer, mannitol, is no longer excluded from the protoplast and lysis results. The survival of the various cellular organelles is then influenced directly by the composition of the medium. Since the vacuole survives the lysis reaction it may be inferred that the vacuole membrane is relatively impermeable to mannitol under these conditions and that its properties differ from those of the protoplast membrane. Other investigators have reported differences between the properties of the protoplast and vacuolar membranes (Bartholomew & Mittwer, 1952; Svihla, Dainko & Schlenk, 1963).

The lysis conditions used in this work allow the preservation of the cell vacuole; unpublished observations in these laboratories have shown that nuclei prepared according to Rozijn & Tonino (1964) were unstable when suspended in the lysis medium. The fate of other subcellular structures, such as mitochondria and the endoplasmic reticulum, during metabolic lysis is unknown. It is possible that other subcellular organelles might be prepared by using the metabolic lysis technique with suitable control of the composition of the medium. The possibility that vacuoles might serve as a model system for the study of transport phenomena was entertained at an early stage in this work. The failure to find conditions which decreased the lysis of the isolated vacuoles has so far prevented work in this direction.

Since the completion of this work Matile & Wiemken (1967) have published an alternative method for the liberation and purification of vacuoles from yeast. Vacuoles prepared by their method are smaller and show a larger variation in size (0·2–1·0 μ diameter) than do vacuoles prepared by metabolic lysis (2–3 μ diam.). The reason for these differences is not clear.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Yeast vacuoles isolated by metabolic lysis. Phase microscopy. × 3500.

Fig. 2. Yeast cells during staining with neutral red 0·01 % (w/v). Note globules of dye around the vacuole wall. × 1900.

Fig. 3. A later stage in the staining of yeast cells with neutral red 0·01 % (w/v). The dye has begun to dissolve in the vacuole sap. × 1700.

PLATE 2

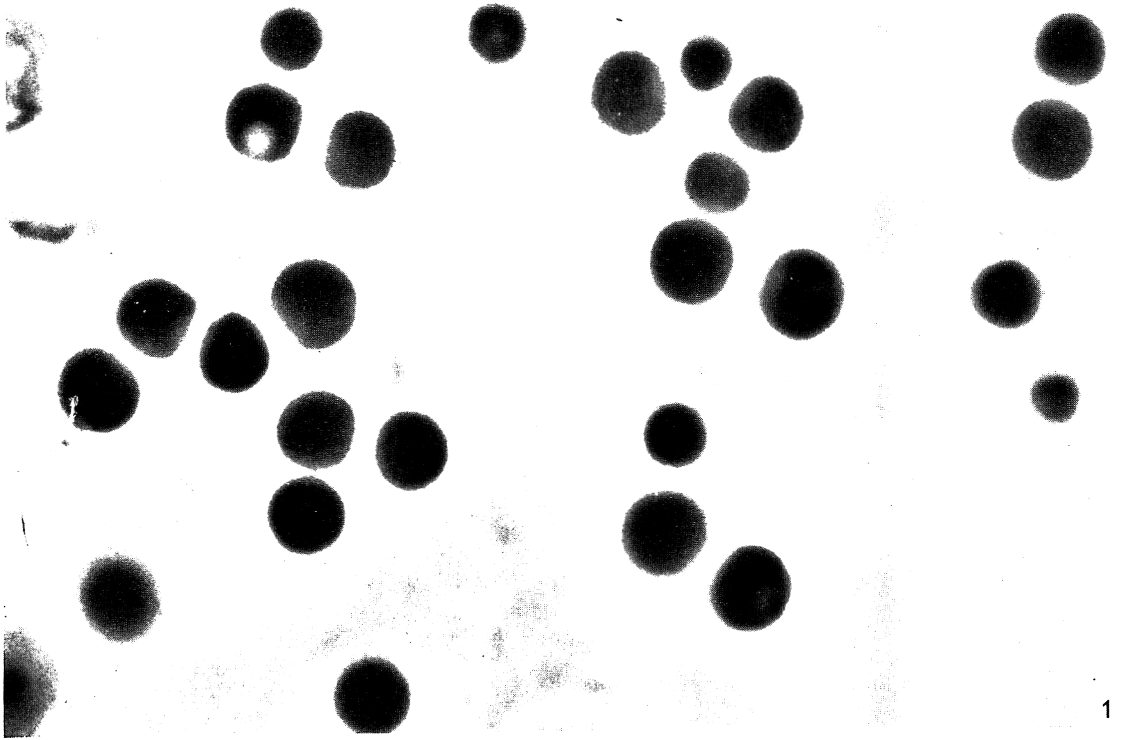
Fig. 4. Isolated vacuoles stained with neutral red 0·005 % (w/v). × 2250.

Fig. 5. Yeast protoplasts stained with neutral red 0·01 % (w/v) × 2250.

Fig. 6. Isolated yeast vacuoles suspended in 10 % (w/v) mannitol solution containing 0·01 M-$MgCl_2$. The vacuolar sap has separated into a dark sphere contained within the vacuole wall. Phase microscopy. × 3000.

PLATE 3

Fig. 7. Electron micrograph of a crude preparation of yeast vacuoles. The preparation was fixed overnight at 0° in 0·01 M-imidazole HCl buffer (pH 6·4) + mannitol to 10 % (w/v) + 5 % (w/v) glutaraldehyde; then stained in 5 % (w/v) aqueous $KMnO_4$ for 2 hr at 0°. × 14,000.



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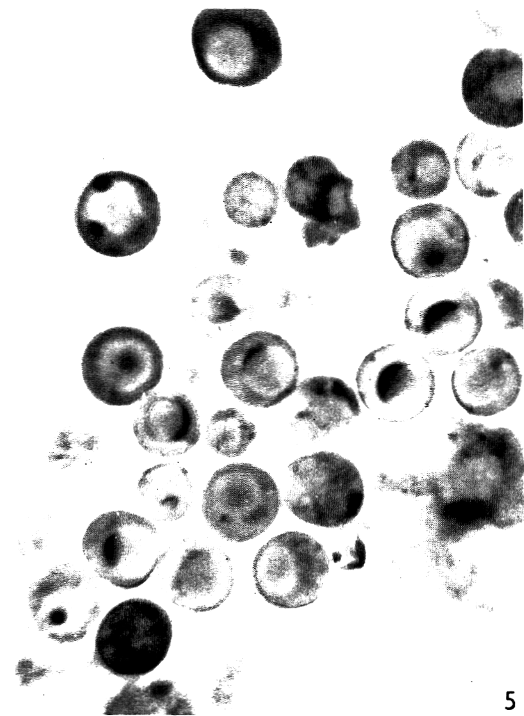

