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THE JOURNAL OF GENERAL MICROBIOLOGY

THE PREPARATION OF PAPERS

'The more words there are, the more words there are about which doubts may be entertained.'
JEREMY BENTHAM (1748–1832)

'What can be said at all can be said clearly; and whereof one cannot speak thereof one must be silent.'
LUDWIG WITTGENSTEIN: *Tractatus Logico-philosophicus* (tr. C. K. Ogden)

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Ainsworth and Bisby's Dictionary of the Fungi, 5th ed. (1961). Kew: Commonwealth Mycological Institute.

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Effect of Actidione, Griseofulvin and Triphenyltin Acetate on the Kinetics of Fungal Growth

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(*Accepted for publication 10 November 1969*)

SUMMARY

The colony radial growth rate and germ tube specific growth rate of *Absidia glauca* decreased linearly with the logarithm of actidione, triphenyltin acetate or griseofulvin concentration. Colony growth rate was directly related to the mould's specific growth rate in submerged culture over a 200-fold range of actidione concentration. The radial growth rate of *Aspergillus nidulans* colonies was not influenced by inoculum concentration but colony diameter was directly related to the logarithm of the inoculum concentration over the lower range.

INTRODUCTION

Although colony diameter and radial growth rate are frequently used to measure fungal growth in bioassays and physiological investigations (Cochrane, 1958), few critical studies have been made to evaluate these growth parameters. The rate of growth of a mould can be defined most precisely in terms of its specific growth rate (α) or doubling time (t_d) in submerged culture. The specific growth rate of an organism may be calculated from the expression:

$$\alpha = \ln 2/t_d,$$

where t_d is the doubling time. The reliability of colony radial growth rate as a parameter of growth may be assessed by determining its relationship to the specific growth rate of the mould in submerged culture under various cultural conditions. Using this criterion the radial growth rate of *Aspergillus nidulans* colonies is directly related to the mould's specific growth rate over a wide temperature range (Trinci, 1969) and may thus be used to determine the optimum growth temperature for this, and presumably other, moulds. However, colony radial growth rate is not a suitable parameter to use to assess the relative growth rates of different mould species (Trinci, 1969) or even mutants of the same species (Bainbridge & Trinci, 1969). Similarly colony extension cannot be used as a meaningful assessment of growth in nutritional studies (Trinci, 1969).

Variation in colony radial growth rate has frequently been used to measure the inhibitory effect of antifungal chemicals and the main object of this study was to determine if there was a correlation between the colony radial growth rate of a mould and its specific growth rate in submerged culture over a range of inhibitor concentration. In a previous study (Trinci, 1969) the value of germ tube specific growth rate as a parameter of mould growth was investigated. We continue this study in the present work.

Three antifungal agents were used in this investigation—griseofulvin, actidione (cycloheximide) and triphenyltin acetate (TPTA). Actidione inhibits protein synthesis in eucaryotic organisms but the mode of action of TPTA and griseofulvin is unknown although it has been suggested that the latter interferes with chitin biosynthesis (Brian, 1949). Triphenyltin compounds have recently been used to control fungal pathogens of plants (Hislop, 1963; Stallknecht & Calpouzos, 1968).

METHODS

Organisms. Aspergillus nidulans BWB 224 (Glasgow) recombinant ve y and *Absidia glauca* (+), Queen Elizabeth College strain z 8 were used; *A. glauca* was grown at 25° and *A. nidulans* at 37°.

Media. Aspergillus nidulans was grown on the DAN medium described previously (Trinci, 1969). The composition of the medium (DPM) for *Absidia glauca* was (g./l.): D-glucose, 10; KH_2PO_4, 3·4; $Na_2HPO_4.12H_2O$, 8·9; $(NH_4)_2SO_4$, 6; disodium ethylenediaminotetracetic acid (EDTA), 0·6; $MgSO_4.7H_2O$, 0·25; $CaCl_2$, 0·05; $ZnSO_4.7H_2O$, 0·2; $MnSO_4.4H_2O$, 0·02; $CuSO_4.5H_2O$, 0·005; $FeSO_4.7H_2O$, 0·1; Na_2SO_4, 0·5; $NaMoO_4(2H_2O)$, 0·005; thiamine hydrochloride, 0·0005. The pH of the medium was 6·8. The media were prepared in the manner described previously (Trinci, 1969).

Preparation of solutions of the growth inhibitors. Stock solutions of griseofulvin (Glaxo), 1000 mg./l., and triphenyltin acetate (kindly provided by Mr R. Barnes, Chemistry Department, Queen Elizabeth College), 100 mg./l., in ethanol were prepared separately. The stock solutions were diluted with ethanol so that the appropriate inhibitor concentration was obtained when 10 ml. was added to 200 ml. of medium. Molten agar was added to the other constituents held at 70° in a water bath. The final concentration of ethanol in the medium was thus less than 5%. The controls contained the same concentration of ethanol as the media with inhibitor. The stock solution (4000 mg./l.) of aqueous actidione (Upjohn, Kalamazoo, Michigan, U.S.A.) was sterilized by membrane filtration and diluted with sterile distilled water so that 20 ml. added to 180 ml of media gave the requisite inhibitor concentration.

Unless stated otherwise, all other methods are the same as those described previously (Trinci, 1969).

RESULTS

Effect of inoculum on colony diameter and radial growth rate

Two methods are widely used to assess the linear growth of fungal colonies on solid media, *viz.* colony diameter at a set time after inoculation (Brancato & Golding, 1953) and colony radial growth rate (Lindenmayer & Schoen, 1967). However, under certain circumstances, as shown below, these two methods do not give the same result.

A suspension of *Aspergillus nidulans* conidia (4×10^7 conidia/ml.) was serially diluted 1/4 in sterile distilled water and each dilution was used to inoculate two plates. The diameters of the colonies 48 hr after inoculation and their radial growth rates between 26 and 48 hr are shown in Fig. 1. Although colony radial growth rate was not influenced by inoculum size, colony diameter increased linearly with the logarithm of the inoculum concentration up to 800 conidia/cm^2 (cf. Gillie, 1968).

Effect of actidione, griseofulvin and triphenyltin acetate on colony radial growth rate

The effects of actidione, griseofulvin and triphenyltin acetate on the radial growth rate (K_r) of *Absidia glauca* colonies are shown in Fig. 2. In each case the radial growth rate of the colonies decreased linearly with the logarithm of the inhibitor concentration.

Effect of actidione, griseofulvin and triphenyltin acetate on germ tube specific growth rate

In these experiments the diameter of the sporangiospore was included in all measurements and if a second germ tube was formed its length was also included (the spore usually produced only one or two germ tubes and rarely three).

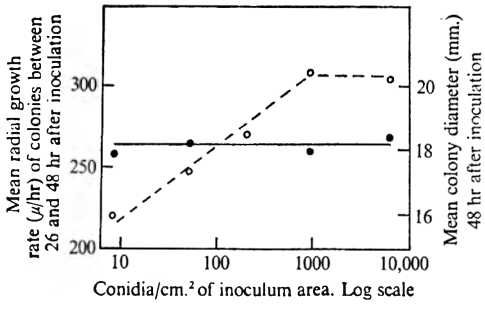

Fig. 1

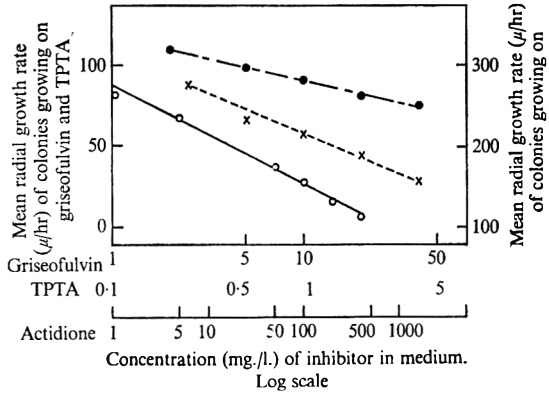

Fig. 2

Fig. 1. Influence of inoculum concentration on the radial growth rate (●) and diameter (○) of *Aspergillus nidulans* colonies.

Fig. 2. Effect of actidione (○), griseofulvin, (●) and triphenyltin acetate (×) on the radial growth rate of *Absidia glauca* colonies.

The germ tubes of *Absidia glauca* sporangiospores, like those of *Aspergillus nidulans* conidia (Trinci, 1969), increased in length at an exponential rate as soon as germination commenced (Fig. 3). The specific growth rates of *A. glauca* germ tubes grown on the three inhibitors are shown in Fig. 4. In each case the germ tube specific growth rate decreased linearly with the logarithm of the inhibitor concentration.

Effect of actidione on colony radial growth rate and germ tube specific growth rate on solid media and on specific growth rate in submerged culture

The specific growth rate (α_s) of *Absidia glauca* grown in submerged culture on media containing various concentrations of actidione was determined in the manner described previously (Trinci, 1969). The results are presented in Table 1 together with the ratios *colony radial growth rate: specific growth rate in submerged culture* (K_r/α_s) *colony radial growth rate*: *germ tube specific growth rate* (K_r/α_g) and *germ tube specific growth rate: specific growth rate in submerged culture* (α_g/α_s) and the corresponding correlation coefficients. The extension rate of *A. glauca* colonies was directly related to the mould's specific growth rate in submerged culture over the range of actidione concentration tested.

The α_g/α_s ratio of 5·1 for *Absidia glauca* growing on media lacking actidione is similar to the value 5·9 obtained with *Mucor hiemalis* (Trinci, 1969), another Phycomycete. It is not known why the germ tube specific growth rate of an organism is consistently higher than its specific growth rate in submerged culture.

The K_r/α_g ratios and correlation coefficients for *Absidia glauca* growing on media containing griseofulvin and triphenyltin acetate are shown in Table 2: for both inhibitors the correlation was statistically significant.

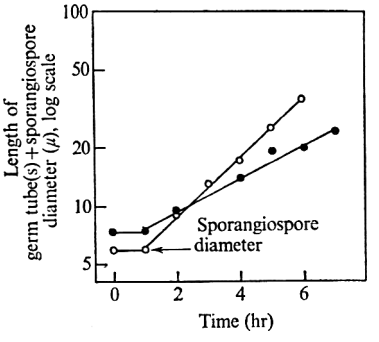

Fig. 3

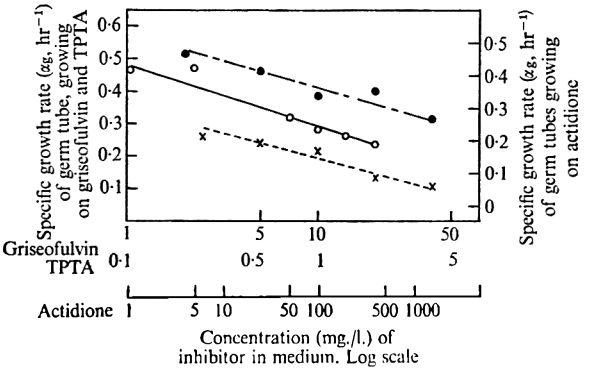

Fig. 4

Fig. 3. Germ tube growth of *Absidia glauca* sporangiospores on media lacking (○) and containing 200 mg./l. actidione (●).

Fig. 4. Effect of actidione (○), griseofulvin (●) and triphenyltin acetate (×) on the specific growth rate of *Absidia glauca* germ tubes.

Table 1. *Effect of actidione on colony radial growth rate and germ tube specific growth rate of Absidia glauca on solid medium and specific growth rate in submerged culture at 25°.*

Actidione concentration (mg./l.)	Colony radial growth rate (K_r, μ/hr)*	Growth in submerged culture: Specific growth rate (α_s, hr^{-1})‡	Growth in submerged culture: Doubling time (t_{ds}, hr)	Germ tube growth: Specific growth rate (αg, hr^{-1})†	Germ tube growth: Doubling time (t_{dg}, hr)	10^{-4} × K_r/α_s	Ratios 10^{-3} × K_r/α_g	α_g/α
0	274	0·075	9·2	0·385	1·8	3·7	7·1	5·1
1	265	0·067	10·4	0·365	1·9	3·9	7·3	5·4
5	236	0·063	11·0	0·385	1·8	3·7	6·1	6·1
50	175	0·059	11·8	0·226	2·7	3·0	7·7	3·8
100	156	0·042	16·4	0·187	3·7	3·7	8·3	4·5
200	132	0·034	20·1	0·169	4·1	3·9	7·8	5·0
400	114	—	—	0·144	4·8	—	7·9	—

* K_r, each rate is the mean of 12 colonies from 2 separate experiments.
† α_g, each rate is the mean of 5 to 11 germ tubes from at least 3 separate experiments.
‡ α_s, each rate is the mean of 3 separate experiments.
Ratio means: K_r/α_s — 3·7; K_r/α_g — 7·5; α_g/α_s — 5·0.
Correlation coefficients, K_r/α_s — 0·84§; K_r/α_g — 0·66; α_g/α_s — 0·96§
§ Significant at 5 % level of probability.

Comparison between the colony radial growth rates on freshly prepared and old media containing actidione

Actidione not only causes a decrease in the radial growth rate of *Absidia glauca* colonies but also increases the lag between inoculation and spore germination (Fig. 5). In the previous experiments there may have been a reduction in the effective antibiotic concentration during the prolonged lag phase at certain actidione concentrations,

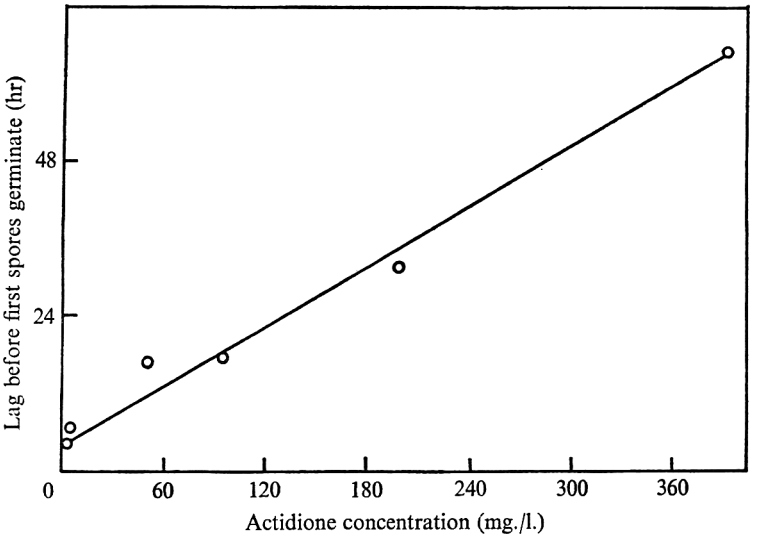

Fig. 5. Effect of actidione on the duration of the lag between inoculation and the germination of the first sporangiospores of *Absidia glauca*.

Table 2. *Values for the ratio colony radial growth rate: germ tube specific growth rate* (K_r/α_g) *for Absidia glauca cultured on various concentrations of griseofulvin and triphenyltin acetate*

Griseofulvin concentration (mg./l.)	$10^{-2} \times K_r/\alpha_g$	Triphenyltin acetate concentration (mg./l.)	$10^{-2} \times K_r/\alpha_g$
0	2·2	0	2·5
2	2·1	0·25	3·4
5	2·1	0·50	2·7
10	2·3	1·0	2·7
20	2·0	2·0	3·5
40	2·4	4·0	2·6

Correlation coefficients: K_r/α_g on griseofulvin = 0·89, K_r/α_g on triphenyltin acetate = 0·90. Both are significant at the 5 % level of probability.

and this in turn may have influenced the type of growth response obtained. The stability of actidione at the incubation temperature was tested by comparing the growth rate of colonies cultured on media (containing 5 to 400 mg./actiodine ml.) which had been kept for 8 days at 25° before inoculation, with colonies grown on freshly prepared media. No significant difference was observed between the degree of growth inhibition,

which indicates that even after 8 days at 25° there was no appreciable degradation of actidione.

DISCUSSION

The present study indicates that colony radial growth rate is preferable to colony diameter as a means of assessing the effect of inhibitors on growth. Actidione influences the lag as well as having an effect on growth rate and thus the diameter of a colony is determined by the sum of these two effects. In this case, at least, the inhibitor influences the kinetics of these processes in different ways. Further, colony diameter, unlike colony radial growth rate, is influenced by the concentration of the inoculum. In the case of inocula containing 800 conidia/cm^2. or above, growth of the colony was probably initiated at the circumference of the inoculum immediately after germination but at lower inoculum concentrations, time would elapse whilst the mycelium first covered the inoculum area. Colony radial growth rate increases linearly with the logarithm of concentration of the growth-limiting nutrient (Gillie, 1968; Trinci, 1969). The present study shows that colony radial growth rate decreases linearly with the logarithm of the concentration of an inhibitor in the medium. The bases of these two relationships is not known. They may, however, be of value in establishing bioassays for vitamins or antifungal chemicals. A linear relationship between colony growth and the logarithm of griseofulvin concentration has previously been reported by Lenhart (1968).

The growth rate of *Absidia glauca* colonies was directly related to the mould's specific growth rate in submerged cultures over a 200-fold range of actidione concentration. Colony radial growth rate would thus seem to be a reliable parameter to use to determine the effect of an inhibitor on fungal growth.

We would like to thank Dr B. W. Bainbridge for helpful discussion during the preparation of this paper.

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Studies on the Group F Antigen of Lactobacilli: Isolation of a Teichoic Acid–Lipid Complex from *Lactobacillus fermenti* NCTC 6991

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(*Accepted for publication 11 November 1969*)

SUMMARY

Membrane glycerol teichoic acid has been extracted from *Lactobacillus fermenti* NCTC 6991 by two different procedures. Phenol extraction gave a complex of teichoic acid with glycolipid and phospholipid (lipoteichoic acid). Trichloracetic acid extraction resulted in degraded lipoteichoic acid and free teichoic acid chains. The teichoic acid is a 1:3 phosphodiester linked glycerophosphate polymer substituted with D-alanine, D-galactose and a disaccharide of D-galactose and D-glucose. The significance of lipoteichoic acid as a membrane constituent is discussed.

INTRODUCTION

Glycerol teichoic acids occur as intracellular components in probably all Gram-positive bacteria. They may be isolated by extraction, with trichloracetic acid (Critchley, Archibald & Baddiley, 1962) or phenol (Burger & Glaser, 1964), of the gelatinous pellet of ribosomes and membrane fragments obtained by high-speed centrifugation of wall-free disrupted organisms. The occurrence of glycerol teichoic acids as contaminants in preparations of bacterial ribosomes (Wicken & Baddiley, 1963) and DNA (Young & Jackson, 1966) has been noted previously. A number of investigators (Hay, Wicken & Baddiley, 1963; Shattock & Smith, 1963; Shockman & Slade, 1964; Smith & Shattock 1964) have suggested that the glycerol teichoic acid which is antigenically specific for group D *Streptococcus faecalis* is located in or on the external surface of the protoplast membrane. These findings have led to intracellular teichoic acids being generally regarded as membrane teichoic acids (Archibald, Baddiley & Blumsom, 1968) although the chemical nature of this association remains undefined. The serological activity of membrane teichoic acids from a number of Gram-positive bacteria in precipitin tests with sera prepared against whole organisms has been well established. Purified teichoic acids have not been shown to act as antigens unless they are first made particulate by complexing with cationic precipitating agents (Burger, 1966).

Sharpe, Davison & Baddiley (1964) suggested that the serological activity of group F

lactobacilli (e.g. *Lactobacillus fermenti*) might reside in an intracellular (membrane) teichoic acid although no analytical data were reported. In experiments designed to confirm this supposition we have isolated the membrane teichoic acid from *L. fermenti* NCTC 6991 as a complex with lipid in a form which retains antigenicity and will sensitize normal sheep red blood cells. This paper describes the isolation procedure and a partial characterization of the complex while following papers (Hewett, Knox & Wicken, 1970; Knox, Hewett & Wicken, 1970) describe the serological properties of this complex.

METHODS

Organism. Lactobacillus fermenti NCTC 6991 was originally obtained from the National Collection of Type Cultures, Colindale, London; this strain was employed by Sharpe (1955) in the serological classification of lactobacilli and belongs to serological group F (Sharpe & Wheater, 1957).

Preparation of teichoic acid extracts. Organisms were grown and disrupted as described previously (Knox & Holmwood, 1968). Cell walls were removed by centrifugation at 12,000 *g* for 20 min. (Servall RC2), and the non-sedimentable material retained as a source of membrane teichoic acids. Centrifugation of this material at 100,000 *g* (Spinco L2-65) yielded a gelatinous pellet and a clear supernatant fraction, both of which were used as a source of teichoic acid. These fractions were freeze-dried and then reconstituted in water (3% (w/v) for the gel fraction and 10% (w/v) for the supernatant fraction). Two procedures were utilized for extracting teichoic acid from these fractions. (i) The crude material was stirred with an equal volume of 20% trichloracetic acid (TCA) at 4° for 24 hr. Insoluble material was removed by centrifugation and re-extracted for 24 hr with a further volume of 10% TCA. The combined extracts were washed repeatedly with ether to remove TCA and then teichoic acid was recovered from the aqueous layer by freeze-drying. (ii) The crude material was shaken with an equal volume of 90% aqueous phenol at 4° for 1 hr. After centrifugation, the aqueous layer was removed and the phenol layer washed with one volume of water. The combined aqueous layers were dialysed against three changes of distilled water and the non-diffusible material concentrated by rotary evaporation and finally freeze dried. The dried material was stirred with chloroform + methanol (2 + 1, v/v) for 2 hr at room temperature and insoluble material recovered by filtration through sintered glass. Nucleic acids in this preparation were degraded by incubation under toluene with deoxyribonuclease and ribonuclease (Sigma Chemical Co.) in 0·05 M-tris buffer, pH 8·0, for 16 hr at 37° (2 mg. of each enzyme + 1 m-mole $MgCl_2$/100 ml. buffer). The digest was extracted with phenol as described above and the aqueous fraction dialysed against distilled water. Teichoic acid was recovered by freeze drying and extracted once more with chloroform + methanol and finally dried *in vacuo*.

Gel chromatography. Gel chromatography was routinely carried out on columns of Sephadex G75 and G200 (Pharmacia) and 6% agarose (Litex, Denmark) at ambient temperatures with 0·2 M ammonium acetate, pH 6·9, as elutant. Fractions (about 5 ml.) were collected automatically by drop-counting using standard LKB equipment and samples were analysed for organic phosphorus and extinction at 260 mμ. Column void volumes were determined by the elution volumes of blue dextran (Pharmacia).

Analytical methods. Conditions for the acidic and alkaline hydrolysis of teichoic acids, enzymic dephosphorylation, characterization of glycosides and the identifica-

tion of products by paper chromatography, unless stated in the text, are essentially as described previously (Forrester & Wicken, 1966; Wicken, 1966). Glucose was determined by the glucose oxidase method of Huggett & Nixon (1957), galactose by the galactostat reagent (Worthington) and phosphorus by the method of Ames (1966). Fatty acid esters were determined by a micro-modification of the method of Snyder & Stephens (1959). Gas liquid chromatography of fatty acids was kindly performed by Dr M. Birmingham, Department of Biochemistry, University of Sydney.

Paper and thin-layer chromatography. The following solvent systems were used for paper chromatography: (A) propan-1-ol + aq. ammonia (sp.gr. 0·88) + water (6 + 3 + 1 by vol., Hanes & Isherwood, 1949), Whatman paper no. 4, ascending; (B) butan-1-ol + pyridine + water (6 + 4 + 3, by vol., Jeanes, Wise & Dimler, 1951), Whatman paper, nos. 1 or 3 MM, descending; (C) ethyl acetate + pyridine + water (5 + 2 + 5 by vol., upper layer, Sastry & Kates, 1964). Whatman paper no. 1 or 3 MM, descending. For thin layer chromatography, silica gel G plates (E. Merck A-G) were prepared and activated at 105° for 16 hr before use. Plates were developed in either (D), chloroform + methanol + water (65 + 25 + 4, by vol.) or (E) light petroleum (b.p. 40 to 60°) + ethyl ether + acetic acid (80 + 20 + 1, by vol.).

The following spray reagents were used where applicable: periodate + Schiff's reagent for polyols and glycosides (Baddiley, Buchanan, Handschumacher & Prescott, 1956); perchloric acid + molybdate reagent for phosphates (Hanes & Isherwood, 1949); alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950) for reducing compounds and modified according to Brundish, Shaw & Baddiley, (1965) for glycosides; ninhydrin for amino acids (Consden & Gordon, 1948); rhodamine G6 (Dittmer & Lester, 1964) and iodine vapour for lipids; dichlorofluorescein + aluminium chloride + ferric chloride (Dudzinski, 1967) for fatty acids.

Isolation of glycolipid. Lipid was extracted from freeze-dried whole organisms with chloroform + methanol (2 + 1, v/v) as described by Brundish, Shaw & Baddiley (1965). Non-lipid contaminants were removed with Sephadex G25 (Wells & Dittmer 1963). A portion of the purified lipid (100 mg.) in chloroform + methanol (9 + 1, v/v) was applied to a column of silicic acid (10 g., Sigma SIL-LC) and eluted successively with 75 ml. amounts of chloroform, acetone and methanol. Thin-layer chromatography showed the bulk of the glycolipid to be present in the acetone fraction. Glycolipids in this fraction were deacylated (Wilkinson, 1968) without further purification and the constituent glycosides separated by paper chromatography in solvent B.

RESULTS

Gel chromatography of teichoic acid extracts

Phenol-extracted teichoic acids were excluded from columns of Sephadex G75 and G200 but were included by 6% agarose columns being eluted as single peaks close to the void volume. Identical elution volumes for teichoic acid were obtained for material extracted from gel or supernatant fractions (see Methods) and contaminating high molecular weight nucleic acid material did not occur in the same peak as teichoic acid. Fractions containing teichoic acid were combined, dialysed and freeze dried. Such preparations were designated as P-teichoic acids with the notations (gel) and (sol), where necessary, indicating origin from gel or supernatant fractions respectively. An elution diagram of P-teichoic acid (gel) from 6% agarose is shown in Fig. 1.

Dr M. W. A. Thomas, Department of Biochemistry, University of Sydney, kindly performed an ultracentrifugal analysis of 1 % (w/v) P-teichoic acid in 0·15 M-NaCl+ 0·15 M citrate pH 7·4 at 20°; the preparation was relatively monodisperse, the sedimentation coefficient S of 9·5 S being indicative of a macromolecule.

After dialysis, the teichoic acids extracted by TCA were separated on columns of Sephadex G75 into two fractions having K_d = 0·0 and 0·5. The excluded fraction was also excluded from Sephadex G200 but included by 6 % agarose. Agarose also gave a better separation of both teichoic acid fractions from contaminating nucleic acid

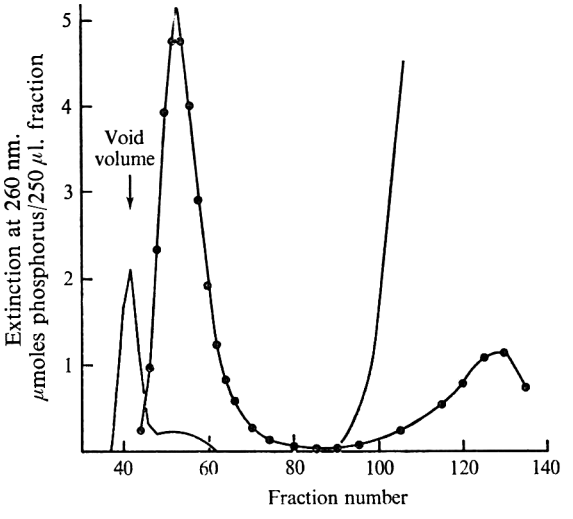

Fig. 1

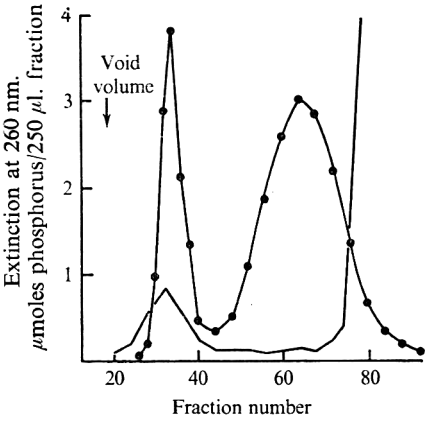

Fig. 2

Fig. 1. Agarose gel (6 %) chromatography of teichoic acid obtained by phenol extraction of gel fraction from *Lactobacillus fermenti*. Column (91 × 2·6 cm.) loaded with 360 mg. crude material and eluted with 0·2 M-ammonium acetate, pH 6·9, at a flow rate of 13 ml./hr; ●, μmoles phosphorus/0·25 ml. fraction; continuous line, extinction at 260 nm.

Fig. 2. Agarose gel (6 %) chromatography of teichoic acid obtained by TCA extraction of gel fraction from *Lactobacillus fermenti*. Column (40 × 2·6 cm.) loaded with 270 mg. crude material and eluted with 0·2 M ammonium acetate, pH 6·9, at a flow rate of 20 ml./hr.; ●, μmoles phosphorus /0·25 ml. fraction; continuous line, extinction at 260 nm.

material and was used routinely in preference to Sephadex. The material with the higher apparent molecular weight was designated as HT-teichoic acid and the material with the lower apparent molecular weight as LT-teichoic acid, the notations (gel) and (sol) again, where necessary, being used to indicate origin. Fig. 2 shows the elution diagram of HT- and LT-teichoic acids (gel) from a column of 6 % agarose. HT-and LT-teichoic acids (sol) showed similar elution patterns although the LT-fraction was contaminated with polysaccharide. Teichoic acids were recovered, after dialysis, by freeze drying.

Composition of the teichoic acids

Paper chromatography of acid hydrolysates of all teichoic acid preparations in solvents A and B showed glycerol, glyceromono- and di-phosphates, inorganic phosphate, glucose and galactose. D-Alanine was evident in HT and LT-teichoic acids and the corresponding amide was obtained from these preparations by ammonolysis.

P-teichoic acids were devoid of all but traces of ester-linked alanine, as would be expected from the incubation at pH 8 during the extraction procedure (see Methods), but showed a range of amino acids in acid hydrolysates indicative of the presence of a small amount of protein. Mole ratios of glucose: galactose and total sugar: phosphorus are shown in Table 1.

Table 1. *Mole ratios of glucose: galactose and total sugar: phosphorus in teichoic acid preparations*

Fraction	Glucose: galactose	Total sugar: phosphorus
P-Teichoic acid (gel)	1·00:2·13	0·17:1·00
P-Teichoic acid (sol)	1·00:1·97	0·18:1·00
HT-Teichoic acid (gel)	1·00:1·15	0·48:1·00
HT-Teichoic acid (sol)	1·00:1·36	0·50:1·00
LT-Teichoic acid (gel)	1·00:1·40	0·32:1·00
LT-Teichoic acid (sol)	*	*

*Values for ratios not reported as analyses indicated that this fraction was contaminated with polysaccharide.

Table 2. *Partial characterization of glycerol glycosides from alkaline hydrolysates of teichoic acid fractions*

	Glycoside *1*	Glycoside *2*	Glycoside *3*
P-Teichoic acid	+	+	+
HT-Teichoic acid	+	+	+
LT-Teichoic acid	—	+	+
R_{glc} (solvent B)	0·46	0·42	1·00
R_{glc} (solvent C)	0·39	0·29	0·88
Reaction with periodate + Schiff's reagent	Rapid	Slow	Slow
Acid hydrolysis	gal, glc*, glycerol	gal, glc, glycerol	gal, glycerol
Partial acid hydrolysis†	gal, glucosyl-1-glycerol and traces of a disaccharide giving a yellow colour with the periodate + Schiff's reagent	—	—
Periodate oxidation‡	No resistant sugar, glycerol only product after reduction	—	—
α-Galactosidase action§	gal, glucosyl-l-glycerol	—	—

* gal = galactose; glc = glucose. † 0·025 N-HCl at 100° for 5 hr. ‡ Glycoside treated with 0·02 M-sodium metaperiodate for 72 hr, reduced with sodium borohydride and acid hydrolysed. § Almond emulsin (Sigma), which contains an α-galactosidase.

All teichoic acid preparations were hydrolysed in alkali to a mixture of neutral components and organic phosphates. P- and HT-teichoic acids gave, in addition, a precipitate which could be extracted into acidified chloroform. Thin-layer silica gel chromatography of this material in solvent E gave a broad band (R_F 0·5 tc 0·6) that reacted with iodine vapour and a specific fatty acid spray reagent. Examination of this fraction by gas-liquid chromatography indicated that palmitic acid was the major component. Quantitative estimation of fatty acid esters showed 3·72% (w/w) and 5·10% (w/w) respectively for P-teichoic acid (sol) and (gel) (as palmitic acid). A similar

analysis of HT-teichoic acid was precluded by the presence of ester linked alanine. Fatty acids were not detected qualitatively as components of LT-teichoic acids.

Alkaline phosphomonoesterase treatment of alkali-hydrolysed teichoic acids gave a mixture of glycerol glycosides, glycerol, organic phosphate esters resistant to the enzyme and diglycerol monophosphate. The latter component was characterized by its chromatographic mobility and comparison with previously reported values, hydrolysis in alkali to glycerol and glycero-monophosphate, and rapid reaction with the periodate + Schiff's reagent on paper chromatograms. Characterization of the other phosphate esters will be reported elsewhere. The neutral fraction of glycerol glycosides was resolved into several components by preparative paper chromatography in solvent C. The composition of these glycosides is shown in Table 2.

Action of TCA on P-teichoic acid

P-Teichoic acid (gel) (40 mg.) was dissolved in 10% TCA (20 ml.) and kept at 4° for 48 hr. Trichloracetic acid was removed by repeated extraction with ether and the

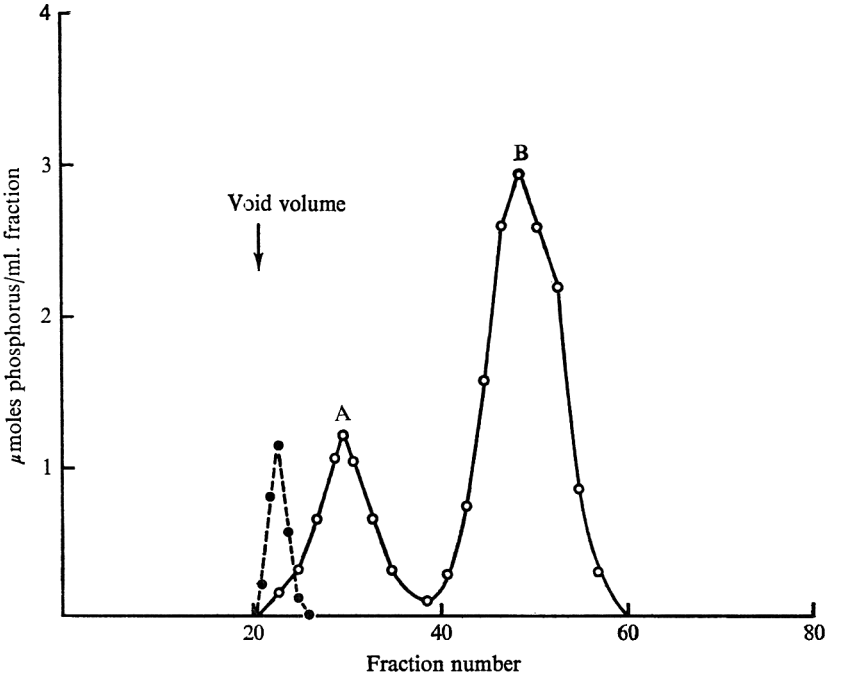

Fig. 3. Agarose gel (6 %) chromatography of P-teichoic acid (gel) from *Lactobacillus fermenti* after treatment with 10 % TCA for 48 hr, column (42 × 2·6 cm.) eluted with 0·2 M-ammonium acetate at a flow rate of 15 ml./hr; ●, P-teichoic acid; ○, P-teichoic acid after TCA treatment,

aqueous layer, after neutralization with ammonia and concentration by rotary evaporation, was chromatographed in 0·2 M-ammonium acetate on a column of 6% agarose. The elution diagram is shown in Fig. 3. Two fractions of teichoic acid (A and B) corresponding in elution volume to HT- and LT-teichoic acids were obtained. Fraction A had a slightly greater elution volume than P-teichoic acid (gel) chromatographed on the same column. Alkaline hydrolysis of fraction A, like that of HT-teichoic acid, gave

glycosides *1*, *2* and *3* (see Table 2) and fatty acids whereas fraction B showed only glycosides *2* and *3* after alkaline hydrolysis. Quantitative fatty acid ester analysis (as palmitic acid) gave 12·52 (w/w) and 0·51 % (w/w) for fractions A and B respectively.

The etheral extract of TCA degraded P-teichoic acid was washed with sodium bicarbonate solution to remove TCA and evaporated to dryness. Extraction of the residue with chloroform + methanol (2:1, v/v) gave a small quantity of a component which had an R_F of 0·91 on silica gel TLC-plates in solvent D. This material reacted with silver nitrate, iodine vapour, rhodamine G and contained phosphorus; all these properties are indicative of a phospholipid.

Major glycoside component of the glycolipid fraction

Deacylation of the glycolipid fraction from a total lipid extract of *Lactobacillus fermenti* (see Methods) and chromatography in solvents B and C gave a major glycoside fraction identical to glycoside *1* in chromatographic mobility, reaction with the periodate + Schiff's reagent, partial acid hydrolysis and treatment with α-galactosidase (see Table 2).

DISCUSSION

It is evident from the gel chromatography of these teichoic acid preparations that phenol extraction gives a product of higher apparent molecular weight than the more commonly used method of cold TCA-extraction. Further, P-teichoic acid, and to a lesser extent HT-teichoic acid, could be obtained virtually free of nucleic acid material (Fig. 1, 2), which suggests that sedimentation of teichoic acid with the ribosomal fraction at 100,000 *g* is not, as originally proposed (Critchley *et al.* 1962; Wicken & Baddiley, 1963), a result of artefactual complex formation between RNA and teichoic acid. An alternative possibility, supported by the association of teichoic acid with membrane lipids (*vide infra*), is that the degree of disaggregation of membrane on disruption of organisms may determine whether or not the teichoic acid complex is sedimented at 100,000 *g* (Knox *et al.* 1970).

The products of alkaline and acidic hydrolysis of these teichoic acid preparations are typical of glycerophosphate polymers substituted with galactose and a disaccharide of galactose and glucose. The isolation of diglycerol monophosphate from phosphomonoesterase treated alkaline hydrolysates is indicative of the normal arrangement of phosphodiester groups between carbon atoms 1 and 3 of each glycerol moiety. Glycosides *2* and *3* (Table 2), from their slow reaction with the periodate + Schiff's reagent, are clearly 2-substituted glycerols and would be among the expected products from a similarly substituted teichoic acid hydrolysed in alkali and treated with phosphomonoesterase. P- and HT-teichoic acids showed, in addition, glycoside *1* and fatty acid(s) as alkali degradation products. Glycoside *1* has the properties (Table 2) of a galactosyl-glucosyl-1-glycerol. A rapid reaction of the glycoside with the periodate + Schiff's reagent together with the production of glucosyl-1-glycerol by partial acid and by enzymic hydrolysis are indicative of substitution of a primary hydroxyl group of glycerol by the disaccharide galactosylglucose. The action of periodate, coupled with sodium borohydride reduction and acid hydrolysis, indicates either a $1 \rightarrow 2$ or a $1 \rightarrow 6$ linkage between the two sugar residues. The yellow colour produced by the periodate + Schiff's reagent acting on the disaccharide found in the partial acid hydrolysate of glycoside *1* is consistent with a $1 \rightarrow 2$ linkage (Wicken & Baddiley, 1963), and the action

of α-galactosidase shows this linkage to have the α-configuration. Thus the evidence is consistent with glycoside *1* being *O*-α-D-galactosyl-(1 → 2)-D-glucosyl-(1 → 1)-glycerol. Further studies are in progress to confirm this tentative identification.

A 1-substituted glycerol, such as glycoside *1*, could not be a constituent of a glycosidically substituted 1 → 3 phosphodiester linked teichoic acid but could derive from a glycolipid (Brundish *et al.* 1965), the glycoside moiety of the glycolipid from *Lactobacillus casei* ATCC 7469 being identical with that proposed for glycoside *1* (Shaw, Heatherington & Baddiley, 1968). The glycolipid fraction of *L. fermenti* yielded a glycoside indistinguishable from glycoside *1* suggesting that P-teichoic acid contains glycolipid.

In addition to glycolipid, tentative evidence has also been obtained for the presence of phospholipid as a component of P-teichoic acid. P-teichoic acid may therefore be regarded as a complex of teichoic acid with lipids and be referred to as lipoteichoic acid. Extraction with TCA results in the disruption of the complex with the formation of partially degraded lipoteichoic acid (HT-teichoic acid) and teichoic acid chains virtually devoid of lipid components (LT-teichoic acid).

The apparent large molecular size of lipoteichoic acids might be explained by micelle formation. The water solubility of P-teichoic acids and the resistance of the lipid moiety to extraction by chloroform–methanol (see Methods) is indicative of an amphipathic structure in which the hydrophobic lipid portion of the complex is 'buried' as well as firmly bound to the hydrophilic teichoic acid chains. Such micellar formations have been reported for lipopolysaccharides and phospholipids from Gram-negative organisms (Rothfield & Horne, 1967). The presence of teichoic acid as a lipid complex is also consistent with its being present as an integral component, with phospholipid, of the common leaflet structure of the cell membrane. In Gram-negative organisms, which lack teichoic acid, it has been proposed that lipopolysaccharide fulfils a similar role in the outer cell envelope (Rothfield & Horne, 1967).

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Studies on the Group F Antigen of Lactobacilli: Antigenicity and Serological Specificity of Teichoic Acid Preparations

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SUMMARY

The membrane glycerol teichoic acid of group F lactobacilli (e.g. *Lactobacillus fermenti*) has been identified as the group antigen. The most active preparations, obtained by phenol extraction, reacted readily with antisera and were antigenic when injected into rabbits with Freund's adjuvant. The products obtained by the action of trichloracetic acid were separable into a high molecular weight fraction, which reacted well with antiserum but was not a very effective antigen, and a low molecular weight fraction which was not antigenic and reacted weakly with group antisera. Specificity of the group antigen depends primarily on galactose with glucose being a minor contributor. However, the ability of galactose and glucose to inhibit the precipitin reaction differs considerably with different antisera. Teichoic acids from other strains of *L. fermenti* cross-reacted with the group antisera to varying extents but preparations from *L. casei* strains reacted only weakly.

INTRODUCTION

Most strains of lactobacilli can be divided into one of six groups, designated A to F, on the basis of the serological reaction of Lancefield acid extracts (Sharpe, 1955; Sharpe & Wheater, 1957). The specific antigens defining groups B and C are cell-wall polysaccharides (Knox, 1963), while teichoic acids have been identified as responsible for the specificities of groups A, D and E (Sharpe, Davison & Baddiley, 1964). Group F comprises strains of *Lactobacillus fermenti* and Sharpe *et al.* (1964) suggested that the specificity of this group might also depend on a teichoic acid. Teichoic acids occur as both wall and intracellular components and the tentative evidence for the group F antigen placed it in the latter category. It is generally considered that intracellular teichoic acid is associated with the cell membrane (Archibald, Baddiley & Blumsom, 1968), and the isolation from *L. fermenti* NCTC 6991 of a teichoic acid–phospholipid—glycolipid complex, referred to as lipoteichoic acid, is consistent with such a location (Wicken & Knox, 1970). The teichoic acid moiety of this complex has been shown to contain glucose and galactose substituents. Serological studies on lipoteichoic acid are described in this paper, and have led to the conclusion that it is the group antigen, with specificity primarily depending on galactose substituents.

Further, the complex is antigenic, when injected with Freund's adjuvant into rabbits whereas lipid-free teichoic acid is not antigenic.

METHODS

Organisms. Strains were obtained originally from the National Collection of Type Cultures, Colindale, London (NCTC), the National Institute for Research in Dairying, Reading (NIRD), and from collections of salivary organisms at the Institute of Dental Research. These organisms were *Lactobacillus fermenti* NCTC 6991 and laboratory strains 77 and 126 representing serological group F (Knox & Holmwood, 1968), *Lactobacillus casei* NIRD H831 (group B) and *L. casei* NIRD R094 and *Lactobacillus helveticus* (*L. casei* var. *rhamnosus*) NCTC 6375 (group C) (Knox, 1963).

Isolation of bacterial components. Teichoic acid was isolated from disrupted bacteria by the procedures previously described (Wicken & Knox, 1970). Two fractions from disrupted *Lactobacillus fermenti* NCTC 6991 were examined, namely the fraction sedimented at 100,000 *g* and the supernatant solution. Teichoic acid was extracted from a portion of each by either cold aqueous phenol, or cold trichloracetic acid (TCA), followed by further fractionation by column chromatography. Phenol-extracted material is designated P-teichoic acid; TCA yielded a high molecular weight fraction (HT-teichoic acid) and a low molecular weight fraction (LT-teichoic acid). Chemical studies on these products showed differences depending on the mode of extraction, though there were no significant differences between the products obtained by a particular procedure from the pellet of ribosomes and membrane fragments and from the supernatant solution (Wicken & Knox, 1970). Teichoic acids were isolated from *L. fermenti* strains 77 and 126 and *L. casei* strains NIRD H831, NIRD R094 and NCTC 6375 by the phenol procedure. The cell wall of *L. fermenti* was digested with a muralytic enzyme preparation from Streptomyces to yield a polysaccharide–peptidoglycan complex (fraction S3, Knox & Holmwood, 1968). This fraction will be referred to as cell-wall polysaccharide. Dr M. McCarty, Rockefeller University, New York, kindly provided a sample of polyglycerophosphate (glycerol teichoic acid) prepared from a group A streptococcal strain D58 (type 3) (McCarty, 1959).

Preparation of antisera. All rabbits used for the production of antisera were 4- to 5-month-old male New Zealand Whites. Antisera to *Lactobacillus fermenti* NCTC 6991 were obtained by the intravenous injection of a suspension of bacteria into three rabbits (Sharpe, 1955). Rabbit 99 received two subsequent booster series of injections; antiserum collected after each series of injections is designated 99/1, 99/2 and 99/3 respectively. The antigenicity of teichoic acid preparations was examined by injecting an emulsion containing equal volumes of teichoic acid solution and Freund's adjuvant, complete or incomplete (Bacto Adjuvant 0638–60 and 0639 respectively from Difco Laboratories, Detroit, Mich., U.S.A.). The emulsion contained 1 mg. teichoic acid/0·75 ml.; 0·25 ml. was injected subcutaneously into one site on the back and into two footpads; the injection procedure was repeated twice at 8-day intervals and the rabbits bled after a further 8 to 10 days (cf. Burger, 1966).

Quantitative precipitin reaction. Antiserum and antigen in 0·85% (w/v) NaCl were mixed in a final volume of 0·6 ml. and incubated at 37° for 1 hr followed by 2 to 3 days at 2 to 4°. The precipitate was deposited by centrifugation and washed twice at 2 to 4° with 0·85% NaCl. It was then dissolved in 0·6 ml. of 0·1 N-NaOH and 0·4 ml.

used in the estimation of protein with Folin-Ciocalteu's phenol reagent (Heidelberger & MacPherson, 1943). By this procedure a sample of serum containing 120 μg. of antibody (using human IgG as the standard) gave an extinction at 750 mμ in a 1 cm. cell of about 0·50. Experiments were carried out in duplicate when determining precipitin curves, and in triplicate when inhibitions were being tested.

RESULTS

Comparison of products from Lactobacillus fermenti NCTC 6991

Reaction with antisera to whole L. fermenti. The antisera from three rabbits (designated 97, 98, 99) injected intravenously with strain NCTC 6991 were examined for antibodies reacting with preparations of teichoic acid and cell-wall polysaccharide from this strain. HT- and P-teichoic acid preparations precipitated approximately equal amounts of antibody while LT-teichoic acid and cell-wall polysaccharide reacted only weakly (Fig. 1.) No significant differences were observed in the reactivity of products isolated from the 100,000 *g* pellet and the supernatant solution. The results in Fig. 1 and for most of the reported experiments refer to preparations from the supernatant solution. From Fig. 1. the amount of antibody precipitated from antiserum 99/3 by P-teichoic acid corresponds to 6·3 mg./ml. compared with 1·4 mg./ml. when the cell-wall polysaccharide was added. For antisera 99/1 and 99/2 the corresponding values for P-teichoic acid were 3·5 and 4·5 mg./ml.; for antisera 97 and 98 the values after one series of injections were 3·5 and 2·2 mg./ml. respectively.

Reaction with antisera to teichoic acid preparations. Antisera obtained by injection of products in Freund's adjuvant were examined for their reactivity with the different teichoic acid preparations (Fig. 2). A comparison of the reactivities of the teichoic acid

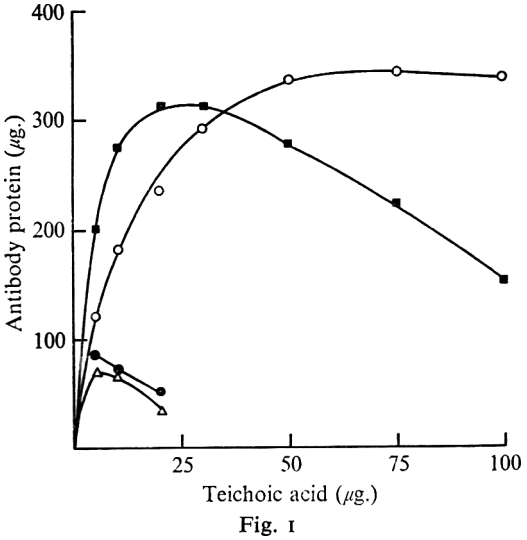

Fig. 1

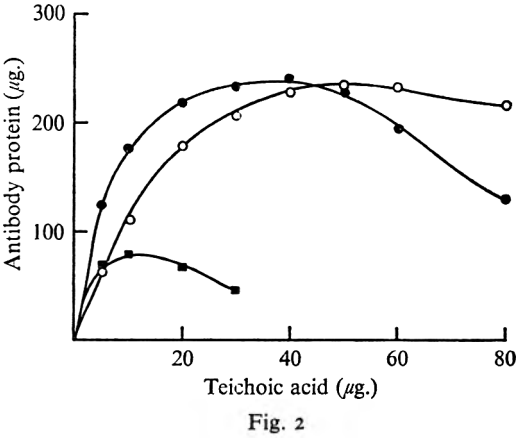

Fig. 2

Fig. 1. Precipitation of teichoic acids and cell-wall polysaccharide by antiserum (50 μl.) against *L. fermenti* (rabbit 99/3) ■, P-teichoic acid; ○, HT-teichoic acid; ●, LT-teichoic acid; △, polysaccharide.

Fig. 2. Precipitation of teichoic acids by antiserum (0·1 ml.) against P-teichoic acid (rabbit 148); ●, P-teichoic acid; ○, HT-teichoic acid; ■, LT-teichoic acid.

preparations with those previously observed with serum 99/3 (Fig. 1) indicated close similarities, but there was no cross-reaction with the cell-wall polysaccharide.

Similar results were obtained with other antisera against P-teichoic acid injected with complete and incomplete adjuvant, and with HT-teichoic acid in complete adjuvant, though antisera differed in the maximum amount of antibody precipitated. Table 1 summarizes the values for the antibody content of these antisera as determined by their reactivity with the injected fraction. Also included for comparison are the results for one series of intravenous injections of whole cells, and for disintegrated cells and LT-teichoic acid in complete adjuvant; in these cases the values were obtained with P-teichoic acid as the reactant in the precipitin test. The results indicate that P-teichoic acid was a more effective antigen than HT-teichoic acid, while LT-teichoic acid was not antigenic. It would also appear that alanine is not a major antigenic determinant as P-teichoic acid, which lacks alanine, was an effective antigen and also reacted with antiserum to HT-teichoic acid, which contains alanine (Wicken & Knox, 1970).

Table 1. *Antibody content of antisera as determined by the quantitative precipitin method*

Values were determined from the maximum amount of antibody precipitated by P-teichoic acid.

Preparation injected	Procedure*	Antibody (mg./ml.)
Whole bacteria	Intravenous	3·5, 2·3, 3·5
Disintegrated bacteria	Complete adjuvant	1·5, 1·6
P-teichoic acid	Incomplete adjuvant	0·7, 1·5
	Complete adjuvant	1·5, 2·4, 5·9
HT-teichoic acid	Complete adjuvant	0·8, 0·8, 0·6
LT-teichoic acid	Complete adjuvant	0·1, 0·1

* Details of injection procedures given in Methods.

Reactivity of HT-teichoic acid after degradation by alkali. Wicken & Knox (1970) showed that a high molecular weight product of the action of TCA on P-teichoic acid contained a considerable amount of lipid while the corresponding low molecular weight fraction was lipid free. These results suggested that the differences in properties of HT- and LT-teichoic acid may relate to the lipid component of HT-teichoic acid. To remove fatty acids (and alanine) from HT-teichoic acid, a solution (500 μg./0·5 ml.) was mixed with one volume of aqueous ammonia (sp.gr. 0·88) and left for 16 hr at room temperature. The solution was then evaporated to dryness *in vacuo* and reconstituted in 0·85% NaCl. The serological reactivity of the degraded material was compared with that for HT- and LT-teichoic acid, using antiserum to HT-teichoic acid in complete adjuvant. The results showed that ammonolysis yielded material with serological reactivity similar to that of LT-teichoic acid (Fig. 3). The removal of fatty acids by the ammonia treatment was confirmed by the procedures used previously (Wicken & Knox, 1970).

Specificity of antisera

Inhibition of the precipitin reaction by component sugars. The ability of D-alanine, D-glucose and D-galactose to inhibit the precipitin reaction was examined. The amounts of teichoic acid to be added were determined from quantitative precipitin curves and the volume of serum was such as to contain 100 to 150 μg. antibody protein. D-Alanine (100 μmoles) did not inhibit the precipitin reaction between HT-teichoic acid, which

contains D-alanine, and homologous antisera, while the results with D-glucose and D-galactose (100 μmoles) depended on the serum used. The abilities of glucose and galactose to inhibit the precipitation of HT- and P-teichoic acid by antisera prepared against whole cells or P-teichoic acid also varied, depending on the serum used. Table 2 summarizes the values obtained with P-teichoic acid. Similar results were obtained for the inhibition of the precipitation of HT-teichoic acid; for example, the percentage inhibitions by glucose and galactose with antiserum 132 were 9 and 31 % respectively.

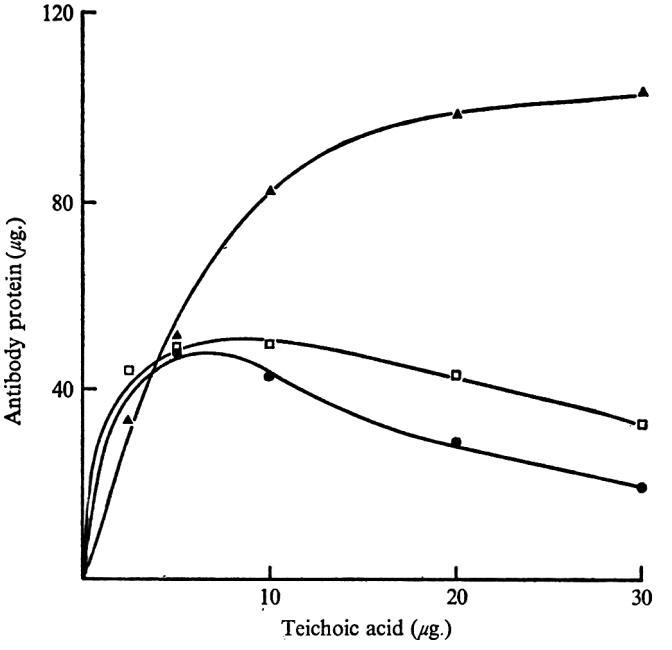

Fig. 3. Effect of alkali on serological reactivity of HT-teichoic acid with homologous antiserum (0·2 ml. from rabbit 136); ▲, HT-teichoic acid; □, alkali-degraded HT-teichoic acid; ●, LT-teichoic acid.

