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Multiple Forms of D(—)3-Hydroxybutyrate Dehydrogenase in Rhizobium

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SUMMARY

3-Hydroxybutyrate dehydrogenase was studied in 14 Rhizobium strains representing six species. Cell-free extracts of the bacteria were subjected to electrophoresis in starch or polyacrylamide gels and 3-hydroxybutyrate dehydrogenase was located *in situ* on the gels. Also, the rate of reaction of the enzyme, from different Rhizobium strains, with NAD was compared with its rate of reaction with various analogues of NAD. Most of the Rhizobium strains had characteristic 3-hydroxybutyrate dehydrogenase patterns on gel electropherograms and these patterns were highly reproducible. Multiple forms of 3-hydroxybutyrate dehydrogenase were detected in many of the Rhizobium strains.

INTRODUCTION

3-Hydroxybutyrate dehydrogenase (HBDH) (D-3-hydroxybutyrate:NAD oxidoreductase, EC 1.1.1.30) catalyses the reversible oxidation of 3-hydroxybutyrate to acetoacetate in the presence of NAD^+. The enzyme occurs in micro-organisms (Shuster & Doudoroff, 1962) and in mitochondria of mammalian cells (Green, Dawan & Leloir, 1937). We recently showed, in a preliminary report (O'Hora & Fottrell, 1968), that multiple forms of HBDH could be detected in rhizobia with the aid of gel electrophoresis. The HBDH electrophoresis patterns, in some instances, were species and strain specific. In the present paper gel electrophoresis and coenzyme analogues (Kaplan & Ciotti, 1961) were used to compare HBDH from six Rhizobium species.

METHODS

Organisms. Fourteen Rhizobium strains representing six species were used. The bacteria, all of which nodulated their respective host plants, are listed in Table 1. All strains were streaked on yeast extract (Oxoid) mannitol agar (Allen, 1957), to confirm purity before being inoculated into growth medium. The bacteria were grown in 2-litre Erlenmeyer flasks containing yeast extract and mannitol, on an orbital shaker at 28°, for 4 days in the case of *Rhizobium leguminosarum*, *R. trifolii*, *R. meliloti* and *R. phaseoli* and for 8 days in the case of *R. japonicum* and *R. lupini*. The bacteria were harvested by centrifugation, washed with 0·9% NaCl and stored at −20° until required.

Soybeans (*Glycine max.*) were grown in nitrogen-free medium and inoculated with the appropriate Rhizobium strain as described previously (Fottrell, 1966). Bacteroids were isolated from soybean nodules as described by Bergersen (1960).

Preparation of cell-free extracts. Suspensions of bacteria and bacteroids (1 g. organisms/1·5 ml. buffer) were disrupted for 2 min. at a temperature less than 5° in 0·05 M-phosphate buffer ($Na_2HPO_4 + NaH_2PO_4$), pH 7·6, with an M.S.E. sonic disintegrator. HBDH was also extracted from organisms by grinding with alumina in 0·05 M-phosphate buffer (pH 7·6) for 30 min. at 4°. After disruption, the suspension was centrifuged for 90 min. at 35,000*g* at 0° and the clear supernatant used for enzyme assays and electrophoresis. Approximately 85% of the total cellular HBDH activity was present in the supernatant after centrifugation.

Assay of HBDH. Enzyme activity was estimated spectrophotometrically at 340 mμ as described by Bergmeyer *et al.* (1967). Specific activity was expressed in international units per mg. protein (Bergmeyer, 1965). Protein was estimated by the biuret reaction (Gornall, Bardawill & David, 1949).

Table 1. *Physiological characteristics and source of Rhizobium strains*

Species	Strain no.	Test host	N-fixation*	Source†
Rhizobium japonicum	392	*Glycine max.*	+	A
	311b59	*Glycine max.*	+	C
	3401	*Glycine max.*	−	B
R. leguminosarum	1001	*Vicia hirsuta*	+	B
	1003	*Vicia hirsuta*	−	B
R. trifolii	7	*Trifolium pratense*	+	B
	6	*Trifolium pratense*	±	B
	5	*Trifolium pratense*	++	B
	4	*Trifolium pratense*	−	B
R. phaseoli	3601	*Phaseolus vulgaris*	+	B
	3602	*Phaseolus vulgaris*	−	B
R. meliloti	3 Doa30	*Medicago sativa*	+	C
R. lupini	3206	*Lotus corniculatus*	−	B
	3001	*Lotus corniculatus*	+	B

* ++, Highly effective in nitrogen fixation; +, normally effective; ±, poorly effective; −, ineffective.