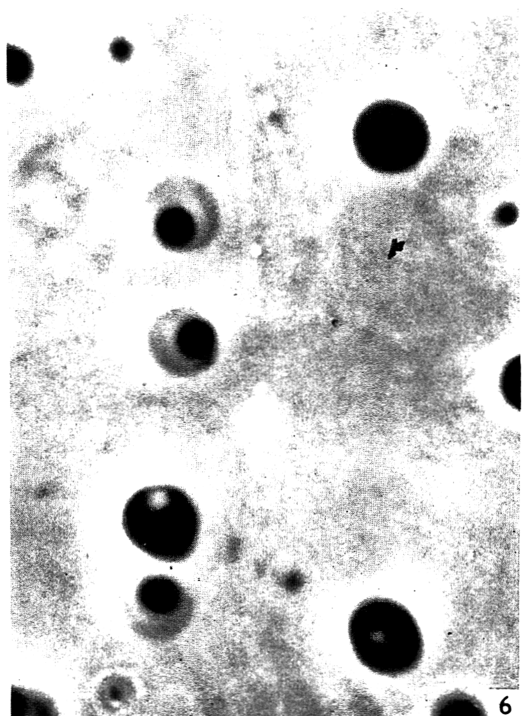

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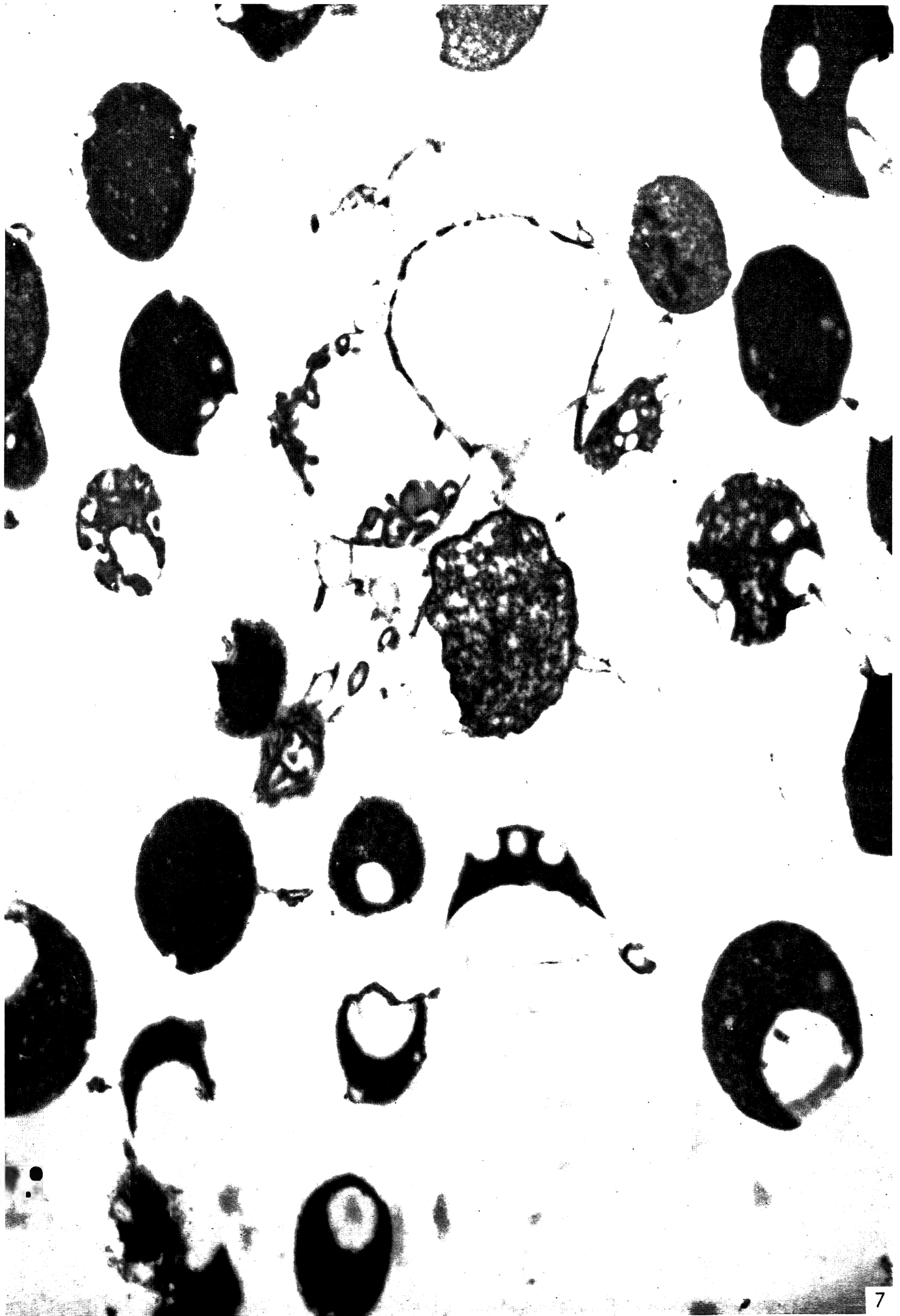

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Polyphosphates of the Yeast Cell Vacuole

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SUMMARY

The distribution of phosphorus compounds in fractions isolated from metabolically lysed yeast protoplasts by centrifugation at 2000 *g* was examined. About 40% of the acid-soluble phosphorus compounds was associated with a particulate fraction which sedimented at 2000 *g* and which was rich in vacuoles. This phosphorus was present largely as polyphosphates of relatively high molecular weights. Evidence is presented to support the view that this polyphosphate fraction is contained in the vacuole sap. The phosphorus of the crude vacuole fraction was labelled only slowly when the protoplasts were allowed to take up ^{32}P-orthophosphate. The phosphorus entering this fraction appeared to be derived from precursors present in the 2000 *g* supernatant fluid fraction.

INTRODUCTION

Since the development of a method for isolating the yeast cell vacuole (Indge, 1968*b*) it has become possible to examine directly the composition of the vacuolar sap. Progress in this field has hitherto been limited by the relatively indirect techniques available, although significant advances have followed the application of the ultraviolet microscope to this problem (Svihla & Schlenk, 1959, 1960; Svihla, Dainko & Schlenk, 1963, 1964). Previous investigators, who used staining and other cytochemical techniques, have in some cases concluded that the yeast cell vacuole contains polyphosphate, but there appears to be no general agreement on this point (Nagel, 1948; Widra, 1959). Pfeiffer (1963) concluded that the vacuole contains phospholipids. In view of this evidence an analysis of the distribution of phosphorus compounds in the crude vacuole fraction of metabolically lysed protoplasts was undertaken.

METHODS

Preparation of protoplasts. Stock suspensions of protoplasts of the yeast *Saccharomyces carlsbergensis* NCYC 74 were prepared as described previously (Indge, 1968*a*). For the preparation of protoplasts labelled with ^{32}P, 0·04 μc./ml. of [^{32}P]orthophosphate was added to the yeast growth medium.

Preparation of vacuoles. Vacuoles were prepared by metabolic lysis of protoplasts (2×10^7/ml.) at 30° in media containing mannitol (8·5%, w/v), glucose (0·5%, w/v), 0·01 M-imidazole HCl buffer (pH 6·4) and the tris salts of citric acid (0·006 M) or EDTA (0·005 M). At the end of the lysis period (about 20 min.) samples (8 ml.) of the suspensions were centrifuged at 2000 *g* during 4 min. In this way the lysis products were separated into the crude vacuole fraction (2000 *g* pellet) and the 2000 *g* supernatant

fluid fraction. The latter fraction is termed the cytoplasmic fraction in the text. The crude vacuole fraction was suspended in 8 ml. of the above solution, and the acid-soluble compounds of both fractions then extracted into trichloracetic acid (TCA) (5%, w/v) at 0° during 4 hr.