Table 2. *Inhibition of precipitin reaction between P-teichoic acid and antisera*

Preparation injected	Rabbit (no.)	Inhibition (%) produced by sugar (100 μmole)	
		Glucose	Galactose
Whole bacteria	97	16	38
	98	10	32
	99/1*	8	5
	99/3*	0	5
P-teichoic acid	132	5	35
	146	6	10
	147	7	36
	148	22	44
HT-teichoic acid	136	8	47
	149	4	11
	150	6	12

* Sera after the first and third series of injections respectively.

More detailed studies of the inhibition by different amounts of glucose and galactose were carried out for some of the sera. Fig. 4 gives the results obtained with antiserum 148 (50 μl.) and P-teichoic acid (10 μg.). Fig. 5 compares the inhibitions by galactose of the reaction between antiserum 136 (0·25 ml.) and each of the two preparations, P-teichoic acid (12·5 μg) and HT-teichoic acid (25 μg.).

Glucose and galactose are also the components of the cell-wall polysaccharide (Knox & Holmwood, 1968). D-Glucose and D-galactose (50 μmoles) inhibited the precipitin reaction between 10 μg. cell-wall polysaccharide and 0·1 ml serum 99/3 by 62 and 10% respectively. In agreement with these results the major disaccharide obtained from the polysaccharide on mild acid hydrolysis has been shown (unpublished observations) to be indistinguishable in its properties from 3-*O*-β-D-glucosyl-D-galactose, previously identified as a component of a streptococcal polysaccharide (Montague & Knox, 1968).

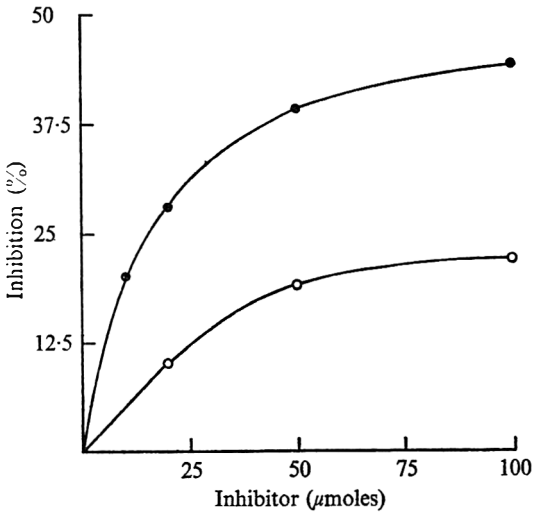

Fig. 4

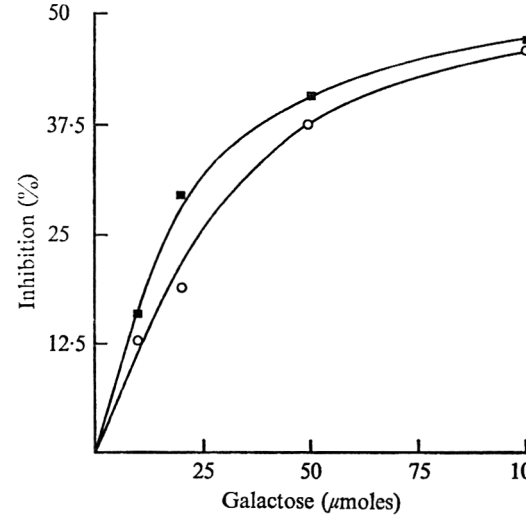

Fig. 5

Fig. 4. Inhibition of precipitin reaction between P-teichoic acid and homologous antiserum (rabbit 148) by D-glucose (○) and D-galactose (●).

Fig. 5. Inhibition of precipitation of P-teichoic acid (■) and HT-teichoic acid (○) by D- galactose.

Reactivity of antisera with teichoic acid preparations from other lactobacilli. P-teichoic acid preparations from *Lactobacillus fermenti* strains 77 and 126 and three strains of *L. casei* were examined for their reaction with antisera obtained by injecting whole organisms (97, 99/3), P-teichoic acid (132, 146, 147, 148) and HT-teichoic acid (149, 150). In each case the teichoic acid preparations were tested between 5 and 30 μg. to obtain maximum precipitation, and the amount of serum used contained 200 to 250 μg. of antibody precipitable by P-teichoic acid from *L. fermenti* NCTC 6991. In Table 3, the maximum amount of antibody precipitated by each of the heterologous reactions is expressed as a percentage of that precipitated in the homologous reaction. The results are grouped in four categories depending on the antiserum: category I comprises the antisera to whole organisms while the remaining categories separate the antisera to teichoic acid according to the abilities of the component sugars to

inhibit the precipitin reaction (see Table 2). When grouped in this manner, the degree of cross-reaction with antisera to teichoic acid is shown to be greatest with those antisera for which glucose and galactose are poor inhibitors (category II), and to be least for antiserum 148 (category IV) where both glucose and galactose are good inhibitors; intermediate cross-reactions are given with antisera in category III, for which galactose alone is a good inhibitor. These distinctions are also evident with antisera to whole organisms (category I). Table 3 also includes for comparison the results of experiments described below on the extent of cross-reaction of streptococcal polyglycerophosphate with the antisera.

Reactivity of antisera with streptococcal polyglycerophosphate

Polyglycerophosphate at a concentration of 1 mg./ml. gave a strong reaction with antiserum 99/3 by the qualitative ring precipitin test. However, when tested quantitatively by the procedure used above for lactobacilli, the cross-reaction corresponded to only 7% (320 μg. antibody precipitated by P-teichoic acid). Other antisera gave low results though values with antisera in category II of Table 3 were apparently the highest.

Polyglycerophosphate was purified from an acid extract of group A streptococci (McCarty, 1959), and its molecular size is probably comparable with that for LT-teichoic acid rather than P-teichoic acid. LT-teichoic acid reacts weakly with antisera compared with P-teichoic acid (Fig 1, 2) and therefore the reactions of polyglycerophosphate and LT-teichoic acid were compared; sufficient antiserum was used to precipitate 200 to 250 μg. antibody in the homologous reaction. The values obtained

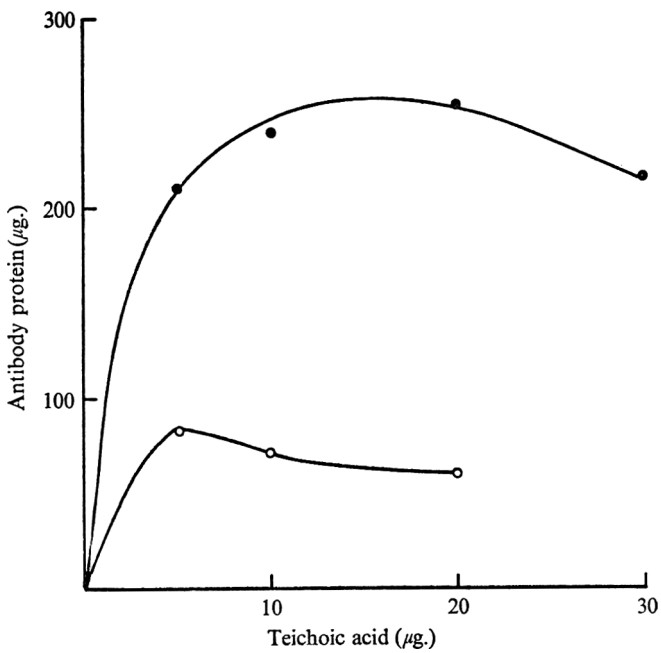

Fig. 6. Precipitation of LT-teichoic acid (●) and streptococcal polyglycerophosphate (○) by antiserum to P-teichoic acid (rabbit 146).

for antiserum 146 are given in Fig. 6 and the results for all antisera are summarized in Table 3.

When the results for the cross-reaction by polyglycerophosphate are compared with the other results in Table 3, they support the conclusion that the antisera differ in their specificity. Further support has been provided by preliminary experiments using sera absorbed with polyglycerophosphate for immuno-electrophoresis (unpublished method, with Miss R. Mollenhauer); absorption of antisera in category II (146, 150) almost completely removed the reaction with P-teichoic acid, whereas antisera in categories III and IV (147, 148) retained most of their reactivity and there was no change in the immuno-electrophoretic pattern.

Table 3. *Cross-reaction of antisera to Lactobacillus fermenti* NCTC *6991 with teichoic acid preparations from other organisms*

For strains of lactobacilli, values are expressed as a percentage of the amount of antibody precipitated by P-teichoic acid isolated from strain NCTC 6991; for polyglycerophosphate from a group A streptococcus, the values are expressed relative to LT-teichoic acid isolated from strain NCTC 6991. The antisera have been grouped into four categories depending on the material injected and the specificity of the antisera (Table 2).

		Source of antigen					
		L. fermenti		*L. casei*			
Category	Antiserum	77	126	H 831	R 094	6375	Streptococcus
I	97	47	81	15	13	16	<5
	99	47	72	22	20	24	24
II	146	67	74	39	26	28	32
	149	58	58	52	41	44	32
	150	46	60	44	34	39	25
III	132	42	35	24	26	23	8
	147	38	38	22	22	24	15
IV	148	29	33	16	15	17	20

DISCUSSION

The suggestion that the group F antigen of lactobacilli is an intracellular glycerol teichoic acid (Sharpe *et al.* 1964) has been confirmed, while the isolation of the antigen as a lipid complex (P- or HT-teichoic acid) is consistent with its being a component of the protoplast membrane (Wicken & Knox, 1970). The poor reaction of LT-teichoic acid with antisera probably relates to its low molecular weight, as Goodman & Kabat (1960) have noted a similar relation between the amounts of antibody precipitated by dextrans of high and low molecular weights. Loss of reaction could also arise from loss of serological determinants, but this is less likely as LT-teichoic acid inhibited the agglutination of red blood cells sensitized with HT-teichoic acid (Hewett, Knox & Wicken, 1970). The first suggestion is also supported by the observation that aqueous ammonia, which would disrupt the HT-teichoic acid complex by liberating fatty acids, yields a product with serological properties similar to those of LT-teichoic acid.

The antigenicity of teichoic acid preparations was determined by injecting products in Freund's complete adjuvant. If the results for P-teichoic acid are compared with those for disintegrated cells injected with adjuvant, it may be concluded that the antigen has been isolated without any significant decrease in activity. Previous studies on

teichoic acids as group antigens showed that antisera could be obtained with whole cells or crude extracts but not with the products obtained by TCA extraction even if adjuvant were used (see Burger, 1966). From the results with *Lactobacillus fermenti* these earlier observations could be explained by concluding that the lipoteichoic acid complex had been degraded by TCA to products which were too small to elicit antibody response in the rabbit. The injection procedure used for obtaining antibodies to lipoteichoic acid was similar to that employed by Burger (1966), who examined teichoic acids obtained from various organisms by a phenol extraction method. Burger (1966)

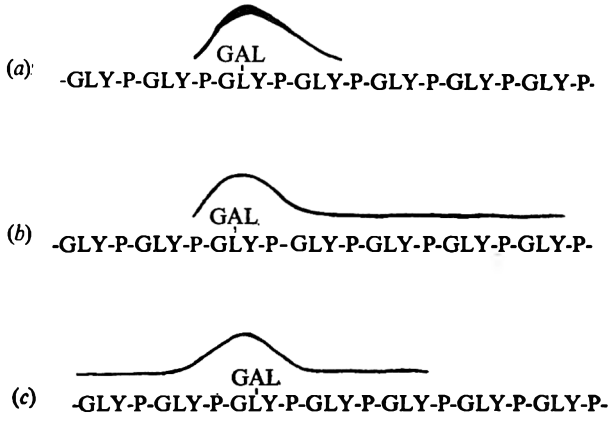

Fig. 7. Three types of antigenic determinants involving a galactose substituent on a sequence of glycerophosphate residues: (*a*) a small determinant group in which galactose is dominant; (*b*) a larger determinant group in which galactose plays a minor role and specificity is shown by glycerophosphate; (*c*) similar to (*b*) except that sequence of unsubstituted glycerophosphate residues is shorter. Gly = glycerol, P = phosphate, Gal = galactose. The lines represent the affinity between the antibody combining sites and the determinant groups; the thicker the line the stronger the affinity for that portion of the determinant (cf. Lüderitz, Staub & Westphal, 1966).

found that purified products did not elicit an antibody response whereas complexing with cationic precipitating agents to give particles of defined size rendered the preparation antigenic.

The specificity of teichoic acids generally depends on the substituent sugars (Staub, 1968), though when sugar is absent D-alanine can be the determinant (McCarty, 1964). Studies on the inhibition of the precipitation of teichoic acid from *Lactobacillus fermenti* indicate that in this case also the sugars rather than alanine are responsible for serological specificity. The abilities of D-glucose and D-galactose to inhibit the precipitin reaction vary considerably, with the values apparently depending on the rabbit injected rather than the source of antigen or the injection procedure. Thus galactose was an effective inhibitor (32 to 47% inhibition by 100 μmoles) for six of the ten rabbits treated, while the results for glucose exceeded 10% only twice.

Differences in specificity of antisera are also evident from a comparison of the degree of cross-reaction of antisera to *Lactobacillus fermenti* NCTC 6991 with other strains of *L. fermenti*. The laboratory strains had been defined as group F by Knox & Holmwood (1968) from the reaction of acid extracts with antiserum against strain NCTC 6991

(Sharpe, 1955). It is now apparent from a quantitative comparison that the cross-reaction is variable, and less than would be expected if the structures of the teichoic acid from each of the three strains were essentially the same. Information on the glycerol teichoic acids from strains 77 and 126 is limited to the observations (unpublished) that each contains glucose and galactose substituents. Serological differences have also been noted for the group D antigens from two strains of streptococci, and in this case differences in structure were detected (Wicken, Elliott & Baddiley, 1963). It thus appears that when the group antigen is a membrane teichoic acid, the grouping of strains may depend on the relative degree of cross-reaction of strain-specific teichoic acids. In contrast, group-specific components of the wall, such as the polysaccharides of lactobacilli of groups B and C (Knox, 1963), are sufficiently similar in structure for there to be a strong cross-reaction between those of the same group.

As indicated by differences in sugar inhibitions (Table 2) and cross-reactions with other teichoic acids (Table 3), antisera to *Lactobacillus fermenti* NCTC 6991 exhibit variable specificity. A diagrammatic representation of a model structure showing three types of reaction involving galactose as part of an antigenic determinant is provided in Fig. 7. This representation is analogous to that used in depicting the specific serological factors of Salmonella where a particular sequence of sugars has been shown to represent a number of antigenic determinants varying in size from two to four or more sugar residues (Lüderitz, Staub & Westphal, 1966). Figure 7*a* depicts a specific grouping in which galactose is immunodominant, and may be regarded as representing the specificity of antisera in category III of Table 2, and part of the specificity of antiserum 148 (category IV) and 97 (category I). Figure 7*b* provides an example of a determinant grouping in which galactose would be a poor inhibitor and where a greater cross-reaction would occur with other teichoic acids containing unsubstituted sequences of glycerophosphate; this would account for the specificity of antisera in category II. Figure 7*c* illustrates a specificity for which galactose is a minor component but the antisera might be expected to be more specific than for the structure shown in Fig. 7*b* owing to the shorter sequence of unsubstituted glycerol units; antiserum 99 (category I) would be in this category. Such representations of antigenic determinants are therefore consistent with the observed properties of the antisera.

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Studies on the Group F Antigen of Lactobacilli: Detection of Antibodies by Haemagglutination

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SUMMARY

Teichoic acid–lipid complexes from *Lactobacillus fermenti* NCTC 6991 absorb to normal sheep red blood cells. Sensitized cells are agglutinated by specific antisera, the titres of representative sera being 1600 to 3200. Lipid-free teichoic acid does not sensitize red blood cells but inhibits haemagglutination. Haemagglutination is inhibited completely by intact cells of *L. fermenti* NCTC 6991, but only partially by other strains of *L. fermenti*. Strains of lactobacilli of other serological groups absorb a variable proportion of antibody. The group F antigen is a membrane teichoic acid and the significance of the absorption by intact bacterial cells is discussed.

INTRODUCTION

The serological classification of group F lactobacilli has been shown to depend on a glycerol teichoic acid associated with the cell membrane and having glucose and galactose substituents: by using a mild phenol extraction procedure the isolated product contained both glycolipid and phospholipid, and retained its antigenicity (Knox, Hewett & Wicken, 1970; Wicken & Knox, 1970).

The presence of lipid as an integral component of the antigen, referred to as lipoteichoic acid, suggested the application of passive haemagglutination as a procedure for the detection of antigen and the corresponding antibodies. Lipid-free polysaccharides do not generally absorb to red blood cells, and Hämmerling & Westphal (1967) have suggested that absorption depends on the formation of hydrophobic bonds between the lipid component of the erythrocyte membrane and the lipid–polysaccharide complex. Such a requirement would account for reports of the failure of preparations of purified teichoic acid to absorb to erythrocytes (Hofstad, 1965; Yoshida & Ekstedt, 1968). It has now been shown that lipoteichoic acid from *Lactobacillus fermenti* NCTC 6991 will absorb to normal sheep red blood cells, whereas the corresponding lipid-free product does not. The reaction can be used as a sensitive means of detecting antibodies to *L. fermenti*.

METHODS

Organisms. Cultures of Lactobacillus of serological groups B, C and F were obtained from the sources previously described (Knox *et al.* 1970). Cultures of organisms of

groups A, D and E were kindly supplied by Dr M. E. Sharpe, National Institute for Research in Dairying, Reading, England, and corresponded to those used by Sharpe, Davison & Baddiley (1964). The cultures listed under serological groups were: group A, *L. helveticus* NCIB 8025; group B, *L. casei* NIRD H831; group C, *L. casei* NIRD R094 and strain NCTC 6375; group D, *L. plantarum* NCIB 7220; group E, *L. bulgaricus* B9 and *L. lactis* NCIB 7278; and group F, *L. fermenti* NCTC 6991 and laboratory strains 77 and 126. Organisms were grown under the conditions described by Sharpe (1955).

Isolation of cell components. Cell wall from *Lactobacillus fermenti* NCTC 6991 was prepared as described previously (Knox & Holmwood, 1968). A similar procedure was used for obtaining wall from *L. plantarum* NCIB 7220. Teichoic acid was isolated from disrupted cells of *L. fermenti* NCTC 6991 (Wicken & Knox, 1970) and the same fractions used as in previous serological studies (Knox *et al.* 1970). Phenol-extracted teichoic acid is designated P-teichoic acid; extraction with trichloracetic acid yielded a high molecular weight fraction, HT-teichoic acid, and a low molecular weight fraction LT-teichoic acid. To remove fatty acids from HT-teichoic acid a solution (500 μg./0·5 ml.) was mixed with one volume aqueous ammonia (sp.gr. 0·88) and left for 16 hr at room temperature (Knox *et al.* 1970). Partial removal of fatty acids was achieved by treating with 15 % (v/v) aqueous ammonia, and leaving for 30 min. at room temperature (cf. Jackson & Moskowitz, 1966).

Preparation of antisera. Antisera to whole cells were obtained by intravenous injection of a cell suspension, and to teichoic acids by injection in Freund's adjuvant (Knox *et al.* 1970). Antisera used in the present investigation were obtained as follows: antiserum 99/3, three series of intravenous injections of *Lactobacillus fermenti* NCTC 6991; antisera 132 and 148, injection of P-teichoic acid with incomplete and complete Freund's adjuvant respectively; antisera 150 and 151, injection in complete adjuvant of HT-teichoic acid and LT-teichoic acid respectively.

Detection of teichoic acid in extracts of disintegrated cells. Suspensions of *Lactobacillus fermenti* NCTC 6991 (28 mg./ml.) were disintegrated for 5, 10, 20 and 35 min. (Shockman, Kolb & Toennies, 1957). After removal of cell wall by centrifugation at 12,000 *g* for 20 min. the supernatant fraction from each period of disintegration was centrifuged further for 90 min. at 100,000 *g*. The supernatant solution was removed and the pellet reconstituted in 0·85 % (w/v) NaCl and neutralized. Tenfold dilutions of the residue and supernatant fractions were prepared in 0·066 M phosphate buffer, pH 7·4, containing 0·85 % NaCl and 0·05 to 0·2 ml. of solution examined for reactivity with antiserum 148 (0·1 ml.) by the quantitative precipitin method (Heidelberger & MacPherson, 1943; Knox *et al.* 1970). By comparison with P-teichoic acid standards, the teichoic acid content of the extracts was estimated. To follow the release of cell components, the extinction of the whole extract (diluted 20-fold) was read at 260 mμ.

Determination of haemagglutination titre. Sheep red blood cells were washed five times with 0·85 % NaCl. Packed cells (0·2 ml.) were mixed with a solution of teichoic acid in 0·85 % NaCl to give a final 2 % (v/v) suspension of red blood cells and a teichoic acid concentration of 2·5 to 50 μg./ml. Controls containing no added teichoic acid were also prepared, and the mixtures rolled at 37° for 30 min., followed by standing at 2 to 4° for 16 hr. The erythrocytes were washed three times with 0·85 % NaCl and finally suspended to 2 % (v/v) in 0·85 % NaCl. In order to remove naturally occurring haemagglutinins, antisera were absorbed with 10 % (v/v) normal sheep red blood cells, and complement was destroyed by heating the absorbed sera at 56° for 30 min. Serial

dilutions of serum were prepared in 0·85 % NaCl containing normal absorbed rabbit serum (0·2/100 ml.). The addition of normal serum prevented non-specific lysis and stabilized the haemagglutinated cells (Mrs B. Humphrey, personal communication). To each serial dilution of antiserum (0·1 or 0·2 ml.) was added an equal volume of sensitized erythrocytes. The reactants were mixed and incubated for 3 hr at 37°. The reciprocal of the greatest dilution showing agglutination on macroscopic inspection was recorded as the end-point or titre.

Inhibition of haemagglutination by teichoic acid preparations was examined by adding inhibitor (10 μg. in 10 μl.) to each serial dilution of antiserum. After 1 hr at 37°, one volume of sensitized cells was added and the above procedure followed.

RESULTS

Ability of teichoic acid preparations to sensitize erythrocytes

Preliminary experiments using 50 μg. teichoic acid/ml. indicated that P- and HT-teichoic acid absorbed to normal sheep red blood cells but that there was no detectable absorption of LT-teichoic acid; subsequent studies indicated that 10 μg. teichoic acid/ml. was sufficient for sensitization. The titres of active sera only vary from 1600 to 3200 (Table 1) whereas the antibody content by the precipitin method showed a

Table 1. *Haemagglutination titres of antisera*

Sheep red blood cells were sensitized with an appropriate amount of teichoic acid (2·5 to 50 μg.) and tested against different antisera. HT = high molecular weight product of TCA action; P = isolated by phenol extraction. Values determined by the quantitative precipitin method are included for comparison.

Antiserum (no.)	Teichoic acid fraction		Titre	Precipitating antibody (mg./ml.)
	Type	Amount (μg.)		
99/3	HT	50	3200	6·3
	P	50	3200	
	P	10	3200	
132	HT	50	1600	1·5
	P	50	1600	
	P	10	1600	
	P	5	800	
	P	2·5	400	
148	P	10	3200	2·4
150	HT	10	1600	0·8
151	HT	10	100	0·1

greater variation (Knox *et al.* 1970). The specificity of haemagglutination was shown by the lack of agglutination in the absence of teichoic acid, and the complete inhibition of agglutination on prior incubation of the serial dilutions of antiserum with HT-teichoic acid (10 μg.); LT-teichoic acid (20 μg.) caused a partial inhibition, the titre in the reaction between antiserum 148 and P-teichoic acid decreasing from 3200 to 400.

It was previously shown that aqueous ammonia degraded HT-teichoic acid with the release of fatty acids and a decreased serological reactivity (Knox *et al.* 1970). It has now been shown that HT-teichoic acid subjected to these conditions partially lost its

ability to sensitize erythrocytes. The titre of antiserum 148 with erythrocytes sensitized by 10 μg. HT-teichoic acid was 3200; with 10 μg. degraded teichoic acid the apparent titre was 800.

Inhibition of haemagglutination by bacterial cells

A washed suspension (11 mg. dry wt/ml.) of *Lactobacillus fermenti* NCTC 6991 was prepared from a 24 hr culture. Suspensions of bacteria (0·25 to 2 ml.) were centrifuged and 2 ml. of dilute serum (1 ml. diluted to 12·5 ml. with 0·85 % NaCl) added to the pellet; the antisera examined were 99/3 and 148. After shaking and then standing for 15 min. at 37° the bacteria were deposited by centrifugation, and the antiserum tested by the haemagglutination procedure, using erythrocytes sensitized with P-teichoic acid (10 μg./ml.). In each case 1 ml. bacterial suspension decreased the titre from 3200 to 100; continued incubation of antisera and bacterial cells for 2 hr at 37° and overnight at 2 to 4° did not decrease the titre further. The decrease in titre related to the dry weight of organisms used, being 400 with 0·25 ml. of suspension and < 50 with 2 ml. suspension.

There was no detectable decrease in titre when antiserum (2 ml. of 50-fold dilution) was shaken with cell walls (10 mg.) from strain NCTC 6991.

Estimation of proportion of membrane teichoic acid of intact organisms reacting with antibodies

Determinations were made of the teichoic acid content of disintegrated organisms by the quantitative precipitin method. After disintegration for 20 min. very few organisms stained Gram-positive but release of cell constituents was not complete even after 35 min. disintegration. Expressed as a percentage of bacterial dry weight the amount of teichoic acid estimated after 35 min. was 1·0 % for the 100,000 *g* residue and 1·55 % for the supernatant solution (Fig. 1). The results are expressed in terms of the reactivity of P-teichoic acid with antisera. It is probable that teichoic acid in disintegrated cells is associated with protein; however, from estimates of the protein content of the bacterial extracts (Folin–Ciocalteu method) the error resulting from expressing all the protein in the antigen–antibody precipitate as antibody protein would not account for more than 0·1 % teichoic acid. The observation (Fig. 1) that the major proportion of the teichoic acid is not deposited with the membrane fraction at 100,000 *g* may be attributed either to the fragility of the protoplast membrane (cf. Shockman & Slade, 1964) or to the presence of a labile attachment of lipoteichoic acid to membrane.

Assuming that the total teichoic acid content of the cell membrane represented 2·5 % of the cell mass before disintegration, a calculation may be made of the proportion of the teichoic acid of intact cells reacting with antibody. From the results reported in the previous section, 11 mg. of bacteria of strain NCTC 6991 lowered the titre of antiserum 148 from 3200 to 100, or almost complete absorption. From previous studies by the quantitative precipitin method (Knox *et al.* 1970), the amount of precipitating antibody in the antiserum was 380 μg., which was sufficient to precipitate 15 μg. of P-teichoic acid. From the results in Fig. 1 the total amount of teichoic acid in 11 mg. bacteria is 275 μg. If it is assumed that precipitation of antibody can be equated with absorption of antibody then the results indicated that only 5·5 % of the teichoic acid content of whole cells was reactive. A comparable estimation for antiserum 99 showed

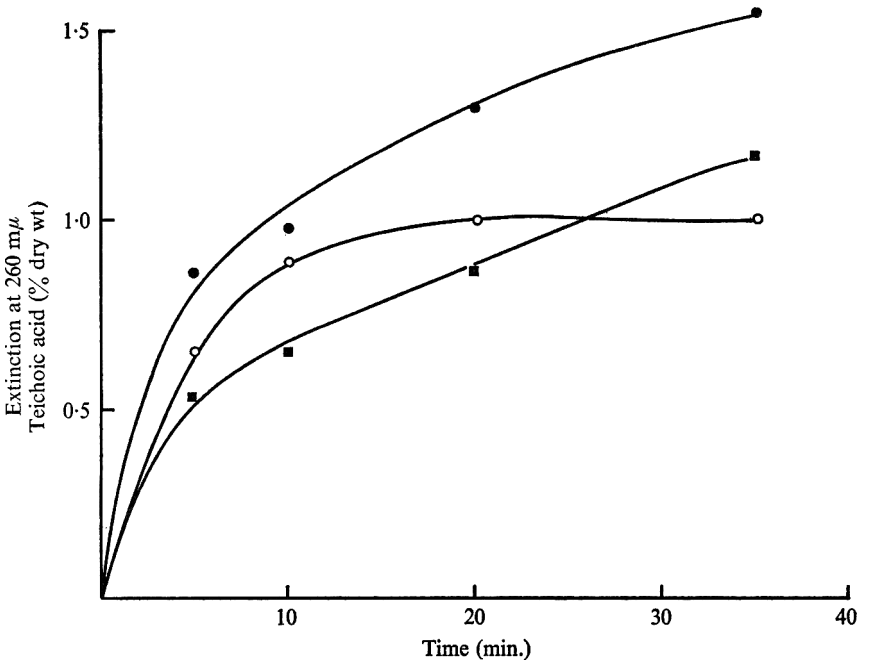

Fig. 1. Release of u.v.-absorbing material (■) and teichoic acid on disintegration of organisms; the teichoic acid was separated into the fraction sedimented at 100,000 *g* (○) and the fraction remaining in the supernatant solution (●). Absorption at 260 mμ was measured on the whole extract. The teichoic acid content of the extracts was estimated serologically (using P-teichoic acid as standard) and the results are expressed in terms of per cent of the dry wt of bacteria from which the extracts were obtained.

Table 2. *Effect of absorption of antiserum with bacteria on the haemagglutination titre*

Comparable amounts of bacteria of each strain were shaken with 2 ml. of diluted antiserum 148. The titre of the serum before absorption was 3200 and the table records the values after absorption; two concentrations of serum were tested: (*a*) 1 ml. diluted to 12·5 ml. and (*b*) 1 ml. diluted to 50 ml.

Serological group	Species of Lactobacillus	Strain	Titre (*a*)	Titre (*b*)
A	helveticus	8025	3200	800
B	casei	H 831	3200	1600
C	casei	R 094	3200	1600
	casei	6375	3200	1600
D	plantarum	7220	1600	400
E	bulgaricus	B 9	800	200
	lactis	7278	800	800
F	fermenti	6991	100	< 200
		77	1600	800
		126	1600	800

that 11 mg. of bacteria lowered the titre from 3200 to 100, though in this case the amount of precipitating antibody was 1·0 mg. This was sufficient to react with 40 μg. teichoic acid or 14·5 % of that present in the intact organisms.

Cross-reactions with other strains of lactobacilli

The ability of other strains of lactobacilli to absorb antibodies to P-teichoic acid was investigated by the procedure used above for NCTC 6991. Laboratory strains of *Lactobacillus fermenti* were examined, and also representative strains of each of the other serological groups. The suspensions of these bacteria were adjusted so as to contain a bacterial concentration similar to that used for *L. fermenti* NCTC 6991. To detect weak cross-reactions, the bacteria were shaken with two dilutions of antiserum 148, (*a*) 1 ml. diluted to 12·5 ml. and (*b*) 1 ml. diluted to 50 ml. The results showed that other strains of *L. fermenti* only partially absorbed antibodies to strain NCTC 6991 (Table 2). Organisms from groups A, D and E absorbed a significant amount of antibody from the more dilute serum. Cell wall (10 mg.) from *L. plantarum* NCIB 7220 also absorbed antibodies, the titre of the more dilute serum decreasing to 800. A number of the strains was tested against other antisera but no significant variations in reaction were observed.

DISCUSSION

The observation that lipoteichoic acid (P- or HT-teichoic acid) from *Lactobacillus fermenti* NCTC 6991 can be absorbed to normal sheep red blood cells enabled the application of haemagglutination to a study of teichoic acid antigens. LT-teichoic acid, the low molecular weight product of TCA action, is devoid of lipid and is not able to sensitize erythrocytes for haemagglutination, though it does cause a partial inhibition of haemagglutination of erythrocytes sensitized by lipoteichoic acid. The lack of reactivity of LT-teichoic acid and the loss of activity of HT-teichoic acid under conditions which remove fatty acids probably relate to the role of the lipid component in sensitizing erythrocytes. The fatty acid content of the lipoteichoic acids (4 to 5 % as palmitic acid) is similar to that found to be effective in rendering polysaccharides capable of absorbing to erythrocytes. Hämmerling & Westphal (1967) first demonstrated this effect when they showed that the addition of 5 % *O*-stearoyl groups to a number of polysaccharides gave optimal erythrocyte-sensitizing properties. Application of the procedure to streptococcal group-specific polysaccharides was studied in detail by Slade & Hämmerling (1968), and the present results for the amount of antigen required to sensitize erythrocytes and the resultant titres are comparable to those reported by these investigators.

Although lipid has not previously been reported as occurring in association with teichoic acids, the studies of several groups of workers on a common erythrocyte-sensitizing antigen occurring in a variety of Gram-positive organisms is consistent with its being a lipoteichoic acid. This antigen, first detected in phenol extracts of organisms by Rantz, Randall & Zuckerman (1956), is an intracellular glycerol teichoic acid with D-alanine substituents (Gorzynski, Neter & Cohen, 1960; Stewart, 1961; Jackson & Moskowitz, 1966). Jackson & Moskowitz (1966) concluded that alanine was responsible for the attachment of antigen to red blood cells, though the conditions used in the experiments that led to this conclusion would also have removed fatty acids

Further, the ability of alanine-free P-teichoic acid from *Lactobacillus fermenti* to absorb on to erythrocytes is indicative that alanine is not required for sensitization.

Antibodies to the lipoteichoic acid of *Lactobacillus fermenti* were absorbed completely from diluted serum on standing with apparently intact organisms for 15 min. Cell wall did not absorb antibodies nor did the cell-wall polysaccharide react with antibodies to purified teichoic acid (Knox *et al.* 1970). It may, therefore, be concluded that absorption of antibodies implies their penetration of the cell wall to react with membrane teichoic acid. However, the quantitative studies indicate that only a small proportion of the membrane teichoic acid of intact organisms is apparently able to react with antibody, suggesting that access of antibody to the membrane teichoic acid is a limiting factor. The absorption by intact group D streptococci of antibodies to the group antigen also implies penetration of the wall by antibody, as investigations by a number of workers (see Shockman & Slade, 1964) have led to the conclusion that the antigen is associated with the membrane.

Of the other strains of lactobacilli that were examined, those in groups A, D and E absorb a significant amount of antibody from antisera to lipoteichoic acid from *Lactobacillus fermenti* NCTC 6991. The reactions by strains of groups A and E may relate to the presence of glucosyl–glycerol teichoic acids as cell-wall components, with strain B9, which is the most effective, also containing galactose (Sharpe *et al.* 1964). A cross-reaction with glucosyl–ribitol teichoic acid of group D cell wall would be considered less likely but is suggested by the ability of purified cell wall to absorb antibodies.

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Inactivation of Bacterial Spores by Hydrostatic Pressure

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SUMMARY

Spores of various species of the genera *Bacillus* and *Clostridium* were inactivated by hydrostatic pressures up to 8000 atmospheres. Inactivation was a function of holding time at pressure rather than of the compression and decompression stages. Inactivation generally proceeded more rapidly at high than at low temperatures; below about 50° there was a well defined optimum pressure for inactivation, but above about 50° an increase in pressure up to 8000 atmospheres caused progressively more inactivation.

Inactivation was decreased at extremes of pH value and by high ionic strength solutions. A proportion of the spores pressurized under certain conditions became heat-sensitive. These observations, and the chemical, phase-contrast, and electron-microscopic changes seen in pressurized spores, suggested that pressure caused inactivation of spores by first initiating germination and then inactivating the germinated forms.

INTRODUCTION

Hydrostatic pressure above 1000 atmospheres (atm.) causes a rapid inactivation of many vegetative bacteria (Hite, 1899; Hite, Giddings & Weakley, 1914; Heden, 1964) whereas spores are more resistant and may survive pressures above 12,000 atm. (Larson, Hartzell & Diehl, 1918; Basset & Macheboeuf, 1932). Recently we noticed, however, that under certain conditions inactivation of spores occurred more rapidly at lower hydrostatic pressures than at higher pressures. This observation was surprising, and was difficult to reconcile with the expected effect of a physical treatment on a biological system: an increase in the intensity of the treatment ought to result in an increase in the degree of inactivation of the bacteria (cf. heat, ultraviolet and ionizing radiation, electric shock, ballistic disintegration; see Roberts & Hitchins, 1969). This anomalous effect of pressure led us to study and define the effects of environmental variables on pressure inactivation of spores more closely, with a view to explaining, first, how spores are inactivated by pressure and, secondly, why lower pressures can cause greater inactivation than higher pressures.

METHODS

Organisms. Bacteria used were *Bacillus coagulans* NCTC 3991 and *B. cereus* NCTC 8035 (National Collection of Type Cultures, Colindale), *B. cereus* strains T and PX, *B. polymyxa* M 1, *B. subtilis* var. *niger* (syn. *globigii*), *B. subtilis* A (rough strain; Edwards, Busta & Speck, 1965) and *Clostridium sporogenes* PA 3679.

Production of spores. Spores of the aerobes were grown on potato yeast-extract

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glucose agar (Gould & Ordal, 1968) at 30° (*Bacillus cereus* strains) or 37° (*B. coagulans*, *B. polymyxa* and *B. globigii*) or on the Fortified Nutrient Agar of Edwards *et al.* (1965) at 45° (*B. subtilis* A). Spores of *Clostridium sporogenes* were grown at 37° in the liquid Reinforced Clostridial Medium (Oxoid) of Gibbs & Hirsch (1956). When sporulation and lysis of sporangia were complete the spores were washed six times with cold distilled water (using a centrifuge) and stored at 4° in water at a concentration of approximately 20 mg. dry wt spores/ml. Suspensions of 'superdormant' spores of *B. cereus* PX and *B. polymyxa* (Gould, Jones & Wrighton, 1968) were prepared as follows: Spores were activated by heating at 70° for 30 min., then incubated at a concentration of about 10^8 spores/ml. for 1 hr in yeast glucose broth at 37°. The suspension was then heated at 70° for 30 min. to inactivate germinated spores whilst leaving viable the superdormant forms. The spores were finally recovered, washed and stored as above. Mixed spores from soil were prepared by extracting 10 kg. portions of soil with 1 l. portions of cold distilled water, filtering through glass wool to remove coarse debris and concentrating the spores by repeated differential centrifugation. The final suspension was heated at 70° for 30 min. to inactivate non-spore forms and stored in water, as above.

Application of pressure. Suspensions (2 ml.) were enclosed in sachets made from 1 in. broad polythene Layflat Tubing (Transatlantic Plastics Ltd., 45 Victoria Road, Surbiton, Surrey), using an impulse heat sealer (A. H. Bland Ltd., Winchelsea Road, Harlesden, London N.W.10). The final seal was made through the suspension in order to exclude air bubbles. The sachets were immersed in a container of water or oil, to which the pressure was transmitted by hydraulic oil.

The high pressure intensifiers were designed following conventional techniques (Manning, 1963), and pressures up to 8000 atm. could be reached. Pressure was measured by a manganin gauge, and temperature close to the sachets by a mineral-insulated metal-sheathed thermocouple. Temperature was controlled by circulating heated glycol from a thermostated bath through a jacket on the outside of the pressure cylinder. Pressure was normally raised in less than half a minute and lowered in less than 5 sec., unless otherwise stated. Adiabatic heating of the samples amounted to approximately 3° per 1000 atm.; the temperature transient decayed in 10 min.

Radiation. Suspensions (1 ml.) were sealed into glass ampoules and subjected at ambient temperature to γ-radiation from a Co 60 source at a dose rate of about 0·6 Mrad./hr.

Estimation of viability. Spores surviving the various treatments were enumerated by poured plate viable counts using nutrient agar, and incubation temperatures as listed above. Heat-sensitive forms were inactivated by heating samples at 70° for 30 min. before plating as indicated in the Results section.

Chemical estimations. Calcium was estimated by flame photometry on samples of bacterial pellets dry-ashed (500°, 16 hr) and extracted with 0·1 N-HCl. Dipicolinic acid in bacterial pellets was assayed by the method of Janssen, Lund & Anderson (1958). For estimation of hexosamine, bacterial pellets were first hydrolysed (6 N-HCl, 5 hr, 100°) in sealed evacuated ampoules. Hexosamine was then determined by the Boas (1953) modification of the Elson and Morgan reaction using sodium carbonate blanks to lower non-specific colour due to sugars and amines, as suggested by Immers & Vasseur (1950); glucosamine hydrochloride was the standard (Hamilton & Stubbs, 1967).

Electron microscopy. Bacterial suspensions were centrifuged and the pellets re-suspended, for fixation, in potassium permanganate (2 %, w/v) at 22° for 90 min. (spores) or 60 min. (germinated spores) (Mollenhauer, 1959). The 'fixed' pellets were washed at least six times by centrifugation prior to embedding in Epon 812, wet-sectioned and stained with uranyl acetate (1 %, w/v) for 10 min. and examined in a JEM 6S electron microscope.

RESULTS

Lethal effect of hydrostatic pressure

When suspensions of spores of different species were subjected to different pressures for a constant time at 20° the pattern of survivors (Table 1) showed that pressure had inactivated the spores, but the greatest inactivation was not caused by the highest pressure. Intermediate pressures, i.e. between about 1000 and 3000 atm. (depending on the organism), were most lethal.

Table 1. *Inactivation of spores of different species by hydrostatic pressure*

	Survivors (%)* following pressurization† at 20° for 1 hr at (atm.)								
Organism	1000	1500	2000	2500	3000	4000	5000	6000	8000
Bacillus cereus NCTC 8035	18	2·8	8·0	11	11·5	34	28	n.t.‡	11
B. cereus PX	98	0·35	0·90	6·4	8·5	11·3	11	7·8	5·7
B. subtilis var. *niger* (syn. *globigii*)	100	10·2	9·8	2·5	22	31	74	84	92
B. polymyxa	100	18	1·8	5·7	34	56	n.t.	68	42

* Survivors were enumerated by poured plate viable counts.

† Spores (*c.* 10^8/ml.) were suspended in sodium phosphate buffer (0·1 M, pH 8·0) during pressurization.

‡ Not tested.

Table 2. *Comparison of continuous and discontinuous application of pressure on viability of spores*

		Survivors (%)* following pressurization	
Organism	Application of pressure†	Unheated spores	Heated spores‡
Bacillus subtilis var. *niger* (syn. *globigii*)	Continuous	3·5	1·6
	Discontinuous	7·7	4·6
B. cereus PX	Continuous	1·6	0·30
	Discontinuous	0·45	0·30

* Spores (*c.* 10^8/ml.) were suspended in sodium phosphate buffer (0·1 M, pH 8·0). Survivors were enumerated by poured plate viable counts.

† Pressure (2000 atm., 20°) was applied either continuously for 1 hr, or discontinuously as a series of twelve 5 min. exposures.

‡ Spores were heated for 30 min. at 70° following pressurization.

Spores in suspensions subjected to pressure for 1 hr (as in Table 1) were inactivated to about the same extent whether held under pressure continuously or discontinuously, i.e. compressed and decompressed twelve times, each treatment being for a period of 5 min. (Table 2). This, and other experiments, showed that the extent of the inactivation

depended on the duration of pressure treatment and not on the compression or decompression steps.

Spore concentration did not affect the extent of the inactivation, e.g. the survivor levels of spores of *Bacillus coagulans* pressurized in sodium phosphate (0·1 M, pH 8·0) at 2000 atm., 45° for 1 hr, then heated at 70° for ½ hr were 0·13, 0·23, 0·21, 0·23 and 0·24 respectively for initial viable spore concentrations of $2{\cdot}6 \times 10^9$, $2{\cdot}7 \times 10^8$, $2{\cdot}5 \times 10^7$, $2{\cdot}5 \times 10^6$ and $2{\cdot}6 \times 10^5$ per ml.

Effect of temperature on the inactivation of spores by pressure

Inactivation of spores by pressure was strongly influenced by temperature. Figure 1 *a* summarizes the results of experiments with spores of *Bacillus coagulans*. Similar but less exhaustive experiments were performed with spores of *B. cereus* strains T, PX

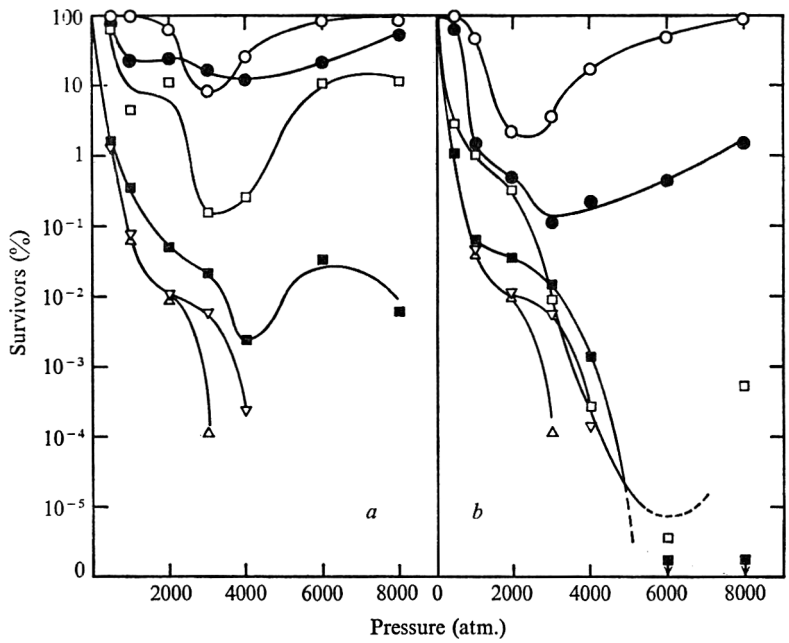

Fig. 1. Inactivation of spores of *Bacillus coagulans* at different pressures and temperatures. (*a*) Spores (*c.* 10^8/ml.) were heated at 70° for 30 min., then pressurized at the indicated temperatures and pressures for 30 min. The suspending medium was sodium phosphate buffer (0·1 M, pH 8·0). Survivors were enumerated by viable counts using poured plates. Temperatures of pressurization were: 25° (○); 35° (●); 45° (□); 55° (■); 65° (▽); 75° (△). (*b*) As Fig. 1 *a*, but spore suspensions were additionally heated at 70° for 30 min. following pressurization in order to inactivate forms which had been heat-sensitized but not inactivated by pressure. (Arrows indicate that survivors were not detected and were therefore less than indicated by the symbol.)

and NCTC 8035, *B. subtilis* var. *niger* (syn. *globigii*), *B. polymyxa* M 1 and *Clostridium sporogenes* PA 3679. The same qualitative pattern was seen with all these organisms. Four well-defined regions of response to pressure could be recognized (Fig. 1 *a*): (1) a region of increased inactivation with increased pressure up to about 1000 atm.; (2) a tendency to plateau around 2000 to 3000 atm.; (3) a region of further increased

inactivation; (4) a region above about 4000 atm. where the inactivation decreased at temperatures below 55° but increased at higher temperatures.

When samples of spores from the experiment reported in Fig. 1 *a* were heated at 70° for 30 min. following pressurization below 65°, the inactivation was increased (Fig. 1 *b*); the increase was particularly evident in the lower temperature and pressure regions (compare Fig. 1 *a*, *b*). Since the temperature of 70° (for 30 min.) was well below that which inactivates untreated spores, pressure, in addition to inactivating spores, increased the sensitivity of a proportion of the survivors to heat.

The storage temperature following pressure treatment was unimportant, except as indicated in Fig. 1 *a*, *b*. For instance, the survivor levels of spores of *Bacillus coagulans* following pressurization at 1000 atm. (at 70° for 30 min.) and incubation for 1 hr at 0, 25, 37, 45, 55 and 65° were respectively 1·2, 1·2, 1·1, 1·1, 1·1 and 0·9 %.

Kinetics of inactivation of spores by pressure

Figure 2 shows the rates of inactivation of spores of three species by pressure at 70°. No single response was apparent: semilog inactivation curves were typically concave, but with an initial rapid drop in numbers of survivors for *Bacillus coagulans* at the

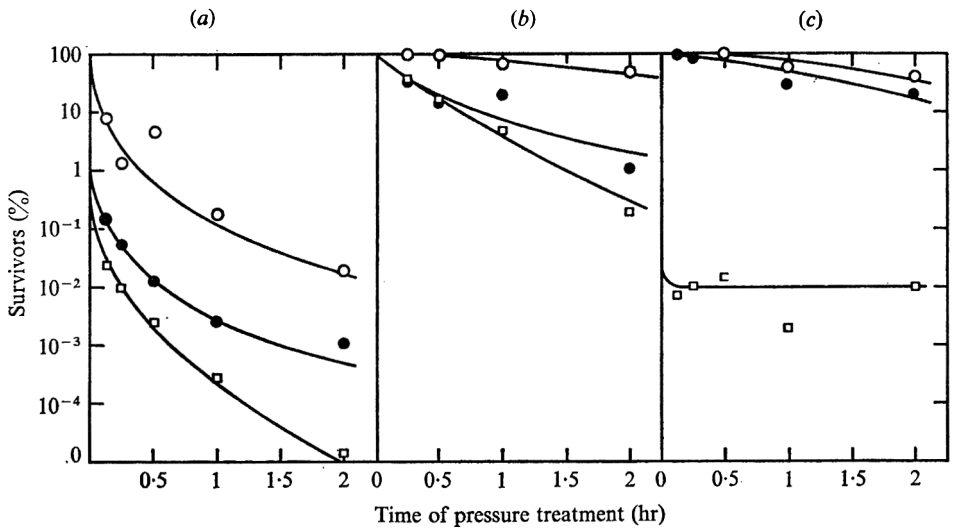

Fig. 2. Kinetics of inactivation of spores by pressure. Spores (*c.* 10^8/ml.) were heated at 70° for 30 min., then pressurized at 70°. The suspending medium was sodium phosphate buffer (0·1 M, pH 8·0). Survivors were enumerated by viable counts using poured plates. Organisms were: (*a*) *Bacillus coagulans*; (*b*) *Bacillus subtilis* A; (*c*) *Clostridium sporogenes*. Pressures were: 500 atm. (○); 1500 atm. (●); 3000 atm. (□).

higher pressures (Fig. 2 *a*); the rate of inactivation of the more pressure-resistant spores of *B. subtilis* A was slow but approximately exponential (Fig. 2 *b*); spores of *Clostridium sporogenes* held at 3000 atm. were about 99·99 % inactivated within a few minutes, but the surviving 0·01 % of the population resisted further inactivation for at least 2 hr (Fig. 2 *c*).

Appearance of spores

The optical density of spore suspension fell when the spores were inactivated, or merely sensitized to heat by pressure. On examination by phase-contrast microscopy,

spores which had been inactivated or sensitized to heat by pressure always had changed from bright to dark, as happens when spores germinate. Examination with the electron microscope revealed structural changes which were typical of the changes seen in germinating spores (Plate 1).

Chemical changes

The chemical changes that occur on germination were sought and found to occur when spores were subjected to pressure. Pressure caused spores to excrete calcium, dipicolinic acid and hexosamine-containing material (Table 3).

Table 3. *Phase-darkening and release of dipicolinic acid, calcium and hexosamine from spores during pressurization*

Organism	Pressure* (atm.)	Time (hr)	Constituent released (%) Dipicolinic acid	Calcium	Hexosamine	Phase-dark spores (%)
Bacillus cereux PX	2000	1	91·7	92	—	—
B. polymyxa M 1	2000	1	86	69·5	48	—
B. subtilis var. *niger* (syn. *globigii*)	1000	½	72·8	—	—	81
		1	74·2	—	—	83
		2	85	—	—	88
	6000	½	87·3	—	—	97
		1	98·2	—	—	97
		2	91·7	—	—	96

* Temperature during pressurization was 20°. Spores (*c.* 10^8/ml.) were suspended in water.

Effect of heat and of γ-radiation prior to pressurization

When spores of some species were sublethally heated prior to pressurization, their sensitivity to pressure at the lower temperatures and pressures increased. However, spores pressurized at higher temperatures (e.g. 65°) could be rendered more resistant to pressure by prior heating above about 70° (Fig. 3). (The low survivor level at 90° in Fig. 3 was due to inactivation by the high temperature alone.)

Exposure to γ-radiation sufficient to inactivate a proportion of the spores in a suspension caused an increase in the pressure sensitivity of the survivors (Table 4).

Inactivation of spores by pressure at different pH values

Figure 4 shows the effect on survivor levels of pressurizing spores in various buffers at different pH values. (Although pressure will change the pH value of buffer solutions, the changes in these experiments were estimated not to exceed about 0·4 pH units; Distèche, 1959.) The nature of the buffer seemed unimportant, and spores were inactivated by pressure over a wide pH range. However, the inactivation was greatest near neutrality and was definitely lowered at extreme pH values.

Decrease in pressure inactivation of spores by ions

Spores were pressurized in various solution of known water activity (a_w). Figure 5 summarizes four experiments which suggested that non-ionic solutes (sucrose, glycerol) at low a_w had little effect on the inactivation of spores by pressure, whereas ionic

solutes (NaCl and, more effectively, $CaCl_2$) decreased the inactivation. The spores were therefore more likely protected from the inactivating effects of pressure by high concentrations of ions rather than by low a_w *per se*. Below a_w about 0·96, additional salt had proportionally less effect than above a_w 0·96.

Spores of different species

When suspensions of spores of different species were subjected to pressure, the same phenomena were observed as recorded above, i.e. some spores were inactivated

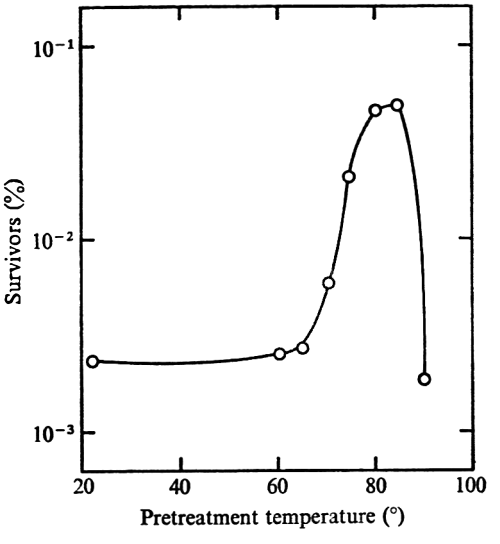

Fig. 3

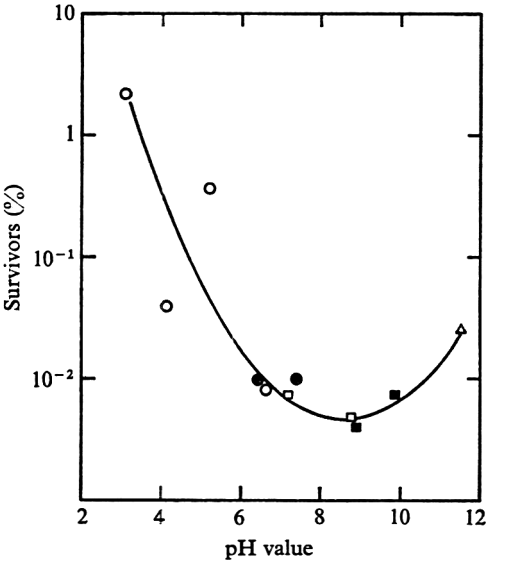

Fig. 4

Fig. 3. Decreased pressure-sensitivity of preheated spores of *Bacillus coagulans*. Spores (*c.* 10^8/ml. in sodium phosphate buffer; 0·1 M, pH 8·0) were heated for 30 min. at the temperatures indicated prior to pressurization at 1000 atm. for 30 min. at 65°.