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Electrophoresis. Polyacrylamide gels, containing 5·5% (w/v) polyacrylamide were prepared with 0·05 M-tris + glycine buffer (pH 8·4) in 6 × 64 mm. glass tubes as described by Davis (1964). Vertical starch gels were prepared with 14% starch (Connaught Laboratories, Toronto) in 5 mM-tris + maleate buffer (pH 8·4) (Smithies, 1959). Occasionally starch gels were prepared in 5 mM-tris + maleate buffer (pH 7·4).

Extracts were assayed for HBDH before being added to the gels. Depending on the activity of the extract, 20 μl. to 30 μl. was added to polyacrylamide gels and 40 μl. to 50 μl. to vertical starch gels. In a typical experiment a 20 μl. sample of extract contained 12 mg. protein and 0·32 i.u. of HBDH per ml.

For polyacrylamide gels, 50 v was applied for 90 min. which gave 2·5 m.amps per glass tube. Vertical starch gel electrophoresis was carried out at 4 v per cm. of gel for 16 to 20 hr.

After electrophoresis, the enzyme was located by immersing the gels in a solution containing the following reagents:

(*a*) 1·0 ml. DL-3-hydroxybutyric acid (M) (British Drug Houses Ltd.); (*b*) 1·0 ml. NAD (10 mg./ml.) (Boehringer & Son, Mannheim); (*c*) 0·25 ml. phenazine methosulphate (1·0 mg./ml.) (Sigma Chemical Co.); (*d*) 2·5 ml. nitro-blue tetrazolium (1 mg./ml.) (Sigma Chemical Co.); (*e*) 2·5 ml. phosphate buffer (0·5 M, pH 7·4); (*f*) 1·0 ml. $MgCl_2$ (5 mM); (*g*) 1·0 ml. NaCl (0·1 M).

About 20 min. at 37° was usually allowed for development of the gels and after staining was completed starch gels were stored in the dark in methanol + acetic acid + water (5 + 1 + 5) and polyacrylamide gels in 7·5 % (v/v) acetic acid.

For detecting non-specific proteins, starch gels were stained with Coomassie Blue (G. T. Gurr Ltd., London) for 15 min. and then rinsed in methanol + acetic acid + water (5 + 1 + 5). Coomassie Blue was more efficient than Naphthalene Black 10B for revealing protein bands.

Coenzyme analogues. The rate of reaction of HBDH from different Rhizobium strains was measured with NAD and with various analogues of NAD. The ratio of the rate of reaction with NAD/coenzyme analogue was determined for the following analogues: Acetylpyridine NAD, Pyridinealdehyde NAD, Deamino NAD and Acetylpyridine Deamino NAD (Sigma Chemical Co.).

Table 2. *Relative mobilities of major 3-hydroxybutyrate dehydrogenase bands from various Rhizobium strains*

Strain no.	HBDH bands									
	a	b	c	d	e	f	g	h	i	j
1001	—	—	0·41*	—	—	0·76	0·83	0·88	0·94	1·00
1003	—	—	—	—	0·62	0·77	—	—	—	—
4	0·19	—	—	—	0·67	0·78	—	—	—	—
5	—	—	—	—	—	0·78	—	—	—	—
6	—	—	—	0·53	—	0·78	—	—	—	—
7	0·19	—	—	—	0·67	0·78	—	—	—	0·97
3601	—	—	—	—	—	—	—	—	—	1·10
3602	—	—	—	—	—	0·80	—	—	—	—
3001	—	—	—	—	—	0·79	—	—	—	1·08
3206	—	—	—	—	0·65	—	0·82	—	—	—
3D0a30	—	—	—	—	—	—	—	0·87	—	—
3401	—	—	—	—	—	—	—	—	—	1·40
311b59	—	—	—	0·53	0·62	—	—	—	—	—
311b59B**	—	—	—	0·52	0·68	—	—	—	—	—
392	—	0·30	0·44	—	—	—	—	—	—	—
392B**	—	0·26	0·44			—	—	—		—

* Extracts were separated by vertical starch gel electrophoresis at pH 8·4. Strain 1001 was used as a reference strain and the mobilities of the other bands were expressed as a fraction of the mobility of band j of 1001, which was given an arbitrary value of 1. Each figure represents a mean measurement for three separate extracts.