Analytical methods. Phosphorus analyses were done radiochemically with fractions derived from ^{32}P-labelled protoplasts. The total acid-soluble P was assayed by counting samples of the TCA extracts. Orthophosphate was counted as the molybdate complex after extraction of the TCA extracts into isobutanol + benzene according to the method of Martin & Doty (1949). Seven-minute phosphorus, i.e. P compounds hydrolysed to orthophosphate during 7 min. at 100° in N-HCl, was similarly extracted into isobutanol + benzene and counted. Stable phosphorus compounds were estimated as the difference between the total P count and the 7 min. P + *ortho*-P-count. This assay scheme was based on the methods of Ehrenberg (1961), Langen & Liss (1958) and Wiame (1949).

Since mannitol can complex with molybdate and interfere in the extraction technique only 1 ml. samples (10–15 μg. P) of the TCA extracts could be assayed by this method. In view of this complication the results were related to those obtained by using TCA extracts of centrifuged pellets of whole protoplasts. Such extracts, which contained only traces of mannitol, served as internal standards to monitor the assay techniques. Carrier orthophosphate (40 μg. KH_2PO_4) was added to each 1 ml. sample extracted into isobutanol + benzene solution.

The TCA-insoluble fractions were dissolved in 0·05 N-sodium hydroxide for counting. ^{32}P was assayed by conventional liquid counting techniques by using an M6 tube (20th Century Electronics Ltd., New Addington, Surrey), and total phosphorus was estimated colorimetrically according to Ehrenberg (1961). Samples (0·5 ml.) containing ^{14}C were mixed with 8 ml. of DEM phosphor (Panax Ltd., Redhill, Surrey) and counted in a Panax SC-LP scintillation counter. Potassium was estimated by flame photometry (Evans Electroselenium Ltd., Halstead, Essex) after digestion of the samples in boiling 8 N-nitric acid. RNA was determined by the method of Mejbaum (1939), and amino acids by the method of Rosen (1957).

Gel filtration. TCA was removed from the acid extracts of the protoplast fractions by ether extraction and the samples then analysed on Sephadex G. 25 and G. 50 columns (1·5 × 12 and 1·5 × 25 cm., respectively). The samples were eluted with 0·05 N-sodium chloride. The ability of Sephadex gels to separate polyphosphates was initially tested by using mixtures of dialysed hexametaphosphate (Allgén & Norberg, 1959) and pyrophosphate.

^{32}P-orthophosphate uptake. The protoplasts (5×10^7/ml.) were incubated with trace amounts of [^{32}P]orthophosphate (2 μc./ml.) in a solution which contained mannitol (12%, w/v), 0·01 M-imidazole-HCl buffer (pH 6·4), potassium chloride (0·01 M) and glucose (0·5%, w/v). These conditions appeared to be optimal for decreasing metabolic lysis to a minimum while allowing phosphate uptake to proceed. Samples of the suspensions were removed at intervals and the protoplasts harvested and washed in unlabelled medium at 0°. The washed protoplasts were then metabolically lysed as described above.

Materials. ^{32}P-orthophosphate (specific activity > 1000 C/g.) [U-^{14}C] L-lysine (5·9 mC./m-mole) and [3-^{14}C]DL-serine (5·25 mC/m-mole) were obtained from the Radiochemical Centre, Amersham, Buckinghamshire.

Dry weights. Certain quantities are reported in terms of the equivalent dry weight of whole yeast. This was calculated from the protein contents of the protoplast samples, assuming no loss of protein from the yeast during protoplast formation.

RESULTS

Distribution of phosphorus in protoplasts

The yeast was grown in ^{32}P-orthophosphate labelled medium and converted to protoplasts which were then lysed metabolically by the addition of citrate (4 mM) and glucose (0·5 %, w/v) to the medium. In Fig. 1 the flow of labelled phosphorus into the 2000 *g* supernatant fluid fraction during the metabolic lysis of the protoplasts is shown. About 20 % of the total acid-soluble P was present in the 2000 *g* supernatant fluid fraction before glucose was added. This was presumably a result of lysis following

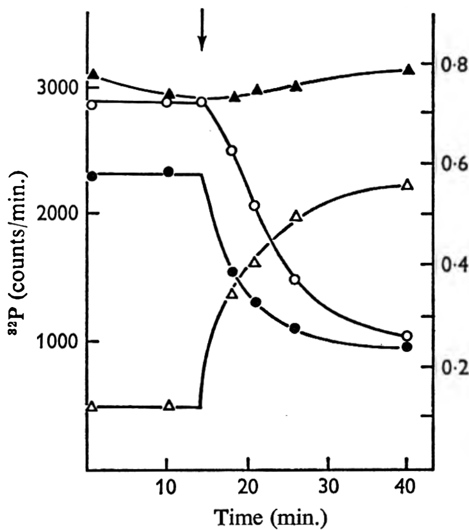

Fig. 1

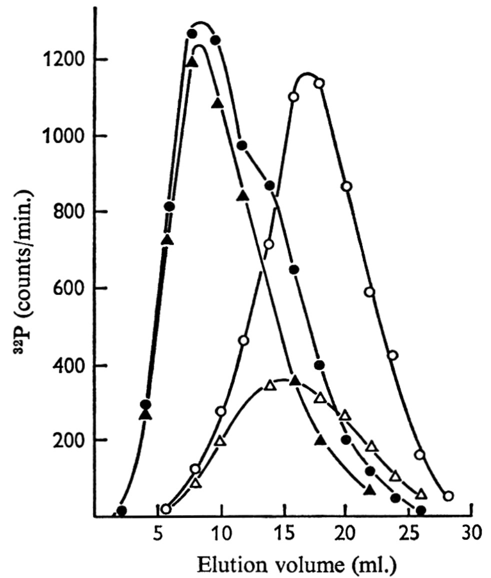

Fig. 2

Fig. 1. Distribution of acid-soluble ^{32}P between the 2000 *g* supernatant fluid and pellet fractions during metabolic lysis of yeast protoplasts. ^{32}P-labelled protoplasts were incubated at 30° in a solution containing mannitol (8·5 %, w/v), citrate (4 mM), and 0·01 M-imidazole HCl buffer (pH 6·4). Samples of the mixture were centrifuged at 2000 *g* at intervals, the fractions extracted into TCA (5 %, w/v) and the acid extracts counted. The arrow indicates the time of addition of glucose (0·5 %, w/v). Total P, ▲——▲; 2000 *g* supernatant fluid P, △——△; 2000 *g* pellet P, ●——●; extinction (Hilger Spekker, 1 cm. cells neutral filters), ○——○.

Fig. 2. Fractionation of cytoplasmic and vacuolar acid-soluble phosphorus compounds on Sephadex G-25. Total P (○——○) and 7 min. P (△——△) of cytoplasmic fraction. Total P (●——●) and 7 min P (▲——▲) of vacuolar fraction.

resuspension of the protoplasts in the lysis medium. After the addition of glucose and the subsequent lysis of the protoplasts, 30 % of the total acid-soluble P was retained in the 2000 *g* pellet fraction. In this experiment the citrate concentration in the lysis medium was reduced to 4 mM in order to lower the rate of metabolic lysis of the protoplasts.