Fig. 4. Inactivation of spores of *Bacillus coagulans* by pressure at different pH values. Spores (*c.* 10^8/ml.) were heated at 70° for 30 min., then pressurized at 1000 atm. for 30 min. at 65°. The symbols refer to different buffers (0·1 M) as follows: sodium citrate/phosphate (○); sodium phosphate (●); tris (hydroxymethyl) aminomethane/HCl (□); sodium bicarbonate/carbonate (■); sodium carbonate/hydroxide (△).

Table 4. *Increased pressure sensitivity of γ-irradiated spores of Bacillus coagulans*

Pressure and temperature	Survivors (%)* following pressurization of spores surviving γ-irradiation†		
	Control (no irradiation)	0·2 Mrad	0·5 Mrad
1500 Atm., 45°	$5{\cdot}5 \times 10^{-2}$	$8{\cdot}0 \times 10^{-3}$	$3{\cdot}1 \times 10^{-4}$
1000 Atm., 65°	$1{\cdot}9 \times 10^{-2}$	$3{\cdot}5 \times 10^{-3}$	$1{\cdot}0 \times 10^{-4}$

* Survivors were enumerated by poured plate viable counts. Radiation alone inactivated spores to the following extent: 0·2 Mrad, 20% survivors; 0·5 Mrad, 2·2% survivors.

† Spores (*c.* 10^8/ml.) were irradiated and pressurized in sodium phosphate buffer (0·1 M, pH 8·0), and were heated at 70° for 30 min. prior to counting.

and some became heat-sensitive in the manner shown in Fig. 1 *a*, *b*, and individual spores became phase-dark as if germinated. However, the extent of inactivation varied greatly with different organisms (Table 5). Those spores which are most easily germinated by 'physiological' germinants at 1 atm. (e.g. *Bacillus polymyxa*, *B. cereus*) were in general more pressure-sensitive than those spores which are most dormant at 1 atm. (e.g. *B. subtilis* A, and mixed spores from soil; Table 5). 'Superdormant' spores of *B. polymyxa* and *B. cereus* (i.e. those spores in a population which remained ungerminated in a medium which caused germination of the majority) were likewise more resistant than the less dormant ones to the lethal effect of pressure (Table 5).

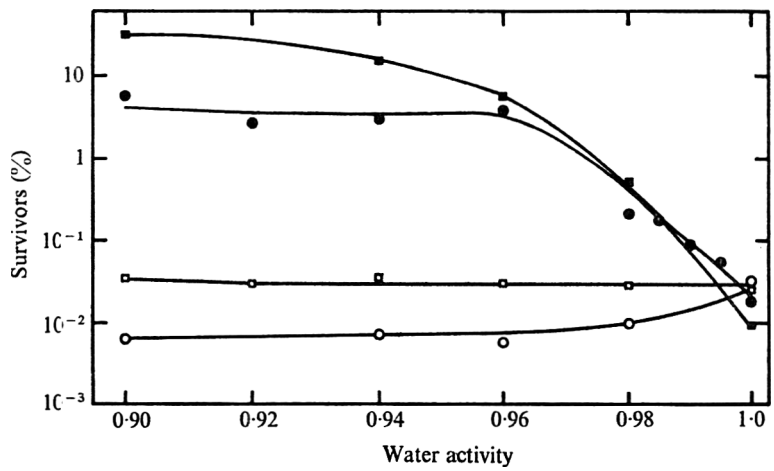

Fig. 5. Effect of ionic and non-ionic solutes on inactivation of *Bacillus coagulans* spores by pressure. Spores (*c.* 10^8/ml.) were heated at 70° for 30 min., then resuspended in media of differing water activity (a_w) and pressurized at 1000 atm. for 30 min. at 65°. Compounds used to adjust a_w were: sucrose (○); glycerol (□); sodium chloride (●); calcium chloride (■). In addition, the suspensions contained buffer (0·1 M, pH 8·0; not taken into account in a_w estimation) which was tris (hydroxymethyl) amino methane/HCl when calcium chloride was present, or otherwise sodium phosphate.

Table 5. *Inactivation of 'superdormant' spores by pressure*

	Survivors (%)* following pressurization† for 1 hr at			
Organism	2000 atm. 20°	2000 atm. 50°	2000 atm. 70°	3000 atm. 70°
Bacillus cereus PX	0·037	—	—	—
B. cereus PX 'superdormant' fraction	0·75	—	—	—
B. polymyxa M 1	0·094	—	—	—
B. polymyxa M 1 'superdormant' fraction	15	—	—	—
B. cereus NCTC 8035	0·08	—	—	—
B. subtilis var. *niger* (syn. *globigii*)	1·7	0·09	0·0015	0·0003
B. coagulans NCTC 3991	11·5	0·1	0·0075	0·0037
Mixed soil spores	—	—	0·28	0·23
B. subtiis A	100	64	3·3	4·6

* Survivors were enumerated by poured plate viable counts.

† Spores were suspended in sodium phosphate buffer (0·1 M, pH 8·0) during pressurization.

DISCUSSION

The lethality of hydrostatic pressures in excess of about 1000 atm. for vegetative forms of bacteria is well documented. The most effective pressure depends on the organism, its habitat (e.g. terrestrial, or marine barophile) and stage of growth (see Introduction, and Zobell & Johnson, 1949). Inactivation of vegetative bacteria, by pressure, also depended upon environmental factors; for instance, being most rapid at extreme pH values and lowered by the addition of protective substances such as salts or sugars (Timson & Short, 1965).

The mechanism of inactivation of vegetative bacteria by pressure was suggested by Heden (1964) and Rutberg (1964) to involve damage to the processes of DNA replication, transcription or translation (Landau, 1967). Timson & Short (1965) reasoned that damage may arise *via* electrostriction and the increase in ionization of weak electrolytes and proteins which is known to result from increase in pressure. Indeed, many studies with pure proteins have shown that high hydrostatic pressures initiate conformational changes which may result in denaturation (Johnson, Eyring & Pollissar, 1954; Kalckar, 1962). Thus the increase in inactivation of vegetative bacteria that occurs with increase in pressure may result from denaturation of proteins.

In contrast to vegetative bacteria, spores resisted pressures well above those which cause the denaturation of free proteins. Furthermore, the pattern of inactivation of spores has been found in this study to differ from that of vegetative forms in a number of fundamental ways. For instance, inactivation of spores by pressure was most rapid near neutral pH values rather than in acid or alkali. In some instances, the denaturation of proteins by pressure, when it involves a decrease in protein molecular volume, can be counteracted by increasing the temperature, which generally causes an increase in protein molecular volume (Eyring, Johnson & Gensler, 1946; Johnson *et al.* 1954). For example, Morita & Haight (1962) reported that malic dehydrogenase was active at 101° under 1300 atm. pressure. Analogous protection by pressure of vegetative bacteria (Johnson & Lewin, 1946) and spores (Johnson & Zobell, 1949) from inactivation by heat, and *vice versa*, has been reported. Results in the present paper, however, show that the temperature coefficient for inactivation of spores by pressure is very high, and that, far from counteracting the effect of pressure, heat that was insufficient alone to inactivate spores increased the inactivation caused by pressure.

The discovery of optimal values of pH, temperature and pressure for inactivation of spores by pressure argues against the inactivation having resulted mainly from effects on nuclear material, translation, denaturation of proteins or increased solvation (like the lethal mechanism postulated for vegetative forms by Heden, 1964; Timson & Short, 1965; and Landau, 1967, or for spores by Clouston & Wills, 1969). These mechanisms should generally cause progressively more inactivation as the pressure is raised. The possibility that hydrostatic pressure physically distorted spores sufficiently to cause inactivation was also unlikely since again one would expect high pressures always to cause greater inactivation than low pressures, and one would not expect the high temperature coefficient that was observed. Furthermore, hydrostatic pressure is not vectorial and therefore would not cause distortion of the sort that can disrupt and inactivate spores; i.e. squeezing under a microscope slide cover glass (Lewis, Snell & Burr, 1960), shaking with glass beads (Curran & Evans, 1942) or scraping with a wire loop (Knaysi & Curran, 1961). The regions of pressure and temperature

investigated were such that transitions through different ice phases under pressure (Bridgman, 1937), which can disrupt vegetative bacteria (Edebo & Heden, 1960), did not occur.

The observations that pressure caused heat sensitization of spores, initiated leakage of calcium, dipicolinic acid and hexosamine-containing material, and resulted in fall in optical density of suspensions and phase-darkening of individual spores, together with the electron-microscopical evidence, showed that pressure initiated changes in spore structure and function identical to those observed on germination (Hamilton & Stubbs, 1967). Further evidence is provided by the pressure inactivation being greatest near neutral pH values. The effects of heat (Curran & Evans, 1945) and γ-radiation (Gould & Ordal, 1968) as 'activating' treatments for germination could explain their potentiation of the lethality of pressure for spores. Germination (at 1 atm.) is inhibited by ionic and non-ionic solutes in a similar manner to the inhibition of pressure inactivation of spores (Fig. 5). Spores which are most difficult to germinate completely with 'physiological' germinants at 1 atm. (e.g. soil spores; 'superdormant' spores, Table 5; Gould *et al.* 1968) were also the most resistant to inactivation by pressure. Taken altogether, the evidence leads to the conclusion drawn by Clouston & Wills (1969) that pressure initiates germination (Gould & Sale, 1970).

Presumably the shapes of the inactivation–pressure curves reflect the sensitivity to pressure of the germination as well as the sensitivity of the germinated spores to pressure inactivation. At lower temperatures the lowest pressures caused germination and heat sensitization but were insufficient to cause appreciable inactivation of the resulting germinated forms. Medium pressures caused considerable germination and were high enough to inactivate a large proportion of the resulting germinated population. The overall effect was therefore to inactivate many but not all of the spores. The highest pressures caused less germination and again only a proportion of the germinated spore population was inactivated, so the overall effect was that the highest pressures caused less inactivation than medium pressures. At higher temperatures the germination was increased and most of the germinated spores were inactivated; always more spores were germinated and became heat-sensitive than were inactivated. Above 65° the germination and inactivation curves coincided, suggesting that the pressure-germinated spores were directly inactivated by heat.

Although pressure induces germination, evidently not all the germinated spores are inactivated by pressure. It is also evident that at near room temperature, spores, whether germinated or not, have a remarkable resistance to extreme pressure.

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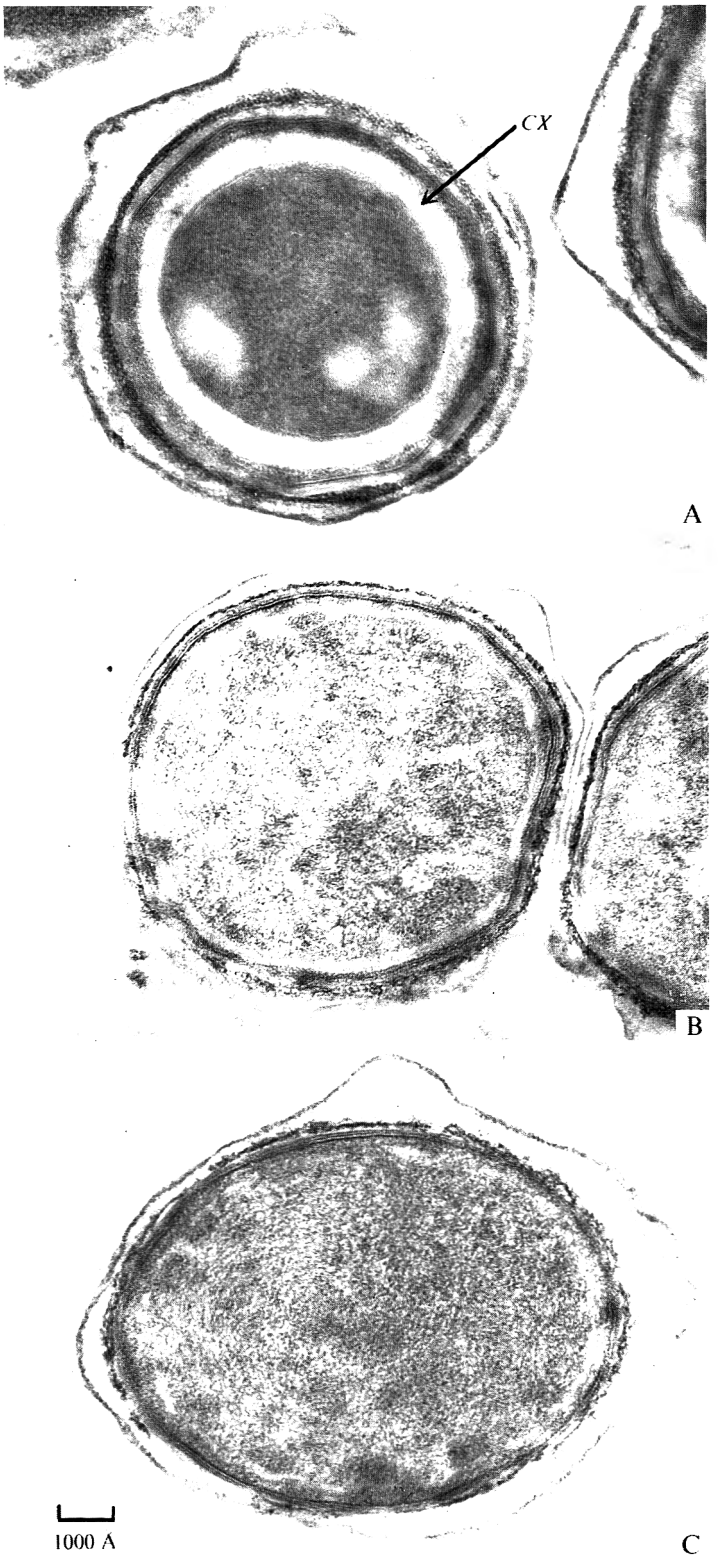
EXPLANATION OF PLATE

Plate 1. Micrographs of thin sections of spores fixed with potassium permanganate. The scale mark represents 1000 Å.

A. Ungerminated spore of *Bacillus cereus* PX, showing wide cortex zone (*CX*).

B. Spore of *Bacillus cereus* PX germinated by incubation at 37° for 10 min. in buffer (sodium phosphate, 0·1 M, pH 8·0) plus the germinants inosine (100 μM) and L-alanine (1 mM).

C. Spore of *Bacillus cereus* PX germinated by pressurization at 2000 atm. (20°, 1 hr) in nutrient-free buffer (sodium phosphate, 0·1 M, pH 8·0).



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Initiation of Germination of Bacterial Spores by Hydrostatic Pressure

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SUMMARY

Hydrostatic pressure initiated germination of bacterial spores in nutrient-free media. Those spores which were most dormant towards chemical germinants at 1 atmosphere pressure were also the most resistant to germination by pressure treatment. Germination by high pressure treatment was characterized by temperature and pH optima, like germination at atmospheric pressure. Germination initiated by pressure was inhibited by metabolic poisons and was potentiated by low concentrations of various nutrients including some of those which are normally germinative (at higher concentrations) at atmospheric pressure. In particular, L-alanine and closely related α-amino acids, but not their breakdown products, potentiated germination initiated by pressure. Study of potentiation by D-alanine (which strongly inhibits germination initiated by L-alanine at 1 atmosphere pressure) revealed that high pressures caused an increase in the rate of racemization of alanine by spores. Germination by pressure probably resulted from acceleration of some germination reaction which is normally negligibly slow at a pressure of 1 atmosphere, and also from an increase in permeability of some barrier within the spore to L-alanine and related α-amino acids.

INTRODUCTION

Inactivation of bacterial spores by hydrostatic pressures, unlike the inactivation of vegetative bacteria, occurred in two stages (Clouston & Wills, 1969; Sale, Gould & Hamilton, 1970). First, pressure caused germination of the spores, and then it inactivated the germinated forms. However, pressures of only a few hundred atmospheres could still cause germination, but were too low to inactivate the resulting germinated forms. The overall effect of such pressures was therefore solely to cause germination, i.e. heat-sensitization of the spores, optical, structural and chemical changes which normally occur during germination. Study of environmental factors showed that pressure inactivation of spores was strongly influenced by temperature, and less strongly by pH value, water activity and ionic strength.

Pressure germination is of interest as a potential step in preservation procedures aimed at eliminating spores from perishable materials. It is also worth studying as a novel initiator of germination, because the way in which spore germination is initiated by any germinant system is not yet understood.

Germination and inactivation of spores of *Bacillus pumilis* by pressures up to 1700 atmospheres (atm.) was originally described by Clouston & Wills (1969). The present paper is concerned with the germinant action of hydrostatic pressure on spores of

various Bacillus and Clostridium species, and in particular describes the strongly synergistic effect of pressure with some of the more often studied 'physiological' germinants. The pressure range was limited to 1000 atm. when we found that the synergistic effect disappeared at higher pressures.

METHODS

Organisms and production of spores. Bacillus subtilis MARBURG ATCC6051, *B. brevis* NCTC7577 and *B. pumilis* S3 (laboratory isolate) were grown at 37° on the surface of potato, glucose, yeast-extract agar. Procedures for recovery and 'cleaning' of spores were as described previously (Sale *et al.* 1970). Suspensions were stored at 4°, in water, and were activated by heating (70°, 30 min.) before use unless stated otherwise in Results.

Pressure treatment. Preparation of samples and the method of application of pressure were as described by Sale *et al.* (1970). Pressures up to 600 atm. were generated directly by an electrically driven hydraulic pump connected by a pipe to the vessel in which the sample sachets were treated. The vessel was immersed in a water bath for control of temperature. The pressure was measured by a Bourdon tube pressure gauge.

Measurement of germination. Spores for germination studies were suspended at a concentration of about 10^8/ml. in 0·1 M-sodium phosphate buffer (pH 8·0) unless stated otherwise in Results. Germination was measured in three ways. (1) The optical density (O.D.) of suspensions was measured using an absorptiometer ('Biochem'; Hilger & Watts Ltd., Camden Road, London), fitted with a 580 mμ peak transmission filter; germination was accompanied by a fall in O.D. (2) Samples were examined microscopically using phase contrast optics, when ungerminated spores appeared bright and germinated spores appeared dark. About 250 spores were examined from each sample and the percentage germination recorded. These two methods were insensitive when the amount of germination exceeded 98 % or so, in which case the third method was used. (3) Samples (1 ml.) were sealed in thin walled glass ampoules which were heated by total immersion in a water bath at 70° for 30 min. The surviving heat-resistant (i.e. ungerminated) spores were then enumerated by poured plate viable counts using nutrient agar (Oxoid).

Chemicals. Amino acids, nucleosides and related compounds were obtained from British Drug Houses Ltd. (Poole, Dorset) or Koch-Light Laboratories Ltd. (Colnbrook, Bucks.). *O*-Carbamyl-D-serine was a gift from Dr P. H. Hidy (Commercial Solvents Corp., Terre Haute, Indiana). Other chemicals were of Analar grade.

Alanine racemase. Racemization of alanine by whole spores was measured in the direction L → D by the method of Yoshimoto (1958) using D-amino acid oxidase (British Drug Houses Ltd.; Wood & Gunsalus, 1951) as described by Jones & Gould (1968).

RESULTS

Initiation of germination by pressure: optimum temperature and pressure

The optimum temperature for initiation of spore germination differed at different pressures. As the pressure was increased, the optimum temperature for germination, as measured by phase-darkening of spores or by viable counts of heat-resistant survivors, increased also. This trend is shown in Fig. 1 for spores of *Bacillus coagulans*, *B. subtilis* and *B. cereus*.

Germination was minimal at low temperatures whatever the pressure, but at high pressures germination occurred at temperatures well above those that supported germination at 1 atm. pressure (e.g. at 60° and 70°).

In general, those spores most dormant towards nutrient germinants at 1 atm. were

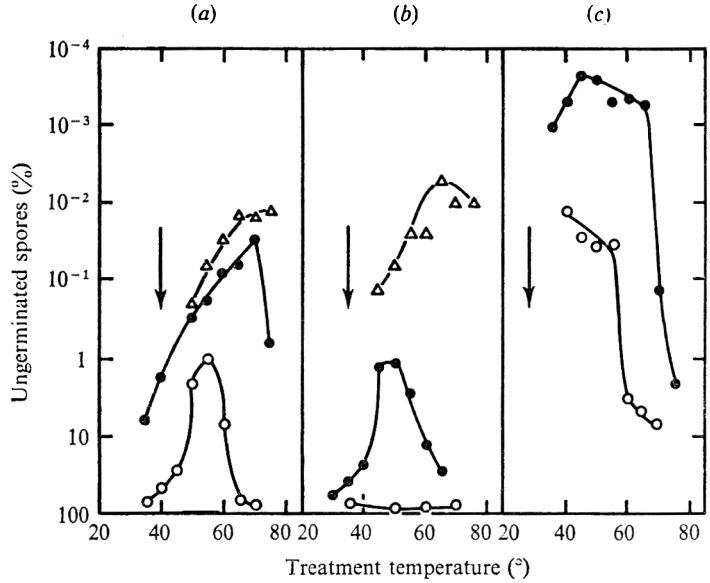

Fig. 1. Pressure germination at different temperatures. Spores of *Bacillus coagulans* (*a*), *B. subtilis* var. *niger* (syn. *globigii*) (*b*) and *B. cereus* T (*c*) were heat activated at 70° for 30 min., then suspended in 0·1 M-sodium phosphate (pH 8·0) and subjected to pressures of 250 atm. (○), 500 atm. (●) and 1000 atm. (△) for 30 min. periods. The germination was measured by heating samples (70°, 30 min.) to kill germinated forms and estimating the numbers of ungerminated survivors by viable counting. The vertical arrows indicate the temperature optima for germination of these spores by L-alanine at 1 atm. pressure.

Table 1. *Increase in pressure germination of heat-activated spores*

Organism	Pressure (atm.)	Time (min.)	Germination (%)* Unactivated spores	Activated† spores
Bacillus cereus T	250	30	60	95
	500	5	—	10
		15	—	50
		30	75	99
	1000	0·25	20	40
		30	>99	>99
B. subtilis MARBURG	250	30	30	60
B. brevis	250	30	0	99
B. coagulans	1000	30	20	80
B. subtilis var. *niger* (syn. *globigii*)	1000	30	60	90
B. pumilis S 3	1000	30	80	>99

* Incubation temperature was 25°. Spores were suspended in 0·1 M-sodium phosphate (pH 8·0). Germination was estimated by counting the percentage of phase-dark spores: incubated but not pressurized controls were all less than 5% phase-dark.

† Activation was at 70° for 30 min.

also most dormant towards pressure (e.g. *Bacillus coagulans* and *B. subtilis* var. *niger* (syn. *globigii*) in Fig. 1 *a*, *b*, compared with the less dormant spores of *B. cereus* T in Fig. 1 *c*).

Stimulation of pressure germination by heat activation

Heat-activated spores were germinated more completely by pressure than were unheated spores (Table 1). However, germination initiated at higher pressures, i.e. above about 1000 atm., was much less influenced by activation than germination initiated at lower pressures.

Influence of pH value on pressure germination

Pressure initiated the germination of spores optimally near neutral pH, but the optimum was much broader than for germination of spores initiated by nutrients at 1 atm. pressure (Fig. 2).

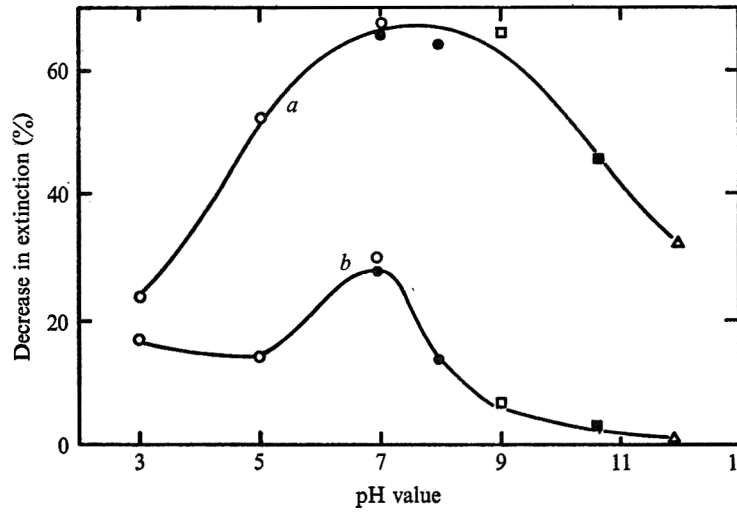

Fig. 2. Pressure germination at different pH values. Spores of *Bacillus cereus* T were activated at 70° for 30 min., then suspended in the following buffers (0·1 M) and subjected to pressures of 1000 atm. (curve *a*) or 250 atm. (curve *b*) for 30 min. at 25°: sodium citrate/phosphate (○); sodium phosphate (●); tris (hydroxymethylaminomethane)/HCl (□); sodium bicarbonate/carbonate (■); sodium carbonate/hydroxide (△). Germination was estimated by measuring the fall in extinction of the treated samples.

Effect of anaerobiosis on pressure germination of aerobic spores

Spores of *Bacillus coagulans* could be germinated by pressure as effectively in media containing reducing agents as in aerobic media (Table 2). Germination of spores of aerobes by nutrients at 1 atm. is well known to be similarly unaffected by anaerobiosis.

Stimulation of pressure germination by amino acids and other compounds

Germination initiated by the lower pressures (below about 1000 atm.) was markedly affected by constituents of the suspending medium (see, for example, the effect of alanine in Table 3). The influence of alanine decreased with rising pressure and became negligible between 1000 and 2000 atm. Following the observations that germination

at such high pressures was in general relatively unaffected by changes of environment or pretreatment of the spores, the experimental pressure range was restricted to 1000 atm. maximum.

Of a variety of substances tested, amino acids were clearly the most effective potentiators of pressure germination. For example, inspection of the results summarized in

Table 2. *Pressure germination of Bacillus coagulans spores in aerobic and anaerobic media*

Medium	Ungerminated spores (%) following pressurization* at 2000 atm.	Ungerminated spores (%) following pressurization* at 3000 atm.
Aerobic		
Water	$1{\cdot}7 \times 10^{-4}$	
Buffer†		$1{\cdot}2 \times 10^{-3}$
Yeast glucose broth	$1{\cdot}2 \times 10^{-4}$	
Anaerobic		
Buffer plus thioglycollate†		$1{\cdot}0 \times 10^{-3}$
R C M‡	$1{\cdot}3 \times 10^{-4}$	

* Pressurization was for 30 min. at 70°. Ungerminated spores were estimated by viable counts.
† Buffer was 0·1 M-sodium phosphate (pH 8·0); sodium thioglycollate was used at 0·1 % (w/v).
‡ Reinforced Clostridium medium (Gibbs & Hirsch, 1956).

Table 3. *Initiation of spore germination by pressure at different temperatures*

Organism	Addition‡	Germination (%)* after pressurization† at 0°	20°	30°	40°	50°	60°	70°
Bacillus coagulans	None	0	0	1	0	50	60	0
	L-Alanine (250 μM)	0	0	5	>95	>95	>95	50
	L-Alanine (1 mM)	0	0	12	>95	>95	>95	50
B. subtilis var. *niger* (syn. *globigii*)	None	1	1	2	3	10	10	8
	L-Alanine (250 μM)	1	1	50	50	75	30	25
	L-Alanine (1 mM)	1	1	68	>90	70	36	13
B. cereus T	None	0	0	21	40	64	5	—
	L-Alanine (250 μM)	12	>95	>99	>99	>99	>99	95

* Spores were activated (70°, 30 min.) before use; germination was measured by recording the percentage of phase-dark spores.
† Pressure was 250 atm. maintained for 30 min.
‡ Suspending medium was 0·1 M-sodium phosphate (pH 8·0).

Table 4 indicated that spores of *Bacillus cereus* could be caused to germinate at 1 atm. by a variety of amino acids, particularly at the higher concentration used (10 mM) and in the presence of inosine. Germination of *B. cereus* spores caused by pressure was similarly potentiated by a variety of amino acids. In contrast, spores of *B. coagulans* were much less responsive than those of *B. cereus* to amino acids at 1 atm., germinating rapidly only in L-alanine, and much less rapidly in L-α-aminobutyric acid and L-valine; similarly, the range of amino acids potentiating germination at 200 atm. pressure was much more restricted than the range potentiating germination of spores of *B. cereus*. Amino acids were effective germinants at 200 atm. at concentrations well below those effective at 1 atm.

In general the amino acids most effective in potentiating germination by pressure were those which were also most effective as germinants, either alone or with inosine, at 1 atm. However, there were exceptions to this generalization, e.g. L-serine was not germinative in any of the systems tested at 1 atm. and yet strongly potentiated germination of spores of *Bacillus cereus* at 200 atm., and L-leucine, L-isoleucine and L-aspartic

Table 4. *Potentiation of pressure germination of spores by amino acids*

	Germination (%)* following incubation† as indicated below						
	Bacillus cereus					*Bacillus coagulans*	
	1 atm. additions at						
Amino acid	1 mM for 1 hr	10 mM for 1 hr	10 mM for 5 hr	1 mM + 10 μM inosine for ½ hr	200 atm. additions at 1 mM for ½ hr	1 atm. additions at 1 mM for ½ hr	200 atm. additions at 1 mM for ½ hr
Control (no addition)	0	0	0	0	0	0	0
L-Alanine	80·5	100	100	100	100	44	92·5
L-α-Aminobutyric acid	95	95	100	100	99	0	90
L-Cysteine	0	99	100	17	96	0	2
L-Phenylalanine	0	92	100	73	100	0	0
L-Glutamine	47	7	20	100	88	n.t.‡	n.t.
L-Threonine	6	0	42	95	81	0	0
L-Valine	2	1	42	60	4	0	9
L-Tryptophan	0	10	67	89	88	0	0
L-Methionine	0	19	20	67	35	0	0
β-Alanine	0	2·5	29	96	100	0	1
L-Leucine	0	5	22	86	10	0	0
L-Isoleucine	0	0	11	100	19	0	0
Glycine	0	0	4	100	100	0	0
L-Tyrosine	0	n.t.	n.t.	63	100	0	0
L-Histidine	0	0	6	59	37	0	0
L-Aspartic acid	0	n.t.	n.t.	68·5	0	0	0
L-Serine	0	0	0	0	95·5	0	0
L-Lysine	0	0	5	0	0	0	0
L-Glutamic acid	0	n.t.	n.t.	0	0	0	0
D-Alanine	0	47	78	100	100	0	0
D-Methionine	0	0	0	15	5	0	0
D-Tryptophan	0	0	0	2	1	0	0
D-Cysteine, D-Phenylalanine, D-Threonine, D-Valine, D-Leucine	n.t.	n.t.	n.t.	n.t.	0	0	0

* Spores were activated before use (70°, 30 min.); germination was measured by recording percentage of phase-dark spores.

† Spores were incubated at 30° in 0·1 M-sodium phosphate (pH 8·0) plus the indicated amino acids.

‡ Not tested.

acid were less effective at 200 atm. than one would expect from their potentiation, with inosine, of germination at 1 atm.

Only in the case of alanine was the D-isomer about as effective as the L-isomer in potentiating pressure germination. Other D-isomers were relatively ineffective, even when the corresponding L-isomers were strong potentiators (e.g. cysteine, phenylalanine).

Role of alanine racemase in pressure germination

The anomalous situation, that D-alanine (which inhibits germination at 1 atm.) potentiated pressure germination, suggested that pressure might cause racemization to the L-form. This was so (Fig. 3). Consequently, under pressure there was a rapid formation of L-alanine (which stimulated germination) from the inhibitory D-enantiomorph. Furthermore, rate studies showed that pressure germination was in fact initially

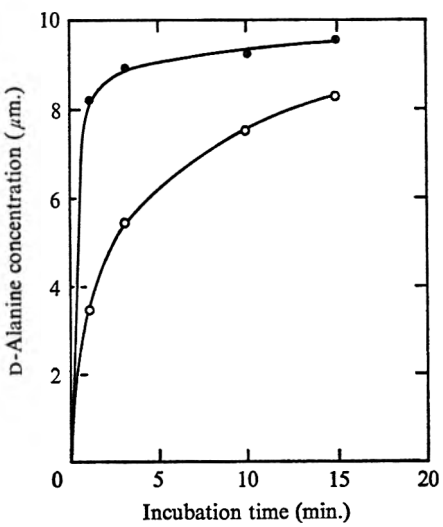

Fig. 3

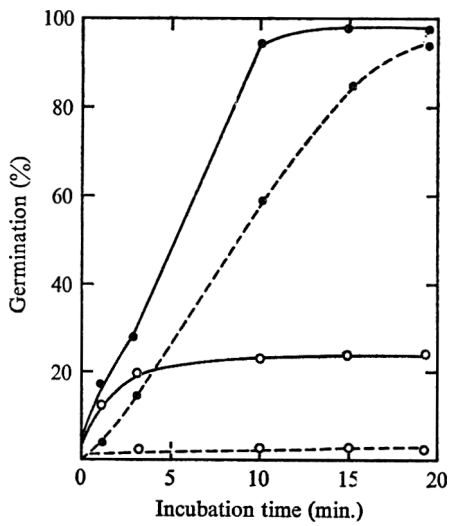

Fig. 4

Fig. 3. Racemization of alanine under pressure. Spores of *Bacillus cereus* T were incubated (25°) with L-alanine (25 μM) in sodium phosphate (0·1 M, pH 8·0) at 1 atm. (○) or 250 atm. (●) pressure. Samples were assayed for racemization by measuring formation of D-alanine as described in Methods.

Fig. 4. Germination of spores in L- and D-alanine. Spores of *B. cereus* T were incubated (25°) in 0·1 M-sodium phosphate (pH 8·0) containing 250 μM of L-alanine (continuous lines) or D-alanine (dashed lines) at 1 atm. (○) or 250 atm. (●) pressure. Samples were assayed for germination by determining the percentage of spores which had become phase-dark.

potentiated more rapidly by L- than by D-alanine, although the difference was small after the first few minutes of pressurization (Fig. 4). The hypothesis that D-alanine potentiated pressure germination *via* formation of L-alanine was further substantiated by using an inhibitor of alanine racemase, *O*-carbamyl-D-serine (OCDS). This substance strongly *antagonized* the potentiating action of D-alanine (presumably by arresting racemization to L-alanine), whilst *increasing* the potentiating action of L-alanine (presumably by arresting racemization to D-alanine) (Table 5). Interestingly, with the exception of phenylalanine, OCDS had little effect on the potentiation of pressure germination by other amino acids, suggesting that alanine was not involved as an intermediate in these instances (Table 5).

Stimulation of pressure germination by ribosides and related compounds

Ribosides, which are strong potentiators of the germination of spores of certain species initiated by amino acids at 1 atm., were much less effective than amino acids

as potentiators of germination under pressure (Table 6). Of the ribosides tested inosine (which is the most effective riboside germinant at 1 atm.) most strongly potentiated the germination of spores of *Bacillus cereus* caused by pressure. Of the other ribosides, adenosine, guanosine and deoxyinosine caused weak potentiation; these three were also weakly active as germinants at 1 atm. (i.e. when used at higher concentrations than in Table 6).

The ribosides, which do not germinate *Bacillus coagulans* spores at 1 atm., did not potentiate the germination by pressure either.

None of the possible metabolites tested, which could be formed more or less directly by breakdown of inosine or alanine, was a potentiator of germination caused by pressure.

Table 5. *Effect of O-carbamyl-D-serine (OCDS) on the germination of spores of Bacillus cereus by pressure*

		Germination (%)* following incubation† in			
		Absence of OCDS		Presence of OCDS‡	
Amino acid§	Concentration	1 atm.	200 atm.	1 atm.	200 atm.
Control (no addition)		0	0	0	0
L-Alanine	50 μM	9	100	85·5	100
	1 μM	0	7	0	100
D-Alanine	50 μM	0	100	0	4
	10 μM	0	14	0	0
L-Phenylalanine	50 μM	0	22·5	0	81·5
β-Alanine	250 μM	0	5	0	5·5
Glycine	20 μM	0	7·5	0	8
L-α-Aminobutyric acid	10 μM	0	5·5	0	6
L-Cysteine	500 μM	0	74	0	74

* Spores were activated before use (70°, 30 min.); germination was measured by recording the percentage of phase-dark spores.

† Spores were incubated at 30° for 30 min. in 0·1 M-sodium phosphate (pH 8·0) plus the indicated amino acids.

‡ *O*-Carbamyl-D-serine was used at a concentration of 100 μM.

§ In addition to those amino acids in the Table, OCDS did not influence the potentiation of pressure germination caused by L-tyrosine, L-serine, L-threonine or L-tryptophan.

Inhibition of pressure germination

In addition to low temperatures (Table 3) and extreme pH values (Fig. 2), pressure germination was inhibited by various metabolic poisons and antimetabolites known to inhibit germination at 1 atm. (Table 7). Further experiments showed that, just as the potentiators (e.g. amino acids, above) became less effective in *accelerating* germination, so the various inhibitors became less effective in *preventing* germination as the pressure employed was increased.

DISCUSSION

The fact that inhibitors of germination initiated by nutrients (octyl alcohol, mercuric chloride etc.; Table 7) also inhibited the germination initiated by pressure, suggested that pressure caused germination *via* some enzyme reaction(s) rather than by simple physical distortion of the spore (e.g. 'mechanical germination'; Rode & Foster, 1960).

Table 6. *Potentiation of pressure germination of spores by ribosides, products of amino acid metabolism, and related compounds*

		Germination (%)* following incubation†				
		Bacillus cereus		*Bacillus coagulans*		
Addition	Concentration	1 atm.	200 atm.	1 atm.	200 atm.	400 atm.
Control (no addition)		0	0	0	0	27
L-Alanine	1 mM	25	100	40	92·5	94
	100 μM	1	100	11·5	81	92·5
Inosine	1 mM	100	100	0	3	27
	100 μM	81	100	0	0	27
	30 μM	1	99	n.t.	n.t.	n.t. ‡
	10 μM	0	16·5	0	0	26
L-Alanine plus inosine	100 μM + 10 μM	87	100	13	82	95
Deoxyinosine	30 μM§	0	4	0	n.t.	24
Adenosine	30 μM§	0	20	0	n.t.	26
Deoxyadenosine	30 μM§	0	0	0	n.t.	26·5
Guanosine	30 μM§	0	9	0	n.t.	26
Deoxyguanosine	30 μM§	0	0	0	n.t.	25·5
Xanthosine	30 μM§	0	0	0	n.t.	26
Riboside breakdown products ¶	30 μM§	0	0	0	n.t.	24–29
Amino acid breakdown products ¶	30 μM§	0	0	0	0	n.t.

* Spores were activated before use (70°, 30 min.); germination was measured by recording the percentage of phase-dark spores.

† Spores were incubated for 30 min. at 30° (*Bacillus cereus*) or 40° (*B. coagulans*) in 0·1 M-sodium phosphate (pH 8·0) plus the indicated additions.

‡ Not tested.

§ Concentrations were 1 mM for *B. coagulans* spores.

¶ The following riboside and amino acid breakdown products and related metabolites were inactive: hypoxanthine, D-ribose, D-ribose 5-phosphate, 3-phosphoglycerate, 2-phosphoglycerate, pyruvate, ammonium (sulphate), nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate (oxidized and reduced forms), formate, acetate, acetyl phosphate, acetyl coenzyme A, coenzyme A, citrate, succinate, phosphopyruvate, adenosine diphosphate and adenosine triphosphate.

Unlike pressure on gaseous systems, hydrostatic pressure of the magnitude used here causes little compression of the liquid (water is compressed less than 4 % at 1000 atm.) and therefore results in negligible changes in concentrations of solutes. Furthermore, the effectiveness of relatively low pressures (i.e. below 1000 atm.) in causing germination of certain spores suggested that pressure was not acting by causing denaturation of proteins or other macromolecules: such changes characteristically become pronounced at pressures well in excess of 1000 atm., and particularly at elevated temperatures. In fact a high proportion of spores survived pressures as high as 8000 atm. at room temperature (Sale *et al.* 1970). Laidler (1951) showed that at high temperatures the dominant effect of pressure on biological materials was on equilibria between natural and denatured forms of enzymes, whereas at lower temperatures the principal effect was on the rates of enzyme-substrate reactions. Pressures of the order of hundreds of atmospheres, at relatively low temperatures, usually affect the rates of reactions in which reactants and products have different molecular volumes.

Such an effect on a germination reaction which normally has a negligible rate at

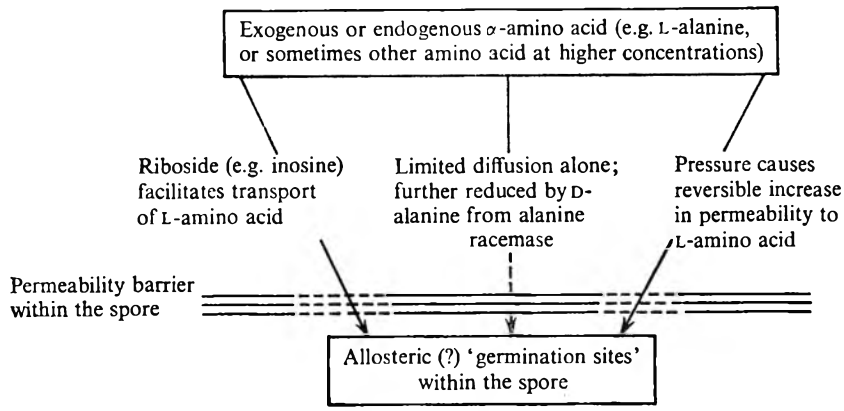

Fig. 5. Diagrammatic representation of the manner in which nutrient (e.g. riboside) or physical (e.g. pressure) potentiators of germination may increase the germinative action of L-alanine or other amino acids. The permeability barrier is regarded as a barrier *within* the spore, separating the 'germination sites' not only from exogenous but also from endogenous germinative amino acids.

Table 7. *Inhibition of pressure germination of spores*

		Germination (%)* following incubation†	
Addition	Concentration	*Bacillus cereus* at 400 atm.	*Bacillus coagulans* at 600 atm.
Control (no addition)		100	87
Ethyl pyruvate	1 mM	100	79
	10 mM	100	0
W 1435‡	1 mM	100	4
	10 mM	100	0
Octyl alcohol	1 mM	53	24
	10 mM	0	0
Cupric sulphate	1 mM	100	89
	10 mM	91	85·5
Mercuric chloride	1 mM	0	0
	10 mM	0	0
Iodoacetamide	1 mM	95	88
	10 mM	84·5	89·5

* Spores were activated before use (70°, 30 min.); germination was measured by recording the percentage of phase-dark spores.

† Spores were incubated for 30 min. at 30° (*Bacillus cereus*) or 40° (*B. coagulans*) in 0·1 M-sodium phosphate (pH 8·0) plus the inhibitors listed.

‡ Bis-1:3-β-ethylhexyl-5-methyl-5-aminohexahydropyridine; an antagonist of pyruvate metabolism.

1 atm. could account for the germinative action of pressure, and also for the observed pH and temperature optima and the effects of metabolic inhibitors. Similar situations are known in biological systems other than spores. Hydrolysis of L-tyrosine ethyl ester by chymotrypsin (Werbin & McLaren, 1951*a*) and of L-arginyl methyl ester by trypsin (Werbin & McLaren, 1951*b*) was accelerated by pressure at moderate temperatures (25° to 55°): the rate of lysis of *Micrococcus lysodeikticus* by lysozyme was increased by moderate pressure (Douglas & Johnson, 1951): in a more complex situation, high

pressure (6500 atm. at 35°) caused an increase in ATP levels in human amnion cells, presumably by stimulating some ATP-generating reaction(s) (Landau & Peabody, 1963).

Nevertheless, the reduction in the degree of germination which occurred below about 40° as the pressure was increased *above* the optimum of 4000 atm. (Sale *et al.* 1970) is less easily explained, unless one postulates that at these higher pressures the conformation of a critical germination enzyme was so changed as to inhibit reversibly the germination reaction.

An alternative explanation of pressure germination was suggested by the observation that pressure accelerated the racemization of alanine catalysed by spores (Fig. 3). Racemization of alanine does not involve a net change in molecular volume of reactants and products, therefore the reaction rate should not be affected by pressure solely by mass action. The observed acceleration by pressure must have some other cause: for instance, a conformational change in the racemase molecule, leading to more rapid action, or (since whole spores were used as the racemase in these experiments) some change in permeability of spores to the substrate alanine could have allowed more rapid racemization. Pressure has been shown to reduce the stereospecificity of an enzyme reaction, for example the hydrolysis of methyl esters of benzoyl L- and D-alanine by α-chymotrypsin (Gonikberg, Prokhorova & El'yanov, 1968). Major conformational changes are not likely below 1000 atm., thus it is more likely that the lower pressures acted by increasing the permeability of spores. The observed synergism of pressure with low levels of various amino acids can be explained on this basis, i.e. if pressure increased the permeability of spores to these germinative compounds.

Interestingly, pressure influenced the germination initiated by amino acids much more than by ribosides. If a permeability change is the correct explanation of pressure germination, it follows that germination at 1 atm. is normally severely restricted by the low permeability of spores to the germinative α-amino acid, which seems to be an essential endogenous germinant even for those spores which may be caused to germinate by addition of ribosides alone (Black & Gerhardt, 1961; Warren & Gould, 1968). Synergism of ribosides, like inosine, with amino acids as germinants for spores of many aerobic species (Hills, 1949; Foerster & Foster, 1966) would suggest that the riboside may function, like pressure, to increase spore permeability to the amino acid as summarized in Fig. 5. At present, the 'germination site' within the spore remains unrecognized, but may be analogous to the allosteric site postulated by Woese, Vary & Halvorson (1968).

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Structure and Composition of Resistant Layers in Bacterial Spore Coats

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SUMMARY

Treatment with mercaptoethanol caused spores of various bacteria to become sensitive to lysis by lysozyme, a spore enzyme and hydrogen peroxide; further treatment with alkali caused greater sensitization to these lytic agents. Alkali removed a protein from the outer coats of mercaptoethanol-treated spores. The protein from *Bacillus cereus*, *B. coagulans* and *Clostridium sporogenes* contained high levels of acidic and basic amino acids; that from *B. coagulans* contained a high proportion (over 7 % of its residues) of tyrosine. Electron microscopy of thin section and freeze-etch samples showed that the alkali-soluble protein contributed to a striking banding pattern containing 57 Å spaced fibres on the outer surface of *B. coagulans* spores. This layer may function as a protection against the enzymes of potential predators.

INTRODUCTION

Spore coats of various bacteria consist mainly of protein (Salton & Marshall, 1959; Warth, Ohye & Murrell, 1963*a*) which contains a disulphide-rich component (Vinter, 1961) with a structure resembling that of keratins (Kadota, Iijima & Uchida, 1965). Also there is a tyrosine-rich component, which can be extracted from isolated coats of *Bacillus megaterium* spores by alkali, and a resistant residue protein associated with a muramyl phosphate polymer (Kondo & Foster, 1967). Although, as yet, the roles of the various components have not been elucidated, they are presumably important in maintaining the survival of spores in adverse environments. We noticed earlier (Gould & Hitchins, 1963) that chemical reduction or oxidation of disulphide bonds, which were probably mostly in the disulphide-rich coat component, could sensitize spores to the enzyme lysozyme. More recently we noticed that exposure of spores to alkali, following the reduction of coat disulphide bonds, caused further changes in sensitivity to lysozyme and to an autolytic enzyme from spores of *B. cereus* (Gould & King, 1969). The observation that the alkali treatment removed one of the coat components prompted the present investigation, which deals with the chemical and cytological nature of the alkali-soluble component and its role in resistance of spores.

METHODS

Organisms and production of spores. Bacteria used included *Bacillus cereus* strains T, PX and NCTC 945, *B. subtilis* strains A and NCTC 3610 (Marburg), *B. coagulans* NCTC 3991, *B. brevis* NCTC 7577, *B. megaterium* ATCC 9885, *Clostridium sporogenes*. These

were from the National Collection of Type Cultures or the American Type Culture Collection, or were described previously (Jones & Gould, 1968).

Spores of the aerobes were grown at 30° on the surface of agar containing Oxoid potato extract (0·4 %, w/v), yeast extract (0·4 %, w/v) and glucose (0·25 %, w/v). Sporulation and lysis of sporangia occurred in 2 to 6 days, after which spores were collected and washed four times by centrifuging suspensions in cold distilled water. Spores of *Clostridium sporogenes* were grown at 37° in Reinforced Clostridial Medium (Gibbs & Hirsch, 1956), then washed as above. Spores were stored at a concentration of about 20 mg. equiv. dry wt/ml., at −20°.

Sensitization of spores to enzymes. The sensitization procedure was based on that described by Gould & King (1969). Conditions were chosen which caused sensitization of spores to lysozyme, and also to hydrogen peroxide, but were insufficiently severe to cause inactivation of spores. Spores (10 mg. equiv. dry wt/ml.) were incubated in 7 M urea (adjusted to pH 2·8 with HCl) containing β-mercaptoethanol (10 % v/v) for 1 hr at 37°, then cooled, centrifuged and washed four times with cold water. Alkali treatment (see Results) consisted of resuspending the pellets in 0·1 N-NaOH and incubating for 15 min. at 4° before washing four times in cold water.

Electron microscopy. Samples for sectioning were fixed by resuspending pellets in freshly filtered $KMnO_4$ solution (2 %, w/v) for 2 hr at 20° (Mollenhauer, 1959). The fixed organisms were then washed ten times by suspending in cold distilled water and separating by centrifugation, dehydrated in a graded ethanol series, embedded in Epon 812 and sectioned on an LKB Ultratome 3. Sections were examined in a JEM 7A electron microscope. Pellets for freeze-etching were resuspended in 20 % (v/v) glycerine immediately before freezing in liquid Freon at −150°. Addition of glycerol immediately prior to freezing was found to minimize formation of ice crystals around the organisms, and thus best preserved their shape. Replicas were prepared in a Polaron/MRC freeze-etching machine, and examined as above.

Isolation of alkali-soluble material. The alkaline extracts of mercaptoethanol-treated spores were dialysed against distilled water to remove NaOH, then dialysed against the appropriate buffer for Sephadex experiments (below) or precipitated by titration to pH 3 with HCl. Samples for amino acid analysis were freeze-dried from aqueous solutions.

Gel filtration. Alkali-soluble protein solutions (1 ml.) from *Bacillus coagulans* spores were eluted from Sephadex G 200 columns (about 1 × 25 cm.) in 0·05 M-sodium phosphate (pH 8·0) + 0·15 M-NaCl and in 0·05 M tris (hydroxymethylaminomethane)-HCl (pH 9·0) + 0·15 M-NaCl. Eluate was monitored by ultraviolet absorption at 254 nm.

Amino acid analysis. Freeze-dried samples of alkali-soluble protein were hydrolysed by refluxing under N_2 in 6 N-HCl for 21 hr. Amino acids were determined by Moore and Stein analysis using a Technicon method based on that described by Thomas (1966). Diaminopimelic acid was determined separately using an autoanalyser method (F. J. Bailey, unpublished data) based on the procedure of El Shazly & Hungate (1966).

Heat and radiation. Aqueous suspensions of variously treated *Bacillus cereus* T spores (about 10^8 in 1 ml.) were sealed into thin walled glass ampoules which were completely immersed in a water bath at 90°. Duplicate ampoules were removed at intervals, cooled, and the survivors counted using pour plates of nutrient agar. Incubation time was 2 days at 30°. Samples of variously treated spores of *B. coagulans* (about

10^8 in 1 ml.) were sealed into glass freeze-drying ampoules and irradiated at room temperature with Co 60 γ-radiation at a dose rate of about 0·7 Mrad./hr. Survivors were enumerated as above except that the incubation temperature was 37°.

RESULTS

Sensitivity of mercaptoethanol and alkali-treated spores to enzymes and hydrogen peroxide

When spores of *Bacillus coagulans* were treated with mercaptoethanol, they became lysozyme-sensitive as expected, but when, subsequent to mercaptoethanol treatment, they were treated with alkali, their lysozyme-sensitivity increased markedly (Fig. 1).

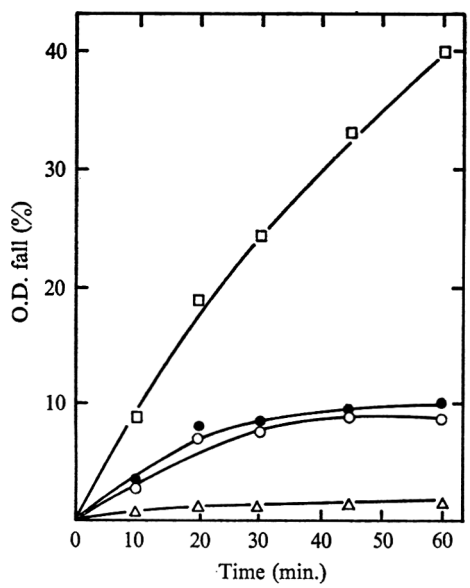

Fig. 1

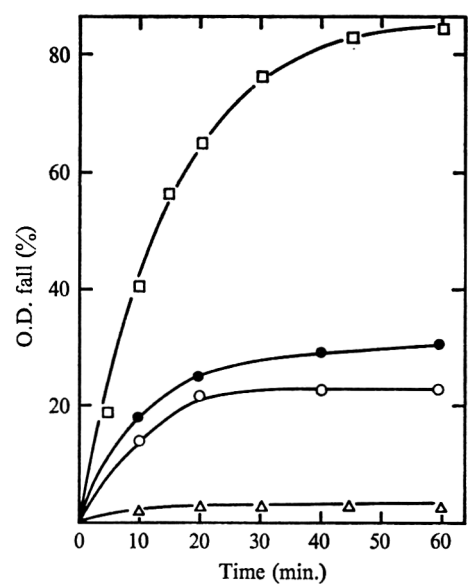

Fig. 2

Fig. 1. Lysis of spores by lysozyme. The figure shows the decrease in optical density (at 580 nm.) that occurred when variously treated spores of *Bacillus coagulans* were incubated at 37° in lysozyme (20 μg./ml. in 100 mM sodium phosphate, pH 8.0). The spores had been previously incubated with mercaptoethanol, as described in Methods, then extracted for 15 min. at 4° with: 0·1 M-sodium phosphate (pH 8·0), ○; 0·01M-NaOH (pH 12), ●; 0·1 M-NaOH, □; 0·1 M-NaOH-treated, lysozyme-free, control, △.

Fig. 2. Lysis of spores by hydrogen peroxide. Details as for Fig. 1 except that spores were incubated in 1·7 M-hydrogen peroxide in place of lysozyme.

Qualitatively similar results were obtained with spores of *B. cereus* T and *B. subtilis* Marburg. Furthermore, the alkali treatment increased the sensitivity of *B. cereus* spores to the *B. cereus* spore autolytic enzyme and also made sensitive to this enzyme the otherwise resistant mercaptoethanol-treated spores of *B. coagulans*.

In addition to causing an increase in sensitivity to enzymes, the alkali treatment also made mercaptoethanol-treated spores more sensitive to lysis by hydrogen peroxide (Fig. 2).

Resistance of treated spores to heat and γ-radiation

Spores were treated with the mercaptoethanol and alkali or left untreated as controls, then washed and subjected to heat or γ-radiation. The survivor curves obtained (Fig. 3 and 4) showed that, although reducing resistance of spores to the lytic enzymes and hydrogen peroxide, the mercaptoethanol and alkali treatments had negligible effects on resistance of spores to heat and ionizing radiation.