** Bacteroids isolated from nodules of soybeans inoculated with either 311b59 or 392.

RESULTS

HBDH patterns of Rhizobium strains. A qualitative comparison of HBDH from 14 Rhizobium strains by polyacrylamide gel electrophoresis showed that various degrees of complexity existed in the HBDH patterns of different strains. Multiple forms of HBDH were detected in several strains, e.g. strains 7, 1001 and 392. Vertical starch gel electrophoresis, at pH 8·4, was used to compare electrophoretic mobilities

of major bands because it was possible to compare ten extracts under identical conditions. Strain 1001 was used as a reference strain and the electrophoretic mobility (distance in cm. from the origin) of the most rapidly migrating band of HBDH from this strain was given an arbitrary value of 1. Mobilities of major HBDH bands from extracts of other strains were expressed as a ratio of this value (Table 2). The values obtained for relative mobilities of HBDH bands, by this procedure, were very reproducible when different extracts of a given strain were compared. The data in Table 2 also showed that individual Rhizobium strains had characteristic patterns although strains of *Rhizobium trifolii* (e.g. 4 and 7) and *R. leguminosarum* (e.g. 1003) showed some resemblance to each other. It was also interesting that the HBDH patterns of strains 392 and 311b59 persisted in bacteroids isolated from soybean nodules. Results similar to those in Table 2 were also obtained when extracts from Rhizobium strains were compared at pH 7·4 instead of pH 8·4, with the exception that extracts of strains 392 and 311b59 were difficult to separate at pH 7·4. Protein patterns of individual Rhizobium strains, in contrast to HBDH patterns, did not show a similar degree of strain specificity.

Table 3. *Ratios of reaction rates of 3-hydroxybutyrate dehydrogenase from various Rhizobium strains with NAD and analogues of NAD*

Strain no.	Specific* activity	NAD. PyAldNAD	NAD. AcPyNAD	NAD. AcPyDeNAD
1001	0·03	4·8	4·3	6·7
1003	0·10	10·9	6·7	11·5
7	0·05	11·9	6·0	12·2
4	0·06	11·9	6·5	> 100
5	0·05	13·0	7·1	23·0
3601		5·3	10·2	> 100
3602	0·07	15·5	5·9	10·0
3D0a30	0·08	11·8	18·8	> 100
392	0·10	9·0	10·3	42·0
392B**	0·08	4·6	10·0	30·2
311b59	0·14	12·8	10·0	53·4
3002	0·05	> 100	21·7	> 100
3206	0·07	14·1	38·4	40·0

* Units, per mg. protein (determined with NAD).

** Bacteroids isolated from nodules of soybeans inoculated with 392.

Each figure represents the mean of at least three separate assays on different extracts of the same strain.

Coenzyme analogues. The values of the ratios for the rates of reaction with NAD and with three coenzyme analogues, for HBDH from different Rhizobium strains, are given in Table 3. These analogues were active with HBDH from all the Rhizobium strains whereas deamino NAD was inactive with enzyme from several strains. In some instances, however, the rates of reaction with the analogues were relatively low, e.g. *Rhizobium lupini*, strains 3002 and 3206 (Table 3). In common with the electrophoresis results, each Rhizobium strain had characteristic values for the coenzyme analogue ratios. In view of the similarity of the electrophoresis patterns of some strains of *R. leguminosarum* and *R. trifolii* it was interesting that one strain from each of these species (i.e. 1003 and 7) had very similar coenzyme analogue ratios. Other workers have also detected similarities between some strains of *R. trifolii* and *R. leguminosarum*

by serological tests (Graham, 1963), DNA homology tests (Heberlein, De Ley & Tijtgat, 1967) and numerical analysis (Graham, 1964; 'tMannetje, 1967).

DISCUSSION

Gel electrophoresis of soluble proteins followed by histochemical methods for locating enzymes *in situ* on gels is becoming an increasingly popular method for characterizing micro-organisms (Cann & Willox, 1965; Williams & Bowden, 1968; Peberdy & Turner, 1968).

Kaplan and co-workers (Kaplan, 1963) have also shown that coenzyme analogues can be used to compare functionally similar enzymes in different strains of the genus *Bacillus* and of the Enterobacteriaceae. These present results show that gel electrophoresis and coenzyme analogues can be used to compare HBDH from different Rhizobium species. Although only 14 Rhizobium strains were studied, interesting findings emerged on the relationship between some strains of *R. trifolii* and *R. leguminosarum*.

HBDH electrophoresis patterns together with coenzyme analogue ratios could be useful procedures for characterizing micro-organisms which contain poly-3-hydroxybutyric acid. The polymeric ester of D(−)3-hydroxybutyric acid is the principal storage product in a wide variety of bacteria (Dawes & Ribbons, 1964) including nitrogen-fixing bacteria and blue-green algae (Carr, 1966). HBDH is one of the enzymes responsible for the conversion of poly-3-hydroxybutyrate to utilizable cell material (Shuster & Doudoroff, 1962). In some Rhizobium species where poly-3-hydroxybutyrate can account for 40% of the dry weight of the bacteria (Hayward, Forsyth & Roberts, 1959; P. F. Fottrell and A. O'Hora, unpublished), the polymer probably serves as a source of energy and reducing power during nitrogen fixation (Klucas & Evans, 1968).