The distribution and nature of the phosphorus compounds of the cytoplasmic and vacuolar fractions (2000 **g** supernatant fluid and 2000 **g** pellet fractions, respectively) was investigated by using five preparations of ^{32}P-labelled protoplasts. It can be seen from Table 1 that the crude vacuole fraction prepared by metabolic lysis contained 39% of the acid-soluble P of the protoplasts, and that this fraction consisted largely of acid labile, 7 min. P. This labile phosphorus was shown to represent inorganic polyphosphate by precipitation with Ba^{2+} at pH 4·5 according to Wiame (1949).

It is known that the acid-soluble fraction of whole yeast cells contains 30–40% of the cell phosphorus (Juni, Kamen, Reiner & Spiegelman, 1948). However, acid extracts of the protoplasts contained 65–70% of the cell phosphorus. About 90% of the acid-insoluble P could be accounted for as nucleic acid P, judging from estimations of the RNA present in this fraction. The crude vacuole fraction prepared by metabolic lysis contained about 18% of the total acid-insoluble P of the protoplasts. In view of the heterogeneity of the crude vacuole fraction the acid-insoluble compounds were not investigated further.

Table 1. *The percentage distribution of acid-soluble phosphorus compounds in the cytoplasmic and vacuolar fractions of yeast protoplasts*

Five separate batches of ^{32}P-labelled protoplasts were disrupted by means of metabolic lysis in solutions containing EDTA (5 mM), glucose (0·5 %, w/v) and mannitol (8·5 %, w/v) at pH 6·4. The lysates were separated by centrifugation at 2000 **g** into the vacuolar and cytoplasmic fractions and extracted with TCA. The results given are the mean values %±S.D. of the five determinations. The total acid-soluble P was about 17 μg. P/mg. dry weight equivalent of whole yeast.

Protoplast fraction	Total P	Ortho-P	7 min. P	Stable P
Whole protoplast	= 100	20·0±2·9	54·9±8·6	26·2±6·7
Vacuole	39·2±6·0	6·0±1·8	30·6±4·5	2·1±1·3
Cytoplasm	60·2±5·0	15·0±2·0	25·3±2·8	21·3±7·0

Table 2. *Release of the phosphorus of the vacuole fraction of yeast protoplasts into the 2000 **g** supernatant solution*

^{32}P-labelled protoplasts were metabolically lysed, the lysate centrifuged at 2000 **g** and the supernatant fluid discarded. The pellet fractions were resuspended in 10% (w/v) mannitol solution at pH 6·4 except where the effects of water and TCA were to be tested, and stored for 1 hr at 20° unless otherwise stated. The mixtures were then centrifuged at 2000 **g**, the supernatant liquids extracted with TCA (5 %, w/v) and the acid extracts then counted.

Treatment of vacuole fraction	^{32}P released to 2000 **g** supernatant fluid fraction (counts/min.)	Lysis of vacuoles
Held at 0°	1320	–
Held at 30°	4433	+
TCA 5% (w/v)	4285	+
Water	4233	+
Neutral red 0·01 %	3456	+

Polyphosphate of the vacuole fraction

The 2000 **g** pellet fraction contained considerable amounts of gelatinous membraneous material as well as intact vacuoles. It was possible, therefore, that the polyphosphate present in this fraction was associated with the vacuoles themselves, or was

present in volutin granules (Widra, 1959) or adsorbed to membrane fragments. However, those conditions which brought about lysis of the isolated vacuoles (Indge, 1968*b*) also liberated the polyphosphate associated with the 2000 *g* pellet fraction into the 2000 *g* supernatant solution (Table 2).

Preliminary experiments using dialysis, precipitation with albumin (Katchman & Wazer, 1954) and paper chromatography (Bernhart & Chess, 1959) indicated that the polyphosphate of the vacuole fraction was of a greater molecular weight than that of the cytoplasmic fraction. This point was investigated in more detail using gel-

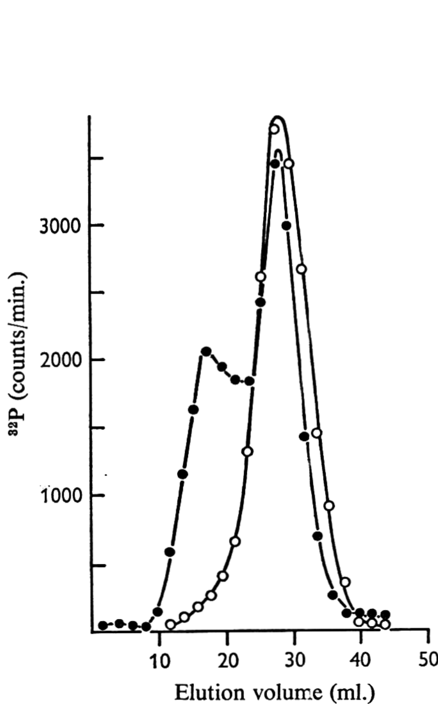

Fig. 3

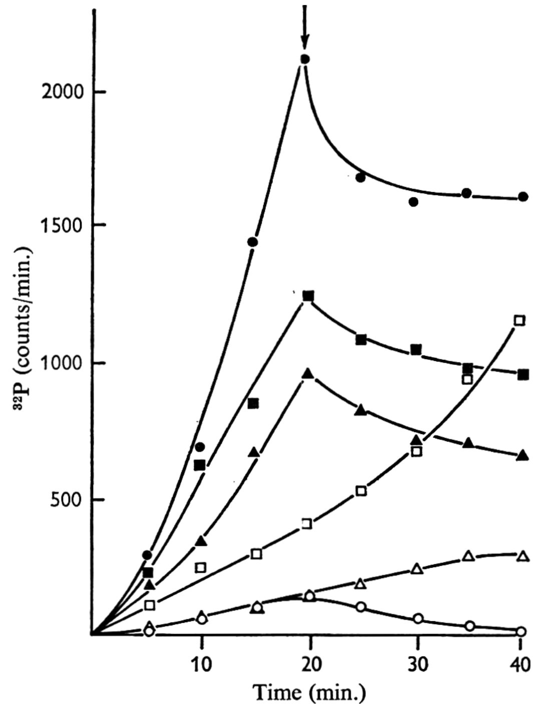

Fig. 4

Fig. 3. Fractionation of cytoplasmic and vacuolar acid-soluble phosphorus compounds on Sephadex G-50. Total P cytoplasmic fraction, ○——○. Total P vacuolar fraction, ●——●.

Fig. 4. Uptake of ^{32}P into cytoplasmic and vacuolar fractions of yeast protoplasts. Protoplasts were allowed to take up ^{32}P-orthophosphate and at intervals samples were metabolically lysed and separated into the vacuolar and cytoplasmic fractions by centrifugation. Further details are given in the Methods section. The isotope was diluted by the addition of 100 μg. P/ml. as KH_2PO_4 at the time indicated by the arrow. Cytoplasmic stable-P, ●——●; 7 min. P, ■——■; and ortho-P, ▲——▲. Vacuolar stable-P, ○——○; 7 min. P, □——□, and ortho-P, △——△.

filtration techniques. Figs. 2 and 3 show that Sephadex G-25 and G-50 gels excluded about 70 and 40% respectively of the phosphorus compounds of the vacuole fraction. There was little evidence of high molecular weight polyphosphate in the cytoplasmic extracts. Thus the long chain-length polyphosphates of the protoplasts appeared to separate solely into the vacuole fraction.