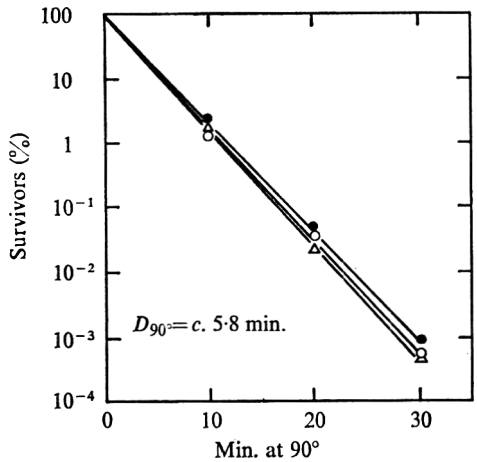

Fig. 3

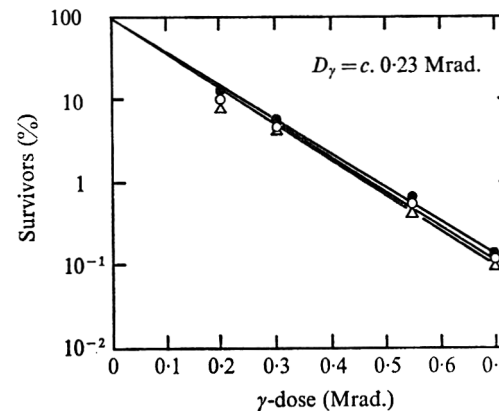

Fig. 4

Fig. 3. Heat resistance of mercaptoethanol- and alkali-treated spores. Variously treated spores of *Bacillus cereus* T were heated at 90°, and survivors enumerated as described in Methods. The figure shows the survivor curves obtained when spores were: untreated, △; treated with mercaptoethanol, ●; treated with mercaptoethanol and alkali, ○.

Fig. 4. γ-Radiation resistance of mercaptoethanol- and alkali-treated spores. Symbols as in Fig. 3.

Alkali-soluble spore protein

The alkali treatment removed some component(s) from mercaptoethanol-treated spores. Acidification of the alkaline extracts from spores of various species caused precipitation as shown in Fig. 5. The pH versus precipitation curves were similar for the eight Bacillus species tested, but precipitation from alkali extracts of mercaptoethanol-treated spores of *Clostridium sporogenes* occurred at lower pH values (Fig. 5 and Table 1). The precipitable material was dialysed to remove low molecular weight solutes, dried at 105° and weighed. It represented between about 1 and 5 % of the dry matter of the different spores.

The dialysed material eluted in about the void volume from a Sephadex G200 column, suggesting it was high molecular weight or aggregated material. The elution peak was symmetrical at pH 9·0 but tailed at pH 8·0, suggesting heterogeneity.

Composition of alkali-soluble protein

Moore and Stein analysis of hydrolysed samples suggested that the alkali-soluble material was predominantly protein with an unusual amino acid constitution (Table 2). In particular, the levels of acidic amino acids (aspartic and glutamic) and basic amino acids (lysine and arginine) were high. The low pH for precipitation of alkali-soluble

protein from *Clostridium sporogenes* (Table 1) may be reflected in its high content of acidic amino acids (Table 2). The level of tyrosine, accounting for over 7 % of the residues in the material from *Bacillus coagulans* spores, was high for proteins, approaching the high level of 11 % previously found in an alkali-soluble protein component isolated from spore coats of *B. megaterium* (Kondo & Foster, 1967). The samples contained no diaminopimelic acid (i.e. less than 0·2 %, w/w) and were therefore probably free of peptidoglycan components.

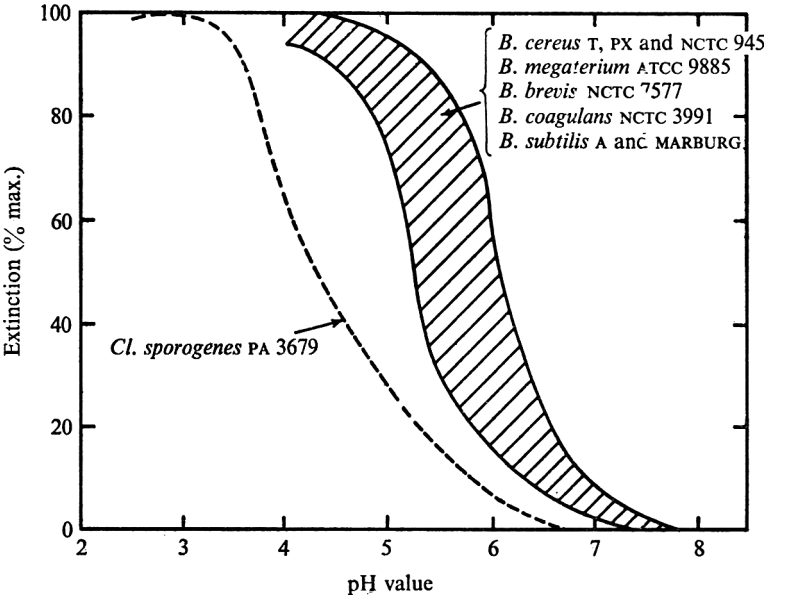

Fig. 5. Precipitation of alkali-soluble spore proteins. Preparations of alkali soluble proteins, in dilute NaOH at about pH 8, were titrated with HCl. The hatched area encompasses the precipitation curves of alkali-soluble protein from *Bacillus cereus* (three strains) and *B. subtilis* (two strains; see Methods), *B. megaterium*, *B. brevis* and *B. coagulans*. The dashed line is the precipitation curve for alkali-soluble protein from *Clostridium sporogenes*.

Table 1. *Yield and properties of alkali-soluble protein from different spores*

Organism	Yield of alkali-soluble protein (μg./mg. dry wt of spores)	pH value for precipitation	
		50% precipitation	Maximal precipitation
Bacillus cereus T	13	5·4	4·5
B. cereus PX	21·5	5·9	4·5
B. cereus NCTC 945	15	6·1	4·6
B. subtilis A	17·5	6·1	4·4
B. subtilis MARBURG	54	6·1	4·2
B. brevis NCTC 7577	12	5·8	4·6
B. coagulans NCTC 3991	13	5·6	4·4
B. megaterium ATCC 9885	12·5	5·5	4·2
Clostridium sporogenes	47·5	4·3	2·8

Table 2. *Amino acid composition of alkali-soluble protein from spores of different species*

	Residues amino acid per 100 residues		
Amino acid	*Bacillus cereus* T	*B. coagulans*	*Clostridium sporogenes*
Half cystine	0·3	0	0
Aspartic acid	11·6	8·2	10·0
Threonine	7·7	6·3	7·1
Serine	3·7	5·6	5·3
Glutamic acid	12·7	12·6	11·1
Glycine	9·7	8·1	10·2
Alanine	9·3	6·1	8·7
Valine	7·0	7·1	8·1
Methionine	1·5	1·5	1·8
Isoleucine	5·5	5·3	4·5
Leucine	8·0	7·2	9·3
Tyrosine	3·5	7·1	4·0
Phenyl alanine	4·2	4·3	5·0
Lysine	6·3	8·8	7·6
Histidine	2·6	5·3	3·6
Arginine	5·4	4·2	3·7
Ornithine	0·9	2·1	0
Overall yield based on weight of sample	67·5%	60·8%	78%

Electron microscopy of treated spores

It seemed likely that the observed increase in sensitivity of mercaptoethanol-treated spores to enzymes and to hydrogen peroxide, following treatment with alkali, resulted from solubilization of the alkali-soluble protein from the spore. In order to detect the location of the alkali-soluble protein in spores, the variously treated samples were examined by electron microscopy.

Examination of freeze etched preparations suggested that the alkali treatment removed a surface layer from spores of *Bacillus coagulans*. The surface layer in untreated spores had a well defined 'bandage' pattern (Pl. 1, fig. 1) which contained a regular substructure consisting of parallel fibrils at about 57 Å spacing (Pl. 1, fig. 2). Treatment of spores with mercaptoethanol in urea at pH 2·8, as described in Methods, hardly influenced this pattern (Pl. 1, fig. 3) but subsequent treatment with alkali caused the pattern to disappear (Pl. 1, fig. 4).

Loss of material from the surface of alkali-treated spores was less apparent in thin-sectioned than in freeze-etched preparations. Nevertheless, some loss of electron density in the outer coat region could be seen (compare Pl. 2, fig. 5, 6).

The alkali-soluble protein was dialysed, negatively stained with phosphotungstic acid and examined by electron microscopy. Plate 2, fig. 7, shows the appearance of the protein sedimenting onto the grid from a solution at pH 8·0, when small particles and also fibres could be seen. When the pH value was lowered to 6·0, the fibres coalesced and precipitated in long strands (Pl. 2, fig. 8).

DISCUSSION

The resistance of bacterial spores to heat, radiation and chemical agents has been intensively studied in the past. In contrast, the resistance of spores to enzymes has

received little attention, even though resistance to enzymes must be an important attribute contributing to the survival of spores in natural environments.

The results in this paper suggest that at least two reasonably well-defined spore components are important in determining their resistance to enzymes. Both are thought to be located in the outer coat regions of spores. The first component is the disulphide-rich protein, originally described by Vinter (1961), and shown by Kadota *et al.* (1965) to have a physical structure resembling keratins in spores of *Bacillus subtilis*. The evidence suggesting involvement of this protein in resistance of spores to enzymes is based on the observations that reagents which break disulphide bonds, by oxidation or reduction, alter the coat sufficiently to allow lysozyme or lysozyme-like enzymes to pass through the altered coat and reach their peptidoglycan substrates, which probably constitute the underlying cortex layer (Warth, Ohye & Murrell, 1963*b*). The second component is the alkali-soluble protein and the evidence for its implication in resistance of spores to enzymes is presented in this paper. Alkali treatment of spores, which had been treated with mercaptoethanol, increased their sensitivity to enzymes and to hydrogen peroxide at the same time as the protein was extracted from the spores. The weight of protein certainly accounted for the bulk of the material extracted by alkali. The alkali may have caused further (undetected) changes affecting enzyme sensitivity, but the important spore properties of resistance to heat and to γ-radiation were unaffected by the various treatments used. The relationship of lysozyme resistance to hydrogen peroxide resistance is not clear. However, hydrogen peroxide is known to primarily attack spore coats by metal-catalysed reactions involving free radicals (King & Gould, 1969).

The striking patterns on the outer surface of *Bacillus coagulans* spores resemble most closely the patterns described on some Bacillus spores by Leadbetter & Holt (1969), on spores of *B. subtilis* and *B. licheniformis* by Holt & Leadbetter (1969) and on *Aspergillus conidia* by Hess & Stocks (1969). The same patterns were not visible on freeze-etch preparations of sporulating *B. cereus* (Remsen, 1966) or *Clostridium perfringens* (Hoeniger, Stuart & Holt, 1968), but Holt & Leadbetter (1969) described a rope-like substructure overlaying a pitted layer in *B. cereus* spore coats, and showed that in other Bacillus spores various similar layers could be recognized. The patterns on spores of *B. coagulans* resemble closely those on vacuoles of *Halobacterium halobium* cells (Stoeckenius & Kunau, 1968). The regularity of the substructure suggests crystallinity. This is also suggested by examination of the areas where 'bandage' patterns meet (see Pl. 1, fig. 2). The 'bandages' do not appear to overlap each other at these points, but rather to meet at an angle, as in a crystal dislocation line.

The patterned alkali-soluble layer is only readily removed from spores following mechanical rupture (Kondo & Foster, 1967) or treatment with a reagent like mercapto-ethanol, which will rupture disulphide bonds. Presumably the disulphide-rich layer holds the alkali-soluble layer onto the spore in some manner; for instance, by physical admixture, or through disulphide bridge from the one component to the other. The latter possibility seems less likely, because the cyste(i)ne contents of the alkali-soluble components were low. Preparations of the alkali-soluble material *in vitro* tended to form fibrils in some respects resembling the fibrils on spores. Presumably the amino acid composition of the material resulted in a tertiary structure which favoured fibril formation. It would be interesting to find whether fibrils were formed and spontaneously coalesced onto the coats of spores during the process of sporulation.

The amino acid composition of alkali-soluble proteins from different spores differed in detail and yet certain similarities were evident. For instance, these samples, and the alkali-soluble protein isolated from *Bacillus megaterium* spore coats by Kondo & Foster (1967), characteristically contained very high levels of acidic and basic amino acids. Unlike that from *B. megaterium* and *B. coagulans*, the alkali-soluble materials from *B. cereus* and *Clostridium sporogenes* were not exceptionally rich in tyrosine. Similarities in chemical composition of the fractions from different spores would suggest a relationship of structure to function of the alkali-soluble proteins, for example, resistance to enzymic degradation or some physical property like hydrophobicity which may be an important property of the outer layers of spores. In this respect, however, it is worth bearing in mind that although the alkali-soluble protein appears to constitute the outer layer of *B. coagulans* spore coats, Kawasaki, Nishihara and Kondo (1969) interpreted their electron microscope study of sectioned spore coats of *B. megaterium* differently. They suggested that the alkali-soluble component was mixed with the keratin-like component in the inner coats, and that the outer coat consisted of a resistant residue containing a phosphomuramic acid polymer. Aronson & Fitz-James (1968) showed that extraction of coats of spores of *B. cereus* T with alkaline thioglycollate resulted in rapid loss of detail from the dense layers and partial removal of the inner coat layers as seen by electron microscopy of thin sections. The extraction procedure caused loss of a dotted fine structure visible on the outer coat surface in negative-stained preparations. In the present study, the freeze-etch technique greatly aided location of the alkali-soluble layer, and in fact revealed details not apparent in thin sections.

There is ample evidence that the keratin-like and alkali-soluble components of spore coats protect spores from lysis by lysozyme-like enzymes: one can speculate that the specialized structures containing these components have evolved as protective layers which guard the spore protoplast against the hydrolytic enzymes of predators.

We would like to thank Mr F. J. Bailey for the amino acid analyses and Mr F. J. Judge for technical assistance.

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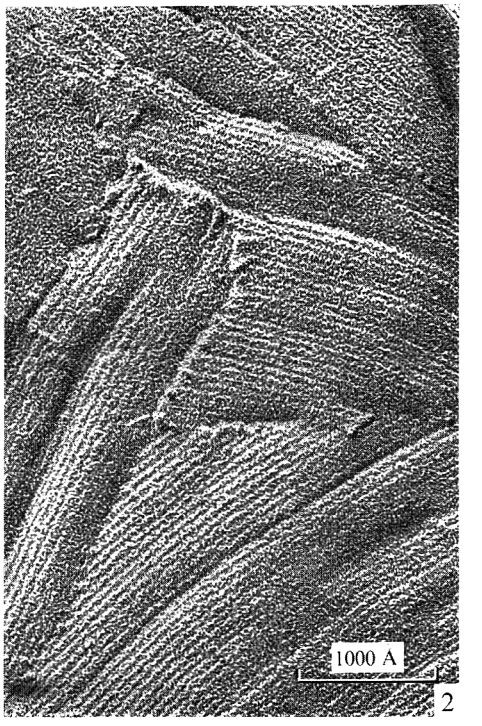

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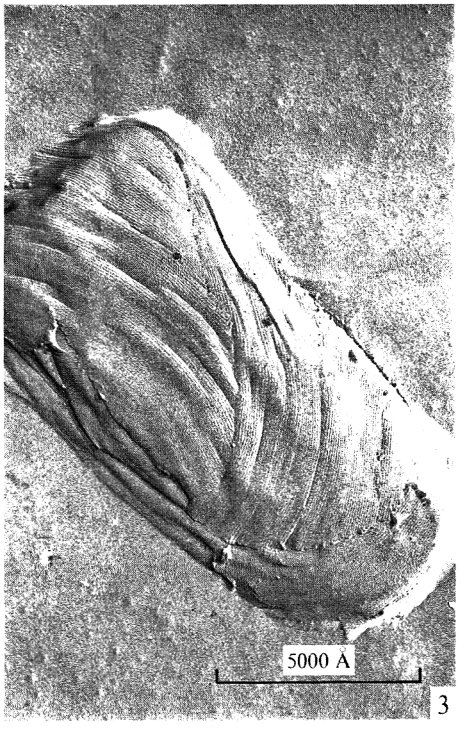

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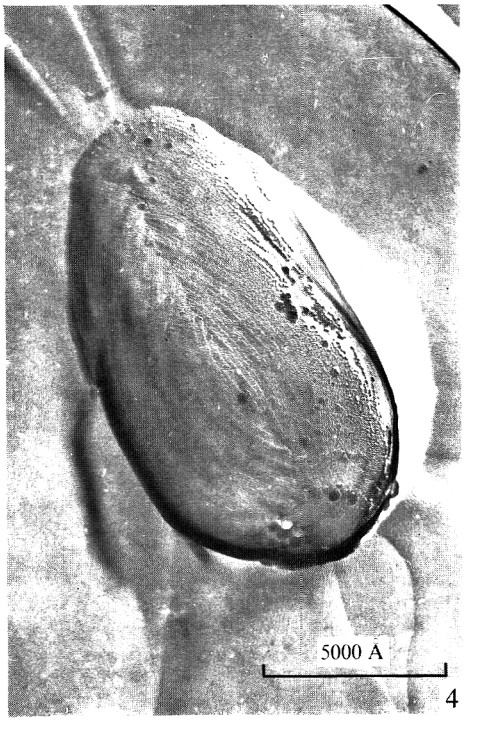

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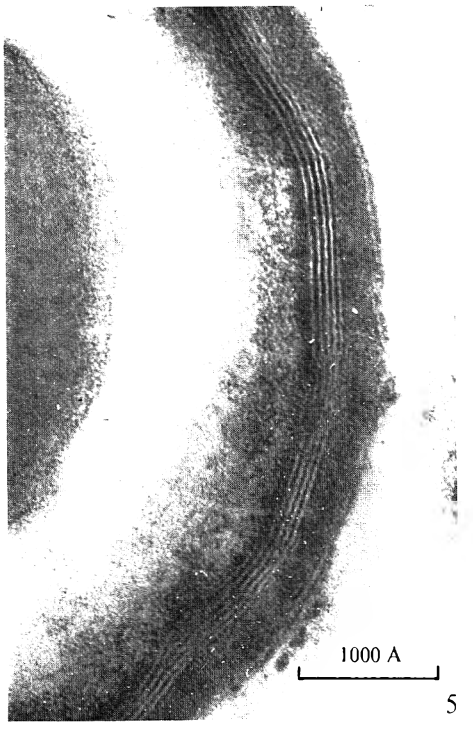

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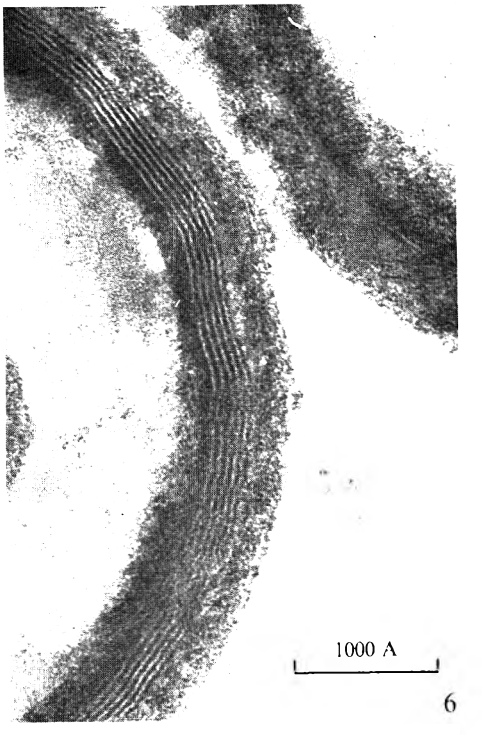

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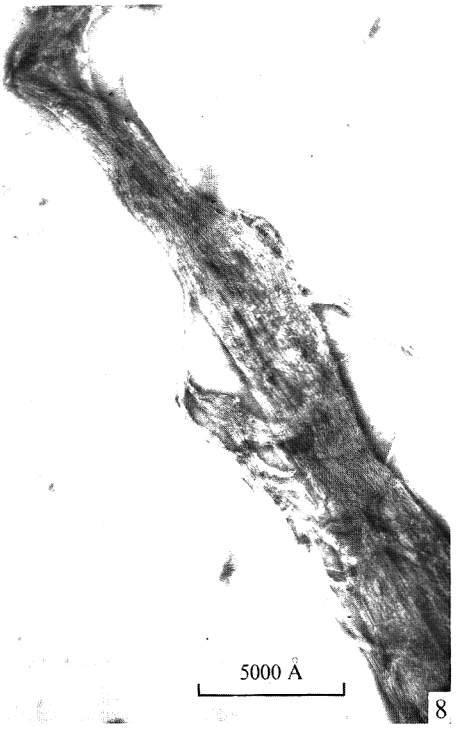

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EXPLANATION OF PLATES

PLATE 1

Electron micrographs of freeze-etched preparations

Fig. 1. Outer surface of a spore of *Bacillus coagulans* showing typical bandage pattern.

Fig. 2. Enlargement of a portion of the surface of *B. coagulans* showing parallel fibrillar structure within the 'bandage' pattern.

Fig. 3. Spore of *B. coagulans* treated with mercaptoethanol, showing little loss of surface detail.

Fig. 4. Spore of *B. coagulans* treated with mercaptoethanol and then with alkali, which strips off the outer 'bandage' coat layer.

PLATE 2

Electron micrographs of thin sections

Fig. 5. Multilayered spore coat of *B. coagulans* in spores treated with mercaptoethanol. (The picture is identical with that seen in untreated controls.)

Fig. 6. Multilayered spore coats in *B. coagulans* spores treated with mercaptoethanol and alkali. The main result of alkali treatment is loss of electron density in the outer coat region (compare Pl. 2, fig. 5).

Electron micrographs of phosphotungstic acid negatively stained preparations

Fig. 7. Alkali-soluble protein at pH 8·0 sedimented onto grid as a network of fibrils and particles.

Fig. 8. Alkali-soluble protein precipitated at pH 6·0 as irregular bundles of fibrils.

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Ultrastructure of the Nuclei and Intranuclear Microtubules of *Paramecium aurelia*

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SUMMARY

Electron microscope observations on the micronucleus of *Paramecium aurelia* showed that the mitotic spindle was organized within the nuclear envelope. At the intranuclear metaphase plate stage some microtubules were attached to chromosomes by localized centromeres and other interzonal microtubules ran between the chromosomes. The interzonal microtubules elongated tenfold during telophase and were tightly packed in a narrow cylindrical membrane-limited stem of the spindle. Some microtubules persisted in the micronucleus during interphase and formed a layer near the nuclear envelope. There were scattered groups of microtubules in the isthmus of the macronucleus during its amitosis. The nuclear envelope remained intact. Large and small bodies persisted throughout the amitosis and the microtubules pass between them. Before the first postconjugal fission there was a stage during which the two macronuclear anlagen were separated by a sheet of cytoplasm only 0·3 μm. wide.

INTRODUCTION

In Paramecium and other ciliates there are two kinds of nuclei, macronuclei and micronuclei, which differ greatly in their size, amount of DNA, structure, function and mode of division at fission. In *Paramecium aurelia* between successive fissions there are normally two micronuclei, each about 3 μm. in diameter, which lie close to a single large ovoid macronucleus, which is about 35 μm. long and 12 μm. across a minor diameter. It has been estimated that the macronucleus contains about 430 times as much DNA as the micronucleus (Woodard, Gelber & Swift, 1961; Woodard, Woodard, Gelber & Swift, 1966). The macronucleus rather than the micronucleus must be concerned with growth and fission since Sonneborn (1938) showed that animals could survive for over a hundred fissions without a micronucleus but could not survive for more than one or two fission cycles without a macronucleus. Sonneborn (1946) also showed that the genes which determine the phenotype of the organism are located in the macronucleus. The micronuclei allow genetic recombination to take place at conjugation and at the first autogamy following conjugation; after autogamy and conjugation new macronuclei are derived from micronuclei.

Many light-microscopists have studied the behaviour of the nuclei of *Paramecium aurelia* during conjugation. Descriptions have been given by Hertwig (1889), Maupas (1889), Sonneborn (1947, 1954), Kimball & Gaither (1955), Jones (1956), Kościuszko (1965) and Jurand & Selman (1969). Prometaphase and metaphase of the first meiotic division of the micronucleus presents the most favourable opportunity for observing

and counting the chromosomes. These are small and numerous, however, and estimates of their diploid numbers in different stocks vary between 70 and 126 (Dippell, 1954; Jones, 1956; Kościuszko, 1965). *Paramecium aurelia* has become popular for certain branches of genetical research (Beale, 1954; Preer, 1967), but cytological observations have not been able to assist these studies to any notable extent. It is possible that electron microscopy may ultimately be able to contribute as much useful information to the study of nuclear events in ciliates as it has already contributed to the study of their cytoplasmic organelles. The present paper includes electron microscope observations on the micronucleus and macronucleus of *P. aurelia* and describes the division of these two kinds of nuclei at fission. The organization of microtubules within the nuclei at division has not been described previously.

METHODS

Paramecium aurelia, mainly of stock 60, was used and mass stocks were cultured by the method of Sonneborn (1950). A modified form of the fixative of Palade (1952) was prepared according to the formula quoted by Jurand & Selman (1969). The addition of 0·03 % calcium chloride to the osmic acid fixative was probably responsible for the preservation of the microtubules. The protozoa were first filtered through a cotton plug and then concentrated into an aqueous pellet by low speed centrifugation. The supernatant liquid was decanted and the fixative added at room temperature to the centrifuge tube which was shaken to disperse the protozoa. Fixation was for 30 min. Dehydration was with graded ethanol + water mixtures and three changes of absolute ethanol (10 min. each). The protozoa were centrifuged down and the supernatant fluid decanted at each stage before fresh ethanol was added. Finally, the protozoa were passed through a 50 + 50 mixture of 1,2-epoxypropane + Araldite and embedded in Araldite by using a rotary shaker (Jurand & Ireland, 1965). For light microscopy, sections were cut at 1 μm. and stained with a 0·5 % aqueous solution of toluidine blue made alkaline by adding 1 % borax. For electron microscopy, sections were cut on a Porter-Blum Sorvall ultramicrotome, mounted on collodion-carbon coated grids and stained for 20 min. with 1 % potassium permanganate solution containing 2·5 % uranyl acetate (Jurand & Selman, 1969).

OBSERVATIONS

The micronucleus and its mitotic stages

When sectioned material stained with a basic dye is observed by light microscopy the interphase micronucleus of *Paramecium aurelia* appears as an achromatic sphere containing a central chromatic core. In thin sections observed by electron microscopy this dense core often appears hollow or cup-shaped and immediately surrounded by a thin moderately dense granular zone (Pl. 1, fig. 1). However, the bulk of the micronucleus contains material of low density outside the central core. In transverse section the two membranes of the nuclear envelope appear to join in some places over a distance of about 65 nm. where there are probably nuclear pores. Immediately within the nuclear envelope there is a layer of microtubules (Pl. 1, fig. 1). The microtubules run parallel to the adjacent nuclear envelope and in directions similar to those of lines of longitude on a globe. Thus they appear to diverge from a polar region of the

nucleus and run round inside the nucleus to the opposite side. In earlier studies (Jurand, Beale & Young, 1962) the type of fixative and embedding medium which were used did not allow these microtubules to be preserved. With the present techniques no interphase micronucleus has been observed which did not contain some microtubules.

When specimens of *Paramecium aurelia* are stained with methyl green and pyronin, the micronucleus appears green and does not stain with pyronin (A. Jurand, unpublished), indicating that the micronucleus contains little or no RNA. Therefore the dense central core of the interphase micronucleus probably consists of condensed chromatin. Similarly the micronuclei of *P. bursaria* and its suctorian parasite *Podophyra parameciorum* studied by Jurand & Bomford (1965) were shown to contain little or no RNA. On the other hand Pasternak (1967) demonstrated autoradiographically that a macronucleate *P. aurelia* synthesized some RNA in the micronucleus.

In mitotic prophase the dense material near the centre of the micronucleus becomes irregularly dispersed (Pl. 1, fig. 2). Microtubules are present near the nuclear envelope. In one case microtubules appeared to pass through the masses of prophase chromatin.

At metaphase many dense chromosomes are attached to parallel groups of microtubules at localized centromeres (Pl. 1, fig. 3). Other interzonal microtubules are not attached to chromosomes but run between them. At the metaphase plate stage the microtubules of the spindle converge slightly toward the poles. However, the spherical nuclear envelope is everywhere intact and the metaphase plate of chromosomes and the spindle are organized entirely within the nuclear envelope. No centrioles have been observed.

At anaphase the interzonal microtubules and the nuclear envelope begin to elongate. The nuclear divisions of the two micronuclei within one protozoon are not always completely synchronous. In one serially sectioned paramecium, one micronucleus was in metaphase and its nuclear form was spherical, while the other was in anaphase, elongated in the direction of the spindle and had a length to breadth ratio of 1·6:1.

During telophase the nucleus continues to elongate until the interzonal microtubules have increased to over 10 times their initial length. The daughter sets of chromosomes are then separated by a long cylindrical tube bounded by the nuclear envelope which is still intact and contains densely packed interzonal microtubules (Pl. 1, fig. 4). The micronuclear mitosis takes place in the last sixth of the interfission interval but it is completed before the fission furrow forms (Woodard *et al.* 1961).

The macronucleus and its amitosis

In thin-sectioned material, the mature macronucleus of *Paramecium aurelia* has been shown to contain two types of dense body (Sonneborn, 1953; Jurand *et al.* 1962; Wolfe, 1967). The large type of body is usually between 0·5 and 1·0 μm. in diameter and may contain a central region of low density between 0·2 and 0·5 μm. in diameter. The large dense bodies are pyronin-positive and were termed nucleoli by Nanney & Rudzinska (1960). For each one of these large bodies there may be about a hundred dense small bodies 0·1 to 0·2 μm. in diameter. The small bodies, termed chromatin bodies by Nanney & Rudzinska (1960), were shown to be Feulgen-positive by Wolfe (1967) who used a technique in which the macronuclei were first expanded by chelation of the divalent ions until the small bodies could be optically resolved. In electron micrographs of material stained with uranyl acetate alone, the small bodies appear to be much denser than the larger bodies (Pl. 3, fig. 8). The small bodies also have a

more fibrillar ultrastructure than the large bodies which are more granular (Pl. 3, fig. 9). Between the large and small bodies there is a low density of fibrillar material with fibril diameters of about 5 nm. The macronucleus is bounded by a nuclear envelope which contains many nuclear pores with an internal diameter of 40 nm. The morphology of the large and small bodies does not appear to change during the fission cycle.

Between fissions, the macronucleus does not normally appear to contain microtubules. In sectioned *Paramecium aurelia*, a group of microtubules has been observed within a non-dividing macronucleus in one instance only, but microtubules have been observed in a small proportion of macronuclei isolated by the method of Stevenson (1967). Vivier & André (1961) observed disorganized microtubules in a macronucleus of *P. caudatum*.

In sections of *Paramecium aurelia* fixed when the fission furrow had formed, the macronucleus is shown constricted in the middle to form a shape like an hour glass (Pl. 2, fig. 5). At this stage the nuclear envelope is complete at all points and there is no evidence that it ever breaks down. The large and small bodies are present and appear to be ultrastructurally identical to the dense bodies in macronuclei at other stages of the fission cycle. In the isthmus, groups of microtubules run between the dense bodies and they are oriented in the direction of elongation of the nucleus (Pl. 2, fig. 6, 7). Fewer microtubules were observed in the main part of the macronucleus away from the isthmus. No attachment has been observed between the microtubules and the large or small bodies. When the fission furrow deepens to divide the protozoon, the macronucleus separates into two halves.

A stage of close approach of the two macronuclear anlagen

As a result of conjugation in *Paramecium aurelia* a synkaryon is formed in each conjugant. The two conjugants then normally separate and the synkaryon undergoes two mitoses to give four nuclei, two of which then enlarge and develop into macronuclear anlagen which are normally distributed one to each daughter animal at the first postzygotic fission so that each contains one new macronucleus. Jurand, Beale & Young (1964) studied the ultrastructure of the macronuclear anlagen during their growth and development and described the origin of the large and small bodies. In the illustrated accounts of Hertwig (1889) and Maupas (1889) there is a stage at which the two macronuclear anlagen seem to press against each other. However, when the stages of conjugation and the nuclear events following conjugation were described and illustrated in the more recent account of Grell (1967) this pairing stage was ignored, which is not surprising because the temporary pairing and subsequent separation is of no known genetic or cytological significance.

The present observations confirm that, at a stage about midway between the separation of the exconjugants and the first postzygotic fission, the two macronuclear anlagen approach each other closely. At this stage the macronuclear anlagen are each about 11 μm. in diameter. By light microscopy, either no gap can be seen between the paired nuclei (Pl. 3, fig. 10) or the nuclei appear to be separated by a fine line. By electron microscopy the anlagen were observed to be separated by a layer of cytoplasm about 0·3 μm. wide (Pl. 3, fig. 11) and each nuclear envelope in this region was flattened over an area of about 13 μ^2. The macronuclear anlagen normally separate after this close approach, but Sonneborn (1947) and Nanney (1957) reported that under conditions

of starvation the two anlagen in the exconjugants became irreversibly fused to form one macronucleus.

DISCUSSION

There are several points of similarity between the micronuclear mitosis in the holotrichous ciliate *Paramecium aurelia* and the heterotrichous ciliate Blepharisma studied by Jenkins (1967) and by Inaba & Sotokawa (1968). In both cases the spindle is organized within the nuclear envelope in the absence of centrioles; the metaphase spindle includes microtubules which attach to chromosomes as well as interzonal microtubules which run between the two poles of the spindle and pass between the chromosomes. The remarkable elongation of the spindle which separates the daughter nuclei appears to take place by an active increase in length of the interzonal microtubules. In the present work, measurement of the external diameter of the microtubules gave results which did not differ significantly from a mean of 25 nm. whatever the mitotic stage. This is in contrast to the finding of Hauser (1968) that in the suctorian *Paracineta limbata* the microtubules have a lesser diameter after the spindle elongates. In Paramecium, Blepharisma, Paracineta and the peritrichous ciliates Epistylis and Vorticella observed by Carasso & Favard (1965), the nuclear envelope of the micronucleus is preserved intact during the entire mitotic division. For Blepharisma, Jenkins (1967) and Inaba & Sotokawa (1968) observed that a new nuclear envelope formed round the daughter masses of chromatin within the old nuclear envelope at telophase. It is not known whether Paramecium is similar in this respect.

In this paper it is accepted that the large dense bodies (0·5 to 1 μm. in diameter) within the macronucleus contain RNA and are nucleoli, while the small dense bodies (0·1 to 0·2 μm. in diameter) contain DNA and consist of condensed chromatin. This represents a re-interpretation of the results of Jurand *et al.* (1962). The observations made by these authors after staining with methyl green and pyronin, and after staining by the Feulgen method, and by ultraviolet microscopy following extraction with ribonuclease or deoxyribonuclease, now appear to support the present interpretation.

There is no evidence that the large or small bodies of the macronucleus change their morphology during the fission cycle of *Paramecium aurelia*. On the other hand in hypotrichous ciliates, both nucleolar material and condensed chromatin becomes temporarily dispersed in a reorganization band associated with the duplication of DNA (Gall, 1959; Inaba & Suganuma, 1966; Kluss, 1962). If the duplication of DNA in the macronucleus of *P. aurelia* took place asynchronously in different parts of the nucleus any associated morphological changes might be difficult to detect. In very young macronuclear anlagen there are no large or small bodies (Jurand *et al.* 1964), and the chromatin appears uniformly dispersed.

In the macronucleus of *Paramecium aurelia*, nucleolar material and condensed chromatin can be distinguished by their ultrastructure without reference to the size of the body in which these materials are aggregated, so that a small nucleolar fragment may be identified by its granular ultrastructure. There are similar fibrillar elements both within the small bodies of condensed chromatin and in the background material between them, so that the background material may contain diffuse chromatin. Wolfe (1967) reached similar conclusions by using a technique in which macronuclei were stretched on an air–water interface. There is no known relationship between the

chromosomes observed within the micronuclei at metaphase and the small bodies found in the macronucleus, except that they both contain DNA.

Oriented bundles of microtubules were observed predominantly in the isthmus of macronuclei during amitosis and they were rarely found in macronuclei from protozoa not in fission, so there is circumstantial evidence that the microtubules play a role in the macronuclear division. However, since no attachment has been observed between the microtubules and any other structure within the macronucleus, there is no evidence that they help to segregate the genomic units. Tamura, Tsuruhara & Watanabe (1969) observed microtubules stretched along the isthmus of a dividing macronucleus of *Tetrahymena pyriformis* and Carasso & Favard (1965) observed microtubules in the dividing macronucleus of the peritrichous ciliate *Campanella umbellaria.*

Sonneborn (1957) showed that the heterozygous and highly polyploid macronucleus of *Paramecium aurelia* retained its heterozygosity after hundreds of fissions and this observation supported his suggestion that the macronucleus is made up of diploid units which are distributed intact between the daughter macronuclei at fission (see Raikov, 1968). However, there are no ultrastructural observations which can be quoted in support of the existence of the diploid units. Amitosis has been regarded as a primitive and perhaps irregular form of nuclear division for which it is difficult to imagine an orderly duplication and equal distribution of daughter genomic units in the absence of any kind of mitosis. A similar conceptual difficulty exists when the duplication of the bacterial chromosome is considered, yet bacterial chromosomes appear to duplicate efficiently.

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EXPLANATION OF PLATES

Scale marks correspond to 1 μm. unless otherwise indicated.

PLATE 1

Fig. 1. The interphase micronucleus has a central core of dense material surrounded by a thin granular layer of intermediate density. The low-density region of the nucleus near the nuclear envelope contains linear elements which are microtubules, most of which are seen here in longitudinal section. The nuclear envelope has inner and outer membranes. The dense material which occurs at intervals in the nuclear envelope represents the position of nuclear pores. Electron micrograph. × 28,000.

Fig. 2. Part of a section through a micronucleus in prophase. The dense material (chromatin, *ch*) is dispersed in irregular clumps. Several bundles of microtubules are seen in transverse section and the microtubules all appear as circular profiles. Electron micrograph. × 37,000.

Fig. 3. The dense chromosomes are arranged in a metaphase plate on an intranuclear spindle. The spindle is viewed laterally and some microtubules appear to terminate at localized regions of the chromosomes, while others run between the chromosomes. The arrowed chromosome has a centromere where microtubules are attached and a distal arm in the plane of the section. At the poles of the spindle, microtubules terminate at the nuclear envelope which remains intact. Electron micrograph. × 18,000.

Fig. 4. A detail of the telophase spindle remnant is shown in the long and narrow region between the daughter nuclei. The cylindrical bundle of tightly packed microtubules has been sectioned longitudinally and is bounded by the nuclear envelope. Electron micrograph. × 37,000.

PLATE 2

Fig. 5. A longitudinal section through a paramecium in fission shows the macronucleus in amitosis. It is elongated and constricted in the middle to form an isthmus at the level of the fission furrow (*f*). Light micrograph of 1 μm. Araldite section stained with toluidine blue. × 880.

Fig. 6. A longitudinal section through part of a macronucleus during amitotic division, to show the region of the isthmus at the level of the fission furrow (*f*). The constricted macronucleus is bounded by the nuclear envelope and large and small dense bodies can be seen. The arrows indicate the position of groups of microtubules. Electron micrograph. × 11,000.

Fig. 7. Part of the field of Fig. 6 at higher magnification, showing a few of the microtubules (*m*) which run in the matrix between the large and small bodies in the direction of elongation of the macronucleus. The double nuclear envelope (*n*) is seen clearly wherever it is perpendicular to the plane of the section. Electron micrograph. × 38,000.

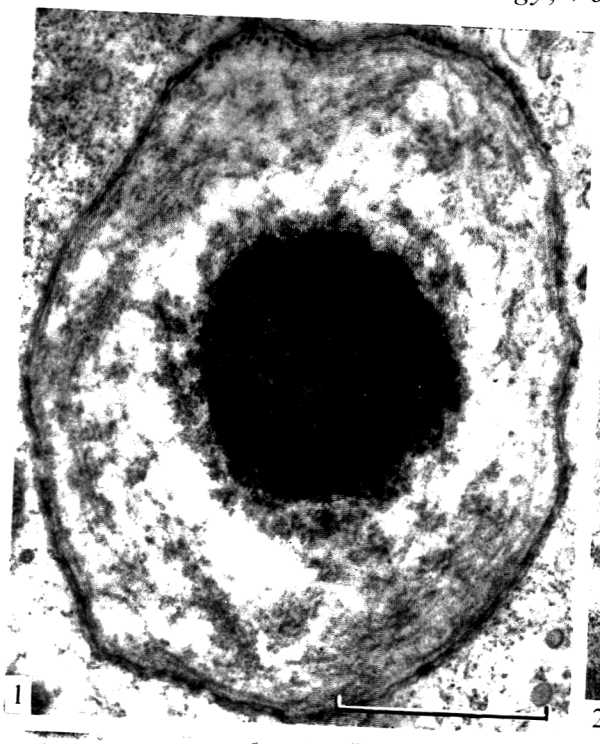
PLATE 3

Fig. 8. Macronucleus from a section stained only with 2·5% uranyl acetate solution after osmic fixation. The small bodies appear darker because of their greater affinity for uranyl acetate. Electron micrograph. × 16,000.

Fig. 9. A detail from a macronucleus showing that the small bodies (*s*) have a fine irregular fibrillar ultrastructure while, between the bodies, there is a matrix of similar fibrillar material of much lower density. The large body (*l*) has a more granular ultrastructure and 20 nm. granules are more distinct near the edges of the large body, while its interior is more fibrillar. Electron micrograph. × 60,000.

Fig. 10. The two macronuclear anlagen (arrowed) are shown at the pairing stage which occurs between the beginning of conjugation and the first postconjugal fission. Light micrograph of 1 nm. Araldite section stained with toluidine blue. × 1200.

Fig. 11. Electron micrograph showing two macronuclear anlagen at the pairing stage. The layer of cytoplasm separating the two anlagen is about 0·3 μm. The anlagen at this stage contain only a few scattered dense bodies. × 6000.



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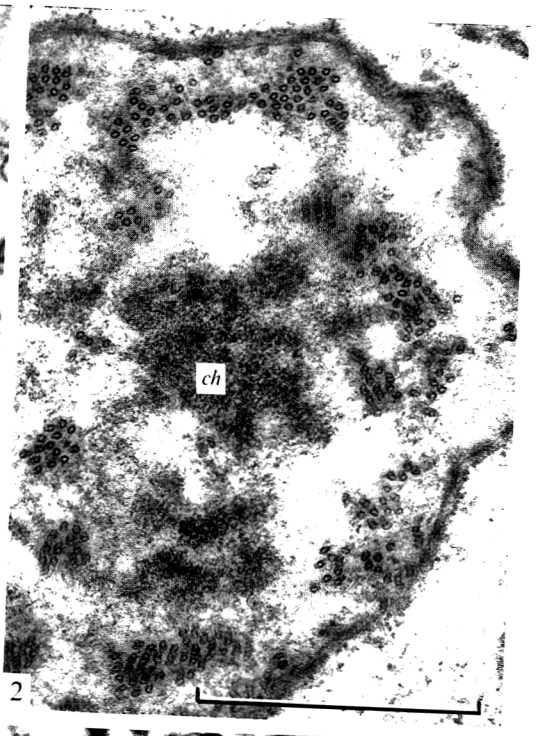

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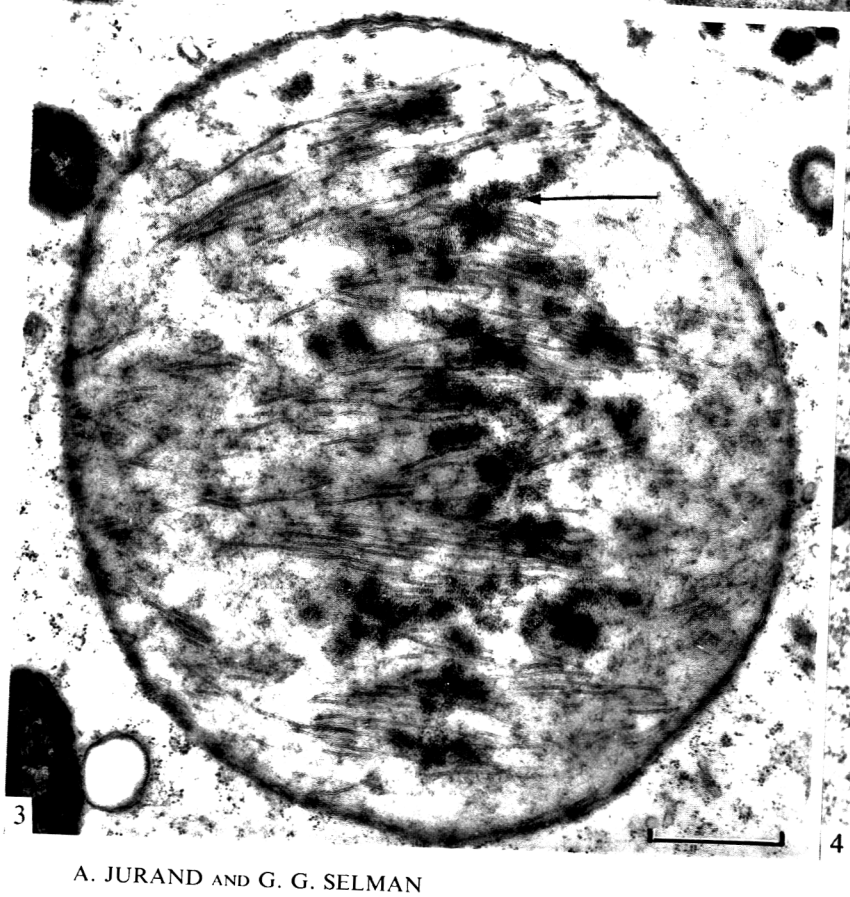

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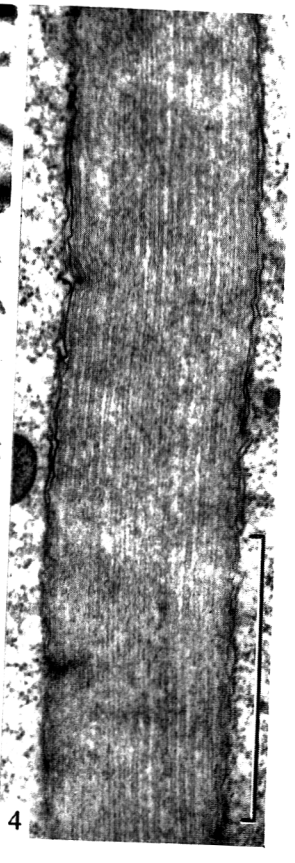

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A. JURAND AND G. G. SELMAN

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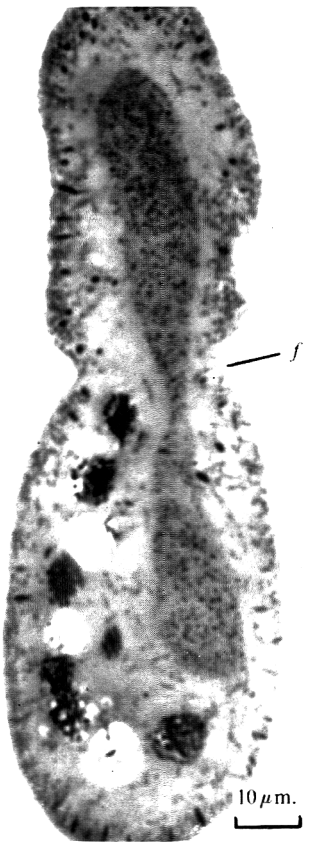

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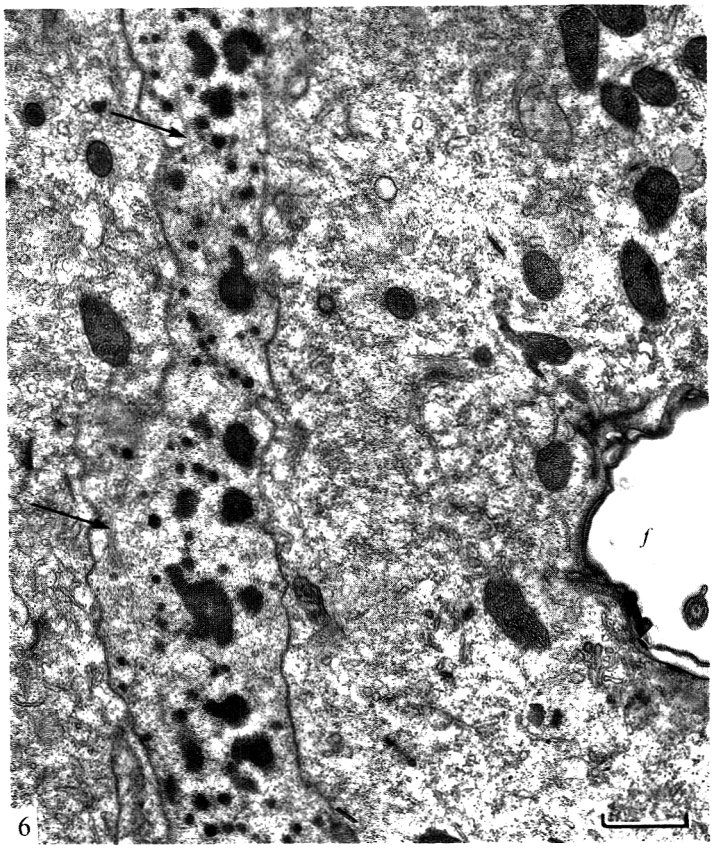

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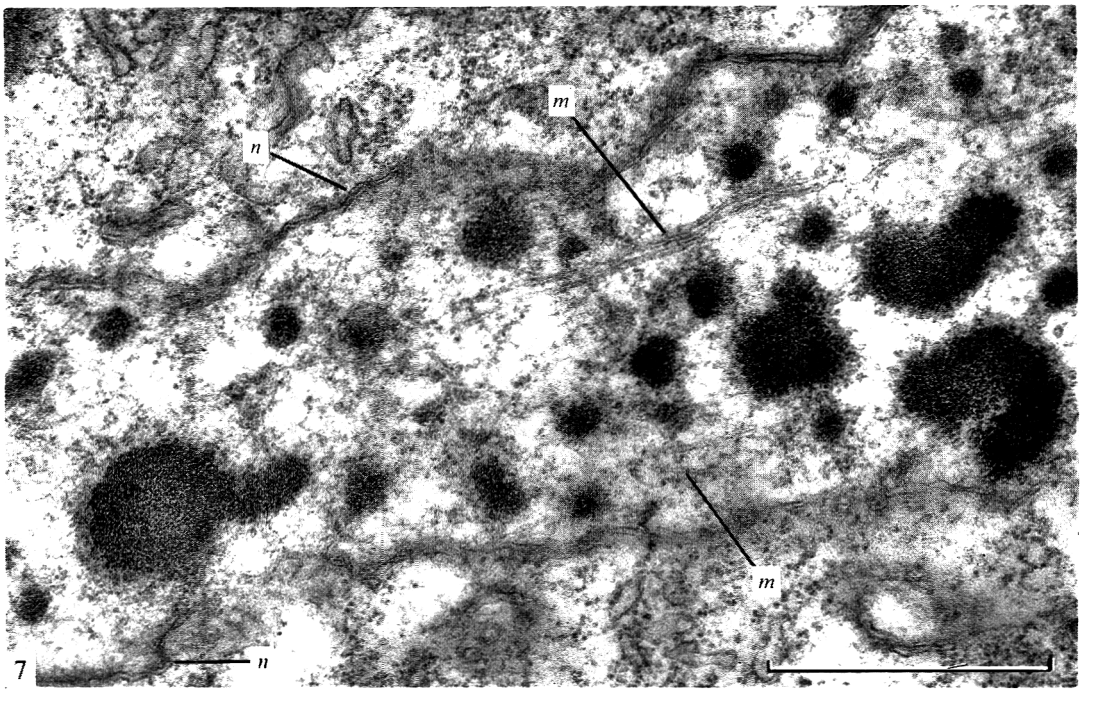

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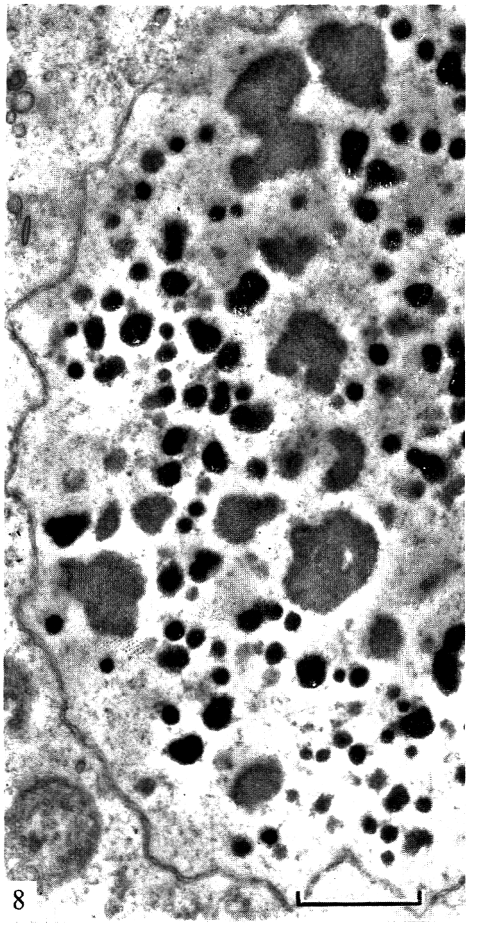

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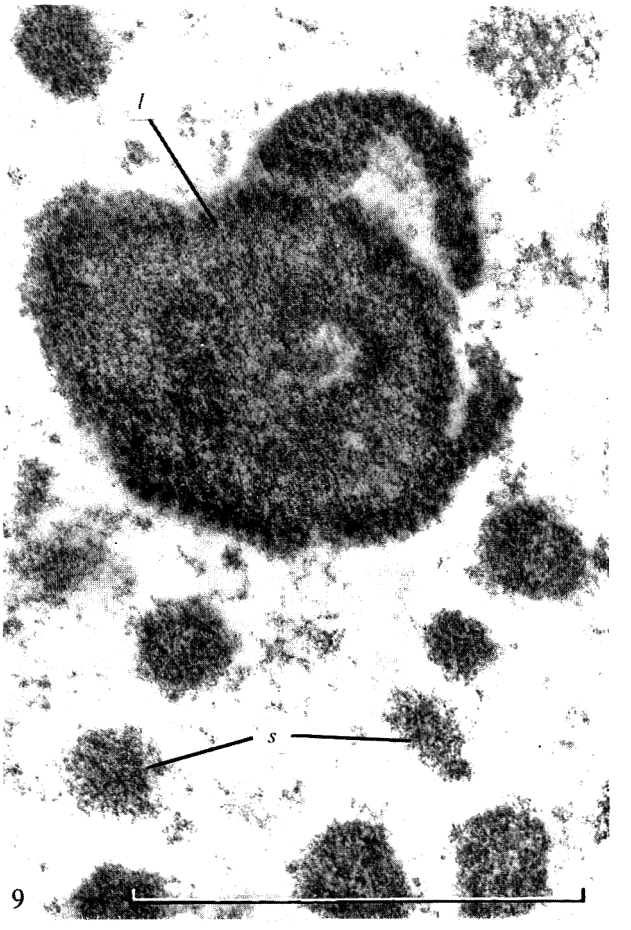

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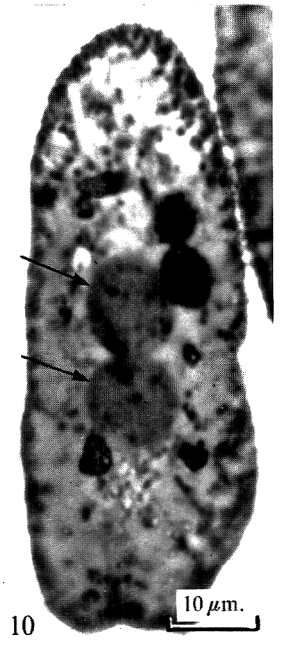

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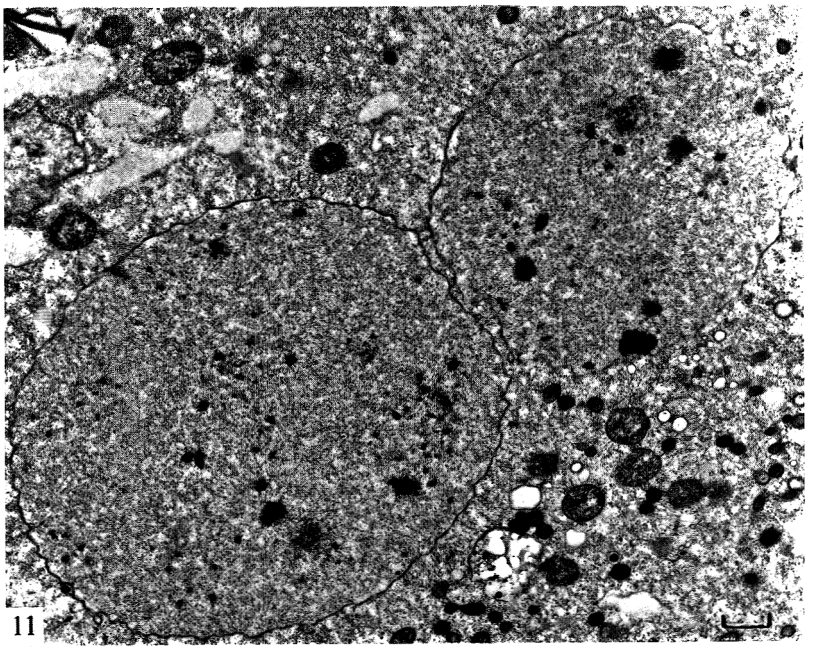

11

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Trichocyst Development During the Fission Cycle of Paramecium

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SUMMARY

In *Paramecium aurelia*, pretrichocysts originate in the endoplasm as undifferentiated vesicles. They develop in the endoplasm and then migrate to sites below the pellicle to become juvenile trichocysts. The maturation process takes place below the pellicle and involves a physical expansion and change in staining affinity of the body material of the trichocyst. Cold fixation usually caused mature trichocysts to be extruded, but the use of osmium tetroxide fixative at 37° allowed them to be fixed without extrusion. Juvenile trichocysts cannot be extruded. In *P. bursaria* and *P. aurelia* after osmic fixation the body of the mature trichocyst, unlike the juvenile trichocyst, had no staining affinity for either potassium permanganate or toluidine blue. During fission in *P. aurelia*, mature trichocysts were present only in small numbers at the poles of the protozoa remote from the furrow. Juvenile trichocysts mature in the first hour of the interfission period.