Although multiple forms of HBDH probably exist in Rhizobium great care was taken to avoid some of the artifacts which can arise in studies of this nature (Kaplan, 1968; Heidrich, 1968). For example, it was possible that multiple forms of HBDH might result from the formation of disulphide bridges in the enzyme molecule during the extraction procedure. However, identical electrophoresis patterns were obtained when Rhizobium strains were extracted by different procedures in the presence and absence of mercaptoethanol.

The physiological significance of the multiple forms of HBDH in Rhizobium is not known at present. Five forms of HBDH were found in extracts of human brain by Van der Helm (1962) and recently in a heterozygote of *Paramecium aurelia* by Tait (1968).

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Methane as a Minor Product of Pyruvate Metabolism by Sulphate-reducing and Other Bacteria

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SUMMARY

Disrupted cells of some Desulfovibrio species, of *Desulfotomaculum ruminis* and of certain other anaerobes produced methane as a minor product of pyruvic phosphoroclasm. In one Desulfovibrio species the reaction, which was not specially sensitive to air, involved vitamin B_{12}, co-enzyme A, thiamine pyrophosphate, magnesium ions and acetyl phosphate. Adenine and other nucleotides stimulated the reaction; a mixture of ATP and AMP was most effective. Methionine stimulated the reaction but the other methyl donors did not. In optimal conditions methane accounted for 0·1 to 0·02 mole % of the pyruvate metabolized, formed at 20 to 30 nl. CH_4/mg. bacterial protein/hr. Tests in D_2O indicated that the methane came from the methyl-carbon of pyruvate; correspondingly, ethane formation from α-ketobutyrate was detected.

INTRODUCTION

Sisler & ZoBell (1951) reported that certain cultures of marine sulphate-reducing bacteria, pure by normal criteria, formed methane during growth in lactate + sulphate media. Though occasional references to this finding have appeared, and Sorokin (1956) failed to confirm it, interest has tended to centre on the disputed question of the oxidation of methane (and other hydrocarbons) by desulfovibrios (e.g. Sorokin, 1957; Davis & Yarbrough, 1966). Methane formation by phosphoroclastic preparations from strains of sulphate-reducing bacteria was reported briefly by Postgate (1969). The present paper describes the reaction in one Desulfovibrio strain in more detail and reports its occurrence in other bacteria.

METHODS

Cultivation. Desulfovibrio desulfuricans variety *azotovorans*, strain BERRE S (NCIB 8388) was grown at 30° in a 600 ml. continuous-culture apparatus (Baker, 1968) at dilution rates ranging from 0·05 to 0·1 hr^{-1}. The medium used was deficient in fixed nitrogen; it contained (g./l.): KH_2PO_4, 0·5; Na_2SO_4 4·5; $CaCl_2.2H_2O$, 0·06; $MgSO_4.7H_2O$, 0·06; $FeSO_4.7H_2O$, 0·004; sodium lactate, 6; sodium citrate, 0·3 (to prevent precipitation of metal hydroxides) in distilled water; plus 10 ml. of a trace element mixture containing (mg.): H_3BO_3, 2·32; $CoSO_4.7H_2O$, 95·6; $CuSO_4.5H_2O$, 8; $MnSO_4.4H_2O$, 8; $NaMoO_4.2H_2O$, 30; $ZnSO_4.7H_2O$, 174 in 1 l. distilled water. The complete medium was at pH 8·2; the atmosphere was N_2 containing 6 to 8% (v/v) CO_2. The CO_2 buffered the culture to between pH 7·6 and 8, and displaced excess sulphide.

Effluent was harvested when 0·75 to 3 l. had accumulated, the organisms washed by centrifugation in 25 mM-tris buffer (pH 7·5 ± 0·1) and disrupted by dropping a cream of washed bacteria (equiv. 50 to 100 mg. dry wt/ml. tris buffer) into liquid nitrogen. Batch cultures of BERRE S and other strains of sulphate-reducing bacteria were grown in long-necked 250 or 500 ml. flasks of medium C (Postgate, 1966) under N_2 and were disrupted in a similar manner.

