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Purine-Requiring Auxotrophs of *Coprinus lagopus* (*sensu* Buller)

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SUMMARY

The allelic relationships between a series of independently-induced adenine mutants of *Coprinus lagopus* were investigated, and limited complementation maps of three of the loci constructed. By means of nutritional tests and a search for aminoimidazole accumulants in the mycelium the adenine loci were correlated with steps in the purine biosynthetic pathway. Four loci can be assigned to (unknown) steps preceding closure of the imidazole ring, two others to specific steps occurring after closure of the ring. One of these latter represents a deficiency in the enzyme adenylosuccinase.

INTRODUCTION

Approximately 70 independently induced mutants exhibiting a response to adenine have been isolated from wild strains of the Basidiomycete *Coprinus lagopus* (Day & Anderson, 1961; D. H. Morgan, personal communication; Moore, 1966; P. R. Day & C. F. Roberts, personal communication), but only a small proportion have so far been allocated to their respective loci. During the course of mapping work designed to increase the linkage data available for *Coprinus lagopus* (to be published) it became imperative that such locus allocations should be known. As an extension to this work the relationships of the mutant loci to the different steps in purine biosynthesis were investigated, primarily by a search for aminoimidazole accumulants in the mycelium.

METHODS

The various strains of *Coprinus lagopus* used were grown on Petri plates from slope stock cultures, most of the stocks being immediately derived from a collection maintained by D. H. Morgan at the John Innes Institute. Many of these were supplied in the first instance by the workers who originally isolated the mutants (see Table 1). As far as can be ascertained all the mutants here mentioned were isolated following ultraviolet irradiation of wild types, with the exception of strain M38 (*ad*-1) which is an ethanemethanesulphonate-induced mutant. The methods and media for general culture described by Day (1959) were used. All plate cultures were incubated at 37°.

Complementation tests. The test for functional allelism between different mutants was for growth of their mutual dikaryon on minimal medium. Confrontations of the strains under test were always made on complete medium. After 48–60 hr growth pieces of surface and aerial dikaryotic mycelium were transplanted to plates of complete and minimal media, together with control inocula of the monokaryotic

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parents. Vigorous growth of the dikaryon on minimal medium showed that the mutants complemented one another, and thus were not allelic. Instances of allelic complementation were rare, only six were observed in a total of 539 tests. Absence of growth on minimal medium indicated that the dikaryon carried allelic mutations. The tests were usually made in triplicate.

Growth tests. Tests for growth of the mutant monokaryons on supplemented minimal media were done on Petri dishes. All supplements were added to a final concentration of 50 μg./ml. Conditions of growth were standardized as far as possible by preparing and autoclaving all media at the same time, using a measured 20 ml. of medium in plastic Petri dishes, standard punch-cut inocula, and incubation in a single incubator for the same time period. Colony diameters were averaged from 12 measurements of three replicates. In all but a few cases individual measurements varied from the mean value by not more than 10%.

Aminoimidazole accumulants. Attempts to determine the possible positions in the purine biosynthetic pathway were centred on a search for aminoimidazole accumulants in the mycelium, the methods used being those of Bernstein (1961) with modifications. To obtain the quantities of mycelium necessary for the extraction procedures the strains were grown in liquid culture. Suspensions of oidia prepared from slope cultures were used, unfiltered, as inocula for 2 l. growth flasks containing 1 l. of complete medium. Flasks were incubated on an orbital shaker at 31° (temperature determined by other users of the machine). After 3–4 days the mycelial mass was filtered off, washed with sterile distilled water, and resuspended in a starvation medium for a further 48 hr. The starvation medium was the normal minimal medium to which casein hydrolysate (0.1%, w/v) was added to increase the total yield of aminoimidazoles (Gollub & Gots, 1959). Mycelium was finally harvested by filtration through cheesecloth, washed with water, blotted and weighed. The yields of mycelium varied with the vigour of the mutants, but suitable choice of the initial incubation period allowed enough mycelium to be obtained even from the slowest growing strain. Eighteen liquid cultures gave amounts of mycelium varying from 20 to 81 g. (pad wet weights); over-all average, 49 g.

Extraction of mycelium was effected by boiling in distilled water (200 ml./20 g. mycelium) for 1–2 min., followed by disintegration in a Waring Blendor. The extracts, after filtration through Whatman no. 1 filter paper, were lyophilized. Lyophilization left a light friable powder (average weight about 0·65 g.) which was completely taken up in 5 ml. of distilled water. Debris was removed by centrifugation; proteins were precipitated by boiling for 15 min. and then centrifuged down. The soluble fraction was then ready for chromatography.

Extracts were spotted on to Whatman no. 1 paper and resolved by descending chromatography in a solvent system of isopropanol + water + ammonia (sp.gr. 0·88); 7 + 2 + 1 by volume. Twelve hr at room temperature gave adequate resolution, and after drying the chromatograms were sprayed lightly with the diazosulphonic acid reagents of Ames & Mitchell (1952). This technique allowed the detection of several aminoimidazole accumulation products not present in extracts of wild-type strains. Identification of the accumulants was attempted with quantities prepared by chromatographing heavy streaks of the extract concerned. The position of the required band was revealed by spraying strips cut from each side of the dried chromatogram, the band cut out and then eluted in water or buffer.

Enzymic activity of mycelia. Since the extracts were prepared from cultures which had been subjected to a long period of starvation, while the method depended on continued enzymic activity producing the accumulants, it was thought desirable to have some indication of enzyme activity at the time of harvesting. Accordingly the optimum assay conditions for glutamic dehydrogenase (GDH) were determined; this enzyme was known to be present in *Coprinus lagopus* (Day, 1962) and assay procedures for Neurospora GDH were available. It was assumed that the activity of this enzyme, which is not directly related to purine biosynthesis, would give some indication of the metabolic status of the starved mycelium. If anything, enzymes of the purine pathway would be expected to be de-repressed and thus to show a higher activity than the measured GDH. The observation that *Coprinus lagopus* GDH is NAD specific was confirmed (Day, 1962). The assay mixture routinely used comprised: NH_4Cl (M) 0·1 ml.; 2-oxoglutarate (0·1 M) 0·25 ml.; $NADH_2$ (2 mg./ml.) 0·1 ml.; tris buffer (pH 8·5, 0·05 M) 2·45 or 2·50 ml.; crude enzyme extract 0·10 or 0·05 ml. The reaction, at 5°, was followed spectrophotometrically by observing the rate of change of extinction at 340 mμ. Crude enzyme extracts were prepared by grinding about 1 g. of mycelial pad in 5 ml. 0·05 M-phosphate buffer (pH 8) containing 10^{-3} M-EDTA. Activities varied from 21% to 133% of the activity of extracts of a wild strain subjected to the same treatment. It was concluded that, with an average of 54% of wild type activity, it was most likely that the enzyme concentrations in all samples were satisfactory.

Abbreviations. The following abbreviations are used (after Bernstein, 1961): AMP, adenosine-5′-phosphate; IMP, inosinic acid; GMP, guanosine-5′-phosphate; AMPS, adenylosuccinic acid ribotide; AICAR, 5-amino-4-imidazolecarboxamide ribotide (or riboside); SAICAR, 5-amino-4-imidazole-*N*-succinocarboxamide ribotide or riboside; CAIR, 5-amino-4-imidazole-carboxylic acid ribotide; AIR, 5-aminoimidazole ribotide or riboside.

RESULTS

Complementation patterns. The adenine mutants could be allocated by means of the dikaryon complementation test into eleven loci (Table 1), six of which have so far been mapped genetically (Day & Anderson, 1961; D. Moore, unpublished). The *ad*-7 locus was designated by P. R. Day; it was represented by a single strain which appears to have been lost from all culture collections. Although there are six alleles of *ad*-8 no allelic complementation was observed for the pairs that were tested. However, allelic complementation was observed among alleles of *ad*-1, *ad*-2 and *ad*-3. The limited complementation maps which can be drawn for these three loci are shown in Fig. 1. These maps satisfy all the data to hand, but since these are not yet complete the maps almost certainly represent simplifications of the situation at each locus.

Growth tests and search for aminoimidazoles. All the mutants showed a fair to good response to adenine supplementation, while none responded to guanine, indicating a uniform lack of GMP-reductase which is responsible for the conversion of GMP to IMP (Magasanik & Karibian, 1960). Guanine inhibited the growth of the wild type by about 60% when present alone, and completely inhibited growth of the wild type when adenine or adenosine were also present in the medium. The latter observation can be explained on the assumption that exogenous adenine or adenosine represses the *de novo* synthesis of purines, but that competitive inhibition by guanine prevents the

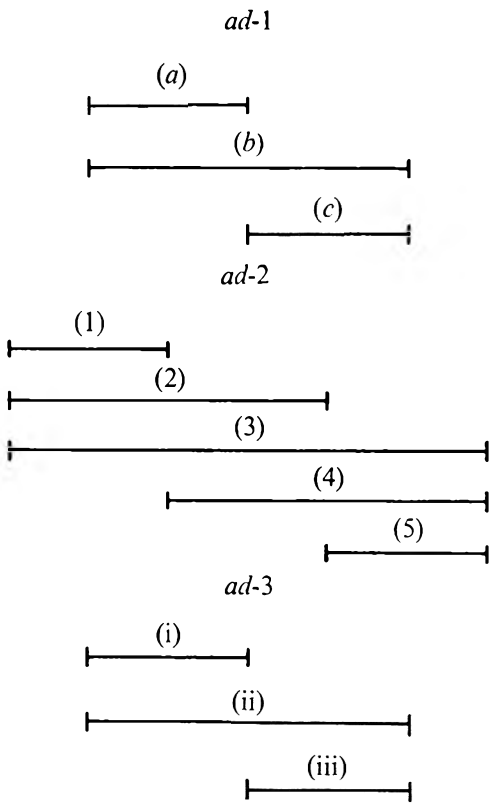

Fig. 1. Complementation patterns at three distinct adenine loci. The figures in parentheses indicate the complementation groups which are composed of the following mutants. *ad*-1: (*a*) G1912, (*b*) C834, (*c*) M38. *ad*-2: (1) G2302, M63, M64, (2) M39, (3) M40, P816, P835, (4) M58, (5) G2215, *ad*-3: (i) M8, (ii) C691, M5, M42, M65, M66, M67, M68, P823, P932, (iii) P819. The maps were drawn using the established principles for expressing complementation data; i.e. where the bars overlap no complementation was observed between the members of the two groups, where the bars do not overlap complementation was observed. Thus, for example, members of group (1) do not complement each other and do not show complementation with members of groups (2) or (3) (the bars overlap), but they do complement with members of groups (4) and (5).

Table 1. *Locus allocations of Coprinus lagopus purine mutants*

ad-1	C834; G1912; M38*
ad-2	P816; P835; G2215; G2302; M39; M40; M58; M63; M64
ad-3	C691; M5; M8; M42; M65; M66; M67; M68; P819; P823: P932
ad-4	P805; P902
ad-5	M36; P838
ad-6	P809
ad-8	C764; M6; M41; G1904; G2242; G3231
ad-9	JR53
ad-10	G2597
ad-11	M15

*Stock numbers prefixed by the letter C indicate mutants isolated by D. Lewis; by G, G. E. Anderson; by M, D. H. Morgan (mutants M63 to M70 isolated by the present author); and by P, P. R. Day.

entry of adenine into the organism in sufficient quantity to support growth. Guanosine showed no such inhibition of the wild type whether alone or in company with adenine or adenosine. Table 2 summarizes the response patterns of the adenine mutants to a number of purines and purine derivatives.

Table 2. *Growth of adenine mutants on purines and derivatives*

Medium	Locus										
	ad-11	*ad*-10	*ad*-9	*ad*-8	*ad*-6	*ad*-5	*ad*-4	*ad*-3	*ad*-2	*ad*-1	Wild type
Complete	14	13	16	21	8	19	8	11	19	19	19
Minimal	—	—	—	—	2	—	2	2	3	—	20
+ Adenine	8	3	15	20	3	9	12	10	13	18	22
+ Adenosine	2	—	16	10	—	—	5	7	7	—	22
+ AMP	—	—	10	—	—	—	—	2	7	—	22
+ Inosine	3	—	13	12	2	—	8	8	12	2	20
+ Hypoxanthine	8	2	19	18	4	—	9	9	13	5	20
+ Adenine and guanine	—	—	14	—	—	—	—	—	—	—	—
+ Adenine and guanosine	12	2	15	20	7	9	10	9	14	20	21
+ Adenosine and guanine	—	—	—	—	—	—	—	1	—	—	—
+ Adenosine and guanosine	5	—	13	9	3	—	4	8	12	3	17

Entries appear as colony diameters (in mm.) after 50 hr growth. Strains used: wild type, BC9/66; *ad*-1, G1912; *ad*-2, P816; *ad*-3, M8 and P823; *ad*-4, P902; *ad*-5, GR100.11 (derived from P838); *ad*-6, P809; *ad*-8, M6 and G1904; *ad*-9, MS165 and JR53; *ad*-10, GR712 (derived from G2597); *ad*-11, M15. All supplements used at a final concentration of 50 μg./ml.

Representative members of each locus were chosen for the determination of response to different degrees of adenine supplementation. After incubation for 50 hr each strain exhibited its maximum growth on medium containing only 5 μg./ml. adenine—the maximum growth rates being essentially the same as those shown in Table 2. The highest concentration of adenine supplementation tested was 100 μg./ml.; there was no evidence of inhibition of growth at the higher concentrations after 50 hr incubation. However, continued incubation to 125 hr revealed that while the maximum growth rate was still attained on medium containing adenine, 5 μg./ml. inhibition of growth was evident in some cases at the highest concentrations. Strains BC6/65 (wild type), *ad*-4, *ad*-5 and *ad*-9 showed this effect; in each case the onset of inhibition was at adenine, 50 μg./ml. and at 100 μg./ml. the growth was about 85% of the maximum.

Of the extracts subjected to the chromatographic search for accumulation products only those of *ad*-1 and *ad*-5 showed definite accumulations. The mutants *ad*-2, *ad*-3, *ad*-4, and *ad*-8 showed no accumulation products distinct from wild type. Alleles of the *ad*-6, *ad*-10 and *ad*-11 loci were alike in showing very weak accumulations which were difficult to interpret.

The main accumulation product of *ad*-1 was a fast-running (R_F 0·35) compound staining bright yellow immediately on spraying with diazosulphonic acid, this colour fading to grey after spraying with sodium carbonate. This compound is very like that produced by the Neurospora 'adenine-purple' mutants of the *ad*-3 locus (Bernstein, 1961). The similarity goes further than this in that the raw extract of *ad*-1 was unique

among the Coprinus extracts in being a completely opaque purple-brown colour, all others were transparent yellow-orange; an extract of Neurospora-*ad*-3 prepared as a control was also an opaque purple colour. Thus despite the lack of appreciable accumulation of pigment in the mycelium of Coprinus *ad*-1 during growth, probably because of the neutral pH value of Coprinus media, such an accumulation was very evident on extraction. It is likely that a change to acid pH values occurring during the extraction procedures allowed the accumulated imidazole to polymerise to the pigment. The fast running Coprinus *ad*-1 accumulant was examined spectroscopically; it had no peak of absorption in the ultraviolet region, but did show the broad end-absorption, starting at about 210 mμ, reported for 5-aminoimidazole ribotide (AIR) by Levenberg & Buchanan (1957). The Bratton-Marshall reaction product (Bratton & Marshall, 1939) of this substance was an orange-red chromophore which absorbed maximally at 500 mμ. These results were consistent with the identification of the major accumulant of Coprinus *ad*-1 as AIR. A slower-running (R_F 0·1) compound also detected in extracts of Coprinus *ad*-1 was not positively identified because of an excessive background of contaminating substances. When spectroscopically compared with wild-type eluate from the same chromatographic position (i.e. the wild-type eluate was used in the reference cuvette position) a distinct absorption peak was observable at 308 mμ, together with end-absorption from 220 mμ. Absorption at 305 mμ is associated with the pigment produced by Neurospora-*ad*-3 (Bernstein, 1961), and is also associated with the raw extract of Coprinus *ad*-1. It is thus possible that the minor 'accumulant' was composed of some residual AIR (which would account for the end-absorption) which had been retained by the pigment (accounting for the 308 mμ absorption peak).

Alleles of the *ad*-5 locus accumulated a compound which stained red after spraying with diazosulphonic acid; this densely coloured area faded to a cluster of grey spots after the sodium carbonate spray. This compound was tentatively identified as 5-amino-4-imidazole-*N*-succinocarboxamide ribotide (or riboside), SAICAR, from this colour reaction. The substance was eluted in acetate buffer (pH 5) and showed a very marked absorption peak at 268 mμ. Published data for SAICAR (Lukens & Buchanan, 1959) gives 267–269 mμ for peak absorption at pH 5. The Bratton-Marshall reaction product of this accumulant absorbed maximally at 560 mμ; this is again characteristic of SAICAR and confirms the identification.

Alleles of the *ad*-6, *ad*-10 and *ad*-11 loci were alike in showing a very weak accumulation of a slow-running (R_F approximately 0.05) substance. No isolation could be made from *ad*-6 extracts; *ad*-10 and *ad*-11 substances were isolated although the poor resolution lead to much contamination, mainly by compounds absorbing maximally at 260 mμ. The use of a wild-type reference abolished the 260 absorption and it became clear that eluates of both Coprinus *ad*-10 and *ad*-11 showed a weak absorption peak (not detectable in the wild type eluate) in the region of 298–310 mμ, coupled with broad end-absorption.

DISCUSSION

The information which can be obtained from growth tests of adenine mutants is limited by the fact that the majority of the intermediates in purine biosynthesis are rather exotic compounds; they are not readily available, and are not readily taken up by the cell. Probably the most useful fact arising from the studies with *Coprinus lagopus*

mutants summarized in Table 2 is that *ad*-5 did not respond to hypoxanthine whereas all other loci did. This places the *ad*-5 'block' after the formation of inosinic acid (IMP), i.e. in one of the two reactions specific to adenosine-5'-phosphate (AMP) synthesis. The status of the other purines is confused by the non-uniformity of response. In some cases adenosine and AMP did not replace adenine, implying that the ribosyl compounds did not enter the organism and were not degraded extracellularly to the free base. On the other hand these two compounds did substitute for the adenine requirement of some mutants; whether this was the result of contamination by the free base or was a genuine genetic difference has not yet been determined. It may be significant that the mutants which responded only to the free base are assumed to be blocked after imidazole ring closure, while most of those which respond to adenosine and AMP are thought to be blocked in steps before closure of the ring.

With regard to the adenine/guanine inhibition it should be noted that *ad*-9 alone among the Coprinus mutants was completely free from any inhibition. The *ad*-9 mutation is possibly caused or accompanied by some change in the permeability of the cell membranes, or in the structure of a regulatory enzyme.

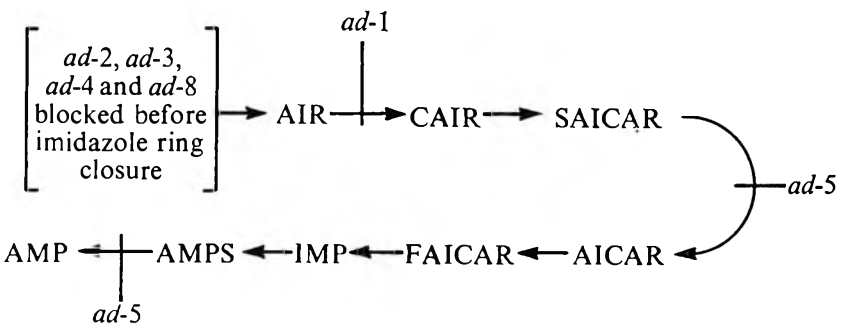

Fig. 2. Correlation of adenine mutants of *Coprinus lagopus* with steps in purine biosynthesis. Key: AIR, 5-aminoimidazole ribotide; CAIR, 5-amino-4-imidazole-carboxylic acid ribotide; SAICAR, 5-amino-4-imidazole-*N*-succino-carboxamide ribotide; AICAR, 5-amino-4-imidazolecarboxamide ribotide; FAICAR, 5-formamido-4-imidazolecarboxamide ribotide; IMP, inosinic acid; AMPS, adenylosuccinic acid ribotide; AMP, adenosine-5'-phosphate.

By basing the conclusions on growth responses and the identities of accumulated aminoimidazoles some of the adenine loci can be correlated with steps in purine biosynthesis (Fig. 2). The *ad*-2, *ad*-3, *ad*-4 and *ad*-8 loci all responded to adenine or hypoxanthine and accumulated no imidazoles. They can be assigned to steps which precede closure of the imidazole ring. Accumulation of 5-aminoimidazole ribotide or riboside (AIR) by *ad*-1 indicates an inability to carboxylate AIR to 5-amino-4-imidazolecarboxylic acid ribotide (CAIR). Since *ad*-5 did not respond to hypoxanthine, and yet accumulated 5-amino-4-imidazole-*N*-succinocarboxamide ribotide (SAICAR) it can be confidently assumed to lack the bifunctional enzyme 'adenylosuccinase'. Some alleles of the *ad*-5 locus not used in this investigation have been shown to lack adenylosuccinase activity (P. R. Day & C. F. Roberts, personal communication). The *ad*-5 locus was termed *adhi*-1 by Cowan (cited in Casselton, 1965) because the growth rate of all *ad*-5 alleles was enhanced when histidine was provided as well as adenine. The data presented here suggest that the histidine 'requirement' is in fact the expression of a sparing reaction in that, if histidine biosynthesis is repressed by an

exogeneous supply of the amino acid, there will be less call on what must be limited amounts of endogenous adenosine-5′-phosphate. The designation *ad*-5 should therefore be maintained. There is not sufficient evidence for any suggestion to be made about the positions of the *ad*-6, *ad*-10 and *ad*-11 mutations in the pathway.

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Lipolytic Activity by Oral Pleuropneumonia-Like (Mycoplasma) Organisms

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SUMMARY

Ninety-five strains of Mycoplasma were isolated from human saliva; all were micro-aerophilic; 72 were actively lipolytic and 23 were non-lipolytic and were inhibited by Tweens. A type culture of human type 4 (*Mycoplasma salivarum*) was culturally identical with the lipolytic strains, and two type cultures of *M. orale* with the non-lipolytic strains. The strains serologically identifiable as *M. orale* were non-lipolytic, whereas those identifiable as *M. salivarium* were of both types. Comparison was also made with type strains from other sources. Those of human origin were non-lipolytic and those of saprophytic or animal origin were slightly lipolytic.

INTRODUCTION

Mycoplasma (PPLO) organisms were first isolated from the human mouth and pharynx by Smith & Morton (1951) and by many workers since. Nicol & Edward (1953) regarded them as distinct from human genital and veterinary strains, and Edward (1954) classified them on cultural grounds as human type 4, the genital strains being type 1. Huijsmans-Evers & Ruys (1956) confirmed the distinction serologically, as also did Edward & Freundt (1956) who named the oral strains *Mycoplasma salivarium*; Pease (1965) showed that, although the oral and genital strains were antigenically distinct, at least four antigens were common to both.

A second antigenic type of oral Mycoplasma was identified and given the name of *Mycoplasma orale* by Herderschee, Ruys & van Rhijn (1963) and by Taylor-Robinson, Canchola, Fox & Chanock (1964).

Lipid metabolism in Mycoplasma has attracted attention: Edward (1950) described the 'film and spots' reaction which he later (Edward, 1954) showed to be associated with lipid metabolism, and specifically to the clearing of egg yolk. Nicol & Edward (1953) showed that some human type 4 strains produced this reaction, whereas others did not. The fatty-acid requirements of Mycoplasma strains were investigated by Razin & Rottem (1963) and lipolytic activity recorded by Rottem & Razin (1964). In the present paper, the lipolytic activity of some oral strains of Mycoplasma is described, and compared with that of named strains.

METHODS

Strains used. Ninety-five strains were isolated from human saliva in this laboratory; 5 strains of *Mycoplasma hominis* type 1, one each of *M. salivarium* (H 110 Edward), *M. Mycoides* (*capri*), *M. gallinarum* and *M. laidlawii* types A and B, all maintained as

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type cultures in this laboratory; two strains of *M. orale*, Ella & Hilverda, obtained from Dr A. C. Ruys. Generic and specific names are used for convenience without prejudice to the problem of their validity.

Media. The basal medium used consisted of 1% (w/v) proteose peptone (Oxoid), 0·25% (w/v) yeast extract (Oxoid), 1% (w/v) Lab-Lemco (Oxoid beef extract) and 0·5% (w/v) sodium chloride. The medium was adjusted to pH 7·8 and sterilized by autoclaving at 120° for 15 min. Any precipitate which developed was removed by filtration, and the clear medium again autoclaved. When a solid medium was required, the broth was supplemented with 1·2% (w/v) New Zealand agar or 1% (w/v) Ionagar no. 2 (Oxoid, L 12). Bovine serum, heated at 60° for 30 min. was added to a final concentration of 10% (w/v). Horse serum was used only for the initial isolations because of the reproduction of the film-and-spots reaction by some mycoplasmas, which would interfere with the tests for lipolysis. The oral mycoplasma strains were normally incubated anaerobically, but aerobic growth was also examined. The remaining strains were grown aerobically. Tests were examined after 7 days of incubation.

Egg-yolk reaction. The basal medium agar was supplemented with 10% (w/v) egg yolk suspension (Oxoid, SR 47). Inoculated plates were examined up to 7 days for lipolysis indicated by the development of a film over the growth and precipitation in the agar, and for proteolysis indicated by a wide zone of clearing of the egg-yolk emulsion.

Lipolysis. Butter fat and castor oil were added to the solid medium in the form of 10% (v/v) emulsions in distilled water, prepared by treatment with a Mullard ultrasonic drill, to give final concentrations of 0·5% (v/v). The breakdown of tributyrin was tested on Oxoid tributyrin agar. Decomposition of butter fat and castor oil was indicated by a clearing of the emulsions and a film over the surface of the growth; the breakdown of tributyrin was indicated by a clearing of the emulsion. Breakdown of Tweens 20, 40, 60 and 80 was tested on base agar containing 2% (w/v) Tween and 0·01% (w/v) calcium chloride. Decomposition was indicated by the production of a film over the growth and precipitation beneath the agar.

Serology. Gel-diffusion precipitin tests were made according to the technique of Pease & Laughton (1965). Growth-inhibition tests (Edward & Fitzgerald, 1954) were done on plates of basal medium agar with filter-paper discs soaked in undiluted rabbit antiserum. Fluorescent antigen antibody tests were observed by the use of fluorescein-linked fowl anti-rabbit serum on colony impression preparations (Chanock, Hayflick & Barile, 1962). Antisera were prepared as described by Pease & Laughton, (1965).

RESULTS

Of the 95 oral Mycoplasma strains isolated, 72 produced a strong reaction on egg-yolk agar and showed lysis of tributyrin, butter fat, castor oil and Tweens 20, 40 and 60, but not Tween 80; these strains grew rather poorly aerobically. The remaining 23 strains were non-lipolytic and were inhibited by Tweens; these strains grew very poorly indeed aerobically. Of the named strains, type 4 (*Mycoplasma salivarium*) was indistinguishable from the lipolytic group; *M. gallinarum*, *M. mycoides* (*capri*) and *M. laidlawii* (A and B) were slightly lipolytic; *M. orale* was non-lipolytic and closely resembled the non-lipolytic group (Table 1).

Twenty strains were examined serologically by gel-diffusion precipitin reactions, growth inhibition and immunofluorescence, against type antisera for type 4 (*Mycoplasma salivarium*) and *M. orale*. Type strains of *M. orale* possessed one antigen (as observed by lines of precipitation) which was absent from type strain of type 4. Five strains were serologically identifiable as *M. orale*, all were non-lipolytic; 15 were identifiable as type 4, of which 12 were lipolytic and three non-lipolytic.

Table 1. *Lipolysis by strains of Mycoplasma*

	Egg-yolk reaction	Castor oil	Butter fat	Tributyrin	Tween 20	Tween 40	Tween 60	Tween 80
Oral strains (72) and *M. hominis*, type 4	+	S	+	S	+	+	+	−
Oral strains (23) and *M. orale*	−	−	−	−	I	I	I	I
M. hominis type 1	−	−	−	−	I	I	I	I
M. gallinarum	+	−	−	−	+	+	+	+
M. mycoides (*capri*)	−	−	−	+	+	+	+	S
M. laidlawii (A and B)	−	−	−	S	I	+	+	S

+ = positive reaction; − = negative; S = weakly positive; I = growth inhibited.

These results show that two types of oral mycoplasmas are distinguishable by their lipolytic activity. Culturally, the lipolytic strains correspond to the type strains of *Mycoplasma salivarium*, and the non-lipolytic correspond both culturally and antigenically to the type strain of *M. orale*; both lipolytic and non-lipolytic strains, however, are found among those antigenically definable as *M. salivarium*. The saprophytic strains and those of animal origin examined are slightly lipolytic, and are not inhibited by the Tweens, whereas the human vaginal strains examined are non-lipolytic and, like *M. orale* strains, are inhibited by the Tweens.

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The Utilization of Propionate by *Micrococcus denitrificans*

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SUMMARY

Suspensions of *Micrococcus denitrificans*, growing with propionate as sole carbon source, incorporated ^{14}C from [1-^{14}C]propionate or from sodium [^{14}C]bicarbonate, initially into succinate and then into intermediates of the tricarboxylic acid cycle and amino acids derived therefrom. In the presence of 4 mM-sodium arsenite, the oxidation of propionate and of L-malate by washed organisms proceeded only to the level of pyruvate, which accumulated: when sodium [^{14}C]bicarbonate was also present, the pyruvate formed from propionate, but not that formed from L-malate, was highly radioactive. Cell-free extracts of propionate-grown *M. denitrificans* catalysed the formation of labelled methylmalonyl-coenzyme A, succinyl-coenzyme A and succinate from sodium [^{14}C]carbonate + ATP + either propionate and coenzyme A, or propionyl-coenzyme A. The evidence thus obtained indicates that propionate enters the tricarboxylic acid cycle of *M. denitrificans*, preponderantly via activation to propionyl-coenzyme A, followed by carboxylation to methyl-malonyl coenzyme A, isomerization to succinyl-coenzyme A and hydrolysis to succinate.

INTRODUCTION

Micrococcus denitrificans is unusual among micro-organisms in that, during growth on acetate, the glyoxylate cycle appears not to be the major route for the maintenance of a functioning tricarboxylic acid cycle (French, Kornberg & Morris, 1964) and, during growth on glycollate or other precursors of glyoxylate, this purpose is achieved via an apparently unique pathway involving the formation and cleavage of *erythro*-β-hydroxyaspartate (Kornberg & Morris, 1965). Since it is known that micro-organisms utilize propionate by a variety of different routes (for brief survey, see Callely & Lloyd, 1964), it was of interest to determine whether the utilization of this C_3-acid by *M. denitrificans* manifested unusual features similar to those exhibited by this organism in its utilization of C_2-acids. The results obtained show that propionate is utilized by a pathway similar to that operating in mammalian systems but which has been observed only rarely to occur in bacteria.

METHODS

Organisms used. The strain of *Micrococcus denitrificans*, obtained from Dr June Lascelles, had been originally supplied by Dr W. Verhoeven.

Growth of the organism. Cultures of *Micrococcus denitrificans* were maintained and grown as described by Kornberg & Morris (1965) except that sodium propionate was substituted for sodium glycollate in all media.

Other experimental procedures. The measurement of the growth of *Micrococcus*

denitrificans, the incorporation of isotope from [^{14}C]-labelled substrates by growing cultures, the assay and identification of labelled compounds, the preparation of cell-free extracts and the determination of protein were performed as previously described (Kornberg & Morris, 1965). Propionyl-coenzyme A was prepared from propionic acid anhydride and coenzyme A (Stadtman, 1957).

Chemicals used. Radioactive materials were obtained from the Radiochemical Centre, Amersham, Buckinghamshire. Coenzyme A, ATP, α-oxoacids and tris were purchased from the Boehringer Corporation (London) Ltd.; L-malic acid from Koch–Light Laboratories Ltd., Colnbrook, Bucks., and all other materials from British Drug Houses Ltd., Poole, Dorset.

RESULTS

Incorporation of [1-^{14}C]propionate by whole organisms

When sodium [1-^{14}C]propionate was added to a suspension of *Micrococcus denitrificans*, which had been grown in a medium containing propionate as sole carbon source and which had been resuspended in fresh propionate medium, isotope was rapidly incorporated into cellular components. Samples, taken from 10 sec. to 3 min. after addition of the labelled material, were received into hot ethanol and were analysed by two-dimensional paper chromatography and autoradiography (Kornberg 1958). Such samples showed the incorporated ^{14}C to be distributed mainly between the tricarboxylic acid cycle intermediates citrate, malate, fumarate and succinate, and the amino acids derived directly from the cycle, aspartate and glutamate (Fig. 1). It was apparent that, after 1·5–2 min., most of the labelled material in any sample was present as malate and glutamate, with the other C_4-acids and citrate each contributing less than 10% to the total radioactivity of the sample; these relative amounts of radioactive substances presumably reflected also the relative amounts of the various intermediates present in the ethanol-soluble 'pools' in the bacteria. However, it was also clear that the distribution of isotope amongst these substances at earlier times was not in accordance with the distribution observed after the isotopic steady state condition had been reached. Initially, over 45% of the total ^{14}C incorporated was present in succinate, and the proportion of labelled succinate in the samples decreased with time, whilst the proportions of other labelled materials remained constant or increased. This showed that succinate was the earliest stable material formed from [1-^{14}C]propionate in the intact organisms, and indicated that propionate was utilized via its carboxylation.

Incorporation of $^{14}CO_2$ by whole organisms

A consequence of this inference would be that the pattern of isotope incorporation observed when sodium [^{14}C]bicarbonate is added to *Micrococcus denitrificans* suspended in propionate growth medium must be closely similar to that observed when [1-^{14}C]propionate is the labelled material. As shown by the results in Fig. 2 this proved to be the case. Glutamate and malate again contained most of the incorporated ^{14}C after 1·5–2 min., with aspartate and succinate contributing less than 15% each (citrate, which contained less than 5% has been omitted from Fig. 2), and this distribution differed markedly from that observed with samples taken at earlier times. Initially, succinate contained a much higher proportion of ^{14}C than did any

other component of the ethanol-soluble fraction. The rapid decrease with time in the proportion of labelled succinate present in the samples suggested that the other isotopically labelled materials were derived from labelled succinate, which was thus the main port of entry of isotope from [^{14}C]bicarbonate. Since, during growth on substances other than propionate, *M. denitrificans* incorporates isotope from [^{14}C]bicarbonate initially into malate and not into succinate (H. L. Kornberg & J. Smith, unpublished observations), this finding supports the view that the utilization of propionate by these organisms necessarily involves its carboxylation to succinate.

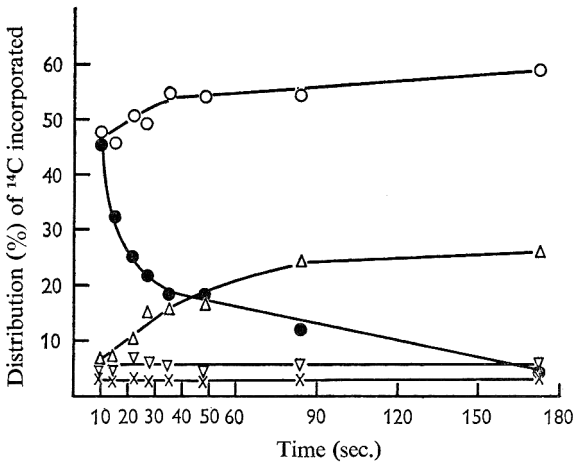

Fig. 1

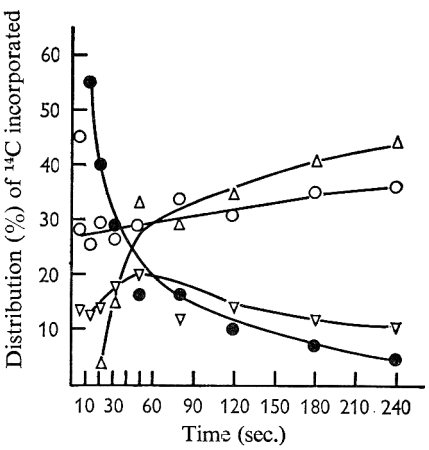

Fig. 2

Fig. 1. Distribution (%) of isotope incorporated from [1-^{14}C]propionate by propionate-grown *Micrococcus denitrificans* into malate (○), succinate (●), citrate (×), glutamate (△) and aspartate (▽).

Fig. 2. Distribution (%) of isotope incorporated from sodium [^{14}C]bicarbonate by propionate-grown *Micrococcus denitrificans* into malate (○), succinate (●), glutamate (△) and aspartate (△).

Effect of arsenite on propionate utilization

A further test of this route of propionate utilization was provided by measurement of the incorporation of sodium [^{14}C]bicarbonate by washed suspensions of propionate-grown *Micrococcus denitrificans*, which were oxidizing either L-malate or propionate in the presence of this isotopically labelled material and sodium arsenite. Manometric experiments showed arsenite to be a powerful inhibitor of propionate oxidation: in its presence, only 10 μmoles of oxygen were absorbed by organisms incubated in air with 10 μmoles of propionate; and over 8 μmoles of pyruvate were found to have accumulated after the uptake of oxygen ceased. This relationship

$$CH_3.CH_2.CO_2H + O_2 \rightarrow CH_3.CO.CO_2H + H_2O$$

cannot by itself reveal the route whereby propionate is converted into pyruvate. However, it would be expected that, if the utilization of propionate involved its prior carboxylation to succinate and the succinate were to give rise to pyruvate via fumarate, malate and oxaloacetate, the pyruvate thus formed and accumulated would be labelled if sodium [^{14}C[bicarbonate were also included in the medium. It would further be expected that routes for propionate utilization not involving an obligatory fixation

of $^{14}CO_2$ would, if operating, yield pyruvate containing little or no isotope; moreover, the oxidation of substances other than propionate—such as L-malate—would, under these conditions, give rise to pyruvate labelled only to the extent that isotopic exchange reactions occurred. As shown in Table 1, these expectations were fulfilled. Whereas the oxaloacetate and pyruvate formed from L-malate, in the presence of arsenite and sodium [^{14}C]bicarbonate, contained a total of only 3000 counts/100 sec., these α-oxoacids derived from propionate under similar conditions contained over 140 times as much ^{14}C. Consequently, the formation of pyruvate from propionate, but not that from L-malate, must have involved a major fixation of carbon dioxide.

Table. 1. *Incorporation of ^{14}C by Micrococcus denitrificans when oxidizing unlabelled L-malate or propionate, in the presence of sodium [^{14}C]bicarbonate and sodium arsenite*

Each of two stoppered flasks contained 1 ml. of 0·1 M-tris buffer (pH 8); 0·4 ml. of 0·01 M-sodium arsenite; 1 ml. of propionate-grown *M. denitrificans* (equiv: 17 mg. dry wt.); 0·9 ml. water and 0·5 ml. of sodium [^{14}C]bicarbonate (100μc) solution. One of the flasks also received 0·2 ml. of 0·1 M-sodium L-malate; the other received 0·2 ml. of 0·1 M-sodium propionate. The flasks were shaken at 30° for 2 h, after which time 1 ml. of the contents was pipetted into 3 ml. of hot ethanol, and 1 ml. into 2 ml. of 0·1% 2,4-dinitrophenylhydrazine hydrochloride in 2 N-HCl. The radioactivity of the ethanol-soluble materials was assayed directly on aluminium planchets, whereas the 2,4-dinitrophenylhydrazones of α-oxoacids were separated and identified by paper chromatography (El Hawary & Thompson, 1953) and their radioactivity assayed directly on the paper.

	Total ^{14}C (counts/100 sec. × 10^{-3}) incorporated into:		
Substrate	Ethanol-soluble fraction	Oxaloacetate	Pyruvate
L-Malate	3·4	1·2	1·8
Propionate	522	4·6	422

Incorporation of ^{14}C from sodium [^{14}C]carbonate by cell-free extracts

The condensation of labelled carbon dioxide and unlabelled propionate, implied to occur by the experiments with intact organisms, was readily demonstrable when cell-free extracts of propionate-grown *Micrococcus denitrificans* were used. A major fixation of isotope from sodium [^{14}C]carbonate into acid-stable materials was observed when such extracts were incubated with either propionate + coenzyme A + ATP, or with propionyl-coenzyme A + ATP. As shown in Table 2, 1 μmole of propionyl-coenzyme A was apparently a more effective acceptor for the isotopic carbon dioxide than were 5 μmoles of propionate. The reaction did not proceed when ATP or propionate were omitted and, with the unpurified cell extract used, omission of coenzyme A caused only a slight decrease in the quantities of labelled carbon dioxide fixed.

When the labelled products obtained from propionyl-coenzyme A and sodium [^{14}C]carbonate were analysed directly by two-dimensional paper chromatography and autoradiography, two major radioactive materials and three minor ones were found. The major labelled products were succinate and a diffuse material running in the position usually occupied by coenzyme A esters; the minor products were malate, fumarate and aspartate. Alkaline hydrolysis of the sample before chromatography, by the procedure indicated in outline in the footnote of Table 2, caused the total dis-

appearance of the diffuse labelled material, the intensification of the succinate spot and the appearance of a different but compact spot of labelled material: this latter compound was identified, by co-chromatography in several solvents (Kornberg, 1958) with the authentic compound, as a salt of methylmalonic acid. It is thus likely that the diffuse labelled material was a mixture of the coenzyme A esters of succinic and methylmalonic acids. Hence, it is also likely that the fixation of carbon dioxide to propionate proceeds predominantly via the enzymic sequence first elucidated for mammalian systems (for details of this sequence, see review by Kaziro & Ochoa, 1964).

Table 2. *Incorporation of isotope from sodium [^{14}C]carbonate by cell-free extracts of propionate-grown Micrococcus denitrificans*

The complete system contained, in a final volume of 1·0 ml., 100 μmoles of tris buffer (pH 8·5); 10μmoles of magnesium chloride; 5μmoles of ATP; ultrasonic extract of propionate-grown *M. denitrificans* (containing 2 mg. protein); 10 μmoles of sodium [^{14}C]carbonate (containing 20 μc of isotope); 5 μmoles of propionate *or* 1 μmole of propionyl-coenzyme A; 0·05 μmole of coenzyme A; water. The mixtures were incubated at 30° for 30 min. and then mixed with 3 ml. of hot ethanol; of the resulting mixture, 0·1 ml. was spotted on to an aluminium planchet. The material was evaporated to dryness, irrigated with 10% (w/v) acetic acid and re-dried to constant radioactivity.

Contents of incubation mixture	Total ^{14}C incorporated (counts/100 sec. $\times 10^{-3}$)
Complete: propionate	127
ATP omitted	4·8
Propionate omitted	6·2
Coenzyme A omitted	96
Complete: propionyl-coenzyme A	323*

*After incubation of the ethanolic solution with 2 N-potassium hydroxide for 30 min. at 23° followed by neutralization with perchloric acid and analysis by two-dimensional paper-chromatography and autoradiography (Kornberg, 1958) of the supernatant solution, the isotopically labelled products formed from propionyl coenzyme A and sodium [^{14}C] bicarbonate were:

Labelled material	Percentage of total
Succinate	63
Methyl malonate	12
Malate	15
Fumarate	4
Aspartate	6

DISCUSSION

The enzymic mechanism whereby propionate is utilized by mammalian tissues has been shown to involve the fixation of carbon dioxide to yield, ultimately, succinate, via isomers of methylmalonyl-coenzyme A and succinyl-coenzyme A (for review, see Kaziro & Ochoa, 1964). Although this sequence of reactions has been reported to occur also in *Rhodospirillum rubrum* (Gibson & Knight, 1961; Knight, 1962) and a Propionibacter species (Stadtman, Overath, Eggerer & Lynen, 1960), many other species of bacteria do not appear to use this route for the utilization of propionate. Rather do such organisms convert propionyl coenzyme A initially to acrylyl-coenzyme A, and thence either to acetyl-coenzyme A and carbon dioxide (via β-hydroxypropionyl-coenzyme A and either malonic semialdehyde or malonyl-coenzyme A; Vagelos,

1960; Callely & Lloyd, 1964), or form pyruvate from acrylyl-coenzyme A, possibly via lactyl-coenzyme A (Vagelos, Earl & Stadtman, 1959). It has also been suggested that propionyl-coenzyme A may be utilized through an initial condensation with glyoxylate to form α-hydroxyglutarate, which is alleged to be cleaved to acetate and pyruvate (Reeves & Ajl, 1963).

The results obtained with *Micrococcus denitrificans* show that propionate utilization by this organism does not proceed via any of the variety of routes peculiar to plants and to other micro-organisms, but occurs through reactions closely similar to those found in mammalian tissues. In addition to the direct evidence to this effect obtained with intact micrococci and extracts derived therefrom, it is also apparent that the operation of other pathways is contra-indicated. Thus, the high degree of labelling in pyruvate, formed from unlabelled propionate and labelled carbon dioxide, argues against any major formation of pyruvate from propionate via acrylyl-coenzyme A; the virtual absence of isocitrate lyase from extracts of propionate-grown *M. denitrificans* also rules out the formation of the glyoxylate necessary for the initiation of the α-hydroxyglutarate pathway proposed by Reeves & Ajl (1963).

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A Kinetic Study of the Mode of Growth of Surface Colonies of Bacteria and Fungi

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SUMMARY

A model for the growth of microbial colonies on the surface of a solid nutrient medium is discussed. The model accounts for the constant rate of increase in the colony radius which is characteristic of a fungal colony growing on the surface of a nutrient medium.

Experiments showed that bacterial colonies after about 12 hr of development showed a virtually constant rate of radial growth over a 12 hr period. Over longer periods (24 hr) a gradual decline in the colony radial growth rate was apparent. The initial rate of radial growth of the bacterial colony was a useful parameter of the growth rate of the organism. The effects on the initial colony radial growth rate of the following factors were determined: initial nutrient concentration depth of agar layer; maximum specific growth rate (ln 2/minimum doubling time); oxygen partial pressure; humidity of gas phase; temperature. Three bacterial types, *Escherichia coli*, *Klebsiella aerogenes* and *Streptococcus faecalis* were studied. With *E. coli* growing in minimal medium in air at 1 atm. pressure when the growth was glucose-limited, oxygen became a limiting factor when the glucose concentration exceeded 0·25% (w/v). With a glucose concentration of 1% (w/v), the growth was strongly inhibited, probably by toxic products.

When the colony growth was glucose-limited and oxygen was present in excess, the relation between initial colony radial growth rate (K_r), the initial glucose concentration (s_0) and the maximum specific growth rate (α_m) was

$$K_r = k_2(\sqrt{s_0} - \sqrt{s_i})\sqrt{\alpha_m},$$

where k_2 is a constant; s_i, called the 'lag concentration', is a value of the glucose concentration which must be exceeded before growth of the colony can occur. The value of s_i was very small or negligible except with a certain type of inhibitory condition, such as an over-optimal concentration of oxygen, which could be overcome by the organism's metabolic activity. Direct proportionality between K_r and $\sqrt{\alpha_m}$ was found by varying the maximum specific growth rate by adding sulphanilamide. When α_m was varied by temperature changes the linear relation between K_r and $\sqrt{\alpha_m}$ did not hold. The implications of these results and their potential applications are discussed.

INTRODUCTION

Although the growth of fungi or bacteria in colonies on the surface of a solid nutrient medium is a general experimental technique and of great ecological importance, the laws which govern colonial growth have not been elucidated. The fact that we expect a colony of a given organism to reach a certain size and shape in a certain

time implies that there are underlying laws. We need to know what factors govern the rate of growth of the colony, how the outward spread of the colony is related to mass growth, what governs the ultimate size of the colony, whether all parts of the colony grow at the same rate and if not, what causes the differential growth. The solution of these problems should extend the usefulness of the colony-growth technique and facilitate quantitative studies of microbial ecology. The object of the present paper is to provide a theoretical and experimental analysis of some of these problems.

The basis on which the concepts of colony growth are built are the growth laws derived from study of the growth of populations of organisms in submerged homogeneous liquid cultures. In such cultures, as long as all nutrients are present in excess and growth inhibitors are not accumulated, the organisms grow exponentially. The law for such growth is:

$$\frac{dM}{dt} = \alpha M, \tag{1}$$

where M = organism mass/unit volume, t = time, and α is a constant known as the 'specific growth rate'. Integration of (1) gives the well-known logarithmic relation for growth

$$\ln M = \alpha t + \ln M_0, \tag{2}$$

where ln = natural logarithm and M_0 = concentration of organisms at zero time. The specific growth rate (α) is a most useful parameter because it is a direct measure of the growth rate of a population in a given environment. It is related to the doubling time (t_d) of the organisms by the expression $t_d = \ln 2/\alpha$. It should be pointed out that exponential growth is characteristic not only of bacteria, yeasts and other non-filamentous organisms, but also of filamentous fungi provided that these filaments are homogeneously dispersed and not aggregated into 'pellets' (Pirt, 1966).

If the flow of nutrients and oxygen into a microbial colony were unrestricted and growth inhibitors did not accumulate, the growth of the colony should conform to (1). Suppose the colony grew as a hemisphere of radius r with a constant mass of organisms ρ/cm^3. Substituting $M = \frac{2}{3}\pi r^3\rho$ in (1), then

$$\ln r = \frac{\alpha}{3}t + \ln r_0, \tag{3}$$

where r_0 is the radius of the colony at zero time. If the colony grew as a disc of constant height h and radius r, then $M = \pi r^2 h\rho$. Substitution of this value in (1) leads to

$$\ln r = \frac{\alpha}{2}t + \ln r_0. \tag{4}$$

In either case, therefore, the radius should increase exponentially and the slope of the plot of ln r against t should be linearly related to the specific growth rate. But it is common experience that microbial colonies of visible size growing on the surface of a solid nutrient do not spread outwards at an exponential rate.

Virtually all the work on colony growth rates has been concerned with filamentous fungi; there seem to be no reports of quantitative studies on the kinetics of growth of bacterial or yeast colonies. It is commonly accepted that colonies of filamentous fungi growing on solid media in Petri-dish cultures spread outwards at a constant rate, often termed the 'linear rate of growth'. Fawcett (1925) apparently was the first who explicitly observed that fungal colonies spread at a constant rate along the sur-

face of a nutrient agar contained in a tube. The linear rate of colony spread along a tube was used by Ryan, Beadle & Tatum (1943) as a parameter of the growth rate of Neurospora. Other workers (e.g. Brancato & Golding, 1953) have used the constant rate of increase in the radius of a fungal colony on a Petri-dish culture as a parameter of the growth rate. A detailed morphological and quantitative study of colonies of a fungus Chaetomium sp. was reported by Plomley (1959), who found that initially the radius of the colony increased exponentially and the transition to a constant radial growth rate occurred at a diameter of about 0·2 mm.

The constant rate of increase in colony radius is represented as follows. If r is the colony radius at time t, and r_0 at time zero, then

$$r = K_r t + r_0, \tag{5}$$

where K_r is a constant. Since K_r represents the increase in the radius per unit time, K_r is here called the 'radial growth rate'.

An estimate of the height of the colony can be derived from the analysis by Hill (1928) of nutrient diffusion into a layer of cells such as a tissue. From this analysis it follows that the thickness (h_g cm.) to which a diffusing nutrient will penetrate a metabolizing tissue is limited to

$$h_g = \sqrt{\frac{2Ds_0}{q}}, \tag{6}$$

where D (cm.^{2}/sec.) is the diffusion constant for the nutrient, s_0 (g./cm.^{3}) is the concentration of nutrient at the surface and q is the metabolic quotient (g. nutrient consumed/cm.^{3} of tissue/sec.). The maximum value of q is given by $q = \alpha_m/Y$ (Pirt, 1957), where α_m(sec.$^{-1}$) is the maximum specific growth rate of the organisms and Y (cm.^{3} tissue formed/g. nutrient) is the yield. The minimum value of q will be the maintenance requirement (Pirt, 1965). The minimum and maximum values for the thickness (h_g) of the growing layer in an *Escherichia coli* surface colony may be estimated as follows. A colony growing on a glucose minimal medium agar with growth limited by the glucose supply is considered. The glucose concentration is taken as 2.5 g./l., that is, $s_0 = 2{\cdot}5 \times 10^{-3}$ g. glucose/cm.^{3}, $D = 0{\cdot}6 \times 10^{-5}$ cm.^{2}/sec. $Y = 4{\cdot}4$ cm.^{3}/g. glucose (calculated from a dry weight yield of 0·44 g./g. glucose, and a population density (concentration of organism) equivalent to 0·1 g. dry wt. organism/cm.^{3}), $\alpha_m = 1{\cdot}94 \times 10^{-4}$ sec.$^{-1}$ (that is, 0·70 hr^{-1}). Hence the minimum value calculated for the thickness of the growing layer of the colony is 260 μ. The maintenance energy requirement may be taken as about one-tenth of the glucose requirement at the maximum growth rate, therefore the maximum value of h_g will be about three times the minimum value, that is, 780 μ. In fact, the concentration of glucose at the colony surface will be lower than the initial concentration, hence the above values for h_g must be overestimates.