INTRODUCTION

Ultrastructural observations on mature trichocysts of Paramecium were made by Sedar & Porter (1955) with *Paramecium multimicronucleatum* and Stewart & Muir (1963) with *P. aurelia*. Mature trichocysts were shown by Yusa (1963, 1965) to be derived from juvenile trichocysts which developed in the endoplasm as pretrichocysts and which were themselves derived from undifferentiated endoplasmic vesicles. The terms pretrichocyst, juvenile trichocyst and mature trichochyst, used in this paper, were used by Yusa (1963, 1965). The developmental stages were illustrated in the electron micrographs of Yusa (1963) for *P. caudatum*, of Ehret & De Haller (1963) for *P. bursaria*, and of Yusa (1965) for *Frontonia vesiculosa*. Frontonia has a close phylogenetic kinship to Paramecium according to Corliss (1961). All the pretrichocyst stages and juvenile trichocysts possess a distinctive crystalline body material which increases in volume as the pretrichocyst grows and develops the shape and structure characteristic of a juvenile trichocyst, so that the developmental sequence has probably been interpreted correctly from the electron micrographs. Moreover, most of the observations of Yusa (1963, 1965) were made on the regeneration of trichocysts at recorded times after the mature trichocysts had all been discharged by the application of an electric current, so that observations on the development of a new set of mature trichocysts were made against a time scale which was initiated by the experimenter. For this reason, however, these observations of Yusa (1963, 1965) cannot be related to the normal fission cycle. Ehret & De Haller (1963) made their observations during the normal fission cycle of *P. bursaria* and showed that pretrichocysts increased in

number during the interfission interval. Although there is good agreement between these three accounts of the pretrichocyst stages, certain difficulties remain unresolved. For instance, Ehret & De Haller (1963) depicted trichocysts with dense body material as mature trichocysts of *P. bursaria*, whereas Yusa (1963, 1965) showed that juvenile trichocysts attained maturity by a process in which the dense crystalline body of the juvenile trichocyst situated below the pellicle was apparently replaced by body material which was of low electron density and in which no crystalline periodicities were detected.

The present observations are mostly of the development and maturation of trichocysts in *Paramecium aurelia*, although some observations have also been made on *P. bursaria*. They confirm that in *P. aurelia* the pretrichocyst stages and juvenile trichocysts resemble those of *P. bursaria* and *P. caudatum*, and new results have been obtained concerning the behaviour of trichocysts during the normal fission cycle. Certain staining properties of juvenile trichocysts and difficulties involved in the fixation of mature trichocysts have been investigated.

METHODS

Cultures of *Paramecium aurelia* were used, mainly of stock 60 syngen 1, but occasionally of other stocks. *P. bursaria* stock 93 syngen 2 was also used. Mass cultures were grown in an aqueous infusion of baked lettuce inoculated with *Klebsiella* (*Aerobacter*) *aerogenes* (Sonneborn, 1950). The protozoa were first filtered through a cotton plug and then concentrated into an aqueous pellet by low-speed centrifugation. The supernatant medium was decanted and the fixative added to the centrifuge tube, which was shaken to disperse the protozoa. A modified form of the fixative of Palade (1952) was prepared according to the formula quoted by Jurand & Selman (1969). To preserve mature trichocysts the fixative was used at about 37° instead of in the cold as normally recommended for electron microscopy. The penetration rate of the fixative is greater at the higher temperature. For comparative purposes fixations were also made by a modification of the method of Afzelius (1962), using a 30 % (w/v) solution of osmium tetroxide in carbon tetrachloride. This method is fully described by Jurand & Preer (1969) and has been proved suitable for the preservation of endosymbionts. Paramecia were fixed for 30 min.; in the case of warm fixations this period allowed the tube containing the protozoa to cool to room temperature. Dehydration was with graded ethanol + water mixtures and three changes of 100 % ethanol (10 min. each). In bulk preparations the organisms were centrifuged down and the supernatant decanted at each stage before fresh ethanol was added. Finally, the protozoa were passed through a 50 + 50 mixture 1,2-epoxypropane + Araldite and embedded in Araldite by using a rotary shaker (Jurand & Ireland, 1965). For light microscopy, Araldite sections were cut at 1 μm. and stained with a 0·5 % aqueous solution of toluidine blue made alkaline by adding 1 % borax. Alternatively the staining procedure of Huber, Parker & Odland (1968) was used. Ultrathin sections were cut on a Porter-Blum Sorvall ultramicrotome, mounted on collodion-carbon-coated grids and stained routinely for 20 min. with a 1 % potassium permanganate solution containing 2·5 % uranyl acetate. The sections were examined with an AEI EM 6 electron microscope.

Individual organisms were grown in medium containing bacteria using depression slides and an incubator at 24°. In this case individual paramecia were fixed, transferred

through ethanol + water mixtures by using micropipettes, and embedded in separate capsules for subsequent examination in sections.

OBSERVATIONS

The ultrastructure of pretrichocysts and juvenile trichocysts in *Paramecium aurelia* may be observed after warm or cold fixation. Pretrichocyst stages are found in the endoplasm in positions which seem to bear no relationship to the trichocyst sites below the pellicle. Pretrichocysts are first recognizable by electron microscopy as irregularly rounded vesicles (Pl. 1, fig. 1) containing amorphous material but with a dense oval core of crystalline material (Pl. 1, fig. 4) with periodicities between 8 and 15 nm. in different sections. The observed periodicity may depend upon the angle between the pretrichocyst and the plane of the section. At later pretrichocyst stages (Pl. 1, fig. 2, 3) the vesicle and its dense crystalline core are larger. The core becomes elongated and develops a tip (Pl. 1, fig. 3). Then a sheath or cap forms round the tip and migration to the pellicle takes place (Pl. 1, fig. 7). Yusa (1963, 1965) did not define the exact developmental stage beyond which a pretrichocyst is termed a juvenile trichocyst, but in the present paper the term juvenile trichocyst is applied after migration to the pellicle has occurred. In the juvenile trichocyst there is no discontinuity between the tip and the body, and both share the same crystalline structure (Pl. 1, fig. 6). In the electron micrographs sets of parallel dark lines run perpendicularly to the axis of the juvenile trichocyst at intervals of 7 to 8 nm. and further sets of dark lines with intervals of 12 nm. intersect the first set at 65° (Pl. 1, fig. 6). Dense peripheral granules, about 13 nm. in diameter, occur outside the body material of juvenile trichocysts and just within their limiting membrane (Pl. 1, fig. 5).

In experimental batches of material juvenile trichocysts and pretrichocysts were observed by electron microscopy after osmic fixation but without additional heavy-metal stain. Under these conditions no crystalline structure was observed in pretrichocysts or in the body or tip of juvenile trichocysts; only a slight darkening was observed round their edges, although in the same sections cytoplasmic membranes and the membranes of mitochondria appeared dense and sharply defined. The dense appearance of the juvenile trichocyst body in material prepared by the normal procedures is not therefore due to combination with osmium tetroxide as was assumed in Selman & Jurand (1968). When osmic fixation was followed by the use of potassium permanganate but without uranyl acetate, the pretrichocysts and juvenile trichocysts showed dense crystalline structure. When osmic fixation was followed by staining with uranyl acetate alone, the density of staining was less than with potassium permanganate and crystalline structure was not observed. Staining with lead citrate alone (Reynolds, 1963) gave a uniform dense stain which was not as intense as after potassium permanganate when the staining times were similar. It may be concluded that whenever juvenile trichocysts appear dense by electron microscopy it is because they combine with the heavy-metal stains which have been used after osmic fixation. The use of these stains has also enabled their crystalline ultrastructure to be observed.

The fixation and embedding of a concentrated mass culture of *Paramecium aurelia* allows a batch of several hundred protozoa in all stages of the fission cycle to be examined rapidly by light or electron microscopy after serial sectioning. When warm fixation was used, the mature trichocysts were not found extruded into the medium

between the protozoa but they were observed in their normal sites below the pellicle, their sheathed tips alternating with the basal granules or pairs of basal granules down each kinety (see also Ehret & Powers, 1959). The body of each mature trichocyst showed no affinity for heavy-metal stains and appeared white and without ultrastructure in electron micrographs (Pl. 2, fig. 8, 9). The tip of the mature trichocyst appeared dense and showed crystalline structure with regular cross-striations (Pl. 2, fig. 9).

When cold fixation was used, the shafts of extruded trichocysts were found in the medium between the protozoa (Pl. 2, fig. 11) and no mature trichocysts, but only immature juvenile trichocysts, were seen below the pellicle. Such juvenile trichocysts are probably incapable of extrusion. In some cases, cold fixation caused mature trichocysts to be extruded backwards into the cytoplasm. In extrusion the trichocyst body becomes transformed into an elongated shaft and then shows transverse striations (see Jakus & Hall, 1946) with a main periodicity of about 50 nm. (Pl. 2, fig. 12) and some fine longitudinal fibrillar ultrastructure with a fibril diameter of about 3 nm. In some cases a trichocyst was observed only partially extruded. In these cases the body was curved and thinner than a mature trichocyst but thicker than a fully extruded shaft.

When mass cultures of Paramecium were sectioned at 1 μm. and stained with toluidine blue for light microscopy it was immediately obvious that mature trichocysts remained unstained and juvenile trichocysts were deeply stained (Pl. 2, fig. 10). Therefore when a juvenile trichocyst becomes a mature trichocyst the result of the change which takes place in its body material can be seen by light microscopy as easily as by electron microscopy (Pl. 2, fig. 10, compare with Pl. 3, fig. 13). No stages of intermediate staining density were observed, however, between the juvenile and the mature trichocyst either by light or electron microscopy. When the staining method of Huber *et al.* (1968) was used, the juvenile trichocysts stained with both basic fuchsin and methylene blue and appeared dark purple against the pink background of the cytoplasm, while mature trichocysts did not stain. When unstained sections, 1 μm. thick, were observed by positive phase-contrast microscopy, the juvenile trichocysts appeared optically denser than mature trichocysts.

Measurements of the body length and the widest cross-sectional diameter of juvenile and mature trichocysts were made on electron micrographs of trichocysts seen in longitudinal section at their sites below the pellicle. Means and standard deviations were calculated. Juvenile trichocysts had a diameter of $0{\cdot}70 \pm 0{\cdot}14$ μm. and body length of $2{\cdot}70 \pm 0{\cdot}53$ μm., whereas mature trichocysts had a diameter of $1{\cdot}27 \pm 0{\cdot}26$ μm. and body length of $5{\cdot}02 \pm 0{\cdot}72$ μm. Therefore the mature trichocyst has a body which is about 84 % greater in its mean linear dimensions than the juvenile trichocyst and has a mean volume six times greater than the mean volume of the juvenile trichocyst. Moreover, the individual values for the corresponding dimension in mature and juvenile trichocysts form two groups which do not overlap. Measurements and observations of staining affinity thus support each other and suggest that the change from a juvenile to a mature trichocyst is rapid and involves both an expansion and a change in the chemical properties of the body material.

Mass cultures of *Paramecium bursaria* were examined after warm fixation by both light and electron microscopy. As with *P. aurelia*, mature trichocysts were fixed without extrusion and these had bodies without affinity for potassium permanganate or

toluidine blue. The juvenile trichocysts densely stained both with potassium permanganate and with toluidine blue (Pl. 2, fig. 10). The only difference was the considerable proportion of partially extruded mature trichocysts which were found in *P. bursaria* after warm fixation. In *P. aurelia* these may occur after cold but not after warm fixation. The difference may indicate that trichocysts of *P. bursaria* are more easily extruded.

A careful examination of sectioned material from mass cultures of *Paramecium aurelia* after warm fixation showed that, except for organisms in fission, the proportion of juvenile to mature trichocysts was usually small but varied with the individual protozoon. Whenever the fission furrow could be seen, the paramecia consistently showed a complete lack of mature trichocysts over about nine-tenths of their surface (Pl. 3, fig. 15). No mature trichocysts were seen near the furrow or in the middle part of the body of these paramecia, although many juvenile trichocysts were observed below these regions of the pellicle. A few mature trichocysts were observed, however, at the extreme ends of paramecia in fission (Pl. 3, fig. 14, 15). The dividing protozoa were not surrounded by extruded trichocysts, so that the lack of mature trichocysts is probably not due to a higher sensitivity of such protozoa to extrusion in the presence of fixative. The majority of mature trichocysts seemed to have been lost immediately before fission. It also appears that the juvenile trichocysts present in the fission animal must attain maturity soon after fission since the mass cultures showed that, except for dividing organisms, most paramecia had a majority of mature trichocysts. Observations were accordingly made on individually fixed organisms where an estimate could be made of the stage with respect to the fission cycle at the time of fixation.

In an experiment at 25° the average fission period was 8 hr 40 min., but individual fission intervals varied over a range of 2 hr. In paramecia fixed 1 hr after the previous fission, 91 % of the trichocysts below the pellicle were mature and the remainder were juvenile. It was concluded, therefore, that the majority of the juvenile trichocysts matured immediately after fission. In paramecia fixed at an estimated time of 1 hr before fission, 98 % of the trichocysts below the pellicle were mature. It was concluded that mature trichocysts are lost immediately before fission takes place. The mature trichocysts may discharge themselves before fission or they may degenerate.

DISCUSSION

The present observations show that mature trichocysts of *Paramecium aurelia* are almost completely absent from paramecia in fission, being normally discharged immediately before fission; the discharge itself has not been observed. Alternatively, the mature trichocysts might degenerate before fission, but this is less probable since degenerating trichocysts have been observed in only a few cases and then in the endoplasm rather than below the pellicle. Juvenile trichocysts are present below the pellicle during fission and subsequently attain maturity soon after fission. These conclusions were unexpected since it had been assumed previously that mature trichocysts are structurally stable components of the pellicle and are distributed between the two daughters at fission, while an equal number of new trichocysts develop from pre-trichocysts during each fission cycle. The work of Ehret, Savage & Alblinger (1964) seems to support the conservation of mature trichocysts through cell fission, since they showed that when trichocysts of *P. bursaria* were labelled with tritiated leucine they retained the label without dilution for at least three fission generations thereafter.

The present work suggests that this may be true only for trichocysts located at the two poles of the paramecia remote from the fission furrow.

The mature trichocysts of *Paramecium bursaria* and *P. aurelia* have bodies composed of material in which no regular structure has been observed. This apparent lack of ultrastructure may be due to the lack of an appropriate stain. It is most unlikely that the mature trichocyst has an amorphous body since its rapid extrusion involves another change of state, after which regular periodicities are observed once more, although the ultrastructure of extruded shafts is quite different from that of juvenile trichochysts. Ehret & De Haller (1963) did not record the maturation of trichocysts in *P. bursaria* and their electron micrographs show only juvenile trichocysts. Ehret & Powers (1957, 1959) published electron micrographs in which mature trichocysts may be recognized. The origin and development of trichocysts in *P. aurelia* is similar to their epigenesis in *P. caudatum* (Yusa, 1963), *P. bursaria* (Ehret & De Haller, 1963) and *Frontonia vesiculosa* (Yusa, 1965). Ultrastructural studies have indicated that trichocysts develop from endoplasmic vesicles, although these vesicles cannot be identified as pretrichocysts unless they possess at least some of the crystalline material of which the body and tip of juvenile trichocysts is composed. It is not known how these vesicles originate and there is no evidence from any work with Paramecium species that they are formed in association with any type of organelle. It is therefore difficult to imagine in what sense trichocysts can continue to be regarded as ultrastructural homologues of cilia (e.g. see Ehret, 1967), although Lwoff (1950), on the basis of observations with the light microscope, considered that the trichocysts of certain parasitic ciliates are derived from kinetosomes.

The body material of trichocysts appears to exist in different physical states, one of which occurs in pretrichocysts and juvenile trichocysts, another in mature trichocysts and a third in extruded trichocysts. Changes between these states appear to take place quickly and so far as is known irreversibly and involve changes in dimensions and staining affinity. Regular periodicities have been directly observed in pretrichocysts, juvenile trichocysts and extruded trichocysts, but their presence in the body material of mature trichocysts seems likely but has not yet been directly observed.

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EXPLANATION OF PLATES

All figures are of *Paramecium aurelia* except for Fig. 10 which is of *P. bursaria*. Fig. 1 to 9 and 11 to 14 are electron micrographs. Fig. 10 and 15 are light micrographs. All scales correspond to 1 μm. unless otherwise indicated.

PLATE 1

Fig. 1. An endoplasmic pretrichocyst at an early stage bounded by a membrane and containing a dense central core of crystalline material. × 28,000.

Fig. 2. A pretrichocyst at a slightly later stage than in Fig. 1. The crystalline core is elongated. × 28,000.

Fig. 3. A pretrichocyst from the endoplasm at a later stage than Fig. 2. The tip has developed in continuity with the body. There has been a decrease in the amount of finely dispersed material between the body and the membrane of the pretrichocyst. × 28,000.

Fig. 4. A detail of the crystalline core of a pretrichocyst at an early stage as in Fig. 1. The regular periodicities are shown. × 93,000.

Fig. 5. A detail of a juvenile trichocyst similar to that shown in Fig. 7 to show the periodicities in the body material. Note also a single row of dense granules between the membrane and the dense body. × 93,000.

Fig. 6. Part of a juvenile trichocyst to show the continuity between its tip and body and the similarity of the crystalline structure in the two regions. × 93,000.

Fig. 7. A juvenile trichocyst in longitudinal section in its position below the pellicle. Round the tip a sheath has developed which was not present at the late pretrichocyst stage shown in Fig. 3. × 28,000.

PLATE 2

Fig. 8. Mature trichocysts are shown in longitudinal section at their sites below the pellicle. The bodies appear unstained and electron-transparent, in contrast to the juvenile trichocyst in Fig. 7. × 7600.

Fig. 9. A detail from a mature trichocyst to show the region of the join between the dense crystalline tip and the electron-transparent body material. Contrast with the juvenile trichocyst in Fig. 6. × 85,000.

Fig. 10. Mature (*m*) and juvenile trichocysts (*j*) are shown at the surface of *P. bursaria* after warm fixation with osmium tetroxide. Algal symbionts (*a*) are present in the cytoplasm. Light micrograph stained with toluidine blue. × 1400.

Fig. 11. In *P. aurelia* fixed with cold osmium tetroxide the shafts (*s*) of discharged trichocysts are present in the medium at the cell surface. Only darkly stained juvenile trichocysts (*j*) remain undischarged below the pellicle. Electron micrograph stained with potassium permanganate and uranyl acetate. × 11,000.

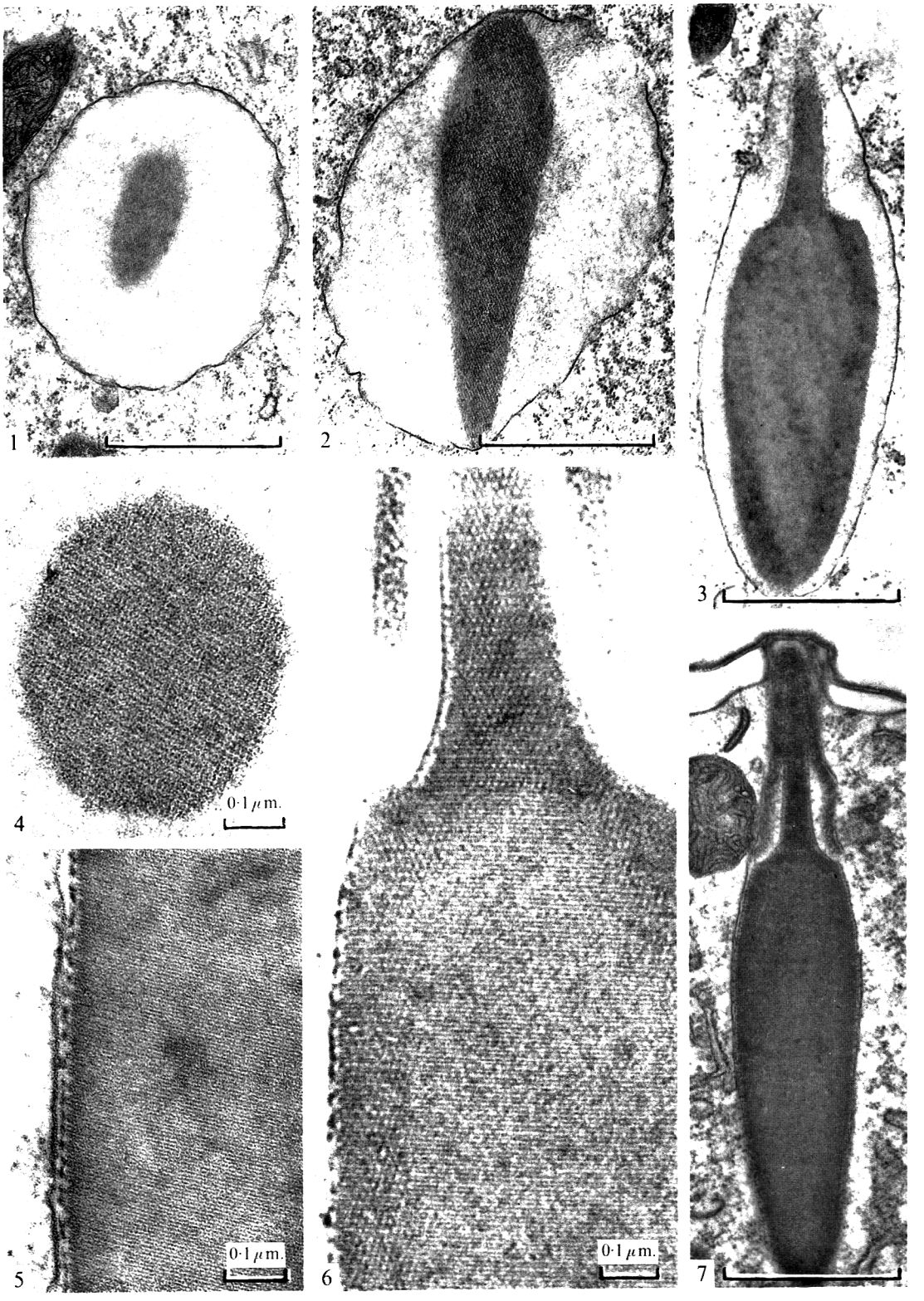
Fig. 12. The shaft and tip of an extruded trichocyst of *P. aurelia*, outside the animal. Transverse striations occur in the shaft at intervals of 50 nm. × 38,000.

PLATE 3

Fig. 13. Both mature (*m*) and juvenile trichocysts (*j*) are shown below the pellicle of *P. aurelia*. The trichocysts are seen in longitudinal and transverse section. The juvenile trichocysts appear smaller and darkly stained. × 4700.

Fig. 14. The polar region of a paramecium in fission shows two mature trichocysts (*m*) in the plane of the section and several juvenile trichocysts (*j*) below the pellicle. × 6600.

Fig. 15. The longitudinal section through a paramecium in fission shows the fission furrow and one polar region remote from the furrow. Only the polar region contains a group of mature trichocysts (*m*), which appear white, while only darkly stained juvenile trichocysts are present below the pellicle over the remainder of the body surface. Warm fixation; stained with toluidine blue. × 1400.



G. G. SELMAN AND A. JURAND

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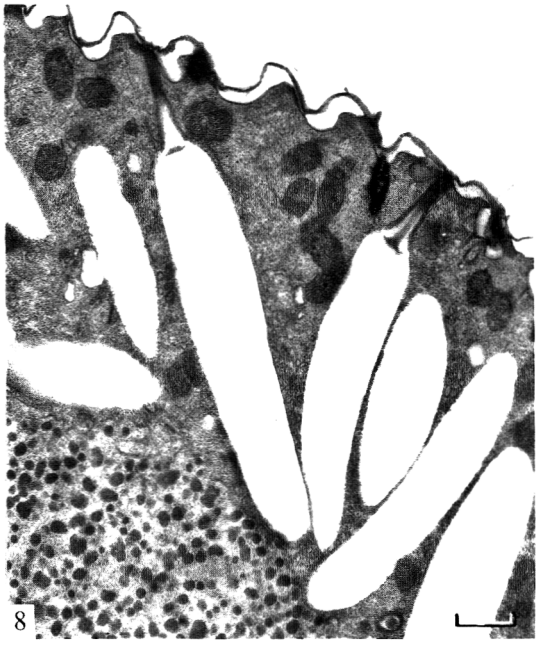

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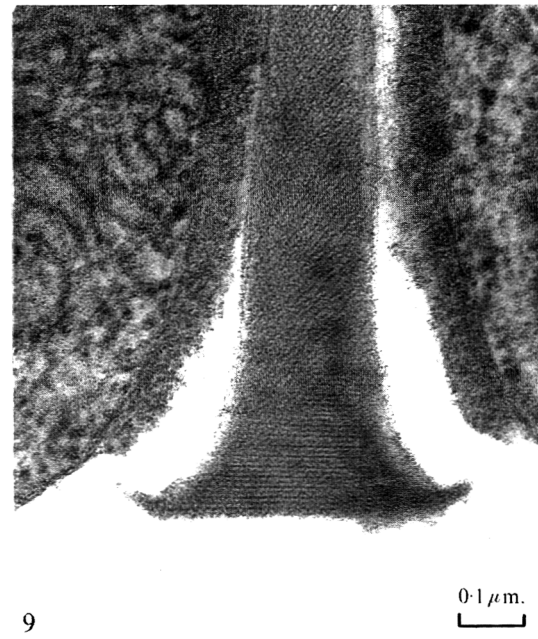

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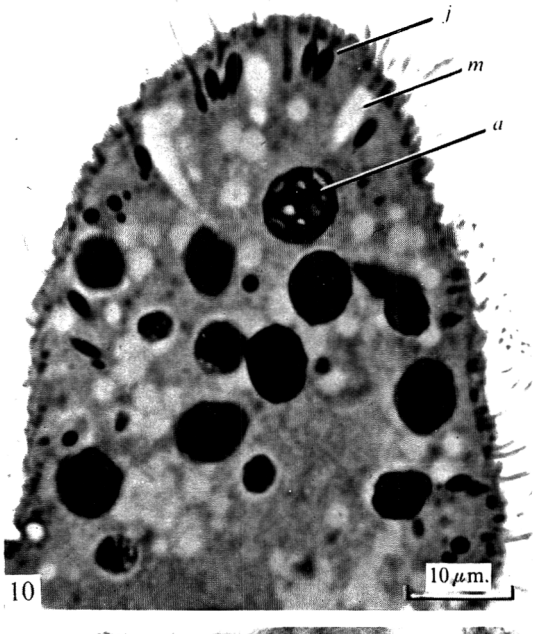

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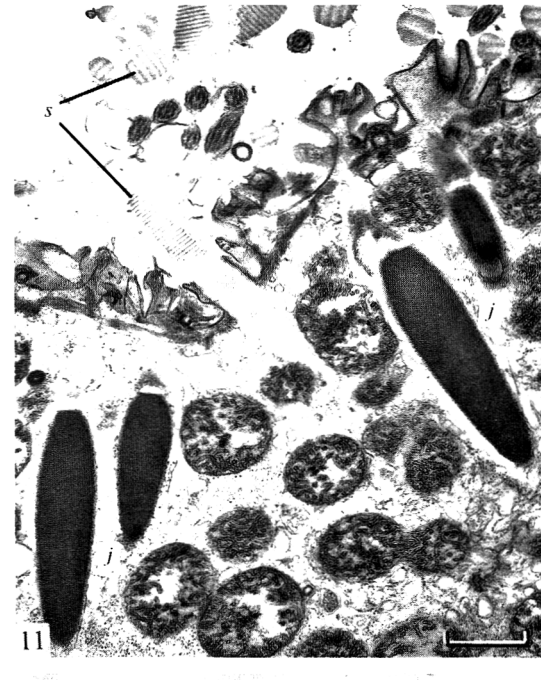

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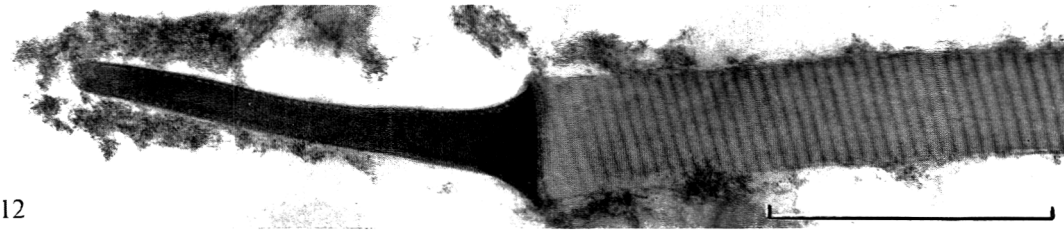

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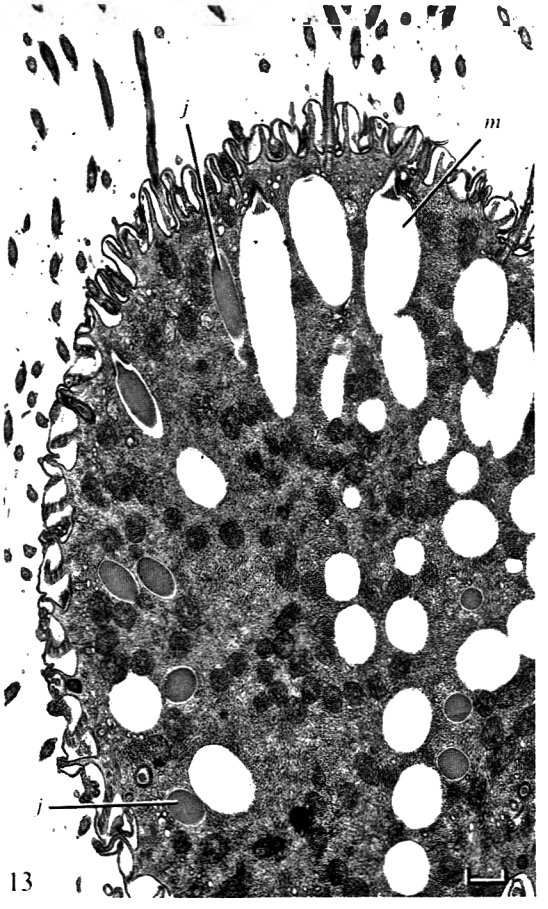

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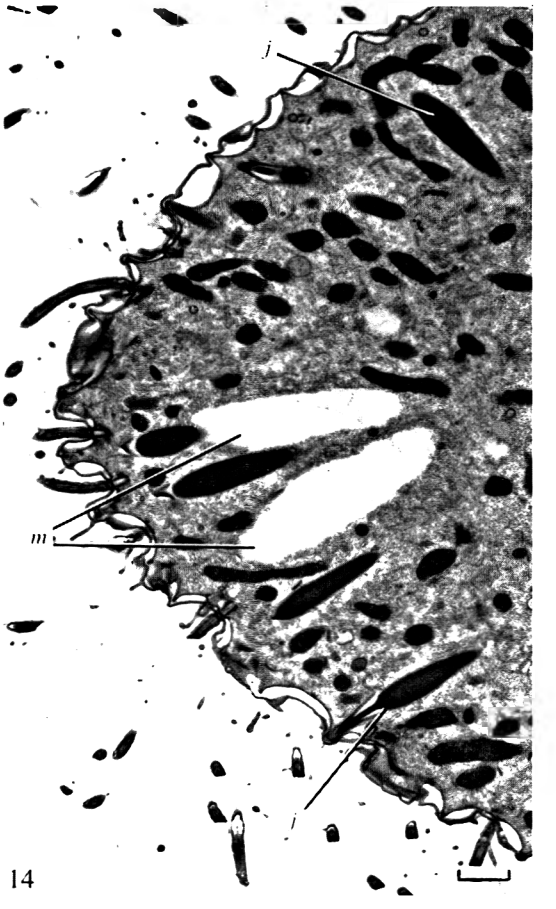

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The Distribution of 2-Keto-3-deoxy-octonic Acid in Bacterial Walls

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SUMMARY

A survey of the walls of over 80 bacterial species has been carried out using chromatographic and colorimetric techniques to demonstrate the presence of 2-keto-3-deoxy-octonic acid (KDO) and or sialic acid. In most Gram-negative bacteria KDO but not sialic acid was found in the wall, whereas the walls of Gram-positive bacteria contained neither compound.

INTRODUCTION

2-Keto-3-deoxy-D-*manno*-octonic acid (KDO) was first recognized as a glycosidic component of the lipopolysaccharide (LPS) of *Escherichia coli* O111B4 (Heath & Ghalambor, 1963; Ghalambor, Levine & Heath, 1966), but subsequently it has been found in the lipopolysaccharides of all the members so far examined of the Enterobacteriaceae (Lüderitz, Staub & Westphal, 1966; Williams & Perry, 1969). Furthermore, some strains of Pasteurella and Brucella contain KDO in the lipopolysaccharide fraction of their cell wall (Ellwood, 1968).

Two other 2-keto-3-deoxy-sugar acids have been reported as structural components of bacteria. 2-Keto-3-deoxy-galactonic acid occurs in an extracellular polysaccharide of *Azotobacter vinelandii*, and sialic acid (*N*-acetylneuraminic acid), an *N*-acetyl amino substituted 2-keto-3-deoxy-sugar acid containing 9 carbon atoms, occurs in a polymeric form in several bacteria (Barry & Goebel, 1957; De Witt & Rowe, 1959). All 2-keto-3-deoxy-sugar acids give a positive reaction with thiobarbituric acid after acid periodate oxidation, and on the basis of this non-specific test it was initially thought that sialic acid was widely distributed in Gram-negative bacteria (Aaronson & Lessie, 1960; Irani & Ganapathi, 1962). Later, on the basis of a similar test, it was suggested that KDO was equally widely distributed (Vincent & Cameron, 1967).

In order to clarify this apparent contradiction a survey has been carried out using both colorimetric and chromatographic analysis for the presence of 2-keto-3-deoxy-sugar acids in the cell walls of a wide selection of bacteria. Since lipopolysaccharides are components of the envelopes of Gram-negative bacteria, KDO is expected to occur in the wall preparations of the Gram-negative bacteria only, nevertheless, both Gram-positive and Gram-negative organisms were examined. A preliminary report has been published (Ellwood, 1966).

METHODS

Organisms. The bacterial species examined are shown in Table 2. They were obtained from the following collections: American Type Culture Collection, Rockville, Md., U.S.A. (ATCC); Microbiological Research Establishment, Salisbury (MRE); National Collection of Industrial Bacteria, Torry Research Station, Aberdeen (NCIB); National Collection of Marine Bacteria, Torry Research Station, Aberdeen (NCMB); National Collection of Type Cultures, Central Public Health Laboratory, London (NCTC); Rothamsted Experimental Station, Harpenden (RES); National Collection of Dairying Organisms, National Institute for Research in Dairying, Reading (NCDO); National Collection of Plant Pathogenic Bacteria, Harpenden, Herts. (NCPPB).

Culture conditions. Bacteria were grown on the surface of a plate count agar (Oxoid) at temperatures facilitating maximum rate of growth. Organisms were harvested by scraping the bacteria from the agar surface with a spatula, washed with saline and disrupted as described below. Three plates (9 cm. diameter) yielded 200 to 300 mg. of bacteria.

Preparation of bacterial walls. Bacteria (*c.* 300 mg. equivalent dry wt in 30 ml. water) were shaken in a vertical shaker (stroke 10 cm.) at 450 strokes/min. in volumes of 10 ml. with Ballotini beads (10 ml. no. 16 grade), for 1 hr. The Ballotini beads were then removed by filtration through a no. 2 glass sinter and the walls were separated by centrifugation (17,000 *g*; 1 hr), washed 3 times in saline (1 %, w/v) and 3 times in water, lyophilized, and weighed. Yield 30 to 60 mg. initial dry wt of bacteria. The optimum conditions for the release of KDO from wall preparations were established using wall samples of *Escherichia coli* MRE600, and *Klebsiella* (*Aerobacter*) *aerogenes* NCTC418. Samples (50 mg.) of these walls were heated in 0·1 N-H_2SO_4 (5 ml.) at 80 and 100°. The release of KDO was followed by analysing samples by the method described below.

Isolation of the 2-keto-3-deoxy-sugar acids. The samples of bacterial walls (50 mg.) were heated with 0·1 N-H_2SO_4 (5 ml.) at 100° for 30 min. The residual walls were sedimented by centrifugation (17,000 *g*; 15 min.). The supernatant fluid was neutralized with a saturated solution of $Ba(OH)_2$, centrifuged to remove $BaSO_4$, and passed through a column (0·5 cm. long and 1·0 cm. diam.) of Dowex 1 (HCO_3^- form). The column was then washed with water (5·0 ml.) and the eluate and washings discarded. The column was eluted with 0·5 N-ammonium carbonate (5 ml.), the eluate treated with an excess of Dowex 50 (H^+ form) and the resin removed by filtration. The filtrate was lyophilized and then dissolved in water (0·1 ml.) for chromatographic analysis.

Determination of 2-keto-3-deoxy-sugar acids. This was a modification of the methods of Weissbach & Hurwitz (1959) and Aminoff (1961). Samples (0·2 ml.) of the acid hydrolysates of the wall samples were diluted with water (0·1 ml.). Periodic acid (0·025 M in 0·125 N-H_2SO_4; 0·25 ml.) was added and incubated at about 20° for 20 min. Sodium arsenite (2 %, w/v in 0·5 N-HCl; 0·5 ml.) was next added, shaken, and allowed to stand until the yellow colour was discharged (2 to 3 min.). Thiobarbituric acid (0·3 %, w/v, pH 2·0; 2·0 ml.) was then added and, after shaking, the solution heated to 100° for 10 min. The pink solution was cooled to 4° (when it became cloudy) and shaken with 5·0 ml. of conc. HCl + *n*-butanol (5 + 95, v/v). After centrifugation the extinction of the coloured upper layer was measured in a spectrophotometer at 550 mμ. Under these conditions an optical extinction of 0·430 was obtained from 0·025 μmole of KDO.

Paper chromatography. This was done on acid-washed Whatman no. 1 paper with the following solvent systems: (A) butan-2-ol + acetic acid + water (8 + 1 + 1, v/v), and (B) butan-1-ol + pyridine + 0·1 N-HCl (5 + 3 + 2, v/v). Paper chromatograms were dried and developed by the technique of Anderson (1966).

Preparation of 2-keto-3-deoxy-sugar acids (chromatographic standards). These were prepared by aldol condensation between the appropriate aldose and oxaloacetic acid in alkaline solutions, as described by Ghalambor *et al.* (1966). Glyceraldehyde gave a mixture of 2-keto-3-deoxy-gluconic acid (KDG) and 2-keto-3-deoxy-galactonic acid. These acids ran as one component in both solvents and were used as a chromatographic standard for KDG. Erythrose also gave a mixture of two 2-keto-3-deoxy-heptonic acids. The major component was used as a chromatographic standard for 2-keto-3-deoxy-heptonic acid (KDH). Authentic KDO was kindly supplied (as the penta-acetyl methyl ester) by Dr E. C. Heath; *N*-acetylneuraminic acid was purchased from Koch-Light Laboratories (Colnbrook), Bucks., England.

RESULTS

The determination of KDO depends upon oxidation with periodate under acid conditions to give β-formyl pyruvate, which is then reacted with thiobarbituric acid to form a chromagen with an absorption maximum at 550 mμ. The assay of Weissbach & Hurwitz (1959) is based upon these reactions but has been modified by extracting the

Table 1. *Separation of various 2-keto-3-deoxy-sugar acids by paper chromatography*

	R_{KDO}			
Solvent	KDG	KDH	KDO	*N*-acetyl-neuraminic acid
A	1·78	1·41	1·00	0·85
B	1·93	1·37	1·00	0·84

Solvent A: butan-2-ol + acetic acid + water (8 + 1 + 1, v/v).
Solvent B: butan-1-ol–pyridine + 0·1 N-HCl (5 + 3 + 2, v/v).

chromagen into acid butanol (cf. the assay method for sialic acid, Aminoff, 1961). This extraction step prevents precipitation which otherwise tends to occur at room temperature. The results obtained in this assay are largely specific for KDO: equimolar solutions of *N*-acetylneuraminic acid give less than $\frac{1}{200}$ the colour reaction. The R_F values of the series of 2-keto-3-deoxy-sugar acids are shown in Table 1 and it is apparent that the acids may be differentiated by paper chromatography in these solvents.

Fig. 1 shows the rate of release of 2-keto-3-deoxy-sugar acids from walls of *Escherichia coli* and *Klebsiella aerogenes* by acid hydrolysis at 80 and 100°. The maximum release of these acids from both wall samples was obtained after heating for 30 min. at 100° and these conditions were used for all other wall samples investigated. Paper chromatography of hydrolysates prepared by the standard procedure showed the presence of KDO in nearly all the wall samples of Gram-negative bacteria examined. *N*-acetylneuraminic acid was found in only a few samples. In wall preparations of Gram-positive bacteria no trace of either KDO or *N*-acetylneuraminic acid was found. The results are summarized in Table 2.

Table 2. *The 2-keto-3-deoxy-sugar acids found in bacterial walls*

Species	Strain number	KDO*	*N*-acetylneuraminic acid*	KDO in the cell wall (%)
		Gram-negative bacteria		
Athiorhodaceae				
Rhodopseudomonas palustris	NCIB 8252	+	−	0·1
R. spheroides	NCIB 8253	+	−	0·1
Pseudomonadaceae				
Pseudomonas aeruginosa	l.s.	+	−	0·6
P. angulata	NCPPB 263	+	−	0·5
P. chlororaphis	NCIB 9402	+	−	0·5
P. fluorescens	KB 1	+	−	0·4
	NCIB 8248	+	−	0·5
P. iodinum	NCDO 613	+	−	0·05
		−		< 0·01
P. primulae	NCPPB 133	+	−	0·1
P. syncyanae	NCDO 759	+	−	0·7
Xanthomonas campestris	NCPPB 528	+	−	0·1
X. hyacinthi	NCPPB 599	+	−	0.1
X. juglandis	NCPPB 362	+	−	0·1
	XJ 107	+	−	0·2
Acetobacter aceti	NCIB 8554	+	−	0·05
Aeromonas hydrophila	NCMB 72	+	−	0·05
A. liquefaciens	NCMB 87	−	−	< 0·01
Protoaminobacter alboflavus	NCIB 8167	−	−	< 0·01
Mycoplana bullata	ATCC 4278	+	−	0·2
Spirillaceae				
Vibrio cuneatus	NCIB 8194	+	−	0·7
V. foetus	ED 148	+	−	0·4
V. percolans	NCIB 8193	+	−	0·6
Rhizobiaceae				
Rhizobium leguminosarum	RES 317	+	−	0·3
Agrobacterium tumefaciens	NCPPB 397	+	−	0·2
Chromobacterium violaceum	NCTC 7150	+	−	0·2
	NCTC 9373	+	−	0·2
Achromobacteraceae				
Alcaligenes faecalis	NCIB 8156	+	−	0·02
A. metaligenes	NCIB 9021	+	−	0·2
Achromobacter lacticum	NCIB 8208	+	+	0·3
Flavobacterium acidificum	NCMB 683	−	−	< 0·01
F. aurantiacum	NCIB 8204	−	−	< 0·01
		+	−	0·02
F. sauveolens	NCIB 8992	−	−	< 0·01
Agarbacterium alginicum	NCMB 886	+	−	0·05
Enterobacteriaceae				
Escherichia coli	MRE 162	+	+	0·4
	NCTC 8164	+	−	0·7
	MRE 600	+	−	0·7
E. freundii	NCTC 8165	+	+	0·5
Klebsiella aerogenes	NCTC 8167	+	−	0·5
Paracolobactrum aerogenoids	NCTC 8105	+	−	0·6
Klebsiella aerogenes	NCTC 418	+	−	0·5
Erwinia amylovora	NCPPB 595	+	−	0·4
E. carnegieana	NCPPB 671	+	−	0·5
Serratia keilensis	NCTC 4619	+	−	0·2
S. marcescens	NCTC 1377	+	−	0·5
Proteus vulgaris	NCIB 8064	+	−	0·4

Table 2 (*cont.*)

Species	Strain number	KDO*	*N*-acetylneuraminic acid*	KDO in the cell wall (%)
	Gram-negative bacteria			
P. mirabilis	PR-27	+	−	0·3
Salmonella typhimurium	MRE LT/2	+	−	0·6
Shigella shigae	NCTC 4837	−	−	< 0·01
S. flexneri	NCTC 8192	+	−	0·3
Brucellaceae				
Brucella abortus	544	+	−	0·3
	45/0	+	−	0·2
	45/20	+	−	0·1
B. melitensis	6015	+	−	0·3
B. suis	PS III KG 25 MRE 43	+	−	0·4
Pasteurella pestis	TS MRE 100	+	−	0·5
	EV 76 MRE 103	+	−	0·4
P. pseudotuberculosis	MRE 321 V	+	−	0·4
Yersinia enterocolitica	l.s.	+	−	0·3
P. multocida	l.s.	+	−	0·1
P. tularensis	MRE 125	−	−	< 0·01
P. septica	l.s.	+	−	0·05
Neisseriaceae				
Neisseria catarrhalis	NCTC 8554	+	−	0·05
	Gram-positive bacteria			
Micrococcaceae				
Micrococcus lysodeikticus	MRE 310	−	−	< 0·01
Staphylococcus aureus	NCTC 6751	−	−	< 0·01
S. epidermidis	l.s.	−	−	< 0·01
S. lactis	NCTC 7944	−	−	< 0·01
Propionibacterium shermanii	l.s.	−	−	
Lactobacillaceae				
Lactobacillus plantarum	l.s.	−	−	< 0·01
Brevibacteriaceae				
Kurthia zopfii	NCTC 404	−	−	< 0·01
Corynebacteriaceae				
Corynebacterium viscosum	NCTC 2416	−	−	< 0·01
C. xerosis	NCTC 9755	−	−	< 0·01
Arthrobacter globiformis	NCIB 8907	+	−	0·02
Bacillaceae				
Bacillus anthracis	NCTC 8234	−	−	< 0·01
B. brevis	NCTC 7577	−	−	< 0·01
B. megaterium	NCTC 2607	−	−	< 0·01
B. subtilis	ATCC 9372	−	−	< 0·01
Mycobacteriaceae				
Mycobacterium smegmatus	NCTC 8159	−	−	< 0·01
Actinomycetaceae				
Actinomycetes bovis	NCTC 9430	−	−	< 0·01

* KDO and *N*-acetylneuraminic acid were characterized and detected by paper chromatograph in solvents A and B while KDO was estimated by a modification of the method of Weissbach & Hurwitz (1959). Laboratory strains are identified as l.s. Strains whose designations are unspecified in the text were gifts from Dr H. E. Wade and Dr J. Keppie of this establishment.

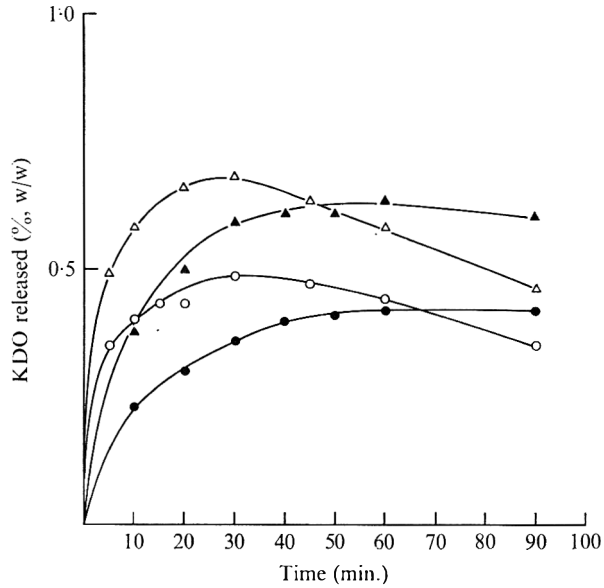

Fig. 1. Release of KDO from walls of *Escherichia coli* MRE600 (50 mg.), at 100° (△) and 80° (▲), and from *Klebsiella aerogenes* NCTC418 (50 mg.), at 100° (○) and at 80° (●), by heating samples with 0·1 N-H_2SO_4 (5 ml.). KDO was estimated by a modification of the method of Weissbach & Hurwitz (1959).

DISCUSSION

Sialic acid occurs either as a polyneuraminic acid (called colominic acid) in several bacterial species or as part of other heteropolysaccharides, such as the K antigen, in other bacterial species (for review see Lüderitz, Jann & Wheat, 1968). A sensitive test for sialic acid is based on the formation of β-formyl pyruvic acid upon periodate oxidation and the subsequent formation of a chromagen formed on heating the β-formyl pyruvate with thiobarbituric acid. This procedure provides the basis of quantitative estimation for sialic acid (Warren, 1969; Aminoff, 1961). The periodate oxidation is carried out in 9 M-phosphoric acid in the Warren procedure and Aminoff used 37° for the oxidation. The high acid concentration or increased temperature is necessary to get a full release of β-formyl pyruvate. However, KDO will also yield β-formyl pyruvate on periodate oxidation and thus give a chromagen with thiobarbituric acid identical to that given by sialic acid.

The Warren procedure was used by Aaronson & Lessie (1960) and Irani & Ganapathi (1962), who considered the test to be sufficiently specific to demonstrate the wide distribution of sialic acid in bacteria. Vincent & Cameron (1967), using the assay system of Weissbach & Hurwitz (1959) in which periodate oxidation is carried out at room temperature, showed that KDO occurred in a similar range of bacteria to those thought to contain sialic acid by Aaronson & Lessie (1960). Our results using room temperature for the periodate oxidation confirm in general the presence of KDO and absence of *N*-acetylneuraminic acid. Recently Hackenthal (1969) showed by ion exchange and paper chromatography that the group-specific polysaccharide of type C meningococci contained both KDO and sialic acid.

A further complication in comparing the present work with that of Aaronson & Lessie (1960) and of Vincent & Cameron (1967) is that they measured material reactive to thiobarbituric acid in hydrolysates of whole bacteria. These preparations would also contain 2-keto-3-deoxy-sugar acids involved in the metabolic pathways of bacteria. We have used wall preparations because this is the location of LPS and capsular fractions of the organisms. The walls were hydrolysed with acid under conditions known to produce maximum yields of thiobarbituric-acid-reactive material in *Escherichia coli* and *Klebsiella aerogenes* and there was no evidence for the presence of 2-keto-3-deoxy-sugar acids with less than 8-carbon atoms.

Nearly all wall samples of Gram-negative bacteria contained KDO. The wide variation in amounts of KDO found may reflect differences in the composition of the walls of different bacteria. However, it is known that the KDO content of the wall of *Klebsiella aerogenes* depends on the way the organisms are grown. The faster they are grown under Mg^{2+} limitation the more KDO is found in the wall, whereas under carbon-limiting conditions the KDO content of the walls falls with increasing growth rate (Ellwood & Tempest, 1967).

Sialic acid was found with KDO in only three organisms, *Achromobacter lacticum* NCIB 8208, *Escherichia coli* MRE 162 and *E. freundii* NCTC 8165, but sialic acid was never found without KDO. Several Gram-negative bacteria appear to have no KDO in their wall, indicating that the LPS may be different in its structure. Kasai (1966) has shown that the LPS of Bordetella species do not contain KDO. None of the wall samples or Gram-positive bacteria examined contained either KDO or sialic acid except for *Arthrobacter globiformis* NCIB 8907, which contained a small amount of KDO. This organism is not unequivocally Gram-positive.

These results indicate that in contrast to the work of Aaronson & Lessie (1960) and Irani & Ganapathi (1962), and in agreement with the work of Vincent & Cameron (1967), KDO is present in most Gram-negative bacteria. The latter authors also found thiobarbituric-acid-reactive material in Gram-positive bacteria. However, KDG and KDH occur as normal metabolites in bacteria and would give a thiobarbituric acid reaction in hydrolysates of whole organisms.

Thanks are due to Mr G. R. G. Moody for expert technical assistance.

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With 1 *plate*
Printed in Great Britain

Characteristics of Cellulolytic Cillobacteria from the Rumens of Sheep Fed Teff (*Eragrostis tef*) Hay Diets

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(*Accepted for publication 20 November 1969*)

SUMMARY

Nine isolates of cellulolytic, Gram-positive motile rods obtained from the rumen contents of sheep fed on teff hay were identified as belonging to the genus *Cillobacterium* Prévot. In most respects the organisms resembled *C. cellulosolvens* and it is proposed that the definition be extended to include the characteristics of the present strains.