Criteria of purity. The strains of sulphate-reducing bacteria were examined microscopically for morphological homogeneity and were tested for both aerobic and anaerobic contamination by using the conditions prescribed by Postgate (1953). No contaminants were found. These procedures might not reveal as contaminants methane-producing bacteria, which are known to associate with sulphate-reducing bacteria (e.g. Stadtman & Barker, 1951) and which could, if present, have been responsible for the phenomenon being investigated. The continuous culture of strain BERRE S, the organisms routinely studied, was therefore examined exhaustively for methane bacteria, by using the media of Stadtman & Barker (1951) with sodium acetate (the main product after growth in the continuous culture), sodium formate, sodium pyruvate or ethanol (0·1%) as carbon sources. Neither 'shake' cultures in such media solidified with agar, nor anaerobic enrichment cultures in stoppered bottles, showed bacterial growth or gas production at 30°. The culture was therefore accepted as free from methane bacteria.

Enzyme assays. Pyruvic phosphoroclastic activity was detected by conventional Warburg manometry at 30°. The preparation under test was set up in a manometer vessel without KOH, gas evolution in response to added pyruvate was observed and finally CO_2 was demonstrated in the gas mixture by introducing 10 N-KOH and observing gas absorption. Methane formation was detected by vapour-phase chromatography, by the following routine procedure. 'Penicillin vials' of about 8 ml. capacity, cooled in ice, were provided with 0·5 to 1 ml. tris buffer containing all necessary co-factors (see Results) except for $MgCl_2$ and the substrate pyruvate. A fresh frozen-cell preparation, after 5 to 20 min. in liquid N_2, was thawed with 1 μg. each of RNase and DNase under nitrogen or argon to between 0 and 10° and homogenized by vortex-stirring with a Whirlimixer (Scientific Industries (U.K.) Ltd.). Volumes of cell preparation equal to the volumes of co-factor solution already present were distributed among the penicillin vials, usually to give equiv. 8 to 15 mg. bacterial protein/vessel. While still cool the vials were flushed with argon and the reaction initiated as soon as possible by injecting pyruvate + $MgCl_2$, transferring the vials to a thermostat bath at 30° and shaking gently (80 to 85 strokes/min. at amplitude 4 cm.). In a few experiments, nitrogen or other gases replaced argon; in some the reaction system was set up complete with substrates and initiated by warming to 30°; in testing new organisms a control without pyruvate was included. Protein was estimated by the biuret method with serum albumin as standard.

Gas chromatography. The gas phase was sampled by filling a syringe with 0·5 ml. argon or other appropriate gas, injecting this and, after a moment for mixing, removing 0·5 ml. for chromatography. CH_4 was detected and estimated on the Pye 104 gas-liquid chromatograph, with a 5 ft. (152 cm.) 'Porapak R' column at 75° or 45°, leading to a flame-ionization detector. The attenuation settings used were 10 to 50. The gas was identified as CH_4 by infrared spectroscopy in a specially scaled-up experiment. In time-course experiments a correction was made for losses and dilution

of CH_4 involved in the sampling procedure; the correction was imprecise because CO_2 and H_2, which were produced simultaneously, altered the pressure within the reaction vessel by amounts which varied according to the conditions. In such experiments, therefore, only wide differences in rates of gas production were taken as meaningful.

The argon used contained small amounts of methane (0·2 to 1 part/10^8, v/v) which were corrected for; the nitrogen contained none. The argon also contained a similar amount of an unidentified gas running just after hydrogen; though the chromatography detector was a hydrogen flame, very large amounts of hydrogen gave a signal just before the argon impurity peak, and the appearance of such a 'spike' during experiments was a useful indication that a conventional pyruvic phosphoroclasm was in progress.

For the separation of CD_4 from CH_4 a 5 Å molecular-sieve column (5 ft., 152 cm., 60 to 80 mesh, from F. & M. Scientific Ltd.) at 25° replaced the 'Porapak R' column. CD_4 (prepared from methyl isocyanide and Azotobacter nitrogenase; Kelly, Postgate & Richards, 1967) ran ahead of CH_4 and was distinguished from it unequivocally by cutting out and superimposing the traces.

Materials. Ordinary chemical reagents were of analytical grade, except some of those used in preparing media. Adenine- and other nucleotides, co-enzyme A, thiamine pyrophosphate, lithium acetyl phosphate, cyanocobalamin, thioctic acid, sodium α-ketobutyrate and sodium pyruvate were Sigma products; the ADP contained variable amounts of AMP and was specially purified chromatographically for critical tests.