Uptake of ^{32}P-orthophosphate

The physiological behaviour of the two phosphorus-containing fractions separated by metabolic lysis was investigated by following the uptake of labelled phosphorus into these fractions. The rate of appearance of radioactivity in the crude vacuole fraction was considerably less than that in the cytoplasmic fraction. Some typical results are shown in the initial phase of the experiment of Fig. 4. The uptake of labelled phosphorus into the cytoplasmic and vacuolar fractions of the protoplasts, and the flow of label after the dilution of the ^{32}P-orthophosphate in the medium with non-radioactive phosphate, is shown in Fig. 4. The results suggested a precursor-product relationship between the phosphorus compounds of the cytoplasmic fraction and those of the vacuole fraction. The phosphorus compounds of the vacuole fraction did not appear to be incorporated directly from the orthophosphate of the medium. It is of interest that the uptake of ^{32}P into the orthophosphate of the vacuole fraction responded only slowly to the dilution of the isotope in the medium. This indicated that this phosphate was a genuine component of the vacuole fraction, and did not represent contamination of the fraction by the 2000 *g* supernatant fluid.

The newly synthesized ^{32}P-compounds formed during these experiments were analysed by gel filtration on Sephadex G-25. There was no evidence of exclusion of the labelled polyphosphates of either the vacuolar or cytoplasmic fractions from the gel. Thus the ^{32}P-polyphosphates synthesized by the protoplasts in these experiments were unlike those formed during the growth of the yeast on ^{32}P-orthophosphate.

Other experiments were done with the aim of observing the turnover of these phosphorus fractions. ^{32}P-labelled protoplasts were incubated with glucose in media either with or without added phosphate. These experiments did not yield useful results since there was apparently only a slow turnover of the cytoplasmic and vacuolar phosphorus fractions and the protoplasts lysed during the extended incubations which had to be used.

Staining of vacuoles

In the preceding paper (Indge, 1968*b*) the observations of Guilliermond (1941) on the staining of the yeast vacuole with neutral red were repeated. Guilliermond attributed the accumulation of the dye in the vacuole to the presence of colloidal materials in the sap. A model experiment was therefore done to see whether polyphosphates present in the vacuole sap could give rise to this phenomenon. A glass microscope slide was coated with agar (Oxoid Ionagar no. 2, 1 %, w/v, in 0·01 M-citrate+phosphate buffer, pH 5) and aqueous solutions of neutral red (0·005–0·01 %, w/v) and polyphosphate (1 %, w/v) were added to cups cut in the agar. The polyphosphate was prepared from hexametaphosphate according to Allgén & Norberg (1959). Precipitation of the dye occurred in regions of the gel where polyphosphate had diffused. Orthophosphate, tripolyphosphate and tetrapolyphosphate did not cause precipitation of the dye in control diffusion tests.

Other solutes present in the vacuole fraction

On the basis of the equivalent dry weight of whole yeast the protoplasts used in this work contained P 25 μg./mg., K^+ 20 μg./mg. and amino acids 0·5–0·6 μmoles/mg. One experiment, similar to that of Fig. 1, was done to measure the distribution of the K^+ and amino acid pools in the 2000 *g* pellet and supernatant fluid fractions separated

after metabolic lysis. It was found that 20% of the total K^+ pool and 23% of the total amino acid pool separated into the 2000 *g* pellet fraction.

In one further experiment the yeast was grown in medium which contained 10 μc./l. of [U-^{14}C]L-lysine or 50 μc./l. of [3-^{14}C] DL-serine. The yeast was converted to protoplasts which were then lysed metabolically. In this case the percentages of the total radioactivity of the protoplasts isolated from ^{14}C serine-labelled yeast and ^{14}C lysine-labelled yeast separated into the crude vacuole fraction were 30% and 70%, respectively.

DISCUSSION

It is suggested that the polyphosphates of the crude vacuole fraction are contained in the vacuole sap, although the evidence for this view is not conclusive. Proof of this hypothesis must await the purification of the vacuoles from the 2000 *g* pellet fraction of metabolically lysed protoplasts. However, it is difficult to account for the liberation of the polyphosphates of this fraction into solution by the various mild treatments applied (Table 2) if this is not the case. It is unlikely that volutin granules would survive exposure to the lysis medium (Harold, 1966). An alternative explanation is that the polyphosphates sedimented at 2000 *g* were adsorbed to particulate fragments of the protoplasts. Since considerable amounts of particulate material would remain in the 2000 *g* supernatant fluid fraction (Boulton, 1965) this explanation seems improbable. Weimberg & Orton (1965), however, have suggested that part (about 30%) of the polyphosphate of the yeast cell may be associated with the cell surface. A further possibility is that the polyphosphates are contained in vesicular structures other than the main vacuole.

The finding that the TCA extracts of the protoplasts apparently contained all the polyphosphates of the cell can be attributed to the low concentration of basic receptor groups in the extracts (Harold, 1966). It is possible that in the extraction of whole yeast cells with acid the cell wall serves to maintain a high concentration of positively charged proteins so that co-precipitation with the 'acid-insoluble' polyphosphate fraction takes place.

About 90% of the total acid-stable phosphorus compounds were found in the acid extracts of the cytoplasmic fractions of the protoplasts. This observation, taken with the results of the ^{32}P-orthophosphate uptake experiments, supports the view that the vacuole serves as a repository for reserve and waste materials (Svihla, Dainko & Schlenk, 1963) and is not a region of high metabolic activity. The small amount of acid-stable P found in the vacuole fraction may represent the occurrence of phosphorylated intermediates in the vacuole sap.

The polyphosphates appearing in the vacuole fraction during the ^{32}P-uptake experiments were not excluded on Sephadex G-25. This finding suggested that relatively low molecular weight polyphosphates were initially synthesised in the cytoplasm and that further chain-building steps took place in the vacuole. The flow of phosphorus in such a reaction sequence would be the reverse of that observed by Langen & Liss (1958) and by Ehrenberg (1961). However, phosphate uptake into the various P-containing fractions of yeast is markedly influenced by the prior starvation of the organisms and by the phosphate concentration in the medium (Ehrenberg, 1961). In the present experiments, with protoplasts from unstarved yeast, it was necessary to use only tracer amounts of phosphate in order to achieve reasonable count rates in the

cell fractions. Therefore these results cannot be compared directly with those obtained with starved organisms. Attempts to prepare protoplasts from such organisms were unsuccessful. The use of mercaptoethanol to prepare protoplasts from cells which are refractory to snail enzyme digestion (Davies & Elvin, 1964; Duell, Inoue & Utter, 1964) would appear to provide a means of overcoming this difficulty and thereby extend the scope of the present approach.

I wish to thank Professor A. A. Eddy for many helpful discussions and encouragement in the course of this work.

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Metabolism of a Plant Wax Paraffin (*n*-Nonacosane) by a Soil Bacterium (*Micrococcus cerificans*)

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SUMMARY

Long-chain paraffins, such as nonacosane, reach the soil from plants and are degraded by micro-organisms. The ability of one soil organism, *Micrococcus cerificans*, to grow on cabbage paraffin (*n*-nonacosane) was studied. Unlike the pattern seen with hexadecane, waxy esters were not produced when nonacosane was the sole carbon source, but esters were formed when a co-oxidation technique of [^{14}C] *n*-nonacosane with hexadecane was employed. The alcohol and acid portions of the radioactive esters formed were primarily of chain length C_{16}, C_{17} and C_{18}; neither nonacosanol nor nonacosanoic acid were detected. Thus the nonacosanol and nonacosanoic acid which might have been produced from *n*-nonacosane must have been oxidized to shorter-chain compounds which in turn were incorporated into waxy esters as well as other lipids.