An estimate of the maximum thickness of the growing layer of a colony of, say, *Escherichia coli* may also be determined from the oxygen requirement. In relation (6) above the following values are inserted: $D = 1{\cdot}9 \times 10^{-5}$ cm.^{2}/sec.; $s_0 = 8 \times 10^{-6}$ g./cm.^{3} (for water saturated with oxygen at 0·21 atm. pressure); $q = 2 \times 10^{-5}$ g. oxygen/cm.^{3} colony/sec. (calculated on the basis that the $q_{O_2} = 500$ ml. oxygen/g. organism dry wt./hr and that there is 0·1 g. organism dry wt./cm.^{3} colony). The value for h_g then is 40 μ. Even if the cells were only respiring at the maintenance level (Pirt, 1965) the q_{O_2} would be about 2×10^{-6} g. oxygen/cm.^{3}/sec. and the maximum

value of the depth to which oxygen would penetrate would be about 127 μ. It is therefore concluded that, since the thickness of the growing layer of bacteria will be small and constant in the steady state, the total height of the colony will not vary greatly.

To account for the growth rate of a microbial colony the following working hypothesis was developed. Growth of a colony on the surface of a solid homogeneous medium such as a nutrient agar was considered, and growth into the medium was excluded. It was supposed that if the inoculum consisted of one or a few organisms, then they would receive nutrients at concentrations above the growth-limiting values and consequently, initially the colony would grow at an exponential rate. In the

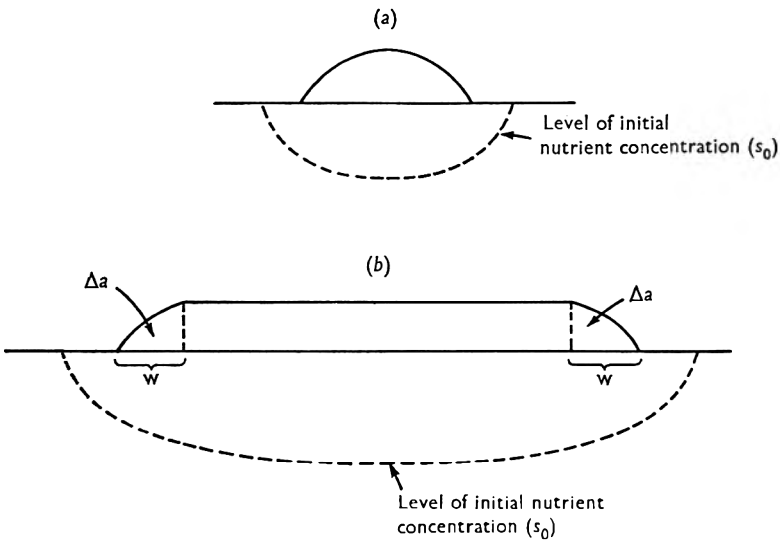

Fig. 1. Vertical cross-sections through model colonies (not to scale): (*a*) during initial exponential growth; (*b*) during phase of constant radial growth rate. The area Δa of width w represents the postulated zone of growth.

exponential phase it is concluded that growth will occur equally in all directions on the surface and lead to a hemispherical shape for the colony (see Fig. 1*a*). A gradient of nutrient concentration will develop under the colony. This means that the level in the agar at which the nutrient concentration has the initial value s_0, will recede from the agar surface as shown in Fig. 1*a*. With increase in size of the colony it was supposed that increase in nutrient demand and decrease in nutrient diffusion rate would occur as the colony grew thicker, and that eventually the concentration of some nutrient, termed the 'growth-limiting nutrient', would decrease virtually to zero in the centre of the colony and stop growth there. Then growth would be restricted to an outer peripheral zone of the colony. This state of the colony is represented in Fig. 1*b*. In the model it was assumed that ultimately a near steady state would be set up in which the width (w) of the growing zone remained constant as shown in Fig. 1*b*. The height of the colony was assumed to have the constant value (h) except in the peripheral growing zone. The colony growth rate according to this model is derived as follows. Let M = total mass of the organisms in the colony; M_g = mass of organisms in the

growing zone with constant specific growth rate, α; then the mass growth rate of the colony is given by

$$\frac{dM}{dt} = \alpha M_g. \tag{7}$$

When the width w of the growing zone is small compared with the colony radius r, to a close approximation, $M = \rho\pi r^2 h$, where ρ = organism mass (g.)/cm.^{3} colony. Also M_g approximates to $\rho 2\pi r \Delta a$, where Δa is the cross-sectional area of the growing zone and is taken to be constant. Substituting these values in (7) leads to

$$\frac{dr}{dt} = \frac{\Delta a}{h}\alpha. \tag{8}$$

Hence

$$r = \frac{\Delta a}{h}\alpha t + r_0, \tag{9}$$

where Δa, h and α are taken to be constants. Thus according to this model the colony should have a constant radial growth rate equal to $(\Delta a)/h$. If Δa approximates to a triangle of area $wh/2$, then (9) becomes

$$r = \frac{w\alpha}{2}t + r_0. \tag{10}$$

Development of the model requires a quantitative analysis of the problem of nutrient diffusion into the colony. However, despite this lack, the present theory has served as a useful guide to experiments designed to elucidate factors which control the growth rate of bacterial colonies.

METHODS

Medium. Defined medium A (DMA) contained the following constituents (amounts/l.): K_2HPO_4, 11·3 g.; NaH_2PO_4, $2H_2O$, 5·4 g.; $MgSO_4.7H_2O$, 200 mg.; $CaCl_2$, 10 mg.; $FeSO_4.7H_2O$, 5 mg.; $ZnSO_4.7H_2O$, 0·5 mg.; $MnSO_4.4H_2O$, 0·5 mg.; $CuSO_4.5H_2O$, 0·1 mg.; $CoCl_2.6H_2O$, 0·1 mg.; sodium borate, 0·1 mg.; sodium molybdate, 0·1 mg.; ethylenediaminetetra-acetic acid (EDTA), 0·26 g.; NH_4Cl, 2·0 g./l.; glucose, amounts separately specified. The final pH value was 7·4.

Medium DMA was prepared in the following manner. The phosphates were mixed at × 10 final strength and adjusted with NaOH to pH 7·50–7·55. The buffer with the other constituents gave finally pH 7·4.

The Mg and Ca salts were dissolved in one solution with an equivalent amount of EDTA and adjusted to pH 7–8 with NaOH; the stock solution was 100 × final concentration. The iron, zinc, manganese, copper, cobalt, borate and molybdate salts were dissolved one by one in an equimolar amount of EDTA adjusted to pH 7–8 with NaOH, and the stock solution was adjusted to 100 × final concentration. There was evidence that when the stock solutions were kept more than 6 months the growth rate of *Escherichia coli* in the medium declined.

The phosphate and the mineral salts were combined for autoclaving at 121° for 15 min. NH_4Cl and glucose were each sterilized separately by autoclaving at 121° for 15 min.

The PYGS medium consisted of the same constituents as medium DMA except that NH_4Cl was replaced by peptone (5 g./l.) + Difco yeast extract (3 g./l.).

Preparation of agar plates. To prepare the solid medium for plate cultures, 10 ml.

of double strength DMA + glucose medium at about 50° and 10 ml. of molten 2% (w/v) agar (New Zealand) were mixed in a tube and poured into a standard glass Petri dish of 9 cm. diameter. The pipette used for the agar was calibrated to allow for the large drainage error (about 0·5 ml.). Before filling the Petri dishes they were levelled on a tripod table with levelling screws. The levelling table consisted of a piece of plate glass held at each side by a slotted piece of wood carrying the levelling screws. After the agar had set, the plates were left with the lids on for 2–4 hr in a 37° constant-temperature room. The plates were then dried in the inverted position, with the lids removed. The drying time in our hot room which had a relative humidity of about 30% was 3–6 min. A useful indication of the completion of drying was the disappearance of the droplets of condensate on the Petri-dish lid. After drying the plates were used immediately.

Inoculation of plates. A satisfactory method of inoculating plates was gradually evolved during the course of the experiments. Initially organisms were spread on the plates as for a plate count but this was abandoned because on the DMA + glucose agar medium there was a lag of one or more days before the colonies were visible. Growth from a mass inoculum was preferred because it eliminated lag due to a small inoculum, and it enabled the colonies to be spaced evenly on the agar. Attempts to produce streaks or pinpoint inocula with inoculating needles did not produce sufficiently even streaks or round colonies. The method finally used was to pipette a small drop of a dense culture on to the agar surface. Fine uniform-bore capillaries which delivered about 2 μl. by means of a teat were at first used. With this type of pipette the variation in volume delivered might be several-fold, but its chief disadvantage was the impossibility of avoiding spray formation which caused many satellite colonies to develop near the main one and thus interfere with its development. A good tool for the inoculation was the Repette (Jensons, Hemel Hempstead, Herts., England) which dispensed μl. amounts by means of a plunger action. The Repette was dismantled for sterilization by autoclaving or by immersion in boiling water and then assembled aseptically. The Repette, adjusted to deliver 3 μl. drops was discharged on to the agar by gently bringing the drop in contact with the agar. Four drops, one at each corner of a square about 2·5 cm. apart, were dispensed on each plate; in some experiments a fifth drop was placed at the middle of the square. The method was rapid and the drop size highly reproducible. After inoculation the plates were placed in the incubator, lids down.

Measurement of colony size. The diameters of colonies were at first measured by means of a low-power miroscope with a micrometer eyepiece. However, this method proved tedious, especially since it had to be done in the 37° room; it also involved removing the Petri-dish lids, with consequent drying of colonies and risk of infection. A most convenient method of measuring the colony size accurately was to use an enlarger with a projection screen (the Shadomaster, supplied by Buck and Hickman, Otterspool Way, Watford, Herts., England). By this means colony size was magnified ten times and the size measured on the screen with a transparent ruler. There was no need to remove the lid of the Petri dish, and since the sizes of the colonies on a plate could be measured within 30 sec. each plate could be removed from the incubator for the duration of the measurement. The mean of two colony diameters at right angles was taken for each colony.

Shake-flask cultures. Homogeneous liquid aerobic cultures were obtained by growth

in 250 ml. conical flasks on a rotary shaker. The flasks initially contained 25 ml. medium + 1 ml. inoculum. Growth was followed by measurement of the culture opacity.

Inocula. The organisms used were *Escherichia coli* (laboratory strain B3), *Klebsiella aerogenes* NCIB8017 (National Collection of Industrial Bacteria, Torrey Research Station, Aberdeen, Scotland) and *Streptococcus faecalis* (laboratory strain, D13).

To obtain reproducible inocula the following procedure was used. First a tube of nutrient broth (5 ml.) was inoculated with a loopful of organisms from a slope culture. When the broth culture was obviously turbid, usually at 3–4 hr, the nutrient broth culture (5 ml.) was transferred to 10 ml. of the final medium + glucose (0·5%, w/v) in a 250 ml. conical flask. This was incubated without shaking for 4–8 hr. From this second stage 0·5 ml. was transferred to 20 ml. final medium + glucose 0·5% (w/v), in a 250 ml. conical flask, and incubated overnight on a rotary shaker at about 200 rev./min. with a 1·5 in. (3·8 cm.) throw. This third-stage inoculum, which contained about 4×10^9 organisms/ml., was used to inoculate either shake-flask cultures or plate cultures.

RESULTS

Radial growth rate of bacterial colonies

DMA nutrient agar plates were inoculated with *Escherichia coli* at four or five evenly spaced points. The plates were incubated for 9–12 hr to produce confluent growth in each colony; the colony diameters were then measured at intervals of 3 or 4 hr. The rate of increase in the diameters of replicate colonies at two different glucose concentrations are shown in Fig. 2. The data for *E. coli* show that over the initial period of measurement, from 12 to 24 hr after inoculation, the diameters increased at a virtually constant rate which was independent of the colony size over the range studied (2–8 mm. diameter). The term 'initial radial growth rate' is used to refer to the rate of increase in the radius (μ/hr) in roughly the first 12 hr after the growth in the colony became confluent. The standard deviation in the initial radial growth rates was 7% of the mean with 0·5% (w/v) glucose and 8% with 0·1% (w/v) glucose (the radial growth rate was a function of the glucose concentration). In each experiment the mean radial growth rate of 8–12 replicate colonies was determined; thus the standard deviation in the mean was about 3%. The variation in the mean radial growth rate from different experiments was about 6%. This increase in the variance is attributed to batch-to-batch variations in media and inocula. The specific growth rate in shake-flask cultures varied to a similar degree with different batches of medium and inoculum.

Over longer periods of time (36 hr after inoculation) a decrease in the radial growth rate was apparent. With *Escherichia coli* the radial growth rate in the period 24–36 hr after inoculation was about 20% less than in the period 12–24 hr, but with increasing time of incubation the rate of decrease in the radial growth rate decreased. Similar results were obtained with colonies of *Klebsiella aerogenes* and *Streptococcus faecalis.*

Effect of agar depth and concentration

It was found that decreasing the agar concentration from 1·0 to 0·6% had no effect on the colony growth rate.

To determine the effect of the depth of the agar layer, plates were poured with differ-

ent volumes of nutrient agar (5, 10, 15, 20, 25 ml., corresponding to depths of 0·86, 1·72, 2·58, 3·44, 4·30 mm., respectively). *Escherichia coli* colonies were grown on the plates and the radial growth rates determined. At each agar depth constant radial growth rates were obtained over a 12 hr period. The results (Fig. 3) showed that the agar

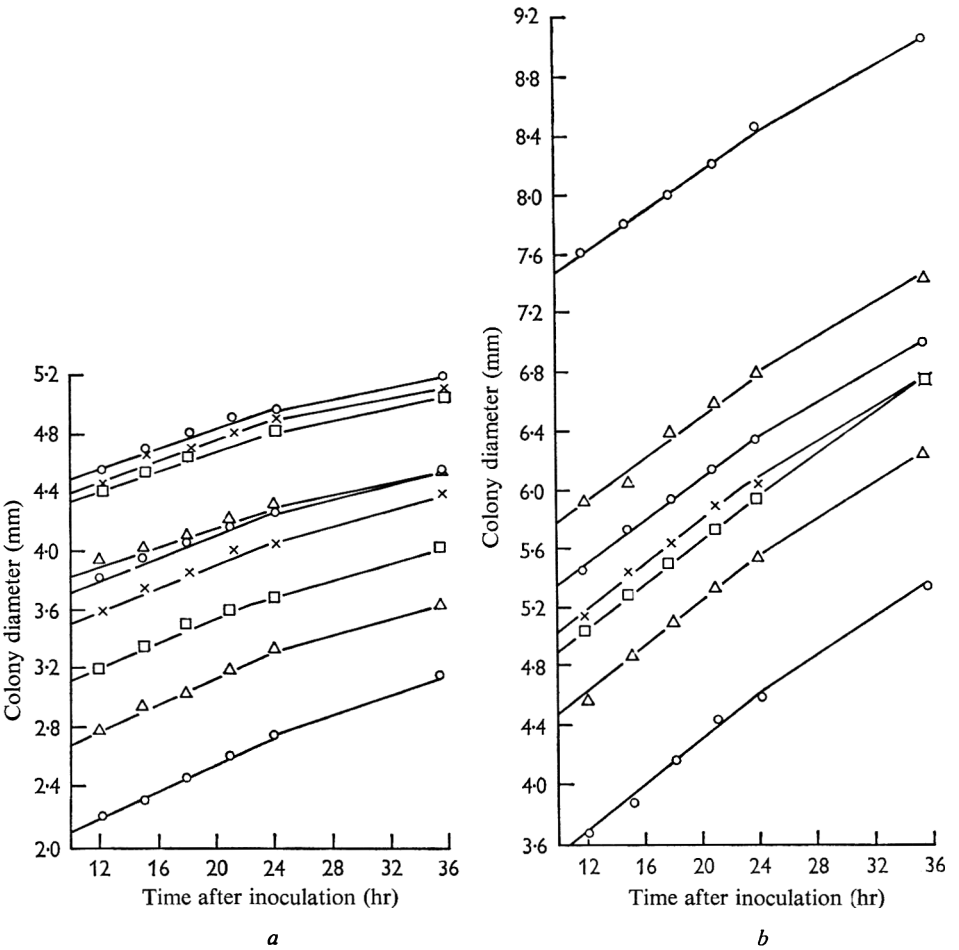

Fig. 2. Rates of increase in the diameters of *Escherichia coli* colonies on nutrient agar: (*a*) glucose, 1·28 g./l.; (*b*) glucose, 5·12 g./l. Medium DMA; temperature 37°.

depth up to 3·44 mm. (20 ml. agar) had a marked effect on the radial growth rate of colonies. With 0·1 % (w/v) glucose there was little further increase in growth rate on increasing the agar depth above 3·5 mm. This result indicated that the glucose concentration gradient in the agar extended to a depth of about 3·5 mm.

Effect of humidity

The good reproducibility of the colony radial growth rates in different plates and different experiments indicated that the degree of drying of the plates was either invariable or immaterial. The effect of the relative humidity of the gas-phase on the colony growth rates over a period of 2–3 days was investigated by growing replicate

plates of *Escherichia coli* colonies both in the hot room with a relative humidity of 30% and in a sealed chamber with a relative humidity of 100%. A layer of water was kept in the chamber (normally a desiccator) to saturate the atmosphere. The water-saturated chamber was kept in the hot room at 37°. The growth rates of *E. coli* colonies over periods of 2 days were virtually the same at both relative humidities. Hence it is concluded that any loss of water from the plates which occurred during drying was immaterial.

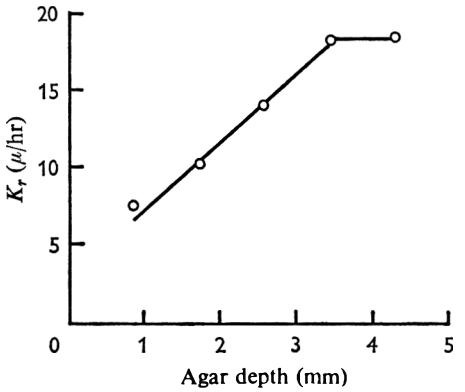

Fig. 3. Effect of agar depth on initial radial growth rates (K_r) of *Escherichia coli* colonies. Temperature 37°; medium DMA + glucose (1·0 g./l.).

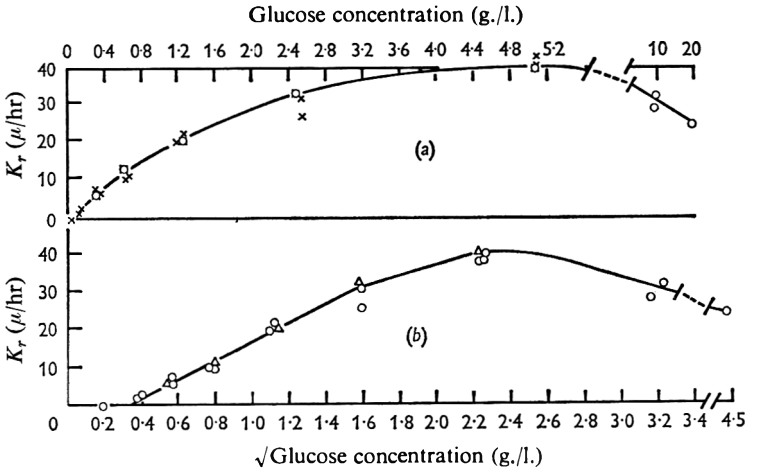

Fig. 4. Initial radial growth rates (K_r) of *Escherichia coli* colonies as functions of the initial glucose concentration. (*a*) K_r plotted against the glucose concn.; ×, in air; ○, in air+4·5% (v/v) CO_2. (*b*) K_r plotted against the square root of the glucose concn.; ○, in air; △, in air+4·5% (v/v) CO_2. Medium DMA; temperature 37°.

Influence of glucose concentration

Plate cultures were set up with different concentrations of glucose, in order to determine the range of glucose concentrations over which the initial radial growth rate was glucose-limited.

The effects of glucose concentration on the initial radial growth rates (K_r) of

Escherichia coli, *Klebsiella aerogenes* and *Streptococcus faecalis* in air are shown in Figs. 4, 5 and 6. The graphs of K_r against glucose concentration show two main features: (1) at the low glucose concentration the radial growth rate was glucose-limited; (2) at glucose concentrations of 10 g./l. or more the colony radial growth was strongly inhibited in the cases of *E. coli* and *S. faecalis*. Colony radial growth of *K. aerogenes* was not inhibited by glucose at 10 g./l.; probably it would be at still higher glucose concentrations.

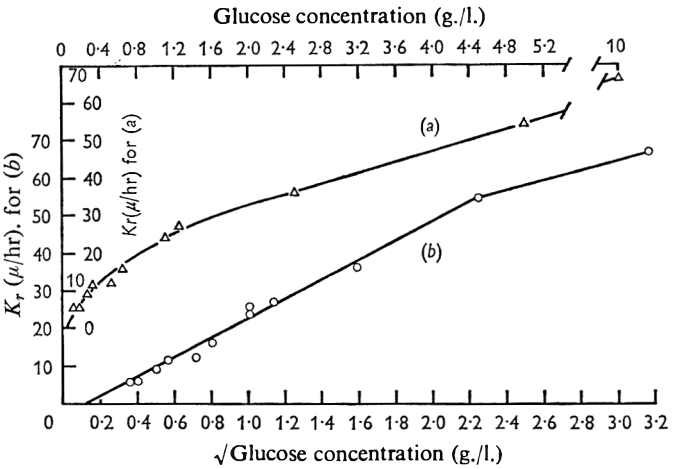

Fig. 5. Initial radial growth rates (K_r) of *Klebsiella aerogenes* colonies as functions of the initial glucose concentration. (*a*) K_r plotted against glucose concentration, △; (*b*) K_r against the square root of the glucose concn. ○, Medium DMA; temperature 30°.

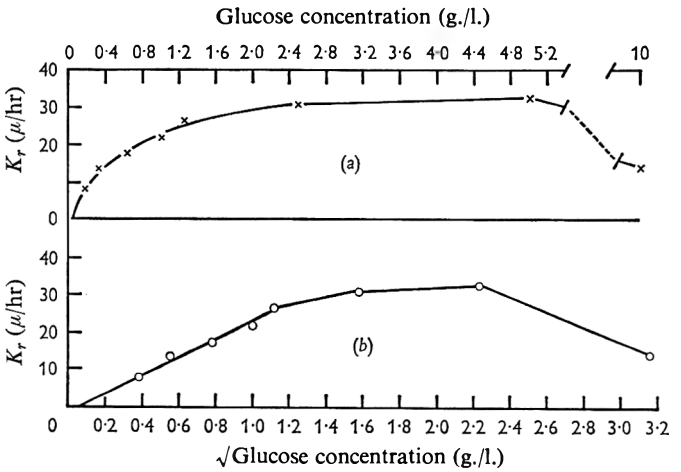

Fig. 6. Initial radial growth rates (K_r) of *Streptococcus faecalis* colonies as functions of the initial glucose concentration. (*a*) K_r plotted against the glucose concentration (×); (*b*) K_r against the square root of the glucose concentration (○). Medium PYGS; temperature 37°.

The relation between the initial glucose concentration and the initial colony radial growth rate is not of the Michaelis–Menten type which one finds in homogeneous liquid cultures (Monod, 1942). This is to be expected because in the developed colony the

initial glucose concentration cannot be the concentration to which the bacteria are exposed, because of the concentration gradient which must develop in the agar. Also it is apparent that, whereas in liquid culture *Escherichia coli* reaches half its maximum growth rate at about 4×10^{-3} g./l. (Monod, 1942) the value of the initial glucose concentration at which the radial growth rate of the *E. coli* colony reached half its maximum value was 1·3 g./l. The relation between the initial colony growth rate and the square root of the initial glucose concentration for each of the three organisms (Figs. 4, 5, 6) was linear over a considerable range. This range for *E. coli* was 0–2·5 g./l., for *K. aerogenes* 0–5 g./l. and for *S. faecalis* 0–1·25 g./l. In the case of *E. coli* it is shown below that at 2·5 g. glucose/l. oxygen became the growth-limiting factor. For similar reasons it is believed that oxygen became the growth-limiting factor for *K. aerogenes* when the glucose concentration reached 5 g./l. In the case of *S. faecalis* the departure from the linear relation between the initial radial growth rate and the square root of the initial glucose concentration may have been due to some nutrient other than glucose, possibly an amino acid or a vitamin, becoming growth-limiting. If this were so, then increasing the concentration of peptone or yeast extract should increase the maximum glucose concentration at which growth is limited.

A surprising feature was the strong inhibition of growth of *Escherichia coli* and of *Streptoccocus faecalis* caused by glucose at 10 g./l. This inhibition is attributed to the formation of toxic products or change in the pH value of the medium. We can compare the colony growth rates of colonies of *E. coli* and *S. faecalis* under glucose limitation when the initial glucose concentration was 1·0 g./l. At the optimum temperature, 37°, the K_r for *E. coli* was 20 μ/hr, and for *S. faecalis* 23 μ/hr. One of the classical distinguishing features of the lactic acid bacteria of which *S. faecalis* is one, is that they normally produce minute colonies as a result of low colony growth rates. It seems probable that this may be due, not so much to an intrinsically lower colony growth rate, but to the strong inhibition caused by the practice of using a high glucose concentration (1 or 2%, w/v) in the normal plate culture media for lactic acid bacteria.

Figures 4, 5 and 6 show that the initial radial growth rates of colonies reached zero at a certain small, but finite, glucose concentration termed the 'lag concentration', s_i. For *Escherichia coli* in air, s_i was 0·090 g./l., for *Klebsiella aerogenes*, 0·013 g./l. and for *Streptococcus faecalis*, 0·005 g./l. A possible cause of this minimum value for the glucose concentration before growth could occur is discussed below. The results show that the relation between the glucose concentration and the initial radial growth rate (K_r) was of the form

$$K_r = k_1(\sqrt{s_0} - \sqrt{s_i}), \tag{11}$$

where k_1 is a constant. The value of s_i in some cases would be small enough to be negligible.

Oxygen and carbon dioxide limitation of colony growth rate

To see whether oxygen was limiting the growth of *Escherichia coli* colonies, growth in air and in oxygen at one atmosphere pressure was compared. To obtain the different gas-phases the plates were put in a sealed jar of the type normally used for anaerobic culture (vacuum desiccator vessels also suggest themselves for this purpose) and the gas in the vessel was changed by evacuation and filling with the appropriate gas mixture. A spacer (Khairat, 1963) was included between the lid and the base of each Petri

dish to ensure that the gas circulated. Colonies were first allowed to develop in air, then after measurement they were transferred to the appropriate gas phase.

The effect of oxygen concentration on the radial growth rate of *Escherichia coli* colonies is shown in Fig. 7. The surprising feature is that at low glucose concentrations (0–1·7 g./l.) oxygen at 1 atm. pressure almost completely inhibited the growth of the colonies. With glucose above the inhibitory concentration the radial growth rate responded to glucose concentration in oxygen (1 atm. pressure) in the same way as in air;

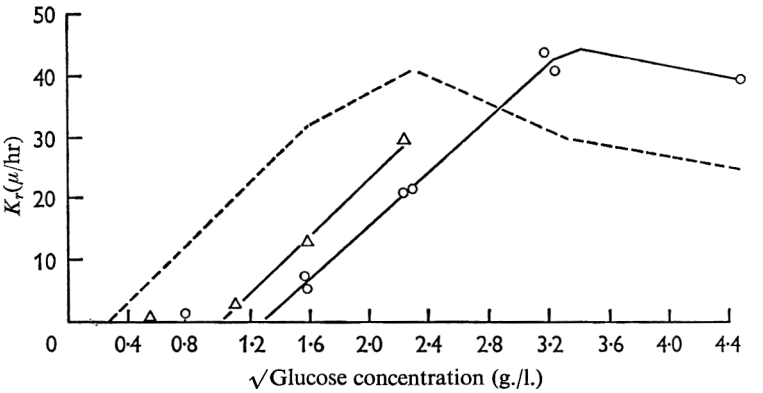

Fig. 7. Effect of increased oxygen and carbon dioxide partial pressures on initial radial growth rates (K_r) of *Escherichia coli* colonies. O, In 100% (v/v) oxygen at 1 atm. pressure; △, in 95·5% (v/v) oxygen + 4·5% (v/v) carbon dioxide at 1 atm. pressure. The broken line (from Fig. 4) shows the radial growth rate in air or air + 4·5% (v/v) carbon dioxide at 1 atm. pressure. Medium DMA; temperature 37°.

that is, K_r was directly proportional to the square root of the glucose concentration, the proportionality factor being the same as in air. However, with oxygen at 1 atm. pressure the initial radial growth rate (K_r) was directly proportional to the square root of the initial glucose concentration (s_0) over a much wider range (1·7–10 g./l.) than in 1 atm. pressure of air (0·1–2·5 g./l.). This result indicates that in air the departure from the linear relation between K_r and the square root of the glucose concentration was due to the oxygen diffusion rate becoming growth-limiting.

The absence of CO_2 from the oxygen gas phase might have been responsible for the inhibition by oxygen at low glucose concentrations. In a repeat experiment an atmosphere of 95% (v/v) O_2 + 4·5% (v/v) CO_2 was used. The results given in Fig. 7 show that the addition of CO_2 partially removed the inhibition, but still a glucose concentration of 0·86 g./l. was necessary to overcome the inhibition by oxygen. In air an increase in the CO_2 partial pressure to 4·5% (v/v) did not affect the colony growth rate when it was glucose-limited (Fig. 4).

It is apparent that with increased oxygen partial pressure the lag concentration (s_i) was increased. One would expect that an inhibitory condition, such as over-optimal oxygen partial pressure, which could be overcome by the culture's metabolic activity, would be made manifest in a liquid culture by a lag period before growth. Hence it is believed that the factor $\sqrt{s_i}$ in equation (11) is related to the lag period which would be produced in a liquid culture.

Effect of decreasing maximum specific growth rate

To determine the influence of specific growth rate on the colony radial growth rate the specific growth rate was varied by the addition of sulphanilamide as an inhibitor. Sulphanilamide (at low concentrations) in aerobic liquid cultures had the effect of decreasing the maximum specific growth rate (α_m) of *Escherichia coli*. The maximum specific growth rate was determined by shake-flask culture in the same batch of DMA medium (+ glucose, 5 g./l.) and with the same inoculum culture as were used for the plate cultures. The glucose concentration for the colony growth was 1 g./l. so that the growth was glucose-limited and not oxygen-limited. In one experiment sulphanilamide alone was added to the medium. In another experiment sulphanilamide + *p*-aminobenzoic acid (PABA) was added to vary the specific growth rate. With the PABA

Table 1. *Effect of sulphanilamide on specific growth rate and on the initial colony radial growth rate of Escherichia coli, laboratory strain B3*

Medium DMA glucose 1 g./l.; incubation temperature 37°.

Sulphanilamide (μg./ml.)	Maximum specific growth rate ($\alpha_m hr^{-1}$)	Initial colony radial growth rate (K_r, μ/hr)	K_r/α_m	$K_r/\sqrt{\alpha_m}$
		p-Aminobenzoic acid absent		
0	0·615	13·4	21·8	17·1
6·25	0·674	12·6	18·7	15·4
12·5	0·510	11·6	22·7	16·2
25·0	0·351	10·2	29·1	17·2
50·0	—	10·4	—	—
100·0	—	6·2	—	—
		p-Aminobenzoic acid (0·25 μg./ml.) present		
0	0·661	14·4	21·8	17·7
200	0·606	14·9	24·6	19·2
400	0·553	14·3	25·9	19·2
800	0·517	12·9	24·9	18·0

addition much greater concentrations of sulphanilamide were, of course, required. The results are given in Table 1. With only sulphanilamide added, specific growth rates could not be measured in liquid cultures with sulphanilamide concentrations greater than 25 μg./ml. because of erratic variations in the growth rate. With sulphanilamide + PABA the range of specific growth rates obtained was much smaller than with sulphanilamide alone. It was concluded that within experimental error the ratio of initial colony radial growth rate to the square root of the maximum specific growth rate ($K_r:\sqrt{\alpha_m}$) was constant. With sulphanilamide alone the mean value of the ratio was 16·3 and the maximum deviation 5·5%. In contrast, the ratio $K_r:\alpha_m$ showed an upward trend in its value and the maximum deviation from the mean value (23·1) was 20·6%. With sulphanilamide + PABA the mean value of the ratio $K_r:\sqrt{\alpha_m}$ was 18·5 and the maximum deviation 4·3%; in contrast, the maximum deviation from the mean value of the ratio $K_r:\alpha_m$ was 9·7%. From these experiments it was concluded that relation (11) for the initial radial growth rate may be modified to

$$K_r = k_2(\sqrt{s_0} - \sqrt{s_i})\sqrt{\alpha_m} \tag{12}$$

where k_2 is a constant.

Effect of temperature on initial colony radial growth rate

Changes in the temperature of incubation were made, primarily as an alternative means of varying the growth rate of the organisms. For these experiments an organism was adapted to the growth temperature by growing the inoculum at the temperature required for the growth measurements. A low glucose concentration (1 g./l.) was used for the colony growth to ensure that there was excess of oxygen and that growth was glucose-limited. The effects of the temperature changes on the specific growth rates and on the colony radial growth rates for glucose-limited growth are shown in Table 2. Only in the case of *Streptococcus faecalis* did the colony radial

Table 2. *Effect of temperature on initial colony radial growth rate and on the specific growth rate of three bacteria*

Medium for *E. coli* and *K. aerogenes*, DMA; medium for *S. faecalis*, PYGS (see text)

Temp.	Maximum specific growth rate (α_m, hr^{-1})	Initial colony radial growth rate (K_r, μ/hr)	$K_r/\sqrt{\alpha_m}$
	Escherichia coli (glucose, 1·1 g./l.)		
37°	0·70	20·0	24·9
30°	0·445	20·4	31·1
25°	0·282	18·0	33·9
	Klebsiella aerogenes (glucose, 1·0 g./l.)		
37°	0·49	23·3	33·3
30°	0·72	26·2	30·9
25°	0·48	28·3	40·8
	Streptococcus faecalis (glucose, 1·0 g./l.)		
37°	1·36	22·8	19·5
31°	1·05	21·7	20·6
25°	0·65	17·8	22·1

growth rate always reflect the change in specific growth rate. The ratio $K_r:\sqrt{\alpha_m}$ did not vary by more than 5% from the mean for *S. faecalis*, but there was an upward trend in the ratio with decrease in temperature of incubation. The upward trend in the ratio $K_r:\sqrt{\alpha_m}$ with decrease in temperature was much more marked in the cases of *Klebsiella aerogenes* and *Escherichia coli*; in fact, so much so, that a decrease in the specific growth rate was not always reflected in the colony radial growth rate. The upward trend in the ratio $K_r:\sqrt{\alpha_m}$ was interpreted to mean that the coefficient k_2 in (12) was temperature-dependent. The results show that the optimum temperature for colony radial growth rate might be up to 5° lower than that for the specific growth rate.

In early experiments with *Escherichia coli* colonies the plates were not dried, and with this condition occasionally a curious temperature effect was noted. At 25° the initial radial growth rate of the colony might be four times greater than the normal value which was obtained on dried plates. Such anomalous behaviour was not shown by *Klebsiella aerogenes* under the same condition or by *Escherichia coli* when the plates were dried or when the temperature was 30°–37°. It is suggested that this more rapid spread of the *E. coli* colony associated with a moist plate and a low temperature was an expression of motility.

DISCUSSION

From this work on colony growth of three diverse types of bacteria (Gram-negative motile rods, Gram-negative non-motile rods, Gram-positive cocci in chains) on the surface of nutrient agar the following principles are formulated. (1) Large colonies (2 mm. or more in diameter) increase their radius over a 12 hr. period at a nearly constant rate. (2) The initial radial growth rate of large colonies (after about 12 hr development) is directly proportional to the square root of the initial concentration of glucose when this is the growth-limiting nutrient and oxygen is in excess. By analogy one would expect the initial radial growth rate of the colony to be proportional to the square root of the concentration of the growth-limiting nutrient in the agar irrespective of its nature, provided the oxygen relation is constant. (3) When the growth rate is varied by an inhibitor such as sulphanilamide, other things being equal, the initial radial growth rate of the colony is proportional to the square root of the specific growth rate (or inversely proportional to the doubling time).

Changes in maximum specific growth rate brought about by temperature change were not always reflected in colony radial growth rates. This effect, attributed to a change in the coefficient k_2 of relation (12) cannot be accounted for. It should be noted that the temperature range investigated was relatively narrow (range about 10° near the optimum growth temperature) and over the full temperature range for growth, the deviation in the responses of colony radial growth rate and of specific growth rate might not be so marked. The measurement of colony radial growth rate might have advantages over liquid cultures for the determination of the temperature range for growth of bacteria.

A gradual decrease in the colony radial growth rate was apparent over longer periods (24 hr). This is taken to be a reflection of a gradual decrease in the rate of nutrient diffusion with time. The recession from the agar colony interface of the level at which the nutrient concentration had the initial value (s_0) means that eventually the agar depth should affect the colony radial growth rate. When the initial glucose concentration was 1 g./l. the initial colony radial growth rate became independent of the agar depth when this exceeded 3·5 mm. (the depth given by 20 ml. of agar in the 9 cm. Petri dish). To ensure better reproducibility of maximum initial colony growth rate it would be advisable to make the agar depth 5 mm., this would require about 35 ml. agar per dish. There appears to be a case for using a Petri dish of 5 cm. diameter in which the optimum depth of agar would be achieved with only 10 ml. agar medium.

Two types of growth inhibition were characterized by the colony growth technique. One was the restriction of specific growth rate by sulphanilamide. The other type of inhibition was made manifest in the colony radial growth rate expression (11) by the term $\sqrt{s_i}$. This effect is thought to reflect the type of inhibition which in liquid cultures causes a lag in growth, that is, an inhibitory condition which the organisms can overcome by their own action.

The almost complete inhibition of the growth of *Escherichia coli* laboratory strain B3 by a partial pressure of 1 atm. oxygen (100%, v/v has not been previously noted. Some inhibition of surface colony growth of *E. coli* and of other bacteria by oxygen at 1 atm. pressure was reported by Wiseman, Violago, Roberts & Penn (1966), but with liquid cultures they obtained conflicting results. These results of Wiseman *et al.* are difficult to interpret because it is not clear what the growth-limiting nutrient was

and whether the inhibition was a reflexion of a growth lag or of a decrease in specific growth rate. If the growth-limiting factors in the surface colony are rigorously defined then measurement of colony radial growth rate might be advantageous for the kinetic study of the influence of gases on microbial growth.

Other applications of the quantitative colony growth technique suggest themselves. Maximum specific growth rates (α_m) of different strains of an organism could be compared by means of the expression,

$$\frac{(K_r)_A^2}{(K_r)_B^2} = \frac{(\alpha_m)_A}{(\alpha_m)_B}, \tag{13}$$

where K_r represents initial colony radial growth rate and the subscripts A and B refer to two strains of an organism. This method would be convenient for growth-rate determinations on large numbers of strains as in taxonomical studies. However, for relation (13) to apply, the growth-limiting nutrient should be known to be the same and the same diffusion conditions should apply.

In conventional routine bacteriological plating media the growth-limiting factor is unknown. To avoid an oxygen lack when glucose is the energy source the concentration of glucose should be much lower than is usually the case in routine media for agar plates. For this purpose, in the case of *Escherichia coli* the concentration of glucose should be less than 2·5 g./l. Again, with routinely used glucose concentrations (e.g. 10–20 g./l.) it appears that growth of colonies is commonly limited by toxic products.

Other applications of the colony growth technique might be based on its ability to estimate growth-limiting substrate (s_0) by relation (12). The method may therefore be useful for the microbiological assay of amino acids, vitamins and other growth factors. The concentration of inhibitors might be determined either by their effect on the initial colony radial growth rate, or on the 'lag concentration' s_i in relation (12).

According to the model for colony growth initially proposed the radial growth rate may, from relation (10), be expressed by

$$K_r = w\alpha/2 \tag{14}$$

For *Escherichia coli* growing on medium DMA + glucose the maximum value of the specific growth rate (α) is 0·70 hr^{-1}. When this value is inserted in (14), then with $K_r = 32\,\mu$/hr (glucose concentration 2·5 g./l.), $w = 91{\cdot}4\,\mu$. Therefore a very small peripheral zone in which the organisms grow at the maximum rate will account for the radial spread of the colony. The model accounts for the constant radial growth rate which is shown by fungal colonies. The gradual decrease with time of the radial growth rate of the bacterial colony is not accounted for. To elucidate the problem further a quantitative analysis of the nutrient diffusion rate would be a great help.

Measurements of the thickness of colonies were not attempted. However, rough visual inspection confirmed that with glucose-limited growth the thickness of the colonies studied here was of microscopic dimensions, as predicted theoretically.

In general, it appears that quantitative growth studies can be extended to microbial colonies in order to obtain knowledge about the reaction of the organism to its environment. The development of a colony is made complex by its possible differentiation into growing and non-growing parts and sometimes into a sporulating part. In the model proposed for the microbial colony, the growing zone may be seen to be analogous to the meristem in a plant tissue, and one could regard the colony of a

micro-organism as a simple model of a differentiating tissue in which one can control the environmental factors and thus discuss their effects in quantitative terms.

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Enzyme and Permeability Changes During Morphogenesis of *Nocardia corallina*

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SUMMARY

Initial growth of *Nocardia corallina* occurred by germination and coenocytic hyphal development which resulted in a large surface-to-volume increase. During this period, endogenous respiration and the ability to oxidize fructose increased rapidly, but the stimulation of oxygen uptake by glucose decreased to zero. This inability to utilize glucose was due to loss of permeability rather than lack of a specific enzyme required for glucose metabolism. This was established by the following data: (1) cell-free extracts of hyphae contained the enzymes for conversion of glucose into fructose-6-phosphate; (2) intact hyphae oxidized fructose; (3) incubation of hyphae with cetyltrimethylammonium bromide increased cell permeability to glucose and oxygen uptake was stimulated. Comparative determinations of enzymes involved in the early steps of glucose metabolism showed quantitative changes associated with the period of impermeability to glucose. The loss of permeability to glucose may represent an initial metabolic change fundamental to morphogenesis in this microbe.

INTRODUCTION

A cyclic series of morphological changes, termed a life cycle by Webb & Clark (1957), has been described for *Nocardia corallina*. The mechanisms which underlie these changes are unknown; however, Brown & Clark (1966*b*) gave evidence that fragmentation was influenced by diffusible metabolic factors which accumulated during growth. One approach toward a better understanding of morphogenesis in this organism is to search for answers at the metabolic level. Evidence for the pentose pathway (Brown & Clark, 1966*a*) and the Krebs cycle (Brown & Clark, 1961) has been reported for this organism, but little is known about metabolic changes potentially correlated with morphogenesis. However, preliminary work showed a loss of ability to utilize glucose which occurred during the growth cycle (Brown, 1964). From the data available, it appeared probable that the block in glucose utilization was the result of a lack of permeability to glucose or a loss of ability to perform one of the component steps in the conversion of glucose to fructose-6-phosphate.

The objectives of this investigation were: (1) to measure throughout the growth cycle, the changes in ability to oxidize glucose; (2) to locate the block in glucose metabolism; (3) to measure the specific activities of those enzymes involved in the early stages of glucose metabolism and to detect changes potentially correlated with the loss of ability to oxidize glucose.

METHODS

Culture. Nocardia corallina ATCC 4273 was grown at 29° on nutrient agar + 0·5% glucose. Inocula for experiments were obtained from cultures which had been transferred consecutively at least twice at 48 hr intervals. Such time was sufficient for completion of the growth cycle and physiological and morphological variations were minimized. Cultures, grown as described above, were harvested at various times by alternate suspension and centrifugation for 15 min. at 18,000*g* and 4° in 0·01 M-potassium phosphate buffer (pH 6·8).

Respiration data. Oxygen uptake was measured by standard Warburg techniques (Umbreit, Burris & Stauffer, 1964). Warburg flasks contained: 0·2 ml. of 10% KOH in the centre well, 0·1 ml. of 0·1 M substrate in a side arm and 2·7 ml. of bacterial suspension in the main compartment. The flasks were equilibrated in air and were incubated at 29° with shaking at 36 excursions/min.

Cetyltrimethylammonium bromide (Cetavlon) a surface-active agent which has been used to increase cell permeability (White, Kell, Suffling & Work, 1964) was tested for ability to induce oxygen uptake by 8 hr hyphae with glucose as substrate. The flasks contained 2·6 ml. of bacterial suspension, 0·1 ml. of 0·1 M-glucose in one side-arm and in a second side-arm, 0·1 ml. of Cetavlon at a concentration of 10, 1, 0·1, 0·01 or 0·001 mM.

The effect of insulin on the permeability to glucose of bacteria from 8 hr cultures was also studied. Insulin for injection (Squibb), 80 and 40 units/ml. and crystalline beef insulin (Lilly) at concentrations of (mg./ml.) 10, 1 and 0·1 were used. The experiments were done as described above except that 0·1 ml of insulin solution was substituted for the Cetavlon.

Warburg experiments were devised to determine if glucose oxidation was induced in 8 hr hyphae by extracts of bacteria from a growth cycle stage capable of glucose oxidation. Experimental flasks contained 1·4 ml. of 8 hr hyphal suspension, 1.3 ml. of 48 hr cell-free extract and 0·1 ml. of 0·1 M-glucose. Five separate controls were run: (1) 1·4 ml. of buffer + 1·4 ml. of 8 hr hyphal suspension; (2) 1·4 ml. of 8 hr hyphal suspension + 1·3 ml. of buffer + 0·1 ml. of 0·1 M-glucose; (3) 1·3 ml. of 48 hr cell-free extract + 1·4 ml. of 8 hr hyphal suspension + 0·1 ml. buffer; (4) 1·3 ml. of 48 hr cell-free extract + 1·4 ml. buffer + 0·1 ml. of 0·1 M-glucose; (5) 1·3 ml. of 48 hr cell-free extract + 1·5 ml. buffer. All flasks contained 0·2 ml. of 10% KOH in the centre well and 0·01 M-potassium phosphate (pH 6·8) was used as buffer.

Cell-free extracts. A 20% (wet wt./v) bacterial suspension in 0·01 M-potassium phosphate buffer at pH 6·8, was disrupted at 4° by sonic treatment with a Branson Sonifier (Model S-125). Bacteria were disrupted for 10, 12, 14, 16, 18 and 20 min. to determine the optimum period of sonic treatment. Extracts were centrifuged twice at 18,000*g* and 4° for 15 min. Approximately 15 ml. supernatant fluid was placed in seamless cellulose tubing (48 Å pore size, 2·54 cm. diameter) and dialysed at 4° against 1 l. of 0·01 M-potassium phosphate buffer (pH 6·8) for 4 hr. The buffer was stirred continuously with a magnetic stirrer and changed for fresh buffer after 2 hr. Protein was determined by the biuret method (Gornell, Bardawill & David, 1949).

Enzyme assays. All extinction measurements were made with a Beckman, Model DU spectrophotometer at 25–26° room temperature using 1 cm, light-path curvettes. The procedures described by McDonald (1955) were used to measure glucokinase and

fructokinase activities. The complete reaction mixtures were incubated at 30° for 10 min. Samples were removed at 2 min. intervals and changes in extinction were measured at 560 mμ. Under assay conditions, the liberation of 1 μmole of acid accompanied the phosphorylation of 1 μmole of sugar and produced a change of 0·032 in extinction. One unit of specific activity is defined as the phosphorylation at 30° of 1 μmole of sugar/min./mg. protein.

The procedure described by Slein (1955) was used to assay glucosephosphate isomerase activity. The reaction was incubated at 30° and samples were removed at 2 min. intervals for a total of 18 min. The resorcinol procedure (Slein, 1955) was used to measure the fructose-6-phosphate formed by measuring changes in extinction at 540 mμ. One unit of specific activity is defined as a change of 0·001 in extinction/min./mg. protein.

The specific activity of glucose-6-phosphate dehydrogenase was determined as described by DeMoss (1955). Extinction changes were measured at 340 mμ. One unit of specific activity for glucose-6-phosphate dehydrogenase is defined as a change of 0·001 in extinction/min./mg. protein.

RESULTS

Oxygen uptake

Figure 1 shows respiratory data for organisms taken from various stages of growth. During the initial 8 hr period the organisms germinated and developed into coenocytic hyphae with a large increase in surface-to-volume ratio. During this same period, endogenous metabolism and the stimulation of oxygen uptake by fructose and caproate increased. However, the ability to oxidize glucose decreased to zero. The highest values for endogenous metabolism and for fructose and caproate oxidation were obtained with organisms from 8 hr cultures which were not stimulated by glucose. Organisms taken from later stages of growth showed a recovered ability to oxidize glucose.

Enzymes

Table 1 shows that protein concentration in cell-free extracts increased through 20 min. of sonic treatment. The specific activity was relatively constant for 12–20 min. of sonic treatment. A 20 min. period of sonic treatment was used for preparing subsequent cell-free extracts since a higher protein concentration with a higher total activity was obtained.

Table 2 shows comparative specific activities of several enzymes which are involved in initial steps of glucose oxidation via the Embden–Meyerhof and pentose pathways. Comparative data are shown for dialysed and non-dialysed extracts. Cell-free extracts of 8 hr hyphae, which did not oxidize glucose, contained the component enzymes necessary for converting glucose into fructose-6-phosphate. Since these 8 hr hyphae were capable of oxidizing fructose (Fig. 1), the enzymes beyond the fructose-6-phosphate stage were not studied.

The specific activities of glucose-6-phosphate dehydrogenase and glucosephosphate isomerase were 48 and 36% lower, respectively, in extracts of 8 hr hyphae than in extracts of 48 hr coccoids (Table 2). The differences are significant at or below the 0·1% value. The specific activities of glucokinase and fructokinase were 17 and 15% lower in extracts of 8 hr hyphae than in extracts of 48 hr coccoids (Table 2). These differences

are significant at the 2% value. Values in Table 2 are averages of a minimum of four separate determinations for each enzyme, performed on each of two separate 48 hr extracts and two separate 8 hr extracts. The non-dialysed extracts show higher activities than the dialysed extracts (Table 2). However, the values for 8 hr hyphae again were lower than those for 48 hr extracts.

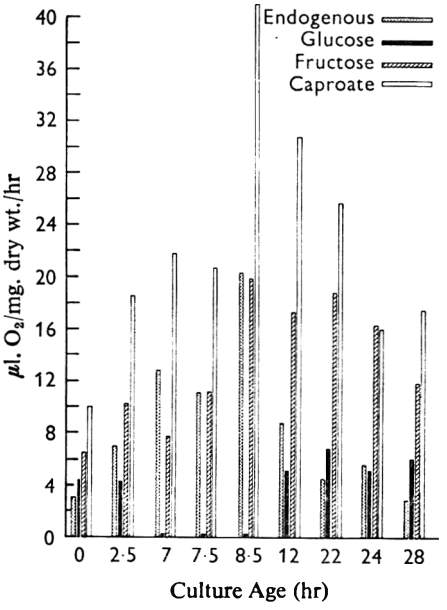

Fig. 1

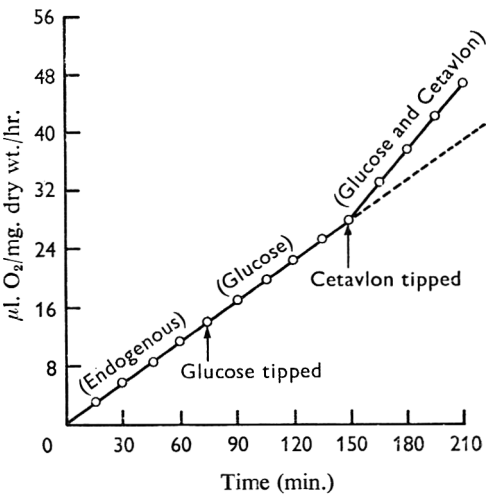

Fig. 2

Fig. 1. Changes in endogenous and exogenous respiration during the growth cycle of *Nocardia corallina*. Endogenous values were substracted from the data shown for glucose, fructose and caproate.

Fig. 2. Effects of Cetavlon (0·003 mM) on the oxidation of glucose by 8 hr hyphae of *Nocardia corallina*.