RESULTS

Conditions for methane formation. Suspensions of whole bacteria of *Desulfovibrio desulfuricans* strain BERRE S, harvested from continuous culture with gaseous N_2 as its sole nitrogen source, showed vigorous phosphoroclastic activity. Traces of methane were detected in the overlying gas amounting to 1 or 2 parts/10^8 formed in 3 hr. Methane was not detected above cultures of this strain when growing in sodium lactate media, nor did the strain utilize methane. No growth in excess of the control occurred when medium C without lactate was inoculated and incubated for 18 days at 30° under 1·5 volumes of N_2 + 7% (v/v) CH_4. 'Blank' growth on the yeast extract in medium C indicated that these conditions were not intrinsically inimical to growth; no utilization of the CH_4 in the atmosphere was detected chromatographically.

Bacterial suspensions disrupted as described in Methods showed much more impressive methane production which varied according to test conditions; suspensions washed with distilled water and dried at 30° *in vacuo* were also active. Methane was also produced in Na phosphate, tris and Na dimethylglutarate buffers (25 mM, pH 7·5). Bacteria grown in medium C, which contained fixed nitrogen, then frozen and thawed as described in Methods, also gave active preparations. Bacteria from the continuous culture were used in most experiments because they were readily available.

Effects of co-factors. Yates (1967) showed that the phosphoroclastic system of strain BERRE S, like those of certain clostridia (Wiesendanger & Nisman, 1954; Biggins & Dilworth, 1968), was stimulated by ATP. Figure 1 illustrates stimulation of methane formation by ATP. Acetyl phosphate occasionally stimulated the system; Yates routinely added certain known co-factors of the phosphoroclastic system;

the following co-factors stimulated methane production by BERRE S preparations in 25 mM tris pH 7·5 (optimal concentrations are given in parentheses): $MgCl_2$ (2·5 mM), KH_2PO_4 (1·6 mM), co-enzyme A (0·5 mg./ml.), thiamine pyrophosphate (0·05 mg./ml.), acetyl phosphate (0·5 mg./ml.), ATP (1 mM). The concentration of co-enzyme A required for maximum activity was unexpectedly large but was indeed necessary; it could not be replaced by equimolar cysteine. No unequivocal effect of α-lipoic acid (0·5 mg./ml.) was detected; K^+ as KCl (5 mM) did not affect the reaction. Ferredoxin, rubredoxin and cytochrome c_3 extracted from strain BERRE S were tested as concentrates after ion-exchange chromatography (50 to 55 μg. carrier/ml.): ferredoxin and rubredoxin depressed methane production slightly; cytochrome c_3 had no effect. No clear effect on hydrogen production by any of these factors was observed.

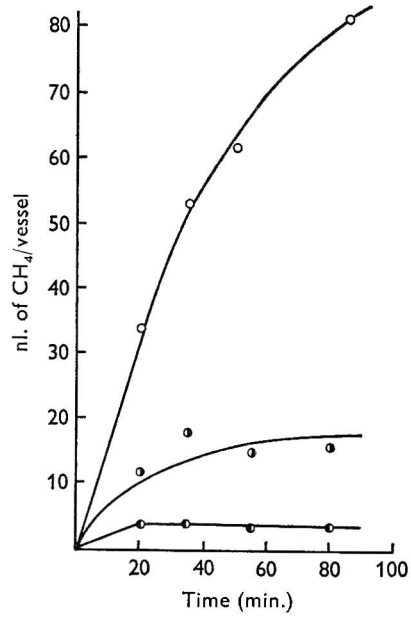

Fig. 1. Effect of ATP on methane production by *Desulfovibrio desulfuricans* strain BERRE S disrupted by freezing in liquid nitrogen. Reaction mixture (2 ml.) contained acetyl phosphate, co-enzyme A, cyanocobalamin, thiamine pyrophosphate and potassium phosphate in tris buffer and equiv. 8·1 mg. bacterial protein at 30° under argon in an 8 ml. penicillin vial; for details see text. ○, ATP (1 mM), $MgCl_2$ (2·5 mM) and sodium pyruvate (7·5 mg./ml.) added to start reaction; ◑, ATP omitted; ◐, pyruvate omitted. A boiled control showed no activity.

Blaylock & Stadtman (1963, 1964) showed that a methane-producing system from *Methanosarcina barkerii* utilized cobalamin derivatives and that methyl B_{12} was an intermediate. Cyanocobalamin stimulated methane production by the BERRE S preparation and showed optimal activity at 5 to 50 μg./ml.; 500 μg./ml. depressed activity by about 60%. During the reaction the pink B_{12} colour became bleached, suggesting that reduced intermediates capable of accepting methyl groups were being formed.