INTRODUCTION

Large amounts of paraffins reach the soil from plants (Stevenson, 1966) and much of this is degraded by micro-organisms present in the soil. These plant paraffins are generally found to have carbon chain-lengths of C_{27}–C_{35}, with C_{29} (nonacosane) one of the most abundant (Silva-Fernandes, Baker & Martin, 1964). Little is known about bacterial utilization of these plant paraffins although the bacterial degradation of petroleum paraffins (C_{12} to C_{18}) has been studied extensively (McKenna & Kallio, 1965). Many species of micro-organisms can grow on paraffins as a sole carbon source and attempts are being made to produce edible microbial proteins from petroleum (Champagnat, 1964). Longer paraffins (C_{20}–C_{34}) are known to be utilized by micro-organisms (ZoBell, 1950) and they can also be utilized by animals (Kolattukudy & Hankin, 1966).

It has been shown (Stevenson, Finnerty & Kallio, 1962; Stewart *et al.* 1959; Stewart & Kallio, 1959) that *Micrococcus cerificans* produces waxy esters from medium chain-length paraffins; the alcohol portion of the ester has the same chain length as the substrate paraffin but the acid portion varies. If this metabolic pattern applies to the very-long-chain plant paraffins then very-long-chain alcohols would be produced. The demonstration that such long-chain alcohols possess androgenic activity (Levin, 1963) raises the possibility that the intestinal flora may produce physiologically active compounds from long-chain paraffins present in the food supply (Kuksis, 1964; Silva-Fernandes *et al.* 1964).

In the present report, the metabolic fate of *n*-nonacosane (from cabbage wax) in *M. cerificans*, a soil organism, is discussed.

METHODS

Organism. Micrococcus cerificans was maintained as a stock culture on agar slants prepared with 3·1 % of nutrient agar supplemented with 0·5 % each of yeast extract and Casamino acids (Difco), and subcultured weekly. The organism was kindly provided by Dr R. E. Kallio, University of Illinois, Urbana.

Media. The basal mineral medium contained per litre of distilled water: $(NH_4)_2SO_4$, 2 g.; KH_2PO_4, 4 g.; Na_2HPO_4, 6 g.; $FeSO_4.7H_2O$, 1 mg.; $MgSO_4$, 200 mg.; $CaCl_2$, 1 mg.; H_3BO_3, 10 μg.; $MnSO_4$, 10 μg.; $ZnSO_4$, 70 μg.; $CuSO_4$, 50 μg.; MoO_3, 10 μg.; calcium pantothenate, 200 mg. pH 7·5.

Test media (except where indicated) were prepared by adding sterile (autoclaved) paraffins (0·5–1·0 % except for radioactive material) to sterile basal mineral medium. To emulsify the hydrocarbon the medium was then subjected to ultrasonic disintegration (Biosonic II, Will Corporation) at 90 % of maximum output for 10 min. The ultrasonic disintegration step was omitted when *n*-hexadecane was the substrate.

Growth studies. In comparative growth tests of different substrates, the same amount of inoculum was placed in each flask. At least 27×10^6 bacteria/ml. were used in each test. Growth was measured by a direct microscopic method (American Public Health Assoc., 1948) after staining the bacteria with a solution of crystal violet. For all tests, the organism was grown in shake culture in a water bath at 30°. When radioactive *n*-nonacosane was used, in addition to shaking, sterile air was bubbled through the incubation mixture to remove radioactive CO_2.

Preparation of substrates and standards. Paraffins other than *n*-nonacosane were a gift from Dr B. J. Humphrey (Humphrey Chemical Co., North Haven, Conn., U.S.A.). Both radioactive and non-radioactive *n*-nonacosane was obtained from the cuticular wax of *Brassica oleracea* as previously described (Kolattukudy, 1965; Kolattukudy & Hankin, 1966). Standard long-chain esters used for gas chromatographic analysis were prepared from stearyl alcohol and acyl chlorides with pyridine as catalyst.

Gas chromatography. Gas chromatographic analyses were made on a Perkin Elmer 810 gas chromatograph with a flame ionization detector. The columns used have previously been described (Kolattukudy, 1967). Radioactive material emanating from the column was measured with a radioactivity monitor (Barber-Coleman).

Isolation and identification of lipids. Total lipids were extracted from the incubation mixture by the method of Folch, Lees & Sloane-Stanley (1957) with a 2+1 (v/v) mixture of chloroform and methanol. After concentration, the extract was placed on a silica gel column (Kolattukudy, 1967; Kolattukudy & Hankin, 1966). The components were eluted successively with hexane, benzene and methanol (containing 10 % of acetic acid). Hexane eluted the paraffins, benzene the esters, and methanol the polar lipids (glycerides, phospholipids). Lipid classes were identified by thin-layer chromatography (Mangold, 1965). Fatty acids from glycerides and phospholipids were isolated after saponification with ethanolic KOH under nitrogen. Methyl esters were prepared with BF_3 as catalyst. The waxy esters were isolated from the benzene eluate of the silica gel columns by thin-layer chromatography on silica gel G with benzene as the developing solvent. The ester fraction was saponified with ethanolic KOH under nitrogen, and the alcohol and acid fractions were isolated by thin-layer chromatography with hexane + diethyl ether + formic acid (40 + 10 + 1, by vol.)

(Kolattukudy, 1967) as the developing solvent. The alcohol fraction was analysed as the acetate, and the acids as the methyl esters by gas chromatography.

RESULTS

Growth of Micrococcus cerificans with long-chain paraffins

Micrococcus cerificans is able to grow on medium-chain-length paraffins (hexadecane, octadecane) as the sole source of carbon (Stevenson *et al.* 1962; Stewart *et al.* 1959; Stewart & Kallio, 1959). We have now observed that this soil organism can also utilize much longer paraffins, such as that from cabbage wax (*n*-nonacosane). Comparable growth was obtained with *n*-docosane (Fig. 1). Since it was noted that with small inocula the organism grew slowly, larger inocula were used in the biochemical studies. In preliminary experiments the paraffin was presented to the organism coated on diatomaceous earth (Celite) since the paraffin is not readily dispersible in aqueous solution. Although it grew with this experimental technique, better growth was obtained when ultrasonic emulsification of the substrate was employed.

Table 1. *Micrococcus cerificans: co-oxidation of n-hexadecane with* [^{14}*C*]*n-nonacosane* (C_{29})

Mineral medium (100 ml.) with 1·0 % of *n*-hexadecane and $23{\cdot}7 \times 10^6$ counts/min. [^{14}C]*n*-nonacosane were inoculated with *Micrococcus cerificans* and incubated for 20 hr at 30°. The water layer contained radioactivity but was not analysed further.