Table 1. *Rate of disruption of Nocardia corallina by sonic treatment, as measured by release of protein and glucose-6-phosphate activity*

Time of sonic treatment (min.)	Protein in non-dialysed cell-free extracts (mg./ml.)	Glucose-6-phosphate dehydrogenase specific activity*
10	2·3	11·3
12	2·8	32·4
14	3·4	30·0
16	4·1	34·6
18	4·3	31·5
20	4·9	32·4

* One unit of specific activity is equivalent to a change of 0·001 extinction unit/min./mg. protein.

Effect of Cetavlon

The effects of Cetavlon upon the ability of 8 hr hyphae to oxidize glucose are shown in Fig. 2. Oxygen intake was not stimulated above the endogenous value by addition of glucose alone. Upon tipping Cetavlon into the system, the oxygen uptake was in-

creased. Separate control experiments, with either glucose or Cetavlon omitted, resulted in no stimulation of endogenous respiration. Cetavlon at concentrations of 0·03 mM or higher, inhibited endogenous respiration.

There was no significant stimulation of oxygen uptake by glucose when 8 hr hyphae were incubated with insulin at various concentrations, as described in Methods.

Stimulation of glucose oxidation by cell-free extracts

Table 3 shows the stimulation of glucose oxidation produced by incubation of 8 hr hyphae with dialysed cell-free extract from 48 hr coccoids. Stimulation of oxygen uptake was not observed in the control systems.

Table 2. *Specific activities of four enzymes in cell-free extracts (supernatant fluid of sonically-treated organisms) of Nocardia corallina*

	Specific enzyme activity* Dialysed extract		Specific enzyme activity*. Non-dialysed extract	
Enzyme	8 hr hyphae	48 hr coccoids	8 hr hyphae	48 hr coccoids
Glucokinase†	1·16±0·06	1·39±0·12	1·26±0·06	1·63±0·04
Fructokinase†	1·29±0·02	1·51±0·07	1·48±0·02	1·78±0·04
Glucosephosphate isomerase‡	15·8±0·66	24·6±0·44	17·9±0·36	27·3±0·10
Glucose-6-phosphate dehydrogenase‡	15·4±0·75	29·4±1·22	29·5±0·65	37·3±0·56

* Mean value±standard error of the mean based on four determinations of 8 hr extract and four determinations of 48 hr extract. Specific activity of each enzyme is defined in Methods.

† The values for these enzymes in the 8 hr and 48 hr extracts were significantly different, with $P < 0·02$.

‡ The values for these enzymes in the 8 hr and 48 hr extracts were significantly different, with $P < 0·001$.

Table 3. *Stimulation of glucose oxidation by 8 hr hyphae in the presence of 48 hr cell-free extracts of Nocardia corallina*

Contents of Warburg vessels*	O_2 values†
48 hr cell-free extract	0·0
48 hr cell-free extract + glucose	0·0
8 hr hyphae	9·9
8 hr hyphae + glucose	9·9
8 hr hyphae + 48 hr cell-free extract	10·0
8 hr hyphae + glucose + 48 hr cell-free extract	15·7

* Quantities are shown in Methods section.

† Q_{O_2} = ml. oxygen uptake/hr/mg. dry wt. organism.

DISCUSSION

Glucose metabolism and cell permeability. The loss of ability to oxidize glucose occurred during the first 8 hr of incubation. There was little cell division during this period and the organisms increased in size and in surface-to-volume ratio as they changed from oval coccoids to tube-like hyphae. This was an active metabolic period as evidenced by the increased endogenous metabolism and increased ability to oxidize caproate and fructose. The transient, but complete, loss of ability to oxidize glucose indicated a temporary block either in the transport of glucose or in phosphorylation

or isomerization of glucose. The enzymes beyond the fructose stage were functional since these organisms oxidized fructose.

Cell-free extracts of hyphae, which did not oxidize glucose, contained the necessary enzymes to convert glucose to fructose-6-phosphate. Thus the defect in glucose metabolism appeared to be due to impermeability of the organisms to glucose. This was confirmed by data which showed that 8 hr hyphae oxidized glucose when the organisms were pre-incubated with Cetavlon. This surface-active agent has been used in amino acid decarboxylase assays to increase cell permeability (White *et al.* 1964). When 8 hr hyphae were incubated with 0·003 mM-Cetavlon in the Warburg flasks, endogenous metabolism was maintained but permeability was increased sufficiently to allow glucose to penetrate, and oxygen uptake was stimulated.

The results of Table 3 suggest the presence in 48 hr organisms of transferable factors involved in glucose transport. A study of this transport mechanism and its possible relationship to morphogenesis in *Nocardia corallina* appears to be a fruitful area for future study.

Enzyme changes. Comparison of specific activities of four enzymes involved in initial steps of glucose and fructose metabolism indicated that the concentrations of glucose-6-phosphate dehydrogenase, glucosephosphate isomerase, glucokinase and fructokinase all decreased during the period when glucose was excluded because of the impermeability of the cells. These differences were found in both dialised and in non-dialysed extracts. The data on non-dialysed extracts were included since such extracts were subjected to a minimum of *in vitro* manipulation which might have resulted in non-uniform inactivation of the enzymes. Since the 8 hr hyphae were several times larger in volume than the 48 hr coccoids, the data suggest that synthesis of the four enzymes occurred during the 8 hr period of germination and coenocytic hyphal growth. If no enzyme synthesis occurred, the specific activities (which are based on total protein concentration) would be decreased by dilution by protein synthesized during this period of increase in cell size.

The site of the block in glucose oxidation in *Nocardia corallina* appears to be similar to the metabolic lesion in the cells of animals with diabetes mellitus. A variety of metabolic sequelae are known to result from the initial lesion in diabetes. The failure of glucose transport in *N. corallina* may also represent a fundamental event preceding other metabolic shifts necessary to accomplish morphogenesis.

We are grateful to Eli Lilly and Company for the generous gift of crystalline zinc insulin.

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With 1 *plate*
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On the Taxonomic Status of 'Quin's Oval' Organisms

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SUMMARY

Purified suspensions of the sheep rumen organism known colloquially as 'Quin's oval' were prepared from sheep rumen liquor. The chemical composition of cell walls prepared from the organisms of these suspensions was found to include protein, lipid and polysaccharide. A fraction from these walls, obtained after removal of much of the protein and polysaccharide, was shown to contain muramic acid, glucosamine and diaminopimelic acid. These findings are consistent with this organism being regarded as a Gram-negative bacterium.

INTRODUCTION

Quin (1943) appears to have been the first to describe an ovoid organism found in sheep rumen, which, because of its yeast-like appearance and its fermentative ability he called *Schizosaccharomyces ovis*. The same organism was observed by McGaughey & Sellers (1948) who found that it became predominant in the rumen of a hay-fed sheep when the diet was supplemented with mangolds. Ingram & McGaughey (1948) could not cultivate the organism in media used for the growth of yeasts and concluded that it was not a yeast. This conclusion was supported by van der Westhuizen, Oxford & Quin (1950) and Oxford (1955). The latter author considered that the organism had not yet been validly named, and workers with rumen micro-organisms have since then contented themselves with reference to it as 'Quin's oval organism' (Q.O.).

We have prepared suspensions of Q.O. from rumen liquor, free from protozoa and virtually free from bacteria, by differential centrifugation. The organisms were obtained in a metabolically active form, and the results of fermentation studies will be reported elsewhere. We present now, as a contribution to the taxonomy of Q.O., some observations on the chemical composition of its cell wall.

METHODS

Isolation of organism. The sheep used in these experiments was fitted with a rumen cannula. It was fed on lucerne hay *ad lib.* and also consumed about 200 g. molasses/day dissolved in the drinking water. Rumen liquor, drawn through the cannula 2 hr after the morning feed, was strained through muslin and incubated at 38° for 1 hr. The scum was carefully removed by suction and glucose was then added to the remaining brown cloudy fluid at a rate of 0·75 g./100 ml liquor. After a further 1 hr period of incubation, the fluid was centrifuged for 5 min. in the cold, $RCF_{max.} = 70g$ (Inter-

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national Refrigerated Centrifuge, model PR2, head no. 276). The supernatant fluid was removed by suction and the greenish residue resuspended in half the original volume of buffer solution (Abou Akkada & Howard, 1960). The suspension was centrifuged as before and the residue discarded. The original supernatant fluid plus washings was centrifuged for 30 min., $RCF_{max.} = 1000g$ (head no. 269) and the cloudy supernatant fluid, containing many bacteria but very few Q.O., was discarded. The creamy-white precipitate was resuspended in buffer solution and centrifuged again. This washing process was repeated until the supernatant fluid was almost clear and microscopic examination of Gram-stained preparations of the precipitate showed it to be virtually free from micro-organisms other than Q.O. The large size of Q.O. in comparison with other bacteria enabled this distinction to be made readily. For the cleanest preparations (< 1 bacterium/500 Q.O.) it was found necessary to centrifuge, during the washing stage, at a $RCF_{max.} = 800g$ with accompanying loss of Q.O. The oval organisms, which averaged $6 \times 4\ \mu$ in size, showed no marked internal features under phase-contrast microscopy, were markedly Gram-negative and the use of nuclear stains did not show a discrete nucleus. In fresh rumen samples, examined microscopically on a warm-stage, the ovals were actively motile. Motility was rapidly lost during the purification procedure but could be partially restored by warming the ovals to 37° in buffer. Refractile granules as reported by McGaughey & Sellers (1948) were not observed. Flagellated crescent forms (McGaughey & Sellers, 1948) have been observed by us in samples of rumen liquor but were completely absent from these preparations of Q.O. Preparations of Q.O. were finally washed with 0·9% (w/v) saline and stored in the frozen state as a 20% (w/v) suspension in saline.

Preparation of cell walls of Q.O. Cell walls were prepared and purified essentially as described by Forrester & Wicken (1966).

Electron microscopy. Suspensions of whole organisms or of cell walls were prepared in distilled water and air dried on 200-mesh carbon-backed collodion grids. Specimens were shadowed at a 30° angle with Au+Pd and examined in a Hitachi HU-11B electron microscope by Mr M. K. Reynolds of the Plant Diseases Division, DSIR, Lincoln, Canterbury, New Zealand.

Chemical methods. Acid hydrolysis of cell walls and chromatographic identification of hydrolysis products was done by methods described previously (Forrester & Wicken, 1966) with the addition of thin-layer chromatography on cellulose+silica gel for amino acid identification (Turner & Redgwell, 1966). Quantitative analyses of phosphorus, lipid and hexosamines were made as previously described (Forrester & Wicken, 1966), neutral sugars by the quantitative paper chromatographic method of Wilson (1959) and nitrogen by combustion analysis (Coleman Nitrogen Analyser).

RESULTS

Final preparations of cell walls of Q.O. were free of cytoplasmic constituents as judged by their appearance under the electron microscope and, although thin, retained the rigid shape of the original organism (Pl. 1, fig. 1). Electron micrographs (Pl. 1, fig. 2) of whole organisms showed clearly the presence of peritrichous flagella. Marked fragmentation of the latter occurred when suspensions of Q.O. were frozen and then thawed; this fragmentation did not occur during storage over several days at 5°.

Amino acids in cell walls. Seventeen amino acids were identified by thin layer

chromatography. Alanine, threonine, glycine, serine, glutamic acid, aspartic acid, methionine, valine, leucine and isoleucine appeared as major components; arginine, histidine, lysine, cysteine, proline, tyrosine and diaminopimelic acid were shown as minor components. Diaminopimelic acid was further characterized by paper chromatography in several solvent systems (Forrester & Wicken, 1966) and by high voltage paper electrophoresis. On paper, but not on thin-layer plates, the characteristic yellow ninhydrin colour of diaminopimelic acid 'spots' was observed.

Table 1. *Composition of the cell walls of Q.O.*

	% by weight of dry cell wall		% by weight of dry cell wall
Nitrogen	9·82	Glucose	0·9
*Hexosamines	7·77	Galactose	1·3
Lipid	9·92	Rhamnose	9·0
Total phosphorus	0·54	Ribose	0·9
Lipid phosphorus	0·19		

* Determined as glucosamine equivalents.

Table 2. *Chromatographic mobility of muramic acid prepared from Q.O. compared with authentic muramic acid*

Chromatographic system	$R_{glucosamine}$ Muramic acid from Q.O.	$R_{glucosamine}$ Muramic acid (authentic)*
1. Phenol + water (3 + 1 by vol) TLC†	2·95	2·95
2. Butan -2- ol + formic acid + water (7 + 1 + 2 by vol.) TLC†	2·05	2·00
3. Propan-2-ol + water (4 + 1 by vol.), paper	1·10	1·10
4. Butan-1-ol + acetic acid + water (4 + 1 + 5 by vol., upper layer), paper	1·92	1·98
5. Butan-1-ol + pyridine + water (6 + 4 + 3 by vol.), paper	1·18	1·20
6. Ethylacetate + pyridine + H_2O + acetic acid (5 + 5 + 3 + 1 by vol.), paper	1·14	1·12

* Authentic muramic acid from Sigma Chemical Co., U.S.A.
† TLC = thin-layer chromatography on cellulose + silica gel (Turner & Redgwell, 1966).

Neutral sugars in cell walls. Glucose, galactose, rhamnose and ribose were positively identified in acid hydrolysates by paper chromatography in several solvent systems. Traces of an unknown sugar with an R_F value corresponding to arabinose, but which was not a pentose, were also found. The Dische reaction (Dische & Shettles, 1948; Salton, 1964) for aldoheptoses carried out with whole cell walls and with material eluted from the hexose region of paper chromatograms of wall acid hydrolysates all proved negative.

Hexosamines in cell walls. Glucosamine was identified in acid hydrolysates of whole cell walls by its R_F value on paper chromatograms, ninhydrin oxidation product and the spectrum of the chromogen formed in a modified Elson-Morgan reaction (Boas, 1953).

The results of quantitative analyses are recorded in Table 1.

Degradation products characteristic of teichoic acids were not found in acid hydrolysates of wall preparations.

A fractionation scheme, involving tryptic digestion and extraction with sodium dodecylsulphate solutions, devised for the preparation of glycosaminopeptides from the cell walls of Gram-negative bacteria (Grula, Smith & Grula, 1965), was applied to cell walls from Q.O. Much of the protein and polysaccharide components of the wall were solubilized by this method, while the residual particulate matter retained the rigid shape of the original cell-wall fragments. Chromatograms of acid hydrolysates of the latter still showed traces of both neutral sugars and the full range of amino acids but the hexosamine fraction was greatly enriched, as were the amino acids, alanine, glycine, glutamic acid and α, ϵ-diaminopimelic acid. Chromatography on Whatman 3MM paper in butan-1-ol+acetic acid+water (4+1+5, by vol. upper layer) gave glucosamine and a component having the mobility of muramic acid in this solvent ($R_{\text{glucosamine}} = 1{\cdot}92$). This material had an R_F value identical with that of authentic muramic acid in five other paper and thin-layer chromatographic systems (Table 2). The spectral curves of the chromogens produced in a modified Elson-Morgan reaction (Boas, 1953; Strominger, Park & Thompson, 1959) with muramic acid from Q.O. and authentic muramic acid were identical. Peak absorption occurred at 505 mμ compared with 525 mμ and 526 mμ for glucosamine and galactosamine respectively, and an increase of absorption at 505 mμ was noted after 24 hr.

DISCUSSION

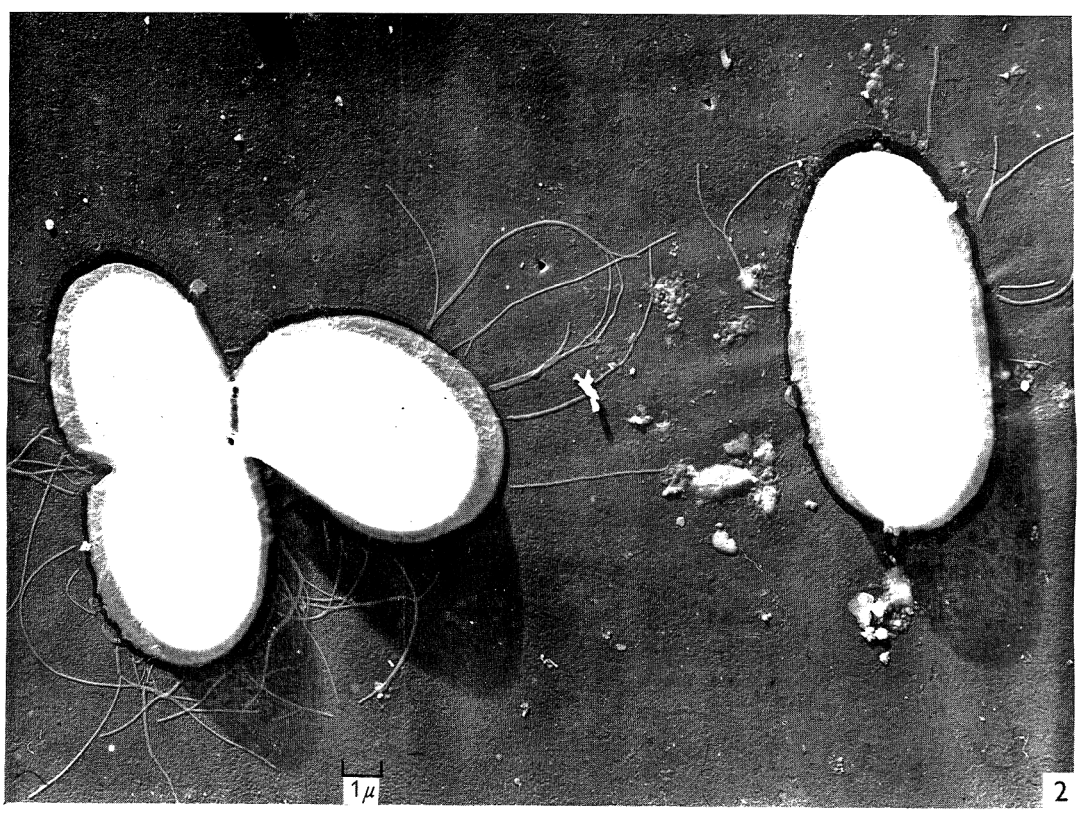
Q.O. appears 'featureless' under phase-contrast microscopy and in stained preparations, a property in keeping with it being a lower rather than a higher protist. That it is Gram-negative might also be considered at variance with the original view that this organism was a yeast, yeasts being generally Gram-positive. Later views (van der Westhuizen, Oxford & Quin, 1950) that Q.O. is not a yeast appear to have been based on a failure of laboratory culture in media which usually grow yeasts. As far as we are aware Q.O. has not been grown in any medium outside the rumen (see McGaughey & Sellers, 1948) and thus such conclusions might be considered premature.

In recent years extensive chemical and biochemical studies of microbial cell walls have been made. It is now evident (Salton, 1964) that the cell walls of algae, yeasts and fungi are predominantly polysaccharide in nature, although protein and lipid are significant constituents in some species. Bacteria, on the other hand, are further distinguished in that the bacterial walls contain glycosaminopeptide heteropolymers, which lend structural rigidity to the wall, and, in many cases, also teichoic acids.

Glycosaminopeptides are characteristically composed of the two amino sugars, glucosamine and muramic acid and a limited number (4–5) of amino acids. α-ϵ-Diaminopimelic acid is a widely occurring constituent of bacterial glycosaminopeptide and the cell walls of most Gram-negative bacteria thus far examined have been shown to contain this amino acid (Salton, 1964). Muramic acid has been found in all bacterial cell walls thus far examined as well as in the blue green algae and appears to be confined to the lower protists (Salton, 1964). More recently the finding of muramic acid in the cell walls of rickettsias (Allison & Perkins, 1960) and spirochaetes (Ginger, 1963) has been used as the main evidence for justifying the inclusion of these groups of micro-organisms among the bacteria.

The isolated cell walls of Q.O. have been shown to contain protein (from the wide range of amino acids found in acid hydrolysates), polysaccharide and lipid, which are


1μ
1


1μ
2

components typical of Gram-negative bacteria (Salton, 1964). In addition, a partially purified fraction of the wall, obtained after removal of much of the protein and polysaccharide and which retained the rigidity of the original cell wall, contained glucosamine and muramic acid. The amino acid composition of this impure glycosaminopeptide could not be determined unequivocally owing to incomplete removal of wall protein but the enrichment, in this fraction, of alanine, glycine, glutamic acid and, particularly, diaminopimelic acid is consistent with the amino acid composition of glycosaminopeptides from other Gram-negative bacteria. Teichoic acid appears to be absent from these cell walls as is the case with most Gram-negative bacteria but it is interesting to note that only a third of the low phosphorus content of the wall is associated with wall lipid. The organic nature of the remaining phosphorus is not known.

In view of the chemical composition of the cell wall of Q.O. we conclude that it is a member of the bacteria (Schizomycetes).

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EXPLANATION OF PLATE

Fig. 1. Electron micrograph of a cell-wall preparation from 'Quin's oval'.

Fig. 2. Electron micrograph of whole organisms of 'Quin's oval' showing the type of peritrichous flagellation.

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The Morphology and Ultrastructure of the Spore and Exosporium of Some Clostridium Species

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(*Accepted for publication* 10 *December* 1966)

SUMMARY

The spores of original isolates of Clostridium species obtained from fresh and processed marine fish and from marine bottom deposits were compared with the spores of culture collection strains of the same species. These strains were *Clostridium botulinum* type E, *C. sporogenes*, *C. bifermentans* and *C. sordellii*. Spores of unclassified, non-toxigenic isolates which resemble *C. botulinum* type E, of 'OS mutants', of culture collection strains of *C. botulinum* types A and B and an isolate of type F were also examined. The gross morphology and ultrastructure of the spores and exosporia were studied in electron microscopic preparations by using the techniques of metal shadowing, carbon replica, negative-staining and ultra-thin sectioning. Spores of some strains were found to possess tubular appendages. The morphology of the spores and the ultrastructural detail of the spore appendages and exosporia are described.

INTRODUCTION

Information on the fine structure of the spores of Clostridium species is limited. Franklin & Bradley (1957) used the carbon replica technique to study the surface structure of the spore of certain Bacillus species and of *C. welchii*. Most of the electron microscopic studies on clostridial spores have been carried out on ultra-thin sections. Hashimoto & Naylor (1958) used stained preparations for light microscopy, and ultra-thin sections for electron microscopy, in studies of the sporulation process and the structure of the spores of *C. sporogenes*. The processes of sporulation and germination in *C. butyricum* and *C. botulinum* type E were studied by Takagi, Kawata & Yamamoto (1960) and in *C. tetani* and *C. histolyticum* by Takagi, Kawata, Yamamoto, Kubo & Okita (1960) using ultra-thin sections. This technique was also used by Fitz-James (1962) to follow spore development in *C. pectinovorum*. Takagi, Nakamura & Ueda (1965) used both ultra-thin sections and the negative-staining technique to study the role of the intracytoplasmic membrane system during sporulation in *C. tetani* and *C. botulinum* type E. In a comprehensive study of sporulation and spore germination in strain NCA 62A (ATCC 7948) of *C. botulinum* type A, Stewart (1963) used ultra-thin section and metal-shadowing techniques.

Walker (1963), in a study of spore antigens, published micrographs of metal-shadowed entire spores of *C. sporogenes* and *C. bifermentans* which showed the presence of distinct exosporia on the spores of these species. Krassil'nikov, Duda & Sokolov (1964) presented micrographs of metal-shadowed entire spores of 20 soil isolates of Clostridium species, demonstrating that these spores possessed elaborate and varied surface protrusions. Subsequently, Hodgkiss & Ordal (1966), using metal-shadowing,

negative-staining and carbon replica techniques, showed that the spore of *C. botulinum* type E possesses unusual tubular appendages and a delicate exosporium. Hodgkiss, Ordal & Cann (1966) then carried out a comparative study of the morphology and ultrastructure of the spore and exosporium of *C. botulinum* type E and of the 'opaque sporulating (OS) mutants' of Dolman (1957).

These latter studies have been extended to investigate the morphology and ultrastructure of the spores of representative strains of certain Clostridium species isolated during surveys of the incidence of *C. botulinum* type E in fresh and processed marine fish and marine mud (Hobbs, Cann, Wilson & Shewan, 1965; Cann *et al.* 1965; Cann, Wilson, Shewan & Hobbs, 1966). For comparison, some Culture Collection strains of Clostridium species have also been studied.

METHODS

The spores of 38 strains of Clostridium species have been examined. The details of the strains are as follows:

TRS = Torry Research Station.
NCIB = National Collection of Industrial Bacteria.
NCTC = National Collection of Type Cultures.
ATCC = American Type Culture Collection.
FDA = Food and Drug Administration, U.S.A.

Strains of C. botulinum type E.

Strain 211: isolated from a sludge sample, Lake Abashiri, Japan (Nakamura *et al.* 1956).

NCIB 4288, NCIB 4299 } TRS isolates from sea-bottom deposits, Scandinavian coast.

FT 4: TRS isolate from Norwegian herring (*Clupea harengus*)

NCIB 4248: Beluga strain. Isolated from 'muktuk', i.e. flippers of beluga (white whale, *Delphinapterus leucas*), Dolman & Chang (1953).

Unclassified non-toxigenic (NT) strains which culturally and biochemically resemble C. botulinum type E

(*a*) NCIB 4266: TRS isolate from vacuum-packed, smoked haddock (*Gadus aeglefinus*).
NCIB 4289: TRS isolate from sea-bottom deposit, Scandinavian coast.
NCIB 4271: TRS isolate from cockles (*Mytilus* sp.), South Wales.

(*b*) NCIB 4268, NCIB 4269, NCIB 4270, NCIB 4277 } TRS isolates from sea-bottom deposits, Scandinavian coast.

FT 3: TRS isolate from Norwegian herring (*Clupea harengus*).

OS strains (Dolman, 1957; Hobbs, Roberts & Walker, 1965).

TRS EOS: TRS isolate from a culture of NCTC 8266, *C. botulinum* type E.

AJMAL OS: isolated by M. Ajmal, Central Public Health Laboratory, Colindale, from a culture of a FDA tuna strain of *C. botulinum* type E.

24 AS: isolated from vacuum-packed kippers (i.e. smoked herring, *Clupea harengus*).

Strains of C. sporogenes

FT23: isolated from vacuum-packed smoked haddock, (*Gadus aeglefinus*).
NCIB 532
NCIB9383
PA3679 (= ATCC7955, NCIB8053): Putrefactive anaerobe.

Strains of C. bifermentans

244B, 247B, 275B, 288B } TRS isolates from vacuum-packed smoked haddock and kippers.
NCIB506, 1341, 2929, 6800, 6928.

Strains of C. sordellii

35B: TRS isolate from vacuum-packed kippers.
NCIB2914, 6801 and 6929.
CN1734: Burroughs Wellcome Culture Collection, Beckenham, England.

C. botulinum type A.

NCTC7272
ZK3: Unilever Research Laboratories, Colworth House, Sharnbrook, Bedford, England (isolated from groundnuts, *Arachis hypogea*).

C. botulinum type B

ATCC7949

C. botulinum type F

610B: Craig & Pilcher, (1966).

Spore suspensions.

Spore crops of the organisms were produced in a variety of nutrient media including Hartley's digest broth, Robertson's meat broth, blood-agar, egg-yolk agar (Willis & Hobbs, 1958) and in the medium of Schmidt, Nank & Lechowich (1962) or in that medium with added manganese and calcium. Spores were harvested, washed by low-speed centrifugation (2000*g*) and resuspended in 0·85 % sodium chloride solution. The saline spore suspensions were then examined in the light microscope by phase contrast. If it was found that sporulation was not complete, vegetative debris was removed by treating the saline suspension with trypsin for 12—24 hr.

Electron microscopy.

The spores were further washed three times by low speed centrifuging (2000*g*) in freshly distilled, sterile water. Droplets of this distilled water suspension were placed on formvar coated 200-mesh copper grids, air-dried and shadowed with gold + palladium (40 + 60) at an angle of 20°. Carbon-coated 200-mesh copper grids were used for negative stained preparations. A droplet of the spore suspension was allowed to air-dry on the grid. The grid was then treated for 1 min. with 1 % aqueous ammonium molybdate or 1 % aqueous phosphotungstic acid (PTA) adjusted to

pH 7·0 (Brenner & Horne, 1959) Carbon replicas were prepared by the method of Bradley & Williams (1957) and were shadowed with gold + palladium (40 + 60) at an angle of 20°. For sectioning, spores were fixed in 2·5 % glutaraldehyde in 0·1 M-phosphate buffer (pH 7·0) for 4 hr at 4°, post-fixed in osmium tetroxide in veronal buffer for 2 hr at 4° and stained in 1 % (w/v) aqueous uranyl acetate for 18 hr at 4°. The fixed and stained spores were then dehydrated by passage through increasing strengths of ethyl alcohol, transferred to propylene oxide, thence to 50 + 50 (v/v) propylene oxide and Epon and finally embedded in pure Epon (Luft, 1961). Ultra-thin sections were cut on a Cambridge Huxley Microtome using glass knives.

Preparations were examined in a Siemens Elmiskop I electron microscope using single- or double-condenser illumination, a 200 μ condenser aperture, a 50 μ objective aperture and accelerating voltages of 60 kV (for sections and metal-shadowed preparations) or 80 kV. Micrographs were recorded at initial magnifications of $\times$8000 to $\times$40,000 on Ilford N50 plates.

RESULTS

During the surveys carried out in this laboratory (Hobbs, Cann, Wilson & Shewan, 1965; Cann *et al.* 1965; Cann *et al.* 1966), some isolates identified as *Clostridium sporogenes* were obtained. The morphology of the spores of one of these strains, FT 23, was compared with that of the spores of two Culture Collection strains of *C. sporogenes*, NCIB 532 and 9383, and that of the spores of the well-documented strain commonly referred to as Putrefactive Anaerobe PA 3679 (ATCC 7955, NCIB 8053). The fine structure of the spores of all four strains was essentially identical (Pl. 1, fig. 1). The spore here appears as an electron-dense, ovoid body loosely enveloped in an exosporium, typical of what might be termed an orthodox bacterial endospore as described previously in many species (review by Robinow, 1960). The spore of *C. sporogenes* has a relatively smooth coat (Pl. 1, fig. 2), being devoid of surface ornamentation of the type found on the spores of certain Bacillus species (Hooff & Aninga, 1956; Bradley & Williams, 1957; Franklin & Bradley, 1957; Dondero & Holbert, 1957; Bradley & Franklin, 1958) and devoid of protrusions or appendages (Krassil'nikov *et al.* 1964; Hodgkiss & Ordal, 1966; Hodgkiss *et al.* 1966). The exosporium of the four strains of *C. sporogenes* may, for the purposes of this paper, be categorized as 'thick' as opposed to the thin, membranous structure which envelopes the spore of *C. botulinum* type E (Hodgkiss & Ordal, 1966). In metal-shadowed preparations it measures approximately 120–150 Å thick. No ultrastructural detail was discernible in micrographs of metal-shadowed or negatively stained preparations. This type of exosporium will therefore herein be described as homogeneous.

Included in the present study were four toxigenic strains of *Clostridium botulinum* type E which we had not examined previously (NCIB 4288, 4299, Nakamura 211 and FT 4). The spores of all strains showed the same morphology and ultrastructure as that found in the Beluga strain, NCIB 4248 (Hodgkiss & Ordal, 1966), and the FDA tuna strain (Hodgkiss *et al.* 1966). The outstanding features of these spores are the presence of numerous tubular appendages (160–240) per spore and the delicate fibrillar ultrastructure of the membranous and large exosporium. With most strains the appendages appeared to be uniformly distributed over the spore surface as in the Beluga strain. However, in some strains the appendages appeared to be most numerous at the poles of the spore, e.g. FDA tuna strain (Hodgkiss *et al.* 1966).

A number of isolates were obtained which were non-toxigenic (NT) but which in other characteristics closely resembled *Clostridium botulinum* type E. These NT cultures could be divided into two groups by spore morphology. First, there were those strains (NCIB 4266, 4271 and 4289) in which the morphology and ultrastructure of the spore and exosporium were identical with that of toxigenic strains of *C. botulinum* type E (Pl. 1, fig. 3). The spores of the second group of NT isolates (NCIB 4268, 4270, 4277, 4269 and FT 3) were all similar to each other in morphology but were quite different from the first group just described. The spores were devoid of appendages and were enveloped in a thick, homogeneous exosporium (Pl. 1, fig. 4).

Non-toxigenic 'mutants' of *Clostridium botulinum* type E which produce opaque sporulating (OS) colonies were described by Dolman (1957). Following the detailed studies of Hobbs, Roberts & Walker (1965) in which three of the isolates (E 60 S, E 70 S and E 1040 S) were compared to a number of toxigenic strains of *C. botulinum* type E, we examined and reported on the spore morphology of the same three strains (Hodgkiss *et al.* 1966).) Since then we have examined the spores of three more strains (TRSEOS, AJMAL OS and 24 AS). These possessed the same morphology and ultrastructure as the three already reported on. For comparative purposes we include a typical electron micrograph of a spore of strain 24 AS (Pl. 1, fig. 5) and will briefly summarize the pertinent characteristics. Each spore possesses from one to four open-ended tubular appendages which normally arise from the poles of the spore. The appendages vary in length (usually 1–2 μ) but are of relatively constant diameter. The overall diameter of the tubule is 480 Å and that of the lumen 320 Å. Negatively stained preparations of the exosporium show a hexagonal pattern of holes, the centre to centre distance of these holes is approximately 90 Å (Pl. 1, fig. 6).

The spores of the several *Clostridium bifermentans* strains were of two types. The spores of the five Culture Collection strains were of orthodox appearance; but the spores of the four strains (244 B, 247 B, 275 B and 288 B) isolated from vacuum-packed smoked haddock and kippers were striking and distinctive in morphology possessing appendages (usually from 10 to 20) which projected from the poles of the spore (Pl. 1, fig. 7; Pl. 2, fig. 8). These were approximately 2 μ in length and the distal portion of the appendage was thicker than the proximal portion. Negatively stained preparations demonstrated the tubular nature and the distinctive fine structure of these appendages (Pl. 2, fig. 9). The overall diameter of the proximal portion of the tubule is 480 Å and that of the lumen 240 Å. The distal portion of the tubule wall is thickened by an outer sheath which is composed of microfibrils, about 500 Å in length and 25–40 Å in diameter, arranged in a parallel array and inserted into the tubule wall at an angle of 40° to the long axis of the tubule. In this respect they resemble the manner in which the barbs are inserted on the calamus of a bird's feather. The sheath of microfibrils thickens the distal portion of the tubule wall and thus increases the overall diameter of the appendage to the order of 1120 Å. The lumen of the tubule is of constant diameter throughout the entire proximal portion and along most of the thicker, distal portion of the appendage. However, at the extreme distal end the diameter increases gradually and the lumen terminates in a funnel-shaped orifice. The exosporium is of the thick homogeneous type. Suggestive evidence that the appendages arise from the spore coat is supplied in Pl. 2, fig. 10. The short shadow of this spore indicates that the spore contents have been lost, but the appendages are still in their normal position. Further evidence that the appendages are extensions of the spore coat is presented below.

A typical spore of one of the five Culture Collection strains of *Clostridium bifermentans* is presented in Pl. 2, fig. 11. No appendages are present and the thick exosporium is of the homogenous type.

The five strains of *Clostridium sordellii* had spores with three types of morphology. Spores of strains NCIB 2914 and CN 1734 possessed a thick, homogeneous exosporium and lacked appendages (Pl. 2, fig. 12). Spores of strain 35B (Pl. 2, fig. 13) had unusually long (up to 4 μ) open-ended tubular appendages which in many respects were similar to the appendages found on the OS spores, the overall diameter of the tubule (600 Å) and the lumen diameter (400 Å) being slightly greater than that of the OS strains. They were commonly seen as a coiled structure within the disintegrating fine-meshed fibrillar exosporium (Pl. 2, figs. 14, 15). The exosporium appeared to be even more delicate than that of the type E strains, the individual fibrils were comparable in diameter (60 Å) to those of the type E exosporium and their arrangement indicated a more loosely formed meshwork structure.

The spores of strain 6929, *Colstridium sordellii* (Pl. 3, fig. 16) possessed simple open-ended tubular appendages similar in dimensions to those of strain 35B but the exosporium was quite different. The exosporium was similar to that of strains NCIB 2914 and CN 1734, i.e. it was of the 'thick', homogeneous type (Pl. 3, fig. 17).

Because of our original interest in the spores of *Clostridium botulinum* type E, we examined the spores of representative strains of types A, B and F. The strains we studied sporulated poorly in the media tested, and so our findings on these strains must be considered to be of a preliminary nature. A typical type-A spore as found in our preparations is presented in Pl. 3, fig. 18. Although the spore preparations appeared to be 'clean' as viewed by dark-phase contrast optics at a magnification of $\times 1250$, most spores seen in the electron microscope showed the tenacious attachment of sporangial fragments. Similar results were obtained with the strain of type B (ATCC 7949) although occasionally we did find a free spore (Pl. 3, fig. 19). The spores of both the type-A and type-B strains were devoid of appendages. With both strains, when the cell sporulates, the sporangium assumes an almost diamond shape and the spore is located in the more obtuse end of the cell. When the spore reaches maturity, the tip of the sporangium furthest away from the spore begins to lyse, and lysis continues slowly toward the end containing the spore (Pl. 3, fig. 20–22). While several sporulating cultures were incubated for longer periods of time before harvesting and cleaning, lysis of the sporangial fragments adhering to the spore was not further improved.

A spore suspension of *Clostridium botulinum* type A, strain ZK 3, prepared by Dr A. C. Baird-Parker, showed the same morphological characteristics as did the preparations of strain NCTC 7272 (Pl. 3, fig. 23).

Spores of *Clostridium botulinum* type F were devoid of appendages but were enveloped in a distinct and voluminous, thick exosporium (Pl. 4, fig. 24). Negatively stained preparations of the exosporium showed an ultrastructure with a definite hexagonal pattern similar to that found in the exosporium of the OS strains (Pl. 4, fig. 25). The centre-to-centre distance of the hexagonal units was approximately 100 Å.

In addition to this survey of the morphological characteristics of some clostridial spores we have also initiated investigations to further describe the attachment of the appendages to the spore and ultimately to follow their development during sporulation and their fate during spore germination. While this effort is still in its initial stages we consider it pertinent to present some of our findings on the Beluga strain of

C. botulinum type E (NCIB 4248). The appearance of an ultra-thin section of a liberated spore is presented in Pl. 4, fig. 26. The spore proper consists of a relatively dense core surrounded by a less-dense cortex with a multi-layered spore coat (cf. Robinow, 1960). The thin, inner layer of the spore coat is very electron-dense but the outer layers are less dense. The tubular appendages appear to be extensions of the outer spore coats as they do not penetrate through the coat into the core or cortex. The whole of the

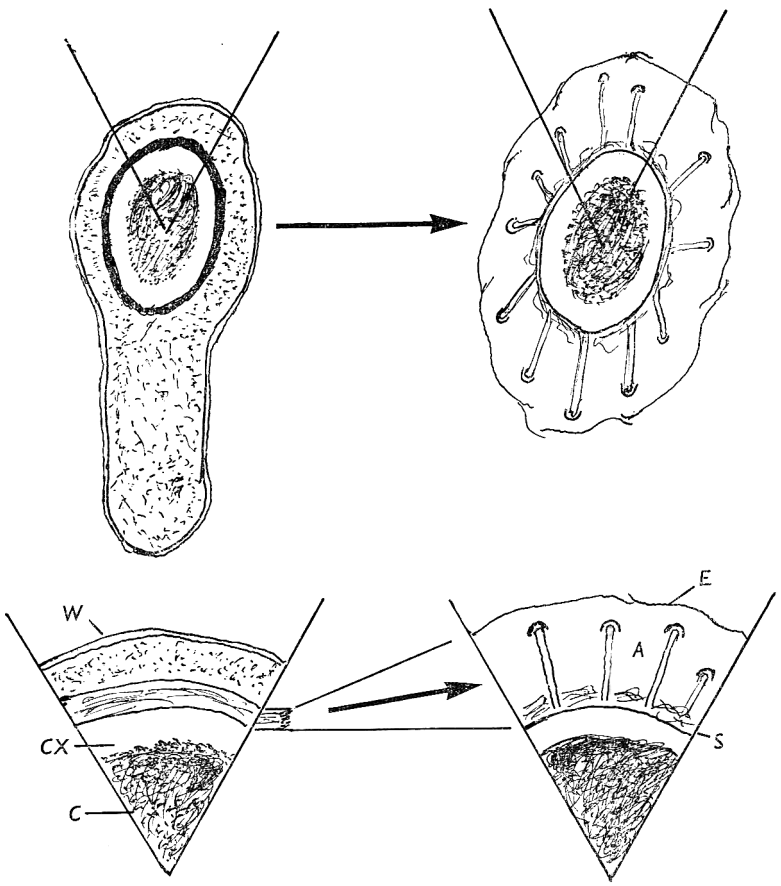

Fig. 1. *Clostridium botulinum* type E. A diagrammatic representation of the appearance, in ultra-thin sections, of a developing spore in a sporangium and a mature, liberated spore within its exosporium, *C* = core, *CX* = cortex, *W* = wall of sporangium, *S* = spore coat, *A* = appendages, *E* = exosporium.

spore is encased in a thin voluminous exosporium which is composed of microfibrils (Hodgkiss & Ordal, 1966). The relative size of the complete liberated spore in its exosporium and that of a normal vegetative cell is presented in Pl. 4, fig. 27. Our observations indicate that whilst the spore is within the sporangium the appendages and exosporium are tightly wrapped around the spore. When the mature spore is liberated from the sporangium, fluid is absorbed through the exosporium causing it to become turgid and swollen. During this process the appendages unfold and become everted. These events are diagrammatically represented in Fig. 1.

DISCUSSION

Our findings of varied and elaborate appendages on the spores of certain strains substantiate those of Krassil'nikov *et al.* (1964), who described elaborate protrusions on the surface of spores of Clostridium species. On the basis of their studies these authors consider that, in this genus, spore morphology is a species-specific characteristic. Our studies were carried out on a very narrow range of species so that our results can be discussed only within this limitation.

Morphologically the spores of the strains studied may be divided into two main groups: those with appendages; and those without—the orthodox spores. The appendage-bearing spores may be further characterized on the basis of the ultrastructure of the appendages. The constant and outstanding feature of the appendages is that they are micro-tubules. Those of the spores of *C. botulinum* type E are sealed at the distal end (Hodgkiss & Ordal, 1966) but in the other strains the distal end of the tubule is open. Again, in the type-E strains the tubules remain within the intact exosporium, whereas in the other strains the tubules eventually protrude through the fractured exosporium.

In all except three of the strains (*Clostridium botulinum* type A, NCTC 7272; ZK 3 and type B, ATCC 7949) the spore was enveloped in an exosporium. Three main structural types of exosporium were found: a thin membranous type composed of microfibrillar elements, a thick type in which a hexagonal pattern may be detected and a thick homogeneous type.

The above, then, are the points of morphological differentiation of the spore and exosporium. The orthodox or plain spores could not be further differentiated morphologically as they were all devoid of the type of surface ornamentation found in some Bacillus species and all strains showed variation in spore size (see also Robinow, 1960).

In addition to four strains of *Clostridium botulinum* type E listed above, five other strains have previously been examined (Hodgkiss & Ordal, 1966; Hodgkiss *et al.* 1966). All the strains were found to have the same morphology of spore appendages and exosporium.

The relationship between the unclassified NT strains and strains of *Clostridium botulinum* type E is not clear. Repeated cultural and biochemical tests fail to distinguish between the two. Similar strains have been isolated, along with type-E strains, by Dolman, Darby & Lane (1955) from post-mortem stomach contents in a case of botulism and by Nakamura *et al.* (1956) from three samples of 'Izushi', all of which had caused outbreaks of botulism. 'Izushi' is a food prepared in the coastal districts of Hokkaido. Raw fish patties mixed with cooked rice, diced vegetables, salt, vinegar and red pepper, and placed in wooden tubs under pressure, are allowed to ferment for 3–4 weeks. Details of the preparation are given by Nakamura *et al.* (1956). Chapman & Naylor (1966) isolated two similar NT strains, as well as two type-E strains, from Cayuga Lake fish during a survey of the incidence of type E in lake water and in fish. Kautter, Harmon, Lynt & Lilly (1966) and Bott, Defner, McCoy & Foster (1966) have also isolated NT strains from fish from the Great Lakes.

The serological relationships of different strains of *Clostridium botulinum* type E are complex (Nakamura *et al.* 1956). It is not surprising therefore that serological tests in this laboratory have failed to provide a key to the relationship of the NT strains to strains of *C. botulinum* type E. However, in view of the uniformity of spore morpho-

logy in type-E strains it is tempting to speculate that the three NT strains which possess spores of exactly this type are non-toxigenic mutants of type E. In the case of those NT strains which possess orthodox spores, spore morphology would not itself suggest a close relationship to type E.

Studies on three additional OS strains have substantiated our original findings on Dolman's strains (Hodgkiss *et al.* 1966). In all these OS strains there is uniformity of spore morphology and of appendage and exosporium ultrastructure. The gross morphology of the OS spore resembles that of the spore of *Clostridium sporosetum* sp.nov. as described by Krassil'nikov *et al.* (1964). In its ultrastructure the exosporium of the OS strains is similar to that of *Bacillus cereus* (Gerhardt & Ribi, 1964). It is our view that the marked differences in spore morphology between the type E strains and the OS strains would not point to a close relationship between the two (Dolman, 1957). Rather, it would seem to be evidence to support the views of Muriel Robertson (quoted on p. 56 by Dolman, 1964) and to add weight to the contention of Hobbs *et al.* (1965) that the OS strains are commensal organisms.

The speciation of organisms which belong to the *Clostridium bifermentans–C. sordellii* group is complex and has been the subject of some controversy (Brooks & Epps, 1959; Huang, 1959, Walker, 1963; Nishida, Tamai & Yamagishi, 1964; Tamai & Nishida, 1964; Huang, Tamai & Nishida, 1965). Within this group of organisms we have demonstrated that strains with very different spore morphology are to be found. The morphological characteristics of the four fish isolates of *C. bifermentans* were constant and contrasted sharply with the orthodox spores found in the five Culture Collection strains. These four strains were arbitrarily selected from approximately 100 such isolates which had been collected as representative clostridial strains found in marine fish products. Their cultural and biochemical reactions were typical of *C. bifermentans* as defined by Brooks & Epps (1959). The gross morphology of the spores of these four strains resembles that of the spore of *C. sporopenitum* sp. nov. of Krassil'nikov *et al.* (1964). The biochemical reactions and ultrastructural details of the spore of this organism have not yet been published so we cannot make further comparison. Three morphological types of spores were found in the five strains which, according to the scheme of Brooks & Epps (1959), could be classified as *C. sordellii*. The fish isolate, strain 35B, and a Culture Collection strain, NCIB 6929, were similar in that they had spores upon which long, open-ended appendages were present but the exosporia of the two strains were quite different in ultrastructure. The other three Culture Collection strains had orthodox spores enveloped in homogeneous exosporia.

The micrographs of spores of *Clostridium botulinum* types A, B and F record our initial observations on these three toxin types. In our preparations the spores of all three types were very clearly differentiated morphologically from those of the type E strains. We found no evidence of an exosporium in types A and B. For the two type-A strains (NCTC 7272 and ZK 3) this finding is in contrast to the findings of Stewart (1963) who demonstrated the presence of an exosporium in strain NCA 62A (ATCC 7948). The spore of the type-F strain is distinguishable from those of the strains of types A and B studied here by the presence of an exosporium which has a hexagonal-pattern ultrastructure.

The orthodox or plain type of spore with a homogeneous, thick exosporium was common to the four strains of *Clostridium sporogenes*, the five Culture Collection strains of *C. bifermentans*, three Culture Collection strains of *C. sordellii* and five of

the NT strains. Walker (1963) also found this type of spore in strains of *C. sporogenes*, *C. bifermentans* and *C. sordellii*. The morphology of this type of spore is therefore of little aid, except as a negative feature, as a determinative tool. However, the elaborate spores described above, which are of consistent morphology, may well come to be regarded as a valuable aid in determinative and taxonomic studies. Clearly a wider range of species must be investigated to assess fully the value of spore morphology within the genus *Clostridium*.

Structures as elaborate as the clostridial appendages have not been described on spores of Bacillus nor on spores of Streptomyces species. In both these genera characteristic spore surface ornamentation has been described and the taxonomic significance thereof outlined (Bradley & Franklin, 1958; Tresner, Davies & Backus, 1961).

The origin, the function and the eventual fate of the microtubular appendages during spore germination have yet to be demonstrated and elucidated. Preliminary observations on ultrathin-sections of sporulating cells and mature spores indicate that the microtubular appendages are a part of the outer coats of the spore. Our studies show that during development of the spore within the sporangium the long appendages are tightly wrapped around the spore and are coiled up within the exosporium. Upon lysis of the sporangium with its concurrent freeing of the spore the exosporium absorbs fluid, enlarges in volume, and the appendages uncoil. With spores of the *Clostridium botulinum* type E the appendages are normally contained within the exosporium whereas in the other types that we have observed, the long tubules eventually break through the exosporium and protrude out into the surrounding medium. The consistent tubular nature of the varied appendages that we have described is probably the most interesting and puzzling aspect of the entire study. What is their function? At this stage of our studies nothing can be added to the conjecture previously advanced (Hodgkiss & Ordal, 1966). Clearly this is a challenge for future studies on spore formation and germination.

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EXPLANATION OF PLATES

Except where stated otherwise the electron-micrographs are of gold–palladium shadowed preparations and the graduation mark is 1·0 μ.

PLATE 1

Fig. 1. *Clostridium sporogenes* strain FT23, spore and exosporium.

Fig. 2. Putrefactive Anaerobe, PA3679. Single stage carbon replica of a spore.

Fig. 3. NT strain NCIB4271. The spore and its appendages are enclosed in a delicate, voluminous exosporium.

Fig. 4. NT strain NCIB4270. Spores and exosporia.

Fig. 5. OS strain 24AS. A spore with a single appendage protruding through the exosporium.

Fig. 6. OS strain 24AS. Ammonium molybdate preparation of exosporium showing a hexagonal pattern structure.

Fig. 7. *C. bifermentans*, strain 247B. The appendages of the spore are coiled up within the exosporium at one end and protrude through the ruptured exosporium at the other end.

PLATE 2

Fig. 8. *C. bifermentans*, strain 288B. Three spores showing the typical morphology.

Fig. 9. *C. bifermentans*, strain 288B. Negatively stained preparation showing the distal end of two appendages. The micro-fibrillar sheath and the funnel-shaped orifice of the tubule are demonstrated.

Fig. 10. *C. bifermentans*, strain 288B. One spore is empty (arrow: note short shadow) and the appendages remain attached to the spore coat.

Fig. 11. *C. bifermentans*, strain NCIB506. Spore and exosporium.

Fig. 12. *C. sordellii*, strain NCIB2914. Spores and exosporia.

Fig. 13. *C. sordelli*, strain 35B. Note the delicate exosporium and the long, coiled appendages.

Fig. 14. *C. sordellii*, strain 35B. Ammonium molybdate preparation. The tubular nature of the appendages and the micro-fibrillar structure of the exosporium are demonstrated.

Fig. 15. *C. sordellii*, strain 35B. Appendages coiled within the exosporium in which micro-fibrillar structure is apparent.

PLATE 3

Fig. 16. *C. sordellii*, strain NCIB6929. The spores possess long appendages and a homogeneous exosporium.

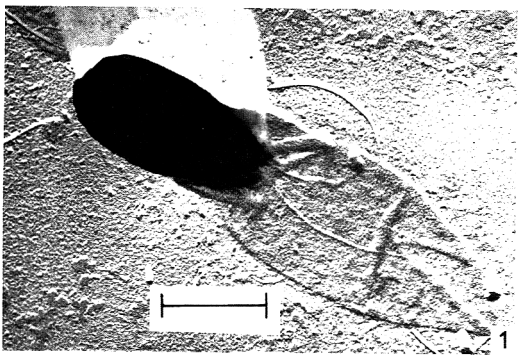
Fig. 17. *C. sordellii*, strain NCIB6929. Ammonium molybdate preparation showing tubular appendages protruding through the ruptured, homogeneous exosporium.

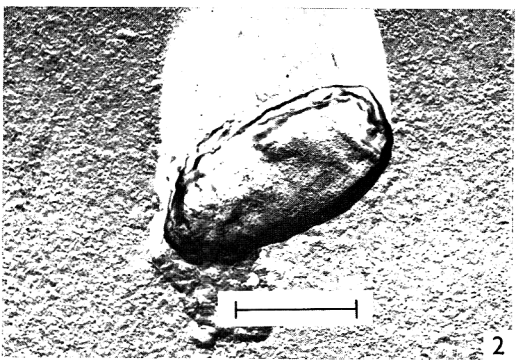
Fig. 18. *C. botulinum* type A, strain NCTC7272. A spore within the remnants of the sporangium.