INTRODUCTION

During a study of the cellulolytic flora in the rumens of sheep fed on diets of teff hay supplemented in different ways (van Gylswyk, 1969) Gram-positive, motile cellulolytic rods in addition to cocci and Butyrivibrio-like rods were isolated. In no case were these rods as plentiful as the other cellulolytic organisms. However, their consistent occurrence indicates that they probably form part of the normal cellulolytic flora of the rumen when low-protein teff hay is fed, with or without certain supplements. Preliminary examination of the isolates revealed several points of resemblance to *Cillobacterium cellulosolvens*, a cellulolytic bacterium from the rumen of cattle first described by Bryant, Small, Bouma & Robinson (1958). Since, to our knowledge, no further isolations of this organism have been reported, and since the description of this species was based on the characteristics of a single strain it was decided to characterize in detail a group of nine isolates resembling *C. cellulosolvens*.

METHODS

Isolation. The nine isolates studied were obtained from the rumen of four sheep fed differently supplemented teff hay diets (van Gylswyk, 1969).

Culture techniques. These were adapted from those used by Bryant & Burkey (1953) and Kistner (1960). Anaerobic conditions were obtained by purging with oxygen-free gases. Traces of oxygen in the commercial gases were removed by passing the latter, together with about 2% hydrogen, through a 'Deoxo' catalyst cartridge (Engelhard Industries Ltd., London, U.K.). Inocula were transferred with sterile syringes. Incubation temperature was $38 \pm 1°$, except in the case of temperature-tolerance tests. Time of incubation was generally about 1 week, except for the cellulose-containing medium, which was incubated for about 1 month. Two separate runs were conducted for all the tests, except the determinations of succinate and C_2 to C_5 volatile fatty acids. The organisms were cultured in media with initial pH values of $6{\cdot}8 \pm 0{\cdot}1$.

Media. Except where otherwise stated the media contained the following constituents (g./l.): K_2HPO_4, 0·225; KH_2PO_4, 0·225; NaCl, 0·45; $(NH_4)_2SO_4$, 0·45; $CaCl_2$, 0·045; $MgSO_4.7H_2O$, 0·09; $NaHCO_3$, 6·37; cysteine hydrochloride, 0·25; $Na_2S.9H_2O$, 0·25; cellobiose, 5; indigo carmine, 0·005 and rumen fluid from sheep fed on lucerne hay, 400 ml. (partly clarified by centrifuging at 1500 *g* for 20 min.). The basal medium was sterilized by autoclaving (121°/25 min.) and $NaHCO_3$ added as heat-sterilized and cellobiose as filter-sterilized, concentrated oxygen-free solutions. The cysteine hydrochloride and sodium sulphide were prepared as a combined, concentrated solution under nitrogen, made alkaline and heat-sterilized before addition to the medium. This medium was equilibrated with a gas phase containing 98% CO_2 and 2% H_2 (Medium 1). For the preparation of maintenance slopes the sodium sulphide was omitted, the cysteine hydrochloride was increased to 0·5 g., and 15 g. agar was added.

For the fermentation tests a medium containing one tenth of the above concentration of $NaHCO_3$ and 20% rumen fluid was used. This medium was equilibrated with 88% N_2/10% CO_2/2% H_2. In the second run the rumen fluid was clarified by centrifuging at about 23,000 *g* for 2 hr to facilitate visual detection of growth. The soluble sugars were added as sterile millipore-filtered solutions while insoluble substances were heat-sterilized together with the basal medium. The final concentration in each case was 5 g./l. For the temperature-tolerance tests the medium was similar to that used for the fermentation tests except that it contained 5 g. cellobiose. The medium for determining the relation of the organisms to free oxygen was as medium 1, but contained 0·637 g. $NaHCO_3$, 15 g. agar, 10 g. Trypticase (Baltimore Biological Laboratories), 1 g. cellobiose (5 g. in the first run), and 1 mg. resazurin instead of indigo carmine. The medium was prepared under 90% N_2/10% CO_2 and after inoculation was incubated under air/10% CO_2. To test for growth in the absence of CO_2, $NaHCO_3$ was omitted from medium 1 and the gas phase consisted of 98% N_2/2% H_2. For testing growth in the absence of rumen fluid, this constituent was replaced in medium 1 by 15 g. Trypticase and 5 g. yeast extract (Difco) in an equivalent volume of water. For the gelatin liquefaction test 50 g. gelatin and 15 g. Trypticase were added to medium 1. One gram KNO_3 and 20 g. Trypticase were added to medium 1 to test for the formation of nitrite from nitrate. Trypticase (20 g.) was also added to the test media for indole and acetylmethyl-carbinol production. In the case of the latter three media indigo carmine was omitted because the colour of the oxidized indicator hampered the interpretation of these tests. For testing for hydrogen sulphide production the organisms were grown in medium 1 to which was added 0·5 g. ferric ammonium citrate, 5 g. agar and 15 g. Trypticase. Hydrogen production was determined after growth in medium 1 plus 12 g. cellobiose, equilibrated with hydrogen-free CO_2. Production of volatile fatty acids was determined in medium 1 containing 12 g. Whatman No. 1 filter-paper cellulose (which had been ball-milled for 72 hr) as the only added carbohydrate. The organisms were examined for motility after growth on a medium similar to that for maintenance except that it contained only 1 g. of cellobiose.

Identification tests. In the tests for temperature tolerance, ability to ferment carbohydrates and ability to grow in the absence of CO_2, a change in pH of more than 0·3 was taken as indicative of growth. For testing the susceptibility of the organisms to free oxygen deep stabs were made in test tubes containing the solid medium. Growth in the absence of rumen fluid was assessed by the development of turbidity. For the

gelatin liquefaction test (Conn, 1951) cultures were incubated for about 2 weeks. In one series of tests residual gelatin was precipitated with $HgCl_2$ while in the second, solidification at low temperature was used as criterion of non-liquefaction. Acetyl-methyl-carbinol was detected by a method employing α-naphthol (Conn, 1951). The production of indole was assessed by the method of Kovács (1928). The detection of hydrogen as a fermentation product of cellobiose, and the quantitative measurement of C_2 to C_5 volatile fatty acids as fermentation products of cellulose (after extraction with ether), were done by gas chromatography. Formate was determined by the method of Rabinowitz & Pricer (1962), lactate by the method of Elsden & Gibson (1954), succinate by a method described by Cohen (1959) and ethanol by the method of Bonnichsen (1962). Flagella stains were made according to the method of Leifson (1951) using basic fuchsin instead of pararosaniline and a staining time of 14 min.

RESULTS

Colony morphology. Surface colonies on 0·5% cellobiose, 40% rumen fluid, agar slopes were circular, convex entire, and about 1 mm. in diameter at 18 hr after inoculation. Surface colonies of the organisms within the clearings produced on cellulose agar films after 2 to 4 weeks were usually entire, flat and translucent and often extended to the outer limit of the zone of clearing. Deep colonies were generally lenticular. Cellulolysis within the clearings was complete.

Morphology and Gram reaction. In young cultures grown on 0·1% (w/v) cellobiose rumen fluid, agar medium, the organisms occurred as Gram-positive rods with pointed ends, arranged in chains (Pl. 1, fig. 1). As the cultures became older, the chains broke up and the proportion of organisms showing a Gram-negative reaction increased markedly (Pl. 1, fig. 2). A proportion of the organisms showed a coccoid morphology. At this stage the rods were 0·5 μ to 3 μ long (typically 0·9 μ to 2 μ) by 0·5 μ to 1 μ wide (typically 0·6 μ to 0·9 μ). In still older cultures the organisms occurred singly and in pairs, and comprised a mixture of Gram-positive and Gram-negative rods (Pl. 1, fig. 3).

Motility and flagella. In young cultures, where chain formation was abundant, no motility could be observed and there was no evidence of flagella. In somewhat older cultures, where the chains had largely broken up, organisms often showed restricted movement, which was more than could be attributed to Brownian movement alone. In such cultures were organisms with developing flagella or 'buds' (Pl. 1, fig. 4). With older cultures very active 'tumbling' motility was observed in all the strains and flagella could be demonstrated. The flagella occurred in numbers of 2 to 9 per organism and the arrangement was peritrichous (Pl. 1, fig. 5, 6, 7, 8, 9).

Fermentation tests. All the isolates fermented the following carbon sources, the figures in parentheses indicating the final pH values after incubation for at least 7 days; glucose (5·1 to 5·5), galactose (5·3 to 6·4), maltose (5·0 to 5·5), lactose (5·6 to 6·1), sucrose (5·1 to 5·6), inulin (5·4 to 6·4), aesculin (5·3 to 5·9), salicin (5·2 to 5·6), pectin (5·6 to 6·4). Ball-milled Whatman No. 1 filter-paper cellulose was always vigorously attacked and almost completely solubilized. None of the isolates fermented L-arabinose, D-xylose, D-mannose, L-rhamnose, trehalose, dextrin, starch, glycerol, mannitol, inositol, or DL-sodium lactate. Fructose was fermented by isolates 1, 5, 6, 7 and 8, but, with the exception of isolate 5, the pH change was very slight. Raffinose was

fermented by all isolates except 1 and 8; the final pH values ranging from 5·4 to 6·0. Xylan was not fermented by isolates 1, 3, 4, 6 and 8, while the reaction of the remaining strains was weakly positive in one run and negative in the other.

Other tests. All the strains were obligate anaerobes. None of the strains produced catalase, indole, H_2S or ethanol. Gelatin was not liquefied, and no nitrite was found after growth in nitrate-containing medium. The Voges–Proskauer reaction was weakly positive. Good growth occurred in CO_2-free medium (final pH 5·0 to 5·4), and in medium containing Trypticase/yeast extract instead of rumen fluid. The strains showed very poor growth at 45° and no growth at 22°. At temperatures of 37° to 39° good growth was always obtained. No significant growth was noted in a medium containing peptone as sole carbon source. Hydrogen was produced by all the strains, although in small quantities (2·0% to 3·3% (v/v) of the gas phase). Results showing the net production or utilization of formate, lactate and C_2 to C_5 volatile fatty acids are given in Table 1. Figures for formate and lactate are mean values for the two separate runs, whereas the figures given for the other acids represent determinations done on the first run only. The mean recovery of acids in the first run for the nine strains was 89% of the titratable acid obtained after ether extraction of the cultures. The corresponding figure for the uninoculated culture medium was 97%. None of the strains produced succinate.

Table 1. *Acids produced* (+) *or utilized* (−) *by nine strains of cellulolytic, motile Gram-positive rods isolated from the rumen of sheep fed teff hay diets*

Acid produced or utilized	Strain no.								
	1	2	3	4	5	6	7	8	9
Formate	+1·22	+1·58	+1·14	+0·99	+1·07	+1·12	+1·31	+1·47	+1·61
Acetate	−0·78	−1·07	−1·22	−1·43	−0·26	−0·83	−0·82	−1·21	−2·37
Propionate	−0·62	−0·64	−0·72	−0·69	−0·63	−0·55	−0·62	−0·65	−0·90
*Iso*butyrate	+0·01	−0·01	0	−0·01	−0·01	0	0	+0·01	−0·02
Butyrate	+2·04	+1·55	+2·25	+1·55	+1·77	+1·85	+2·52	+2·13	+1·21
*Iso*valerate	0	−0·01	−0·01	−0·01	−0·01	−0·01	0	0	−0·03
Valerate	+0·49	+0·41	+0·58	+0·41	+0·45	+0·46	+0·60	+0·55	+0·31
Lactate	+3·28	+4·86	+4·65	+4·79	+4·73	+4·85	+4·04	+3·62	+4·53

Values expressed as m-mole/100 ml. medium.
The uninoculated medium contained the following (m-mole/100 ml.): formate, 0·19; acetate, 5·50; propionate, 1·57; *iso*butyrate, 0·06; butyrate, 0·79; *iso*valerate, 0·07; valerate, 0·11; lactate, 0·11.

DISCUSSION

In so far as the nine isolates were heterotrophic, Gram-positive anaerobic rods, actively motile by means of peritrichous flagella and fermenting a wide range of carbohydrates, the end products including hydrogen, formate, butyrate, valerate and lactate, they belong to the genus *Cillobacterium* Prévot (*Bergey's Manual* 1957). The fact that they did not produce gas in peptone broth medium may have been due to the deficiency of some growth factor in this medium.

Of the seven species that have been described as belonging to the genus *Cillobacterium* (*Bergey's Manual* 1957; Bryant *et al.* 1958) the present isolates most closely resemble *C. cellulosolvens* (Bryant *et al.* 1958), particularly in so far as they were isolated from the rumen and were cellulolytic, in contrast to other species of the genus

for which cellulolysis has not been reported. The principal differences between the type culture of *C. cellulosolvens* and our isolates lie in the nature and proportions of end products formed in the fermentation of carbohydrates. The type culture was practically homofermentative; the lactic acid produced constituted 94% of the carbon of the products recovered, and only small amounts of formate and acetate were produced. Hydrogen, formate, butyrate, valerate and lactate were produced, while acetate and propionate were utilized, by the present strains. In this respect they conform more closely to the definition of the genus *Cillobacterium* than does the type strain. Lesser differences include the ability of the present strains to ferment lactose as opposed to the type culture which did not. Organisms of the type culture had a maximum of four flagella whereas the present strains had up to nine. Even though the latter may differ from the type culture to the extent that the creation of a new species might be warranted, it is felt that the species name *C. cellulosolvens* is very apt as the organisms are very actively cellulolytic whereas the other members of the genus thus far described do not attack cellulose.

On the basis of the present work it is suggested that the description of the species *Cillobacterium cellulosolvens* (Bryant *et al.* 1958) be amended to include organisms that produce hydrogen, butyrate and valerate in addition to formate and lactate; that can utilize acetate and propionate; that have up to nine flagella per rod; that ferment galactose and pectin; that do not ferment D-mannose, L-rhamnose, trehalose and inositol and may or may not ferment fructose, lactose, raffinose and xylan; that may show slight production of acetyl-methyl-carbinol; that grow in the absence of carbon dioxide and that may show a little growth at 45°.

Cillobacterium cellulosolvens was often found in the 10^{-7} dilution of rumen ingesta from sheep conditioned to diets containing low-protein teff hay (van Gylswyk, 1969). In cases where the organisms were found they occurred in numbers representing 2 to 13% of the mean level of the cellulolytic counts. It is quite possible that the organisms would have been found more regularly if a greater number of cellulolytic isolates had been obtained. The available data suggest that *C. cellulosolvens* normally occurs in numbers of at least 10^6 per g. rumen contents from sheep fed on diets containing teff hay. In spite of the relatively low number of these bacteria in the rumen, when compared with, for instance, the number of cellulolytic ruminococci, they may nevertheless contribute significantly to cellulose breakdown as they have a high rate of cellulose digestion *in vitro*.

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EXPLANATION OF PLATE

Cells and flagellated cells of *Cillobacterium cellulosolvens*.

Fig. 1. Gram-stained preparation of young culture. × 1200.

Fig. 2. Gram-stained preparation of somewhat older culture. × 1200.

Fig. 3. Gram-stained preparation of a culture in which abundant growth had occurred. × 1200.

Fig. 4. Leifson stain of a cell showing 'bud' formation. × 2400.

Fig. 5, 6, 7, 8 and 9. Leifson stain of flagellated cells. × 2400.


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Production of Single-walled Cysts of *Schizopyrenus russelli* by Sonication and their Behaviour Towards Excystment-inducing and Excystment-blocking Agents

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SUMMARY

Ultrasonic treatment of double-walled cysts of *Schizopyrenus russelli* led to the removal of the outer wall. These single-walled cysts excysted readily in aqueous extract of *Escherichia coli*, as did normal double-walled cysts. There was a marked delay in the excystment of single-walled cysts obtained by ultrasonic treatment of the normal cysts treated with *p*-chloromercuric benzoate (*p*-CMB) or emetine, similar to that obtained with normal cysts. The action of *p*-CMB was annulled by reduced glutathione. These findings suggest that the sites of action of excystment-inducing and excystment-blocking agents are on or inside the inner cyst wall and that these agents are able to permeate the outer cyst wall.

INTRODUCTION

Earlier work has shown that aqueous extracts of either Aerobacter species or *Escherichia coli*, or certain amino acids, are able to cause excystment of double-walled cysts of the amoeba *Schizopyrenus russelli* Singh (Singh, Mathew & Anand, 1958). Emetine inhibited the induction of excystment by these agents (Imam, Dutta & Agarwala, 1968) and *p*-chloromercuric benzoate (*p*-CMB) delayed the emergence of trophozoites from the cysts (Rastogi, Sagar & Agarwala, 1969). This action of *p*-CMB was annulled by reduced glutathione. The present paper deals with the production of single-walled cysts of *S. russelli* by ultrasonic treatment and their behaviour towards excystment-inducing and excystment-blocking agents.

METHODS

Schizopyrenus russelli was grown in monobacterial culture with *Escherichia coli*. Viable sterile cysts, free from bacteria, were obtained by the method reported earlier (Rastogi *et al.* 1969). A suspension of 10×10^6 cysts/ml. was made and stored in a refrigerator. Ultrasonic treatment was done for periods up to 10 min. in 8 ml. lots of cyst suspension in distilled water containing 2×10^7 cysts in a 50 ml. beaker, chilled in crushed ice, by using a Mullard ultrasonic generator fitted with 20 kc. transducer (output current 1·5 amperes). The treated cysts were centrifuged at 650 *g* for 5 min. and suspended in distilled water to 10×10^6 cysts/ml.; 0·1 ml. of this suspension was added to 0·9 ml. aqueous extract of *E. coli* and the excystment followed as reported earlier (Rastogi *et al.* 1969).

RESULTS

Table 1 shows that ultrasonic treatment of double-walled cysts (Pl. 1, fig. 1) removed the outer wall and increased the number of single-walled cysts (Pl. 1, fig. 2). A decrease in the total count (normal and single-walled cysts) was also observed, indicating cyst disintegration. After 2 min., 59% of the cysts were converted to single-walled structures, and 29% of the cysts were completely disrupted. After more than 3 min. treatment, the residual population comprised almost entirely single-walled cysts. The single-walled cysts obtained by ultrasonic treatment excysted readily in *Escherichia coli*

Table 1. *Effect of ultrasonic treatment of cysts of Schizopyrenus russelli*

Exposure time (min.)	% of cysts		
	Double-walled	Single-walled	Disrupted completely
0	100	0	0
½	75	17	8
1	30	47	23
2	12	59	29
3	2	53	45
5	1	25	74
10	0	5	95

Table 2. *Effect of p-chloromercuric benzoate on excystment of ultrasonically treated single-walled cysts of Schizopyrenus russelli*

Cysts treated with	% count of different stages after varying periods of treatment															
	Hr...	2				4				8				20			
	Stage...	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
E. coli extract (normal cysts)		75	0	5	20	40	0	7	53	10	0	20	70	2	0	2	96
E. coli extract (ultrasonically treated cysts)		12	50	4	34	0	45	0	55	0	35	0	65	0	5	0	95
Ultrasonically treated cysts treated with *p*-CMB (10^{-4} M) in *E. coli* extract		10	80	6	4	10	78	4	8	4	76	0	20	0	50	0	50
Ultrasonically treated cysts treated with *p*-CMB (10^{-4} M) for 1 hr; excystment with *E. coli* extract after the removal of *p*-CMB		10	89	0	1	8	72	5	15	4	69	6	21	0	50	0	50
Ultrasonically treated cysts treated with *p*-CMB (10^{-3} M) in *E. coli* extract		12	88	0	0	12	88	0	0	12	88	0	0	12	88	0	0
Ultrasonically treated cysts treated with *p*-CMB (10^{-3} M) for 1 hr; excystment in *E. coli* extract after the removal of *p*-CMB		14	86	0	0	8	86	6	0	7	85	8	0	6	83	11	0

A, Double-walled cysts; B, single-walled cysts; C, cysts in the process of excystment; D, trophozoites. Ultrasonic treatment time 3 min.

extract, as did normal double-walled cysts (Tables, 2, 4 and 6). The majority of them did not take eosin staining (0·125%, w/v), suggesting that the cysts were viable.

Effect of p-CMB on excystment of single-walled cysts

The excystment of single-walled cysts of *Schizopyrenus russelli* was similar to that of the double-walled cysts (Table 2). When the single-walled cysts were treated with *p*-CMB (10^{-4} M) in *Escherichia coli* extract, there was a marked delay in excystment. Similar results were obtained when the cysts were treated with *p*-CMB (10^{-4} M) in water, washed in distilled water and then put for excystment in *E. coli* extract.Under both these conditions cysts treated with 10^{-3} M *p*-CMB failed to excyst in 20 hr. Reduced glutathione (GSH) annulled the *p*-CMB effect, both when added with *p*-CMB and after removal of the latter (Table 3). There was a marked delay in excystment of the single-walled cysts of *S. russelli* obtained by sonication of the normal cysts treated with *p*-CMB (Table 4). These findings suggest that *p*-CMB is able to permeate the outer cyst wall of double-walled cysts.

Table 3. *Annulment of p-CMB delayed excystment of ultrasonically treated Schizopyrenus russelli cysts by reduced glutathione*

	% count of different stages after varying periods of treatment															
Hr...	2				4				10				20			
Cysts treated with Stage...	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
E. coli extract (normal cysts)	56	0	11	33	45	0	0	55	8	0	2	90	5	0	0	95
E. coli extract (ultrasonically treated cysts)	7	49	5	39	0	37	0	63	0	6	0	94	0	6	0	94
Ultrasonically treated cysts treated with *p*-CMB (10^{-3} M) for 1 hr; excystment in *E. coli* extract after the removal of *p*-CMB	11	89	0	0	12	88	0	0	12	88	0	0	12	88	0	0
Ultrasonically treated cysts treated with *p*-CMB (10^{-3} M) + GSH (2×10^{-3} M) for 1 hr; excystment in *E. coli* extract after the removal of *p*-CMB and GSH	7	55	4	34	0	41	0	59	0	9	0	91	0	10	0	90
Ultrasonically treated cysts treated with *p*-CMB (10^{-3} M) for 1 hr; washed and then treated with GSH (2×10^{-3} M) for 1 hr; excystment in *E. coli* extract after the removal of GSH	12	88	0	0	0	56	0	44	0	40	0	60	0	30	0	70

A, B, C and D as in Table 1. Ultrasonic treatment for 3 min.

Effect of emetine on excystment of normal and ultrasonically treated cysts

The results in Table 5 indicate that, even after the removal of emetine, there was a delay in excystment which was proportional to the concentration of emetine. Ultrasonically treated single-walled cysts were also sensitive to the action of emetine (Table 6) and the delay in excystment increased with its concentration. The maximum

excystment obtained in 24 hr after incubation and removal of emetine (10 mg./ml.) was around 50% (Tables 4 and 5). The residual cysts, however, excysted almost completely in another 24 hr when incubated with fresh bacterial extract.

When emetine-treated normal cysts were subsequently treated ultrasonically, a

Table 4. *Delay in excystment of single-walled cysts of Schizopyrenus russelli (obtained by ultrasonic treatment of normal cysts treated with p-CMB or emetine)*

	% count of different stages after varying periods of treatment															
	Hr...	2				4				8				20			
Cysts treated with	Stage...	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
E. coli extract (normal cysts)		58	0	4	38	53	0	0	47	15	0	0	85	5	0	0	95
E. coli extract (ultrasonically treated cysts)		0	80	0	20	0	62	0	38	0	30	0	70	0	8	0	92
Normal cysts treated with *p*-CMB (10^{-3} M) for 1 hr; excystment with *E. coli* extract after the removal of *p*-CMB		100	0	0	0	100	0	0	0	79	0	21	0	70	0	20	10
Normal cysts treated with *p*-CMB (10^{-3} M) for 1 hr, and washed; excystment with *E. coli* extract after ultrasonic treatment		0	100	0	0	0	100	0	0	0	75	0	25	0	70	0	30
Normal cysts treated with emetine (10 mg./ml.) for 1 hr; excystment in *E. coli* extract after removal of emetine		100	0	0	0	100	0	0	0	100	0	0	0	50	0	0	50
Normal cysts treated with emetine (10 mg./ml.) for 1 hr, and washed; excystment with *E. coli* extract after ultrasonic treatment		0	100	0	0	0	100	0	0	0	100	0	0	0	55	0	45

A, B, C, and D as in Table 1.

Table 5. *Delay in excystment of normal cysts of Schizopyrenus russelli treated with emetine*

	% count of different stages after varying periods of treatment								
Hr...	4			8			24		
Cysts treated with Stage...	A	B	C	A	B	C	A	B	C
E. coli extract (normal cysts)	20	33	47	16	8	76	0	0	100
Emetine (0·1 mg./ml.) for 1 hr; excystment in *E. coli* extract after removal of emetine	17	36	47	13	22	65	0	0	100
Emetine (1 mg./ml.) for 1 hr; excystment in *E. coli* extract after the removal of emetine	34	32	34	10	32	58	0	0	100
Emetine (10 mg./ml.) for 1 hr; excystment in *E. coli* extract after the removal of emetine	100	0	0	91	0	9	52	0	48

A, Double-walled cysts; B, cysts in the process of excystment; C, trophozoites.

delay in excystment was still observed in the resulting single-walled cysts, as found with *p*-CMB (Table 4), showing that emetine also was able to permeate the outer cyst wall of normal double-walled cysts.

Table 6. *Delay in excystment of ultrasonically treated cysts of Schizopyrenus russelli treated with emetine*

	% count of different stages after varying periods of treatment															
Hr...	2				4				8				20			
Cysts treated with Stage...	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
E. coli extract (normal cysts)	10	0	8	82	5	0	0	95	1	0	0	99	1	0	0	99
E. coli extract (ultrasonically treated cysts)	0	70	0	30	0	39	0	61	0	26	0	74	0	4	0	96
Ultrasonically treated cysts treated with emetine (0·1 mg./ml.) for 1 hr; excystment in *E. coli* extract after the removal of emetine	0	100	0	0	0	90	0	10	0	36	0	64	0	10	0	90
Ultrasonically treated cysts treated with emetine (1 mg./ml.) for 1 hr; excystment in *E. coli* extract after the removal of emetine	0	100	0	0	0	100	0	0	0	84	0	16	0	70	0	30
Ultrasonically treated cysts treated with emetine (10 mg./ml.) for 1 hr; excystment in *E. coli* extract after the removal of emetine	0	100	0	0	0	100	0	0	0	96	0	4	0	85	0	15

A, B, C and D as in Table 1. Ultrasonic treatment for 3 min.

DISCUSSION

Ultrasonic treatment of *Schizopyrenus russelli* cysts gave single-walled structures. These single-walled cysts excysted in *Escherichia coli* extract as readily as the normal double-walled cysts. This suggests that the site of interaction with the excystment factors is located either on or within the inner cyst wall and that the excystment agents are able to permeate the outer cyst wall to trigger the excystment mechanism in normal cysts. The marked delay in excystment of single-walled cysts after treatment with *p*-CMB or emetine was similar to that obtained with normal cysts, and suggests that these agents are able to permeate the outer cyst wall. The delay in excystment depended on the concentration of *p*-CMB and emetine used.

The fact that *p*-CMB- and emetine-treated cysts were viable and could excyst after longer incubation in the bacterial extract shows that the damage caused by these agents is repairable by factors inherent within the cysts or in the excystment medium.

The authors' thanks are due to Dr B. N. Singh, Scientist-in-charge of the Microbiology Division, for helpful suggestions in the preparation of the manuscript. The authors are also thankful to Mr L. M. P. Singh and Mr R. B. Lal for technical assistance throughout these investigations.

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EXPLANATION OF PLATE

Fig. 1. Normal double-walled cysts of *Schizopyrenus russelli*.

Fig. 2. Ultrasonically treated single-walled cysts of *S. russelli* after the removal of the outer wall.

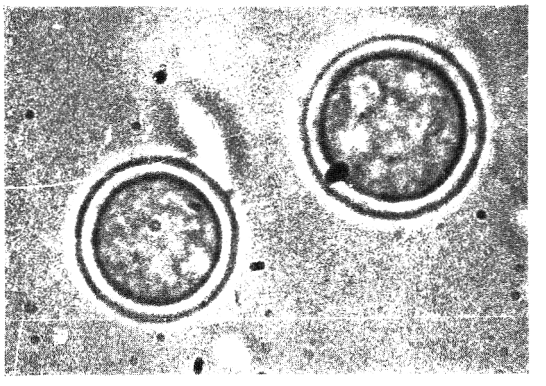

Fig. 1

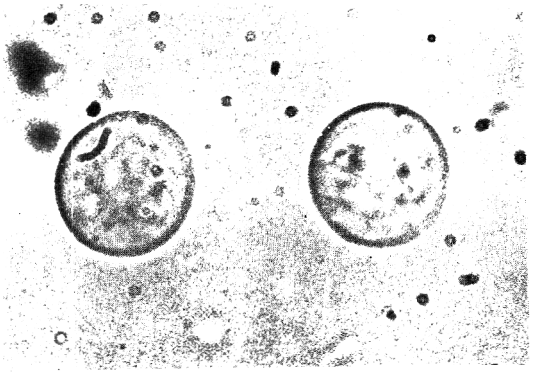

Fig. 2

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Control of Respiration and Nitrogen Fixation by Oxygen and Adenine Nucleotides in N_2-grown *Azotobacter chroococcum*

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SUMMARY

Oxygen uptake by *Azobacter chroococcum* (NCIB 8003) grown in continuous culture without fixed nitrogen and with a low mannitol concentration (2·5 g./l.) and treated with lysozyme and EDTA was inhibited by ATP but not by ADP; ADP frequently prevented inhibition by ATP. In preparations obtained by disrupting bacteria suspended in a mixture of defatted bovine serum albumin, sucrose and $MgCl_2$ in the French press, ATP inhibited oxygen uptake with either sodium succinate or sodium isocitrate as substrates and ADP prevented this inhibition; oxygen uptake with glucose-6-phosphate was inhibited by ATP or ADP. A form of respiratory control by nucleotides may thus occur in Azotobacter. Acetylene reduction (a measure of nitrogenase activity) by bacteria treated with lysozyme and EDTA was inhibited by ATP; this was attributed to inhibition of oxygen uptake by ATP causing inhibition of nitrogenase by oxygen. High oxygen solution rates inhibited nitrogenase in whole bacteria or in bacteria treated with lysozyme and EDTA; when the oxygen solution rate was lowered nitrogenase functioned immediately. These observations are probably expressions of processes which protect nitrogenase in whole bacteria from damage by oxygen.

INTRODUCTION

Inhibition by ATP of oxygen uptake and the prevention of this inhibition by ADP has been observed in mitochondria and described by Klingenberg & Schollmeyer (1960) and by Chance & Hollunger (1961). A similar type of respiratory control has not been found in bacteria, although stimulation of oxygen uptake by inorganic phosphate in particulate preparations from the following bacteria has been reported: *Micrococcus lysodeikticus* (Ishikawa & Lehninger, 1962); *Alkaligenes faecalis* (Scocca & Pinchot, 1965) and *Mycobacterium phlei* (Revsin & Brodie, 1967). Revsin and Brodie also reported that oxygen uptake was inhibited by ATP or ADP and stimulated by AMP in their preparations from *M. phlei* provided inorganic phosphate was present. Oxygen uptake by a particulate NADH oxidase from *M. tuberculosis* was stimulated by AMP or ADP; this stimulation did not depend upon added inorganic phosphate and was attributed to allosteric activation of the NADH oxidase by AMP (Worcel, Goldman & Cleland, 1965).

Respiratory control by ATP in mitochondria depends upon a high degree of physical intactness (see Lehninger, 1964); it is possible that a similar degree of intactness is necessary to show respiratory control by ATP in bacteria. Inhibition by ATP of

oxygen uptake in N_2-grown *Azotobacter chroococcum* treated with lysozyme and ethylene diamine tetracetic acid (EDTA) is reported in the first part of this paper.

Experiments with cell-free extracts indicate that nitrogen fixation, even in anaerobic organisms, is a reductive process (see Postgate, 1969). Dalton & Postgate (1967) found that moderate oxygen tensions inhibited the growth of N_2-grown but not NH_4-grown *Azotobacter chroococcum* and they suggested that oxygen inhibited nitrogen fixation in the growing organism. Aerobic nitrogen fixing bacteria must, when growing, possess mechanisms that protect the nitrogenase from oxygen damage; Dalton & Postgate (1969) suggested two mechanisms: oxygen removal by respiration (Azotobacter has exceptionally high QO_2 values: Williams & Wilson, 1954) and a conformational state that protects the nitrogenase from oxygen damage. Nitrogenase reduces acetylene to ethylene (Dilworth, 1966, Schöllhorn & Burris, 1967); this reaction can be used as a sensitive assay of nitrogenase activity. The action of ATP on acetylene reduction, together with its effect on oxygen uptake, by *A. chroococcum* grown in continuous culture, is described in the second part of this paper.

METHODS AND MATERIALS

Growth of organism. Azotobacter chroococcum (NCIB 8003) was grown in continuous culture (Baker, 1968) using a nitrogen-free medium containing 2·5 g. mannitol/l. and the trace elements prescribed by Dalton & Postgate (1969) at a dilution rate of 0·1 hr^{-1} at 30°. The culture had a working volume of approximately 500 ml. and was aerated by a 4 cm. magnetic stirring bar at 300 rev./min. under a stream of air (800 ml./min.). Under these conditions the population in the culture was oxygen-limited.

Acetylene reduction by whole bacteria or by bacteria treated with lysozyme and EDTA. Nitrogenase activity in growing *Azotobacter chroococcum* was very sensitive to environmental change: for example, organisms allowed to stand unaerated at room temperature for 5 min. showed severalfold less activity than organisms that were tested immediately after sampling; J. Drozd & J. R. Postgate (personal communication) observed a comparable 'switch-off' of activity which they attribute to aeration. Care was therefore taken that culture samples were handled identically throughout. Even so, no significance was attached to different acetylene reduction rates between experiments and only complete inhibition of acetylene reduction was accorded significance within experiments.

Acetylene reduction and oxygen uptake were assayed with 10 ml. samples from the continuous culture injected immediately after sampling, without aseptic precautions, through a side-arm into 50 ml. double side-armed Warburg manometer flasks stoppered with 'Suba Seal' closures, and containing the atmospheres and reagents described under Table 2 and Fig. 1, 3 and 4. The flasks and reagents were pre-incubated at 30° for 10 min. before adding the culture. Acetylene (1·0 ml.), produced by adding water to CaC_2, was added last; setting up took 5 to 10 sec. from the start of sampling the culture. Oxygen uptake was measured manometrically: 2 ml. gas samples from the manometer flasks (replaced with air pre-incubated at 20°) were assayed for ethylene by vapour phase chromatography on a 5 ft column of Porapak R (4 mm. internal diameter) at 45° with N_2 (50 ml./min.) as the carrier gas using a Pye 104 gas chromatography instrument with a flame ionization detector head.

Mechanically disrupted preparations. Azotobacter chroococcum (300 ml.) was

harvested at 30,000 *g* for 15 min. and resuspended in 3 ml. of 50 mM-tris (hydroxymethylaminomethane buffer (pH 7·4), containing 2% (w/v) bovine serum albumin, 0·3 M-sucrose and 0·1 M-$MgCl_2$ (a mixture that protects oxidative phosphorylating systems in adipose tissue: Aldridge & Street, 1968) and disrupted in the French pressure cell from 8000 lb./sq. in. Preparations contained approximately 5 mg. of bacterial protein/ml. Oxygen uptake in these preparations was measured manometrically or with a Clark oxygen electrode.

Dehydrogenase activities. These were measured by the rate of reduction of tetrazolium salt (Fahmy & Walsh, 1952): assays contained tetrazolium (2·4 mg.) in 50 mM-tris buffer (pH 7·4) with substrate (50 μmoles) and protein (0·5 mg.) and were run under argon at 30° in a shaker.

P/O ratios. These were determined by measuring the oxygen uptake and the esterification of inorganic phosphate in the presence of an ATP trap (glucose and hexokinase) over 20 min. Esterification of inorganic phosphate was measured as the amount of radioactive ^{32}P incorporated according to the method of Nielsen & Lehninger (1955). The radioactivity was measured with an IDL end window counter (lead castle type 710) at 540 volts.

Cytochrome spectra. These were measured in an oxygen atmosphere at room temperature on a Unicam SP 700 using a reflectance head. Approximately 2·4 mg. bacterial protein/ml. 0·1 M-tris (pH 7·4) were used.

Materials. All biochemicals, including hexokinase (E.C.2.7.1.1) and lysozyme (E.C.3.2.1.17) were purchased from Sigma Chemical Co. (London) Ltd.

RESULTS

Effects of nucleotides on oxygen uptake

Whole bacteria. ATP or ADP had no effect on oxygen uptake in whole organisms (Fig. 1*b*).

Bacteria treated with lysozyme and EDTA. ATP inhibited oxygen uptake but ADP did not (Fig. 1*a*). In some experiments ADP stimulated oxygen uptake while in 60% of cases equimolar ADP wholly, or partly, prevented inhibition by ATP.

Disrupted preparations. NADH and sodium succinate were used as key substrates to the respiratory chain to test the effect of adenine nucleotides on oxygen uptake with disrupted preparations. Two other dehydrogenases reported to be affected by adenine nucleotides were also tested: glucose-6-phosphate dehydrogenase (this enzyme, from several organisms, was inhibited by ATP; Schindler & Schlegel, 1969); iso-citric dehydrogenase (in mammalian systems this enzyme is inhibited by ATP and stimulated by ADP; see Mahler & Cordes, 1966). NADH and glucose-6-phosphate dehydrogenases were the two most active in disrupted preparations, reducing tetrazolium chloride five to 10 times as rapidly as succinic, isocitric and other dehydrogenases. The effects of ATP, ADP and phosphate ions on oxygen uptake with these four substrates are shown in Table 1. NADH oxidase appeared to be unaffected by ATP, ADP or a mixture of both over 20 min. However, observations with manometric experiments suggested that oxygen uptake in the first 3 min. was inhibited by ATP and this inhibition was prevented by ADP. When the system was examined with the oxygen electrode ATP completely inhibited oxygen uptake only over the first 30 sec. (Fig. 2). The presence of 5 mM-KF extended the inhibition by ATP to between 1 and

2 min. Oxygen uptake with succinate or with isocitrate was partly inhibited, usually between 20 and 50%, by ATP; this effect was prevented by ADP. Glucose-6-phosphate dehydrogenase was inhibited by ATP, ADP or inorganic phosphate. Inorganic phosphate had no effect upon oxygen uptake with any of the other three substrates.

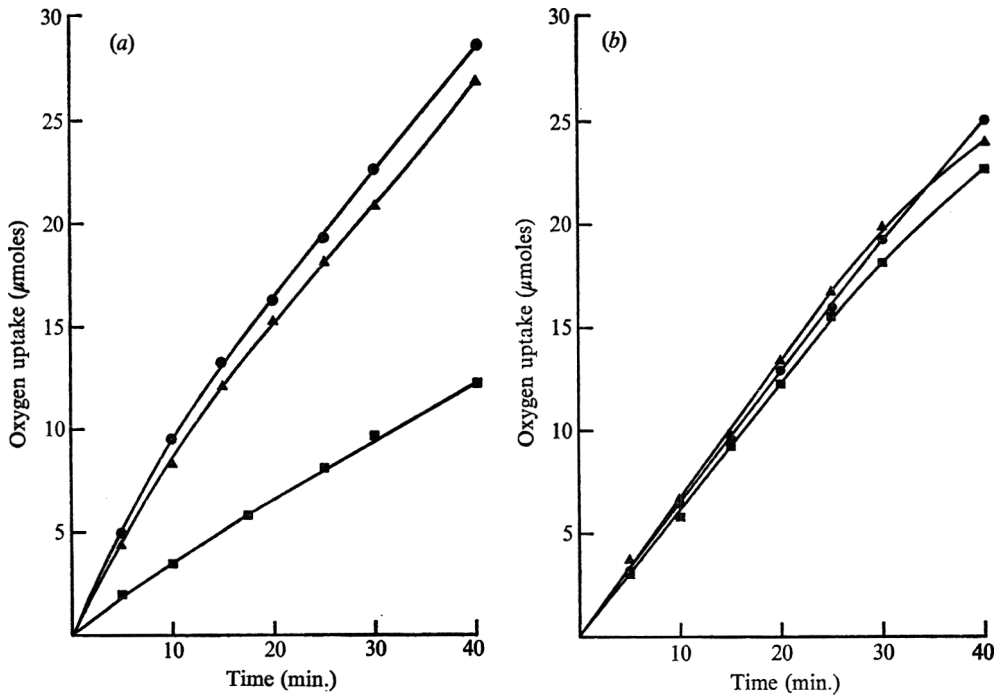

Fig. 1*a*. Effect of ATP or ADP upon oxygen uptake by *Azotobacter chroococcum* treated with lysozyme and EDTA. Conditions: as described under Table 2 but in addition the main compartment contained lysozyme (1 mg. in 1 ml. H_2O), 0·2% EDTA (0·25 ml.) and nucleotides (10 μmoles). ▲——▲, control; ■——■, control + ATP; ●——●, control + ADP.

Fig. 1*b*. As for 1*a* but water (1·25 ml.) replaced lysozyme and EDTA.

Table 1. *Effect of adenine nucleotides on oxygen uptake with disrupted preparations from Azotobacter chroococcum*

	μmole O_2 absorbed in 20 min.			
Substrate (sodium salts)	Glucose-6-phosphate	Isocitrate	Succinate	NADH
(Additions)	6·6	5·8	6·1	6·9
ATP	3·25	4·4	4·8	6·5
ADP	3·4	5·7	6·8	7·25
AMP	3·6	6·0	6·4	6·8
ATP + ADP	2·3	5·2	5·6	7·25
Phosphate ions	2·15	5·7	5·7	6·3

Conditions: Experiments were run in 15 ml. Warburg manometer flasks at 30° under air in water (2·4 ml.) containing Tris buffer (pH 7·4) (120 μmoles) NAD (1 μmole) and disrupted *A. chroococcum* in the following concentrations: 0·75 mg. and 0·83 mg. with isocitrate or succinate as substrate, 0·3 mg. and 0·28 mg. with glucose-6-phosphate or NADH. Substrates (50 μmoles, except NADH, 10 μmoles) and nucleotides (10 μmoles) or phosphate ions (50 μmoles) were added from the side-arm to start the experiment. The centre well contained 40% KOH (0·1 ml.).

Esterification of inorganic phosphate. Azotobacter chroococcum treated with lysozyme and EDTA esterified inorganic phosphate; this esterification was stimulated approximately 2-fold by added ADP (10 μmoles) although no significant increase in oxygen uptake occurred. The highest P:O ratio was 0·25 measured from the total oxygen uptake and total esterification with ADP. Disrupted preparations also esterified inorganic phosphate with a P:O ratio of 0·52. It seems unlikely at face value that mechanically disrupted cells should yield higher P:O ratios than cells suffering more gentle damage by lysozyme and EDTA. Perhaps some of the oxygen was taken up by cells that were not sufficiently damaged to allow access of ADP; this would yield an exaggerated oxygen uptake compared with the stimulation of phosphate esterification by ADP.

Effects of nucleotides on cytochrome spectra. The effect of ATP and/or ADP on the steady level of cytochromes in disrupted preparations was measured with NADH or sodium succinate as the electron donor. ATP (20 μmoles) caused up to 20% reductions in the α and β peaks of the *b* and *c* cytochromes; ADP did not have this effect. Adding ADP to preparations to which ATP had previously been added did not significantly change the heights of these peaks. Cytochromes of the *a* type were not observed in these preparations.

Table 2. *Effect of shaking rates upon oxygen uptake and acetylene reduction by Azotobacter chroococcum*

Amplitude (cm.)	Sulphite oxidation value, m-mole O_2 l./hr	Oxidation absorbed, μmoles in 40 min.	Ethylene formed, μmoles in 40 min.
0	5	4·25	32
1·7	9	12·7	74
4·7	17	23·1	645
7·9	22	25·2	5

10 ml. of culture containing 0·015 mg. bacterial protein/ml. was injected into a manometer flask containing glucose in the main compartment and KOH in the centre well under argon-oxygen (4:1) containing 4% acetylene at 30°. The shaking rate was 90 strokes/min.; gas-uptake measurements were started after 2 min.

Effect of oxygen and nucleotides on acetylene reduction

Whole bacteria. Oxygen uptake and acetylene reduction were measured at four shaking rates giving sulphite oxidation rates (Cooper, Fernstrom & Miller, 1944) between 5 and 22 mmoles O_2/l./hr. (Table 2). While oxygen uptake increased at each shaking rate, acetylene reduction first rose and then dropped to zero at the highest shaking rate. In a second experiment, bacteria tested at the highest shaking rate produced no ethylene, but on changing to the second highest shaking rate acetylene reduction started immediately (Fig. 3). ATP or ADP had no effect upon acetylene reduction by whole organisms.

Organisms treated with lysozyme and EDTA. These preparations behaved as did whole bacteria when subjected to different shaking rates in the absence of added nucleotides. In addition, oxygen uptake and acetylene reduction were inhibited by ATP (Fig. 4): at the higher shaking rate (sulphite oxidation rate of 17m-moles O_2/l./hr) ATP partly inhibited oxygen uptake but completely inhibited acetylene reduction (Fig. 4*a*); at the lower shaking rate (sulphite oxidation rate of 9 m-moles O_2/l./hr)

neither of these parameters was much affected (Fig. 4*b*). ADP, on the other hand, occasionally stimulated oxygen uptake, but in most experiments it had no effect even when the same concentration of ATP completely inhibited oxygen uptake. ADP had no effect on acetylene reduction. Equimolar ADP, when added with ATP, partially prevented the inhibition by ATP of oxygen uptake and occasionally partly restored acetylene reduction. The ADP used in these experiments usually contained 10 to 20% AMP (judged by visual survey of thin layer chromatograms under ultraviolet light); however, increasing the amount of ADP did not significantly alter the situation: AMP was ineffective in comparable experiments.

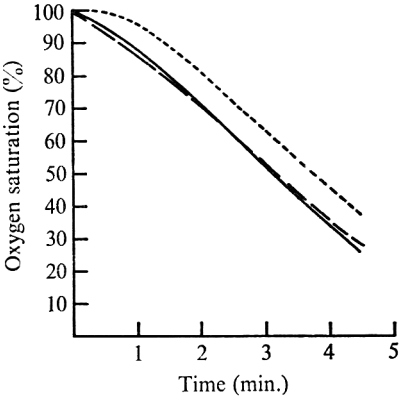

Fig. 2

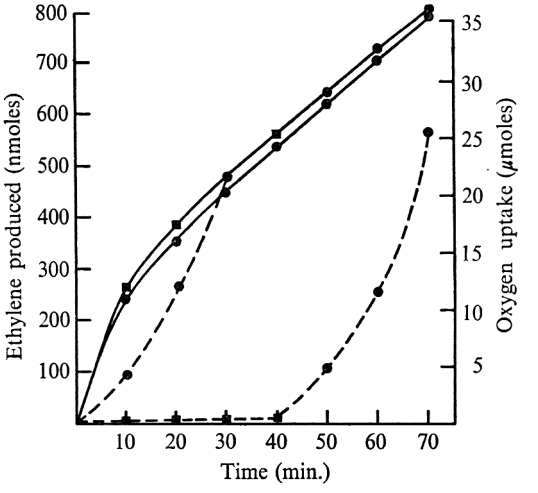

Fig. 3

Fig. 2. Effect of ATP or ADP on oxygen uptake with disrupted preparations from *Azotobacter chroococcum*. Conditions: NADH (10 μmoles) tris buffer (pH 7·4) (80 μmoles) and nucleotides (10 μmoles) were incubated in water in the electrode cup at 30° for 5 min. Enzyme protein (0·3 mg.) in tris buffer, bovine serum albumin, sucrose, $MgCl_2$, mixture (0·5 ml.) was incubated at 30° in a water bath for 5 min. and added to start the reaction. —— Control or control + ATP + ADP; – – –, control + ATP; — — —control + ADP.

Fig. 3. Effect of changing the shaking amplitude on oxygen uptake and acetylene reduction in *Azotobacter chroococcum*. Conditions: as described under Table 2 using the two highest amplitudes (4·7 and 7·9 cm.). After 40 min. the amplitude of 7·9 cm. was reduced to 4·7 cm. ■——■, O_2 uptake at amplitude 7·9 cm.; ●——●, O_2 uptake at amplitude 4·7 cm.; ■– – –■, ethylene produced at amplitude 7·9 cm.; ●– – –●, ethylene produced at amplitude 4·7 cm.

Disrupted preparations. Acetylene reduction by these preparations was low and variable and the effect of ATP was inconclusive.

Effect of cell treatment and carbon source. When bacteria were treated with hyaluronidase (50 μg./ml.) sodium lauryl sulphate (0·1% w/v) or sodium deoxycholate (0·1% w/v) under conditions similar to those used with lysozyme and EDTA, acetylene reduction was enhanced 3- to 4-fold over an untreated control. However, only bacteria that were treated with hyaluronidase or sodium lauryl sulphate showed inhibition of acetylene reduction by ATP. The concentration of carbon substrate in the growth medium influenced the concentration of EDTA necessary to allow ATP inhibition of

acetylene reduction: organisms grown with 2·5 g. and 5 g. mannitol/l. needed 0·005% and 0·01% respectively; organisms grown in batch culture with 20 g. sucrose/l. needed 0·02% EDTA.

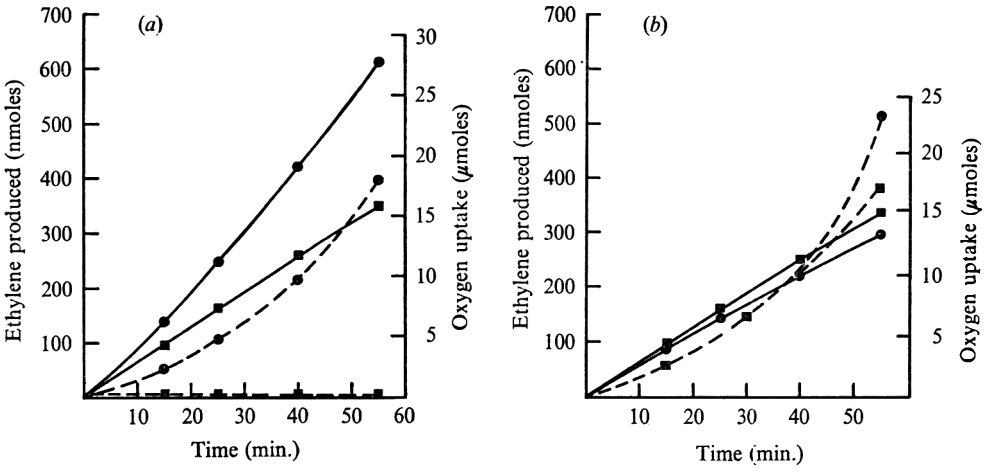

Fig. 4. Effect of ATP on oxygen uptake and acetylene reduction by *Azotobacter chroococcum* treated with lysozyme and EDTA when shaken at two different amplitudes. Conditions: as described under Fig. 1*a* and using shaking amplitudes of 4·7 (Fig. 4*a*) and 1·7 cm. (Fig. 4*b*). ●——●, O_2 uptake without added nucleotide; ■——■, O_2 uptake with added ATP (10 μmoles); ●- - -●, ethylene formed without added nucleotide; ■- - -■, ethylene formed with added ATP (10 μmoles).

DISCUSSION

Adenine nucleotides and respiration in Azotobacter. In relatively intact preparations of *A. chroococcum*, i.e. organisms treated with lysozyme and EDTA, ATP showed some of the properties to be expected if it is involved in a control process analogous to respiratory control in mitochondria; it inhibited oxygen uptake and its action was antagonized by ADP. The converse effect, stimulation of oxygen uptake by ADP was not unequivocally demonstrated. The absence of an effect of added phosphate ions upon oxygen uptake could be because phosphate was already present in the test medium. These experiments provide some evidence that ATP and ADP exert a control analogous to that in mitochondria; convincing evidence for the analogy would require experiments with more highly disrupted bacterial preparations: attempts to obtain such preparations were unsuccessful and are not reported here. If the principle of ATP/ADP control in *A. chroococcum* is accepted the question which then arises is how does ATP inhibit oxygen uptake in relatively intact *A. chroococcum*? In preparations obtained by disrupting bacteria suspended in bovine serum albumin, sucrose and $MgCl_2$ in a French pressure cell, ATP affected oxygen uptake in three different ways: it inhibited (*a*) glucose-6-phosphate dehydrogenase; (*b*) isocitric dehydrogenase and (*c*) succinoxidase and, to a lesser extent, the NADH oxidase system. Any of these systems would be affected by ATP in more intact preparations and the relevant one for respiratory control would depend on whichever system was rate-limiting to oxygen. A fourth possibility arises from the work in part 2. ATP markedly inhibited nitrogenase activity in bacteria treated with lysozyme and EDTA, so one could argue that this was

the primary effect of ATP; that inhibited nitrogenase might, in some way, lead to reduced oxygen uptake. This possibility is not unreasonable if respiration is regarded as partly concerned with protecting nitrogenase from oxygen damage (Dalton & Postgate, 1969) but is made unlikely because (i) at a high shaking rate nitrogenase was completely inhibited but oxygen uptake increased over that at a lower shaking rate (see Table 2); (ii) ATP did not inhibit acetylene reduction or oxygen uptake in conditions where the oxygen solution rate was low, suggesting that the effect of ATP was not primarily upon acetylene reduction. Control via glucose-6-phosphate dehydrogenase is attractive because this is a key enzyme in Azotobacter; sugars are metabolized through the hexose monophosphate shunt and the Entner–Douderoff pathway (Mortenson & Wilson, 1954; Johnson & Johnson, 1961; Still & Wang 1964). However, the inhibition by ATP of oxygen uptake in relatively intact preparations is unlikely to be due to inhibition of glucose-6-phosphate dehydrogenase since there was no comparable inhibition by ADP. NADH oxidase may be the relevant site because so many dehydrogenases are linked to this enzyme and it was one of the most active dehyrogenases in *A. chroococcum*. In disrupted preparations it was apparently inhibited by ATP only during the first minute of oxygen uptake; this could be because ATPase rapidly produced sufficient ADP to relieve ATP inhibition; the prolonged inhibition by ATP in the presence of KF supports this suggestion. However, ATPase activity was not observed in preparations treated with lysozyme and EDTA, so if inhibition does occur through NADH oxidase one must make the additional assumption that the sites of ATPase and ATP inhibition are physically separated in these preparations. ATP inhibition of oxygen uptake was more often observed than stimulation by ADP and significant reversal of inhibition occurred only with equimolar amounts of ADP. These observations are in sharp contrast to respiratory control experienced in mitochondria and make it unlikely that the two systems are similar.

Control of aerobic nitrogen fixation. Accepting that acetylene reduction is a measure of nitrogen-fixing ability of the population studied, then the inhibition of acetylene reduction at the highest oxygen solution rate agrees with the evidence of Dalton & Postgate (1967) that high oxygen tensions inhibit nitrogenase activity. If the primary effect of ATP in organisms treated with lysozyme and EDTA is upon oxygen uptake rather than on nitrogenase as was argued earlier, then ATP inhibition of acetylene reduction was a consequence of its lowering the rate of oxygen uptake; this view agrees with the proposal by Dalton & Postgate (1969) that oxygen uptake protects against oxygen inhibition of nitrogenase. Nitrogenase activity in cell-free extracts of *Azotobacter chroococcum* can be damaged by exposure to oxygen (Kelly, 1969) and this damage is not reversed by replacing the oxygen with argon; however, oxygen inhibition of nitrogenase in whole bacteria or in organisms treated with lysozyme and EDTA was reversed without lag by reducing the oxygen solution rate. This indicates that the nitrogenase was undamaged, though inactive, at high oxygen tensions, and that synthesis of new nitrogenase was unnecessary for restoration of acetylene reduction at lower oxygen tensions. These findings support the suggestion made by Dalton & Postgate (1969) that a second, perhaps physical, nitrogenase-protecting mechanism exists which causes the enzyme to become 'switched off' at high oxygen tensions.