Product	% of administered ^{14}C
Respiratory CO_2	8·4
Hydrocarbon fraction	64·0
Ester fraction	0·81
Polar lipids	2·7
Wax acids	0·28
Wax alcohols	0·30
Cell residue	2·5

Ester production on paraffin substrates

The cultures were assayed for waxy ester production by thin-layer chromatography. Ester production was readily detected when hexadecane was the substrate, lesser amounts when docosane was used and none when nonacosane was the sole carbon source. To increase the chances of detecting a metabolite of nonacosane, such as a 1-nonacosanol, [^{14}C]nonacosane was presented to the organism in the presence of relatively large amounts of a readily oxidizable hydrocarbon, namely hexadecane. This co-oxidation technique with non-radioactive hexadecane and radioactive nonacosane resulted in small but significant amounts of radioactive ester being detected and isolated by the column and thin-layer chromatographic methods used (Table 1). Of necessity the growth period was shortened in order to avoid extensive degradation of the presumed intermediate, nonacosanol. Gas chromatography of these esters showed that the major radioactive esters had chain lengths of 31–34 carbon atoms.

Products formed from [^{14}C]n-nonacosane by co-oxidation with hexadecane

When the ester fraction was hydrolysed, the radioactivity was found to be equally distributed between the alcohol and acid parts (Table 1). The gas-chromatographic analysis of the alcohols (as the acetate) and the acids (as methyl esters) revealed that neither had a chain length comparable to that of the substrate hydrocarbon. The

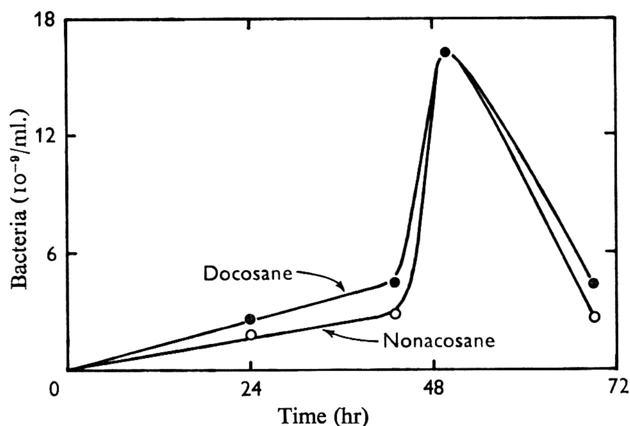

Fig. 1. Growth of *Micrococcus cerificans* on *n*-nonacosane and *n*-docosane as sole source of carbon; 30°.

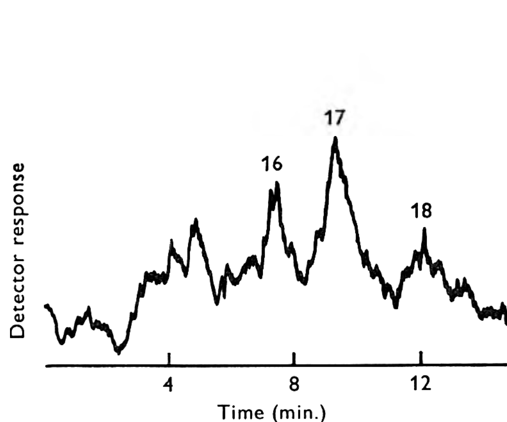

Fig. 2

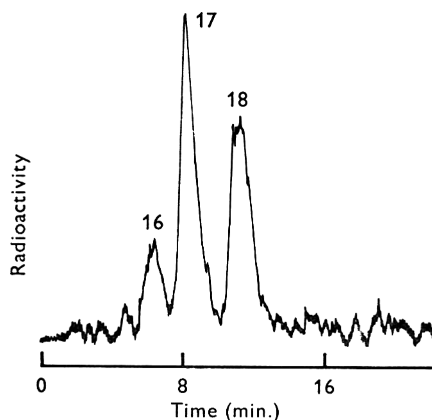

Fig. 3

Fig. 2. Radio gas–liquid chromatogram of alcohols (as acetates) derived from waxy esters produced by *Micrococcus cerificans* during co-oxidation of [^{14}C]*n*-nonacosane with *n*-hexadecane. Essentially all the mass was represented by C_{16} alcohol as measured by the flame ionization detector. Experimental conditions: 183 cm. (0·6 cm. o.d.) coiled copper column, 12 % Apiezon L on 50–60 mesh Anakrome A (Analabs, Hamden, Conn.), temperature of column and injector 230° and 350° respectively. Carrier gas was argon at 75 ml./min. Analysis on a 5 % SE-30 column at 300° did not show significantly radioactive alcohols longer than C_{18}.

Fig. 3. Radio gas–liquid chromatogram of fatty acids (as methyl esters) derived from polar lipids produced by *Micrococcus cerificans* during co-oxidation of [^{14}C]*n*-nonacosane with *n*-hexadecane. Experimental conditions same as in Fig. 2 except the column temperature which was 220°. Most of the mass was represented by approximately equal quantities of saturated and unsaturated C_{16} acids.

major alcohol was C_{17} with smaller amounts of C_{16} and C_{18} (Fig. 2). The wax acids were of similar chain lengths. The polar lipid fraction contained radioactivity several times higher than the ester fraction (Table 1). The major radioactive fatty acids were C_{17}, C_{18} and C_{16}, in decreasing order of magnitude (Fig. 3). This distribution pattern is almost identical to that seen with the wax acids and alcohols.

DISCUSSION

A possible explanation for the absence of ester production with *n*-nonacosane is that the rate of oxidation of the very-long-chain paraffins (C_{29}) is much slower than that of the medium-chain-length paraffins. Therefore, the first stable intermediate in very-long-chain oxidation (namely the alcohol) undergoes oxidative degradation as soon as it is formed. Consequently, unlike the case of the medium-length paraffins, the alcohols of the long-chain paraffins do not accumulate, and hence no ester formation occurs. If such be the case, dilution of the alcohol pool might be accomplished by

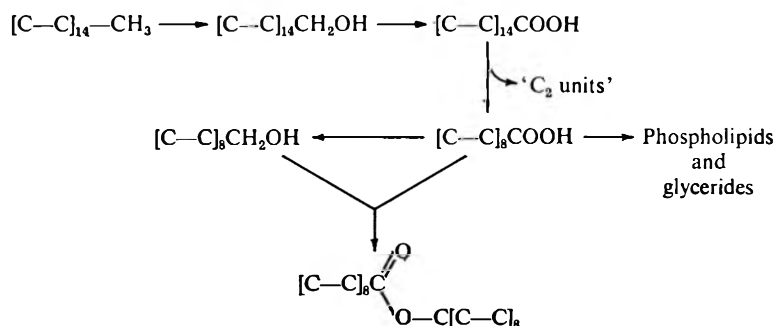

Fig. 4. Scheme of proposed metabolic fate of *n*-nonacosane in *Micrococcus cerificans.*

providing the organism with the readily oxidizable C_{16} paraffin along with C_{29} paraffin. Under these conditions sufficient nonacosanol (C_{29}) might accumulate for its esterification to occur. However, the absence of C_{29} alcohol from the ester fraction after co-oxidation indicates that in spite of the dilution of the alcohol pool that must have occurred as a result of the co-oxidation, the C_{29} alcohol underwent preferential oxidation. The enzymes that synthesize the waxy esters may prefer the medium-chain-length alcohol (C_{16} alcohol) and thus leave the C_{29} alcohol for oxidative degradation. However, the possibility of an oxidative attack on an internal carbon (such as C_{17}) of the paraffin cannot be ruled out. The radioactive alcohols detected in the ester fraction were probably formed by reduction of fatty acids derived from the oxidation of C_{29} paraffin. This hypothesis is consistent with the fact that the chain lengths of the radioactive acids and alcohols were similar. Stewart *et al.* (1959) did find ester formation when this organism was grown on palmitic acid, although the ester production was very much less than with hexadecanol (C_{16}). This indicates that palmitic acid did undergo reduction to the alcohol.