On the basis of these findings a standard reaction mixture was formulated for other

tests containing ATP, 1 mM; KH_2PO_4, 2·5 mM; thiamine pyrophosphate, 0·1 mg./ml., co-enzyme A, 1 mg./ml.; lithium acetyl phosphate, 1 mg./ml.; cyanocobalamin, 0·01 mg./ml. tris buffer (0·025 M, pH 7·5). Sodium pyruvate (15 mg./ml.)+$MgCl_2$ (5 μmole) were generally injected in 0·1 ml. buffer to initiate reaction; in certain tests ATP or substitute nucleotides were omitted from the reaction mixture and injected with the substrates.

Effect of pH value, pyruvate and enzyme concentration. Methane was produced more rapidly at pH 7·5 than at pH 7 or 8. Pyruvate was necessary for methane formation (Fig. 1). Between 10 and 100 mg. sodium pyruvate/ml. the rate of production of CH_4 decreased, though the final amount did not necessarily do so; the initial rate was constant between 1 and 10 mg. pyruvate/ml. but the rate always decreased with time (see Fig. 1). The reaction rate with 5 mg. pyruvate/ml. was proportional to 'enzyme' concentration between equiv. 7 and 25 mg. bacterial protein/ml. but decreased at 35 mg./ml.

Effect of air. Extracts of *Methanobacterium omelianskii* are extremely sensitive to air (Wolin, Wolin & Wolfe, 1963). Preparations of *Desulfovibrio desulfuricans* strain BERRE S were not unduly sensitive: equiv. 6·3 mg. bacterial protein/vessel (2 ml. fluid) yielded 12·6 to 17·4 nl. CH_4/mg. protein/hr under air, nitrogen+air to 0·02 atm. O_2, or pure N_2. CO_2 was formed in all conditions. A preparation of equiv. 8·5 mg. protein/ml. retained 65 % of the activity of a control in argon after stirring vigorously in air for 1 min. with a vortex mixer. This relative insensitivity to oxygen makes it unlikely that the stimulating effect of cyanocobalamin was due to scavenging of residual oxygen by the catalytic effect of vitamin B_{12} on thiol oxidation (Peel, 1963). Nevertheless, this possibility was tested by using sodium dithionite to remove oxygen; cyanocobalamin stimulated the reaction equally well whether $Na_2S_2O_4$ (0·5 mg./ml.) was present or not.

Effects of reaction products. The major products of pyruvic phosphoroclasm in desulfovibrios are acetate (by way of acetyl phosphate), CO_2, H_2 and/or ethanol (Millet, 1954; Sadana, 1954). Acetyl phosphate yielded no methane alone but, in the presence of pyruvate, was a strong stimulant of CH_4 production. It was routinely present in the reaction mixtures. Sodium acetate (0·5 mg./ml.) had no effect; phosphoenol pyruvate (0·5 mg./ml.) was also inactive. CH_4 was formed under helium or N_2 as well as under argon, but an atmosphere of H_2 decreased the rate of production and yield by about 55 %. CO_2, either 3 % (v/v) in the atmosphere or as 0·5 mg. $NaHCO_3$/ml. in the reaction mixture, decreased the amounts of methane formed by 60 to 75 %. Ethanol (0·5 mg./ml.) inhibited methane production by 95 %.

Other substrates and inhibitors. Sodium formate, which undergoes the hydrogenlyase reaction in Desulfovibrio (Ishimoto, Yagi & Shiraki, 1957), yielded no methane at 0·5 mg./ml. Sodium α-ketobutyrate was metabolized by preparations of *D. sulfuricans* strain BERRE S with a nominal Q_{CO_2} of 4·8 μl. CO_2/mg. bacterial protein/hr as compared with a control Q_{CO_2} value of 2·14 without substrate and of 40 with pyruvate (for calculating these values all the gaseous product was taken to be CO_2 though, with pyruvate, some was H_2); α-ketobutyrate depressed the 'blank' methane production by such preparations but ethane was detected in the overlying gas, formed at about 0·6 nl./mg.bacterial protein/hr. Methanol or propanol (0·5 mg./ml.) decreased the rate and amount of methane formation by 58% and 90 %, respectively; butanol, at a similar concentration, had no influence on the reaction. Methylviologen (0·25 mM) completely

inhibited methane formation but stimulated formation of hydrogen. Methylviologen has a comparable effect on methane formation by washed *Methanobacterium omelianskii* (Wolin, Wolfe & Wolin, 1964).

Effects of biological methylating agents. Choline chloride (0·5 mg./ml.) had no effect on methane formation. Methionine (0·05 to 1 mM) stimulated the reaction moderately in five tests out of six; S-adenosyl methionine (0·5 mg./ml.), which might have been formed from ATP and methionine, had no effect when tested as the iodide, in place of both ATP and methionine.