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Autolysis of Extracellular Glucans Produced *in vitro* by a Strain of *Claviceps fusiformis*

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SUMMARY

Claviceps fusiformis usually produces a stable viscous glucan during submerged culture fermentations. A new strain 139/2/1 G of the fungus, which subsequently autolysed this glucan to glucose, was studied and the autolysis ascribed to a constitutive $\beta 1 \rightarrow 3$ glucanase and a β-glucosidase which were detected as soon as the fungal hyphae differentiated to a sclerotial form. The glucanase production followed a sigmoid pattern, reaching a maximum within 12 days, and the liberated glucose contributed to renewed growth towards the end of the fermentation. A sucrase and a maltase were also detected. Maximum glucan autolysis was achieved by using a large spore inoculum. This maintained minimal viscocity throughout the fermentation and, by maintaining adequate aeration, has since facilitated ergot alkaloid production by the organism.

INTRODUCTION

Davis, Rhodes & Shulke (1965) described the significant decrease of extracellular glucan in submerged cultures of *Plectania occidentalis* and a *Helotium* sp. towards the end of the fermentation and they presumed that this was due to the secretion of an 'exocellular glucanase' by the organism. However, no autolysis was observed during studies on the structurally similar glucan produced by *Claviceps fusiformis* (Buck, Chen, Dickerson, & Chain, 1968). The production of alkaloids in submerged cultures by *C. fusiformis* is adversely affected by the formation of the viscous glucan, and during investigations on the alkaloid fermentation process a new variant strain (139/2/1 G) arose spontaneously and was selected for its ability to autolyse the extracellular glucan. The usual non-autolysing strains yielded a stable and highly viscous growth within 7 days, whereas the new variant autolysed completely after a further 7 to 10 days. In view of the structure of the extracellular glucan, its autolysis by strain 139/2/1G has been investigated to determine whether this is due to the activity of glucanases similar to those reported by Reese & Mandels (1959) for a variety of fungi.

METHODS

Origin of the Claviceps fusiformis isolate used. The non-autolysing isolate designated 139/2/1 was obtained from *C. fusiformis* sclerotia collected in Senegal in November 1965 and kindly supplied by the Director, Centre de Recherches Agronomiques, Senegal.

Submerged culture procedures

Media. Cultures were maintained on agar (2% w/v) slopes of the following medium modified from Stoll *et al.* (1954): sucrose, 100 g.; L-asparagine, 10 g.; $Ca(NO_3)_2.4H_2O$, 1 g.; KH_2PO_4, 0·25 g.; $MgSO_4.7H_2O$, 0·25 g.; KCl, 0·125 g.; $FeSO_4.7H_2O$, 0·033 g.; $ZnSO_4.7H_2O$, 0·027 g.; L-cysteine hydrochloride, 0·01 g.; yeast extract, 0·1 g.; distilled water, 1 l.; pH, 5·2 adjusted with NaOH. For liquid culture 100 ml. of the standard sucrose + asparagine liquid medium was sterilized in 500 ml. Erlenmeyer flasks at 106° for 15 min. A modified medium with 10% mannitol (w/v) instead of the sucrose was also used.

Seed cultures. A spore suspension (2 to 6×10^9 spores/ml.) was prepared from 14-day agar cultures and 1 ml. inoculated into each 500 ml. flask. Usually the fermentation was completed without further transfer, but in some experiments the first (seed) stage flasks were subsequently used as a source of inoculum for a second stage. Where mycelial inocula were used an aqueous suspension was prepared by crushing the sparsely sporing mycelium from 3-day agar cultures.

Flasks were incubated at 27° on a rotary shaker (Buck *et al.* 1968). For sampling, duplicate flasks were removed at intervals from the shaker and the course of fermentation was followed by the following parameters: microscopic examination and measurement of pH value, sugar utilization, nitrogen utilization, dry weight of mycelium and polysaccharide. All experiments were at least duplicated.

Analytical techniques

Mycelial dry weight. A sample of culture was centrifuged at 2500 *g* for 20 min. and the supernatant fluid removed. The mycelium was washed twice with distilled water, filtered onto a weighed filter paper and dried to constant weight at 70°. Highly viscous cultures were diluted 10 times before processing.

Polysaccharide. The supernatant fluid and washings from mycelial dry-weight determination were stirred with an equal volume of absolute ethanol and the precipitated polysaccharide collected on a glass rod and dried at 70° to constant weight.

Nitrogen. Samples obtained after distillation in a micro-Kjeldahl apparatus were assayed in a Technicon autoanalyser at 625 nm. using the alkaline phenol reagent of Garza & Weissler (1967).

Glucose was specifically determined by a glucose oxidase method modified for the autoanalyser by Marks & Lloyd (1963).

Sucrose was estimated as glucose by the above method after it had been hydrolysed at 37° for 30 min. by invertase (British Drug Houses, Poole, Dorset; 0·5 units/ml. of incubate).

Other analytical techniques used were substantially as described by Buck *et al.* (1968). The following changes and additions were made.

Protein estimation. Protein in solution was estimated by determining the extinction of the sample at 260 and 280 nm. The concentration was then obtained from a nomograph compiled by Adams (California Corporation for Biochemical Research) from the data of Warburg & Christian (1942).

Column chromatographic procedure. The method used was similar to that described by Chesters & Bull (1963). An aqueous slurry of 10 g. diethylaminoethyl (DEAE) cellulose was poured into a glass column and eluted with 200 ml. of N-NaOH to

remove impurities. The column was then washed until free from NaOH and equilibrated at 4° with 6 l. of 1 mM-phosphate buffer (pH 5·6); the final column size was 1·4 cm. diam. × 28 cm. The acetone powder containing the enzymes (60 mg.) was solubilized at 4° for 2 hr in 5 ml. of 1 mM-phosphate buffer (pH 5·6) and applied to the column. The column was eluted with a linear gradient of buffer at pH 5·6 increasing in ionic strength from 1 to 150 mM, prepared according to the method of Bock & Ling (1954); the flow rate was approx. 30 ml./hr; 5 ml. fractions were collected. The fractions were dialysed overnight at 4° against 1 mM-phosphate buffer (pH 5·6) and, after their protein content had been determined, were lyophilized.

Enzymic methods. Acetone powders of the enzymes in the culture medium were prepared at 4° as follows. After the mycelium had been removed, the culture medium was dialysed for 24 hr against 5 mM-acetate buffer (pH 5·2, 10 l./100 ml. of medium was used). The dialysed medium was then mixed slowly with two volumes of ice-cold acetone; glucan, when present, was removed at this stage with a glass rod. The protein from the resulting mixture was allowed to sediment overnight and was then isolated by decantation and centrifugation at 2500 ***g***, and dried in vacuum over $CaCl_2$.

All enzyme reactions were made according to the recommendations of the International Union of Biochemistry. Reactions were done at 30° in 0·03 M-acetate buffer (pH 5·2). Substrate concentrations were 1% w/v, unless otherwise stated.

Glucanase activity was assayed by its action on the [U-^{14}C]glucan and determining the radioactivity of the products (Buck *et al.* 1968).

The activity of β-glucosidase was determined by its action on *p*-nitrophenyl-β-D-glucopyranoside and measuring the liberation of *p*-nitrophenol at 410 nm. Also, β-glucosidase activity was assayed by its action on [U-^{14}C]gentiobiose (prepared by the action of the glucanase on [U-^{14}C]glucan) and on unlabelled gentiobiose, whence the product was determined by radioactivity and by glucose oxidase, respectively.

Sucrase activity *in vivo* was assayed by harvesting the fungal tissue, washing it briefly in 0·03 M-acetate buffer containing 5, 10 or 30% sucrose and then suspending it in its original culture volume in the same sucrose + buffer solution as used for the washing. The flasks were replaced on the shaker and 10 ml. samples taken over a period of 24 hr, filtered, and the sugars in the filtrate separated and identified by chromatography (Buck *et al.* 1968).

Preparative and counting techniques with ^{14}*C.* In addition to counting from paper, liquid samples were also counted. These samples, obtained by fractionation procedures made on tissue (see below), were placed in liquid scintillant (6 g. of CIBA butyl-PBD scintillator + 50 g. naphthalene/l. toluene) and converted to a single-phase system by adding 2-methoxyethanol (methyl cellosolve).

Procedure for extraction of fungal tissue. The procedure followed was a modification of that described by Roberts *et al.* (1955) for *Escherichia coli*, using a ratio of 5 ml. extractant to 1 g. fresh weight of fungal tissue. The tissue was washed twice briefly in 0·01 M-acetate buffer, (pH 5·2); the washings were discarded and the washed tissue crushed in an X-press (AB Biox, Sweden). The crushed material was then extracted successively as follows: (*a*) For 10 min. at 4° in 5% trichloracetic acid (TCA) to remove low molecular weight substances. (*b*) For 30 min. at 45° in 75% ethanol in water to remove lipid (and some protein). (*c*) For 15 min. at 45° in 75% ethanol (v/v) in water + ether (1 + 1) to remove remaining lipid. (*d*) For 30 min. at 95° in 5%

TCA to remove nucleic acid. (*e*) The residue, containing insoluble protein and polysaccharide, was washed in ether to remove TCA and dried in vacuum over $CaCl_2$. The TCA was removed from the extracts by shaking them twice with ether.

Hydrolysis of solid residue. This was done at 110° overnight in 6 N-HCl in sealed tubes. Approx. 1 ml. HCl was used/5 mg. sample. The hydrolysates were neutralized with NaOH.

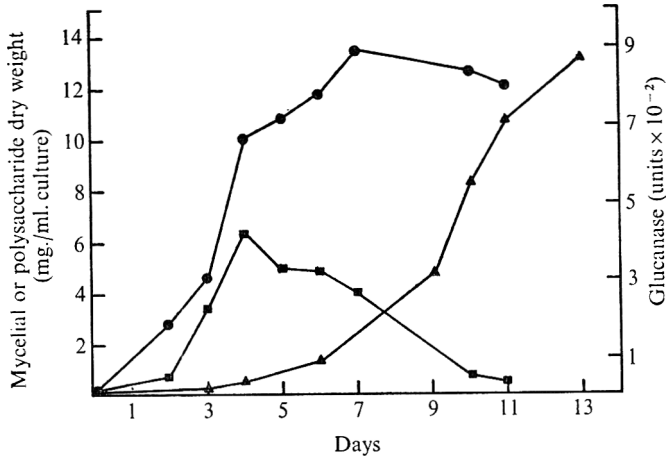

Fig. 1. *Claviceps fusiformis* strain 139/2/1 G. Course of fermentation in shake-flasks: ●, mycelium; ■, polysaccharide; ▲, β-glucanase.

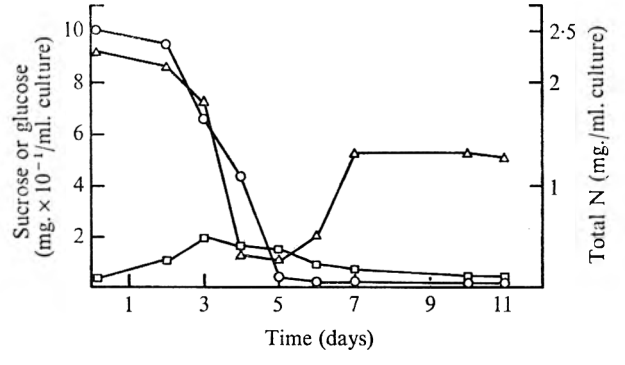

Fig. 2. *Claviceps fusiformis* strain 139/2/1 G. Course of fermentation in shake-flasks (continued from Fig. 1): ○, sucrose; □, glucose; △, total nitrogen.

RESULTS

Course of fermentation of Claviceps fusiformis

The course of a typical fermentation of the *Claviceps fusiformis* strain used, during which glucan was autolysed, is shown in Fig. 1 and 2. The well-buffered medium remained in the range pH 5·2 to 5·8. Preliminary studies indicated an optimum spore inoculum of 2 to 8 × 10^9 spores/100 ml. medium; this gave well replicated growth and, subsequently, the most rapid glucan autolysis. Mycelial growth, as determined by dry weight, progressed through the usual lag and exponential phases, but within 1 to

2 days of entering the stationary phase a significant resurgence of growth occurred which coincided not only with decreasing viscosity related to glucan autolysis but also with release into the medium of nitrogenous products from the normal autolysis of the oldest cells. It is suggested that the nitrogen source, being no longer limiting, allowed a further short phase of growth for which ample glucose was available (see below).

During the first 3 to 4 days of growth germinating spores formed long thin hyphae which sporulated to give both micro- and macro-spores. Fragmentation of these sphacelial hyphae (Fig. 3), rapid growth, and the synthesis of glucan resulted in a thick

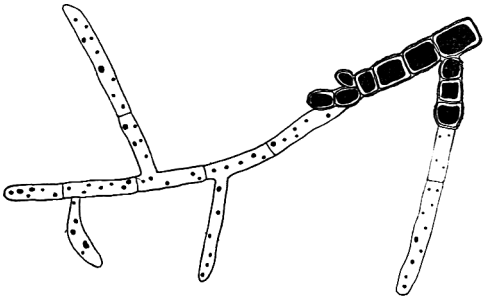

Fig. 3

Fig. 4

Fig. 3. *Claviceps fusiformis* strain 139/2/1 G. Typical mycelial fragment from 4-day submerged culture showing few fat droplets in younger sphacelial cells and large fat reserves in older cells which have differentiated to a sclerotial form.

Fig. 4. *Claviceps fusiformis* strain 139/2/1 G. Typical mycelial fragment from 7-day-old submerged culture composed solely of sclerotial cells.

viscous culture within 4 days of inoculation, after which sphacelial growth and sporulation declined. About 3 days after inoculation the older cells at the centre of the small groups of sphacelial hyphae became thicker, filled with fat droplets and progressed morphologically to resemble the compact plectenchymatic tissue of natural sclerotia, suggesting that this represented a diffuse change in growth form of this plant parasite from sphacelial to sclerotial similar to that occurring *in vivo*. Further, hyphal fragments also developed directly to form abundant short-branched sclerotial hyphae (Fig. 4). The initial autolysis of extracellular glucan was closely associated with the appearance of sclerotial cells in the culture, suggesting that the production of the factor responsible for glucan autolysis may be associated with sclerotial cells, whereas the glucan synthesis was clearly associated with sphacelial cells.

The sucrose content of the culture filtrate decreased rapidly, greatly in excess of that which one might expect to be required for growth and respiration alone. This discrepancy was investigated and the rapid disappearance of sucrose was shown to be due to the activity of a cell-associated sucrase. This enzyme split sucrose, liberating glucose only and forming oligofructosides by transfer. The liberation of free glucose was associated with the increase in mycelial dry weight and glucan. While the sucrose was being depleted, the glucose also diminished after the third day but did not disappear completely, because growth was then being limited by the very low asparagine content. Concurrently, glucose was also released by glucan hydrolysis which later provided the carbon source for the secondary growth phase from day 7, noted above.

Incorporation of glucose from the medium during the secondary growth phase was further demonstrated by injecting into flasks 150 μC of [U-^{14}C]glucose (3 ml.; 2·9 mC/mM), sterilized by Millipore filtration, on the 7th day and harvesting the flasks at

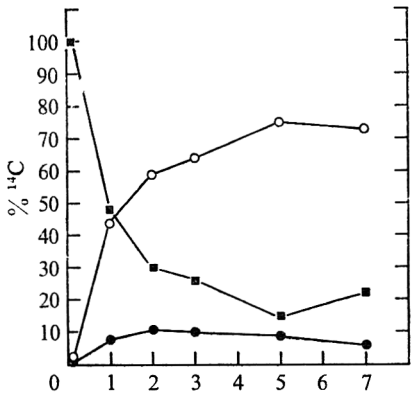

Fig. 5

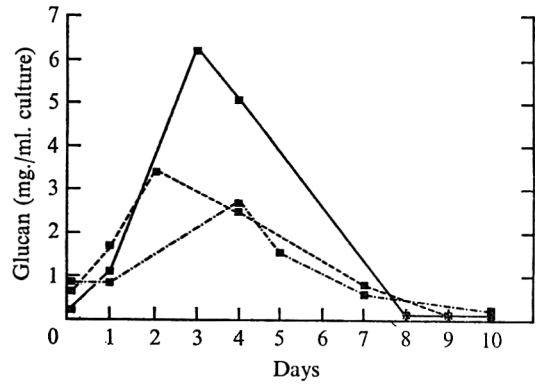

Fig. 6

Fig. 5. Incorporation of ^{14}C from [U-^{14}C]glucose into the mycelium of *Claviceps fusiformis* strain 139/2/1 G during late fermentation (autolysis of glucan): ■, culture filtrate; ●, mycelium and glucan; ○, respired (by substraction).

Fig. 6. *Claviceps fusiformis* strain 139/2/1 G. Effect of age of seed stage inoculum on glucan content of second-stage fermentation: ■——■, 3-day-old inoculum; ■ – – – ■, 5 day-old inoculum; ■ – · – · – ■, 7-day-old inoculum.

Table 1. *Claviceps fusiformis, strain 139/2/1 G: incorporation of* ^{14}C (μC) *from* [*U-*^{14}C]-*glucose into various mycelial fractions during late fermentation (glucan autolysis).*

[U-^{14}C]glucose (3 ml. containing 150 μC) was aseptically introduced into each flask during culture on the seventh day. Flask contents were harvested and fractionated as described in Methods. Respired values were obtained by subtraction.

	Time after introduction of [U-^{14}C]glucose (days)					
Fraction	0	1	2	3	5	7
a	0	4·9	6·0	4·3	5·7	1·7
b	0	1·7	2·4	2·6	2·1	1·6
c	0	0·67	1·0	1·2	1·1	1·0
d	0	0·72	1·4	1·7	1·7	0·80
e	0	1·4	1·8	2·3	2·0	1·8
Spores	0	0·71	0·71	0·70	0·23	0·10
Glucan	0	1·1	0·70	0·43	0·21	0·34
Filtrate	150	71	46	40	24	33
Respired	0	68	90	97	113	110

intervals up to 14 days. The tissue was extracted as described above and assayed together with the culture medium for ^{14}C content. The results are given in Table 1, and Fig. 5, which show that the isotope was rapidly incorporated into all parts of the tissue to an extent that exceeded 10% by the 9th day.

Effect of age of seed stage on the glucan content during the second stage of fermentation

Seed flasks, inoculated as usual with spores, were grown for 3, 5 and 7 days and 10 ml. samples were transferred to duplicate second-stage flasks. Flasks inoculated with a 3-day seed showed an increase and then a decrease of glucan during fermentation (Fig. 6) similar to that occurring in the first stage (Fig. 1), whereas older inocula yielded progressively less glucan.

Comparison of spores and mycelia of different strains as inocula for optimum glucan autolysis

An optimum spore concentration ($> 10^9$ spores/ml.) for the inoculum of seed or first-stage flasks had been determined in preliminary investigations and this ensured reproducibility, good growth and a decline in glucan content after 5 days. An optimum (2×10^9 spores/ml.) and a low (2×10^4 spores/ml.) spore inoculum level were compared with non-sporing mycelial inocula comprising similar numbers of constituent cells/ml. For comparison the high glucan-yielding parent strain (139/2/1) was used at the same high inoculum levels (Table 2). A low spore inoculum had a longer lag phase and

Table 2. *Claviceps fusiformis strain 139/2/1G: growth and glucan content of 13-day shake cultures inoculated with spores or mycelial fragments at various concentrations*

Inocula prepared as described in Methods

Strain	Inoculum	Mycelium (mg./ml. culture)	Polysaccharide (mg./ml. culture)	pH value	Morphology
139/2/1G	2×10^9 spores	14·9	0·90	6·2	sclerotial
	2×10^4 spores	11·2	1·44	5·5	sclerotial where growth good: sphacelial where growth poor
	2×10^9 mycelial	13·4	1·14	5·8	sclerotial
	2×10^4 mycelial	16·3	2·08	5·9	sclerotial
139/2/1	2×10^9 spores	24·0	7·7	5·4	mainly sphacelial

yielded results not reproducible, whereas a similar number of cells in mycelial form had a much shorter lag phase and subsequently grew well. The viscocity of fermentations of strain 139/2/1 G after 5 to 7 days incubation indicated vigorous glucan formation and, whether established from spore or mycelial inocula, the glucan content after 13 days represented the residue remaining after considerable autolysis of the polysaccharide. There was a correlation between the existence of a sclerotial growth-form during the latter stages of the fermentation and the autolysis of glucan. Conversely, strain 139/2/1, in which no noticeable glucan autolysis occurred, grew principally in a non-sclerotial or sphacelial form in spite of an inoculum which, in the autolysing strain, was optimum for glucan autolysis. The ability to autolyse glucan during the latter stage of fermentation was principally a characteristic of the new strain but was best expressed where a high inoculum was used.

Enzyme investigations

Action of extracellular enzymes on carbohydrates. The extracellular enzymes extracted as an acetone powder were incubated at a final concentration of 0·25% with several carbohydrates individually, and samples were taken over periods up to 24 hr. The extracellular enzymes hydrolysed maltose, β-glucosides and β 1→3 linked glucans; 10% of the maltose was hydrolysed per hour, the β-glucosides were almost completely hydrolysed in 2 h; the glucanase activity was 5 to 10% of that of the exo-β 1→3 glucanase (Reese & Mandels, 1959; see Buck *et al.* 1968) when incubated under the same conditions. There was also a noticeable action of the enzyme mixture on sucrose; by 24 hr approx. 50% of the original substrate had been broken to glucose and oligosaccharides. Since no fructose was detected, the action appeared to be that of a sucrase giving fructosylsucroses by transfer (see above) rather than an invertase which produced mainly glucose and fructose. The pH optimum was broad in each case: for the maltase, β-glucosidase, and β-glucanase it was between pH 5·0 and 5·5 and for the sucrase between pH 5·5 and 6·0.

Table 3. *Claviceps fusiformis 139/2/1 G: comparison of the effect of sucrose and mannitol as carbon sources on the yields of mycelium and polysaccharide and on the activity of extracellular enzymes*

Cultures were harvested at 8 days and acetone powders of the extracellular enzymes prepared. The enzymes were assayed at 30° with 1% (w/v) substrate and 0·1% (w/v) acetone powder. Samples were taken at intervals over 5 hr and assayed as in Methods. Results are expressed as % of substrate (cellobiose for β-glucosidase and glucan for β-glucanase) hydrolysed/hr/mg. acetone powder.

Carbon source	Mycelium (mg./ml. culture)	Polysaccharide (mg./ml. culture)	Extracellular material (mg. acetone powder)	β-glucosidase activity	β-glucanase activity
Sucrose	10·9	2·52	5·0	38	3·3
Mannitol	8·06	0·39	12·5	35	3·5

Activity of extracellular enzymes from cultures producing no glucan. The acetone powders used above were all prepared from 'sucrose' cultures which produced glucan. To determine whether or not the enzymes were induced or constitutive the organism was grown on the mannitol medium in which little or no glucan was produced (Table 3). These were compared with glucan-producing cultures grown concurrently and the acetone powders were assayed as above for the relevant enzymes: β-glucosidase and β-glucanase. The results are given in Table 3. Low polysaccharide production did not correspond to low enzyme production. No significant differences in the specific activities of the two enzyme preparations were noted, although the yield of acetone powder from 100 ml. of each culture was more than double for the mannitol medium (in a typical case: 5 against 12·5 mg.). This suggests that part of the difference in unit weight of glucan produced in the two cultures may have been due to a greater rate of breakdown in the mannitol medium, and indicates that the enzymes are constitutive.

In preliminary investigations with two *Claviceps purpurea* strains in which no glucan was detected when cultured under conditions similar to *C. fusiformis*, amounts of β-glucanase activity even higher than those found in *C. fusiformis* were found in the culture medium after only 2 to 3 days growth.

Polysaccharide bound enzyme. During these experiments it was observed that when glucan isolated from the cultures was incubated alone with β-glucosides, the latter were slowly broken down ($\simeq$ 10% substrate hydrolysed/hr). This phenomenon was investigated further. The polysaccharide from above was heated in aqueous suspension for 5 min. in a water bath at 100°. Following this treatment all activity towards β-glucosides was lost, indicating the presence of polysaccharide-bound enzyme. To determine the intensity of the binding the polysaccharide was first subjected to a rigorous re-purification by the procedure described by Buck *et al.* (1968): six precipitations were done. The final product had substantially the same specific β-glucosidase activity as above.

Secondly, 50 mg. polysaccharide was suspended and gently stirred for 6 hr at room temperature in 5 ml. 0·03 M-acetate buffer (pH 5·2). The polysaccharide was then precipitated from the resulting viscous solution with ice-cold acetone as in the purification procedure above. The precipitated polysaccharide was removed and lyophilized; the precipitate from the slightly cloudy supernatant fluid left was isolated by centrifugation at 500 *g* and dried in vacuum over $CaCl_2$ to yield about 1 mg. product. The resulting polysaccharide had substantially the same activity as initially; the precipitate from the supernatant fluid had only a trace of β-glucosidase activity (< 0·5% substrate hydrolysed/hr). A sample of the precipitate itself was then subjected to the action of the exo-β1→3 glucanase (see above); it was not digested and no glucose or gentiobiose was detected by thin-layer chromatography. This indicated that the precipitate was not composed of small fragments of polysaccharide and that its activity was probably due to traces of enzyme freed from the polysaccharide. Repeating this extraction procedure in the presence of 2% cellobiose, a substrate for the enzyme, did not increase the yield or activity of free enzyme.

Lastly, in an attempt to free the enzyme from polysaccharide and to determine whether it remained active when in free solution and had properties similar to the soluble β-glucosidase, a sample of the polysaccharide (20 mg.) was treated with the exo-β1→3 glucanase (2 mg.) in 2 ml. 0·03 M-acetate buffer, (pH 5·2), for 6 hr at 30°. The product was then dialysed at 4° overnight against 1·5 l. of the same buffer, centrifuged to remove any debris, and the soluble protein content determined. No volume change occurred during dialysis and the protein content, over and above that due to the exo-β1→3 glucanase, had risen by about 100 μg. Acetone precipitation was then done as before to remove any residual polysaccharide. Cellobiose was over 50% hydrolysed by the resulting acetone powder within 1 hr, which was slightly slower than the free enzyme (β-glucosidase activity of the exo-β1→3 glucanase alone was undetectable at 1 hr). With *p*-nitrophenol-β-glucoside as a substrate, the enzyme had a K_m of $6{\cdot}6 \times 10^{-4}$ M; in contrast the free enzyme had a K_m of $2{\cdot}9 \times 10^{-4}$ M (with the 'natural' substrate, namely gentiobiose, the K_m obtained was $3{\cdot}1 \times 10^{-3}$ M). In the latter case marked substrate inhibition occurred at 4 mM with the *p*-nitrophenol salt, and at 2·5 mM with gentiobiose. Bound β-glucosidase was considerably more stable than the unbound enzyme. This was shown by repeated (4 to 6) precipitations with ethanol at room temperature. Under these conditions the unbound material lost more than 50% of its activity, whilst the bound enzyme was virtually unaffected.

Development of extracellular enzymes during culture. Acetone powders of the extracellular enzymes were prepared at intervals during the course of a fermentation starting on the 3rd day and finishing on the 13th day. Other variables in the culture,

namely: pH value, glucose, sucrose, asparagine, total nitrogen and dry weight of mycelium, polysaccharide and acetone powder were assayed concurrently (see Fig. 1, 2). The specific glucanase activity of the acetone powder was determined by the use of [^{14}C]glucan prepared as by Buck *et al.* (1968). Hydrolysis of the [^{14}C]glucan at 2·5% (w/v) was done with 0·5% of acetone powder in each case. Initial rates of reaction were determined by assaying [^{14}C]glucose liberated during the hydrolysis at intervals up to 20 hr. As the only product was glucose in all cases, the results indicated that β-glucosidase was being formed concomitantly with the glucanase, the hydrolytic step of the latter being rate-limiting; a correction for β-glucosidase activity was not applied in this case (see below). The results are included in Fig. 1 and Table 4 and show that enzyme activity appeared early in the culture and consistently increased, specifically and totally.

Table 4. *Claviceps fusiformis strain 139/2/1 G: development of β-glucanase activity during culture*

Cultures were harvested and acetone powders of the extracellular enzymes prepared. The β-glucanase was assayed at 30° using 2·5% (w/v) [^{14}C]glucan and 0·5% (w/v) acetone powder in each case.

Day	Extracellular material (mg./ml. of culture)	Specific activity of β-glucanase (μg. glucose produced/min./mg. extracellular material)	Total activity (units*/100 ml. culture)
3	0·04	2·7	10·8
4	0·06	5·6	33·6
6	0·16	5·7	91·2
9	0·48	6·6	316·8
10	0·79	7·0	553·0
11	0·90	8·0	720
13	0·80	11·0	880

*1 unit = 1 μg. glucose produced/min.

Characterization of the glucanase in the extracellular enzyme mixture. To characterize the glucanase further it was necessary to separate it from the β-glucosidase which was also present in the enzyme mixture. This was done by column chromatography. The specific activity of the protein in the column fractions obtained was determined by assaying with [^{14}C]glucan for the glucanase as described above. As with the exo-β 1→3 glucanase (see above), two products were obtained, which were identified as glucose and gentiobiose by the methods described by Buck *et al.* (1968). Hence the enzyme activity was most conveniently expressed as μmole product liberated/hr/ml. incubate/mg. protein. The specific activity of the β-glucosidase in the column fractions was determined by its action on 1·5 mM-*p*-nitrophenyl-β-glucoside as described above. The results are shown in Fig. 7.

Assaying the enzymes at 30° increased the ratio of glucose to gentiobiose over that obtained at 50° with the exo-β 1→3 glucanase for the same sample of glucan (from 2·0 to 2·1:1). The lowering of the temperature therefore appeared to give the β-glucosidase, known to be present in the exo-β 1→3 glucanase, a selective advantage with respect to activity. As the lowest ratio of products, obtained at 50°, was maintained for over 5 hr, it may be concluded that β-glucosidase activity was suppressed and that this ratio is the correct value, particularly since it also agrees with the non-enzymic estimations (Buck *et al.* 1968). Therefore to obtain a true value for the glucan-

ase activity (total number of product molecules liberated) of all samples for *Claviceps fusiformis* it was necessary to correct for the β-glucosidase activity, assuming a true value of 2:1 for the products; this was done for Fig. 7. The lowest product ratios obtained were in fractions 7 and 8 (2·1 and 2·4:1, respectively) which were therefore substantially free from β-glucosidase activity. The activity of the β-glucosidase for fractions 9 and above, whilst specifically very low, was, in terms of total activity, significant. By fraction 16 the ratio had risen to over 7:1.

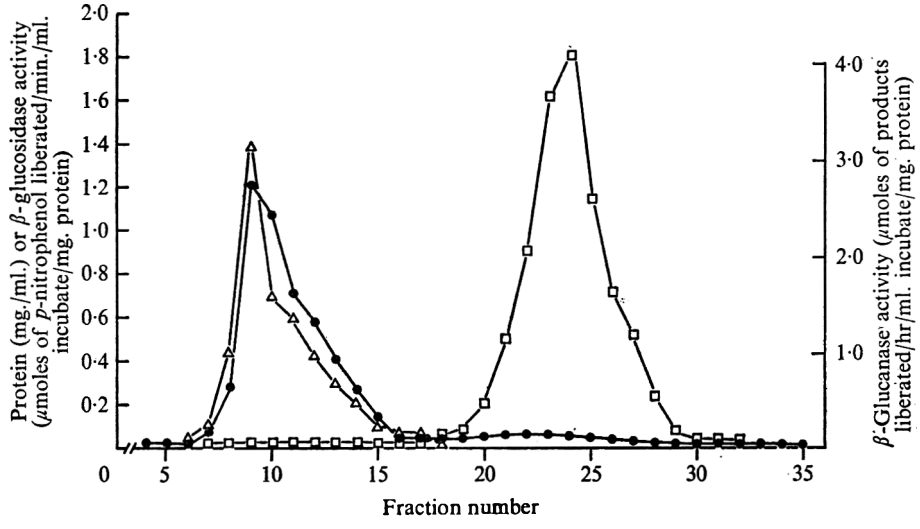

Fig. 7. Column chromatography, on DEAE cellulose, of extracellular enzymes produced by *Claviceps fusiformis* strain 139/2/1 G during fermentation: ●, protein; △, β-glucanase; □, β-glucosidase.

Table 5. *Claviceps fusiformis strain 139/2/1 G: comparison of activities of the crude and purified enzyme from C. fusiformis with the exo-β1→3 glucanase*

The enzymes (0·5%, w/v) were incubated at 30° with [^{14}C]glucan (2·5%, w/v) and the products separated as described in Methods. The product levels at 6 hr for the exo-β1→3 glucanase indicate 80% substrate utilization.

	Time of incubation (hr)			
	1	2	3	6
	μmoles of product liberated/ml. incubate/mg. enzyme			
Crude enzyme				
Glucose	0·95	2·08	3·32	6·60
Gentiobiose	0·005	0·02	0·05	0·09
Purified enzyme (fraction 9)				
Glucose	2·60	4·85	6·95	11·50
Gentiobiose	0·85	1·55	2·20	3·31
Exo-β 1→3 glucanase				
Glucose	8·14	10·93	12·02	13·28
Gentiobiose	3·72	4·83	5·12	5·56

Comparison of the purified glucanase with the exo-β1→3 glucanase. Since the purified glucanase (fraction 9 was used) gave the same products as the exo-β 1→3 glucanase of

Reese & Mandels (1959) when acting upon the glucan from *Claviceps fusiformis*, a direct comparison of the two enzymes, together with the original unpurified acetone powder mixture was made. Each of the three was incubated in the usual way with [^{14}C]glucan. Samples were taken over a period of 6 hr; the results are given in Table 5. The difference in the ratios of glucose to gentiobiose between exo-β 1$\rightarrow$3 glucanase and the purified glucanase from *C. fusiformis* may be ascribed to the greater β-glucosidase activity of the latter. From Table 5 it can be seen that, after applying the correction value as above, the column chromatography of the acetone powder mixture resulted in a purification of the glucanase by 4·5 times for fraction 9.

DISCUSSION

The subsequent autolysis of the extracellular glucan produced during the early stage of fermentation of the novel strain 139/2/1G of *Claviceps fusiformis* has been shown to be due to the activity of an exo-β 1$\rightarrow$3 glucanase of the type described by Reese & Mandels (1959) and is the first reported occurrence of this phenomenon in *Claviceps* spp.; a similar phenomenon has also been detected in *C. purpurea*. The extent of the enzyme activity, which began 2 to 3 days after inoculation and was paralleled by the concentration of the enzyme in the culture filtrate, followed a sigmoid pattern reaching a maximum within about 12 days. Characterization of the glucanase has confirmed the speculations of Davis *et al.* (1965) that an 'exocellular glucanase' could account for the hydrolysis of extracellular glucans observed in fermentations of their fungi. Production of the glucanase was accompanied by the formation of several other enzymes, the most notable of which was a highly active β-glucosidase; this occurred both free and glucan-bound. The latter form rendered the enzyme more stable though decreasing its affinity for the substrate. However, the K_m of both forms was sufficiently low to make it impossible to detect in the culture medium any products of glucan hydrolysis other than glucose. The glucose, thus liberated from the glucan, mixed with that which was liberated from sucrose during the first 4 days of incubation by the activity of another enzyme, a cell-associated sucrase. Towards the end of the fermentation this free glucose was partly utilized for a second growth phase.

This mechanism for the release of glucose from β-glucans, which have also been shown to be present in the naturally occurring sclerotia of *Claviceps fusiformis* (Buck *et al.* 1968), may enable these polysaccharides to constitute a reserve carbon source for the sclerotial germination process in addition to the large reserves of triglyceride oil (Mantle, 1968). Not only is the glucanase of intrinsic interest as a mould metabolite but its activity in restoring low viscosity during *C. fusiformis* fermentations has already facilitated the production and easy extraction of large yields of clavine alkaloids in 300 l. fermenters in this department. The usual *C. fusiformis* strains which do not produce the glucanase (Buck *et al.* 1968) are limited early in the fermentation by aeration deficiency, and any alkaloid product can only be extracted after the glucan has been precipitated from the culture and filtered off with considerable difficulty.

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SHORT COMMUNICATIONS

Growth of *Arthrobacter citreus* in a Chemically Defined Medium and its Requirement for Chelating Agents

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Previous studies on the physiology of *Arthrobacter citreus* have been made with either complex or partially defined media. To further our studies on the roles played by cations following the observation that certain metallic ions promote growth of the organism at elevated temperatures (Wong, Chan & Page, 1966), it was necessary to determine the exact nutritional requirements of *A. citreus* NCIB8915. We report the formulation of a chemically defined medium suitable for the growth of this organism.

The basal medium used was described by Chan (1964); it was adjusted to pH 6·8 before autoclaving at 121° for 15 min. Glucose was autoclaved separately as a 50 % (w/v) solution and was added aseptically to the sterile medium to a final concentration of 1 % (w/v). Cultures were grown in 50 ml. volumes in 250 ml. nephelo-culture flasks (Bellco Glass, Inc., Vineland, N.J., U.S.A.) and incubated on a rotary shaker (Psycrotherm Incubator Shaker, New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) at 25°.

Cultures used for inocula were grown on Trypticase Soy Agar (Baltimore Biological Laboratory, Baltimore, Maryland, U.S.A.) slopes at 25° for 24 hr. Bacteria were then suspended in basal medium salt solution, washed three times by centrifugation at 7710 × *g* and suspended to a density of 100 Klett units. Two-tenths ml. of this suspension was used to inoculate 50 ml. of medium. Growth was followed turbidimetrically with a Klett–Summerson photoelectric colorimeter (Klett Mfg. Co., New York) with a no. 42 blue filter (400 to 450 nm.).

All chemicals used were of purest grade available commercially. Inorganic salts and chelating agents (other than sideramines) were from Fisher Scientific Co., Montreal, Canada, with the exception of 2,3-dihydroxybenzoic acid from Aldrich Chemical Co., Milwaukee, Wisconsin, U.S.A. Casamino Acids and vitamin-free Casamino Acids were from Difco Laboratories, Detroit, Michigan, U.S.A.; vitamins, purines, pyrimidines and amino acids from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Samples of sideramines were from Dr J. B. Neilands, University of California at Berkeley (ferrichrome), Dr Hans Bickel, Ciba Ltd., Basel, Switzerland (ferricrocin, ferrirubin); Dr I. H. Zähner, Universität Tübingen, Tübingen, Germany (ferrioxamine); Dr J. Gelzer, Ciba Pharmaceutical Co., Summit, N.J., U.S.A. (desferal), and Dr G. A. Snow, I.C.I. Ltd., Alderley Park, Macclesfield, Cheshire (mycobactin P, mycobactin S).

Preliminary experiments indicated that *Arthrobacter citreus* grew in the basal medium + 1 % (w/v) Casamino Acids + thiamine-HCl (0·2 μg./ml.), but only after a lag phase

of 3 to 4 days. No decrease of the lag nor stimulation of growth was observed on adding purine and pyrimidine mixtures, both separately and together, or with the ash of Trypticase Soy Broth.

Lankford, Kustoff & Sergeant (1957) reported that several compounds having only one property in common, i.e. ability to chelate metal ions, were effective in initiating and maintaining exponential growth of a strain of *Bacillus subtilis*, and suggested that these compounds performed a function in common with a natural bacterial metabolite. We tested several chelating agents for capacity to initiate growth of *Arthrobacter*

Table 1. *Effect of chelating agents on the growth of Arthrobacter citreus*

Compound tested	Concentration* (μg./ml. medium)	Reference used	Bacterial concn. in Klett units Time (hr) 0	48	96
Control†	—	—	0	0	52
Disodium EDTA	8	*a*	0	20	595
Oxalic acid	1000	*a*	0	36	750
2,3-Dihydroxybenzoic acid	50	*a*	0	10	410
Sodium citrate	2000	*a*	0	0	0
Sodium salicylate	150	*a*	0	0	11
Salicylaldehyde	4	*b*	0	16	483
Acetylacetone	50	*b*	0	10	175
8-Hydroxyquinoline	1	*b*	0	19	335

* The concentrations used were found to be biologically active in the references cited.
† Basal medium + 1 % (w/v) Casamino Acids + 0·2 μg./ml. thiamine-HCl.
(*a*) Denotes Arceneaux & Lankford (1966); (*b*) denotes Morrison, Antoine & Dewbrey (1965).

citreus. The results (Table 1), indicated that all the compounds tested were active in initiating and maintaining exponential growth, with the exception of sodium citrate and sodium salicylate, which were inhibitory in the concentrations used. Similar positive results were obtained with various sideramines, a group of microbially synthesized ferric hydroxamates with chelating and growth-factor activity (Bickel *et al.* 1960). These were ferrichrome and ferricrocin (tested at 1 and 1000 mμg./ml. medium); ferrirubin (20 and 1000 mμg./ml.); ferrioxamine (0·5 and 1000 mμg./ml.); desferal, the iron-free derivative of ferrioxamine (50 mμg./ml.); mycobactin P and mycobactin S (at 1500 mμg./ml.).

To establish a chemically defined medium from a partially defined one for growth of *Arthrobacter citreus*, the Casamino Acids had to be replaced. The medium did not support growth when either vitamin-free Casamino Acids or an amino acid mixture equivalent to casein hydrolysate replaced Casamino Acids suggesting a requirement for some vitamins. This was confirmed by replacing the Casamino Acids with vitamin mixtures + amino acid mixture. The specific amino acid requirements were determined by using an amino acid block procedure. The essential ones were found to be tyrosine, cystine and methionine. In the amino acid experiments, vitamin mixtures had been included in the medium in addition to thiamine–HCl. In the presence of thiamine, the vitamin mixtures could be replaced by nicotinic acid + biotin. The optimal concentrations of biotin and nicotinic acid were 1 mμg./ml. and 0·2 μg./ml., respectively, and the vitamin requirements were confirmed by failure of the organism to grow when any one of the three vitamins, thiamine, biotin, nicotinic acid, was deleted from the medium.

Thus the basal medium supplemented with a chelator, tyrosine, cystine, methionine,

thiamine–HCl, biotin, nicotinic acid, and sodium chloride was able to support growth of *Arthrobacter citreus*. However, the amount of growth obtained was far less than that obtained with the amino acid mixture; another amino acid, while not essential, appeared to be required to obtain maximum growth. Since *A. citreus* is reported to be unable to utilize nitrates as a N source (Sacks, 1954), KNO_3 was removed from the medium and replaced by monosodium glutamate. (Monosodium glutamate cannot support growth of *A. citreus* without the essential amino acids so far determined, and, although probably purer, glutamic acid is as effective as monosodium glutamate in replacing nitrate.) The composition of the medium as finally constituted is shown in Table 2.

Table 2. *Composition of a chemically defined medium supporting good growth of Arthrobacter citreus*

	Quantities/l.
Inorganic salts, Solution A	5 ml.
Solution B	5 ml.
Sodium chloride	5·85 g.
Glucose	10 g.
Desferal*	50 μg.
L-Monosodium glutamate	1 g.
L-Tyrosine	0·1 g.
L-Cystine	0·1 g.
L-Methionine	0·1 g.
Thiamine–HCl	0·2 mg.
Biotin	0·001 mg.
Nicotinic acid	0·2 mg.

* May be replaced by any other effective chelator mentioned in text.

Solution A: K_2HPO_4, 25 g.; KH_2PO_4, 25 g.; distilled water to 250 ml.

Solution B: $MgSO_4.7H_2O$, 10 g.; $FeSO_4.7H_2O$, 0·5 g.; $MnSO_4.4H_2O$, 0·5 g.; distilled water to 250 ml.

Three other species, *Arthrobacter terregens*, *A. flavescens* and *Arthrobacter* sp. JG-9, require sideramines for growth (Lochhead & Burton, 1953; Lochhead, 1958; Burnham & Neilands, 1961, respectively); our results indicate that *A. citreus* is sideramine-dependent. We found that the sideramine requirement of *A. citreus* can be replaced by other chelating agents; similar observations have been made by other workers for a strain of *Bacillus megaterium* (Arceneaux & Lankford, 1966) and for *A. terregens* (Morrison, Antoine & Dewbrey, 1965).

At present, it is assumed that sideramines act as iron transfer agents (Neilands, 1967; Koser, 1968), and there is evidence to support the hypothesis that at least one sideramine, ferrichrome, is responsible for the insertion of iron into protoporphyrin (Burnham & Neilands, 1961; Burnham, 1962, 1963). Since ferrichrome binds Fe^{3+} tightly but shows only a weak affinity for Fe^{2+}, and since Fe^{2+} is the valence state more readily inserted into protoporphyrin (Lascelles, 1964), the iron bound by ferrichrome could be released upon its reduction to the ferrous form.

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The Nutrition of *Zymomonas anaerobia*

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The genus *Zymomonas* includes two species, *Zymomonas mobilis* and *Zymomonas anaerobia*. Millis (1956) has described some of the biochemical characteristics of *Z. anaerobia* and presented evidence for an essentially quantitative conversion of glucose to ethanol and CO_2. McGill, Ribbons & Dawes (1965) and McGill (1966) demonstrated the operation of the Entner–Doudoroff (1952) pathway and studied the metabolism of glucose and fructose by this organism and the molar growth yields.

The first nutritional studies were carried out with *Zymomonas mobilis* by Belaïch & Senez (1965), who showed that either an amino acid mixture or, less effectively, ammonium salts, supported growth with glucose as the energy source if supplemented with calcium pantothenate. The present report concerns the nutritional requirements of *Z. anaerobia* which differ significantly from those of *Z. mobilis*.

METHODS

Organism. Zymomonas anaerobia, strain NCIB 8227, was maintained as stab cultures in nutrient agar and by subculture in liquid medium under O_2-free nitrogen in a Fildes–McIntosh jar; growth was at 30°.

Media. The standard liquid medium for maintenance contained: Difco Bacto-peptone, 10 g., Difco yeast extract, 10 g. and D-glucose, 20 g./l. distilled water; solid media were prepared with 15 g. New Zealand agar substitute (Hopkin & Williams Ltd.)/l. This standard medium was modified in various ways to define the nutritional requirements of the organism.

Defined medium. Belaïch & Senez (1965) described a defined medium which, plus calcium pantothenate, supported growth of *Zymomonas mobilis*. It contained, per l. of tris-maleate buffer, pH 6·8: KH_2PO_4, 10 mg.; NH_4Cl, 1 g.; $MgSO_4.7H_2O$. 238 mg.; $CaCl_2$, 1·1 mg.; $FeSO_4.7H_2O$, 50 mg.; $ZnSO_4.7H_2O$, 7·2 mg.; $MnSO_4.H_2O$, 4·2 mg.; $CuSO_4$, $5H_2O$, 1·4 mg.; $CoSO_4$, $7H_2O$, 1·4 mg.; KCl, 50 mg. and NaCl, 50 mg., plus the following amino acids at 60 mg./l.: L-alanine, L-arginine, L-aspartic acid, L-cysteine, L-glutamic acid, glycine, L-histidine, L-hydroxyproline, L-isoleucine, L-leucine, L-lysine, DL-methionine, L-ornithine, DL-phenylalanine, L-proline, DL-serine, L-threonine, DL-tryptophan, L-tyrosine and L-valine. This medium was used in place of the Bacto-peptone of the standard medium, and glucose, together with either yeast extract or the appropriate vitamin solutions, were added to it.

Energy source. The standard medium was varied by substituting 2% (w/v) fructose, sucrose, gluconate, glycerol or pyruvate for the glucose. All energy sources were sterilized separately by Millipore filtration, and added to the otherwise complete medium.

Vitamins. Sterile stock solutions (100 μg./ml.) were prepared of each of the following

eleven vitamins, the pH being adjusted to 7·0 where necessary: *p*-aminobenzoic acid, biotin, cyanocobalamin, folic acid, inositol, lipoic acid, nicotinic acid, calcium pantothenate, pyridoxine hydrochloride, riboflavin and thiamine hydrochloride. A solution of haem was similarly prepared. The vitamins were added aseptically to either Bacto-peptone (1%)–glucose (2%) medium to a final concentration of 1 μg./ml., or were added to the defined amino acids–glucose medium to give a final concentration of 1 or 20 μg./ml.

Amino acid requirements. The amino acid requirements were investigated by the methods described by Seaman (1963) and also by adding individual amino acids to media containing 2% (w/v) glucose, 20 μg./ml. of biotin and lipoic acid, and inorganic salts.

Purines and pyrimidines. Adenine, guanine, hypoxanthine, xanthine, cytosine, thymine and uracil were added singly or in combination to the defined medium to give each a final concentration of 60 μg./ml.

Fatty and keto acids. n-Caproic, α-oxobutyric and α-oxoisovaleric acids were added singly or together to give each a final concentration of 17 μg./ml.

Inocula. To avoid the carry-over of nutrients, cells grown in 12·5 ml. of either standard or defined media were harvested under aseptic conditions, washed and resuspended in 12·5 ml. of sterile 0·067 M-Na K-phosphate buffer (pH 7·1); 0·1 ml. of the resulting suspension was used to inoculate 12·5 ml. of medium.

Growth conditions and measurement. Growth was obtained in $6 \times \frac{1}{2}$ in. pyrex tubes, containing 12·5 ml. of the appropriate medium, in a Fildes–McIntosh jar under O_2-free nitrogen. When growth was complete, the cultures were transferred quantitatively to 25 ml. volumetric flasks and made to the mark with 0·067 M-phosphate buffer (pH 7·1). The extinction was measured at 570 nm., and the dry weight read from a calibration curve relating extinction to bacterial dry weight over a range of 0 to 200 μg./ml. Results are recorded as the mean of replicate determinations, the range of which did not exceed 10 μg./ml., carried out in at least three independent experiments.

Chemicals. The chemicals used were of analytical reagent quality wherever possible.

RESULTS

Energy source. Peptone-yeast extract medium cannot support the growth of *Zymomonas anaerobia* in the absence of glucose. While fructose can replace glucose, giving altered fermentation products (McGill *et al.* 1965; McGill, 1966), neither sucrose, gluconate, glycerol nor pyruvate did so.

Nitrogen requirements. Replacement of peptone with ammonium chloride (0·1%) gave a medium capable of supporting some growth (57 cf. 172 μg./ml.). The Seaman (1963) groups of amino acids individually also supported some growth in the range 48 to 60 μg./ml., but the complete mixture of 18 amino acids was necessary to secure the greatest effect (121 μg./ml. increased to 139 in the presence of ammonium chloride). The Seaman (1963) medium, which differs from that of Belaïch & Senez (1965) in having three fewer amino acids and lacking ammonium chloride, supported less growth (121 cf. 146 μg./ml.); the addition of either these amino acids (lysine, ornithine and proline) or ammonium chloride enhanced the total growth to 133 and 139 respectively, the maximum effect (145 μg./ml.) being achieved with supplements of both, when growth equivalent to that in the Belaïch & Senez medium was recorded. Maximum

growth in the defined medium was, however, less than that in peptone (146 cf. 172 μg./ml.).

The net growth on single amino acids at a much higher concentration than in the Seaman medium (1·35% cf. 0·067%, w/v) revealed that arginine, tryptophan, glutamic acid and cystine were the most effective, giving yields of 87, 87, 83 and 76 /μg.ml. respectively. Similar results were obtained with an amino acid concentration of 0·27%.

Vitamin requirements. The mixture of 11 vitamins, each at 1 μg./ml., could replace 1% yeast extract in peptone–glucose medium, and despite the disparity in concentration give 75% of the growth with the yeast extract (311 cf. 418 μg./ml.). The addition of haem did not increase the cell yield. Screening experiments indicated that lipoic acid and biotin were the most effective growth factors and this was confirmed by a comparison of the effect of the addition of single vitamins.

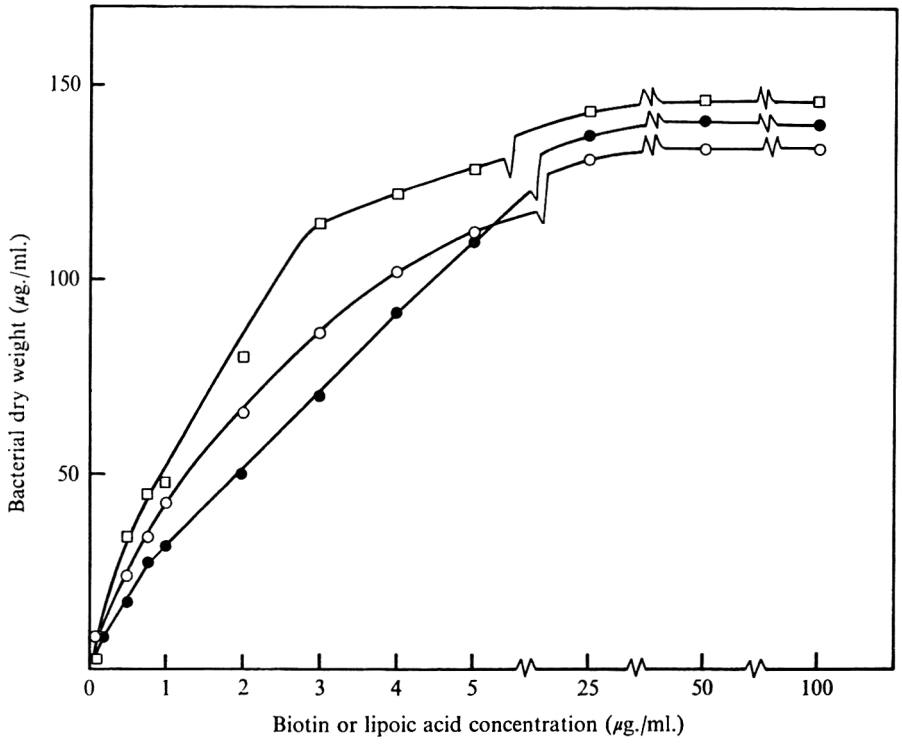

Fig. 1. Relationship between total growth of *Zymomonas anaerobia* and biotin and/or lipoic acid concentration in an amino acid–glucose–salts medium. Biotin, ○; lipoic acid, ●; biotin plus lipoic acid, □.

When similar experiments were conducted with defined amino acid media replacing peptone the vitamin mixture was only about 60% as effective as yeast extract (226 cf. 379 μg./ml.). *p*-Aminobenzoic acid was the only other vitamin to show some effect at 1 μg./ml. concentration, but at 20 μg./ml. the growth was very much less marked (40 μg./ml.) than that with lipoic acid (131 μg./ml.) or biotin (132 μg./ml.).

The growth response to increasing concentrations of biotin and lipoic acid, singly and combined, is shown in Fig. 1. It will be noted that the combined effect of these two vitamins is not additive.

Purines and pyrimidines. The addition of purines and pyrimidines, singly or combined, to the basal amino acid medium severely depressed the growth yield (e.g. 55 to 67 cf. 148 μg./ml.).

Fatty and keto acids. The fatty and oxo acids used did not have any significant effect on the growth of the organism.

DISCUSSION

The two species of the genus *Zymomonas* display many similarities in their metabolism but may be distinguished nutritionally. Both *Z. mobilis* and *Z. anaerobia* require an energy source for growth in complex media although they display a very restricted utilization of carbohydrates. While *Z. anaerobia* can utilize only glucose and fructose, *Z. mobilis* additionally utilizes sucrose (Millis, 1956). Dawes, Ribbons & Rees (1966) found that growth of *Z. mobilis* on sucrose gave appreciably lower molar growth-yield coefficients than those for the equivalent concentrations of glucose plus fructose and observed that growth on sucrose gave rise to a levan, which they characterized. The utilization of sucrose in a peptone–yeast extract medium thus serves readily to distinguish between *Z. mobilis* and *Z. anaerobia*. The molar growth yield of *Z. anaerobia* in peptone-glucose media is 5·89 (McGill, 1966), a value substantially lower than those of 8·3, 7·95 and 8·7 respectively recorded for *Z. mobilis* in comparable medium by Bauchop & Elsden (1960), Belaïch & Senez (1965) and Dawes *et al.* (1966). It seems, therefore, that *Z. anaerobia* utilizes glucose less efficiently than *Z. mobilis*. Further, unlike *Z. mobilis*, the growth yield on fructose is less than on glucose, a finding which was correlated with a different fermentation pattern when growth occurs on the ketohexose (McGill, 1966). It has also been observed throughout our work with these organisms that *Z. anaerobia* appears more granular in liquid peptone-yeast extract culture than does *Z. mobilis* and there is no difficulty in distinguishing between them visually under these growth conditions.