These results suggest the metabolic fate of C_{29} paraffin with *Micrococcus cerificans* as depicted in Fig. 4. Although no direct evidence was obtained from the present experiments, it is likely that the end carbon of C_{29} paraffin would undergo oxidation

to the corresponding alcohol presumably as described in the case of octane (Gholson, Baptist & Coon, 1963) and hexadecane (Stewart *et al.* 1959; Stewart & Kallio, 1959). This alcohol in turn is presumably oxidized to the corresponding acid which appears to undergo rapid 'chain shortening' to the level of common fatty acids of the organism (C_{16}–C_{18}). Such a conversion could be accomplished by a β-oxidation system; but in that case the β-oxidation would have to cease at the C_{17} stage. Although it is thought that β-oxidation of a fatty acid proceeds to completion, C_{18} acid has been shown to be converted into C_{16} acid in the rat by a 'leakage' in the β-oxidation system (Elovson, 1965). However, a 'chain-shortening' system (counterpart of the chain-lengthening system) similar to, but not identical with the β-oxidation system, could have been responsible for the formation of C_{17} acid from C_{29} acid. If it is a 'leakage', then it must be specific enough to cause 'leakage' primarily at the C_{17} stage. The complete conversion of C_{29} acid into 13 acetate units and a propionate unit, and the resynthesis of C_{17} from such units, cannot be ruled out conclusively. However, the relative magnitude of radioactivity in the various metabolic products makes this unlikely. The medium-chain-length acids, primarily C_{17}, derived from C_{29} paraffin are then incorporated mostly into phospholipids; smaller quantities undergo reduction to the alcohol which in turn is esterified. In the rat, *n*-nonacosane underwent a similar oxidative degradation, resulting in primarily C_{17} acid which was incorporated into phospholipids and glycerides (Kolattukudy & Hankin, 1966).

The organism used in this study, *Micrococcus cerificans*, was isolated by enrichment culture with the use of hexadecane (Finnerty, Hawtrey & Kallio, 1962) from soil. It might be possible to isolate an organism from soil which would more readily utilize, or even prefer, plant wax paraffin, if such paraffins were used in the enrichment procedures.

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The fifty-first meeting of the Society was held jointly with the Biochemical Society at the University of Leicester on Thursday and Friday, 14 *and* 15 *December* 1967. *The titles of the papers that were presented were as follows. Abstracts of the papers are published in the Biochemical Journal* (*Proceedings of the* 477*th Meeting*).

SYMPOSIUM ON 'CONTROL OF GENE FUNCTION IN MICRO-ORGANISMS'

Control of the Enzymes of the β-Oxoadipate Pathway in Bacteria. By R. Y. Stanier (*Department of Bacteriology and Immunology, University of California, Berkeley, Calif.* 94720, *U.S.A.*)

Regulation of the Synthesis of the *Escherichia coli* Pyruvate Dehydrogenase. By U. Henning, J. Dietrich and G. Deppe (*Max Planck-Institut für Biologie, Tübingen, Germany*)

The Regulation of Nitrate Reduction in the Fungus, *Aspergillus nidulans*. By D. J. Cove (*Department of Genetics, University of Cambridge*)

Mutations which affect the Synthesis and Function of Ribosomes. By G. Turnock (*Department of Genetics, University of Leicester*)

Temporal Control of Gene Expression in Bacteria. By W. D. Donachie (*M.R.C. Microbial Genetics Research Unit, Hammersmith Hospital, Ducane Road, London, W.* 12)

Cell Differentiation in the Cellular Slime Mould *Dictyostelium discoideum*. By J. M. Ashworth (*Department of Biochemistry, University of Leicester, Leicester*)

Early Biochemical Events in Sporulation of *Bacillus subtilis*. By J. Mandelstam, W. W. Waites, S. C. Warren and J. M. Sterlini (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

Developmental Changes During Outgrowth of Bacterial Spores. By H. Halvorson (*Department of Molecular Biology, University of Wisconsin, Madison, Wis., U.S.A.*)

ORIGINAL PAPERS

Regulation of Secondary (Phenolic) Biosynthesis in *Penicillium urticae*. By J. D. Bu'Lock and and D. Shepherd (*Microbial Chemistry Laboratory, Department of Chemistry, The University of Manchester*)

Onset of a Secondary Biosynthesis in Some Penicillia. By J. D. Bu'Lock, M. A. Hulme, A. J. Powell and D. Shepherd (*Microbial Chemistry Laboratory, Department of Chemistry, University of Manchester*)

Regulation of Convergent Pathways for Shikimate and *p*-Hydroxybenzoate Oxidation by Moraxella. By J. L. Cánovas (*Department of Bacteriology and Immunology, University of California, Berkeley, Calif.,* 94720, *U.S.A.*)

Regulation of Enzymes of the Mandelate Pathway in Bacterium NCIB 8250. By C. A. Fewson and S. I. T. Kennedy (*Department of Biochemistry, University of Glasgow*)

Consequences of Derepression in the *lac* Operon. By W. H. Holms (*Department of Biochemistry, University of Glasgow*)

Induction and Repression of Arginase and of Ornithine Transaminase in Baker's Yeast. By W. J. Middelhoven (introduced by K. S. Dodgson) (*Laboratorium voor Microbiologie der Landbouwhogeschool, Wageningen, Netherlands*)

β-Galactosidase Messenger in *Escherichia coli* Lysates. By J. G. EDWARDS, D. R. JAMES, A. P. MATHIAS and M. J. EVANS (*Department of Biochemistry and Department of Anatomy and Embryology, University College, London*)

Positive Control of Sulphate Reduction in *Escherichia coli.* By M. C. JONES-MORTIMER (introduced by C. A. PASTERNAK) (*Department of Biochemistry, University of Oxford*)

Control of Chloroplast Formation in *Euglena gracilis*: Antagonism between Carbon and Nitrogen Sources. By R. C. HARRIS and J. T. O. KIRK (*Department of Biochemistry and Agricultural Biochemistry, University College of Wales, Aberystwyth*)

Regulation of Enzyme Profiles in *Escherichia coli*: The Effects of Oxygen, Nitrate or Nitrite. By J. W. T. WIMPENNY (*M.R.C. Group for Microbial Structure and Function, c/o Mining Department, Newport Road, Cardiff*)

Vancomycin and Mucopeptide Precursors. By H. R. PERKINS (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

Genetic Analysis of Some Mutations Causing Resistance to Tetracycline in *Escherichia coli* K 12. By E. C. R. REEVE (*A.R.C. Unit of Animal Genetics, Institute of Animal Genetics, Edinburgh, 9*)

Enzymic Properties of a Mutant of *Escherichia coli* K 12 lacking Nitrate Reductase. By W. A. VENABLES, J. W. T. WIMPENNY and J. A. COLE (*Department of Microbiology, University College of South Wales and Monmouthshire, Cardiff*)

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Difficulties Experienced in the Hybridization of *Penicillium patulum.* By C. T. CALAM and L. B. DAGLISH (*Imperial Chemical Industries Ltd., Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire*)

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