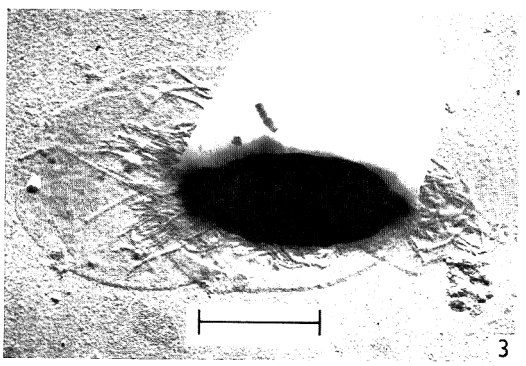
Fig. 19. *C. botulinum* type B, strain ATCC7949. A free spore.

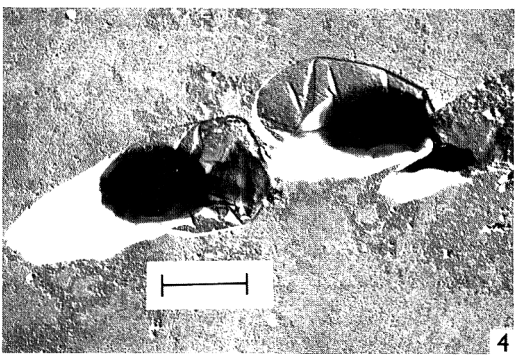
Fig. 20 to Fig. 22. *C. botulinum* type A, strain NCTC7272. Three morphological forms seen in spore preparations. A wholly electron-dense sporangium, an almost entirely electron-dense diamond-shaped stage of the sporangium and the last stage in which the electron-dense spore is seen within the less electron-dense remnants of the sporangium.

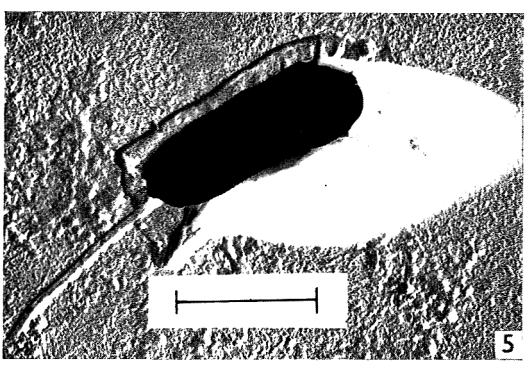
Fig. 23. *C. botulinum* type A, strain ZK3. Typical field, spore preparation; the spores are seen within the remnants of sporangia.

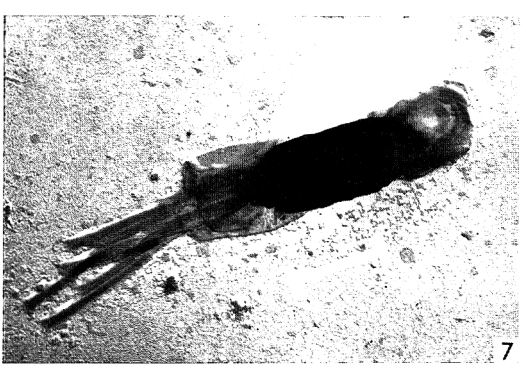












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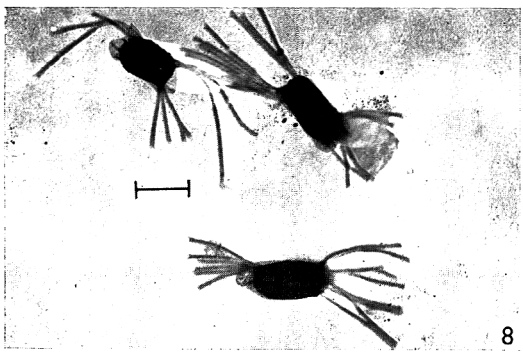

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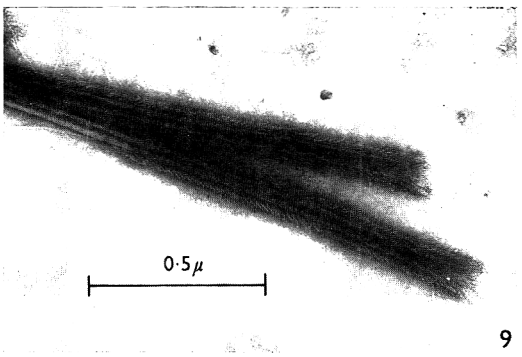

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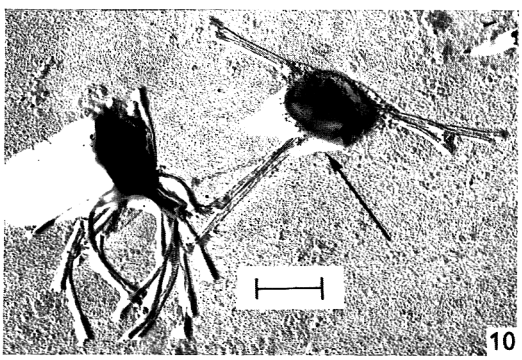

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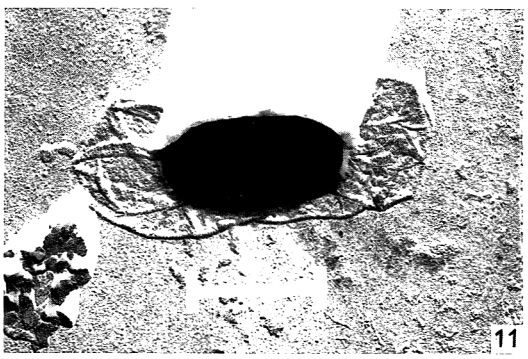

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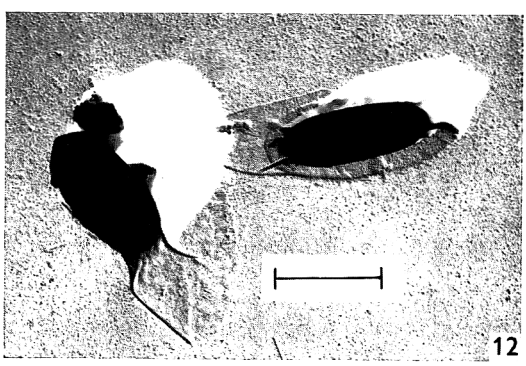

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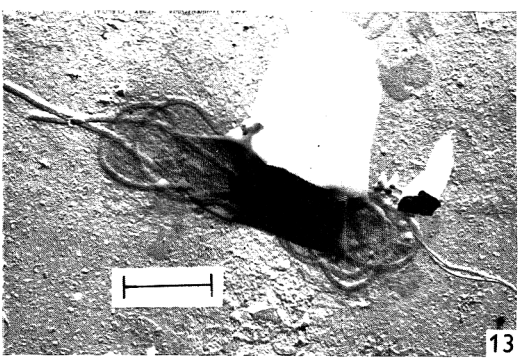

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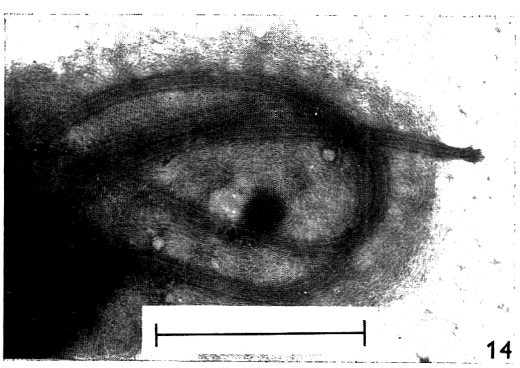

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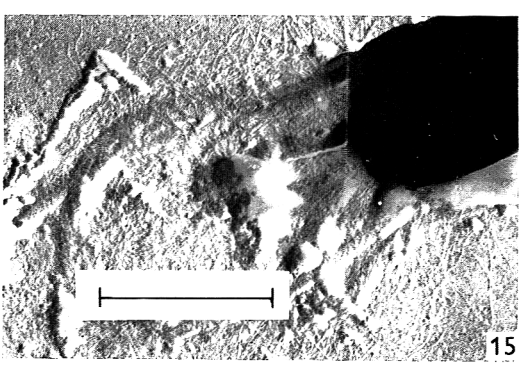

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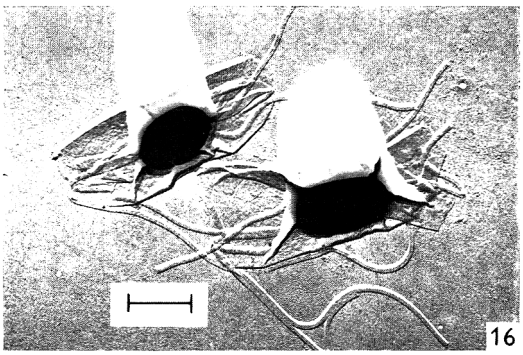
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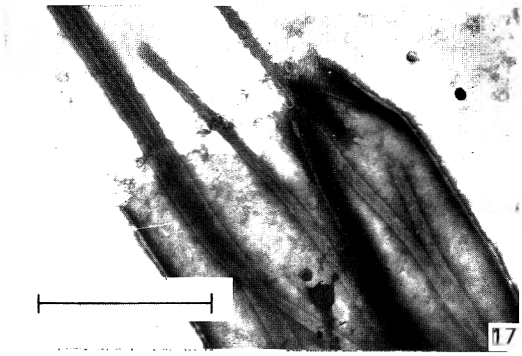
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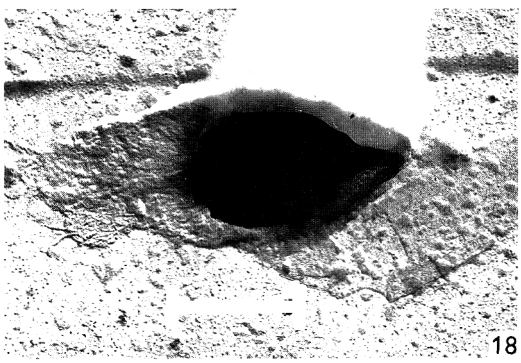
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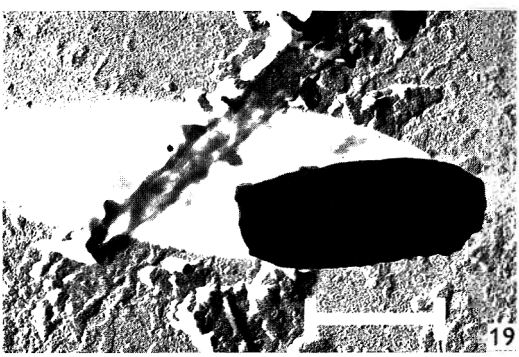
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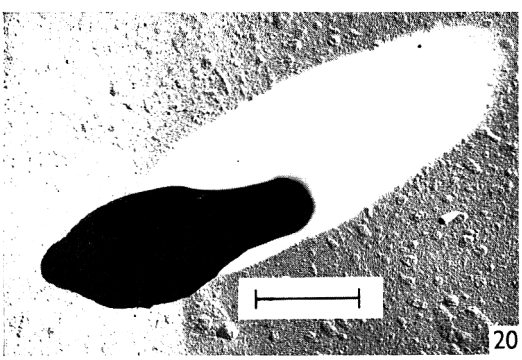
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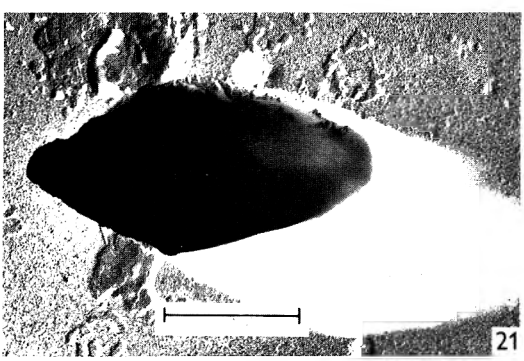
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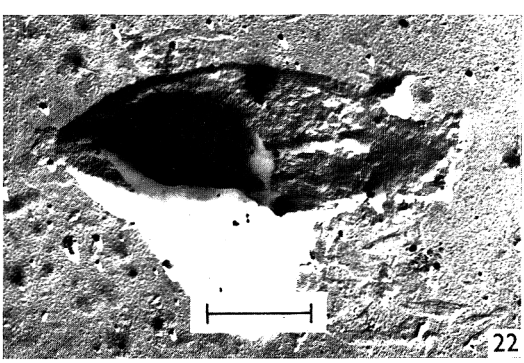
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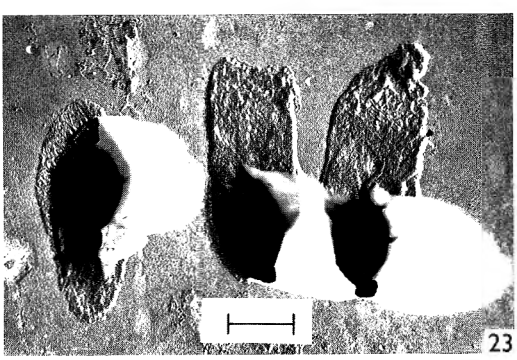
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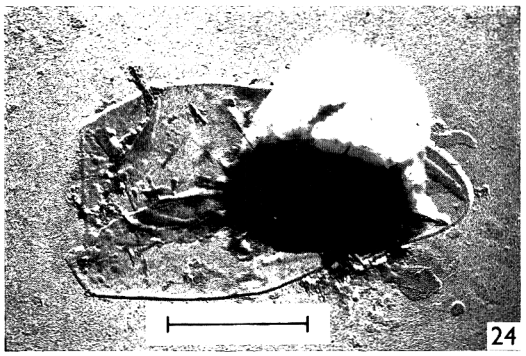
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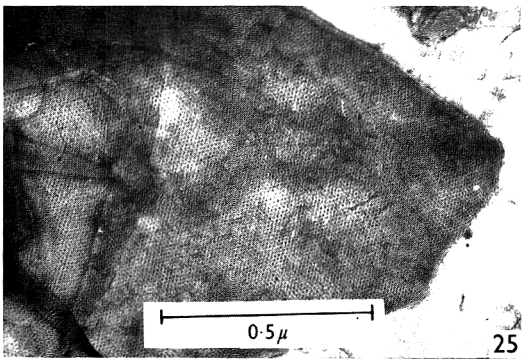
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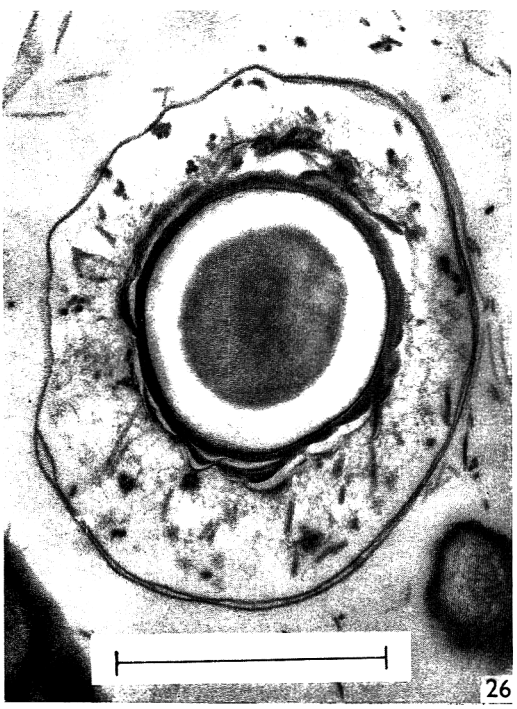
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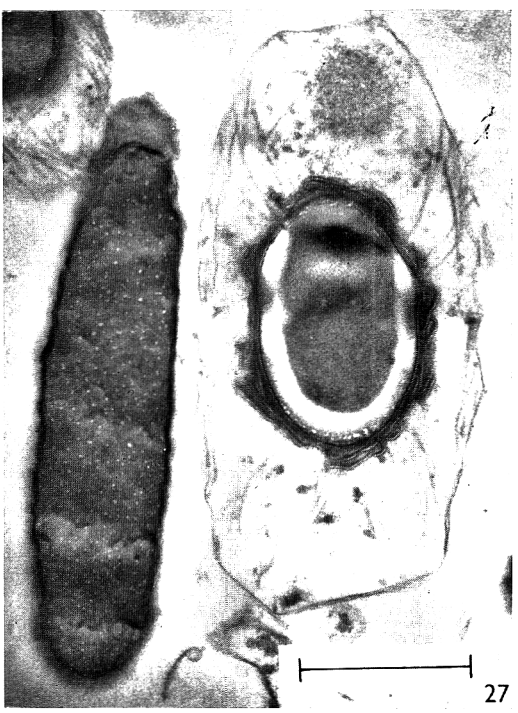
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PLATE 4

Fig. 24. *C. botulinum* type F, strain 610B. Spore and voluminous exosporium.

Fig. 25. *C. botulinum* type F, strain 610B. Ammonium molybdate preparation showing the hexagonal pattern structure of the exosporium.

Fig. 26. Section through a mature spore of *C. botulinum* type E, Beluga strain, NCIB4248. Proceeding from the centre of the spore the section shows the dense core, the clear cortex, a thin electron-dense inner spore coat, a multilayered outer spore coat and appendages surrounded by the exosporium.

Fig. 27. *C. botulinum* type E, Beluga strain, NCIB4248. Section showing the relative size of a vegetative cell and a mature spore and its exosporium.

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Studies on the Virulence of Hospital Strains of *Pseudomonas aeruginosa*

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SUMMARY

The virulence of 36 strains of *Pseudomonas aeruginosa* was studied by intravenous injection into mice; the strains were found to fall into three broad groups, of high, medium and low virulence. This difference could not be related to any biochemical property studied, pyocine type nor antibiotic sensitivity pattern. It was found that 24 of the 30 strains of the high and medium virulence group produced large colonies on agar while 5 of the 6 strains of the low virulence group produced small colonies when plated under similar conditions. To test this observation a further series was collected and the strains were allocated to the high or low virulence category on colonial appearance. Mouse challenge with these fresh strains showed that the prediction was accurate. The large colony type had a faster growth rate on agar and in broth than the small. When a strain was grown in agar it was found to be more virulent than when harvested from broth ($P = 0{\cdot}02$–$0{\cdot}01$). Chromatography studies on slime derived from all three categories showed that they were apparently chemically similar. The amount of slime produced by a standard number of bacteria was measured for agar and broth grown cultures. The yields for the two media were approximately the same with large-colony forms. Small-colony forms produced similar amounts to the large forms on plates, but up to 15 times as much when grown in broth.

The ability to kill mice was derived in part from early toxic death; the remainder died of renal disease. Study of the initiation of kidney infection showed that large-colony types were more successful in maintaining their numbers in the kidneys over the first 5 hr. Strains grown on nutrient agar did not give higher counts in the kidney than those from broth in the first 5 hr but after 24 hr they grew faster, infected more kidneys and killed more mice.

INTRODUCTION

The human pathogen *Pseudomonas aeruginosa* (*pyocyanea*) is found frequently in hospital-acquired infections (Darrell & Wahba, 1964; Bassett, Thompson & Page, 1965; Wahba, 1965). Certain sites such as the urinary tract and burnt skin are particularly susceptible, as are patients with lowered resistance and children. *Pseudomonas aeruginosa* infections are difficult to cure owing to their failure to respond to most antibiotic treatments. The epidemiology of *P. aeruginosa* cross-infection has shown that organisms can survive in moist conditions even in the presence of some antiseptics (Rogers, 1960; Emmanouilidou-Arseni & Kommentaleou, 1964; Ayliffe *et al.* 1965). Recent reports have indicated that this organism can survive and multiply in unexpected places such as jet fuel (Bushnell & Hass, 1941; Edwards, 1965) and it has been found in this laboratory in the silicone fluid used as a syringe lubricant. In addition to being

pathogenic for man *P. aeruginosa* is an insect pathogen (Angus, 1965) and a plant pathogen (Bullock, 1965). Members of the genus are known to be involved in plant disease (Paton, 1960) and to contribute to the spoilage of meat, poultry and eggs (Ayres, 1960).

Despite its importance as a human pathogen there is relatively little agreement as to the characters which confer virulence. To study this problem it was proposed to devise an experimental model in which virulence and a number of characters could be related. It is always difficult to produce a successful animal model of human disease but the following argument may be put forward.

Pseudomonas aeruginosa is found in human pyelonephritis (Seneca, Lattimer & Peer, 1964; Ambrose & Hill, 1965) and experimental murine pyelonephritis can be produced in the laboratory (Gorrill, 1965; Gorrill, Klyhn & McNeil, 1966), hence experimental infection in mice would be not too far removed from the human disease pattern. Earlier work (Gorrill, 1952) had shown that strains derived from different sorts of lesions were efficient at producing mouse-kidney infection, so it would be possible to compare in this model a number of isolates made from a variety of hospital infections.

One of the fractions believed to be important in the virulence of *Pseudomonas aeruginosa* is the outer slime layer (Liu, Abe & Bates, 1961; Liu & Mercer, 1963; Callahan, Beyerlein & Mull, 1964; Brown & Lowbury, 1965). Much less often strains appear to be encapsulated and they too are virulent (Çetin, Töreci & Auğ, 1965). In one strain isolated from a case of cystic fibrosis it was found that the mucoid fraction was different from that obtained from strains isolated from other conditions. Hence not only may slime be important in virulence but its composition may influence or be influenced by the type of disease produced (Doggett, Harrison & Wallis, 1964; Doggett, Harrison, Stillwell & Wallis, 1965). It was proposed to investigate the relationship between mouse virulence and other characters of *P. aeruginosa* isolated from clinical cases with particular reference to the role of slime, and the amounts and composition of this fraction in different strains would be compared.

METHODS

Organisms. Pseudomonas aeruginosa strains were received from the Clinical Pathology Department, Guy's Hospital, derived from infected wounds, sputa, tracheal suckings and urines; 50 strains were initially collected. Later a further 40 were used to verify the findings on colony size and virulence. The cultures were maintained on meat digest agar slopes (Southern Group Laboratories, London), and the medium used throughout for culture unless otherwise stated.

Morphology. On isolation the colonial appearance, pigment production (Wahba & Darrell, 1965) motility and Gram reaction were recorded. Capsule and slime stains were also made (Duguid, 1951; McKinney, 1953).

Biochemical reactions. The following tests were made; (*a*) oxidase by Kovacs method (1956); (*b*) fermentation versus oxidation of carbohydrates (Hugh & Leifson, 1953); (*c*) gluconate test (Hayes, 1951); (*d*) growth at 37° and 41° (Hayes, 1951); (*e*) growth on 1 % triphenyl tetrazolium chloride and 0·2 % cadmium sulphate (Wahba, 1965); (*f*) Wahba's modification (1965) of Oakley, Warrack & van Heyningen (1946) test for collagenase; (*g*) urease by Christensen's method (1946); (*h*) haemolysis of horse blood was tested by growing the cultures on 5 % horse blood agar plates;

(*i*) antibiotic sensitivity patterns 3 hr static cultures were used to flood plates of sensitivity test agar (Oxoid Ltd., London). Excess culture was removed and either Oxoid Multodisks (11–14 D) or Evans Sentest high potency tablets applied. The results were read after overnight incubation at 37°.

Pyocine typing. A modification of the method of Darrell & Wahba (1964) was used. The test strain was streaked across a 10 in. (25·4 cm.) plate of Tryptone Soya Agar (Oxoid) containing 5 % (v/v) horse blood and then incubated for 14 hr at 32–34°. The bacteria were then killed with chloroform vapour and scraped off with a microscope slide. Narrow streaks of 4 hr cultures of the 12 indicator strains were then printed on the plate at right angles to the test strain. After incubation for 8–18 hr at 37° the inhibition patterns were read. The indicator strains were printed on to the plate by using 12 stainless-steel blades held in a Perspex block; the blades were charged by dipping into 12 wells cut in a Perspex block $5 \times 3{\cdot}5 \times 0{\cdot}75$ in. ($12.7 \times 8{\cdot}9 \times 1{\cdot}9$ cm.). Each well contained one 4 hr culture of an indicator strain. This machine, described by Wahba & Lidwell (1963) was made in the workshop of Guy's Hospital Medical School.

Animal experiments. LD 50 values were obtained by the methods reported by Gorrill & De Navasquez (1964) and Gorrill (1965), using 6 mice for each dose. The LD 50 values were calculated by the method of Reed & Muench (1938). Since cultures grown in broth are reported to contain little extracellular slime (Doggett *et al.* 1964; Liu *et al.* 1961) it was decided to prepare some strains for intravenous challenge from organisms grown on nutrient agar plates. Dilutions of *Pseudomonas aeruginosa* containing between 50–100 organisms were spread over meat digest agar plates and incubated overnight. Next morning the colonies were washed off with sterile normal saline.

In some experiments the number of bacteria found in the kidney at different times after injection was studied as described by Gorrill (1965).

Chromatography. Chromatography of sugars was done on the extracellular slime layer of the first 36 strains collected. This group included representatives of high, medium and low virulence. Slight modifications were made to the earlier description of this method (Doggett *et al.* 1964, 1965). Digest agar medium was used throughout, the organisms were washed off with distilled water and the slime separated by shaking the suspension with Ballotini beads for 10 min. The extraction of the slime from the supernatant fluid and the preparation of samples for chromatography were as described by Doggett *et al.* (1964).

Cell-wall preparations were autolysed so that their sugars could be compared with those of the slime layer. The bacteria were harvested from digest agar plates, with distilled water, the slime layer removed and the bacteria washed by centrifugation with distilled water until the supernatant fluid was clear. The deposit of bacteria (the yield from a 10 in. (25·4 cm.) plate) was suspended in 10 ml. of boiling distilled water to inactivate any cell-wall-degrading enzymes and also to prevent the formation of an aerosol of living organisms later in the experiment (Salton, 1964). The killed bacteria were then treated with an M.S.E. (Spencer St., London) Ultrasonic Power unit at 1·5 A for 10 min. using a 0·75 in. (1·9 cm.) probe. The cell-wall deposit was washed twice with 10 ml. distilled water and then treated in the same way as the slime fraction. In both cases the chromatograms were run for 16–17 hr, rather than the 20 hr used by Doggett *et al.* (1964), since it was found that some of the components ran off the paper in the longer period.

Slime precipitation. The slime material from several strains was extracted in the same way as described under chromatography techniques. It was collected from known numbers of broth-grown or nutrient agar-grown bacteria and then precipitated, dried overnight and weighed.

RESULTS

Morphology

Considering the initial 50 strains all were found to be motile and Gram-negative. The colonies produced pigment (pyocyanin) on one or more solid media. The majority of strains were of the S and SR types, only two being of R type (Wahba, 1965). However, in our hands there were some differences from the classification of Wahba. The S type was described as being 2–3 mm. irregular shape, effuse elevation with a lobate edge. The SR were smaller, 1–2 mm. diam., circular, of convex elevation and entire edge. The R type were 2–3 mm. diam., of irregular shape, with a raised centre and an undulate edge. When our S strains were plated on digest medium agar to produce 20–60 colonies/plate, the colonies were 9–10 mm. diam. and the SR colonies 2–3 mm. None of the 50 primary isolates showed mucoid, gelatinous or dwarf forms, but on subculture some strains did dissociate (Zierdt & Schmidt, 1964).

With all the strains tested it was noticed that when homogenates of infected kidneys were spread on digest agar the colonial type was always of the dwarf form. However, subculture from these colonies restored the original colonial appearance. Suspensions of liver and spleen and red blood cells also had this effect. Examination of this phenomenon yielded the following observations. The haemoglobin present in the organs appeared to be responsible for the inhibitory effect, which was bacteriostatic rather than bactericidal. Haematin did not have this effect, while serum and plasma annulled the inhibitory effect when excess of these materials was incorporated in the plates.

The effect of the haemoglobin was only seen when it was spread as a surface layer in contact with the dividing bacteria. Blood agar plates were not inhibitory. When the haemoglobin was spread 1 hr or more before the plates were inoculated the inhibitory effect was enhanced. When plates containing a thick layer of agar (i.e. more than 1 cm.) were used the inhibitory effect was less marked. Various chemicals ($MgSO_4$, $MnSO_4$, NaCl and $FeSO_4$) overcame the inhibitory effect to various degrees.

These observations, together, suggest the binding of a nutritional factor. This effect was only seen under a rather limited range of conditions.

By the capsule and slime stains used we were unable to show capsules in our 50 strains and there was doubt about the presence of a slime layer. The method of McKinny (1953) showed, with some strains, organisms held together in strands, possible bound with slime. Unfortunately the original dye used by McKinny was no longer available and the nearest substitute (8 G × 300 I.C.I.) had to be used.

Biochemical reactions

All strains were oxidase-positive, oxidized carbohydrates and gluconate and produced slime, grew at 37° and 41° and grew on media containing tetrazolium and cadmium sulphate. Of 50 strains 6 were collagenase-negative and 35 urease-negative; only 14 produced α-haemolysin on horse blood agar. All strains were resistant to penicillin, erythromycin, oleandomycin and methicillin, and all were sensitive to

colistin. The results were variable with streptomycin, tetracycline, chloramphenicol, sulphonamides and novobiocin.

Pyocine typing

The first 50 strains were distributed among 6 types: 17 in group A, 16 in group B, 8 in group D, 3 in group K, 2 in group F, 2 in group L, and 2 were untypeable.

Animal experiments

The first 36 strains of *Pseudomonas aeruginosa* were tested for virulence by intravenous challenge of mice with broth-grown cultures and the LD50 dose calculated from the deaths which occurred during the first 14 days. Twenty strains killed in doses between 4×10^6 and $2{\cdot}8 \times 10^7$; 10 strains in doses between 3×10^7 and 7×10^7; 6 strains in doses between 1×10^8 and 4×10^8. These three groups were referred to as being of high, medium and low virulence. While this grouping is to some extent arbitrary, it was noticed that 24 out of 30 strains of the high and medium group had colonies of the 'S' type while with the low virulence group 5 out of 6 strains produced SR colonies. A further 40 strains of *P. aeruginosa* were collected and plated to give optimum colony formation: 33 were 'S' type and 7 SR. Seven S and 7 SR strains were taken and used for mouse challenge. Six of S strains were found to be of high to medium virulence, 6 of 7 SR strains were of low virulence. Hence it appears that it was possible to predict mouse virulence from colony appearance.

The growth rates of these two types were different in broth, the 'S' strains being the faster. These experiments were then repeated with nutrient agar-grown bacteria. At intervals after inoculation the plates were washed clear of bacteria and their numbers counted. The growth rates again were different, the S forms growing faster.

Of the 36 strains grown in broth and tested for mouse virulence the first 18 were then grown on nutrient agar plates, harvested and used for mouse challenge. The results are given in Table 1 where it will be seen that 16 strains killed mice with a dose of fewer bacteria derived from agar cultures; one strain was the same; and one strain more virulent when grown in broth. This latter strain was not consistent in colonial morphology, dissociating frequently. Taking all 18 strains and comparing the virulence by the '*t*' test on the log values of the LD50 (Gorrill, 1963) *P* was between 0·02 and 0·01, suggesting a significant difference between the organisms grown in the two media.

When the fate of the bacteria which arrived at the kidney was studied it was found, as reported earlier (Gorrill *et al.* 1966), that there was a marked decrease in numbers from the initial implant. However, when the S and SR strains were compared it was found that the decrease was much greater with the SR form. It was not possible to detect any difference between the strains grown in broth or agar in the initial clearance period. However, after 24 hr the number of nutrient agar-grown bacteria began to increase faster than with the broth-grown bacteria, and the former produced more infected kidneys and killed more mice. In view of the work which suggests that slime may be of importance in the virulence of *Pseudomonas aeruginosa* (Liu *et al.* 1961; Callahan *et al.* 1964) it was considered possible that the difference between broth-grown and nutrient agar-grown strains might be due to differences in the amount of slime material produced under the different cultural conditions.

Slime precipitation

Sixteen of the 18 strains of *Pseudomonas aeruginosa* used for mouse challenge after harvesting from nutrient agar plates were taken and the amount of slime produced by 10^{10} bacteria harvested from nutrient agar was compared with the amount produced by a similar number of bacteria harvested from broth culture. With the S-type colonies an average broth culture yielded 9·4 mg. slime and nutrient agar 6·6 mg.; the SR-type

Table 1. *The $\log_{10}$ LD50 values of 18 cultures of Pseudomonas aeruginosa (strains) grown in broth or nutrient agar, and results of the 't' test: there were both 'S' and 'SR' colony types among the 18 strains.*

	Broth-grown organisms	Nutrient agar-grown organisms
	7·6021	7·2553
	8·0000	7·3979
	8.2041	7·3424
	7·3617	6·9031
	7·6021	7·1139
	7·2553	7·0000
	7·3010	7·0000
	7·4472	7·0000
	7·0000	7·0792
	7·3424	7·0000
	7·3434	7·3010
	6·6021	6·9031
	8·5563	8·3617
	7·7782	7·6990
	7·2041	6·9542
	7·7404	7·3979
	7·6021	7·7404
	7·3979	6·8751
N =	18	18
Mean ($\bar{x}$) =	7·5188	7·1847
$S(x-\bar{x})^2$ =	2·9	2·2

$$t = \frac{\bar{x}_1 - \bar{x}_2}{S\sqrt{([1/N_1]+[1/N_2])}},$$

where $$S = \sqrt{\frac{S(x_1-\bar{x}_1)^2+S(x_2-\bar{x}_2)^2}{N_1+N_2-2}} = \sqrt{\frac{2\cdot9+2\cdot2}{34}} = \sqrt{0\cdot15},$$

$$t = \frac{7\cdot5188-7\cdot1847}{0\cdot3872\times\sqrt{0\cdot1111}} = \frac{0\cdot3341}{0\cdot1290} = 2\cdot59 \quad 34 \text{ degrees of freedom.}$$

P lies between 0·02 and 0·01

colonies broth yielded 74·4 mg. of slime and the agar-grown bacteria 5·1 mg. The agar-grown bacteria were very similar but the results with broth cultures were unexpected: the SR-forms yielded about 8–10 times as much slime as was expected. It appears either that the SR forms produced more slime, or that slime was much more easily detached from the bacteria during harvesting.

Chromatography

The sugars detected in the extracellular slime of 36 strains of *Pseudomonas aeruginosa* in which the LD 50 values of broth-grown organisms had been measured were galactose, glucose, mannose and fucose, thus confirming the results of Doggett *et al.* (1965). Two other sugars were found which corresponded on chromatograms with controls of ribose and rhamnose; these two sugars were found when the chromatogram was run for only 17 hr. Two hexosamines were also found and provisionally identified as galactosamine and glucosamine. All the slime extracts were prepared in the same way, but there were differences in the intensity of the spot colours, possible indicating differences in quantity between strains. However these differences were shared equally between strains of high and low virulence. In the analysis of cell wall extracts of *P. aeruginosa* glucose, fucose and ribose were found; Salton (1964) reported glucose, fucose and rhamnose.

DISCUSSION

The mouse model for the study of the virulence of *Pseudomonas aeruginosa* suffers from the defect that many deaths are due to bacterial endotoxaemia. The stage of pyelonephritis and cystitis leading to renal failure which occurs later is more easily compared with the disease in man. Unfortunately it is not possible to separate the two effects and produce an infection in which death is due solely to renal failure. Despite its gloomy record *P. aeruginosa* infections in man are rarely rapidly fatal with endotoxaemia, and it would be much better for experimental work if one could produce an infection more like that seen in man. The latter part of the experimental infection with pus formation, abscesses and scarring is much more comparable. The main problem is whether one may take the LD 50 dose calculated over the whole period as being a good guide to the severity of the pyelonephritis produced. This seems reasonable since the strains which killed in small doses with smaller doses produced a higher incidence of pyelonephritis than those which killed in large doses. The ability of *P. aeruginosa* to remain in the kidney and grow to large numbers is a good guide to a strain's ability to produce pyelonephritis.

After injection into mice the large-colony virulent type was cleared less efficiently than the small-colony type and grew more rapidly to destroy the kidney. The differences shown by statistical analysis between nutrient agar-grown and broth-grown organisms did not show in the early period, but after 24 hr the difference became apparent, at first in the rate of increase in bacterial numbers, later in the number of kidneys infected and then destroyed, with subsequent death of the mouse.

Attempts to relate the differences in virulence between *Pseudomonas aeruginosa* strains, or between the same strain grown in broth or on nutrient agar, to slime formation, were unsuccessful. The composition of slime seemed the same for all strains tested and differences in intensity of the colour spots on the chromatogram were distributed at random amongst the high-, medium- and low-virulence strains. The estimates of the quantity of slime produced in broth and on agar which showed an excess production in broth-grown small-colony type (SR) organisms, the least virulent form tested, reflect the ease with which slime is detached from the SR bacteria, which would leave them deficient in slime. The other characters investigated were also unhelpful in predicting mouse virulence. However, there was a good correlation between

colony size and virulence in mice as shown by LD 50 values, renal clearance rates and incidence of pyelonephritis in the survivors. This appeared to reflect the difference in growth rate between large-colony and small-colony forms. It was not possible to show a difference in the growth rates of the same bacteria grown in broth and on nutrient agar, but as the animal experiments showed, this difference was not easy to demonstrate during a few hours and was probably within the limit of the error of counting.

The inhibitory effect of organ extracts and red blood cells on the colony size of *Pseudomonas aeruginosa* was apparently a local effect since it was lost on subculture into organ-free media. The findings seem to point to a nutritional effect, possibly the binding of an essential nutrient in the upper layer of the agar by the haemoglobin molecule.

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Chemical Composition of Hyphal Wall of Phycomycetes

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SUMMARY

Isolated hyphal walls from *Phytophthora heveae*, *Pythium butleri* and *Saprolegnia ferax* were hydrolysed and compared in quantitative composition. A high carbohydrate content was found in all these walls (80–90%); also a small amount of glucosamine (1–2%), lipids (1–2·5%) and protein. Quantitatively the cell walls differed appreciably in protein (1–11%). Five neutral sugars (glucose, galactose, mannose, rhamnose, ribose) were detected by chromatography; glucose was always the main monosaccharide. From the results of acid-extraction of walls and enzymic hydrolysis it was concluded that a portion of cell-wall polysaccharide was cellulose (30–45%). The amino acids found were those typical of normal protein hydrolysates and containing also hydroxyproline.

INTRODUCTION

Nabel (1939) found cellulose in cell walls of Pythium species by using microchemical methods. The presence of cellulose in cell walls of other Phycomycetes (Saprolegnia and Phytophthora species) was reported by Frey (1950). Crook & Johnston (1962) made studies on cell walls of *Saprolegnia ferax* and *Phytophthora cactorum* and compared the compositions with those of other fungi and yeasts. Quantitative results on the chemical composition of cell walls of Phycomycetes have been reported recently by Bartnicki-García (1966) for *Phytophthora parasitica* and *P. cinnamomi* and by Mitchell & Sabar (1966) for two Pythium species.

Bartnicki-García & Lippman (1966) obtained protoplasts from two Phytophthora species by using a crude preparation of cellulase from a *Streptomyces* sp. In an attempt to obtain protoplasts of *Phytophthora heveae*, *Pythium butleri* and *Saprolegnia ferax* we have used a preparation of cellulase from *Trichoderma lignorum*. We have found occasionally that vacuoles are liberated but there is always a thin layer of cell wall which prevented the liberation of protoplasts. Our enzyme complex has protease and $\beta(1 \rightarrow 3)$glucanase activities, and hydrolyses crystalline cellulose and carboxymethyl-cellulose (Nicolás, 1965: Villanueva, 1966). Our finding that the cell walls of our Phycomycetes were not completely hydrolysed by our enzyme preparations strongly suggested that some components other than that cellulose were present in the cell walls. To examine this possibility we have studied the chemical composition of *P. heveae*, *P. butleri* and *S. ferax*; the results obtained are reported in this paper.

METHODS

Organisms and growth conditions. The three organisms of which cell walls were studied were *Phytophthora heveae*, *Pythium butleri* and *Saprolegnia ferax* kindly given to us by Commonwealth Mycological Institute, Kew, England. Stock cultures were maintained on agar slopes containing a medium of glucose and potato.

The organisms were grown in the medium GAE which consists of two solutions A and B. Solution A: glucose, 10 g.; asparagine, 1 g.; Difco yeast extract, 0·5 g.; water, 200 ml. This solution was autoclaved separately, three times for 30 min. at 100°. Solution B: K_2HPO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; $FeSO_4.5H_2O$, 0·01 g.; water, 800 ml. This solution was first adjusted to pH 7·2 with NaOH and then autoclaved at 120° for 30 min. Solution B (800 ml.) was added to solution A (200 ml.).

A suspension of mycelium obtained directly from a slope was used to inoculate 750 ml. of GAE medium contained in 3000 ml. conical flasks. Cultures were incubated on a reciprocating shaker for 72 hr at 28°. Organism was harvested by filtration through a sintered glass-filter, washed well with distilled water and stored at 4°.

Preparation of cell-wall material. Various assays were made to select the best methods for breakage of each organism.

Pythium butleri mycelium (2 g. wet wt) was homogenized in a Waring blender with 100 ml. distilled water for 1·5 min. The resulting suspension was passed through a Ribi Cell fractionator (Sorvall) under a pressure of 35,000 lb/sq. in. The organism was centrifuged at 800*g* for 3 min. and the supernatant fluid, consisting mainly of cytoplasmic debris, discarded. The 'cell-wall' fraction was washed five times with distilled water and centrifuged down at 800*g*. To remove remaining impurities from the cell walls the suspensions were again disintegrated as described above, centrifuged at 800*g* for 3 min. and the supernatant fluids discarded. The cell walls were washed five times with 1% (w/v) NaCl and finally five times with water by centrifugation at 800*g*.

Phytophthora heveae (1–2 g. wet wt) was placed in the cup of a Braun cell homogenizer using no. 12 ballotini beads and treated for 7 min. at full speed. The supernatant fluids were separated and the beads washed several times with M-sucrose solution. These washings were added to the first supernatant fluids containing the cell-wall fraction, and the cell walls recovered by centrifugation for 5 min. at 800*g*. The pellet was washed five times with 10% (w/v) sucrose and finally four or five times with water. As this procedure was not effective enough for breakage a further disintegration for 5 min. was done; cytoplasmic debris were removed by centrifugation twice with 10% (w/v) sucrose, then water, 1% (w/v) NaCl and finally water.

Saprolegnia ferax mycelium (1 g. wet wt) was suspended in 13 ml. water and broken by treatment in a MSE ultrasonic disintegrator for 6–7 hr at 1·5 A. In some cases the disintegration was prolonged up to 10 hr to obtain complete breakage of vacuoles. After treatment cytoplasmic debris were removed by centrifugation at 800*g* for 3 min. and the cell walls were washed five times in 1% (w/v) NaCl and five times in water. The cell walls preparations were lyophilized and stored over P_2O_5 at 20°.

The course of breakage and purification of cell walls were followed by phase-contrast microscopy. The examination in the electron microscope of the isolated walls showed that they were practically free from cytoplasmic material.

Chemical analyses

Amino sugars. Three types of hydrolysates were prepared in sealed tubes at 105° with 2 N-HCl for 4 hr, 4 N-HCl for 18 hr, and 6 N-HCl for 24 hr. The HCl was removed in vacuum over NaOH pellets. The residue was dissolved in warm water and any insoluble material was separated by centrifugation. Neutral sugars were removed by passage of the hydrolysate through column of Dowex 50 (H^+) resin and elution with M-NH_4OH. The total hexosamine content was estimated by the method of Rondle & Morgan (1955). Amino sugars were resolved by one-dimensional paper chromatography (Whatman no. 1) with *n*-butanol + pyridine + 0·1 N-HCl (5 + 3 + 2 by vol.) as solvent. Hexosamines were located by using a modification of the Elson–Morgan reagent (Partridge, 1948).

Protein. The protein content of the wall preparations was estimated by three methods: (*a*) By the method of Lowry, Rosebrough, Far & Randall (1951), performed on cold N-KOH extracts of walls, with a standard curve obtained for serum albumin. (*b*) By the ninhydrin method (Moore & Stein, 1954) in 6 N-HCl hydrolysates, with leucine as standard. With the reactive ninhydrin-hydrindantine hexosamine yields a colour similar to that of amino acids. The interference was overcome in the following way: samples of all wall hydrolysates were used for the reaction of Moore & Stein and other samples for the reaction of Rondle & Morgan, as described above to determinate the hexosamine content in these hydrolysates. The reaction of Moore & Stein was performed on glucosamine to make a standard curve. From the total colour (extinction at 570 mμ) obtained with ninhydrin was subtracted the colour corresponding to amino sugars. (*c*) By the micro-Kjeldahl procedure. From the total nitrogen was subtracted the nitrogen corresponding to the amino sugars.

Amino acids. One to 10 mg. dry wt. cell walls were heated in 0·1–1 ml. 6 N-HCl in sealed tubes for 24 hr at 105°. Excess HCl was removed in vacuum over P_2O_5 and NaOH. Whatman paper no. 1 was used for chromatography. One-dimensional chromatograms were run for 18 hr with *n*-butanol + formic acid + water (75 + 15 + 10, by vol.). Arginine, lysine and histidine were eluted with water from chromatograms and separated by electrophoresis on Whatman no. 1 at pH 11·5; 0·5 M-carbonate buffer (750 v; 3 hr), before spraying. In the same way aspartic acid, serine and glycine were separated at pH 2·25 (formic acid + acetic acid buffer) Glutamic acid and threonine were separated at pH 6·5: pyridine + acetic acid + water buffer (100 + 4 + 900, by vol.). Two-dimensional chromatograms were wetted first with phenol + water (80 + 20, by vol.) in the presence of KCN and NH_3; then with *n*-butanol + formic acid + water solvent. To detect the amino acids 0·2% (w/v) of ninhydrin in acetone was used. The intensity of colour and the size of spots of hydrolysate were compared with spots produced by known amounts of amino acids. Cysteine and cystine were detected on chromatograms as cysteic acid after oxidation with molybdate + H_2O_2. To detect proline and hydroxyproline 0·2% (w/v) isatine in 4% (w/v) acetic acid in acetone was used, followed by Ehrlich's reagent for hydroxyproline.

Sugars. The anthrone procedure as described by Chung & Nickerson (1954), with glucose as standard, served to determine the total amount of hexosan present in intact walls. Identification and approximate estimation of monosaccharides was done by paper chromatography of various hydrolysates: N-HCl at 105° for 45 min., 2 hr, 4 hr, 6 hr. Excess HCl was removed in a desiccator over NaOH. The paper chroma-

togram (Whatman no. 1) was run with *n*-butanol + acetone + water (4 + 5 + 1, by vol.) for 24 hr. Sugars were detected with aniline phthalate. Glucose was assayed with a purified glucose oxidase reagent (glucostat 'special' Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.).

Uronic acids. To detect uronic acids, the walls were hydrolysed as described by Gancedo, Gancedo & Asensio (1966). The hydrolysates were chromatographied on Whatman no. 1 paper using ethyl acetate + pyridine + acetic acid + water (5 + 5 + 1 + 3, by vol.; Fischer & Dörfel, 1955). A naphthoresorcinol reagent was used to detect uronic acids.

Lipids. Lipids were isolated by the method of Dr G. Ballesta (personal communication) which involves three extractions. The cell walls were first treated with 80% methanol at 70° for 1 hr. Then, after drying in vacuum the residue was extracted by refluxing with ether, three times, and the extracts combined. This fraction constitutes the lipids of the first extraction. The second extraction was performed with ethanol + ether + HCl (50 + 50 + 1, by vol.), and the third extraction with ethanol + ether + chloroform (10 + 40 + 50, by vol.). The products of the second and third extractions were mixed and this constitutes the lipids of the second fraction.

Phosphorus. Total phosphate was estimated by the method of Fiske & SubbaRow as described by Umbreit, Burris & Stauffer (1957).

RESULTS

Amino sugars. Hexosamines were detected in the three Phycomycetes (Table 1). The greatest hexosamine content was found in hydrolysates with 4 N-HCl at 105° for 18 hr. The drastic hydrolysis 6 N-HCl, 24 hr at 105°, caused 30–35% destruction.

Proteins. The protein content of mycelial walls estimated by the methods described showed important differences for *Saprolegnia ferax* (1–1·8%), whereas similar values were obtained for *Phytophthora heveae* (5–6%) and *Pythium butleri* (11–12%) (Table 1). It is interesting to observe that for the three Phycomycetes the values found with the ninhydrin procedure was smaller than that found with the micro-Kjeldahl procedure. A possible explanation of this finding might be that some destruction of amino acids took place during hydrolysis, especially in presence of sugars. Two-dimensional paper chromatography of cell-wall hydrolysates resolved 17 amino acid (probably 18) spots with variable intensity according to organisms, as shown in Table 2. No attempts were made to separate leucine from isoleucine. *Pythium butleri* contained 5–10% of hydroxyproline, whereas only a small amount was found in *P. heveae* and *S. ferax*. The unknown amino acid spots reported by Crook & Johnston (1962) and Bartnicki-García (1966) with three Phytophthora species were not observed in the present work.

Carbohydrates. The main components (80–90%) of the hyphal wall dry weight of the organisms studied were carbohydrates anthrone positive (glucose as standard; Table 1). Estimation of monosaccharide constituents of wall polysaccharides was done with various types of hydrolysates, but in no case was the sugar recovery complete. Some hydrolysis were insufficient to dissolve the wall completely, whereas in others a part of the wall carbohydrate was destroyed. Paper chromatography of hydrolysates revealed glucose as the main sugar component, and only very small amounts of other hexoses, pentoses and methyl pentoses (sometimes only traces) were detected in the three Phycomycetes (Table 3).

Glucose was further characterized by treating the hydrolysates with purified glucose oxidase. The amount of glucan calculated from the glucose obtained by this method was smaller than the total carbohydrate values obtained by the anthrone procedure performed directly on intact walls. It is interesting that galactose was found in *Pythium butleri*, exclusively in hydrolysates with N-HCl for 45 min. and in *Phytoph-*

Table 1. *Principal components of cell walls of Phytophthora heveae, Pythium butleri and Saprolegnia ferax*

	P. heveae	*P. butleri*	*S. ferax*
	% dry wt. cell wall		
Total-N (micro-Kjeldahl)	1·08	2·02	0·42
Protein (ninhydrin)	4·6	10·7	1·3
Protein (N × 6·25)*	5·6	11·9	1·8
Protein (Lowry)	6·7	11	1·1
Total carbohydrates (anthrone)	90	81	93
Glucosamine (Rondle & Morgan)	2·3	1·3	1·7
Phosphorus (as H_2PO_3)	0·26	0·44	0·36
1st fraction of lipids	1·2	1·8	0·5
2nd fraction of lipids	1·3	0·83	0·5

* (Total-N-hexosamine-N) × 6·25.

Table 2. *Amino acids of cell walls of Phytophthora heveae, Pythium butleri and Saprolegnia ferax*

	Grading of amounts*				
	++++	+++	++	+	Traces
	Amino acid spots				
P. heveae	Ala	Pro, Thr Val Leu-Ileu	Cys, Asp Ser, Gly Glu	Lys, Arg Hypro, Phe Tyr	His Met
P. butleri	Lys, Asp Ser, Thr Ala	Gly, Hypro Pro, Val	Cys, Glu Tyr Leu-Ileu	Arg, Phe	His Met
S. ferax		Arg Glu, Thr Ala, Pro Val Leu-Ileu	Cys, Lys Asp, Ser Gly, Phe	Hypro Tyr	His Met

* Amino acids in percentage: over 10% of wall protein (++++); 5–10% (+++); 2–5% (++); 1–2% (+); less than 1% (traces).

Table 3. *Monosaccharides in cell walls of Phytopththora heveae, Pythium butleri and Saprolegnia ferax*

	Galactose	Glucose	Mannose	Rhamnose	Ribose
	Gradings (%)*				
P. heveae	+	++++++	+	+	+
P. butleri	+	++++++	+	+	—
S. ferax	Traces	++++++	Traces	+	+

* Monosaccharides in percentage: over 80% (+++++), 0·5–1% (+) less than 0·5% (traces)

thora heveae in hydrolysates with N-HCl for 2 hr. It is possible that stronger hydrolysis would destroy galactose.

Some spots with a low R_F appear in hydrolysates assayed in the study of monosaccharides. These spots could be oligosaccharides. To study this possibility we chromatographed the hydrolysates obtained by treatment with N-HCl at 105° for 45 min., 1 and 2 hr using the same solvent described above for sugars but the running time of the solvent was 48 hr. Thus we could detect cellobiose R_G:0·21 and, laminaribiose R_G:0·34. On the other hand, gentibiose R_G:0·11 and laminaritriose R_G:0·09 were not identified by this method, but there was always a spot with R_G 0·10 in the chromatograms. Gentiobiose, laminaritriose, cellobiose and laminaribiose were separated and characterized by chromatography for 36 hr using the same solvent described above to investigate the presence of uronic acids. The R_G values obtained in this system were; gentiobiose 0·56, laminaritriose 0·63, cellobiose 0·67 and laminaribiose 0·83. Small amounts of other oligosaccharides not well resolved appeared in the chromatograms.