The organisms also have different vitamin requirements. Calcium pantothenate is the only growth factor required by *Zymomonas mobilis* (Belaïch & Senez, 1965) whereas biotin and lipoic acid are required by *Z. anaerobia*, although their action is not additive and together they give growth equivalent to 64% of that on a mixture containing nine additional vitamins. Presumably biotin is involved in CO_2-fixation reactions; since lipoic acid is usually associated with α-keto acid dehydrogenases the precise role of such enzymes in this anaerobic organism, which possesses an extremely active pyruvate decarboxylase, requires investigation.

Zymomonas anaerobia is more exacting for amino acids than *Z. mobilis*, which grows reasonably well, although slightly more slowly, on ammonium chloride as the sole nitrogen source, to give a growth yield of 4·09 g. dry weight/mole glucose fermented, compared with a yield of 4·98 in the amino acid medium (Belaïch & Senez, 1965). Maximum growth of *Z. anaerobia* in the defined medium was obtained with the complete mixture of 21 amino acids but arginine, tryptophan, cystine and glutamic acid supported the highest growth when amino acids were presented singly to the organism. *Z. anaerobia*, unlike *Z. mobilis*, did not grow well on ammonium chloride.

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Interference by Oxygen in the Acetylene-reduction Test for Aerobic Nitrogen-fixing Bacteria

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The reduction of acetylene (Dilworth, 1966; Schöllhorn & Burris, 1967) with the detection of ethylene by gas–liquid chromatography is a widely used method of assaying nitrogen fixation (Stewart, Fitzgerald & Burris, 1967; Hardy, Holsten, Jackson & Burns, 1968). Nitrogen fixation by the Azotobacteriaceae, which are important aerobic nitrogen-fixing bacteria in nature, can be inhibited by oxygen, particularly in carbon- or phosphate-limiting conditions (Dalton & Postgate, 1969*a*). We now report that the oxygen sensitivity of Azotobacteriaceae can lead to false assessments of nitrogenase activity by the acetylene-reduction test.

Batch cultures of *Azotobacter chroococcum* (NCIB 8003), *A. vinelandii* (NCIB 8660) and *A. macrocytogenes* (NCIB 8700) were grown in Burk's sucrose medium (Newton, Wilson & Burris, 1953) and 2 ml. samples of growing cultures were shaken at 75 strokes/min. at 30° in 25 ml. flasks under atmospheres of $A+O_2+C_2H_2$; the latter always at 0·03 atm. Maximum rates of acetylene reduction usually occurred at pO_2 values well below atmospheric (Fig. 1 A). Comparable bell-shaped curves relating acetylene reduction to pO_2 in the aerobic nitrogen-fixing bacteria *A. chroococcum* (Dalton & Postgate, 1969*b*) and *Mycobacterium flavum* (Biggins & Postgate, 1969) and the blue-green alga *Anabaena flos-aquae* (Stewart, 1969) have been reported earlier. The optimal pO_2 for each species depended on the population density; only with very dense cultures (e.g. *A. vinelandii* at 2 mg. dry wt/ml.) was the optimal pO_2 for acetylene reduction at or above the atmospheric value.

Shaking samples of the cultures in air on a Griffin flask shaker (Griffin & George, Wembley, England) at 250 vib./min. for 2 to 4 min. before testing normally lowered their maximum acetylene-reducing activity by 25 to 75%; only dense cultures with optimum pO_2 values at or above 0·2 atm. were unaffected by such shaking. The 'switching off' of acetylene-reducing activity in sensitive cultures could be reversed: gentle shaking (75 strokes/min.) at pO_2 0·2 atm. for 10 min. restored the original activity tested at the optimum pO_2. However, prolonged shaking in air (up to 1 hr) caused irreversible loss of up to 60% of the original activity.

It is possible to 'adapt' populations to high oxygen tension (Fig. 1 B). Nitrogen-fixing continuous cultures of *Azotobacter chroococcum* were set up at 30° as described by Dalton & Postgate (1969*b*), and 'adapted' to 0·09 and 0·55 atm. O_2 at $D = 0{\cdot}2\ hr^{-1}$; oxygen electrode measurements showed the ambient oxygen concentration in both cultures to be about 12 μM and the population densities were the same. The respiratory activities of such populations were related directly to pO_2: at pO_2 0·55 the QO_2 was about 2700 μl./mg. dry wt/hr; at pO_2 0·09 it was 1400. The low-oxygen population, like a batch culture, was readily 'switched off' by vigorous shaking in air.

The high pO_2 population was not; it even tolerated shaking in pure O_2, a treatment which caused irreversible suppression of acetylene-reducing activity of the low pO_2 population.

The populations so far discussed were all 'N_2-limited' in the sense used by Dalton & Postgate (1969*b*). Carbon-limited populations are very sensitive to oxygen inhibition of growth; so, to test organisms of a different nutritional status, a carbon-limited continuous culture of *Azotobacter chroococcum* growing in air was studied. Its pO_2

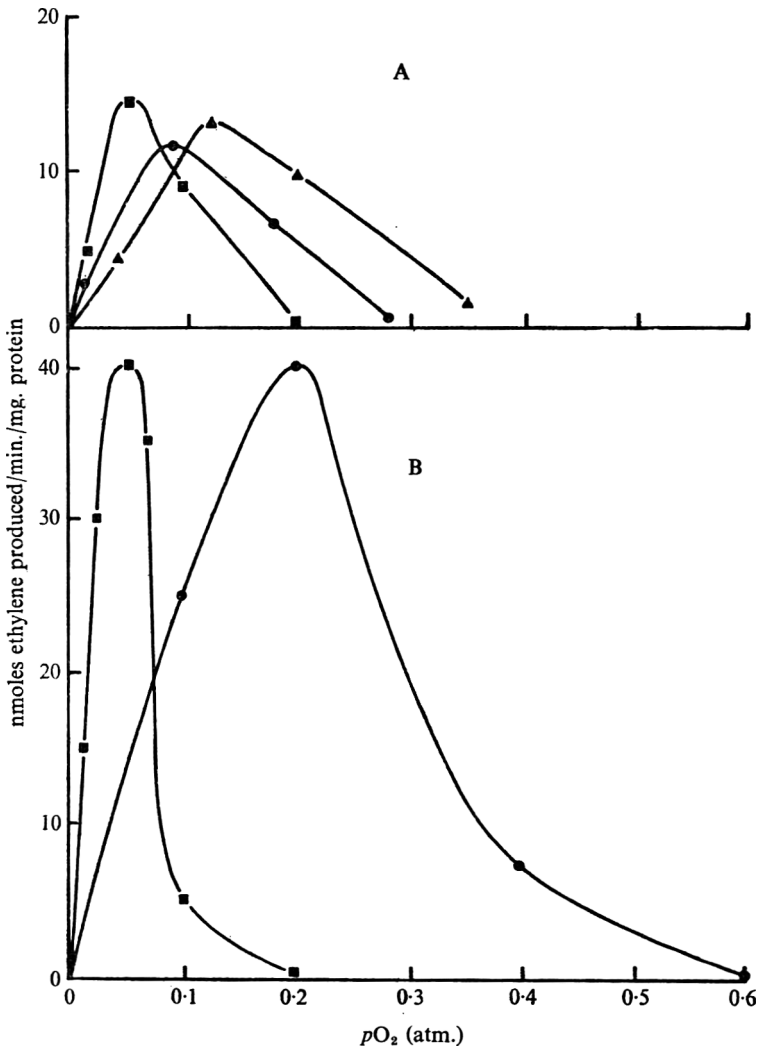

Fig. 1. (A). Effect of pO_2 on acetylene reduction by azotobacters. 2 ml. samples of batch cultures of: ●——●, *A. chroococcum*; ▲——▲, *A. macrocytogenes*; ■——■ *A. vinelandii*; tested at 75 strokes min, 30° under argon + oxygen + acetylene, the latter always at 0·03 atm. (B). Effect of pO_2 during growth, and respiratory activity, on the optimum pO_2 for acetylene reduction by *A. chroococcum*. 1 ml. samples containing 0·5 mg. dry wt organisms from continuous culture at D = 0·2 hr^{-1} tested at 150 strokes/min. ●——●, culture grown at pO_2 = 0·55 atm, QO_2 = 2700 μl./mg. dry wt/hr; ■—■, culture grown at pO_2 = 0·09 atm, QO_2 = 1400 μl./mg. dry wt/hr.

optimum for acetylene reduction was 0·025 atm. and no activity at all was detected a 0·2 atm. Shaking populations comparable to the N_2-limited ones on a vortex mixer in air for 1 min. lowered activity at the optimum pO_2 by 50%.

The present work shows three points relevant to the routine use of the acetylene-reduction test. (i) Aerobic bacteria rarely show their optimal activity at the atmospheric pO_2 value; (ii) aerobic bacteria can undergo, partially or completely, a reversible 'switching off' process when vigorously aerated by shaking; (iii) the sensitivity to 'switching off' depends on population density and nutritional status.

Field samples are usually tested at the atmospheric pO_2 of 0·2, which is reasonable because, even if the oxygen inhibits some of the bacteria present, this condition fairly represents their natural state. Soil and water organisms are likely to be growing under carbon-limited conditions and adapted to sub-atmospheric pO_2 values; conditions which may be expected to maximize oxygen damage on handling. Therefore, if natural samples are vigorously shaken in air or otherwise unduly oxygenated, it is likely that aerobic nitrogen-fixing bacteria will partly or wholly 'switch off' their nitrogenase systems and the test will give a falsely low estimate of the original nitrogenase activity. The nature of the 'switch-off' and inhibition processes will be discussed in more detail elsewhere; we present the above data because of their relevance to the general use of the acetylene-reduction test.

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its fifty-seventh General Meeting at the A.R.C. Food Research Institute, Norwich, on Tuesday and Wednesday, 6 and 7 January 1970. The following communications were made:

SYMPOSIUM: THE PHYSIOLOGY OF ANAEROBIOSIS

Introductory Talk. By S. R. ELSDEN (*A.R.C. Food Research Institute, Norwich*)

Special Features of Catabolism in Anaerobes. By J. L. PEEL (*A.R.C., Food Research Institute, Norwich*)

Most organisms obtain energy by oxidation-reduction processes and when oxygen is available it appears to be the oxidant of choice, and ATP is generated as a result of substrate-level phosphorylation and the transfer of electrons from intermediate acceptors, such as NAD, along a chain of carriers to oxygen. Organisms growing in the absence of oxygen ($E_0' = 815$ mV) are denied the use of such a high potential electron-accepting system. Under anaerobic conditions there appears to be no single oxidant of choice and a wide variety of electron-accepting systems is found among the various anaerobic species. This is one special feature of catabolism in anaerobes.

The electron-accepting systems which are utilized in the absence of oxygen are, in general, of much lower redox potential. This is especially true in the classical fermentations which give rise to reduced organic compounds as end products. Among such organisms, the electron-accepting systems of highest potential are the fumarate/succinate couple ($E_0' = 30$ mV) and the crotonyl CoA/butryryl CoA couple ($E_0'^{a} = 190$ mV). It is perhaps not surprising therefore that some anaerobes have developed electron carriers of lower redox potential than NAD ($E_0' = -320$ mV) and this is a second special feature of anerobic catabolism. The principal such carrier is ferredoxin; this has a redox potential of $E_0' = -420$ mV and in the growing cell is only reduced by a few systems, notably, the phosphoclastic splitting of pyruvate, the oxidation of acetaldehyde to free acetate and photoreduction. The use of these low potential carrier systems is associated with high sensitivity of the organism to oxygen. The formation of ATP coupled with the transfer of low potential electrons may well occur. This has not been demonstrated directly though there is some circumstantial evidence.

A third special feature is that some anaerobes utilize ATP-generating systems which are absent, or do not play a prominent part in the generation of energy, in aerobic organisms. Thus the fermentations of arginine, creatinine and allantoin produce ATP via carbamyl phosphate and in the clostridial fermentation of purines, ATP can arise from formyltetrahydrofolate.

Special Features of Biosynthesis in Anaerobes. By M. C. W. EVANS (*Department of Botany, King's College London*)

The majority of biosynthetic pathways have been investigated in aerobic organisms but comparative studies with anaerobes indicate that many pathways are similar in both groups of organisms. Studies of the incorporation of labelled 1, 2 or 3 carbon substrates by photosynthetic (Cutinelli, C., Ehrensvard, G., Reio, L., Saluste, E. & Stjernholm, R. (1951), *Ark Kemi.* **3**, 315) and non-photosynthetic (Tomlinson, N. (1954), *J. Biol. Chem.* **209**, 597)

anaerobic bacteria did however indicate the operation of different pathways in the synthesis of amino acids particularly glutamate, aspartate and alanine. The synthesis of glutamate from pyruvate or acetyl coenzyme A in aerobes involves the enzymes of the 'top half' of the tricarboxyllic acid cycle. Glutamate synthesized by this pathway contains one carbon derived from carbon dioxide and this is located exclusively in the C-1 position. Labelling data compatible with this pathway have been obtained for *Peptostreptococcus elsdenii* (Somerville, H. J. & Peel, J. L. (1967), *Biochem. J.* **105**, 299) and for photosynthetic bacteria grown on acetate (Hoare, D. S. & Gibson, J. (1964), *Biochem. J.* **91**, 546). The labelling experiments are supported by studies of enzyme activities, which indicate that all the necessary enzymes for the partial operation of the tricarboxyllic acid cycle are present.

In *Cl. kluveri* and several other anaerobes the labelling pattern in glutamate is different, the bicarbonate carbon being incorporated solely into C-5 of glutamate.

This difference in labelling pattern has been shown to be due to a difference in specificity of the citrate synthase in these organisms (Gottschalk, G. & Barker, H. A. (1966), *Biochemistry* **5**, 1125). The specificity of the enzyme can be altered by treatment with mercurials (O'Brien, R. W. & Stern, J. R. (1969), *Biochem. Biophys. Res. Commun.* **34**, 271). The pathway of glutamate synthesis in these organisms is otherwise identical to that in aerobes.

The synthesis of three carbon compounds from acetate and carbon dioxide by photosynthetic bacteria and some other anaerobes involves a type of carboxylation reaction not found in aerobic organisms, in which carbon dioxide is condensed with the carboxyl group of a carboxyllic acid to produce an α-keto acid. This reaction, which may be generalized by Equation 1, depends on the low potential electron carrier ferredoxin.

$$\text{Acyl CoA} + \text{Fd}_{\text{red}} + \text{Co}_2 \rightarrow \alpha \text{ keto acid} + \text{Fd}_{\text{ox}} + \text{CoA}.$$

Although this type of reaction was indicated by labelling experiments in photosynthetic bacteria and *Cl. kluyveri* it was first demonstrated in a cell-free system as a reversal of the phosphoroclastic reaction as extracts of *Cl. pasteurianum* (Bachofen, R., Buchanan, B. B. & Arnon, D. I. (1964), *Proc. Natn. Acad. Sci. U.S.A.* **51**, 690).

$$\text{Acetyl CoA} + \text{CO}_2 + \text{Fd}_{\text{red}} \rightarrow \text{Pyruvate} + \text{Fd}_{\text{ox}} + \text{CoA}.$$

This reaction, pyruvate synthase has been demonstrated in a number of photosynthetic bacteria and in *Cl. kluyveri* (Andrew, I. G. & Morris, J. G. (1965), *Biochim. Biophys. Acta* **97**, 176) and other anaerobes. This type of ferredoxin-dependent carboxylation reaction has been shown to occur with a number of different carboxylic acids and to be involved in photosynthetic carbon dioxide fixation and synthesis of a number of amino acids.

The carboxylation of succinate to α-ketoglutarate by α-ketoglutarate synthase was first shown in *Chlorobium thiosulphatophilum* (Buchanan, B. B. & Evans, M. C. W. (1965), *Proc. Natn. Acad. Sci. U.S.A.* **54**, 1212) and is also present in other photosynthetic bacteria and the rumen bacterium *Bacteriodes succinogenes* (Robinson, I. M. & Allison, M. J. (1969), *Bact. Proc.* p. 72). In the photosynthetic bacteria these two enzymes, pyruvate and α-ketoglutarate synthase replace the irreversible lipoic acid-dependent pyruvate and α-ketoglutarate oxidases in the tricarboxyllic acid cycle and permit the operation of the reductive carboxyllic acid cycle, essentially a reversal of the oxidative cycle, for carbon dioxide fixation (Evans, M. C. W., Buchanan, B. B. & Arnon, D. I. (1966), *Proc. Natn. Acad. Sci. U.S.A.* **53**, 928).

Experiments with *Methanobacillus omelianskii* grown on ethanol and carbon dioxide resulted in the synthesis of glutamic acid containing two ethanol carbon atoms and three from bicarbonate which would be consistent with its synthesis by the reductive carboxyllic acid cycle (Knight, M., Wolfe, R. S. & Elsden, S. R. (1966), *Biochem. J.* **99**, 76).

Three other reactions of this type, the carboxylation of propionate to α-ketobutyrate in isoleucine synthesis (Buchanan, B. B. (1969), *J. Biol. Chem.* **244**, 4218), of phenylacetate to phenylpyruvate in phenylalanine synthesis (Allison, M. J. & Robinson, I. M. (1967), *J. Bact.* **93**, 1269), and of isobutyrate in valine synthesis (Allison, M. J. & Peel, J. L. (1968), *Bact. Proc.* p. 142) have recently been discovered.

It seems likely that other reactions dependent on low potential electron carriers will prove to be of importance in anaerobic biosynthesis, permitting the operation of biosynthetic pathways not available to aerobic organisms.

The Biochemical Basis of Oxygen Sensitivity. By J. GARETH MORRIS (*Department of Biochemistry, School of Biology, University of Leicester, Leicester LE1 7RH*)

Despite an extensive (often reiterative) literature on the harmful effects of oxygen on the cultivation and metabolism of many micro-organisms, exceedingly little is yet known of the primary biochemical events that lie at the root of such expressions of oxygen toxicity; for a contemporary review of the interaction of microbes with oxygen, see Wimpenny, J. W. T. (1969), in '*Microbial Growth*', 19th Symposium of this Society, pp. 161–183. Several attempts have been made to deduce a prime cause of oxygen toxicity which would explain not only why anaerobes differ in their sensitivity to oxygen, but also why excess (hyperbaric) oxygen should prove harmful to even the most avid aerophile. The hypotheses that are, or have been, most influential, are the following.

(1) Oxygen itself is the toxic agent and is lethal to the cell (Pasteur, L. (1861), *C. r. hebd. Séanc. Sci., Paris* **52**, 344).

(2) Products of the interaction of oxygen with the cells or their culture medium are the actual toxic agents.

Although free radicals have been implicated, hydrogen peroxide is more usually assumed to be the culprit, especially in those anaerobes which lack any form of catalase (see Gordon, J., Holman, R. A. & McLeod, J. W. (1953), *J. Path. Bact.* **66**, 527).

(3) Normal growth and metabolism of any organism is only possible within certain stringent limits of redox potential (E_h); the presence of free oxygen is incompatible with the attainment and/or maintenance of the low E_h that is crucial for the growth of an obligate anaerobe.

This hypothesis would attribute the protective action of reducing agents to their ability to poise the E_h of a culture medium at a suitably low voltage, or otherwise to assist the organism to create and sustain the favoured redox state. Quastel, J. H. & Stephenson, M. (1926) (*Biochem. J.* **20**, 1125) favoured this explanation of the especial effectiveness of −SH compounds as promotors of the growth of anaerobes, though Quastel J. H. & Wooldridge, W. R. (1929) (*Biochem. J.* **23**, 115) later questioned whether E_h was the overriding consideration.

(4) The cell contains key compounds bearing −SH groups, e.g. enzymes, whose oxidation, by oxygen, to the −S−S− form, halts growth and metabolism. Added thiol compounds such as cysteine or glutathione might be especially protective of such −SH bearing molecules.

A modern variant of this hypothesis would direct attention to the sulphur-containing, non-haem iron proteins such as ferredoxin which are notably oxygen sensitive.

(5) Oxygen preferentially consumes the cell's 'reducing power', leaving it insufficient to maintain normal biosyntheses. This argument is particularly cogent in the case of anaerobes rich in flavoproteins and possessing potent NADH oxidases.

(6) Oxygen indirectly controls cellular activity by determining the intracellular concentration of a key metabolite which has a major regulatory function. This 'metabolic regulator' is presumably a redox couple which is liable to oxidation by O_2, or is in equilibrium with such a couple.

For example, if the activity of a key enzyme of the ATP-generating fermentative pathway was subject to allosteric activation by NAD(P)H, or to inhibition by NAD(P), an oxygen-provoked abnormal increase in the NAD(P)/NAD(P)H ratio could be the cause of generalized cessation of metabolic activity attributable to the resultant deprivation of ATP.

Although it is intellectually tempting to adopt a unitary hypothesis, it is more likely that there is no single cause of oxygen toxicity that is applicable to all organisms in all media, so that in inferring a single basis for oxygen sensitivity the title of this contribution may well be inappropriate.

The hypotheses listed above were discussed with special reference to some preliminary studies which we have made of effects of oxygen on the growth and metabolism of butyric clostridia.

Aerobic-Anaerobic Transformation in Facultative Organisms. By J. W. T. WIMPENNY (*Microbiology Department, University College, Cardiff*)

Facultative anaerobes are defined as organisms capable of growing in the presence or absence of oxygen. Energy for metabolism is obtained by the passage of electrons from low to high redox potential couples. Cell regulatory processes involving not only feedback control but induction and repression of enzymes ensures the most efficient utilization of available electron acceptors. Changes in respiratory apparatus between fully aerobic and fully anaerobic states have been examined in eucaryotic organisms and in a variety of procaryotes. The literature in this field has been reviewed. (Pichinoty, F. (1965), *Colloques int. du C.N.R.S. Marseilles* 1963, no. 24, p. 507. Wimpenny, J. .W. T. (1969), *Symp. soc. gen. Microbiol.* **19**, 161.) Less attention has been paid to the nebulous area between fully aerobic and fully anaerobic, probably most significant in natural environments. Such data as do exist suggests that in some strictly aerobic and some facultative procaryotes limiting oxygen concentrations lead to increased formation of respiratory carriers and enzymes over the aerobic level.

In continuous culture experiments using carbon-limited cultures of *Klebsiella aerogenes* and *Escherichia coli* distinct phases of growth with respect to oxygen can be detected. Anaerobically tricarboxylic (TCA) cycle enzymes are low, as are cytochrome pigments and over-all cell yield. Hydrogenase, an anaerobic enzyme, is active. At low oxygen concentrations a discrete peak in cytochrome pigments is observed whilst hydrogenase disappears. At higher oxygen concentrations cytochromes are depressed whilst TCA cycle enzymes, growth yield, flavoprotein-dehydrogenase enzymes and steady state ATP pool levels are all at their highest. Finally under fully aerobic conditions a reduction in all these parameters can be measured.

In similar experiments with *Staphlococcus epidermidis* the same general conclusions seem evident. There appears to be a lower optimum concentration of oxygen for cytochrome synthesis than for cell yield; however, respiration on glucose or lactate and membrane bound lactate dehydrogenase are all highest under fully aerobic conditions. We tentatively explain these observations as follows: at low oxygen concentrations with excess reductant and limiting oxidant the cytochromes and cytochrome oxidase proliferate and increase the affinity of the structure for oxygen; at higher oxygen levels reductant supply becomes limiting and the TCA cycle and other aerobic dehydrogenase systems increase, greatly augmenting the supply of reducing equivalents. At the same time, as oxygen is always in excess, the cytochrome system falls to a 'normal' aerobic level with a lower affinity for oxygen. Above this level oxygen is toxic, probably competing for reductant which would otherwise be channelled to biosynthesis. These changes constitute a number of different regulatory steps and indicate relatively complex control. From a structure and function point of view the cytoplasmic membrane appears most versatile as its constitution can vary very significantly at different oxygen concentrations. This plasticity must be of considerable survival value to species living in the hinterland between anaerobiosis and aerobiosis.

Toxigenicity and Sporulation in Strict Anaerobes. By MADELEINE SEBALD (*Laboratoire des Anaérobies, Institut Pasteur, Paris*)

In various species of clostridia, a direct correlation or, more often, an indirect one, has been suggested between ability to produce toxin and either the ability to sporulate, or the spores to be heat resistant (Nishida (1967), p. 147 in Fredette, ed., Laval des Rapides, Canada). The possibility of such relationships and their significance was investigated in our laboratory. Production of toxin was studied in sporulation mutants of *Clostridium histolyticum* and *Clostridium perfringens*; the mutants were easily recognized by the more transparent aspect of their colonies. The stage at which the sporulation is blocked cytologically in mutant strains was characterized with an electron microscope.

From *C. histolyticum* G 54 tox^+ sp^+, *c.* 30 sporulation mutants blocked at all cytological stages of sporulation except stage I were obtained. One component of the toxin (α_A) was lacking in three sp^- strains blocked at stage 0; the other mutants blocked at a later sporulation stage produced a normal amount of α_A toxin. The ability to produce α_A toxin was partially restored in oligosporogenous pseudorevertants obtained from one of the three sp_0^- mutant

strains. The role of α_A toxin in an early stage of sporulation indicated by these results (Sebald & Schaeffer (1965), *C. r. hebd. Séanc. Acad. Sci., Paris* **260,** 5398) is discussed. This situation is somewhat similar to that of protease and antibiotic production in sporulation mutant strains of *Bacillus* (Schaeffer (1969), *Bact. Rev.* **33,** 48).

Mutants with a different colony form were obtained from a wild type strain of *C. perfringens* ATCC 3624 (sp^W α^+ θ^+) (Sebald & Cassier, (1969), p. 306 in Campbell, ed., Spore IV, *Am. Soc. Microbiol., Bethesda*). Fifty per cent of them were sp^- or osp. Whether sp^W or sp^-, they were either hypo (α^-) or hyper (α^{2+}) or normal α toxin producers. According to the results of Nishida, a high incidence of the α^{2+} trait among sp^- mutant strains was obtained. But it is not possible to establish any evident correlation between the α or θ toxin production and the ability to sporulate. Kinetic data suggest that the α^{2+} strains might be regulatory mutants, their α toxin production being derepressed during growth. The existence of α toxin hyperproducers among the sp^- mutants does not support the hypothesis of a sporulation or (and) toxigenic episome in this species.

On the other hand, investigations were made with *C. perfringens* on the heat resistance of α atoxigenic and of α toxigenic strains (food poisoning (F.P.) and classical strains). Under normal conditions, our results are in accordance with those published previously (Weiss & Strong (1967), *J. Bact.* **93,** 21). But a lysozyme requirement is responsible for the so-called heat sensitivity of the classical strains; the heat injury to the spores can be reversed by adding lysozyme to the germination medium. Among various compounds tested, none was a substitute for lysozyme: the germination is lysozyme dependent (lzd^+ spores). The more severe the heat treatment, the greater the lysozyme dependence. The spores of F.P. strains do not require lysozyme for germination (lzd^- spores) (Cassier & Sebald (1969), *Annls Inst. Pasteur, Paris* **117,** 312). So, the inverse correlation between α toxigenicity and heat resistance might be converted into a direct one between α toxigenicity and lysozyme dependence of spores for germination. From an F.P. strain NCTC 8798 which is lzd^-, a lzd^+ mutant was spontaneously obtained. As with the wild type strain, this mutant is α^- (Cassier & Sebald, unpublished). So there exists no causal relationship between α toxigenicity and lysozyme dependence. The correlation observed by comparison with wild type strains might be fortuitous.

Special Techniques for Handling Anaerobic Bacteria. By P. N. HOESON and S. O. MANN (*Rowett Research Institute, Bucksburn, Aberdeen, AB2 9SB*)

Although bacteria may be broadly classified into aerobes and anaerobes, amongst the anaerobic bacteria there is a broad spectrum of sensitivities to oxygen. This range of sensitivities occurs not only amongst genera of bacteria but also amongst species of the same genus.

In the last few years it has been found possible to culture anaerobic bacteria which will not grow under the conventional anaerobic methods. These bacteria naturally exist in mixed cultures where the Eh of the medium is kept at a very low level by the metabolism of the accompanying bacteria, and, apparently having no ability to reduce a medium themselves, in pure culture they need a medium initially highly reduced and from which oxygen is subsequently excluded. Conventional methods of preparing anaerobic media in air and subsequently placing them in an anaerobic atmosphere do not allow the preparation of sufficiently reduced media, and examination of plates from an anaerobic jar involves exposure of these to air. Deep cultures prepared conventionally do not reduce sufficiently to permit growth.

Methods for handling extreme anaerobic bacteria have largely been developed for the study of rumen organisms, but they are now being used and adapted by workers in other fields. The techniques for culture of extreme anaerobic bacteria fall into three main classes, but all have in common that although some constitutents of the medium may be prepared in air final mixing of these constituents takes place in an anaerobic atmosphere and subsequently the medium is never exposed to the slightest trace of oxygen. Commercial gases used to maintain the anaerobic atmosphere usually contain traces of oxygen and must be specially purified for this work. The medium Eh is poised at a suitable value by chemical reducing agents, the most widely used being cysteine, sodium sulphide and dithionite.

Most techniques involve the use of sealed tubes or flasks for growth of bacteria in liquid or solid media. Choice of sealing rubber stoppers can be a critical factor here. In one method the

tube is flushed with an inert gas and the individual constituents of the medium are injected by hypodermic syringe through the stopper into the tube, in which some, or all, of the constituents may be sterilized, so that the medium is prepared in small batches. Inoculation, or removal of medium, is then carried out with a hypodermic syringe inserted through the stopper and the culture is never opened. This method may be the only one to use when reduction of the medium has been initially brought about by growth of a facultatively anaerobic bacterium which is heat-killed, and so looses its reducing power, before the medium is inoculated with the desired culture, but it does not allow of removal of colonies from roll-tube cultures. A more generally useful method involves the preparation of medium in bulk under an inert atmosphere and its transfer to tubes through which a continuous stream of anaerobic gas is passed. The tubes are stoppered under the gas stream but may subsequently be opened for inoculation or transfer of growth to fresh medium, again under a gas stream. This technique has been adapted to handling large volumes of media and to continuous culture of bacteria.

However, it is easier to see and subculture colonies from a plate than a roll-tube culture so methods of handling plate cultures and media in an anaerobic atmosphere have recently been tested. In these methods media are prepared and plates poured and inoculated in an airtight 'box' flushed with an oxygen-free gas. The 'box' can be of varying complexity. The plates are transferred to anaerobic jars in the box and can then be removed through an air-lock for incubation, but must be returned to the box for further examination or sub-culture. This method would appear to give promising results but obviously needs more complicated apparatus than the former ones.

The preparation and use of cell suspensions, and often enzyme extracts of these bacteria, generally needs the use of similar anaerobic techniques, although resting suspensions may be very resistant to oxygen-induced death. Maintenance of cultures may involve difficulties which will also be discussed.

ORIGINAL PAPERS

The Role of Ferredoxin in Formate Metabolism of *Sarcina ventriculi*. By M. P. STEPHENSON and E. A. DAWES (*Department of Biochemistry, University of Hull*)

It is now established that the formation and utilization of hydrogen gas by several anaerobic bacteria involve the enzyme hydrogenase in a ferredoxin-dependent reaction.

The strict anaerobe *Sarcina ventriculi* was found to produce hydrogen from formate by a formate hydrogenlyase system apparently analogous to that of the facultative organism *Escherichia coli*, i.e. involving formate dehydrogenase and hydrogenase (Bauchop, T. & Dawes, E. A. (1968), *J. gen. Microbiol.* **52**, 195). We have therefore investigated the occurrence and possible role of ferredoxin in the evolution of hydrogen from formate by this organism.

Cells harvested from a peptone-yeast extract-glucose medium, with and without supplementation with ferric chloride, contained ferredoxin, which was isolated and purified by a modification of the method of Mayhew, S. G. & Peel, J. L. (personal communication). *S. ventriculi* ferredoxin proved to be spectrally similar to that of *Clostridium pasteurianum* and could replace *Peptostreptococcus elsdenii* ferredoxin in the reduction of sulphite by hydrogen in that organism.

Cell extracts treated anaerobically with DEAE-cellulose to remove ferredoxin retained formate hydrogenlyase activity; this was observed whether or not the growth medium was supplemented with ferric chloride.

Ammonium sulphate fractionation of extracts yielded a fraction (40 to 60% saturation) which contained both formate dehydrogenase and hydrogenase activities but which lacked the necessary electron carrier(s) for the formate hydrogenlyase reaction to occur. Activity could be restored to the inactive fraction by the addition of either the sarcina ferredoxin, methyl viologen or benzyl viologen but FMN, FAD, NAD or NADP were ineffective.

Reduction of NADP by hydrogen or formate in the presence of crude cell extracts was

observed and shown to be ferredoxin-dependent; evolution of hydrogen from dithionite was similarly dependent on ferredoxin.

We are grateful to Dr J. L. Peel for discussion and the gift of dried cells of *Peptostreptococcus elsdenii*.

Effect of Starvation on the Viability and Intracellular ATP Content of *Zymomonas anaerobia*. By P. J. LARGE and E. A. DAWES (*Department of Biochemistry, University of Hull*)

Washed suspensions of *Zymomonas anaerobia* grown on glucose-peptone-yeast extract medium were starved in 67 mM-phosphate buffer, pH 6·8, at 30° under oxygen-free nitrogen. Viability, as determined by anaerobic slide culture, began to fall exponentially. This fall was accompanied by a parallel drop in the intracellular ATP level as measured by the method of Forrest, W. W. & Walker, D. J. (1965) (*J. Bact.* **89**, 1448). The addition of $MgCl_2$, which prevents degradation of RNA in this organism, decreased this loss in viability. After 175 hr starvation in the presence of 33 mM-$MgCl_2$, the cells were 22 % viable compared with only 3 % in the absence of $MgCl_2$. The presence of Mg^{2+} however did not have a significant effect on the ATP content of the cells.

When a pulse of glucose (final concentration, 2·5 mM) was administered to starved cultures the glucose disappeared from the supernatant in 2 hr. The intracellular ATP concentration qualitatively reflected closely the pattern of glucose utilization. There was a biphasic formation of ethanol, the inflexion point corresponding to 1 mole of ethanol formed/mole of glucose added. Then there followed a second phase reaching a maximum at 2 moles/mole of glucose added. The pattern of glucose utilization and ATP formation did not seem to be impaired even when the viability of the cells, as determined by the slide culture technique, had fallen to 3 %. This suggests that death of the cells (in the sense of failure to grow in slide culture) is not due to loss or disorganization of the energy-yielding enzymes of the Entner–Doudoroff pathway which operates in this organism (McGill, D. J., Dawes, E. A. & Ribbons, D. W. (1965), *Biochem. J.* **97**, 33 P).

The Effect of Metronidazole on Anaerobic Gas Production in *Trichomonas vaginalis*. By D. IWAN EDWARDS and G. E. MATHISON (*Department of Microbiology, Queen Elizabeth College, University of London, Campden Hill, London, W.8*)

Trichomonas vaginalis is a facultative, anaerobic protozoan which causes veneral disease in humans. During growth on a modified Bushby's medium (Bushby, S. R. M. & Copp, F. C. (1955), *J. Pharm. Pharmac.* **7**, 122) the organism evolves carbon dioxide gas, and, it is thought, hydrogen gas, but no experimental evidence has been published to substantiate the latter. This communication deals with the identification of hydrogen as one of the metabolic gases, the possible mechanisms by which hydrogen and carbon dioxide are evolved and the effect the antitrichomonal drug, metronidazole (1-β-hydroxyethyl-2-methyl-5-nitroimidazole), on gas production.

Chromatography of metabolic gas revealed that hydrogen is responsible for about 58 % of the total gas evolved, the remainder being carbon dioxide. Metronidazole inhibits hydrogen production before that of carbon dioxide, indicating that gas production is not the result of a single reaction. The process by which both metabolic gases are evolved is thought to be the phosphoroclastic system, whereby pyruvate, in the presence of phosphate, is cleaved to acetyl phosphate, carbon dioxide and hydrogen. The addition of formate to *T. vaginalis* has no effect on gas production (indicating the absence of a formate hydrogenlyase system), but pyruvate addition increases both hydrogen and carbon dioxide evolution. Moreover, the addition of pyruvate also results in increased production of acetyl phosphate. The synthesis of the latter, however, is unaffected by metronidazole. This indicates that the phosphoroclastic system in *T. vaginalis* consists of at least two stages. The first stage is responsible for the production of acetyl phosphate, carbon dioxide and hydrogen ions (the pyruvate dehydrogenase system); and the second stage is responsible for the production of molecular hydrogen from electrons and hydrogen ions (the hydrogenase system). Since hydrogen gas evolution is intimately concerned with electron transfer, and metronidazole inhibits electron

transfer in *T. vaginalis*, it is postulated that the drug directly or indirectly interacts with components of the hydrogenase system.

Financial assistance from May & Baker Ltd., Dagenham, is gratefully acknowledged.

Effect of Oxygen and Redox Potential on Spore Germination in Clostridia. By DIANA HOLLAND, A. N. BARKER and J. WOLF (*Division of Food and Agricultural Bacteriology, Department of Bacteriology, University of Leeds*)

Bicarbonate and CO_2 are powerful stimulants for the germination of clostridial spores (Holland, Barker & Wolf (1970), *J. appl. Bact.* in the Press) and were used throughout the following study. Spores of *Clostridium sporogenes* PA 3679 S2 were used in actively oxygenated or deoxygenated (passage of nitrogen) conditions over a wide range of Eh (−390 to +590 mV) adjusted with reducing or oxidising agents. In a synthetic medium (L-alanine + lactate) germination was independent of Eh as well as of anaerobic conditions. A similar independence of Eh was evident in a complex medium (brain-heart + yeast extract), but only anaerobically.

A marked inhibitory effect due to oxygen was apparent in the complex medium but could be neutralized or ameliorated by some reducing agents, e.g. thioglycollate (100 mM).

These observations were extended to spores of clostridia reported as apparently not requiring anaerobic conditions for germination. With the closely related strain PA 3679 h germination was even more sensitive to oxygen, in that inhibition also occurred in the synthetic medium.

In contrast, spores of *Clostridium bifermentans* CN 1617 were not affected by oxygen and high rates of germination (> 90 % in 10 min.) were obtained in both synthetic (alanine, phenylalanine, lactate) and complex media (casamino acids, minerals, yeast extract).

It would thus appear that the germination of clostridial spores is largely independent of Eh. Sensitivity to oxygen appears to be a function not only of the particular strain or species but also of the composition of the medium.

Facultative and Anaerobic Organisms which have been called *Bacteroides corrodens*. By F. L. JACKSON, Y. GOODMAN, P. C. WONG and R. WHITEHOUSE (*Department of Bacteriology, University of Alberta, Edmonton, Alberta, Canada*)

The name *Bacteroides corrodens* has been applied to certain agar-pitting Gram negative bacilli (Eiken, E. (1958), *Acta path. microbiol. scand.* **75**, 91). Most strains of these organisms at present available, including seventy-two similar strains isolated at the University of Alberta Hospital, are facultative and form a group of biochemically homogeneous and antigenically closely related organisms (Jackson, F. L., Goodman, Y. & Wong, P. C. (1969), *Bact. Proc.* M. 232). We have found the G + C content of these organisms to be in the 58 to 60 % range. These organisms should probably be assigned to a new genus.

Three oxidase-positive, catalase-negative, nitrate reducing, non-fermentative anaerobic organisms of a type in most features resembling Khairat's description of *Bacteroides corrodens* (*J. Path. Bact.* (1967), **94**, 29) have been investigated. They differ from the Eiken type antigenically and in a number of biochemical reactions. Unlike the facultative organisms, the anaerobes produced urease, would grow in the presence of 0·02 % NaN_3, and showed marked H_2S production. Two of the strains exhibited the 'swarming' phenomenon described by Khairat, but flagella have not so far been detected in electron micrographs. The G + C content of one of these strains, and that of a non-'swarming' strain (kindly provided by Dr S. M. Finegold, Wadsworth Veterans Administration Hospital, Los Angeles) was found to be 28 to 29 %. It is concluded that organisms now being classified as *Bacteroides corrodens* should be re-assessed because on the basis of certain current test schemes the name is being applied to organisms which are genotypically widely dissimilar.

The Greater Activity of Membrane-Bound Compared With Free Polyribosomes—An Artifact of Ionic Environment? By GEOFFREY COLEMAN (*Department of Biochemistry, University of Sheffield*)

Recent work showed that the distribution of ribosomal material between soluble and membrane fractions of lysozyme lysates of logarithmic phase cells of *Bacillus amyloliquefaciens*, in the presence of a fixed concentration of Mg^{2+}, was dependent on the K^+ concentration, and the distribution of ribosomal components in the two fractions at any K^+ concentration were closely similar (Coleman, G. (1969), *Biochem. J.* **112**, 533). It had previously been shown in the same system that amino acid incorporation by free polyribosomes was also influenced by K^+ concentration (Coleman, G. (1969), *Biochim. biophys. Acta* **174**, 395). Thus conditions which favour binding of ribosomal material to membranes were sub-optimal for amino acid incorporation. Considered in relation to the reported greater *in vitro* amino acid incorporating activity of membrane-bound ribosomes compared with free ribosomes (Hendler, R. W. (1965), *Nature, Lond.* **207**, 1053) this observation appeared to present something of a paradox.

A study was therefore undertaken of the effect of K^+ concentration on the amino acid incorporating activity of membrane-bound polyribosomes compared with the corresponding free polyribosome fractions in the *B. amyloliquefaciens* system. The *in vitro* activity of membrane-bound polyribosomes, unlike free polyribosomes, was found to be independent of K^+ concentration and consequently membrane-bound polyribosomes were more active than free polyribosomes at sub-optimal K^+ levels although they were never more active than free material under optimal conditions for amino acid incorporation.

These results will be discussed in relation to the observations of other workers and an explanation in terms of membrane having a non-specific ionic role will be presented.

This work was supported by a grant from the Medical Research Council.

Influence of Environment on the Free Amino Acid 'Pool' Content of Bacteria. By D. W. TEMPEST, J. L. MEERS (*Microbiological Research Establishment, Porton*), and C. M. BROWN (*Department of Microbiology, The Medical School, University of Newcastle-upon-Tyne*)

The free amino acid 'pool' content of *Aerobacter aerogenes* (a representative Gram-negative organism) was studied as a function of the growth condition and the data obtained compared with that from correspondingly grown cultures of *Bacillus subtilis* var. *niger* (a typical Gram-positive Bacillus). The predominant amino acid in the pools of both organisms was glutamic acid, but whereas this amino acid accounted for less that 50% of the total amino acids in the Gram-negative organism it accounted for over 75% of the free amino acids in the Bacillus organism. The pool glutamic acid content of both organisms varied significantly with the growth rate but was consistently much higher in the Gram-positive species. For example, with NH_3-limited *A. aerogenes* cultures it varied from about 4 mM to 7 mM when the dilution rate was increased from 0·1 to 0·7 hr^{-1}; with correspondingly grown cultures of *B. subtilis* the free glutamic acid concentration varied from about 100 mM to 135 mM. Changes in the nature of the growth limitation effected minor changes in pool amino acid composition and content but addition of NaCl to the growth environment had a most marked differential effect. With cultures of *A. aerogenes* the amino acid pool was greatly enlarged, particularly the free glutamic acid content. Thus, when the medium NaCl content was increased from less than 0·1 % to 2 % to 4 % (w/v) the pool glutamic acid content of NH_3-limited organisms increased from 4 mM to 16 mM and then to 67 mM. In contrast, the medium NaCl concentration had little or no effect on the free amino acid content of *B. subtilis* organisms.

With *A. aerogenes* cultures, rapid changes in bacterial glutamic acid content occurred following sudden changes in medium salinity and closely parallelled the changes in bacterial potassium content. It seems probable, therefore, that glutamic acid functions as the anion which is associated with that potassium serving an osmoregulatory function in the growing bacteria.

A Novel Pathway of Synthesis of Glutamic Acid in *Aerobacter aerogenes*. By J. L. MEERS, D. W. TEMPEST (*Microbiological Research Establishment, Porton*), and C. M. BROWN (*Department of Microbiology, The Medical School, University of Newcastle-upon-Tyne*)

When grown in a chemostat culture, in a simple salts medium in which ammonia provided the sole nitrogen source, *Aerobacter aerogenes* organisms contained glutamate dehydrogenase (EC 1.4.1.4). This enzyme was present in substantial amounts when growth was limited by the supply of glucose, Mg^{2+}, K^+ or PO_4^{3-} but was almost absent from organisms limited in their growth by the supply of NH_3. Since, under these latter conditions, the intracellular ammonia concentration was insufficient even to one-half saturate glutamate dehydrogenase (the K_m for ammonia being about 2 to 3 $\times 10^{-3}$ M) this enzyme could not reasonably account for the rapid rate of assimilation of ammonia into NH_3-limited bacteria. Pulsing ammonia into either growing or non-growing cultures of NH_3-limited *A. aerogenes* led to a rapid transient rise in the bacterial pool glutamine level, and examination of cell-free extracts showed the presence of a highly active glutamine synthetase (EC 6.3.1.2). Since the K_m for ammonia incorporation into glutamine is low (< 1 mM) it seemed possible that ammonia incorporation into NH_3-limited bacteria might be primarily via the synthesis of glutamine. But this assumption required the presence of a hitherto unknown enzyme capable of converting the amide nitrogen to α-amino nitrogen. Such an enzyme has now been located; it reductively converts one molecule each of glutamine and 2-oxo glutarate to two molecules of glutamic acid. This enzyme has a K_m for glutamine of < 2 mM and for 2-oxo glutarate also of < 2 mM; it requires $NADPH_2$ but seemingly no other diffusible cofactors. Magnesium ions are markedly inhibitory as is glutamic acid (the end product of the reaction). Like glutamine synthetase, this enzyme (tentatively named Glutamine (Amide): 2-oxo Glutarate Amino Transferase (Oxido-reductase, NADP)) is virtually absent from glucose-limited organisms and it is concluded, therefore, that its functional significance lies in the assimilation of ammonia under those conditions where the pool ammonia level is considerably depressed.

NADH Dehydrogenases of Photosynthetic Bacteria. By FREDA WHALE and O. T. G. JONES (*Department of Biochemistry, University of Bristol, BS8 1TD*)

Rhodopseudomonas spheroides grows either aerobically, without a light requirement, or anaerobically in the light (photosynthetically). Using a dual wavelength spectrophotometer fitted with a fluorescence attachment, we have found that light-induced reduction of NAD by whole cells of a carotenoid-less mutant of *R. spheroides* lags behind the rapid oxidation of cytochrome c_2 and is inhibited by uncoupling agents and by HOQNO, indicating that NADH formation is by energy-dependent reversal of electron flow, possibly via the aerobic electron transport chain, as in *Rhodospirillum rubrum* (Keister, D. T. & Yike, N. J. (1967), *Arch. Biochem. Biophys.* **121**, 415) and not by a non-cyclic pathway involving ferredoxin. We have examined the metabolism of NADH in the anaerobic sulphur bacterium *Chlorobium thiosulphatophilum*, which like higher plants contains low potential ferredoxin, to learn if NADH was linked to electron flow in this organism in a way similar to *R. spheroides* or to higher plants.

Chromatophores and soluble fraction were prepared from light-grown cells of *R. spheroides* and *C. thiosulphatophilum* after breakage in a French press; particles were washed once. Addition of NADH to either supernatant caused rapid reduction of endogenous flavins and cytochromes of the *c*-type, and both soluble fractions catalysed the reduction of added cytochrome *c* by NADH in an NAD-inhibited reaction.

The particulate fraction from *C. thiosulphatophilum* contained about 3 % of the NADH-cytochrome *c* reductase activity of the supernatant and its properties (Km for substrates; inhibitor insensitivity) were the same as those of the soluble fraction. The particulate NADH cytochrome *c* reductase of *R. spheroides*, but not the soluble, was inhibited by rotenone, piericidin A, antimycin A and HOQNO. NADH caused reduction of endogenous cytochromes of the *b* and *c*-types in particles, with an antimycin A sensitive crossover point between. No cytochromes of the *b*-type were found in *C. thiosulphatophilum* by spectroscopy at 77 °K or in pyridine haemochrome preparations.

Our results suggest that it is unlikely that NADH can be formed by *C. thiosulphatophilum* by a mechanism similar to that found in the photosynthetic heterotrophs *R. rubrum* and *R. spheroides*.

We are grateful to the Agricultural Research Council and the Science Research Council for their support of this work.

The Use of Fructose Diphosphate Aldolase as a Parameter in Comparative Microbiology. By G. H. FLEET, G. J. MANDERSON and H. W. DOELLE (*Department of Microbiology, University of Queensland, Medical School, Herston, Queensland, Australia*)

Comparative enzymological studies are becoming of increasing importance in microbiology. As it is impossible to assay every enzyme of a pathway during a large taxonomic survey, a few so-called 'key enzymes' are selected for this purpose. FDP aldolase (E.C. 4.1.2.13) is one of those key enzymes, being used as one of the criteria for separating homo- from heterofermentative lactobacilli (Buyze *et al.* (1957), *Antonie van Leeuwenhoek* **23**, 345). We have critically investigated each step of the procedure used in such studies, using *Escherichia coli* 518, *Lactobacillus casei* ATCC 7469 and *L. salivarius* ATCC 11741.

The investigations demonstrated that from the comparative microbiological point of view, FDP aldolase analyses made at the onset of the deceleration phase of growth are stable and taxonomically useful. However, rigid standardization of all growth conditions, cell-free extract preparations and enzyme assays are absolutely necessary. The kinetic behaviour of the enzyme extracted from *E. coli* and *L. casei* was very different. A rapid deterioration occurred if *E. coli* FDP aldolase assays were carried out in solutions containing mercaptoethanol, whereas lactobacilli aldolase was stabilized by the same compound. Similar results were obtained in cell and cell-free extract storage experiments. *E. coli* aldolase activity reached its peak after 3 min. sonication, whereas in the case of lactobacilli 10 to 15 min. were required. Complex media decreased the efficiency of sonication for the rupture of *E. coli* cells. The pH optimum for *E. coli* aldolase was 9·0, whereas that of lactobacilli was 6·8 to 7·0. Significant variations in FDP aldolase activity occurred during the deceleration and stationary phases of growth in both *E. coli* and lactobacilli, thus giving variable and misleading results.

Factors Influencing the Activity of Succinic Dehydrogenase in Membrane Preparations from *Micrococcus lysodeikticus*. By P. OWEN and J. H. FREER (*Department of Microbiology, University of Glasgow*)

Differences in the distribution of respiratory chain enzymes, including succinic dehydrogenase (SDH), between cytoplasmic and mesosomal fractions of Gram-positive bacteria have been reported. The SDH activity appeared largely in the mesosome fraction (Ghosh, B. K. & Murray, R. G. E. (1969), *J. Bact.* **97**, 426), or solely in the cytoplasmic membrane (Rogers, H. J., Reaveley, D. A. & Burdett, I. D. J. in *Protides of the Biological Fluids*, Vol. 15, p. 303. Amsterdam: Elsevier; Ellar, D. J. 2nd meeting of the North West European Microbiological Group Symposium, 1969; Reaveley, D. A. & Rogers, H. J. (1969), *Biochem. J.* **113**, 67). Cytochemical evidence suggested a distribution of activity throughout the cell membranes, but predominating in the internal membranes (Sedar, A. W. & Burde, R. M. (1965), *J. Cell Biol.* **27**, 53).

The present work reports several factors which influence the SDH activity in membrane preparations, with some comments on the spectrophotometric assay.

Preparation of membranes: SDH activity in membrane preparations is reduced by exposure to temperatures above 10°. A loss of 25 to 30 % of activity occurs after exposure to 30° for 10 min.

The specific activity of SDH is dependent upon membrane concentration. At 0·3 to 0·4 mg. membrane/ml. the specific activity is 2- to 3-fold greater than that for concentrations of 10 to 15 mg. membrane/ml. Washing with sodium deoxycholate results in a similar increase in activity. Activation by dilution is largely inhibited if the diluent includes bovine serum albumin (1·0 mg./ml.) and totally eliminated in membranes mildy fixed with glutaraldehyde. In all cases activity remains in the sedimentable fraction.

Spectrophotometric assay: The assay method used was based upon the method of Ells, H. A. (1959, *Archs Biochem. Biophys.* **85**, 561). Factors influencing the observed SDH activity are temperature, age of membrane and light.

Results suggest that SDH is firmly bound to the membrane, and that its activity is controlled to some extent by the presence of an 'inhibitor', the dissociation of which leads to an increase in activity.

Multiplicity Dependent Infectivity of Incomplete Adenovirus Particles. By GÖRAN WADELL and MARIE-LOUISE HAMMARSKJÖLD (*Department of Virus Research, Karolinska Institutet, Stockholm*)

Nonsoluble components of adenovirus type 16 were separated by three consecutive isopycnic centrifugations in CsCl. Six distinct populations of particles could be demonstrated at the following densities: (*a*) 1·301, (*b*) 1·308, (*c*) 1·314 to 1·323, (*d*) 1·327, (*e*) 1·331 to 1·337, (*f*) 1·344. Bands (*c*) and (*e*) were heterogeneous with regard to their density. Band (*f*) represents the intact virions which comprise about one half to two thirds of all hemagglutinating particles. Electron microscopy of the different populations of virus particles demonstrated that the major portion of the particles had a morphology indistinguishable from infectious virions. The content of internal components of different particles was analysed by treatment of the virus particles with diethylpyrocarbonate. A slight amount of stain penetrated into treated virions, whereas treated particles from band (*b*) were partially filled by stain. This observation indicated that internal components occur in low density particles but in smaller amounts than in virions. Specific infectious activity could be demonstrated in all the different populations. The particles in bands (*f*), (*d*), (*c*) and (*b*) displayed ratios of infectious titers over hemagglutinating activities of 33,000, 200, 6 and 1, respectively. These results seem to indicate that low density virus particles, presumably containing reduced amounts of viral DNA, can initiate a complete viral replication by means of multiplicity dependent complementation.

Demonstration of the Gaspak Anaerobic System. By G. SIROCKIN (*Chelsea College of Science and Technology, Botany Department, Hortensia Road, S.W.10*)

A simple method developed for the growth of anaerobic bacteria which dispenses with the need for hydrogen cylinders.

The system consists of a Gaspak anaerobic jar made of polycarbonate resin, a durable rigid and dimensionally stable thermoplastic. The Gaspak jar is lightweight, transparent and will withstand autoclaving. The lid clamps on to the jar and has on its inner surface a catalyst chamber. An anaerobic environment is produced by using a Gaspak Disposable hydrogen and carbon dioxide Generator envelope. The gas is generated by adding water into the envelope. A Gaspak indicator added to the jar enables the worker to see that anaerobic conditions have been obtained. This system dispenses with the need of vacuum pumps, pressure, regulators, manometers and electrical attachments.

Index of Authors

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

Index of Subjects

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

CORRIGENDUM

In T. D. THOMAS & R. D. BATT (1969),
Journal of General Microbiology **58**, 371–380.

Page 373, Fig. 1:

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