Uronic acids were not detected in the special hydrolysates made as described under methods.

Phosphorus. Determination of total phosphorus (as metaphosphate) indicated a low phosphate content (Table 1). The nature of the phosphate components of the cell walls is not known.

Lipids. Small amounts of lipids in the first and second fractions were detected in the walls of the three Phycomycetes studied.

Cell-wall degradation with β-glucanases. Three β-glucanases preparations were used to investigate the presence of glycosidic linkages: β(1 → 3) glucanase, β(1 → 6) glucanase and cellulase. β(1 → 3) glucanase from *Streptomyces* RA was kindly given to us by Dr Rodríguez Aguirre. This preparation had been filtrated through a column of DEAE-cellulose and was free from β(1 → 6) and β(1 → 4) glucanases and β-glucosidase activities. Crude β(1 → 6) glucanase was a gift from Dr E. T. Reese; this preparation was obtained by precipitation with acetone from culture fluids of *Penicillium brefeldianum* QM 1872 and it had high β(1 → 3)-glucanase, β-glucosidase and low β(1 → 4) glucanase activities. A crude preparation of cellulase with a low β-glucosidase activity was obtained by us from culture fluids of *Trichoderma lignorum*. The products of enzymic hydrolysis of walls were investigated by paper chromatography as described above for acid hydrolysates. Controls were used in all these assays, consisting of cell-wall suspensions in buffer and the given enzyme preparation previously boiled for 10 min. Liberation of sugars could not be observed in any of these controls.

Only small amounts of glucose and laminaribiose could be detected when cell walls of *Phytophthora heveae*, *Pythium butleri* and *Saprolegnia ferax* were treated with β(1 → 3) glucanase in 0·05 M-citrate buffer, pH 4·5 at 40°, for 7 or 14 hr. On the other hand, glucose and gentiobiose were obtained by treating the walls with the β(1 → 6) glucanase for 4 hr in a similar way to that described by Reese, Parrish & Mandels (1962). The highest yield of glucose and gentiobiose was obtained from the walls of *S. ferax*. Treatment with the crude preparation of cellulase (2 or 12 hr at 40° in 0·05 M-citrate-phosphate buffer, pH 5·4) was more dramatic and nearly complete hydrolysis of the walls was obtained after 12 hr of treatment; paper chromatograms of 2 hr hydrolysates always revealed high amounts of cellobiose and glucose and a low amount of laminari-

biose. Under identical experimental conditions the amounts of cellobiose and glucose increased in the order: *P. butleri*, *P. heveae* and *S. ferax*. Thus, the enzymic hydrolysis shows also the existence of at least three types of glycosidic linkages: $\beta(1 \rightarrow 3)$, $\beta(1 \rightarrow 4)$ and $\beta(1 \rightarrow 6)$.

Hydrolysis of hyphal walls polysaccharides. The rate of hydrolysis of the cell walls of the three fungi considered in this work was compared with the data obtained in a similar hydrolysis of crystalline cellulose (Whatman cellulose powder) as described by Bartnicki-García (1966). The proportions soluble carbohydrate: dry cell wall are shown in Table 4. Soluble carbohydrate was estimated by the anthrone method after hydrolysis at 105° with N-HCl for different times. It can be concluded that there were at least two different types of glucan in the walls studied. One type of glucan was quickly solubilized by hydrolysis in N-HCl and the other one, like cellulose, was very resistant to hydrolysis. The insoluble material after 2, 4 and 6 hr hydrolysis was soluble in Schweizer's reagent and was precipitated by acidification. From these results it can be concluded that this material is cellulose. The approximate proportions of glucan non-cellulosic in the walls studied can be obtained subtracting the value of hydrolyzed crystalline cellulose after 6 hr from the approximate values of total glucan solubilized during the same time. These values expressed as % dry weight of wall are: *Phytophthora heveae* 54% (36); *Pythium butleri* 58% (23), and *Saprolegnia ferax* 51% (42); (figures in brackets are the corresponding ratio cellulose: dry wall).

Table 4. *Estimation of soluble carbohydrates after hydrolysis of cell walls of Pythium butleri, Phytophthora heveae and Saprolegnia ferax*

Cellulose and cell walls of *P. butleri*, *P. heveae* and *S. ferax* were hydrolysed with N-HCl for different times. Soluble carbohydrate was then estimated by the method of the anthrone (Chung & Nickerson, 1954).

	Time of hydrolysis (hr)						
	0·25	0·50	1	2	4	5	6
	% soluble carbohydrate: dry wall						
Cellulose	0·9	2·0	3·3	4·1	5·5	6·1	6·4
P. butleri	40·1	49·2	55·9	59·4	61·9	63·2	63·7
P. heveae	41·1	49·4	54·2	56·8	58·2	59·0	59·8
S. ferax	40·8	50·2	53·5	54·8	55·2	56·1	57·2

DISCUSSION

Three different methods are described in this paper for obtaining cell walls of *Phytophthora heveae*, *Pythium butleri* and *Saprolegnia ferax*. Only the finding that different methods were required for breakage of each one of these fungi suggested that their cell walls have some differences in their structure and chemical composition. However no significant differences between the cell walls of these fungi were found by electron microscopy. The most significant difference in the chemical composition of the cell walls studied was found in their protein content which follows the pattern: protein in wall of *S. ferax* < *P. heveae* < *P. butleri*. The protein(s) of the walls contained all normal amino acids and hydroxyproline. The presence of this amino acid has been described previously in the walls of *S. ferax* and Phytophthora species (Crook & Johnston, 1962; Bartnicki-García, 1966), higher plants (Lamport & North-

cote, 1960) and algae (Punnet & Derrenbacker, 1966). These findings suggested that hydroxyproline is characteristic of cellulosic cell walls.

The amounts of glucosamine, ribose, rhamnose, mannose and galactose in the cell walls studied were very small and it is difficult to evaluate the structural importance of these components in the wall. These minor components might be important in haptens of the cell walls antigens. Mannose, glucosamine and ribose have been also detected in small amounts by Crook & Johnston (1962) and Bartnicki-García (1966) in walls of different species of cellulosic fungi. The presence of rhamnose and galactose has not been reported in these fungi.

Three different sorts of glycosidic linkages were detected in the cell walls studied by partial hydrolysis with HCl or with three β-glucanases. The low activity of the $\beta(1 \rightarrow 3)$ glucanase on the walls could be due either to the special disposition of $\beta(1 \rightarrow 3)$ glycosidic linkages in the polysaccharide or to the low activity of the partially purified enzyme (Potgieter & Alexander, 1966) or to the existence of only small quantities of $\beta(1 \rightarrow 3)$ glycosidic units. However, the last hypothesis can be discarded due to the laminaribiose and laminaritriose formed in the mild acid hydrolysis and it can be concluded that they constitute an important part of the polysaccharide. On the other hand the only liberation of glucose and gentiobiose by crude $\beta(1 \rightarrow 6)$ glucanase can be due to either a higher activity of the $\beta(1 \rightarrow 6)$ glucanase than of the other glucanases or to a high β-glucosidase activity of our enzyme preparation. If this was the case, gentiobiose might be more resistant to β-glucosidase hydrolysis as the activity of this enzyme differs very much on the oligosaccharide used as substrate (Reese & Mandels, 1963). The liberation of laminaribiose, cellobiose and glucose by crude cellulase preparation was as expected from the enzymic activities shown by our preparation.

The presence of cellulose in the walls of the three fungi was shown by the high proportion of cellobiose liberated by a crude cellulase. It was confirmed by the finding of one sort of glucan very resistant to HCl hydrolysis, soluble in Schweitzer's reagent and precipitable from this solution by acid. Bartnicki-García (1966) has also detected cellulose, $\beta(1 \rightarrow 3)$ and $\beta(1 \rightarrow 6)$ glycosidic linkages in the walls of Phytophthora species. In addition Mitchell & Sabar (1966) have shown cellulose in the cell walls of *Pythium myriotilum*.

The complete chemical composition and structure of the walls studied is not yet known. However we know that there are at least three sorts of linkages in their polysaccharide(s). The hydrolysis studies showed that there are two sorts of glucans. One of them soluble and the other one insoluble (cellulosic-like) under our conditions of hydrolysis; it is possible that the soluble glucan is linked to the cellulosic-like glucan. On the other hand, the whole wall protein was solubilized under the conditions required to solubilize the soluble glucan; this finding does not discard the possible existence in cellulosic fungal cell walls of a glucan-protein complex, as it has been reported in the walls of yeasts (Korn & Northcote, 1959). More work is needed to clarify these hypotheses and to determinate the number and disposition of $\beta(1 \rightarrow 3)$, $\beta(1 \rightarrow 6)$ and $\beta(1 \rightarrow 4)$ glycosidic linkages.

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Growth and Characterization of Nocardiophages for *Nocardia canicruria* and *Nocardia erythropolis* Mating Types

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SUMMARY

Two nocardiophages were isolated from soil samples: one, called ϕC, was specific for the *Nocardia canicruria* mating type; the other, called ϕEC, proliferated in *N. canicruria* and *N. erythropolis* mating types. Inactivation of phage ϕC was rapid in NaCl solutions, with chloroform, 0·3% hydrogen peroxide and 30% (v/v) ethanol in water. Phage ϕEC behaved similarly but was less labile in NaCl solutions. Both phages were resistant to diethyl ether. Phage ϕC could be propagated to titres of 10^{10} plaque-forming units/ml. on a medium containing peptone, yeast extract and calcium nitrate; the highest observed titres of phage ϕEC were also produced in this medium. A chemically defined medium for proliferation of phage ϕC consisted of inorganic salts, valine, isoleucine, leucine, nicotinamide, glycerol. Other variables which affected the growth of these phages were the amount of phage and host inocula and the age of the host before infection. Phage ϕC was distinct from other nocardiophages in its ability to attach quickly and efficiently to its host; 95% of the phage attached, and attachment was time and temperature dependent. Attachment of phage ϕEC was slow and inefficient, reaching only 34%. The latent period for phage ϕC was about 25 min., the increase period was 35 min. and the burst size about 60 particles/infective centre. The latent period of phage ϕEC was 180 min. and the burst size about 20 particles. Both these nocardiophages possessed the typical actinophage morphology.

INTRODUCTION

Genetic recombination between nocardias of heterologous origin has been demonstrated (Adams, 1964). In general, substrains derived from *Nocardia erythropolis* do not recombine with each other nor will substrains derived from *N. canicruria*, but 'interspecific' combinations are fertile. These observations indicate a mating factor(s) governing compatibility in nocardial recombination. In further investigations of hereditary phenomena in nocardias, characters have been sought for use in the development of linkage maps. Specific phage-resistance characters are deemed appropriate for such purposes.

Two nocardiophages were isolated from soil. One (phage ϕC) specifically infected the *N. canicruria* mating type while the second (phage ϕEC) infected both the *N. canicruria* and *N. erythropolis* mating types. Although mating-type specific phages have been described for *Escherichia coli* (Loeb, 1960; Dettori, Maccacaro & Piccinin,

1961), they are largely unknown for other bacterial systems. To exploit nocardiophages as genetic markers and to ascertain their exact relationships to mating type, it has been necessary to grow and characterize them. In this report, the conditions necessary for propagating and assaying two nocardiophages are defined, thereby allowing for their further characterization. Special attention has been given phage ϕC because of its potential application in studies of compatibility phenomena in the nocardias.

METHODS

Cultures. The hosts used were *Nocardia canicruria* strain 3 (Adams, 1964) and *N. canicruria* strain 57 (Anderson & Bradley, 1961). These strains were originally designated *Jensenia canicruria* (Bisset & Moore, 1950). *Nocardia erythropolis* strain 2 (Adams, 1964) was also used. Stock cultures were maintained on nutrient agar (Difco) or on peptone + yeast extract (Difco) medium (PY) composed of 0·5% (w/v) peptone and 0·3% (w/v) yeast extract solidified, when necessary, with 1·5% (w/v) agar. Cultures were maintained at 30°.

Plaque assay medium and phage propagation medium. The plaque-forming capacities of lysates containing phage ϕC or phage ϕEC were determined by the conventional soft-agar overlay technique (Jones & Bradley, 1962) with nutrient agar or PY agar as the nutrient. In some tests, the assay media were supplemented with 0·01 M-NaCl, 0·1 M-NaCl, or 0·01 M-$Ca(NO_3)_2$. Plaque numbers were not appreciably affected by supplementation and PY agar containing 0·01 M-$Ca(NO_3)_2$ was arbitrarily chosen as the routine plaque assay medium. To determine the effects of salts on nocardiophage growth, nutrient broth or PY broth supplemented with NaCl or $Ca(NO_3)_2$ was inoculated with *Nocardia canicruria* to give an extinction of 0·1 at 620 mμ. After incubation for 2 hr at 30°, phage ϕC or phage ϕEC was added to a final concentration of 1×10^7 plaque-forming units (p.f.u.)/ml. The phage-infected cultures were assayed for increase in p.f.u. values after incubation for 4 or 12 hr.

Determination of nocardiophage stability. To discover suitable diluents for phages ϕC and ϕEC, 0·1 ml. samples of stock phage preparations were added to various diluents and maintained at 25° for 2 hr. The stability of phages ϕC and ϕEC to materials commonly lethal for other bacteriophages was tested by incubating mixtures of nocardiophage with different reagents at 25° for 2 hr.

Determination of factors which affect nocardiophage attachment. Several factors were tested for their effect upon attachment of nocardiophage. A 2 hr culture of *Nocardia canicruria* was harvested and resuspended in different media to an extinction of 0·4 at 620 mμ. Phage was added and 5 min. later the mixture was sampled, diluted into 4 volumes of broth, centrifuged and the titre of the supernatant fluid determined. Attachment was expressed as the ratio of free phage particles present in the supernatant fluid to the total phage count. Alternatively, a sample of the whole culture was shaken with an equal volume of diethyl ether and the titre of the aqueous layer measured. In this case, attachment was expressed as the ratio of the number of phage particles present in the aqueous phase of the ether-treated culture to that of the untreated sample. Another method used to measure phage to host attachment involved the addition of specific antiphage serum (Bradley, Papermaster, Watson & Good, 1961). Phage able to form plaques after the addition of antiserum was considered to be attached to the host.

Effect of host age and inoculum sizes on phage propagation. The effect of host age on phage propagation was tested by adding suitable concentrations of phage to the host which had been incubated for various times. Twelve hr after the addition of phage, the phage titre was determined. The effect of the size of the phage inoculum on propagation was examined by mixing phage inocula at different concentrations with a standard host concentration and measuring the p.f.u. after incubation for 3 hr and 24 hr. The effect of the host-inoculum size on phage proliferation was determined by varying the host inoculum concentration and assaying plaque numbers.

Propagation dynamics. A 2 hr host culture having an extinction of 0·1 at 620 mμ in PY broth supplemented with 0·01 M-$Ca(NO_3)_2$ was inoculated with phage ϕC, 5×10^8 p.f.u./ml. or phage ϕEC, $1{\cdot}9 \times 10^6$ p.f.u./ml. At intervals, after allowing 10 min. for adsorption, samples were removed, diluted in fresh medium and assayed for plaque numbers.

Minimal medium. A 3 hr culture of *Nocardia canicruria* was harvested and resuspended in 0·001 M-$Ca(NO_3)_2$ (pH 7) to an extinction of 0·2 at 620 mμ. To this suspension various supplements were added and phage ϕC added to obtain $2{\cdot}5 \times 10^6$ p.f.u./ml. in the mixture. After incubation for 3 hr at 30°, the mixtures were assayed for plaque numbers.

Host range. The host ranges of phages ϕC and ϕEC were determined using methods described previously (Anderson & Bradley, 1961). The host ranges of ϕC and ϕEC were compared to the host range of phage MJP1. This phage, MJP1, was isolated on *Nocardia canicruria* and its host range previously described (Anderson & Bradley, 1961).

Morphology. The structural features of phages ϕC and ϕEC were determined by examining negatively-stained preparations with a Siemen's electron microscope according to the methods of Painter & Bradley (1965).

RESULTS

Although the nutrient and salt composition of the plaque assay medium did not markedly affect the titres of phages ϕC and ϕEC, the composition of the propagation medium was critical. Peptone+yeast extract broth was the best routine nutrient source (Table 1). The addition of $Ca(NO_3)_2$ stimulated phage production but 0·1 M-NaCl was deleterious. The highest routine phage titres were obtained in PY broth supplemented with 0·01 M-$Ca(NO_3)_2$.

Phage ϕC was rapidly inactivated in many salt solutions (Table 2); it was susceptible to M-NaCl, but was stable for 2 hr at 25° in 0·01 M-$Ca(NO_3)_2$, $MgSO_4$ or tris buffer, and in nutrient broth or PY broth at pH 7. Phage ϕEC followed the stability pattern of phage ϕC except that ϕEC was somewhat more stable in NaCl solutions.

The two phages were similar in their patterns of resistance to deleterious agents (Table 3). Chloroform and thymol (frequently used to render phage lysates free from living bacteria) decreased the titre of phage ϕC 10,000-fold; chloroform was somewhat less lethal to phage ϕEC, decreasing the titre only 100-fold. Both phages were rapidly inactivated by ultraviolet irradiation.

Table 1. *Complex medium for propagation of nocardiophages φC and φEC*

Nocardia canicruria, at an extinction of 0·1 at 620 mμ, was incubated for 2 hr at 30°. After host incubation, phages φC or φEC were added to give a final phage concentration of 10^7 p.f.u./ml. After 4 or 12 hr further incubation, plaque numbers were determined.

Medium	Supplement	φC (p.f.u./ml.) 4 hr	φC (p.f.u./ml.) 12 hr	φEC (p.f.u./ml.) 12 hr
Nutrient broth	—	$2·0 \times 10^7$	$3·3 \times 10^8$	$2·0 \times 10^9$
	0·1 M-NaCl	$< 1 \times 10^5$	$< 1 \times 10^5$	$3·0 \times 10^3$
	0·01 M-NaCl	$1·8 \times 10^7$	$4·4 \times 10^8$	$3·0 \times 10^6$
	0·01 M-$Ca(NO_3)_2$	$3·5 \times 10^9$	$5·3 \times 10^9$	$1·8 \times 10^8$
	0·001 M-$Ca(NO_3)_2$	$3·9 \times 10^9$	$6·4 \times 10^9$	$6·9 \times 10^9$
	—	$4·4 \times 10^8$	$5·1 \times 10^9$	$2·3 \times 10^9$
PY broth	0·1 M-NaCl	$< 1 \times 10^5$	$< 1 \times 10^5$	$1·0 \times 10^4$
	0·01 M-NaCl	$4·7 \times 10^8$	$3·9 \times 10^9$	$9·0 \times 10^7$
	0·01 M-$Ca(NO_3)_2$	$8·2 \times 10^9$	$1·6 \times 10^{10}$	$3·3 \times 10^9$
	0·001 M-$Ca(NO_3)_2$	$1·6 \times 10^{10}$	$9·0 \times 10^9$	$2·4 \times 10^9$

Table 2. *Diluents for nocardiophages φC and φEC*

A 0·1 ml. sample of stock preparations of phages φC or φEC was added to 10 ml. of the diluent to be tested. All solutions were previously adjusted to pH 7·0. After 2 hr incubation at 25°, the diluent-phage mixtures were assayed for viable phage.

Diluent	Phage φC (p.f.u./ml.)	Phage φEC (p.f.u./ml.)
Deionized water	$9·9 \times 10^5$	$9·4 \times 10^5$
NaCl (0·001 to 0·1 M)	$8·6 \times 10^5$	$1·2 \times 10^6$
NaCl (M)	$1·0 \times 10^2$	$6·7 \times 10^5$
PY broth	$2·3 \times 10^6$*	$3·1 \times 10^6$*
Nutrient broth	$3·3 \times 10^6$*	$3·7 \times 10^6$*
$MgSO_4$ (0·001–0·01 M)	$2·7 \times 10^6$*	—
$Ca(NO_3)_2$ (0·001–0·01 M)	$2·4 \times 10^6$*	$1·1 \times 10^6$*
$KH_2PO_4 + K_2HPO_4$ (0·1 M)	$5·1 \times 10^3$	—
$KH_2PO_4 + K_2HPO_4$ (0·01 M)	$5·3 \times 10^5$	—
Tris (0·01 M)	$2·9 \times 10^6$*	—
0·01 M-tris in 0·001 M-$Ca(NO_3)_2$	$2·5 \times 10^6$*	—
0·1 M-NaCl + 0·001 M-$Ca(NO_3)_2$	$2·6 \times 10^6$*	$1·1 \times 10^6$*

* Satisfactory diluent. —, Not done.

Nocardiophage attachment

Attachment of phage φC was markedly affected by medium composition (Table 4). When water was used as the attachment medium, only 5% of phage φC particles were effectively attached; the addition of 0·01 M-Ca^{2+} to PY broth facilitated its attachment. Other media tested were less satisfactory for attachment.

Three methods were used to determine attachment (Table 5). The methods based on the use of antisera or ether sensitivity were equally useful, and indicated slightly greater degrees of attachment than did the centrifugation method.

Attachment of phage φC was retarded at 5° (Table 6). The best attachment of phage φC was at the highest temperature tested (37°). In contrast, phage φEC attached inefficiently; for example, after 30 min. incubation at 30° in PY broth containing 0·01 M-Ca^{2+}, phage φC attached with an efficiency of 95%, whereas phage φEC attached with an efficiency of 34%.

Table 3. *Effects of chemical and physical agents on nocardiophages φC and φEC*

To determine the effects of physical treatments, PY broth grown preparations of phages φC or φEC were diluted 1 to 10 in PY broth. A 0·1 ml. sample was added to 10 ml. PY broth, the physical treatment applied, and viable phage numbers were estimated immediately after the treatment. A similarly diluted but untreated PY broth preparation served as a control. To determine the effects of the chemical agents, PY broth grown preparations of the phages were diluted 1 to 10 in water. A 0·1 ml. sample of the dilution was added to 10 ml. of the reagent to be tested at the specified concentration in water. After 2 hr incubation at 25°, the mixtures were assayed for phage numbers. *Nocardia canicruria* was used as the host in all cases.

Treatment	Phage φC (p.f.u./ml.)	Phage φEC (p.f.u./ml.)
Untreated control	$2{\cdot}7\times10^{8}$	$3{\cdot}7\times10^{6}$
Chloroform*	$1{\cdot}2\times10^{4}$	$1{\cdot}8\times10^{4}$
Thymol (0·1 g./added to 10 ml. water)	$1{\cdot}0\times10^{3}$	—
Diethyl ether†	$1{\cdot}6\times10^{8}$	$3{\cdot}2\times10^{6}$
Hydrogen peroxide (3 %, v/v, in water)	$< 1\times10^{3}$	$< 1\times10^{3}$
Hydrogen peroxide (0·3 %, v/v, in water)	$1{\cdot}6\times10^{4}$	$1{\cdot}5\times10^{3}$
Mercaptoethanol (1·5 M in water)	$9{\cdot}0\times10^{4}$	$5{\cdot}0\times10^{5}$
Mercaptoethanol (0·15 M in water)	$9{\cdot}4\times10^{7}$	$1{\cdot}0\times10^{6}$
Ethanol (30 %, v/v, in water)	$1{\cdot}0\times10^{3}$	$< 1\times10^{3}$
Freezing	$2{\cdot}7\times10^{8}$	$3{\cdot}1\times10^{6}$
45° for 15 min.	$1{\cdot}7\times10^{7}$	$4{\cdot}2\times10^{4}$
56° for 15 min.	$8{\cdot}0\times10^{3}$	$1{\cdot}0\times10^{3}$
Ultrasonic treatment (20 kc./sec.) for 60 sec.	$2{\cdot}4\times10^{6}$	—
French press (4000 lb./sq.in.)	$1{\cdot}7\times10^{6}$	—
Ultraviolet irradiation for 30 sec.	$2{\cdot}2\times10^{6}$	$1{\cdot}0\times10^{4}$
Ultraviolet irradiation for 90 sec.	$1{\cdot}3\times10^{5}$	$1{\cdot}7\times10^{3}$
Ultraviolet irradiation for 150 sec.	$2{\cdot}3\times10^{4}$	$4{\cdot}1\times10^{2}$
Ultraviolet irradiation for 210 sec.	$2{\cdot}3\times10^{2}$	$< 10^{1}$

* Biphasic: 1 vol. chloroform to 2 vol. water. † Biphasic: 1 vol. ether to 3 vol. water. —, Not done.

Table 4. *Total attachment of nocardiophage φC to washed Nocardia canicruria organisms*

A 2 hr culture of *Nocardia canicruria* was harvested and resuspended in the indicated media to an extinction of 0·4 at 620 mμ. Phage was added and after 5 min. to allow for attachment, the mixture was sampled, diluted into 4 vol. of broth, centrifuged and the viable titre of the supernatant fluid was assayed. Alternatively, a whole culture and a sample were shaken with an equal volume of diethyl ether and the phage titre of the aqueous layer was determined.

Attachment medium	Titre × 10^{-3}: Total phage φC	free phage φC	% attached	% ether-sensitive
Water	339	321	5	11
0·1 M-NaCl	96	50	48	50
Nutrient broth	295	138	53	62
0·001 M-$Ca(NO_3)_2$	255	108	58	63
0·01 M-$Ca(NO_3)_2$	281	101	64	63
PY broth	268	115	57	68
PY broth + 0·01 M-$Ca(NO_3)_2$	248	24	90	93
0·1 M-NaCl + 0·1 M-$Ca(NO_3)_2$	170	88	48	56

Table 5. *Comparison of three criteria for measuring attachment of nocardiophage ϕC to Nocardia canicruria cells*

A 2 hr culture of *Nocardia canicruria* was harvested and resuspended in the indicated media to an extinction of 0·4 at 620 mμ. Phage ϕC was added to a concentration of $1{\cdot}9 \times 10^7$ p.f.u./ml. After 10 min., attachment was determined by the indicated criteria.

	Attachment medium	
Attachment index	0·003 M-$Ca(NO_3)_2$	Peptone + yeast extract medium + 0·01 M-$Ca(NO_3)_2$
	% phage attached in	
Ether sensitivity	88·5	99·3
Antiserum resistant	92·1	100
Free phage	80·6	92·4

Table 6. *Effects of time and temperature on attachment of nocardiophage ϕC to Nocardia canicruria*

A culture was equilibrated to the indicated temperature and phage ϕC added to a final concentration of $3{\cdot}3 \times 10^6$ p.f.u./ml. At the indicated times, 1 ml. samples were mixed with 1 ml. of diethyl ether; the aqueous layer was assayed for viable phage.

	Temperature		
	5°	30°	37°
Time (min.)	Free phage (temperature variable)		
0+	$3{\cdot}3 \times 10^6$	$1{\cdot}3 \times 10^8$	$4{\cdot}8 \times 10^5$
2	$5{\cdot}6 \times 10^6$	$1{\cdot}0 \times 10^6$	$1{\cdot}8 \times 10^5$
4	$4{\cdot}3 \times 10^6$	$8{\cdot}5 \times 10^5$	$1{\cdot}5 \times 10^4$
6	$2{\cdot}9 \times 10^6$	$1{\cdot}3 \times 10^5$	$1{\cdot}1 \times 10^4$
8	$2{\cdot}4 \times 10^6$	$2{\cdot}0 \times 10^5$	$6{\cdot}0 \times 10^3$
10	$2{\cdot}6 \times 10^6$	$2{\cdot}1 \times 10^5$	$2{\cdot}5 \times 10^3$

Nocardiophage propagation

From the results of the preceding experiments, it was evident that several factors affected the phage to host interaction. When host age before infection was examined, it was found (Table 7) that young cultures gave the best yields of phage. However, the time at which the phage + host mixture (Table 8) was sampled also determined phage yield. In a 3 hr period, the phage output was directly related to the size of the phage inoculum. With an 18 hr incubation time, the final phage yields were practically identical regardless of the multiplicity of infection. Upon varying the amount of the host (Table 9), an increased yield was observed with an increase of host. These data indicate that a maximum yield of phage was dependent upon the absolute number of host organisms, the growth stage of the host, and ratio of phage to host.

Under standard conditions, with young host cultures, the growth curves of phages ϕC and ϕEC were distinct from each other, but typical of phage growth curves. The latent period for phage ϕC was about 20 min., the increase period was 40 min. and the burst size about 60 particles/infective centre. Phage ϕEC had a latent period extending to 180 min. and the burst size was lower than that for ϕC, approaching only 20 particles/infective centre.

Table 7. *Effect of host age on production of nocardiophages ϕC and ϕEC*

Peptone+yeast extract medium was inoculated with *Nocardia canicruria* to an initial population density of 5×10^7 cells/ml. At intervals, phage was added to a final concentration of 2×10^6 p.f.u./ml. and the mixture incubated for 12 hr. After incubation, the cultures were centrifuged and the supernatant fluid assayed for plaque numbers.

Phage	Host	Time before phage added (hr)	Titre
ϕC	*N. canicruria*	0	$6{\cdot}0 \times 10^8$
	N. canicruria	3	$1{\cdot}5 \times 10^9$
	N. canicruria	5	$2{\cdot}2 \times 10^8$
	N. canicruria	12	$< 1 \times 10^7$
ϕEC	*N. canicruria*	0	$1{\cdot}0 \times 10^9$
	N. canicruria	3	$2{\cdot}0 \times 10^9$
	N. canicruria	12	$< 1 \times 10^7$
	N. erythropolis	0	$7{\cdot}1 \times 10^7$
	N. erythropolis	3	$7{\cdot}5 \times 10^8$
	N. erythropolis	12	$< 1 \times 10^5$

Table 8. *Effect of size of nocardiophage ϕC inoculum on yield*

A 2 l. flask containing 500 ml. of peptone+yeast extract+0·01 M-$Ca(NO_3)_2$ broth was inoculated with 10 ml. of an 18 hr. culture of *Nocardia canicruria*. The host was incubated for 2 hr before phage ϕC was added at the indicated concentrations. After incubation for 3 or 18 hr the cultures were assayed for plaque numbers.

Phage inoculum size (p.f.u./ml.)	Output of phage at (p.f.u./ml.) 3 hr	18 hr
10^8	5×10^9	3×10^9
10^7	3×10^9	5×10^9
10^6	3×10^8	9×10^9
10^5	2×10^7	9×10^9
10^4	4×10^6	$1{\cdot}8 \times 10^{10}$
10^3	3×10^5	$1{\cdot}6 \times 10^{10}$
10^2	1×10^5	$1{\cdot}1 \times 10^{10}$
10^1	1×10^5	$1{\cdot}1 \times 10^{10}$

Table 9. *Effect of size of the host inoculum on proliferation of nocardiophage ϕC*

Nocardia canicruria was incubated for 2 hr at 30°, harvested and suspended in fresh medium to various concentrations. After 1 hr incubation at 30°, phage ϕC was added and the mixture incubated for 200 min. at 30°.

Amount of host colony count/ml.	Plaque-forming units/ml. yield (p.f.u./ml.) Phage input $3{\cdot}2 \times 10^2$	Phage input $3{\cdot}2 \times 10^4$
$4{\cdot}3 \times 10^7$	$6{\cdot}7 \times 10^5$	$2{\cdot}9 \times 10^7$
$4{\cdot}3 \times 10^6$	$2{\cdot}4 \times 10^4$	$5{\cdot}2 \times 10^5$
$4{\cdot}3 \times 10^5$	$7{\cdot}8 \times 10^2$	$1{\cdot}3 \times 10^5$
$4{\cdot}3 \times 10^4$	$2{\cdot}1 \times 10^2$	$1{\cdot}2 \times 10^4$

By the method of group and single additions or omissions, a chemically defined propagation medium was devised (Table 10). For maximum production of phage ϕC, a medium composed of glycerol, nicotinamide, basal salts and three amino acids was most effective. The omission of any one of these supplements or groups of supplements (e.g. vitamins) resulted in lowered production of phage. The omission of glycerol, which resulted in a somewhat smaller production of phage, was the least necessary of the defined medium constituents.

Table 10. *Simplified medium for the propagation of nocardiophage ϕC in Nocardia canicruria*

Supplement	Concentration	Phage yield (p.f.u./ml.)
Salts*	1 ml./11 ml.	$3{\cdot}6\times10^{8}$
Vitamins†	0·1 ml./11 ml.	
Peptone	5 mg./ml.	
Yeast extract	3 mg./ml.	$1{\cdot}2\times10^{10}$
Casamino acids	1 mg./ml.	$3{\cdot}6\times10^{8}$
Casamino acids	1 mg./ml.	
Salts	1 ml./11 ml.	$1{\cdot}3\times10^{9}$
Casamino acids	1 mg./ml.	
Salts	1 ml./12 ml.	
Vitamins	0·1 ml./12 ml.	$1{\cdot}0\times10^{10}$
Amino acids‡	1 ml./12 ml.	
Salts	1 ml./12 ml.	
Vitamins	0·1 ml./12 ml.	$8{\cdot}1\times10^{9}$
Valine	1 ml./12 ml.	
Salts	1 ml./12 ml.	
Vitamins	0·1 ml./12 ml.	$3{\cdot}1\times10^{9}$
Isoleucine	1 ml./12 ml.	
Salts	1 ml./12 ml.	
Vitamins	0·1 ml./12 ml.	$3{\cdot}1\times10^{9}$
Leucine	1 ml./12 ml.	
Salts	1 ml./12 ml.	
Vitamins	0·1 ml./12 ml.	$2{\cdot}0\times10^{9}$
Nicotinamide	0·1 ml./12 ml.	
Salts	1 ml./12 ml.	
Amino acids	1 ml./12 ml.	$2{\cdot}2\times10^{10}$
Glycerol	0·1 ml./12 ml.	
Nicotinamide	0·1 ml./12 ml.	
Salts	1 ml./12 ml.	
Amino acids	1 ml./12 ml.	$7{\cdot}0\times10^{10}$

* The salt solution consisted of KNO_3, 1 g.; K_2HPO_4, 1 g.; $MgSO_4$, 0·5 g.; distilled water, 100 ml.
† The vitamins consisted of 0·1 g. each of thiamine, pyridoxine, nicotinic acid and riboflavin; distilled water, 20 ml.
‡ The amino acids consisted of 0·1 g. each of isoleucine, leucine and valine; distilled water, 20 ml.

Host range

Nocardiophage ϕC attacked two strains of *Nocardia canicruria* and an organism designated *N. corallina* strain N305. Phage ϕC was unable to lyse any other of the strains tested (Table 11). *N. canicruria*, *N. erythropolis*, *N. opaca* and *N. corallina* strain N305 were lysed by phage ϕEC; other nocardias tested were not susceptible to

the action of this phage. Thus the range of increasing lytic capability extends from phage ϕC to ϕEC to MJP1.

Morphology of nocardiophages ϕC and ϕEC

Negatively stained phage ϕC was found to have a head 52 ± 2 mμ in diameter and tail 10 mμ wide $\times$ 192 ± 8 mμ long (Pl. 1, A). The head size of phage ϕEC (Pl. 1, B) was 52 mμ in diameter and the tail 10 mμ wide $\times$ 197 mμ long.

Table 11. *Host ranges of nocardiophages ϕC, ϕEC and MJP1*

Stock PY preparations of the phages, containg *ca.* 1×10^9 p.f.u./ml., were diluted for three ten-fold serial dilutions. 0·1 ml. samples of the dilutions were added to ca. 5×10^7 host cells/ml. contained in 2·5 ml. soft overlay agar at 45°. The phage-host mixtures were poured on the surface of PY agar plates. The production of plaques after 24 hr. incubation for any dilution tested was considered as a positive result.

Organism and strain	ϕC	ϕEC	MJP1
Nocardia canicruria 1574	+	+	+
N. canicruria 57	+	+	+
N. corallina 2747	−	−	−
N. corallina W5	−	−	−
N. corallina 107	−	−	−
N. corallina	−	−	nt
N. corallina W3408	−	−	nt
N. corallina 78	−	−	−
N. corallina S5	−	−	−
N. corallina N305	+	+	+
N. brasiliensis 301	−	−	+
N. opaca 76	−	−	−
N. opaca 765A	−	+	nt
N. erthropolis	−	+	+
N. farcinica	−	−	nt
N. rubra 1	−	−	−
N. rubra 74	−	−	−
N. asteroides 58	−	−	nt
N. asteroides 133	−	−	nt
N. asteroides 300	−	−	nt
N. asteroides 162	−	−	nt
N. asteroides 92	−	−	nt
N. rhodnii N 6117	−	−	nt
Mycobacterium rhodochrous 370	−	−	−
M. rhodochrous 482	−	−	−

nt: not tested.

DISCUSSION

Nocardiophage ϕC was routinely propagated to titres of 10^{10} p.f.u./ml. in this work. Attempts to purify the phage further by centrifugation or column chromatographic separation procedures were unsuccessful; phage ϕC was sensitive to these processes. Inactivation under these conditions apparently resulted from damage to the long, delicate phage tail. Nocardiophage ϕEC is similar to ϕC in susceptibility to chemical and physical agents; but ϕEC is easily concentrated by centrifugation without loss of titre.

Unlike phage ϕEC and other actinophages, phage ϕC attached efficiently to its host. The observed attachment was irreversible and dependent upon time and

temperature. Attachment proceeded best in a nutrient medium; phages ϕC and ϕEC both proliferated in a peptone+yeast extract broth medium containing 0·001 M-$Ca(NO_3)_2$. These results suggest that either organic cofactors or host metabolism are required for attachment. The composition of the defined medium for phage ϕC propagation indicated that proliferation was not solely a question of host nutrition because the host, *Nocardia canicruria*, is easily grown in a simple defined glucose+basal salts medium (Adams, 1964). Nocardiophage ϕEC also resembles other actinophages more closely than does ϕC with respect to their latent periods.

Host populations which had been incubated for more than about 5 hr before infection with phage ϕC or ϕEC gave low yields of phage. These results may be correlated with the developmental cycles of the host (Adams & McClung, 1962; Adams, 1964). The interval from 0 to 5 hr appears to represent the lag and germination phase of *Nocardia canicruria*. From the present work, it appears that the host is most susceptible to viral infection of these phages ϕC and ϕEC during this period. With further growth after incubation for 5 hr, a well-defined coenocytic development stage of the nocardias was noted. Multiple attachment of phage particles to the filaments may account for the decrease in nocardiophage proliferation. Unfortunately, unlike the Eubacteriales, synchronous growth of the actinomycetes in general has not been successfully achieved; only under conditions of synchronous growth of the host organisms will the more subtle of the nocardiophage+host interactions be easily explored.

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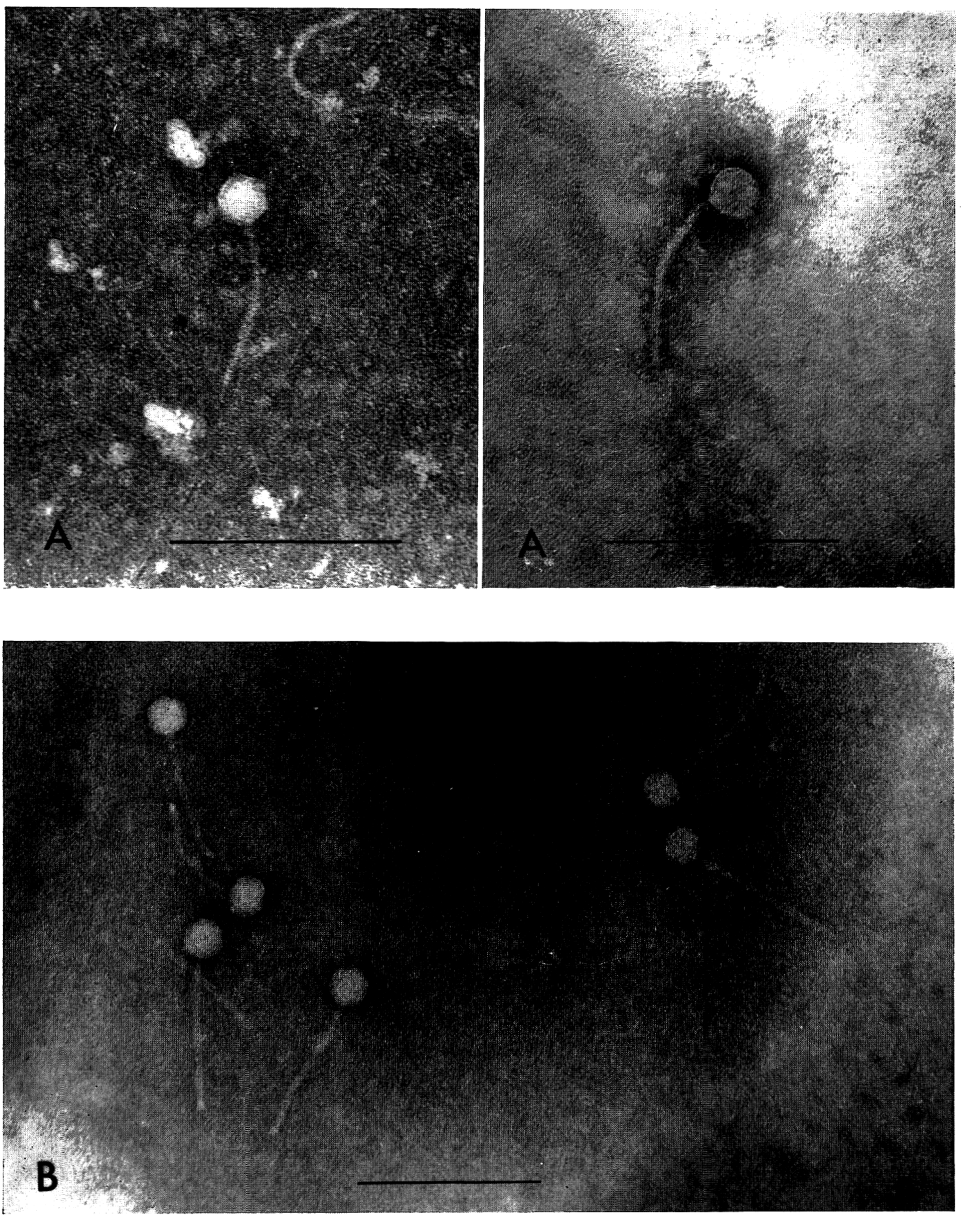
EXPLANATION OF PLATE

Electron micrographs of nocardiophages ϕC (A) and ϕEC (B). The marker bars denote 250 mμ.


A
A
B

J. gen. Microbiol. (1967), **47**, 257–271
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Chemical and Electron Microscope Studies on Fractions Prepared From Coats of Bacillus Spores

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SUMMARY

'Coat fractions' were prepared from spores of six Bacillus species by mechanical disruption, centrifugation, and digestion with lysozyme. The coats were successively extracted with dilute NaOH, treated sonically (mildly), and digested with the proteolytic enzyme preparation Pronase. *Bacillus megaterium* QM B1551 was studied in detail. Electron microscopic studies were made at the various stages. Three fractions were obtained and partly characterized chemically. The alkali-soluble fraction consisted mainly of protein(s). The paracrystal fraction solubilized by sonic treatment consisted mainly of protein with a chemical composition and physical characteristics similar to those of keratins. The final resistant residue fraction of the coats after hydrolysis contained amino acids, phosphorus and muramic acid, and may be composed of a phosphomuramyl polymer to which a peptide and/or protein is linked. Electron microscopic observation suggested that the spore coat of *B. megaterium* QM B1551 consisted of at least three components: a middle, paracrystal fraction is sandwiched between or 'cemented' with the alkali-soluble fraction on one side and the resistant residue layer on the other. Large differences were found in the composition of the coats and in the P contents of the resistant residue of the various organisms.

INTRODUCTION

The spore coat constitutes a major part of the bacterial spore, in terms of both anatomy and mass. The coat is an important structure which probably plays a role in the resistance of the bacterial spore; it is also the initial contact site for germinative substances. The knowledge of chemical and physical constitutions of this complicated architecture will facilitate the studies on resistance and germination mechanisms in bacterial spores. The bulk of this coat has an amino acid composition similar to proteins (Salton & Marshall, 1959; Hunnel & Ordal, 1961; Warth, Ohye & Murrell, 1963; Snoke, 1964). It has been also reported that wide differences are found in the proportions of amino acids and in the phosphorus contents of coats from different species (Fitz-James, 1955*a*, *b*; Strange & Dark, 1956; Salton, 1964). Spore coats consist of at least two distinct layers (Robinow, 1960; Ohye & Murrell, 1962; Warth *et al.* 1963) but only the overall composition has been reported, inasmuch as no one has yet succeeded in stripping the individual layers; furthermore, the criteria of 'pure' coats are arbitrary. The present report describes the isolation of individual layers from the spore coat and their chemical and physical characteristics.

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METHODS

Bacteriological. Most of the work was done with *Bacillus megaterium* QM B 1551 (Levinson & Sevag, 1953). Other strains used were: *B. megaterium* ATCC 19213 (formerly known as 'Texas strain'); *B. megaterium* ATCC 13368; *B. polymyxa* ATCC 842; *B. subtilis* ATCC 6633; *B. licheniformis* ATCC 9259. Bacilli from an overnight nutrient agar slope suspended in sterile de-ionized water were spread on the surface of agar of the following composition. Solution A: glucose, 1·0 g.; Basamine (Anheuser-Busch, Inc., St Louis), 0·5 g.; $MgSO_4.7H_2O$, 0·2 g.; NaCl, 0·1 g.; L-glutamic acid, 1·0 g.; $CaCl_2.2H_2O$, 6·6 mg.; $MnSO_4.5H_2O$, 7·8 mg.; $ZnSO_4.7H_2O$, 17·8 mg.; $FeSO_4.7H_2C$, 18·3 mg.; agar (Difco), 25 g.; de-ionized water, 800 ml.; adjusted to pH 6·9. Solution B: $(NH_4)_2HPO_4$, 1·0 g.; KH_2PO_4, 5·0 g.; de-ionized water, 200 ml.; adjusted to pH 6·9. Four hundred ml. of solution A were mixed with 100 ml. of solution B after autoclaving each, and the resulting medium poured to a depth of about 0·5 cm. into aluminum-foil-covered sterile aluminum dishes (17 × 25 cm.). After 4 days of incubation at 37°, the surface growth was rinsed off with a small volume of cold sterile de-ionized water and the small proportion of vegetative organisms was removed by biphasic washing (Sacks & Alderton, 1961). The spores were washed further by eight changes in cold sterile water, lyophilized, and stored in vacuum.

Spore coats were prepared by mechanical rupture of spores in a Nossal disintegrator (Nossal, 1953). The glass tube in the metal cylinder was charged with 4 g. of Ballotini beads (size 12) and 7 ml. of water containing 200 mg. lyophilized spores. The cylinder was chilled in ice water after each 10 sec. This treatment was repeated until no intact spores were visible microscopically, usually a total of 6–10 min. The beads were removed by low-speed centrifugation and the coat particles were recovered and washed by centrifugation at 10,000 *g* in cold de-ionized water. Microscopic examination revealed no unbroken spores. Although consisting predominantly of pieces of integument, the 'coat fraction' probably also contained remnants of spore membranes (Fitz-James, 1960; Ohye *et al.* 1962; Sacks & Thomas, 1965). Before use, the stock coat fractions were dialysed against de-ionized water overnight in a cold room.

Chemical. Organic and inorganic phosphorus (P) were determined by the Fiske–SubbaRow method (Fiske & SubbaRow, 1925). Hexosamine, including muramic acid (Strange, 1960) was analysed by the Rondle–Morgan method (Rondle & Morgan, 1955) in samples hydrolysed with 6 N-HCl in a sealed tube for 8 hr at 105°. The HCl was removed in vacuum before analysis. Muramic acid was detected (Bar[illegible] & Summerson, 1941) on samples eluted from thin-layer chromatograms. Amino acid[illegible]re determined by the ninhydrin procedure (Moore & Stein, 1948), with L-alanine as a standard, after hydrolysis with 6 N-HCl in a sealed tube for 25 hr at 105° and vacuum removal of the HCl. Cystine was determined according to Kassel & Brand (1938). The molar ratios of amino acids were obtained by means of a Beckman model 120-G amino acid analyser.

Chromatography. Most of the chemical components of the spore-coat fractions were identified by thin-layer chromatography on silica gel G (Warner-Chilcott Laboratories, Richmond, California, U.S.A.). Solvents used were as follows: for 2-dimensional separation of amino acids, *n*-butanol + acetic acid + water (4 + 1 + 1, by vol.) followed by phenol + water (4 + 1, by vol.); for water-soluble dinitrophenylamino acids (DNP-amino acids), *n*-butanol + acetic acid + water (4 + 1 + 1, by vol.); for

ether-soluble DNP-amino acids, chloroform + methanol + acetic acid (95 + 5 + 1, by vol.); for polyols, *n*-propanol + ammonium hydroxide (sp.gr. 0·88) + water (6 + 3 + 1, by vol.).

Presumptive identification of lysine was made with two-dimensional chromatography on Whatman no. 1 paper, with the solvents: *n*-propanol + 24% ammonium hydroxide (sp.gr. 0·88) (2 + 1, by vol.) followed by methanol + water + 10 N-HCl + pyridine (80 + 17·5 + 2·5 + 10, by vol.). Amino sugars were chromatographed on Whatman no. 3 paper, with *n*-butanol + pyridine + 0·4% (w/v) glacial acetic acid in water (60 + 35 + 25, by vol.) followed by phenol + water (4 + 1, by vol.). On occasion, both hexosamine and amino acids were detected on 2-dimensional thin-layer chromatograms developed with *n*-butanol + glacial acetic acid + water (4 + 1 + 1, by vol.) and phenol + water (4 + 1, by vol.).

Other determinations. *N*-Terminal amino acids were identified by Sanger's fluorodinitrophenylation method (Sanger, 1945, 1949), substituting $NaHCO_3$ + KOH for trimethylamine; the bacterial DNP-derivatives were compared with authentic DNP-amino acids. Partial hydrolysis of the resistant residue was done in 2 N-HCl for 3 hr at 100°.

For the X-ray diffraction of paracrystal fraction, a model ADP-101 B Toshiba X-ray diffraction apparatus was employed. The sample powder filled into capillary tubing was placed in the apparatus at the distance of 3 cm. from the camera. The time of exposure was 10 hr (30 kV, 15 mA).

Electron microscopy. A model EMU 3-G RCA electron microscope was employed. Specimens were freeze-dried on freshly cleaned mica, shadowed with platinum palladium (80 + 20, w/w) and coated with carbon. The carbon-coated preparation was floated with distilled water from the mica onto a 200-mesh wire grid.

RESULTS

Fractionation of spore coats

Figure 1 depicts the sequence of steps for separating individual components from spore coats. The fractionation was done on approximately 1 g. samples of coats.

Lysozyme digestion. This treatment eliminates mucopeptide regarded as cortical in origin and not an integral part of the spore coat (see Warth *et al.* 1963; Warth, 1965). Nearly one-third of the weight of the coat fraction was removed in this step.

NaOH extraction. Lysozyme-digested coats were suspended in de-ionized water to an extinction of 0·8 and brought to 50°. Upon adding NaOH the extinction rapidly decreased by about 50% (Fig. 2). The alkaline extract was neutralized with HCl and concentrated in vacuum to a small volume in a rotary evaporator; a considerable amount of a white amorphous material precipitated. This water-insoluble material was dialysed against de-ionized water for 2 days in a cold room; it is designated as the 'alkali-soluble fraction'.

Sonic treatment. When the sediment undissolved by the NaOH was agitated with glass rod to make a homogeneous suspension, the sediment had a strong tendency to form aggregates difficult to disperse. A homogeneous suspension was obtained by mild sonic treatment. NaOH-extracted coats (150 mg.) were suspended in 15 ml. de-ionized water in a 20 ml. beaker (diam. 3·5 cm., height 4·5 cm.). Sonic treatment was done in the cold with a Branson Sonifier (Branson Instruments, Inc., Danbury,

Conn., U.S.A.) at power setting 3 for 5 min. The supernatant liquid obtained by centrifugation of the sonically treated suspension at 10,000 *g* for 20 min. was faintly opalescent. Upon concentration in vacuum, a considerable quantity of crystalloid

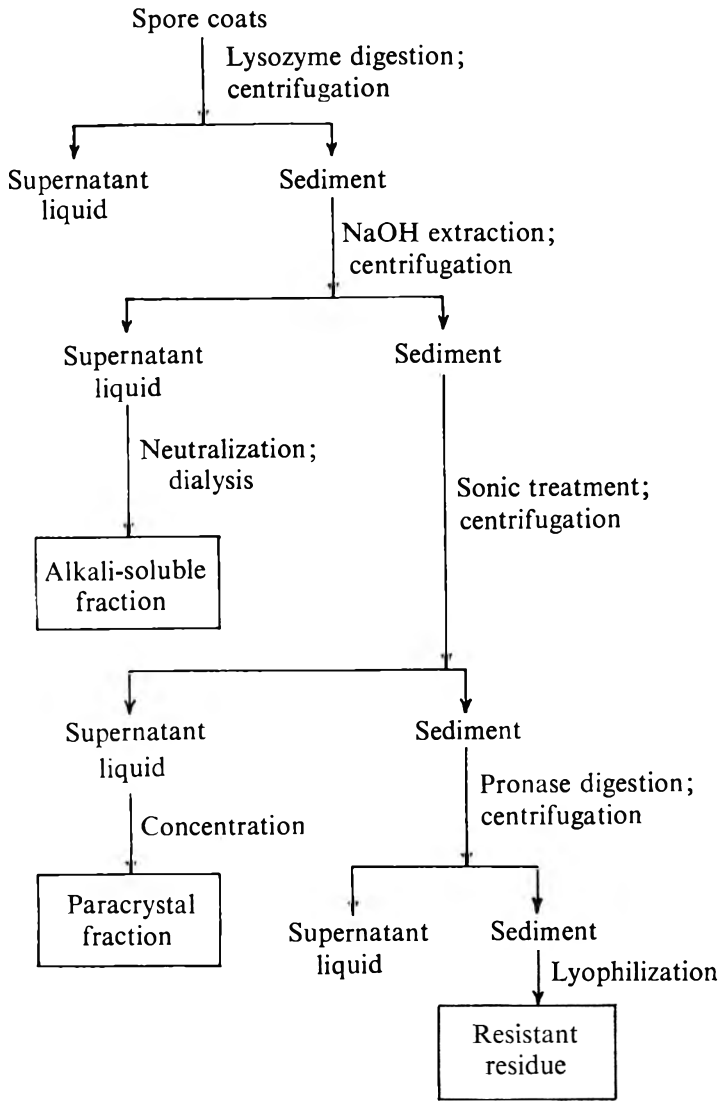

Fig. 1. Scheme for fractionation of spore coats into three products (denoted by boxes). Details of each step are given in the text. Centrifugation was at 10,000 *g* for 20 min.

organic material precipitated (Pl. 1, figs. 1–4) along with some amorphous matter and was recovered on a sintered-glass filter. The content of this fraction in the lysozyme-treated spore coat of *Bacillus megaterium* QM B1551 was 30%. The fraction was designated as the 'paracrystal fraction'.

The coat residues not solubilized by mild sonic treatment represented, in different strains, from one-half to four-fifths of the original coat weight (Table 1). They were

digested with the proteolytic enzyme preparation Pronase (California Biochemical Corp., Los Angeles, Calif., U.S.A.); the Pronase-insoluble residues, amounting to about one-quarter to one-half of the weights of the original lysozyme-digested coats (Table 1), were washed thoroughly by centrifugation in water at 10,000 *g* for 20 min., dialysed against distilled water overnight in the cold room, and designated as 'resistant residue'.

Thus, the spore coats were resolved into three fractions: (1) alkali-soluble fraction, (2) paracrystal fraction, (3) resistant residue.

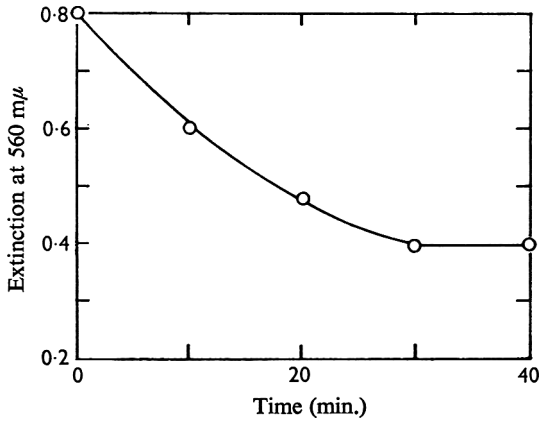

Fig. 2. Change in extinction (at 560 mμ) of lysozyme-treated spore coats of *Bacillus megaterium* QM B1551 during extraction with 0·06 N-NaOH at 50°.

Phosphorus contents of spore coat fractions

The P content of the coat fractions differed widely in the various strains, from a low of 0·23 to 3·2% (see Fitz-James, 1955*a*, *b*; Fitz-James & Young, 1959*b*; Warth *et al.* 1963; Levinson & Hyatt, 1964). Coats with the higher P contents evidently contained most of their P in the resistant residue in which it became concentrated through the successive digestions. However, the other three strains showed no such P enrichment. From these and the findings of others (Fitz-James, 1955*a*, *b*; Warth *et al.* 1963), it is obvious that high coat-P is not an essential attribute of the spore. The final resistant residue from *Bacillus megaterium* QM B1551 containing the surprisingly high content of coat-P has been correlated with the presence of a dense outer coat (Fitz-James & Young, 1959*a*, *b*; Warth *et al.* 1963).

Electron microscopy of spore coats

Coats of *Bacillus megaterium* QM B1551 (Pl. 2) were not strikingly altered in appearance by lysozyme digestion (Pl. 3, figs. 6 and 7). Indications of heterogeneity in the construction of the coats are seen in Pl. 3, especially fig. 7. Two layers can be seen: one appears to be composed of more or less uniform spherical particles (Pl. 3, fig. 7), and the other smooth and relatively featureless, although this also may be layered (Pl. 3, fig. 6). The smooth layer disappeared after alkali-digestion and aggregated material can be seen on or around the treated coats (Pl. 4, fig. 8). An interpretation possible from the photographs is that one of the coat layers is composed of

Table 1. *Weight loss and P-contents of successive residues during fractionation of spore coats of various strains of Bacillus*

Bacillus strain	Coat fraction: P content of starting spores (%)	Coat fraction: Preparation of starting spores (%)	Coat fraction: P content of insoluble residue (%)	Lysozyme digestion*: Weight loss of lysozyme-digested coats (%)	Lysozyme digestion*: P content of insoluble residue (%)	Alkali extraction† and sonic treatment‡: Weight loss of lysozyme-digested coats (%)	Alkali extraction† and sonic treatment‡: P content of insoluble residue (%)	Pronase digestion§: Weight loss of lysozyme-digested coats (%)	Pronase digestion§: P content of resistant residue (%)	Pronase digestion§: Resistant residue of lysozyme-digested coats (%)
B. megaterium QM B1551	2·2	45·0	3·2	32·5	4·80	50·8	6·30	8·3	7·00	40·9
B. megaterium 19213	1·1	30·6	0·42	33·2	0·43	28·7	0·48	20·0	0·40	51·3
B. megaterium 13368	2·0	40·0	0·68	39·0	0·76	45·3	0·35	29·6	0·60	25·1
B. licheniformis 9259	0·83	47·3	0·23	43·0	0·28	21·2	0·05	37·9	0·14	40·9
B. subtilis 6633	2·39	43·9	3·17	30·3	3·83	34·4	4·00	9·4	4·54	56·2
B. polymyxa 842	1·72	38·6	1·54	39·5	1·56	41·8	2·10	20·9	3·80	37·3

* Coats suspended in a solution of crystalline lysozyme in 10 mg. 50 ml. of 10 mM-tris buffer (pH 8·0), with stirring for 24 hr at 37°; toluene as preservative.

† 0·06 N-NaOH for 3 hr at 50°.

‡ 5 min.

§ Residue from the sonic treatment suspended in 40 ml. of solution of 10 mg. Pronase in 0·01 M-tris buffer (pH 8·0) for 25 hr at 50°; toluene as preservative.

discrete particles which can be dissociated in conjugated form from their normal array by alkali treatment. The particle layer is probably overlayered with the alkali-soluble material; removal of the latter permits the release of the particles and this is expedited by mild sonic treatment. The particles are believed to constitute the bulk of the paracrystal fraction and were obtained readily by sonic treatment only after extraction with alkali. The resistant residue of the coats after sonic treatment and sedimentation in the centrifugation had a distinct ultramicroscopic structure (Pl. 4, fig. 9) and was almost devoid of the larger particles described above. These particles probably represent the major portion of the paracrystal fraction which, as already seen, spontaneously associate into regular macrostructures.

From the results of the electron microscopy, one can imagine, as a working hypothesis, that the spore coat of *Bacillus megaterium* QM B1551 consists of at least three components: a middle, particulate layer (the paracrystal fraction) is sandwiched between or 'cemented' with the alkali-soluble layer on one side and the resistant-residue layer on the other.

Table 2. *Amino acid composition* of fractions from spore coats of Bacillus megaterium* QM B1551

	Residues per 100 total residues		
Amino acid	Alkali-soluble fraction	Paracrystal fraction	Resistant residue
Lysine	7·5	2·5	18·4
Histidine	4·8	2·4	3·1
Arginine	6·2	5·6	4·5
Aspartic acid	9·8	15·0	11·0
Threonine	4·0	2·9	5·1
Serine	5·1	6·6	6·1
Glutamic acid	7·5	8·3	10·0
Proline	6·5	1·2	4·8
Glycine	12·1	16·0	6·2
Alanine	5·6	4·7	4·7
Cystine (half)	1·3	8·2†	1·9
Valine	4·0	2·3	3·8
Methionine	1·1	1·0	3·5
Isoleucine	3·2	2·0	5·8
Leucine	5·1	·27	Trace
Tyrosine	11·6	2·1	4·9
Phenylalanine	4·7	6·7	5·4

* Determined with a Beckman model 120 B amino acid analyser.

† Determined as cysteic acid on a separate sample of protein oxidized with performic acid (Moore, 1963).

The alkali-soluble fraction of Bacillus megaterium QM B1551

This material was insoluble in water at pH 7 but was soluble at pH 9·5. It did not move from the origin in thin-layer chromatography with *n*-butanol + acetic acid + water as solvent and it gave a strong reaction with ninhydrin. The material was treated again with lysozyme to eliminate contamination with mucopeptide. After acid hydrolysis, numerous amino acids were identified, by combinations of one- and two-dimensional thin-layer chromatograms, suggestive of protein. The P content was relatively low (0·9%). The amino acid analysis showed all of the usual amino acids (Table 2) of proteins. Thus, the alkali-soluble fraction appeared to consist predominantly of one

or more protein(s) distinct in amino acid composition (Table 2) from that in the other fractions described below.

The alkali-soluble protein had more glycine than most proteins, with the notable exception of the collagens, which are extremely rich in this amino acid. Collagens also lack cystine; there was a very low cystine content in the bacterial alkali-soluble product. Perhaps the most outstanding finding was the very high content (12·0%) of tyrosine. In distinction from the collagens where this tyrosine occurs in very low concentrations, the tyrosine content of the alkali-soluble proteins from the spore coats was higher than that of any of the 253 proteins whose analyses were compiled by Tristam & Smith (1963). Only papain has a tyrosine content approaching that of the protein from these spore coats.

Other major differences between the three spore-coat protein fractions (Table 2) are in the amounts of aspartic acid, lysine, and proline.

During the alkali extraction, the spore coats agglutinated strongly. This suggested that surface charges, either absent or masked by the alkali-soluble protein before its extraction, were generated by disruption of the normal combination (possibly a weak ester linkage involving e.g. the tyrosyl hydroxyls) between the protein and the rest of the spore coat.

Table 3. *Effect of various agents on the solubility of the paracrystal fraction from spore coats of Bacillus megaterium* QM B1551

Agent and final concentration	Effect on colloidal suspension of paracrystal fraction (5 mg./ml.) in water
Na laurylsulphate, 0·1 %	Clearing
Polyethylene lauryl alcohol, 0·1 %	Partial clearing
Na ethylenediamine tetracetate, 0·1 %	Partial clearing
Na bisulphite, 0·1 %	White precipitate
Na hydrosulphate, 0·1 %	White precipitate
β-Mercaptoethanol, 0·1 %	White precipitate
Ascorbic acid, 0·1 %	White precipitate
Cysteine, 0·1 and 1·0 %	Tan precipitate
KCl, 0·5 and 1·0 M	Tan precipitate
NaOH, 0·1, 0·5 and 1·0 N	Tan precipitate
HCl, 0·1, 0·5 and 1·0 N	Tan precipitate
H_2O_2, 0·1 % (w/v)	None
Na iodacetate, 0·01 and 0·1 M	None
Cetyl trimethylammonium bromide, 0·1 and 0·5 %	None

The paracrystal fraction of Bacillus megaterium QM B1551

The lyophilized material was a slightly tan-coloured fluffy product; in water it formed a light amber opalescent solution. The colloidal solution was reactive with several reagents (Table 3). Sodium lauryl sulphate (SLS) strikingly enhanced the solubility of the paracrystal fraction. Sonic treatment of the NaOH-extracted coats in 0·1 (w/v) SLS was a much more efficient means of obtaining this fraction than treatment in water alone. A cationic surface-active agent, cetyl trimethylammonium bromide, did not have this effect.

Several compounds, notably some reducing agents, elicited the opposite reaction, namely, a precipitation of the paracrystal fraction. The solution turned colourless and yielded a copious white amorphous precipitate (Table 3). Other reagents, including

cysteine, also induced precipitation although less effectively and the products were tan-coloured. These observations suggest that the paracrystal fraction had easily reducible groups and that, while reduced, these reacted inter- and possibly intramolecularly to form an insoluble polymer.

The behaviour in SLS also suggested that the paracrystal fraction broke into smaller components, resulting in increased solubility. The principal effect of the reducing agents also appeared to be one of solubilization, as suggested by the clearing of a colloidal suspension. The precipitation developed secondarily, presumably from

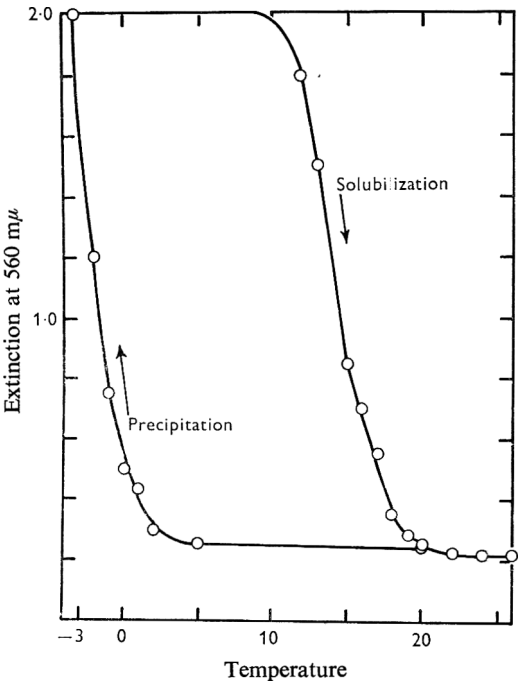

Fig. 3. Temperature-dependent, reversible 'precipitation solubilization' of paracrystal fraction, 5 mg./ml. distilled water.

a random interaction of the reduced fragments or subunits. This precipitation of the reduced units was markedly hindered by SLS. Consequently, conducting the reduction in the presence of SLS led to the formation of stable solutions of much higher concentrations than were otherwise obtainable. Comparable effects of reducing compounds and detergents on proteins dissociable into subunits is well known (Putnam, 1953; Reithal, 1963). The precipitation and solubilization were reversible and temperature-dependent, with curves exhibiting a hysteresis (Fig. 3). There was a 12° differential in the temperatures which initiated precipitation and solubilization in this experiment. The phenomenon was seen in SLS solution as intensely as in water.

Ultracentrifugation in a Spinco Model E instrument of a solution of paracrystal material in 0·1% (w/v) SLS showed the material to be markedly heterogeneous in respect to macromolecular components. In a comparable SLS solution to which 0·1% (w/v) mercaptoethanol was added, the several fast-moving peaks disappeared; there appeared instead a single rather broad peak that moved very slowly. The

heterogenous heavier macromolecular components were dissociated into smaller components by the mercaptoethanol. These ultracentrifugation experiments were only preliminary ones, and it is not yet possible to decide whether the smaller particles are a homogeneous single component. The major slow-moving band was indicative of very small particles of macromolecules, estimated to be in the range of 4 Svedberg units. Mercaptoethanol and sodium lauryl sulphate had a decided effect on 'dissociation' of the paracrystal material, on elimination of much of the macromolecular heterogeneity, and on stabilization of dissociated solutions.

An acid hydrolysate (6 N-HCl for 8 hr at 105°) of the paracrystal fraction contained a variety of amino acids, muramic acid, and P in the ratio of 40 (L-alanine equivalents):6:1. The hexosamine content was 0·8% but the reacting compound could not be identified as acetylglucosamine, glucosamine or galactosamine by using authentic reference samples. The muramic acid and P values may indicate contamination of the main protein component with a small amount of the resistant residue rich in phosphomuramic acid described below. The paracrystal material proved to have an unusual

Table 4. *Cysteine content of representative fractions derived from spore coats of Bacillus megaterium* QM B1551

Fraction	Weight (%)
Whole coats	1·7
Alkali-soluble	nil
Paracrystal material	6·6
Resistant residue	0·8

amino acid composition (Table 2) more than half of the amino acids present were glycine, aspartic acid, glutamic acid and cysteine. More than four-fifths of the residues were accounted for by eight amino acids; more than one of every four residues was glycine. There was a high proportion of cysteine (half)—one of every 12 amino acid residues. Also noteworthy is the high proportion of the dicarboxylic acids: nearly one of every four amino acids residues was an aspartic acid or glutamic acid.

The paracrystal fraction was the only fraction rich in cysteine (Table 4) and it accounted for the bulk of the cysteine in the whole coats.

The X-ray diffraction photograph for the paracrystal fraction showed the following major rings: 10·1, 4·8, 3·0, 2·6, 2·3, 2·1, 1·9 and 1·7 Å. The inner rings corresponding to 10·1 and 4·8 Å were not so sharp as the other six rings, which were very sharp. Thus, two kinds of rings appeared in the X-ray diffraction photographs. The two inner rings (10·1, 4·8 Å) corresponded to those of α-keratin. Only the two inner rings were obtained from the fraction of germinated spore coat.

The above results suggest that the paracrystal fraction consists of at least keratin-like substance as a basal structure.

The resistant residue fraction of Bacillus megaterium QM B1551

The various components in a 6 N-HCl hydrolysate were identified with the aid of thin-layer chromatography and estimated with the amino acid analyser. The composition suggested that the resistant residue contained a phosphomuramyl polymer in combination with peptide and/or protein. However, about one-third of the weight of the resistant residue was unaccounted for. The presence of aspartic acid, glycine,

and glutamic acid in substantial amounts might suggest major contamination with incompletely removed paracrystal fraction, but this was contradicted by the low content of cysteine.

The distinctiveness of the amino acid composition of the resistant residue was emphasized by the exceptionally high proportion of lysine; nearly one of every five of the identifiable amino acid residues was a lysine. The only protein reported to have a comparably high lysine content is cytochrome *C* (Tristam & Smith, 1963). If, alternatively, the high lysine is in the usual kind of side chain of polymucopeptide (Weidel & Pelzer, 1965), correspondingly high proportions of alanine, aspartic acid, glutamic acid and perhaps glycine would be expected. (There was no indication of 2,6-diaminopimelic acid in the results furnished by the amino acid analyser.) Since the amounts of the latter amino acids did not suggest the usual type of mucopeptide, we concluded that the resistant residue contained either a protein or a peptide of composition distinguished by an abundance of lysine. In addition to the known amino acids, three other significant ninhydrin-positive peaks, estimated to comprise less than 15% of the total, were recorded by the amino acid analyser. One of these unidentified components eluted from the analyser column before lysine, where a hexosamine was to be expected. A second peak was located just after the position of cysteic acid and well before aspartic acid. The third component was detected as a relatively broad peak whose apex developed shortly after that of phenylalanine and overlapped it.

Of the organic compounds identified in the hydrolysate, muramic acid was by far the most abundant (Table 5). Contrary to the usual finding with cell walls of vegetative bacteria (Salton, 1964) and despite deliberate attempts to detect it, no significant amounts of hexosamine were found.

The usually high P content was paralleled by the high total ash; more than one-quarter of the weight of the resistant residue was recovered as inorganic matter after ignition. The cations in this ash were not investigated but, since it contained 25% P, a reasonable inference is that the mineral matter was composed primarily of phosphate(s).

Table 5. *Some components of resistant residue fraction of spore coats of Bacillus megaterium* QM B1551

Component	Amount in resistant residue (%, w/w)
Amino acids	27·8*
Muramic acid	14·5†
Glucosamine	Trace
Total-N	5·06
P	7·0
Ash	25·9

* L-Alanine equivalents (Moore, 1963) in acid-hydrolysed sample.
† Glucosamine equivalents (Rondle & Morgan, 1955) in acid-hydrolysed sample.

Partial hydrolysis of the resistant residue. Digestion with 2 N-HCl at 100° for 3 hr dissolved most of the resistant residue. The soluble material was passed through a column of Dowex 50 ion exchange resin (H^+ form); almost none of the P was adsorbed. The solution was then passed through a Dowex 1 (chloride form) column followed by 0·1 M-NaCl as eluent. The several eluate fractions were analysed for

orthophosphate and for organo-P. Three P-containing fractions were separated. The first fraction, containing 50% of the soluble P (all organo-P), was not adsorbed by the Dowex 1 (more details of this fraction below). The second P-containing fraction was discharged in the first 100 ml. of eluent; it represented 38% of the soluble P and was identified as orthophosphorus. The third P-containing fraction contained 12% of the soluble P and was recovered in the second 100 ml. of eluent. No additional P was eluted in three further 100 ml. volumes of NaCl solution. On a thin-layer chromatogram the third fraction yielded a single P-containing spot. After hydrolysis in 6 N-HCl for 8 hr at 105°, the only products detectable were orthophosphorus and muramic acid. The P-containing compound in fraction 3 probably was, therefore, a phosphomuramic acid.

The organo-P in the first fraction showed only one P spot which did not move from the origin in thin-layer chromatograms. The spot reacted very quickly with reagents for amino acids (ninhydrin), for amino sugars (Elson-Morgan reagent) and for reducing sugars ($AgNO_3$). The P-containing material was eluted from a chromatogram, hydrolysed with 6 N-HCl for 12 hr at 100°, and the products subjected to 2-dimensional thin-layer chromatography. Only three components were detected; they were identified as muramic acid, lysine and ortho-P, in the molar ratio of 1:1:4·8. The unhydrolysed material did not react with fluorodinitrobenzene (FDNB), and DNP-lysine was not detected in the acid hydrolysate of an FDNB-treated specimen. The behaviour of the organo-P compound in fraction 1, particularly its very low reactivity when unhydrolysed, suggests that it may be a polymer of phosphomuramic acid and lysine. In hydrolysates of the corresponding fraction obtained from resistant residues of the spore coats of *Bacillus subtilis* 6633 and *B. polymyxa* 842, lysine was not detected; only muramic acid and ortho-P were found and column fraction 1 from these two organisms is considered to be a phosphomuramic acid polymer.

DISCUSSION

This work indicates that the spore coats of some *Bacillus* species are chemically and anatomically heterogeneous. Further, in accord with results of other investigators, substantial differences exist in the proximate composition in different species, even between strains of one species. However, a semblance of the sameness along with diversity which characterizes the walls of vegetative bacterial cells (Salton, 1964; Weidel & Pelzer, 1965) seems likely.

Judging from the exploratory analyses reported here, the coat chemistry suggests adaptation for a specialized function in the spore. Structural rigidity, physical toughness and metabolic inertness in biological systems are commonly provided by polysaccharides and protein. Identification of major components of the coats as a phosphomuramyl polymer [a 'murein' according to Weidel & Pelzer (1965)], probably as a part of a phosphomucopeptide [a 'muropeptide' or a phosphomuroprotein (Weidel & Pelzer, 1965)], and also a protein whose chemical and physical properties resemble those of keratins (Crewther, Fraser, Lennox & Lindley, 1965), provides a rationale for coat structure and exoskeletal function. The high content of glycine, cystine and dicarboxylic acids found in the paracrystal fraction is characteristic of most keratins. It also seems likely that the high tyrosine content and perhaps the amounts of some of the other amino acids in the alkali-soluble protein, and also the high lysine content of the resistant residue, represent an adaptation for specialized function in the coat

structure. The gross spore coats of *Bacillus licheniformis* contain tyrosine and glycine as major amino acids (Snoke, 1964). In all probability the cystine enrichment discovered by Vinter (1961) in bacterial spores and the keratin identified by X-ray crystallography in spore coats (Kadota, Iijima & Uchida, 1965) correspond to the keratin-like protein isolated as our paracrystal fraction.

The electron micrographs and the physicochemical properties presented suggest that in *Bacillus megaterium* QM B1551 at least one coat layer is composed of units more or less selectively dispersible by mild sonic treatment. Similar electron microscope evidence has been obtained with spore coats of *Bacillus megaterium* 988; these have yielded ultramicroscopic particles of various sizes and also discrete smaller units which appear to be uniform and primary (Williams, Holdom & Foster, unpublished data). Recently, we have also succeeded in visualizing *in situ* in spore integuments regular arrays of uniform macromolecular particles in sheet-like layers (see Gerhardt & Ribi, 1964, on ultrastructure in the exosporium of *B. cereus* spores). The layers of *Bacillus megaterium* 998 are composed of parallel chains of the particles (Suzuki, Williams & Foster, unpublished data). Probably the tendency of solutions of the keratin fraction to associate spontaneously to form paracrystal structures (Pl. 1, figs. 1–4; Pl. 4, fig. 8) is a reflexion of the presence of subunits which exhibit a polarity through an abundance of surface carboxyl and sulfhydryl groups (Table 2).

The exchangeable cations known to play significant roles in heat resistance (Alderton & Snell, 1963) and in germination (Rode & Foster, 1966*a*), and which are bound by isolated coat fractions (Foerster & Foster, 1966), possibly interact with the dicarboxylic amino acids contained in abundance in the paracrystal fraction. Of course, interaction with coat muropeptides is also a possibility. Likewise, there is growing evidence of involvement of –SH groups, very likely those of the spore paracrystal keratin fraction, in radiation- and heat-resistance (Vinter, 1961) and in the dormancy–heat activation interplay (Gould & Hitchins, 1963; Keynan, Evenchik, Halvorson & Hastings, 1964; Keynan, Issahary-Brand & Evenchik, 1965).

It may not be without significance that numerous substances, including hydrocarbons, long-chain alcohols, chelating agents, detergents, electrolytes and calcium ions, which have important effects on the germination of bacterial spores, also affect the association/dissociation equilibrium of proteins (Reithal, 1963) and may have implications for dormancy versus germination.

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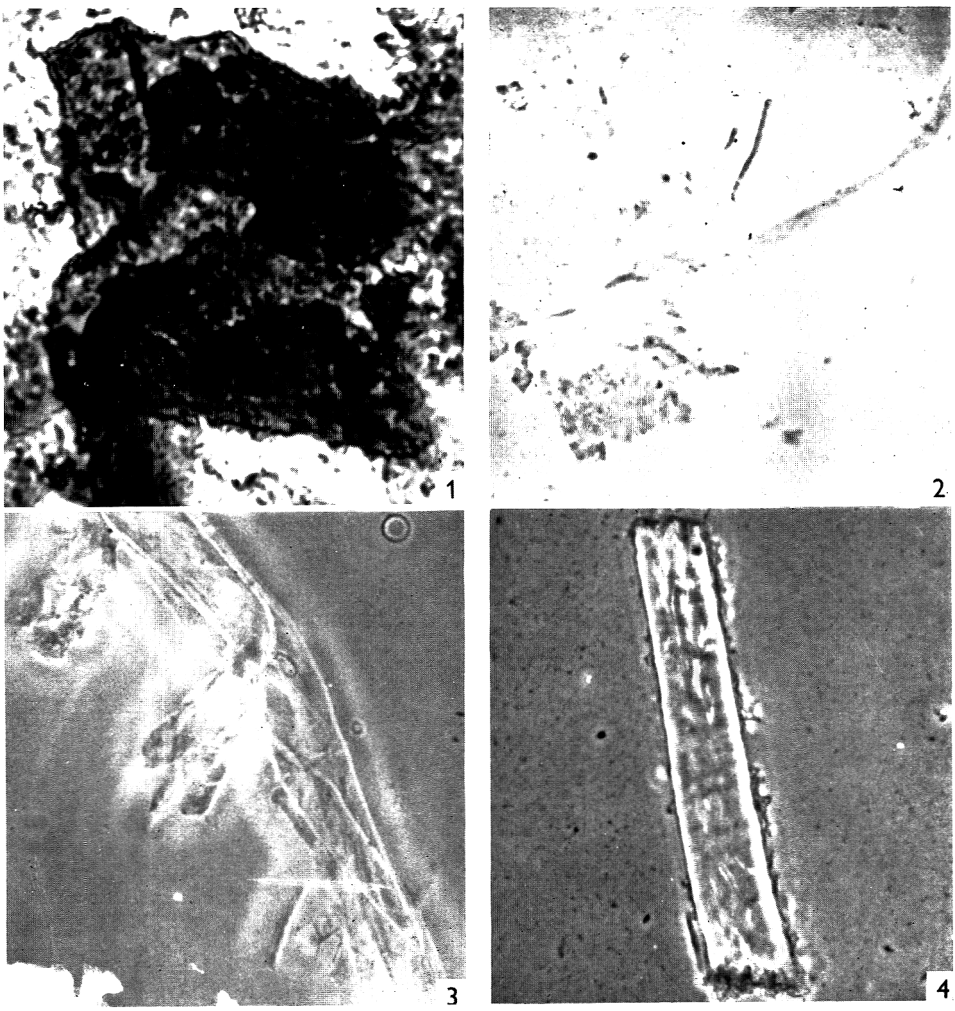
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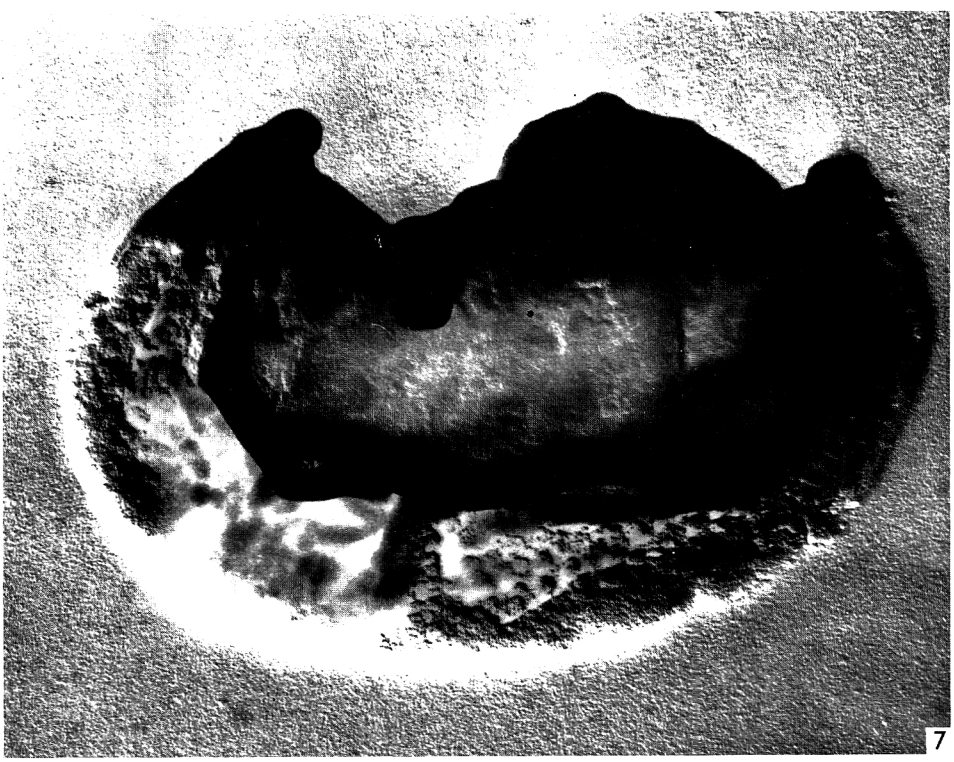

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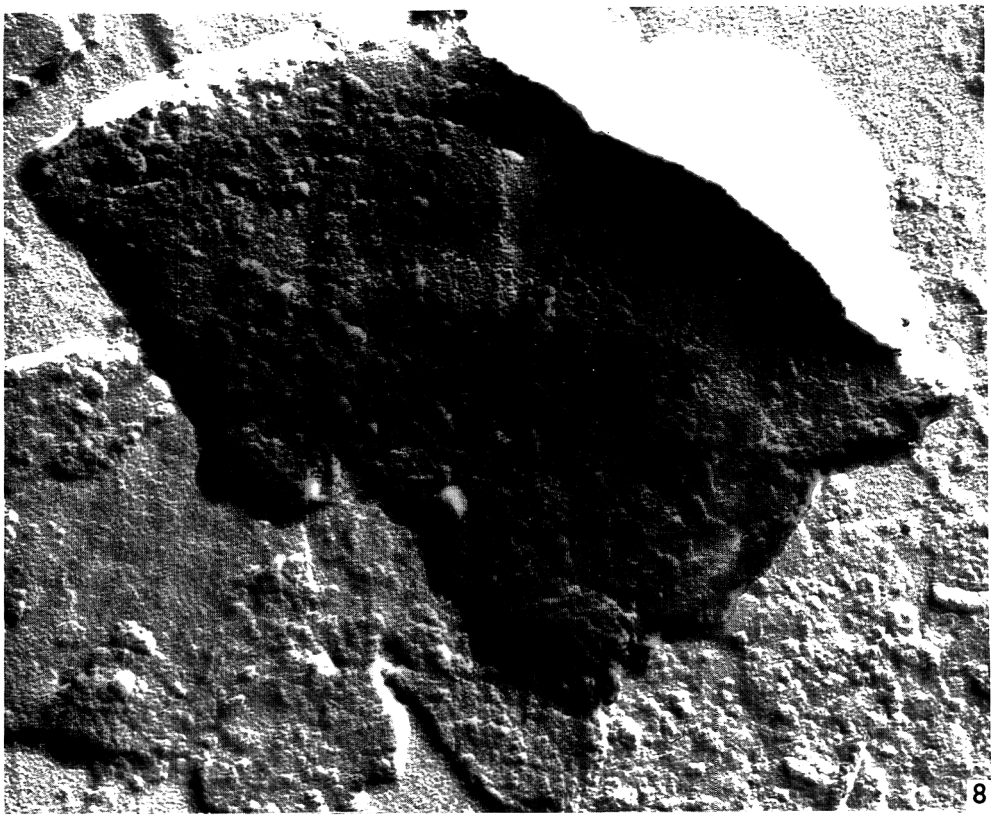
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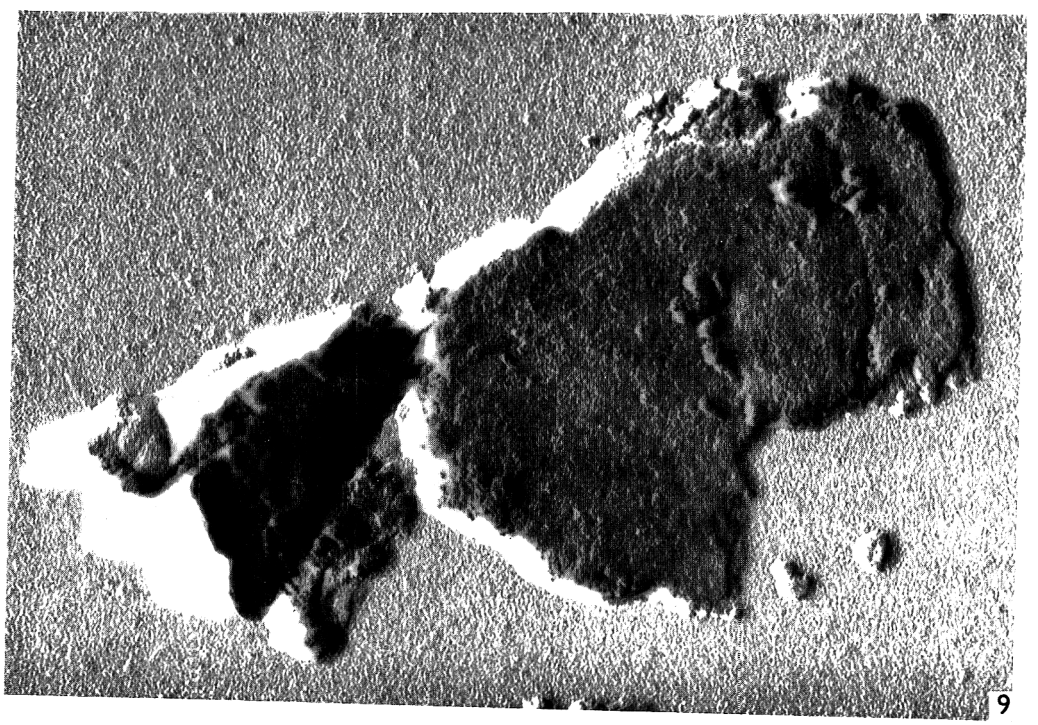
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EXPLANATION OF PLATES

PLATE 1

Figs. 1–4. Photomicrographs of 'paracrystal' solids that formed spontaneously during in-vacuum concentration of the supernatant fluids from sonic treatment (mild) of NaOH-extracted spore coats (see Fig. 1). *Bacillus megaterium* QM B1551; dark-field phase contrast. × 1000.

PLATE 2

Fig. 5. Spore coat fraction of *Bacillus megaterium* QM B1551. Preparation unshadowed. × 35,000.

PLATE 3

Spore coats of *Bacillus megaterium* QM B1551 treated with lysozyme. (200 μg. enzyme/ml. in 10 mM-tris buffer for 24 hr at 37 ; toluene added). Shadow angle 45°.

Fig. 6. The treated coat in this photograph appears to be smooth and relatively featureless, although it may be layered. × 68,000.

Fig. 7. The treated coat in this photograph appears to be composed of more or less uniform spherical particles. × 100,000. (A different field from fig. 6.)

PLATE 4

Fig. 8. Spore coats of *Bacillus megaterium* QM B1551 after successive treatment with lysozyme and extraction with NaOH (0·06 N-NaOH) for 3 hr at 50°. Shadow angle 45°, × 34,000.

Fig. 9. Spore coat particles of *Bacillus megaterium* QM B1551 resistant to sonic treatment after removal by centrifugation of the paracrystal material. A few paracrystal fraction particles adhere to the smooth basal layer which itself shows some indication of a substructure of uniform particles. Shadow angle 45°, × 100,000.

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Differentiation of Strains of *Staphylococcus epidermidis* Isolated From Bovine Udders

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SUMMARY

Strains of *Staphylococcus epidermidis* isolated from bovine udders were differentiated by comparing biochemical tests, serological typing of proteolytic enzymes, colonial morphology, and the spectrophotometric analysis of pigments extracted with methanol. The cultures were divided into proteinase-positive and proteinase-negative groups, based on their proteolytic activity on skim milk agar, staphylococcus medium no. 110 (Difco), and gelatin. Most of the proteinase-negative cultures produced acetoin, whereas the opposite was true for the proteinase-positive cultures. A further subdivision of the cultures in each group was made by using Baird-Parker's (1963) biochemical subgrouping scheme. The proteinase-positive cultures were also subdivided by serological typing of their proteolytic enzymes into five groups, B, F, G, H, and NR, a non-reacting group. Approximately three-quarters of the cultures in the first four proteinase groups could be placed in one of Baird-Parker's biochemical subgroups. The cultures in the NR group, however, were sufficiently distinctive in their biochemical reactions to be considered as a separate biochemical subgroup. The colonies formed by the cultures were classified into five types, each consisting of two to four forms. No absolute relationship was found between the spectral absorption curves of the pigments and proteinase groups, although there were differences in the types of absorption curves that predominated in each proteinase group. Nine cultures which produced coagulase were more closely related to *S. epidermidis* than to *S. aureus* in that they did not utilize mannitol anaerobically or produce α- or β-type haemolysis, they were non-pigmented and were less active biochemically. Also, serological typing of the proteolytic enzymes of three of these cultures resulted in one being classified as group G and two as group H. Group H contained only these two cultures. In contrast 27 cultures of *S. aureus* were classified as group A. Except for coagulase production, the biochemical reactions and the serological grouping of the proteolytic enzymes suggested a classification of *S. epidermidis* for the nine cultures.

INTRODUCTION

Staphylococcus epidermidis was found to be the principal cause of udder infections in a newly assembled research herd of Holstein-Friesian heifers. To study the pathogenicity and spread of the organisms in the herd, a differentiation of the strains was considered to be necessary. Differences in some characteristics of the organisms, such as colonial morphology, haemolysis, pigmentation, and biochemical reactions, indicated that the infections were caused by different strains; however, the tests used

did not provide a good method of classification. Unlike *S. aureus*, *S. epidermidis* organisms are not typable with *S. aureus* bacteriophages, they do not produce specific toxins or haemolysins, and serological classification of cellular antigens has limited value since only two groups have been identified thus far (Losnegard & Oeding, 1963). However, Baird-Parker (1963, 1965) has divided the staphylococci into six subgroups by physiological and biochemical tests, wherein subgroup I was similar to *S. aureus* and subgroups II to VI were similar to *S. epidermidis*. Sandvik & Fossum (1965) have classified certain members of the Family Micrococcaceae by serological differentiation of their proteolytic enzymes. Pigmentation also has been studied as a means of differentiating strains of *S. epidermidis* by analysing the methanol-extracted pigments spectrophotometrically (Sandvik & Brown, 1965) and by determining differences in the colour of their growth on Trypticase soy cream agar by spectral reflectance colrimetry (Brown, 1966).

This report, therefore, is concerned with the differentiation of strains of *Staphylococcus epidermidis* isolated from bovine udders by comparing physiological and biochemical tests, serological typing of proteolytic enzymes, colonial morphology, and the spectrophotometric analysis of methanol-extracted pigments. Strains of *S. aureus* isolated from bovine udders also were studied to provide a basis for comparison.

METHODS

Cultures. One hundred and ninety-two cultures of *Staphylococcus epidermidis* and 52 cultures of *S. aureus* were used in this study. All of the *S. epidermidis* and 42 of the *S. aureus* cultures were isolated from milk samples obtained from the experimental herd of cows sampled at weekly intervals over a 3-year period. The *S. epidermidis* cultures were isolated from 81 quarters of 34 cows and the *S. aureus* cultures from 18 quarters of 12 cows. Seven of the cows were infected in 10 quarters with both species at different times. Each milk sample was streaked fresh (0·1 ml.) on a bovine blood agar plate which was incubated for 24 hr at 37° and for 24 hr at room temperature (about 23°). Each culture was selected from a single representative colony and transferred to two tubes of beef infusion broth. After incubation at 37° for 24 hr, one tube was stored at −65° and the other used to inoculate media for preliminary identification.

Cultures were classified as *Staphylococcus aureus* when they were Gram-positive cocci which produced catalase and coagulase, utilized glucose and mannitol anaerobically and produced α-, $\alpha\beta$- or β-type haemolysis. Cultures were classified as *S. epidermidis* when they were Gram-positive cocci which produced catalase but not coagulase, utilized glucose but not mannitol anaerobically, and produced slight or no haemolysis.

When the detailed study was begun, each frozen culture was thawed and 0·1 ml. transferred to a tube of beef infusion broth and incubated for 20–24 hr at 37°. Each culture was then streaked on 5% (v/v) bovine blood agar and incubated for 24 hr at 37° and for 24 hr at room temperature. The colonies were graded as positive or negative for pigment, and the type of morphology noted for those cultures classified as *Staphylococcus epidermidis*. A representative colony was transferred to a Tryptose agar (Difco Laboratories, Detroit, Michigan, U.S.A.) slope which was incubated at 37° for 24 hr and then stored at 4° and used for subcultures.

Unless otherwise specified, all test media were inoculated from a fresh 24 hr Tryptose Agar slope culture and tests were read directly or performed on 1 ml. samples of culture after 2, 5 and 7 days of incubation at 37°. Enzyme activity on skim milk agar, tributyrin agar, egg yolk agar and staphylococcus medium no. 110 (Difco) was determined by the development of either a zone of clearing or a zone of precipitation around the point of inoculation. In most tests, the reactions are reported as negative or positive for ease in making comparisons, even though various gradations of reactions were observed and recorded.

Blood agar. Washed red blood cells from 10 ml. of bovine blood were added to 200 ml. melted beef infusion agar (beef infusion; peptone, 1%; NaCl, 0·5%; agar, 2%) which had been cooled at 45°.

Acid production from carbohydrates. Anaerobic acid production was determined by making stab cultures in phenol red glucose and mannitol agars (Difco) which contained 1% carbohydrate. Before use, the media were steamed at 100° for 10 min. to drive off oxygen and then cooled at 4°. The cultures were incubated anaerobically in Brewer jars in an atmosphere of hydrogen for 5 days.

Aerobic acid production was determined in phenol red glucose, mannitol, lactose and maltose broths (Difco) each containing 1% carbohydrate.

Coagulase production. One volume of citrated rabbit plasma was diluted with 4 vol. of a sodium chloride solution (0·85%, w/v) and 0·5 ml. of the mixture was used for each test. After inoculation, the tubes were incubated for 4 hr at 37° and a further 20 hr at room temperature. Readings were made after incubation for 1, 4 and 24 hr.

Phosphatase production. A 1% solution of phenolphthalein diphosphate (trisodium salt, Sigma Chemical Co., St Louis, Missouri, U.S.A.) was sterilized by filtration through a Seitz filter (ST-3) and added to sterile beef infusion broth to give a final concentration of 1/10,000. To test for phosphatase activity, one drop of N-sodium hydroxide solution was added to a 1 ml. sample of the broth culture. A crimson red colour was recorded as positive and a pink colour as weak positive.

Urease production. Cultures were grown on urea agar (Difco) slopes and the decomposition of urea graded as positive when the entire medium turned red and weak positive when only a part of the medium turned pink.

Acetoin production. Cultures were grown in MR-VP medium (Difco) and acetoin was detected in 1 ml. samples by adding 1 ml. of modified O'Meara's reagent (0·3% solution of creatine in 40% potassium hydroxide; *Manual of Microbiological Methods*, 1957). All cultures which were negative after incubation for 7 days were also tested by adding 0·5 ml. of 5% α-naphthol in 95% (v/v) ethanol in water, and 0·5 ml. of 0·1% creatine in 16% potassium hydroxide to 1 ml. of culture (Page, 1962). In both tests, the mixtures were incubated at room temperature and visual readings made after 4 hr.

Nitrate reduction. Cultures were grown in nitrate broth (Difco) and 1 ml. of culture tested for nitrites by adding a drop of sulphanilic acid and a drop of α-naphthylamine reagent (*Manual of Microbiological Methods*, 1957). All negative tests were confirmed by adding zinc dust to determine whether nitrate was still present or had been decomposed beyond the nitrite stage.

Asparagine utilization. Cultures were grown on slopes of a medium containing asparagine, 0·1%; glucose, 1%; potassium chloride, 0·02%; magnesium chloride, 0·02%; agar, 1·5% and bromcresol purple, 0·0016% (*Bergey's Manual*, 1957, footnote,

p. 455). Slopes were examined only for acid production and graded as positive when the entire medium was yellow and weak positive when the surface of the slope was partially or completely yellow.

Gelatin liquefaction. Nutrient gelatin (Difco) in screw-capped tubes was inoculated and incubated at 30° for 4 weeks. Examination for liquefaction was made at weekly intervals after the tubes had been refrigerated at 4° for 30 min. Cultures were considered negative for gelatinase activity when there was no liquefaction or only a thin layer of liquid on top, weakly positive when only the top $\frac{1}{4}$–$\frac{1}{2}$ in. (0·7–1·3 cm.) of medium was liquid, and positive for all other degrees of liquefaction.

Skim milk agar. The medium was prepared by adding 25 ml. of fresh skim milk to 100 ml. of hot beef infusion agar, mixing, and then pouring into Petri plates. The milk was obtained aseptically from a non-infected udder, centrifuged, and then skimmed. Sixteen cultures were point-inoculated on each plate. The plates were incubated at 30° for 48 hr and proteinase activity graded according to the size of the zone of precipitation (Sandvik, 1962).

Staphylococcus medium no. 110 (S-110 medium). Four cultures were tested on each Petri plate, containing 12 ml. medium, by making a streak about $1\frac{1}{2}$ in. (4 cm.) long from the periphery toward the centre of the plate with a loopful of a 24 hr broth culture. After the plates had been incubated at 30° for 48 hr, the amount of growth was graded as good (growth confluent), fair (growth not confluent and with a nodular appearance), poor (growth sparse or only to be seen with a hand lens), or negative. Cultures which showed good growth were also graded as smooth or rough according to the type of growth. Smooth growth appeared moist and glossy, and rough growth usually appeared dry with the edges frequently irregular. Final classification of the type of growth was based on its wettability. When the surface of the medium was flooded with a 20% sulphosalicylic acid solution to test for gelatinase activity, the liquid readily flowed over the smooth growth on immediate contact; but the rough growth remained unwetted until about 10 ml. of liquid had been added to each plate. The rough growth frequently separated from the medium and floated in the liquid.

Gelatinase production was determined, 10–15 min. after the addition of the sulphosalicylic acid, by grading the amount of clearing around each culture.

Tributyrinase production. A mixture of 2 ml. of a 1% solution of polysorbate 80 (Tween 80, Atlas Chemicals, Wilmington, Delaware, U.S.A.), 1 ml. of tributyrin (Eastman Organic Chemicals, Rochester, New York, U.S.A.), and 16 ml. of an 0·85% solution of sodium chloride was autoclaved at 121° for 15 min. After being autoclaved, the hot mixture was shaken vigorously for 1 min. to emulsify the tributyrin. The emulsion was then mixed with 80 ml. of melted beef infusion agar and poured into Petri plates. Sixteen cultures were point-inoculated on each plate. The plates were incubated for 48 hr and tributyrinase activity was indicated by a zone of clearing around the culture. This is the medium described by Marks (1952) except that beef infusion agar was substituted for nutrient agar.

Egg yolk agar. A 10% suspension (v/v) of fresh egg yolk was made aseptically in beef infusion broth. The suspension was heated to 50° and mixed with an equal volume of beef infusion medium containing 3% agar. The melted beef infusion agar was cooled to 50° before adding the egg yolk and pouring the mixture into Petri plates. Sixteen cultures were point-inoculated on each plate. The plates were incubated for

48 hr and 'lipase' activity was indicated by the formation of a cloudy zone usually surrounded by a clear or lytic zone. A pearly surface sometimes developed within the cloudy zone.

Colonial morphology. All studies on colonial morphology were made with broth cultures that were spread on blood agar plates and incubated at 37° for 24 hr and at room temperature for a further 24 hr. The classification of colonial types was based on single colonies that were sufficiently separated from other growth to permit maximum development of size and shape. Photographs of representative colonies were used for comparison and the colonial morphology of each culture was designated by the identification number of the culture(s) they resembled.

Pigments. Pigments were extracted from whole organisms with hot methanol and examined spectrophotometrically (Sandvik & Brown, 1965). The spectral absorption curves were classified into seven types, designated I, II, III, IV, V, VI, and a *Staphylococcus aureus* type. Two subtypes were included in each of types I and III; all non-pigmented extracts were classified as type I. The extracts from 95 cultures of *S. epidermidis* were classified by this method.

Serological classification of proteinases. The cultures were classified for proteinase activity on the basis of their reactions on skim milk agar, nutrient gelatin and S-110 medium. Cultures that were positive on any one of the three media were subdivided into groups according to the serological specificity of the proteinase as determined by the inhibition of enzyme activity by a specific antiserum (Sandvik & Fossum, 1965). The specific antiproteinase was separated from normal proteinase inhibitors in immune rabbit sera by paper electrophoresis. The paper strip was then applied to a caseinate medium; after incubation the strip was removed from the medium and replaced by narrower strips of filter paper that had been immersed in solutions containing the proteinases to be tested. The antiproteolytic effect of the antiserum was demonstrated when the specific enzyme was prevented from precipitating the sodium caseinate in the area of the γ-globulin. Liquefied gelatin was used as a source of the enzyme for each culture. When cultures did not liquefy gelatin, enzymes were obtained by growing the organisms on a skim milk agar (agar concentration 0·8–0·9%; Sandvik, 1962). When this study was begun there were available five specific antisera, designated A, B, C, D, E (Sandvik & Fossum, 1965). However, three additional antisera were prepared during the study and designated F, G, H. Cultures were classified proteinase-negative when they showed no reaction with all three of the media used to determine proteinase activity.

RESULTS

Biochemical studies

The biochemical reactions of *Staphylococcus aureus* and *S. epidermidis* cultures grown on various media are compared in Table 1. Eight cultures which were classified as *S. aureus* met all of the requirements that were used for classifying them in this species except that, they did not ferment mannitol (two cultures), or they produced only slight haemolysis (four cultures), or they showed a combination of both characteristics (two cultures). We considered them to be *S. aureus* because they were variants of strains which fermented mannitol and produced $\alpha\beta$-type haemolysis. Nine cultures which produced coagulase were not included with the *S. aureus* cultures in Table 1

because they were more like *S. epidermidis* in growth characteristics and metabolic activity. Consequently, these cultures are treated as a coagulase-positive group in Tables 2–4 with the *S. epidermidis* cultures.

The *Staphylococcus aureus* cultures gave more positive reactions than the *S. epidermidis* cultures in all the tests made. The greatest difference between the two species occurred with those tests used routinely for their differentiation (i.e. coagulase, haemolysis and fermentation of mannitol).

Table 1. *A comparison of biochemical reactions of cultures of Staphylococcus aureus and S. epidermidis*

	Staphylococcus aureus		*Staphylococcus epidermidis*			
			Proteinase positive		*Proteinase negative*	
Substrate or test	No. of cultures	%	No. of cultures	%	No. of cultures	%
Coagulase production	43/43	100	0/137	0	0/55	0
Anaerobic acid production						
Glucose	43/43	100	137/137	100	55/55	100
Mannitol	39/43	91	1/137	0·7	0/55	0
Aerobic acid production						
Glucose	43/43	100	137/137	100	55/55	100
Mannitol	43/43	100	89/137	65	35/55	64
Lactose	43/43	100	127/137	93	50/55	91
Maltose	43/43	100	75/137	55	51/55	93
Milk agar precipitation	43/43	100	137/137	100	0/55	0
Gelatin liquefaction						
Positive	35/43	81	70/137	51	0/55	0
Weak positive	6/43	14	6/137	4	0/55	0
Negative	2/43	5	61/137	45	55/55	100
Staphylococcus medium no. 110 (Difco)						
Gelatinase production						
Positive	20/43	47	125/137	91	0/55	0
Weak positive	20/43	47	0/137	0	0/55	0
Negative	3/43	6	12/137	9	55/55	100
Type of growth						
Good smooth	38/43	88	34/137	25	7/55	13
Good rough	0/43	0	60/137	44	0/55	0
Fair, poor, negative	5/43	12	43/137	31	48/55	87
Acetoin production	41/43	95	7/137	5	47/55	85
Phosphatase production						
Positive (strong)	43/43	100	129/137	94	10/55	18
Positive (weak)	0/43	0	7/137	5	37/55	67
Negative	0/43	0	1/137	0·7	8/55	15
Nitrate reduction	43/43	100	130/137	95	33/55	60
Urease production	43/43	100	131/137	96	33/55	60
Egg yolk agar	33/43	77	10/104	10	6/41	15
Tributyrinase production	38/43	88	49/104	47	23/41	56
Asparagine utilized	41/43	95	69/104	66	39/41	95
Haemolysis						
α-β or β	37/43	86	0/137	0	0/55	0
Slight	6/43	14	24/137	18	51/55	93
Negative	0/43	0	113/137	82	4/55	7

The *Staphylococcus epidermidis* cultures were divided into proteinase-positive and proteinase-negative groups to demonstrate metabolic differences within the species based on this classification (Table 1). Both groups showed comparable results for the aerobic utilization of glucose, mannitol and lactose, and for lipolytic activity on egg yolk agar and tributyrin agar. The proteinase-positive cultures, considered as a group, were able to grow better on S-110 medium and gave approximately one-third (30–40%) more positive reactions than did the proteinase-negative cultures in the reduction of nitrate to nitrite and in the production of urease. However, the proteinase-positive cultures were less active than the proteinase-negative cultures in utilizing maltose aerobically, and in utilizing asparagine as the sole source of nitrogen. The principal differences between the two groups were found for the production of phosphatase, acetoin, and haemolysis.

Phosphatase. All except one of the proteinase-positive cultures were positive for phosphatase. A strong positive test was given by 87% of these cultures after 2 days of incubation and by an additional 7% of cultures after 7 days. The other 5% produced only weak positive reactions. Although 85% of the proteinase-negative cultures were positive for phosphatase, a strong positive reaction for phosphatase was produced by only 18% of the cultures; 13% after 2 days and the other 5% after 5–7 days. Those cultures which made up the 13% were also the only proteinase-negative cultures that developed good smooth growth on S-110 medium. In all instances, when a culture gave a weak positive test at the end of 2 days it always gave a strong positive test by the fifth day. Also, all cultures that were classified as weakly positive for phosphatase after 7 days never produced the weak positive reaction until after 5 or 7 days.

For comparative purposes, 34 proteinase-negative cultures which had been tested previously for phosphatase activity in liquid medium, were tested on an agar medium described by Baird-Parker (1963). The cultures that gave negative (5), weak positive (20), and late strong positive reactions (3) in the liquid medium were all negative on the agar medium. However, the 6 cultures that were strongly positive for phosphatase in the liquid medium after 2 days of incubation were also positive on the agar medium.

Acetoin. The proteinase-negative cultures were more active in the production of acetoin. Only 5% of the proteinase-positive cultures gave a positive test for acetoin with modified O'Meara's reagent, as opposed to 85% of the proteinase-negative cultures. When a more sensitive test was used (Page, 1962), the number of positive reactions increased to 17 and 94% for the proteinase-positive and proteinase-negative cultures, respectively. However, five-sixths of these additional positive reactions were graded as trace or weak. With the more sensitive test, the strong positive reactions developed as soon as the reagents were added to the culture medium, whereas the trace and weak positive reactions developed later. Comparable results with the two tests can be obtained when the less sensitive test is read after 4 hr and the more sensitive test after 30 min.

Haemolysis. The *Staphylococcus epidermidis* cultures were either non-haemolytic or slightly haemolytic. Only 18% of the proteinase-positive cultures were slightly haemolytic as opposed to 93% of the proteinase-negative cultures. The slight haemolysis appeared primarily as either a small zone (< 1 mm.) of complete or partial clearing around the colony, or as a relatively large area of partial haemolysis that was

lighter than the surrounding medium. The latter haemolysis gave the appearance of a halo and was distinguished from β-type haemolysis wherein the zone of affected red blood cells is well demarcated and appears darkened. A larger well demarcated zone of clearing that extended 1–2 mm. beyond the edge of the colony also was produced by 5% of the *S. epidermidis* cultures.

Proteinase grouping

Serology. One hundred and ten of the 137 proteinase-positive cultures of *Staphylococcus epidermidis* and 28 of the cultures of *S. aureus* were classified by serological differentiation of their proteolytic enzymes with specific antiserums. All of the *S. aureus* cultures were classified as group A except one, which did not fall into any group. This culture was coagulase positive, produced $\alpha\beta$ haemolysis but did not utilize mannitol anaerobically and was a weak producer of proteinase. Sixty-six of the *S. epidermidis* cultures were placed in one of three groups; 50 in B, eight in F, and eight in G. The other 44 cultures produced proteolytic enzymes that did not react with any of the antisera and were classified in a non-reacting (NR) group.

A comparison of the cultures of *Staphylococcus epidermidis* separated into the various serological groups showed that the cultures in each group were related in metabolic activity (Table 2). Included in Table 2 are seven of eight tests used by Baird-Parker (1963) as a basis for his biochemical grouping of staphylococci (i.e. coagulase, phosphatase and acetoin production, fermentation of mannitol, and oxidation of mannitol, lactose and maltose) plus gelatin liquefaction and growth on S-110 medium, which we found useful in classifying the cultures. The nine cultures that produced coagulase were placed in a separate group to show their close relationship to *S. epidermidis*. Two of these cultures were typed as group H and one as group G while the other six were not tested serologically. Three cultures in group NR were omitted because their biochemical and growth characteristics were sufficiently different to indicate they were not related to the other 41 cultures.

Proteinase-positive cultures. In Table 2, the cultures in groups B and NR showed the greatest differences in their biochemical reactions and growth characteristics. The group B cultures were the least active and the NR cultures the most active in the aerobic utilization of mannitol and maltose. Although 36% of the B cultures were graded as positive for the aerobic utilization of mannitol, two-thirds of these reactions were weakly positive. Almost all of the B cultures liquefied gelatin (98%) but none of the NR cultures, even though all of the latter group were gelatinase-positive on S-110 medium. Only the B cultures produced fair, poor, or no growth on S-110 medium, whereas all of the NR cultures produced good rough growth. Although approximately half of the B cultures were graded as producing good smooth growth, the surface of the growth was irregular rather than homogeneous, and differed from the nodular appearance of fair growth only in that it was more extensive and coalescent. The cultures in group F were intermediate to those in groups B and NR and were the only proteinase positive cultures that were positive for acetoin with O'Meara's reagent. A close relationship between the cultures in groups G and NR was indicated by the fact that five of the eight group G-cultures gave test reactions identical to those given by the cultures in group NR. The coagulase positive cultures in Table 2 differed from the *Staphylococcus aureus* cultures in Table 1 by not utilizing mannitol anaerobically or mannitol and maltose aerobically, by not producing acetoin, and by not producing

α or β haemolysis. All of these cultures reduced nitrate, produced urease, and liquefied gelatin, except the two group H cultures.

Proteinase-negative cultures. These cultures were divided into two subgroups on the basis of the type of growth on S-110 medium and the production of acetoin and phosphatase (Table 2). The cultures in subgroup 1 gave fair, poor, or no growth on S-110 medium, positive tests for acetoin, and late strong positive, weak positive, or negative tests for phosphatase. The cultures in subgroup 2 gave good, smooth growth on S-110 medium, negative tests for acetoin, and strong tests for phosphatase after 2 days of incubation.

Table 2. *A comparison of biochemical reactions of various groups of proteinase positive and proteinase negative cultures of Staphylococcus epidermidis*

	Proteinase positive					*Proteinase negative*	
	Serological groups				Co-agulase positive	Sub-group 1	Sub-group 2
Substrate or test	B (50)*	F (8)	G (8)	NR (41)	(9)	(48)	(7)
	Percentage of cultures positive						
Coagulase production	0	0	0	0	100	0	0
Phosphatase production							
Positive (strong)	100	100	100	98	100	6	100
Positive (weak)	0	0	0	2	0	77	0
Negative	0	0	0	0	0	17	0
Acetoin production	0	75	0	0	0	98	0
Acid production							
Mannitol (anaerobic)	0	0	0	0	0	0	0
Mannitol (aerobic)	36	38	100	100	0	65	57
Lactose (aerobic)	100	75	62	98	100	90	100
Maltose (aerobic)	4	100	100	100	0	98	57
Gelatin liquefaction	98	76	0	0	78	0	0
Staphylococcus medium 110 (Difco)							
Type of growth							
Good smooth	48	63	12	0	100	0	100
Good rough	0	37	88	100	0	0	0
Fair, poor, negative,	52	0	0	0	0	100	0

* Number of cultures per group.

Colonial morphology

A study of the colonial morphology of *Staphylococcus epidermidis* was made to determine the different colonial forms that developed, to classify into types the forms which showed similarities in their structure and development, and to determine any relationship between the colonial types and proteinase groups. Preliminary studies showed that the colonies formed on blood agar by cultures isolated directly from raw milk were smaller and in some instances rougher, drier, and more irregular in shape than the colonies formed after the cultures were grown in beef infusion broth and re-isolated on blood agar. The effect on the colonial morphology was attributed to the raw milk and not to a change that developed after the cultures had been isolated and grown in broth. This was shown when smaller irregular colonies were produced by suspending the growth from broth cultures in raw milk and then spreading 0·1 ml.

of this milk on a blood agar plate. The effect of raw milk was nullified by diluting the milk 10 times with 0·85% sodium chloride solution before inoculating the blood agar.

The colonies formed by the cultures of *Staphylococcus epidermidis* were classified into five types, each consisting of two to four forms (Plate 1). More than one form of a type might develop on the same agar plate. The surface of most colonies appeared moist (glossy finish) although some appeared dry (mat finish).

The S-1 type consisted of colonies which were smooth and with entire margins. The differentiation into four forms was based on surface variations that consisted of the presence or absence of a raised centre and ring formations caused by differences in coloration between the centre and peripheral zones. When growth of the other S types was heavy, the colonies frequently appeared as S-1 type.

Type S-2 colonies were smooth with entire margins and characterized by a peripheral zone that was well demarcated from the central area of the colony. The two forms were differentiated by the degree of demarcation, with form (*b*) showing the most demarcation and a distinct dome-shaped central area. The peripheral zone was lighter in colour in both pigmented and non-pigmented colonies and in some instances was translucent. Form (*b*) with a translucent peripheral zone was associated more frequently with small colonies. A slightly depressed area developed in the centre of some colonies which gave the appearance of a small dark circle.

Type S-3 colonies were smooth with finely serrate or undulate margins and the two forms were differentiated by the degree of irregularity of the margin. Some colonies showed raised centres while others appeared to have a flat central area surrounded by a peripheral collar.

Table 3. *A comparison of the types of colonies associated with the various groups of Staphylococcus epidermidis*

Colonial morphology type	Serological groups				Coagulase positive	Proteinase negative
	B (50)*	F (8)	G (8)	NR (41)	(9)	(55)
S-1	1	2	1	3	9	44
S-2	49	0	0	0	0	0
S-3	0	0	2	35	0	0
s-1	0	3	0	0	0	11
R	0	3	5	3	0	0

* Number of cultures per group.

Type s-1 colonies were smooth with entire margins and were classified separately from the S-1 type because of their small size. They had the appearance of a flat smooth button and were differentiated into two forms on the basis of the presence or absence of ring formations caused by differences in coloration between the centre and peripheral zones.

Type R colonies were rough with irregular lobate margins. The four forms were differentiated by the degree of lobation and the degree of wrinkling of the surface in the central area of the colony.

A comparison of the colonial types produced by the organisms in each group is presented in Table 3. A relationship in the type of colony produced by the

cultures in each group existed for the cultures in the serological groups B and NR, the coagulase-positive and the proteinase-negative cultures. All of the cultures in group B, except one, produced S-2 type colonies, the majority (85%) of the cultures in group NR produced S-3 type colonies, the nine coagulase-positive cultures produced S-1 type colonies, and the proteinase-negative cultures produced either S-1 or s-1 type colonies. All of the proteinase-negative cultures in subgroup 2 produced S-1 type colonies. No relationship existed between the colonial type and proteinase group for the cultures in groups F and G, although the cultures in group G produced the same three colony types as the cultures in group NR.

Table 4. *A comparison of the types of spectral absorption curves associated with the various groups of Staphylococcus epidermidis*

	Serological groups					
Type of spectral absorption curve*	B (13)†	F (8)	G (8)	NR (26)	Coagulase positive (9)	Proteinase negative (31)
I (non-pigmented)	0	8	4	2	9	4
II	4	0	2	0	0	10
III	0	0	1	20	0	3
IV	2	0	1	4	0	0
V	1	0	0	0	0	12
VI	5	0	0	0	0	0
S. aureus	1	0	0	0	0	2

* Sandvik & Brown (1965).
† Number of cultures per group tested.

Pigments

The spectral absorption curves of the pigments extracted with methanol were determined for 95 cultures of *Staphylococcus epidermidis*. A comparison of the absorption curves produced by the cultures in each proteinase group is given in Table 4. No absolute relationship existed between these two characteristics, although there were differences in the types of absorption curves that predominated in each proteinase group. The predominant types of absorption curves associated with groups B, NR, and proteinase-negative cultures were II and VI, III, and II and V, respectively. In addition, seven of the eight cultures in group F and the nine coagulase-positive cultures were non-pigmented. The one culture in group F was so poor in pigment production that colonies developed only a trace of pigment on tryptose agar and the spectral absorption curve produced with methanol-extracted pigment was classified as type Ib. Only the group B cultures produced the type VI curve, and all of the type V curves, except one, were produced by proteinase-negative cultures. The type II and VI curves were associated with poor pigment production since the extracts had to be concentrated threefold before satisfactory curves could be obtained.

DISCUSSION

All of the cultures of *Staphylococcus epidermidis* and most of the cultures of *S. aureus* used in this study were isolated from the udders of cows in one dairy herd. The results, therefore, may be biased, in that all cultures with similar characteristics,

regardless of their source, could be considered to be the same strain. Consequently, the results with any of the tests performed could have been influenced by how well any one strain spread in the herd and by its frequency of isolation and inclusion in the group of cultures tested. In spite of this, we feel that the cultures used in this study are representative of the strains of *S. epidermidis* that infect bovine udders. However, any conclusions made about the differentiation of this species apply only to bovine strains.

In most instances, the separation of the cultures into *Staphylococcus aureus* and *S. epidermidis* was accomplished on the basis of coagulase production and the anaerobic utilization of glucose and mannitol. However, nine cultures that produced coagulase were more closely related to *S. epidermidis* than to *S. aureus*, for the following reasons. These nine cultures did not utilize mannitol anaerobically; they did not produce α- or β-type haemolysis and were non-pigmented; they gave biochemical reactions, except for coagulase, similar to Baird-Parker's subgroup III rather than subgroup I, which contained the *S. aureus* (Baird-Parker, 1963); the proteolytic enzymes of three of the nine cultures tested were serologically classified as groups G and H, whereas the *S. aureus* cultures in this study and those studied by Sandvik & Fossum (1965) were classified in group A. In addition, the two group H cultures were resistant to lysis with *S. aureus* bacteriophages when tested by Dr E. H. Coles (College of Veterinary Medicine, Kansas State University, Manhattan, Kansas, U.S.A.). The possibility that the nine cultures did not produce coagulase but coagulated citrated rabbit plasma by utilizing citrate was disproved by the fact that they also coagulated heparinized rabbit plasma. These organisms are not strains of *S. aureus* which have lost certain characteristics while being maintained in the laboratory. For instance, two of the cultures were representative of 47 and 50 isolates obtained separately at weekly intervals from two infected quarters. All of these isolates were tested for coagulase, haemolysis, pigment, and anaerobic utilization of glucose and mannitol: all showed the same characteristics as the nine cultures described above.

According to Baird-Parker (1965), these cultures would be classified as *Staphylococcus aureus* since they are positive in two of the three tests he recommended for recognition of this species (i.e. coagulase, phosphatase, fermentation of mannitol). He suggested that further confirmation of diagnosis can be obtained by determining phage typability, haemolysin pattern and ability to grow in normal human serum. The latter test was not used with our cultures, but the results in the other two tests would not add any support to the classification of *S. aureus*. Regardless of their classification, these nine organisms are sufficiently different for them to be easily distinguished from the usual strains of *S. aureus* that infect bovine udders. However, if $\alpha\beta$- or β-type haemolysis is the only criterion used for the identification of staphylococci, as is frequently used in the routine diagnosis of udder infections, these cultures would be diagnosed as non-haemolytic or non-pathogenic staphylococci or just micrococci.

As a result of this work, we feel that the determination of proteolytic activity is valuable in the differentiation of strains of *Staphylococcus epidermidis*. This is best done on skim milk agar; however, testing for the liquefaction of gelatin and gelatinase production on S-110 medium is useful in confirming the proteolytic activity of a culture, if a weak reaction is produced on the milk agar. The liquefaction of gelatin is the poorest indicator of proteolytic activity. The 41 cultures in the NR group were unique, as a group, in that they were unable to liquefy gelatin in the tube yet they

produced gelatinase on S-110 medium. It was thought that this resulted because, either the optimum temperature of incubation was not used, or conditions in the tube were insufficiently aerobic. When six cultures of this group were grown in nutrient gelatin medium in tubes and on Petri plates and incubated at 20°, 30° and 37°, none of the cultures liquefied the gelatin. Apparently this medium is not conducive to the production of proteolytic enzymes by this group of organisms.

Use of the S-110 medium was also of value in showing differences in growth characteristics of the organisms. The difference between the cultures that gave good smooth growth and good rough growth may be caused by a greater amount of lipid in the organisms of the rough-growing cultures. This opinion is based on the observation that the rough type of growth was unwetted by a 20% sulphosalicylic acid solution or by water when either was added to the surface of the solid medium and allowed to flow over the plate, whereas the same type of growth was readily wetted and covered by light petroleum.

Acetoin production was found to be useful as a differential test by Abd-el-Malek & Gibson (1948) and was used by Shaw, Stitt & Cowan (1951) to subdivide the coagulase-negative staphylococci. In some respects, a division of our *Staphylococcus epidermidis* cultures based on acetoin production would give a breakdown similar to our proteinase positive and proteinase-negative groups (with some exceptions), in that most of the proteinase-positive cultures were acetoin negative and most of the proteinase-negative cultures were acetoin positive (Tables 1, 2). Of course, more exceptions occur when the test for acetoin is a more sensitive one. Our experience with two tests of different sensitivity tends to confirm the observation made by Shaw *et al.* (1951) that a test can be too sensitive and lose its differential value. Although we did only a limited study, a similar situation developed when two phosphatase tests were compared. The method which uses a broth medium was more sensitive than a method which uses an agar medium (Baird-Parker, 1963). Nevertheless, the latter method may be better because the weak phosphatase-producing cultures appear negative on the agar medium, which permits a simple positive or negative classification of cultures.

By utilizing seven of the eight tests proposed by Baird-Parker (1963) for grouping staphylococci, a comparison was made of the reactions given by the cultures in our groups with the reaction scheme given for his (B–P) subgroups. The test not made by us and omitted from the comparisons was the determination of growth at 10°.

All of the cultures in the proteinase group A were classified in the B–P subgroup I which contained the *Staphylococcus aureus*. Only four cultures in group F were classified as B–P subgroup II and none of the cultures was classified as B–P subgroup IV. Thirty group B cultures (60%) and three cultures of the proteinase-negative subgroup 2 could be placed in B–P subgroup III. The coagulase-positive cultures, not classified as *S. aureus*, showed agreement with B–P subgroup III in all tests except coagulase. Almost all (91%) of the proteinase-negative cultures of our subgroup I could be placed in B–P subgroups V (33%) and VI (58%) when the weak positive phosphatase reactions were considered comparable to a negative rather than to a positive reaction. This seemed justified in view of the fact that 21 of these cultures gave negative tests for phosphatase when tested by Baird-Parker's method. None of the group G or NR cultures could be placed in any Baird-Parker subgroup.

It would seem that by dividing bovine strains of *Staphylococcus epidermidis* into proteinase-positive and proteinase-negative groups, further subdivision can then be

made by using Baird-Parker's biochemical subgrouping scheme or by serological grouping of the proteolytic enzymes (Sandvik & Fossum, 1965). When Baird-Parker's 8-test scheme is used for classification, consideration should be given to using staphylococcus medium no. 110 (Difco) and the liquefaction of gelatin as supplementary tests. Because the cultures in the proteinase NR group were so distinctive, and because they did not fit any of Baird-Parker's staphylococcal subgroups, it might be desirable to consider them as an additional subgroup, based on the reactions given in Table 2. It should be realized that these biochemical reactions do not represent all of the proteinase-positive cultures that fall into the NR group. Consequently, proteinase-positive NR cultures should be further differentiated into biochemical groups. If we exclude the group NR cultures from consideration, approximately three-quarters of the *S. epidermidis* cultures that were typed could be placed in one of Baird-Parker's biochemical subgroups. However, the difficulty of relying on the biochemical method alone for the differentiation of strains of *S. epidermidis* is indicated by the fact that some of the proteinase-negative subgroup 2 cultures were classified as B–P subgroup III along with proteinase group B cultures. In addition, 36% of the proteinase group B cultures were excluded from subgroup III because they utilized mannitol aerobically.

The colonial types described by us for *Staphylococcus epidermidis* are not unlike the descriptions given by Shaw *et al.* (1951) for the staphylococci. They stated that colonies were round, low, convex and opaque and that most strains produced colonies with a smooth surface and an entire edge; but a minority had rough-surfaced colonies and a few produced colonies with undulate or crenated edges. It would seem that we differ only in further differentiating their most common form into S-1, S-2 and s-1 types. Their rough-surfaced colonies and colonies with undulate edges would correspond to our types R and S-3, respectively. Although colonial morphology may have little value in the classification of staphylococci, it can be useful in differentiating strains of *S. epidermidis*.

The value of determining the spectrophotometric absorption curves of pigments and the colour of pigmented growth as methods of differentiating strains of *Staphylococcus epidermidis* has been presented in other reports (Sandvik & Brown, 1965; Brown, 1966). The data presented in Table 4 further emphasize the problem of using one method for differentiating strains of *S. epidermidis*. The pigment studies, however, have proved valuable in showing the presence of the same type of organism in the same infected quarter of an udder during a long period.

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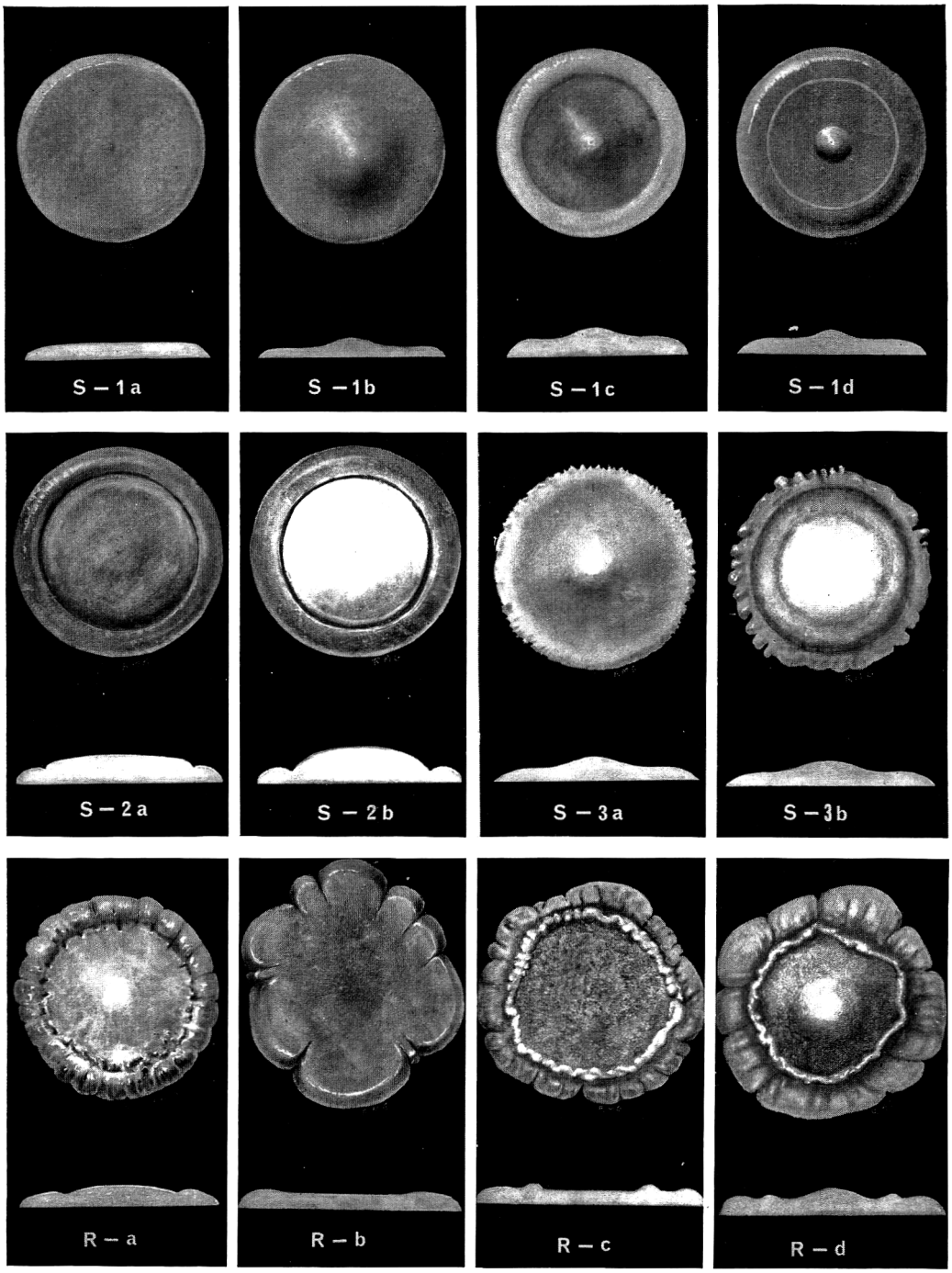

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EXPLANATION OF PLATE

Drawings of the colonial types produced by cultures of *Staphylococcus epidermidis* isolated from the milk of infected bovine udders. All colonial forms were drawn the same size, although isolated colonies of those illustrated were 1·75–2·75 mm. in diameter. S-2b colonies usually were smaller. Type s-1 colonies are not illustrated because they appeared similar to type S-1 a and c except for being smaller (1·0–1·5 mm. in diameter).

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With 1 *plate*
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A Solid Medium Test for Measuring Growth Inhibition and Neutralization of *Mycoplasma mycoides* by Immune Bovine Serum

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(*Accepted for publication* 29 *December* 1966)

SUMMARY

The growth of *Mycoplasma mycoides* var. *mycoides* was inhibited and neutralized by immune serum from cattle which had recovered from infection with *M. mycoides*. Although mycoplasmal neutralization is not necessarily the same as virus neutralization, it is suggested that this terminology be retained since it is accepted and is descriptively appropriate. The effect of immune serum on colony growth was determined by incubating test serum in plastic cylinders on nutrient agar with pre-incubated microscopic *M. mycoides* colonies. Optimal inhibition and neutralization occurred when serum and colonies were incubated at 30° before final incubation at 37°. Inhibition of growth and neutralization also occurred at 37° and 21°; inhibition but no neutralization occurred at 5°.

INTRODUCTION

Blood from cattle affected with contagious bovine pleuropneumonia (CBPP) has been reported to be bactericidal to *Mycoplasma mycoides* var. *mycoides* (Priestley, 1952). Inhibition (Edward & Fitzgerald, 1953; Bailey *et al.* 1961; Cottew, 1963) and 'neutralization' (Edward & Fitzgerald, 1954) of growth of Mycoplasma species by hyperimmune sera incorporated in liquid media have also been reported. The possibility that these phenomena may have been caused by agglutination does not appear to have been excluded.

The specific inhibition of Mycoplasma species by hyperimmune rabbit serum incorporated in the solid growth medium was reported by Edward & Fitzgerald (1953). This phenomenon has been used to elucidate the serological relationship of several Mycoplasma species (Huijsmans-Evers & Ruys, 1956; Clyde, 1964) and to detect specific antibodies in sera of individuals infected with *M. pneumoniae* (Herderschee, 1963).

A review of the literature about 'neutralization' and inhibition of Mycoplasma species by antisera indicates that these phenomena, when observed in liquid media, have not been clearly differentiated from agglutination, that neutralization has not been demonstrated on solid media, and that factors which affect the sensitivity of these phenomena have not been fully investigated. A concurrent report presents a study of mycoplasmal inhibition and neutralization in liquid media (Gourlay &

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Domermuth, 1966). The work described here was designed to elucidate further the nature of mycoplasmal neutralization and inhibition by immune sera on solid medium.

METHODS

Organism. A highly virulent strain of *Mycoplasma mycoides*, referred to as Gladysdale (Turner, 1961), was used as the test organism in this work. Seed culture was grown in modified Newing's tryptose broth (Gourlay, 1964) at 37°. The maximum viable titre (colony count) was reached in 72 hr. At this time, the culture was divided into small samples and stored at −70° until used.

Immune sera and control. A pool of immune sera was prepared from grade cattle (crosses between Zebu and European cattle) which had recovered from subcutaneous infection with the Gladysdale strain of *Mycoplasma mycoides* (Gourlay & Domermuth, 1966). This *M. mycoides* antiserum was selected as a test serum because the donor animals had proved to be immune to subsequent subcutaneous challenge with *M. mycoides*. Pooled freeze-dried normal bovine serum (Nutritional Biochemicals Corporation, batch number 5920) was obtained from the U.S.A., a country free from contagious bovine pleuropneumonia. Both pools of sera were tested for *M. mycoides* antibodies by the complement-fixation test (c.f.t.; Gourlay, 1965). The immune serum had a c.f.t. titre of 1/1280 and the normal serum had no titre to *M. mycoides*.

Preparation of nutrient agar plates for application of test sera. Solid medium (Gourlay, 1964), modified to contain 1000 i.u. penicillin/ml., thallium acetate 0·5 mg./ml. and agar 1·1 % (w/v), was dispensed to a depth of 4·0 mm. in small Petri plates (6·0 cm. internal diameter). The agar surfaces were inoculated by flooding with 1·0 ml. of a 1/10 dilution of seed culture (see above). The inoculum was allowed to remain on the surface of the agar for 15 min., excess of inoculum then poured off and the plates inverted and incubated for 7 or 14 hr at 37°. After this initial period of incubation, the plate tops were flamed and the plates returned to an upright position. Six plastic cylinders (4·5 mm. internal diameter × 6·0–7·0 mm. height) were then inserted 2·0–3·0 mm. in the agar of each plate. The plastic cylinders were sterilized before use by boiling for 20 min. in thallium acetate solution (2 mg./ml.) followed by boiling in sterile distilled water, and then vacuum-dried.

Determination of neutralization and/or inhibition of Mycoplasma mycoides by test sera. The two test sera, anti-*M. mycoides* (immune) and *M. mycoides* negative (normal), described above were heated at 56° for 45 min. and penicillin (1000 i.u./ml.) added to each. Serial doubling dilutions of serum, 1/1 (undiluted) to 1/128 were made in a liquid nutrient medium, prepared by omitting the agar from the solid medium described above. Two drops of each serum dilution were then delivered into each plastic cylinder. Two sets of plates with each of these dilutions were incubated at 5°, 21°, 30° and 37° for 24 hr and two similar sets for 72 hr. After incubation, the test sera were removed from one complete set of plates by flooding each plate with 10 ml. of the nutrient liquid medium which was used as serum diluent. The plastic cylinders were then removed, and the washing medium was removed by decantation and replaced with fresh liquid medium. The plates were then allowed to stand overnight at 21° (ambient), the liquid medium removed, and the plates inverted before and during final incubation. Final incubation of these and identical unwashed plates was at 37° for 24 hr (plates previously incubated with test serum and colonies at 30° and

37°) or 48 hr (plates previously incubated with test serum and colonies at 5° and 21°). After this final incubation, the remaining plastic cylinders were removed and all plates washed for 10 min. with M/15 KH_2PO_4 containing 0·04% (w/v) sodium azide. The plates were then stained with neutral red (equal parts of 0·5% neutral red in 95% ethanol and M/15 KH_2PO_4 plus 0·85% NaCl in distilled water; fresh stain prepared weekly) for 5 min., washed with the $KH_2PO_4 + NaN_3$ solution, and examined with a dissecting microscope (magnification ×12·5) to determine the effect of the test sera on colony growth.

Absorption of test sera with Mycoplasma mycoides. Test sera were twice absorbed with freeze-dried *M. mycoides* (20 mg./ml.) for 1 hr at 37°, followed by 3 hr at 21° and 18 hr at 5°. After absorption the sera were twice centrifuged at 2000*g* for 30 min. The supernatant sera were retained and tested for neutralizing and inhibitory activities as described above except that only the most sensitive form of the test was used (30° incubation of test sera and mycoplasma colonies).

Effect of complement on neutralizing and inhibitory activities of test sera. One per cent of 5% (v/v) of guinea-pig serum (complement-fixing activity of 100 minimal haemolytic doses as determined for the complement-fixation test; Gourlay, 1965) was added to the solid medium and to test serum dilutions. Inactivated (56° for 45 min.) control sera were similarly added to control plates. The effect of these components on neutralization and inhibition was determined by the most sensitive test procedure as above.

Examination for growth-inhibitory antibodies in serum of normal cattle and of cattle which had been infected with M. mycoides. One hundred samples of sera with no c.f.t. titre against *M. mycoides* and 10 sera from cattle endobronchially infected with *M. mycoides* were tested for inhibitory antibodies by the most sensitive test procedure.

RESULTS

The appearance of plates prepared for the application of test sera. Plates inoculated in the manner described produced an average of 25 colonies/mm.^{2}. When incubated at 37°, the organisms in the inoculum developed into colonies which grew into the agar and after 6 hr could not be washed away with test serum or nutrient medium. Such colonies were therefore usable for the test. They became visible, therefore unusable for the test, at 16 hr. As judged by continued incubation and observation of 7 hr (37°) pre-incubated colonies, slow growth of *M. mycoides* occurred at 30° and no growth occurred at 21° during the 2-week observation period.

Determination of neutralization and inhibition of Mycoplasma mycoides by test sera. Absence of growth of colonies in the presence of test serum is called inhibition; absence of growth of colonies after washing the test serum from the agar is called neutralization. The term neutralization is used to conform with previously used and accepted terminology (Edward & Fitzgerald, 1954); however, this terminology does not necessarily conform in meaning to that used in virology.

Growth of *Mycoplasma mycoides* colonies was inhibited by *M. mycoides* antiserum in all variations of the test system used (Fig. 1).

Neutralization occurred when colonies and antiserum were incubated together at 21°, 30° and 37°, but not when incubated at 5° (Fig. 1). No neutralization or inhibition was produced by normal serum; partial and complete neutralization and inhibition

of growth were produced by immune serum. Younger (7 hr) colonies were more sensitive to the inhibitory and neutralizing effects of serum than older (14 hr) colonies (Fig. 1).

Partial inhibition and neutralization were produced by immune serum dilution 1/64, complete inhibition by dilution 1/32 and complete neutralization by dilution 1/2 (Fig. 1). The appearance of uninhibited and partially inhibited colonies is shown in Pl. 1. Colonies which are completely inhibited or neutralized do not grow to visible size.

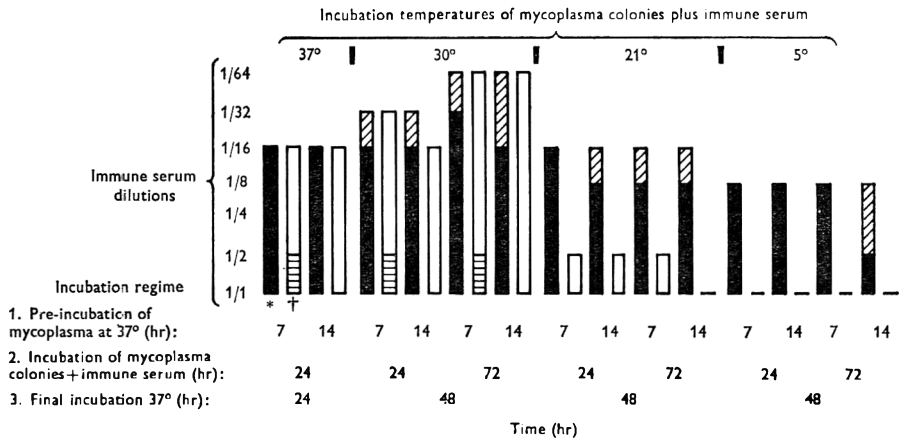

Fig. 1. Effect of physical variables on neutralization and inhibition of *Mycoplasma mycoides* colony growth by homologous antiserum from *M. mycoides* immune cattle.* Serum retained during final incubation: ▨, partial inhibition; ■, complete inhibition. † Serum removed during final incubation: □, partial neutralization; ▤ complete neutralization.

Absorption of immune serum with Mycoplasma mycoides. The neutralizing and inhibitory activities of immune serum were completely removed by absorption with *M. mycoides.*

Effect of complement. The presence or absence of complement produced no detectable change in the neutralizing or inhibitory capacity of immune serum.

Growth-inhibitory antibodies in serum of normal cattle and of cattle infected with M. mycoides. The growth of *Mycoplasma mycoides* was not inhibited by sera from 100 normal cattle but was inhibited by sera from all 10 infected cattle.

DISCUSSION

A sensitive method of detecting *in vitro* neutralization and inhibition of *Mycoplasma mycoides* var. *mycoides* by immune bovine serum has been developed. The method, while utilizing the basic method of Edward & Fitzgerald (1953), has been modified by using plastic cylinders as test serum reservoirs to maintain appropriate serum concentrations. This modification restricts the dilution of serum and permits several sera to be tested on each Petri plate, thus causing the test to be of greater potential value in the diagnosis of contagious bovine pleuropneumonia and other mycoplasmal infections which produce antisera of low inhibitory titre. The use of a temperature of 30° instead of the customary 37° for incubating organisms and sera also significantly increased the sensitivity and thus the applicability of the test.

'Neutralization' of Mycoplasma species by immune serum has been long regarded as a unique characteristic of the Mycoplasmataceae (Edward & Fitzgerald, 1954). The data presented in the present report support and strengthen Edward & Fitzgerald's (1954) findings about 'neutralization', as the present information was obtained by observing the effect of immune serum on microscopic colonies rather than in liquid medium where agglutination may have been partially or wholly responsible for diminution of mycoplasmal titre (see Gourlay & Domermuth, 1966). That all inhibitory antibody was washed from the agar before the final incubation was effectively demonstrated, as no neutralizing effect was observed on colonies incubated with immune serum at 5° (Fig. 1). If an effective antibody concentration had been present after the removal of immune serum by washing, this would have been readily apparent and some inhibition of colonies would have occurred during the final incubation at 37°.

The maximum inhibition and neutralization observed in these tests occurred at 30°, a point very near 27°, which was reported to be the lower growth limit for *Mycoplasma mycoides* (Dujardin-Beaumetz, 1900). This observation suggests that maximum neutralization and inhibition are favoured by slow growth of the test organisms; however, the fact that some neutralization was observed at 21° indicates that the phenomenon was not completely growth-dependent as might be deduced from studies in liquid medium. The reason for this apparent discrepancy is not known.

It would be of interest to determine what antigen(s) are responsible for the production of inhibitory antibody and the nature of the mechanism of inhibition. In addition, the relationship of inhibition to immunity should be elucidated as this knowledge is of great potential value to understanding the pathogenesis of contagious bovine pleuropneumonia. The serious nature of the non-specificity of conventional diagnostic tests for contagious bovine pleuropneumonia has only recently been elucidated (Shifrine & Gourlay, 1966); since conventional mycoplasmal growth inhibition tests indicate that inhibition is species-specific (Clyde, 1964), it is possible that the test described here will eliminate false positive reactions when used in the diagnosis of this disease.

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C. H. Domermuth: visiting scientist at the East African Veterinary Research Organization, while employed by the United States Department of Agriculture, Agricultural Research Service, Animal Disease and Parasite Research Division, Plum Island, Greenport, Long Island, N.Y., U.S.A.

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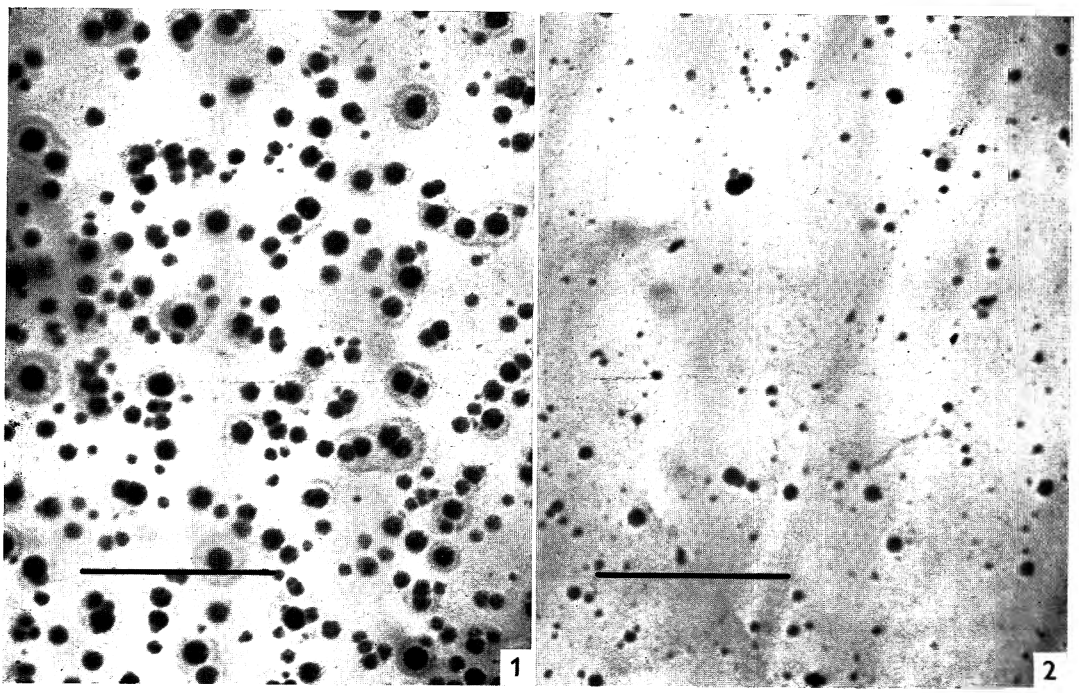
EXPLANATION OF PLATE

Inhibition and neutralization tests on *Mycoplasma mycoides* colonies.

Fig. 1. Normal serum: colonies are uninhibited and normal in appearance.

Fig. 2. Immune serum: colonies are partially inhibited by immune serum as evidenced by reduction in size. When colonies are completely inhibited or neutralized, no colony growth is visible.



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Isolation, Classification and Nutritional Requirements of Cellulolytic Cocci in the Sheep Rumen

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SUMMARY

Twenty-five strains of cellulolytic cocci were isolated from the rumen contents of seven sheep, over a period of two years. Six sheep were maintained at roughly constant body weight on chaffed lucerne hay or a lucerne chaff/oat chaff mixture. One strain was obtained from an animal on a low protein diet.

The bacteria were classified as *Ruminococcus albus* or *R. flavifaciens* by the accepted monothetic classification. Morphological, cultural, nutritional and fermentation data were then used to classify them by a numerical method. It was concluded that the numerical classification of these strains agreed closely with the classification by more established methods, but justified the assignment of relationship between members of the same species.

The nutritional characteristics of 11 strains capable of growth in partially defined medium were investigated. An organic nitrogen source was essential for three strains; the remaining eight strains grew better with casein hydrolysate than with ammonium sulphate as the sole nitrogen source. Although ammonium ions were not essential for growth, only three strains were capable of growth in the absence of volatile fatty acids. CO_2 was essential for 10 of 11 strains.

INTRODUCTION

The development of adequate cultural methods (Hungate, 1950; Sijpesteijn, 1948) has permitted the isolation and classification of cellulolytic rumen bacteria. Two species of cellulolytic cocci have been established, *Ruminococcus flavifaciens* and *R. albus* (Sijpesteijn, 1951; Hungate, 1957; Bryant, Small, Bouma & Robinson, 1958*b*). Nutritional studies of ruminococci in pure culture showed that most strains required branched or straight chain volatile fatty acids as growth factors and utilized ammonium nitrogen in preference to amino acids for protein synthesis (Bryant & Robinson, 1961*b*).

This paper describes the isolation and classification of 25 strains of ruminococci from sheep and the nutritional characteristics of eleven strains capable of growth in chemically defined medium.

METHODS

Sheep. Six merino sheep from three commercial flocks in the New England district of New South Wales were placed on diets which would maintain them at roughly constant body weight. The sheep varied in age from 4 to 8 years and included ewes and

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wethers. A permanent rumen fistula was established in each sheep. The sheep were housed indoors in separate pens on a slatted floor. Sheep C6, C18, C76, C83 and C114 were fed 800 g. lucerne chaff at 9.00 a.m. daily. C2 received 200 g. lucerne chaff + 600 g. oat chaff daily. A seventh sheep, C3, received 500 g. low protein diet (See Table 1). All sheep except C3 had been established on their rations more than 6 months before bacterial isolations were made. Sheep C3 was changed from lucerne to the low protein diet 6 weeks before sampling. The sheep had access to water at all times.

Isolation of ruminococci. Ruminococci were isolated from rumen contents by using the anaerobic roll-tube technique described by Hungate (1950), with the following modifications. Commercial CO_2 was freed from O_2 by passing it over heated reduced copper gauze. Rumen liquor was obtained approximately 8 hr. after feeding, strained through two thicknesses of cheesecloth, and collected in a 150 ml. centrifuge bottle. Air was excluded by completely filling the bottle, and closing it with a rubber stopper. The bottle was then held overnight at 2° and centrifuged at 1200 *g* for 10 min. before use. The medium used was a modification of the enrichment medium described by Halliwell (1957), having the following composition (g./100 ml.): KCl, 0·04; KH_2PO_4, 0·018; $MgSO_4.7H_2O$, 0·03; yeast extract (Difco), 0·03; indigo disulphonate, 0·0005; agar (Bacto no. 1), 1·35; $(NH_4)_2HPO_4$, 0·09; $CaCl_2.6H_2O$, 0·06; $NaHCO_3$, 0·375; cysteine HCl.H_2O, 0·05; $Na_2S.9H_2O$, 0·025; rumen liquor 20·0% (v/v). The first six ingredients were weighed out, made up with a boiled-out water and rumen liquor, then autoclaved at 121° for 15 min. The flask was cooled to 45° and the remaining ingredients added as Seitz-filtered solutions under O_2-free CO_2 or N_2. Sterile medium (9 ml.) was dispensed in 25 mm. × 150 mm. test tubes under CO_2. Each tube contained a piece of sterile filter paper (Ekwip no. 1, Industrial Equipment (A/sia) Pty, Ltd., Sydney). A sterile solution of cysteine HCl and $Na_2S.9H_2O$ was prepared (Bryant & Robinson, 1961*c*) and added to the tubes immediately before inoculation. This medium is referred to subsequently as modified Halliwell agar. When cellobiose agar was required, the filter paper was omitted and cellobiose 0·5% (w/v) was added to the medium as a Seitz-filtered solution before it was dispensed.

A decimal dilution series of eight tubes was set up for each sample of rumen content in modified Halliwell agar. The first tube was opened under a stream of CO_2 and a wide-mouthed pipette was used to transfer 1 ml. of whole rumen contents to it; the tube was then closed and its contents thoroughly mixed. Subsequent transfers were made through the stoppers by using 2 ml. Luer-Lok syringes fitted with $1\frac{3}{8}$ in. no. 18 needles. The plunger was removed and the syringe flushed with sterile CO_2 immediately before use and a fresh syringe was used for each dilution. Cellulolytic organisms were recognized by their ability to produce visible colonies and digestion on filter paper within 4 days at 38·5°. New strains were isolated from 10^{-6} dilutions so that they could be considered true rumen organisms (Gall & Huhtanen, 1951). Strains were purified by passage through the following sequence of subcultures on modified Halliwell agar: filter paper agar → cellobiose agar → filter paper agar → cellobiose agar. At each stage the isolate was serially diluted and subsequent inocula derived from tubes which showed well-isolated colonies. Colonies developing in the second series of cellobiose agar cultures usually showed no cultural or microscopic evidence of impurity and were capable of growth on filter paper strips.

Maintenance of cultures. Cultures in current use were maintained on filter paper

agar and subcultured every 4–8 days. For prolonged storage, infected filter paper was used to inoculate cellobiose agar slopes which were incubated for 48 hr at 38·5°, sealed with a mixture of 3 parts petroleum jelly to 1 part paraffin wax, quick-frozen by immersion in liquid air, and stored at −25° to −30° in a deep-freeze cabinet.

Gram reaction and morphological arrangement. Slides were prepared from the water of syneresis exuded from cultures on modified Halliwell agar containing 0·25% cellobiose after 15–24 hr. incubation at 38·5°.

Pigment production. Pigment production was recorded after 4 days incubation on filter paper strips in modified Halliwell agar. The pigment was classified as light yellow (LY), dark yellow (DY), or absent (DZ).

Production of gas. The formation of bubbles in solid media or excessive pressure in vessels containing liquid media was recorded as evidence of gas production

Fermentation of carbohydrates and the final pH values of cultures. The basal medium for these tests was the weakly buffered 20% (v/v) rumen fluid medium under 10% (v/v) CO_2 in N_2 described by Bryant *et al.* (1958*a*).

With the exception of xylan, the substrates were freshly prepared as 5% (w/v) solutions in boiled-out distilled water under N_2. A Luer-Lok syringe fitted with a Swinney adaptor containing a membrane filter (code no. G.S., Millipore Filter Corp., Bedford, Mass., U.S.A.) was used to sterilize each solution as it was added to the medium. Xylan (50 mg.) was weighed out in 15 mm. × 150 mm. test-tubes which were plugged with cotton wool and autoclaved at 121° for 15 min. The basal medium was prepared, autoclaved and dispensed into the appropriate number of xylan tubes or empty sterile tubes under an atmosphere of 10% (v/v) CO_2 in N_2. Reducing agents (Bryant & Robinson, 1961*c*), sodium carbonate solution (0·06%, w/v) and substrate were added just before inoculation.

The final pH value was determined in basal medium containing 1% (w/v) cellobiose. Duplicate 5 ml. quantities were inoculated for each strain and incubated for 7 days at 38·5°. After incubation the pH value of each tube was determined by using a Cambridge meter.

The following carbohydrate substrates were tested in basal medium at a final concentration of 0·5% (w/v) analytical reagent grade D(+)-xylose, L(+)-arabinose, fructose, glucose, cellobiose, sucrose, lactose (Oxoid, bacteriological grade) and xylan (Mann Research Labs., Inc.). The inoculum for each strain was the drop of a 15–24 hr. culture in cellobiose broth. Controls containing inoculum but no substrate, and substrate (cellobiose) but no inoculum, were set up with each series. Cultures were incubated 7 days at 38·5°. A marked decrease in pH value relative to the controls indicated that a substrate had been utilized.

Fermentation products. Small fragments of infected filter paper were used to inoculate flasks containing 100 ml. quantities of 20% (v/v) rumen liquor broth (Bryant *et al.* 1958*a*) modified to obtain a higher buffer capacity by using 100% CO_2 and 0·4% (w/v) Na_2CO_3. Each strain was inoculated into medium with and without substrate (1%, w/v, Ekwip no. 1 filter paper). After incubation at 38·5° for 4 weeks the products of cellulose fermentation were measured, corrections being made for the volatile fatty acids produced in the control flasks in the absence of substrate.

Reducing sugars were determined qualitatively on the filtered fermentation liquor by Benedict's method (Hawk, Oser & Summerson, 1954).

Organic acids were determined by silicic acid column chromatography as described by Leng & Annison (1963).

Ethanol was distilled from fermentation liquor (Neish, 1952) and estimated by using alcohol dehydrogenase (Bücher & Redetski, 1951).

Growth in the absence of rumen liquor. This was usually examined by transferring one drop of a 48 hr culture in 20% (v/v) rumen liquor broth under 10% (v/v) CO_2 in N_2 to 5 ml. of the complete, partially defined medium devised by Bryant & Robinson (1961*a*), but the organisms LY 2, LY 3 and LY 4 were tested on modified broth medium as described by Dehority (1963). Bryant & Robinson (1961*a*) medium contained: cellobiose, casein hydrolysate, $(NH_4)_2SO_4$, $CH_3COONa.3H_2O$, branched chain volatile fatty acids, vitamins, trace minerals, resazurin, cysteine HCl and $NH_2S.9H_2O$ as reductants and a 100% CO_2-bicarbonate buffer system.

Growth in the absence of CO_2. Bryant & Robinson medium was prepared and handled under 100% N_2. Sodium carbonate was omitted and the pH value was adjusted with sterile CO_2-free NaOH solution.

Growth in the absence of organic nitrogen. Casein hydrolysate was omitted from the Bryant & Robinson medium containing B vitamins and cysteine HCl prepared under 100% CO_2.

Growth in the absence of $(NH_4)^+$. Aqueous solutions of casein hydrolysate were freed from traces of $(NH_4)^+$ by adjustment to pH 10 with 20% NaOH and vigorous gassing with a stream of N_2 for 90 min.; the solution was then readjusted to pH 7·0 by adding a few drops of concentrated HCl and the remaining solid ingredients of Bryant & Robinson medium were added. Na_2SO_4 replaced $(NH_4)_2SO_4$.

Growth in the absence of volatile fatty acids. Bryant & Robinson medium was prepared under 100% CO_2 omitting acetate, isobutyrate, α-methyl-*n*-butyrate, valerate and isovalerate.

Batches of six to eight cultures were inoculated in duplicate for each of the deficient media and a complete medium control. After inoculation each tube was dipped in a mixture of petroleum jelly and paraffin wax (1 + 1) to prevent the entry of oxygen. Cultures were incubated at 38·5° and inspected daily for turbidity. Tubes which became turbid were examined microscopically and recorded as positive when the expected morphological type was found.

RESULTS

Table 1 relates the strains isolated to the diets of the sheep from which they were isolated, and to the date of isolation.

The microbiological characteristics of these strains are recorded in Table 2. The Gram reaction was variable in nine of the twenty-five strains; changes of this kind were reported by Bryant (1963). Some strains showed a series of changes with age. For instance, strains DZ 2 and D 24 changed from strongly Gram-positive to Gram-variable and became Gram-negative after 7 days of incubation.

Approximately half the strains (14/25) were able to grow in Bryant & Robinson (1961*a*) medium. This permitted a more detailed study of the nutritional requirements of eleven strains recorded in Table 3. All strains grew in the absence of $(NH_4)_2SO_4$ and utilized casein hydrolysate as a source of nitrogen, but three strains failed to grow when $(NH_4)_2SO_4$ was the sole nitrogen source, casein hydrolysate being omitted.

A majority of strains required volatile fatty acids; only strains LY10, LY11 and DZ4 were able to grow in their absence. Only one strain (LY10) grew when carbonate was omitted from the medium.

Table 1. *Origins of the cellulolytic bacteria isolated from sheep rumens*

Sheep diet	Sheep identification code	Date sampled	Time sampled (p.m.)	Strain code no.
Group I. Gram-positive or Gram-negative cocci in pairs or singly, 11 strains				
Lucerne chaff (800 g./day)	C.6	29. v. 63	—	LY2
		17. x. 63	—	LY3
		17. x. 63	—	LY4
		18. xi. 64	4.20	LY5
		18. i. 65	5.00	LY11
		18. ii. 65	4.30	LY23
	C.18	29. i. 65	3.00	LY16
		1. ii. 65	3.00	LY17
	C.76	14. i. 65	3.00	LY9
		18. i. 65	8.00	LY12
	C.114	8. ii. 65	9.00	LY21
Group II. Gram-negative cocci in long chains frequently over 20 units, 8 strains				
Lucerne chaff (800 g./day)	C.6	18. xi. 64	4.20	LY7
		14. i. 65	4.15	LY10
	C.18	3. xii. 64	9.30	LY8
		22. ii. 65	7.30	LY24
	C.76	18. i. 65	8.00	LY13
		18. ii. 65	4.30	LY22
	C.83	8. ii. 65	8.30	LY20
	C.114	27. i. 65	4.15	LY14
Group III. Gram-variable cocci in short chains				
Lucerne chaff (800 g./day)	C.6	4. ii. 65	7.30	LY18
		4. ii. 65	7.30	LY19
Group IV. Gram-negative cocci in short chains 8–10 units				
Oat chaff (600 g./day)+lucerne chaff (200 g./day)	C.2	17. viii. 64	9.30	DY2
Low protein diet* (500 g./day)	C.3	19. viii. 64	3.00	DY1
Group V. Large Gram-positive cocci in clumps and pairs				
Oat chaff (600 g./day)+lucerne	C.2	9. viii. 64	3.30	DZ2
chaff (200 g./day)	C.2	18. xi. 64	4.30	DZ4

* Low protein diet, 1,450 g. wheat straw; 50 g. lucerne chaff; 7·5 g. calcium phosphate; 1·5 g sodium chloride; 5·0 g. Premix (Fe, I, Cu, Co, Zn, Mn, vitamins A and D).

The carbohydrates fermented by six strains of ruminococci are recorded in Table 4. Each strain was tested in duplicate on more than one occasion. The results with xylose, arabinose, cellobiose and xylan were reproducible for all strains, but the fermentation of glucose, sucrose, lactose and fructose was not a reproducible property of these strains.

Table 2. *Microbiological characteristics of twenty-five strains of ruminococci from sheep rumens*

Characters	Strain											
	DZ2	DZ4	LY2	LY3	LY4	LY5	LY9	LY11	LY12	LY16	LY17	LY21
Morphological												
Gram reaction*	+	+	−	−	±	±	±	±	−	±	−	±
Arrangement†	diplo	diplo	diplo	diplo	diplo	diplo	diplo	diplo	diplo	diplo	diplo	diplo
Cultural												
Yellow pigment produced on filter paper‡	none	none	ly	ly	ly	ly	ly	ly	none	ly	ly	ly
Production of gas §	−	+	−	+	−	+	−	+	±	±	−	+
Final pH value in cellobiose broth	5·3	5·6	5·5	5·4	5·4	5·6	5·6	5·5	5·6	5·6	5·5	5·7
Nutritional												
Ability to grow without rumen liquor or growth in Bryant & Robinson medium	+	+	+	+	+	+	+	+	−	−	−	+
Fermentation end products ‖												
Butyric (mmole/100 ml.)	0·11	0·04	0·08	0·09	0·03	0	0·10	0·04	0·03	−0·06	−0·11	0·07
Propionic (mmole/100 ml.)	0	0	1·22	0·16	0·11	0	0·06	0·08	0·05	−0·22	−0·16	0·06
Acetic (mmole/100 ml.)	−0·43	0·07	0·92	0·50	0·43	0·08	0·32	1·23	0·10	0·06	0	0·76
Formic (mmole/100 ml.)	1·31	0·61	1·22	1·24	1·12	1·62	0·90	0·52	0·80	1·60	1·40	1·11
Lactic (mmole/100 ml.)	0·10	0·71	0·09	0	0·13	1·04	0·40	0	0·30	1·00	0·60	0·40
Succinic (mmole/100 ml.)	0	0	0·44	0	0	0·60	0·10	0	0	0	0	0·20
Ethanol (mmole/100 ml.)	1·38	1·56	1·43	1·43	1·57	2·60	1·23	1·45	1·57	1·11	1·25	0·67
Reducing sugars ¶	+	+	−	−	−	−	+	−	+	−	−	+

Table 2 (*cont.*)

Characters	Strain												
	LY23	DY1	DY2	LY18	LY19	LY7	LY8	LY10	LY13	LY14	LY20	LY22	LY24
Morphological													
Gram reaction*	±	−	−	−	−	−	+	±	−	±	−	−	−
Arrangement†	diplo	short	short	short	short	long	long	long	long	long	long	long	long
Cultural													
Yellow pigment produced on filter paper‡	ly	dy	dy	dy	dy	dy	dy	dy	dy	dy	dy	ly	dy
Production of gas §	−	−	−	±	±	−	±	−	±	+	−	±	−
Final pH value in cellobiose broth	5·7	5·4	5·4	5·6	5·4	5·4	5·8	5·4	5·4	5·4	5·5	5·3	5·4
Nutritional													
Ability to grow without rumen liquor or growth in Bryant & Robinson medium	+	−	−	−	+	+	−	+	−	+	−	−	−
Fermentation end products ‖													
Butyric (mmole/100 ml.)	0	−0·09	0	−0	−0·07	0	0·04	0	−0·14	−0·09	0	0	0
Propionic (mmole/100 ml.)	0·08	0	0·15	−0·06	−0·20	0	0·09	0	−0·17	−0·14	0	−0·13	0
Acetic (mmole/100 ml.)	−0·18	0·78	1·14	−0·24	0	0·64	1·80	0·08	0·66	1·16	0·76	1·00	0·20
Formic (mmole/100 ml.)	1·90	0·29	1·11	0·60	0·20	0·67	0·42	0·54	0·60	0·20	1·14	1·10	1·00
Lactic (mmole/100 ml.)	0·20	0	0·34	1·50	0·20	0·26	3·80	0·40	2·10	0·15	0·60	0·20	0·36
Succinic (mmole/100 ml.)	0·36	0·75	1·00	0·45	0·15	0·78	0	0·90	0	1·06	0·50	0·20	0·36
Ethanol (mmole/100 ml.)	0·98	0	0	0	0	0	0	0	0	0	0	0	0
Reducing sugars ¶	+	+	+	−	−	−	+	−	+	+	+	+	−

* +, Gram-positive after 15–24 hr at 38.5°; −, Gram-negative; ±, Gram-variable.

† 'diplo' = diplococci and single cocci; 'short' = chains of 4–8 cocci; 'long' = chains of 20–50 cocci.

‡ ly = light yellow; dy = dark yellow.

§ −, no gas produced; ±, very slight gas production; +, definite gas production.

‖ Negative values represent dissimilation.

¶ +, presence, and −, absence, of reducing sugars in the media after incubation with excess cellulose substrate for 30 days.

Table 3. *The growth of sheep-rumen ruminococci in various nutritionally deficient media*

	Bryant & Robinson medium				
Strain no.	Complete	Without $(NH_4)+$	Without casein hydrolysate	Without volatile fatty acids	Without $(CO_3)^{2-}$
DZ2	+*	+	+	−	−
DZ4	+	+	+	+	−
LY4	+	+	+	−	−
LY5	+	+	+	−	±
LY9	+	+	−	±	±
LY10	+	+	+	+	+
LY11	+	+	+	+	±
LY14	+	+	−	±	−
LY19	+	+	−	±	−
LY21	+	+	+	−	−
LY23	+	+	+	−	−

* A positive result denotes that the strain grew freely in the medium. Negative results denote a failure to produce a significant increase in turbidity. In the case of results recorded ±, the result is uncertain. The growth in these cases was sparse and frequently did not occur in both duplicates.

Table 4. *Carbohydrate fermented by strains of sheep-rumen ruminococci*

	Strain					
Sugar	LY2	LY3	LY4	DZ2	DY1	DY2
Xylose	− − −	− − −	− − −	− −	− − −	− − −
Arabinose	− − −	− − −	− − −	− −	+ + +	± + +
Fructose	+ + ±	+ − ±	+ − −	− −	+ + +	− − −
Glucose	+ + +	± − −	+ − −	− −	− − +	− − +
Lactose	+ + −	− − −	− − −	− −	− − +	− − −
Sucrose	± + ±	± − −	+ − −	− −	− ± +	± − −
Cellobiose	+ + +	+ + +	+ + +	+ +	+ + +	+ + +
Xylan	+ . .	+ + +	+ + +	+ +	+ + +	+ + +

* Each result represents a pair of duplicates. The three columns for each strain indicate the results obtained on three separate occasions. A definite decrease in pH value relative to the controls is denoted by a positive sign. A decrease in pH value in one member of a pair is recorded as ±. A negative sign indicates that no change in pH occurred. . = no test

Nutritional requirements of ruminococci

In preliminary trials Bryant & Robinson (1961*a*) found 10 of 15 strains capable of growth in their complete, partially defined medium. Seven of these were *Ruminococcus albus* and three *R. flavifaciens*. In the present study 14 of 25 strains grew in Bryant & Robinson medium. Of these, 10 strains were shown to be *R. albus* and four *R. flavifaciens*. These observations suggest that strains classifiable as *R. flavifaciens* are more dependent upon special growth factors in rumen fluid than those classed as *R. albus*.

The existence of ruminococcal strains which utilize $(NH_4)^+$ in preference to amino-nitrogen is well established. $(NH_4)^+$ was reported to be an essential nutrient for some of the strains which have been examined (Bryant & Robinson, 1961*b*; Bryant & Robinson, 1962; Bryant & Robinson, 1963; Dehority, 1963). Strains which required organic nitrogen have also been reported. Fletcher (1956) and Ayers (1958) considered casein hydrolysate to be essential for the growth of a strain of *Ruminococcus albus* and

one of *R. flavifaciens*. Allison, Bryant & Doetsch (1962) suggested that organisms capable of utilizing inorganic nitrogen in the rumen have a competitive advantage because branched chain volatile fatty acids and ammonia are more abundant in rumen contents than are amino acids and thus more readily available for protein synthesis. It seems likely that the ecological significance of these considerations is decreased in animals fed a high protein (lucerne chaff) diet, especially as the ruminococci are often closely applied to particles of ingesta which are likely to be relatively rich in organic nitrogen. The 11 strains considered here all grew readily when vitamin-free casein hydrolysate was the sole nitrogen source. In the case of strains LY4 and DZ2, growth on Bryant & Robinson medium without $(NH_4)^+$ was equivalent to that in the complete Bryant & Robinson medium after 48 hr, although at this time no growth was apparent in Bryant & Robinson medium without casein hydrolysate. However, on further incubation, a well marked turbidity appeared in this latter medium. Strains LY4 and DZ2 grew in Bryant & Robinson medium with an amino acid mixture as the sole nitrogen source apart from B-group vitamins and cysteine (amino acids %, w/v: glutamic acid, 0·4; L-leucine, 0·2; DL-valine, L-lysine, DL-aspartic acid and DL-serine, 0·16 each; DL-phenylalanine, L-arginine HCl, 0·12 each; L-histidine, 0·06). Turbidity was evident after incubation for 48 hr for strain LY4 and after 72 hr for strain DZ2 indicating that with this restricted range of amino acids growth still occurred more readily than it did when all cellular nitrogen had to be synthesized from $(NH_4)^+$. One strain of *R. albus* (LY9) and two strains of *R. flavifaciens* (LY14, LY19) were dependent on organic nitrogen for growth. It is concluded that the genus *Ruminococcus* includes strains which have an absolute requirement for organic nitrogen and strains which have a preference for organic nitrogen, as well as those for which inorganic nitrogen is an essential nutrient (Bryant & Robinson, 1961*b*).

A majority of the strains were capable of synthesizing amino acids from $(NH_4)^+$ and $(CO_3)^{2-}$ ions and volatile fatty acids. Presumably the mechanism was analogous to that shown to exist in *Ruminococcus flavifaciens* strain C94 (Allison *et al.* 1962; Allison & Bryant, 1963). The strains described here could also utilize preformed amino acids and thus resembled strain B34b of Dehority (1963); consequently volatile fatty acids were not required for amino acid synthesis. Nevertheless, only three out of the 11 strains were capable of growth in media which did not contain volatile fatty acids.

Rumen bacteria are characterized by their dependence on exogenous CO_2 for growth (Bryant, 1959). Strains of ruminococci have been shown to require CO_2 (Bryant & Burkey, 1953; Dehority, 1963) and utilize it for amino acid synthesis (Allison & Bryant, 1963). All but one of the eleven strains conformed to this pattern.

Taxonomy of ruminococci

Bryant (1963) distinguished between *Ruminococcus albus* and *R. flavifaciens* on the basis of morphology and fermentation product data. In the present work CO_2 was not determined and production of H_2 was determined only for a few strains. With these limitations the results for each strain can be compared with Bryant's specification.

The strains examined here were classified as follows:

Ruminococcus albus: 10 strains: DZ4, LY2, LY3, LY4, LY5, LY9, LY11, LY12, LY16, LY21.

Ruminococcus flavifaciens: 8 strains: DY1, DY2, LY7, LY10, LY14, LY20, LY22, LY24.

Intermediates similar to *Ruminococcus albus*: 3 strains: DZ2, LY17, LY23.

Intermediates similar to *Ruminococcus flavifaciens*: 4 strains: LY8, LY13, LY18, LY19.

Intermediates classed as 'similar to *Ruminococcus albus*' were grouped together on the basis of morphology, pigment produced and production of ethanol; these characteristics are held in common with *R. albus*. These intermediates were not classed as *R. albus* because they did not produce acetate as a fermentation product.

Intermediates classed as 'similar to *Ruminococcus flavifaciens*' grew in chains and, in common with other strains of *R. flavifaciens* considered here, did not produce ethanol, produced little or no gas, and showed dark yellow pigmentation. Strains LY18 and LY19 could not be accepted as *R. flavifaciens* because acetate was not produced. Strains LY8 and LY13 were not acceptable because they did not produce succinate.

Ruminococcus albus. Hungate (1957) and Bryant *et al.* (1958*b*) placed some streptococci in the *R. albus* group, but in the present study only diplococci were classed as *R. albus* since only diplococci were found to produce ethanol. The *R. albus* strains were similar to those described by other workers in that the yellow pigment was pale or absent. All three sets of data agree in that most *R. albus* strains produce ethanol, acetate and formate as fermentation end products. Hungate (1957) found one strain which metabolized acetate, which was not included as typical of the *R. albus* group. In the present study three of 13 'albus'-type strains did not produce acetate. Hungate found no *R. albus* strains which produced succinate but the present results agree with Bryant *et al.* (1958*b*) who found strains which produced succinate. Strains were also found which produced lactate, a character observed by Hungate but not by Bryant *et al.* Hydrogen was identified as a gaseous end product for strains LY11, LY12, LY16, and gas production was also observed for strains DZ4, LY3, LY5, LY7; but gas production was not as general among these isolates as among those reported by Hungate (1957) and Bryant *et al.* (1958*b*). The additional strains studied here support the conclusions of Bryant *et al.* (1958*b*) about the fermentation end products produced by *R. albus* except in regard to acetate which may or may not be produced by our strains. They do not show the similarity observed by Kistner & Gouws (1964) among 10 strains isolated from sheep on a lucerne hay diet.

Ruminococcus flavifaciens. Comparison of our strains accepted as *R. flavifaciens* with those described by Sijpesteijn (1951), Hungate (1957) and Bryant *et al.* (1958*b*) indicated that they fit the previous descriptions except that three of our strains did not produce reducing sugar when fermenting excess cellulose. The absence of ethanol from the end products was the most consistent feature of the streptococcal strains and extended to those intermediates which, on other grounds, could not be regarded as *R. flavifaciens*.

Numerical classification of ruminococci

The classification of ruminococci applied in the preceding paragraphs is based on their biochemical and morphological characteristics. Successive investigators have relaxed these criteria to accommodate strains which exhibited all but one or two of the characteristics required of the species. It has never been shown that the two 'species', *Ruminococcus albus* and *R. flavifaciens*, are genetically independent or that they are ecologically distinguishable, so that the possibility of an exchange of genetic

material between them cannot be excluded. Again, the importance to be attached to characters such as morphology and production of ethanol is not clear. These characters are consistently associated but their significance in the life of the bacterial cell is obscure. In these circumstances a technique which classifies strains on the basis of their overall similarity is of value because it embodies all the information without weighting it. Such an Adansonian classification is particularly suited to the present study, where the relationship between overall biochemical type and serological type was investigated. Numerical taxonomy provides the methods necessary for this treatment of the data.

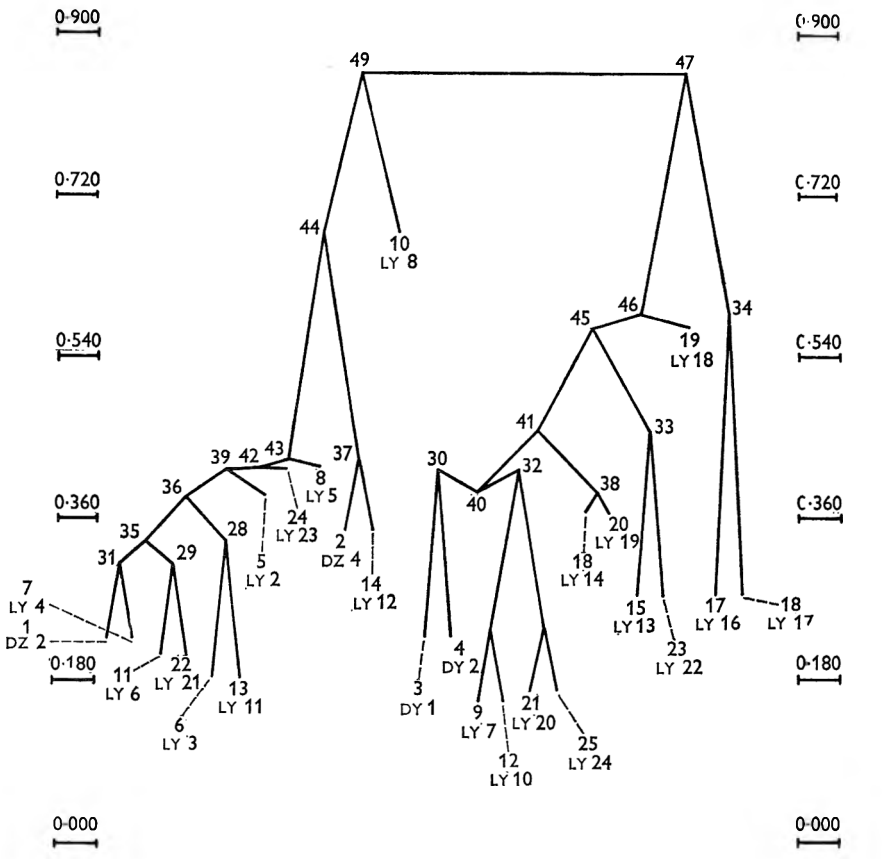

Fig. 1. Numerical classification of twenty-five strains of the genus *Ruminococcus*.

The data presented in Tables 2 and 3 together with a summary of those in Table 4 were analysed by using a Fortran program 'MULTIST' for Control Data 3600 computer (C.S.I.R.O. Computing Research Section, technical note no. 14). The rationale of the method and the formulae used are given by Williams & Lance (1965). This program accepted qualitative, multi-state and quantitative data, calculated a non-metric coefficient and utilized centroid sorting. Gram-reaction results were treated as three-state multi-state attributes since Gram-variable (±) is no less a feature of the organism than is Gram-positive or Gram-negative. Arrangement and pigment production were

similarly treated. Gas production was treated as a multi-state variable because here ± represented 'slight gas production'. In the nutritional data results indicated as ± were treated as unknown because it was doubtful whether the medium had been used for growth. These data were treated as qualitative in common with 'growth in Bryant & Robinson medium', 'sugar fermented' and 'production of reducing sugars'. Final pH value and 'fermentation end products' were treated as quantitative data. The affinities which were demonstrated by this treatment of the data are presented as a dendrogram in which the ordinate represents the value of the similarity coefficient (Fig. 1). Here groups 9 and 12 represent the two most similar strains, their degree of similarity being represented by their positions relative to the ordinate scale. Together they constitute a new group 26. At the 0·238 degree of similarity group 27 can be added to group 26, making a new group 32. The perpendicular distance between 26 and 32 represents the decrease in similarity which must be accepted before further strains can be clustered with group 26. This process is continued until at a low degree of similarity all strains can be grouped together.

The dendrogram comprises two main branches corresponding to the existing species and two intermediate groups. The branch represented by group 44 includes all the strains classified as *Ruminococcus albus* except LY16, which is now classed as an intermediate in group 34. It is evident that the differences which prevented strains DZ2 and LY23 from being considered members of this group were minor. The dendrogram shows that these two strains closely resembled accepted members of the species and should logically be included with it. The branch represented by group 45 comprises all strains classed as *R. flavifaciens* and includes strains LY13 and LY19. Strain LY18 can also be included for a small change in similarity coefficient. Hence group 46 is considered to represent *R. flavifaciens* strains. It is concluded that numerical classification of these strains agrees closely with that derived by the established methods. But the numerical method justifies the assignment of intermediate strains to appropriate species and permits an assessment of the degree of relationship between members of the same group. Thus *R. albus* (group 44) is, in general, more homogeneous than *R. flavifaciens* (group 46) although a considerable increase in similarity index is required to accommodate group 37 within group 44. Comparison of Table 2 with Fig. 1 permits the extraction of characters typical of the two species.

Ruminococcus albus (11 strains). All were diplococci. When grown on cellulose they produced butyrate, propionate, formate and ethanol. In addition 10 of 11 produced acetate and 9 of 11 produced lactate. Most strains (10 of 11) were capable of growth without unidentified growth factors which occur in rumen liquor.

Ruminococcus flavifaciens (11 strains). All were streptococci. Grown on cellulose they produced formate but not ethanol. Most strains (9 of 11) produced succinate and lactate, and 10 of 11 produced acetate. Butyrate and propionate were usually not produced. A minority (4 of 11) of strains were capable of growth without unidentified factors which occur in rumen liquor.

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Antigenic Relations of Cellulolytic Cocci in the Sheep Rumen

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SUMMARY

Twenty-five strains of ruminococci were isolated from seven sheep fed chaffed lucerne hay, or mixtures of cereal chaff and lucerne chaff, for 18 months. Antiserum to each strain was produced in rabbits and used to classify the strains by cross-agglutination. Two main groups and a subgroup were identified which served to characterize half (12 of 25) of the strains isolated. Group 1 (7 strains) and the subgroup (2 strains) were all classified as *Ruminococcus albus*. Group 2 comprised 3 strains of *R. flavifaciens*. An indirect fluorescent-antibody technique was also used to show antigenic relationships amongst the isolates. Fluorescence cross-reactions were obtained among 13 strains of *R. albus* using group 1 antisera. Similarly a group 2 antiserum labelled 7 of the 12 strains of *R. flavifaciens* isolated. The persistence of these serotypes in the rumen was demonstrated by the isolation of cross-agglutinating strains of *R. albus* from one animal at intervals throughout the experimental period. Recovery of cross-agglutinating strains of *R. albus* and *R. flavifaciens* from more than one animal suggests that a given serotype becomes disseminated among animals which are housed together. Furthermore, the isolation of group 1 and group 2 ruminococci from the same sheep indicates that the group-specific determinants were not dependent on the rumen environment for expression. It is concluded that selection was rigorous enough to maintain recognizably different genetic strains in the rumen against mutation pressure.

INTRODUCTION

Investigations of the serology of rumen organisms include many studies of streptococci, in sheep and cattle (Perry, Wilson, Newland & Briggs, 1955; Perry, Newland & Briggs, 1958; Medrek & Barnes, 1962*a*, *b*). Medrek & Barnes used a ring precipitation test to study the relationships between 149 strains of *Streptococcus bovis*. They established 12 groups, but these only served to type half the strains. Perry *et al.* (1958) also observed serological heterogeneity among strains of rumen streptococci. Serological heterogeneity has also been reported among strains of the genus *Butyrivibrio* (Margherita & Hungate, 1963; Margherita, Hungate & Storz, 1964). On the other hand, Briggs (1951) studied the spread of pathogenic strains of *Escherichia coli* during an epidemic of white scours in calves and showed that serologically identical strains could be recovered from other calves in the same environment. Strains isolated from field cases of the disease were not serologically related. Hobson, Mann & Oxford (1958) isolated 250 strains of *Peptostreptococcus elsdenii*. Three agglutination types were distinguished and a few isolated strains belonged to a fourth group. Two of these

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serotypes were detected in additional calves and sheep by means of an immunofluorescence technique.

The immunological composition of these bacterial species could be influenced by environmental factors such as the host animal, diet, geographical location and lapse of time. Limitation of these variables may restrict the variety of serotypes so that investigators dealing with animals from one locality encounter only a few serotypes. This paper describes the use of two serological tests, agglutination and immunofluorescence, to characterize strains of sheep rumen cellulolytic cocci, and establish the existence of serological relationships which would permit the detection of ruminococci *in situ*.

METHODS

Preparation of suspensions of ruminococci. This investigation was concerned with the relationship between 25 strains of ruminococci whose isolation is described by Jarvis & Annison (1967).

Antigen suspensions were prepared from cocci grown in 1500 ml. clarified rumen liquor broth (20 %, v/v) which contained cellobiose (0·5 %, w/v). This medium was prepared and maintained under an atmosphere of 100 % CO_2. Each flask was inoculated with a small piece of infected filter paper from a 4-day-old culture on modified Halliwell agar (Jarvis & Annison, 1967). An increase in turbidity was usually evident after 24 hr at 38·5°. Growth was stopped, after 3–4 days, by injecting formalin (0·25 %, v/v) and incubating a further 12 hr. The cocci were collected by centrifugation of successive portions at 10,800 *g* for 20 min., washed three times in formol saline, concentrated to a final volume of about 30 ml. and stored at 2°. Antigen concentration was estimated from duplicate Kjedahl nitrogen determinations on each suspension.

Preparation of suspensions for inoculation. Suspensions given without adjuvant were diluted from stock in formol saline and standardized against McFarland's turbidity scale. Freund's incomplete adjuvant (Freund, 1947) was used when an adjuvant-treated antigen was required. The antigen (50 mg. wet-weight) was prepared in formol saline (3 ml.) and homogenized with anhydrous lanolin (1 g.). Liquid paraffin B.P. (2 ml.) was added and the mixture homogenized again. The adjuvant mixture was transferred to a bijou bottle and stored at 2°. In a few instances the triolein–Tween adjuvant described by Barrie & Cooper (1964) was used.

Injection schedules. Two rabbits were used for each antigen. One received a saline suspension intravenously, consisting of a total of 5 ml. saline suspension (opacity 3, McFarland's scale) given in seven daily doses increasing from 0·2 to 1·0 ml., followed by an interval of 7 days and a second course using a suspension with an opacity of 8 (Hobson, Mann & Smith, 1962). The second rabbit was given adjuvant-treated antigen in three doses intramuscularly in different sites at 7-day intervals (Kabat & Mayer, 1961). Each rabbit was bled 7 days after its last injection. A further course of injections was given if the titre at this bleeding was not at least 1/640. Usually the route and vehicle in the second course were not the same as that used in the first. The triolein adjuvant mixture (Barrie & Cooper, 1964) was used where difficulty had been experienced in getting a response by other methods.

Bleeding for serum. Approximately 5 ml. blood were drawn from each rabbit before injections commenced. Normal serum obtained from these bleedings was used as a control to reveal non-specific agglutination. After a course of injections 30–40 ml.

of blood was drawn from the marginal ear vein, allowed to clot and held at 2° overnight. The immune serum was separated by centrifugation and stored at −10°.

Determination of antibody concentration in immune serum. The tube agglutination test was used for this purpose. Antigen suspensions were prepared by diluting formolized stock suspensions in saline (0·85%, w/v, NaCl in distilled water). These suspensions were made as required and standardized with a Spectronic 20 colorimeter (Bausch & Lomb Inc., N.Y.) to an extinction of 0·200 ± 0·005 at 600 mμ. Opacity standards were prepared with each antigen suspension used for each series of tests. These standards, representing 0, 25, 50 and 75% agglutination, were prepared by diluting 1·0, 0·75, 0·50 and 0·25 ml. of standard antigen suspension to 2 ml. in saline. The standards were incubated with the test series. Doubling dilutions of antiserum were prepared in unit volumes (0·5 ml.) of saline. Each antigen was also set up with normal serum diluted from 1/10 to 1/1280. After the serum had been diluted, an equal volume (0·5 ml.) of homologous standard antigen was added to each tube and racks of tubes were incubated at 37° for 2 hr in a water bath and then at 2° overnight. Tests were read at room temperature. Each tube in a test series was compared with opacity standards prepared using the homologous antigen and scored as: −(negative), 1 (25%), 2 (50%), 3 (75%), 4 (100%). The reciprocal of the highest dilution having a score of 2 was recorded as the titre.

The similarity of different strains was estimated by comparing the titre of each antiserum with homologous and heterologous antigens.

Detection of relationship between strains of ruminococci using an indirect fluorescent antibody technique. Unlabelled rabbit antibody was reacted with antigen and became attached to its surface. This effect was revealed by a second-stage reaction in which the antibody acted as antigen for an anti-rabbit fluorescein-labelled sheep γ-globulin (Baltimore Biological Laboratory, Inc., Maryland). Indirect labelling was used for comparative purposes because the fluorescein:protein ratio remained constant, although various antigens and antisera were used. Thus the intensity of fluorescence indicated the relative amount of unlabelled rabbit antibody absorbed on the antigen cocci.

Anti-rabbit fluorescein-labelled γ-globulin was reconstituted in distilled water, and chromatographed on Sephadex G25 (Peters, 1963) to ensure that no free fluorescein was present. An ultraviolet striplamp was used to locate the protein peak and any unbound fluorescein. The protein peak was collected for use as conjugated γ-globulin. This conjugate (7·5 ml.) was absorbed once with mouse-liver powder (750 mg.). This mixture was shaken for 1 hr at room temperature and then centrifuged (22,000 *g* for 30 min.). The supernatant-absorbed conjugate solution was recovered, preserved with Thiomersal (B.D.H., England), and stored at −10° until required.

Slides of each antigen were prepared from suitable dilutions of the concentrated formolized suspensions in phosphate-buffered normal saline. One drop of each diluted suspension was spread on an ultraviolet-transmitting slide (Shandon Scientific Co., Cromwell Place, London S.W. 7) and allowed to dry in air. Subsequent treatment of the slides followed Cherry, Goldman & Carski (1960) and Hobson *et al.* (1962). Smears were fixed by immersion in redistilled acetone for 20 min., removed, allowed to dry in air, then moistened with buffered saline. A large Petri dish containing wet filter paper and several glass rods was used as a moist incubation chamber. Slides were arranged, smear uppermost, on the glass rods. One drop of undiluted rabbit

Table 1. *Agglutination cross-reactions among ruminococci from sheep*

Antisera to strain	Antigens of strain												
	DZ2	DZ4	LY2	LY3	LY4	LY5	LY9	LY11	LY12	LY16	LY17	LY21	LY23
DZ2	2,500	160	—	—	—	—	—	—	—	—	—	—	—
DZ4	20	10,000	—	—	—	—	—	—	—	—	—	—	—
LY2	—	10	640	640	640	640	80	—	20	—	—	—	80
LY3	40	—	2,500	2,500	2,500	10	(tr)	—	—	—	—	—	20
LY4	40	—	640	640	640	20	80	—	40	—	—	—	160
LY5	—	20	1,300	320	1,300	2,500	1,300	—	1,300	—	—	—	2,500
LY9	20	—	640	160	160	1,300	1,300	—	1,300	—	40	—	5,000
LY11	—	20			—	—	—	320	—	—	—	—	—
LY12	—	—	640	320	320	1,300	1,300	20	1,300	—	—	—	5,000
LY16										40			
LY17	—	—			—	—	—	—	—	—	5,000	—	—
LY21	—	—			—	—	—	—	—	—	—	5,000	—
LY23	40	—	2,500	2,500	2,500	5,000	25,00	—	5,000	—	—	—	10,000
DY1	80	—	40	—	—	—	—	—	—	—	—	—	—
DY2	80	—	—	—	—	—	—	—	—	—	—	—	—
LY18	(tr)	—			—	—	—	—	—	—	—	—	—
LY19	—	—			—	—	—	—	—	—	—	—	—
LY7	20	10	—	—	—	—							
LY8	80	10	—	—	—	—							
LY10	—	—			—	—	—	—	—	—	—	—	—
LY13	—	—			—	—	—	—	—	—	20	—	—
LY14	—	—			—	—	—	—	—	—	—	—	—
LY20	—	—			—	—	—	—	—	—	—	—	—
LY22													
LY24	—	—	80	40	40	80	40	—	20	—	—	—	20

Table 1 (*cont.*)

Antisera to strain	Antigens of strain											
	DY1	DY2	LY18	LY19	LY7	LY8	LY10	LY13	LY14	LY20	LY22	LY24
DZ2	—	—	20	—	—	—	—	—	—	—	—	—
DZ4	—	—	—	—	—	—	—	—	—	—	—	—
LY2	—	—	—	—	—	—	—	—	—	—	—	160
LY3	—	—	—	—	—	—	—	—	—	—	—	—
LY4	—	—	—	—	—	—	—	—	—	—	—	—
LY5	—	—	—	—	—	—	—	—	—	—	—	160
LY9	—	—	—	—	—	—	—	—	—	—	—	40
LY11	—	—	20	—	—	—	20	—	—	—	—	—
LY12	—	—	—	—	—	(tr)	—	—	—	—	—	40
LY16												
LY17	—	—	—	—	—	—	—	—	—	—	—	—
LY21	—	—	—	—	—	—	—	—	—	—	—	—
LY23	—	—	—	—	—	—	—	—	—	—	—	—
DY1	1,300	—	—	—	—	—	—	—	—	—	—	—
DY2	—	1,300	—	—	—	—	—	—	—	—	—	—
LY18	—	—	10,000	—	—	—	—	—	—	—	—	—
LY19	—	—	—	5,000	—	—	—	—	—	—	—	—
LY7	—	—			80	—						
LY8	—	—			—	80						
LY10	—	—	40	—	—	(tr)	160	—	—	—	—	—
LY13	—	—	—	—	—	—	—	20,000	20,000	20,000	—	(tr)
LY14	—	—	—	—	—	—	—	20,000	20,000	20,000	—	(tr)
LY20	—	—	—	—	—	—	—	20,000	5,000	20,000	—	(tr)
LY22											zero	
LY24	—	—	—	—	—	—	—	40	40	80	—	5,000

The table records to a 50% agglutination end-point.

In a few cases where a titre was not corroborated when the roles of antigen and antibody were reversed, for the same strains, evidence of agglutination to a 25% level is recorded (tr).

Absence of cross-agglutination is recorded by a negative — sign.

Combinations which were not set up are indicated by gaps in the table.

A measure of agglutination in the presence of normal serum was observed with some antigens, principally from strains DZ2, DZ4, LY19 and LY24. The extent of these reactions is recorded in Table 2.

antiserum was added to each slide and the whole batch incubated for 30 min. at 37°. The protein not absorbed at the surface of the cocci was completely rinsed from each slide by three successive 5 min. washes in fresh buffered saline. Slides were individually rinsed with buffered saline between each wash. The slides were then rearranged in the moist chamber and a drop of undiluted absorbed fluorescein-conjugated γ-globulin applied to each. After incubation for a further 30 min. at 37° the slides were rinsed with the same care as before, a drop of mountant (Univert aqueous mountant; G. T. Gurr Ltd., London) was added and a coverslip applied to each slide whilst still moist. Slides were examined within 2 hr of preparation. Positive and negative controls were set up with each batch of slides. These comprised homologous antigen against rabbit antiserum and antigen against normal rabbit serum.

The preparations were examined by using a standard G.F.L. microscope and multi-purpose microscope illuminator (Carl Zeiss). The lamp and microscope were mounted on a hardboard base. The illuminator was fitted with a high-pressure mercury lamp, H.B.O. 200 W. (Osram) and connected to the main power supply through a choke 39 26 22 (Carl Zeiss). Exciter filters comprised KG. 1 heat protection filter (permanently in the light path), BG 12, BG 3, and UG 5, UG 1. BG filters were used for blue-light fluorescence, whilst the U.G. series were used when ultraviolet excitation was required. On the microscope, the diaphragm insert of the standard built-in illuminator was replaced by a mirror and an optical system comprising a 1·3 NA bright-field condenser; ×10, ×40, ×100 achromatic objectives and P×10 eyepiece were used. The binocular head was replaced by a straight monocular tube. A barrier filter intermediate tube was located between the objective nose-piece and monocular tube. It contained two neutral filters (N 0·1 and N 0·01) and six barrier filters (designated 41, 44, 47, 50, 53, −65). Estimates of fluorescent intensity were made with ×40 objective and blue-light fluorescence obtained with BG 12 and BG 3 exciter filters coupled with 53 and 44 barrier filters. When several slides were to be examined, the fluorescent image of the positive control was sharply focused so that other slides could then be slipped in with only minor focusing adjustment, thus facilitating comparisons. When no fluorescent image was visible the presence of cocci in the field was verified by bright-field observation. Estimates of fluorescent intensity were recorded according to the following scale after Cherry *et al.* (1960): 4−, maximal fluorescence, brilliant yellow-green; 3+, bright yellow-green fluorescence; 2+, less brilliant but definitely fluorescent, 1+; fluorescent but dull; tr., a faint fluorescent glow.

RESULTS

Twenty-five strains of rumen cellulolytic cocci were used to immunize rabbits and produce specific antisera of high titre. A majority of strains (18 of 25) provoked titres of 1/1200 or more after one course of injections. This result was obtained in a second group (2 of 25) after a second course of injections. But a third group (5 of 25) appeared to be incapable of eliciting a satisfactory agglutinin response. Antigen was the major factor determining the titre of immune serum. In 21 of 25 strains the mode of immunization had no significant effect on the agglutinin titre of the antiserum collected from two differently treated rabbits. To some extent these differences in antigenicity could be related to species differences. Among *Ruminococcus albus* and *R. flavifaciens* 10 of 13 and 8 of 12, respectively, were good antigens, but whereas a

Table 2. *Extent of agglutination of sheep ruminococci by normal rabbit serum*

Strain as antigen	Agglutination titre at three end-points: 25% agglutinated	50% agglutinated	75% agglutinated
LY14	10	—	—
LY18	20	—	—
LY20	20	—	—
LY22	20	—	—
LY13	40	—	—
DZ4	20	10	—
LY24	160	10	—
LY19	160	40	—
DZ2*	—	160	—
DZ2	—	160	—
DZ2	320	160	—
DZ2	—	320	—
DZ2	—	—	—

* Strain DZ2 was compared against several samples of normal serum from different rabbits.

Table 3. *A comparison of cross-reactions detected by indirect immunofluorescence (F.A.) with those detected by agglutination (Ag), for three antisera and strains of sheep ruminococci*

Strain as antigen*, †	LY9 antisera: Ag titre	LY9 antisera: F.A.‡	LY23 antisera: Ag titre	LY23 antisera: F.A.‡	LY14 antisera: Ag titre	LY14 antisera: F.A.‡
DZ2	20	3+	40	1+	—	2+
DZ4	—	2+	—	1+	—	1+
LY2	640	3+	2,500	2+		—
LY3	160	4+	2,500	3+		—
LY4	160	2+	2,500		—	—
LY5	1,300	3+	5,000	3+	—	—
LY9	1,300	4+	2,500	3+	—	—
LY11	—	3+	—	3+	—	—
LY12	1,300	4+	5,000	2+	—	1+
LY16	—	2+	—	4+	—	tr
LY17	40	2+	—	4+	—	tr
LY21	—	2+	—	2+	—	tr
LY23	5,000	2+	10,000	4+	—	4+
DY1	—	—	—	—	—	—
DY2	—	—	—	—	—	4+
LY18	—	—	—	—	—	—
LY19	—	—	—	—	—	—
LY7	—	—	—	—	—	4+
LY8	—	—	—	—	—	—
LY10	—	—	—	—	—	2+
LY13	—	—	—	—	20,000	4+
LY14	—	—	—	—	20,000	4+
LY20	—	—	—	—	20,000	4+
LY22	—	—	—	tr	—	—
LY24	40	—	—	—	(tr)	4+

* Antigens DZ2 to LY23 were diplococci; DY1 to LY19 were short-chain streptococci, whilst LY7 to LY24 were long-chain streptococci.

† Group I comprised LY2, LY3, LY4, LY5, LY9, LY12, and LY23. Group II comprised LY13, LY14 and LY20.

‡ The method of scoring fluorescence is described in the text.

further 2 of 13 *R. albus* strains gave titres of 1/1200 or more when the course of injections was repeated, and only one was classed as a poor antigen, 4 of 12 of *R. flavifaciens* strains did not provoke an adequate antibody response after repeated injection.

Agglutination cross-reactions are recorded in Table 1. Titres of 1/80 and above are considered to represent effects which could not be confused with non-specific agglutination. In the case of strain DZ2 the titres obtained with immune serum frequently did not exceed those with normal serum (Table 2) and their significance is doubtful. On the other hand, those antigens not listed in Table 2 did not agglutinate with normal serum and cross-reactions in which they were involved may be significant below 1/80. Table 1 indicates that half the strains (13 of 25) showed some relationship with other strains. A further group (5 of 25) were poor antigens, a feature which would tend to prevent the establishment of an agglutination cross-reaction. The remainder (7 of 25) were serologically distinct and showed no cross-reactivity. Table 1 is arranged so that similar morphological types are brought together. Two well-defined groups are evident. Group 1 consisted of strains LY2, LY3, LY4, LY5, LY9, LY12, LY23 (all diplococci). Group 2 comprised strains LY13, LY14, LY20 (all streptococci). The DZ series formed a third minor group unrelated to groups 1 and 2. Strain LY24 was of interest because, although a large part of its antigenic character was probably unique, it had features in common with both group 1 and group 2 strains and represented an intermediate type.

Table 4. *Validity of the indirect fluorescent-antibody technique used to label antigens of sheep ruminococci*

	Components of the labelling reaction			
Strain as antigen		Rabbit serum	Fluorescein-labelled anti-rabbit γ-globulin	Result
LY23	+	None	+label	No fluorescence
LY23	+	1/10 normal serum	+label	Very faint glow, no detectable organisms by blue-light fluorescence
LY23	+	1/10 absorbed specific antiserum*	+label	No fluorescence
LY23	+	1/10 specific antiserum	+label	Bright spherical cocci on a black background

* Concentrated homologous antigen suspension (1 ml.) shaken with undiluted antiserum (1 ml.) at room temperature for 1 hr. Then centrifuged for 20 min. at 22,000*g* and 2°. The supernatant fluid was used in this test.

Results obtained when the indirect fluorescent-antibody technique was used to detect cross-reactions among ruminococci are recorded in Table 3. It is evident that antisera to group 1 strains reacted with a wider range of antigens than was revealed by agglutination. In spite of this they exhibited a remarkable specificity for diplococci. Group 2 was represented by LY14 antiserum. In this case the strains showing cross-fluorescence did not correspond so well with the morphological type. Some strepto-

coccal strains (5 of 12) did not fluorescence with LY14 antiserum, whilst some diplococcal strains (4 of 13) were labelled by LY14 antiserum.

Table 4 demonstrates that the fluorescent labelling observed was dependent on the presence of specific antibody.

DISCUSSION

The conclusion that most taxonomic groups of rumen bacteria include a variety of serotypes (Bryant, 1963) can be extended to ruminococci. Three groups were identified by cross-agglutination and these served to classify 13 of 25 strains. The agglutination data do not suggest that the members of this genus are any more closely related than are members of the genus *Butyrivibrio* (Margherita & Hungate, 1963) or *Peptostreptococcus elsdenii* (Hobson *et al.* 1958), but a broader relationship was observed by using immunofluorescence. Agglutination is concerned with surface antigens, but the existence of antigenic determinants which normally take no part in agglutination can be detected by using cell-wall fractions in place of intact organisms (Cummins, 1954). Such fractions cross-agglutinate more extensively than intact organisms, because determinants associated with the inner structure of the cell wall are exposed when the walls are broken. Some of these antigenic groups can also be demonstrated by means of fluorescent antibodies. Moody, Ellis & Updyke (1958) used the fluorescent-antibody technique to detect group A streptococci. They found marked cross-reactions between members of Lancefield's groups A and C, and overcame this by absorbing group A antisera with group C organisms. In this case the group-specific determinants are terminal groups on rhamnose side-chains attached to the bacterial surface. Cross-reactivity is associated with rhamnose-rhamnose linkages, common to the side-chains of both groups. These are normally masked by the terminal groups (McCarty, 1956; Karakawa, Krause & Borman, 1965). The cross-fluorescence observed among ruminococci may be due to common structural features such as those described for streptococci.

Two species of ruminococci have been described, *Ruminococcus albus* (Hungate, 1957) and *R. flavifaciens* (Sijpesteijn, 1948). These are distinguished on the basis of morphology and fermentation products. The physiological characteristics of the strains considered here were used to construct a dendrogram which had two main branches corresponding with the accepted species (Jarvis & Annison, 1967). Comparison of the relationships established by serological methods with those based on physiological data shows that strains which cross-agglutinated are clustered in the same branches of the dendrogram. Thus group 1 strains are all classified as *R. albus* and the three group 2 strains are classified as *R. flavifaciens*. The DZ strains did not cross-agglutinate with group 1 strains, although classified as *R. albus*. Cross-fluorescence data generally revealed additional serological relationships with members of the same physiological group. Thus group 1 antisera reacted with all the strains classified as *R. albus* but not with strains classified as *R. flavifaciens*. There are two anomalies. Strains LY16 and LY17, which occupied an intermediate position on physiological grounds, fluoresced brilliantly when labelled with group 1 antisera, indicating that they were related to group 1 strains. Conversely, strains DZ2 and DZ4, each of which was related to an authentic *R. albus* strain on physiological grounds, reacted as intermediates and cross-fluoresced with antisera from group 1 (*R. albus*) and group 2 (*R. flavifaciens*). The lack of serological relationship between

R. albus and *R. flavifaciens* provides independent evidence for the existence of two species of rumen cellulolytic cocci and corroborates the existing monothetic classification.

A study of these serological data in relation to the origin of each strain (Jarvis & Annison, 1967) indicates that organisms showing group 1 antigenic character were repeatedly isolated from sheep C6 over a period of 18 months. This supports the conclusion that an established rumen population is relatively stable although it is in a state of dynamic equilibrium (Kay & Hobson, 1963). Strains LY2, LY3 and LY4 were isolated from sheep C6 several months before strains LY5 and LY23. Agglutination cross-reactions between strains LY2, LY3 and LY4 indicate a close relationship. But the titres of antisera to strains LY2, LY3 and LY4 with their homologous antigens were noticeably higher than those with either LY5 or LY23 antigen, suggesting that their relationship with the later isolates was less marked. In the reverse situation LY5 and LY23 antisera reacted strongly with LY2, LY3 and LY4 antigens. These results indicate that group 1 ruminococci were relatively stable during this experiment and provide only slight evidence of the progressive changes in intestinal flora postulated by Emslie-Smith (1961) and Medrek & Barnes (1962*b*).

The detection of group 1 ruminococci in sheep C76 as well as sheep C6 suggests that an interchange of ruminococcal strains between animals occurs. Such a transmission of microbial species between animals has been reported by other workers (Briggs, 1951; Eadie, 1962).

The isolation of strains LY9 (group 1) and LY13 (group 2) from sheep C76 indicates that group-specific determinants were not dependent on the rumen environment, and it can be inferred that the genetic capacity to produce group 1 antigenic determinants persisted in the intestinal flora of sheep C6 over a period of 18 months. This may have been due, as Cummins (1962) suggested, to the relative stability of cell-wall structures compared with those parts of the organism which must adapt to changing external conditions, but it is possible that selection was rigorous enough to maintain a recognizable genetic strain against mutation pressure.

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