Effects of nucleotides. Reaction mixture made up without ATP was tested with ADP or AMP at 1 mM or pairs of adenine nucleotides at 0·5 mM each. For these tests specially purified ADP was used. In four separate tests, no single nucleotide was more effective than ATP and ADP was generally the least effective. But mixtures of nucleotides had profound effects, though test-to-test variations were considerable. ATP + AMP had maximum effect on one occasion but did not exceed ATP alone on another. Equimolar amounts of other nucleotide triphosphates replaced ATP: guanosine triphosphate and uridine triphosphate were about as effective as ATP, cytidine triphosphate showed 50% activity, inosine triphosphate was inactive.

Table 1. *Stoichiometry of methane production by Desulfovibrio desulfuricans strain* BERRE S *during pyruvic phosphoroclasm*

Washed organisms were disrupted in liquid N_2 and incubated under argon in Warburg manometers at 30° with 7·5 to 10 mg. sodium pyruvate/ml. reaction mixture (see text). Seven different bacterial preparations are recorded.

mg. bacterial protein/ml.	Incubation (min.)	CO_2 (μmole)	H_2 (μmole)	CH_4 (nmole)	CH_4 as mole % CO_2
35*	23	8·8	9·3	4·7	0·053
24	30	16·1	—	38·0	0·24
50	45	12·4	15·5	7·0	0·56
33†	50	5·0	0·56	7·2	1·4
9·2‡	55	9·1	2·2	0·48	0·053
9·2‡	65	7·05	4·5	0·38	0·051
7	110	11·4	0·7	2·87	0·025
7·7§	155	20·4	0·75	2·57	0·0126

* 0·5 mg. DL-methionine/ml. reaction vessel present.
† Bacteria grown with fixed-nitrogen (note relatively low CO_2 and H_2 output).
‡ Same bacterial preparations.
§ Experiment in penicillin vial, not Warburg manometer; all gases analysed by gas chromatography.

Stoichiometry. Blaylock & Stadtman (1963) noted that a considerable consumption of pyruvate was required to generate relatively little methane by extracts of *Methanosarcina barkerii*. Preparations of *Desulfovibrio desulfuricans* strain BERRE S were studied in 2-side arm Warburg flasks with a 'Suba-seal' closure on one arm to permit removal of gas samples and injections of alkali for gasometric CO_2 analysis. Table 1 quotes selected results from eight experiments and illustrates three points. (1) Methane was formed in greater proportions early in the reaction. (2) The theoretical CO_2:H_2 ratio of 1 for pyruvic phosphoroclasms was rarely obtained, and the H_2 yield was generally low. This finding was checked by experiments using gas-chromatographic analysis for CO_2 and H_2 with a gas-density detector: the situation was attributed to

hydrogen consumption by endogenous hydrogen acceptors in the strongly proteinaceous preparations necessary for this work; freshly disrupted strain BERRE S preparations had initial $-Q_{H_2}$ values of about 16 μl. H_2/mg. protein/hr. (3) Methane accounted for between 0·02 and 0·2 mole % of the pyruvate utilized, considerably less than the 3 to 7 mole % methane which Blaylock & Stadtman (1963) observed with *Methanosarcina barkerii* and methyl B_{12} as co-factor.

Origin of the methane. The experiments in which ethane was formed from α-ketobutyrate suggested that the ethane originated from the alkyl chain of the substrate, not by CO_2 reduction. This conclusion was checked by tests in D_2O, which would give CD_4 by a CO_2-reduction mechanism but CH_3D (chromatographically indistinguishable from CH_4 in my test system) by a reductive decarboxylation mechanism. Bacteria pre-equilibrated 20 min. at 30° with D_2O buffer and vacuum-dried bacteria re-constituted in D_2O buffer were tested; both yielded peaks indistinguishable from CH_4. Insufficient gas was found for identification of CH_3D by infrared spectroscopy.

Table 2. *Methane-producing activity of disrupted sulphate-reducing and other bacteria*

Washed organisms were disrupted in liquid N_2 and shaken under argon at 30° with 5 to 20 mg. sodium pyruvate/ml. reaction mixture (see text). Bacterial protein 3 to 30 mg./vessel according to activity; negative or 'trace' tests ran for 90 to 180 min., positives ran for 30 to 50 min. All preparations showed CO_2 and/or H_2 production from pyruvate; *Clostridium pasteurianum* and *Bacillus polymyxa* were not tested.

Species and strain with N.C.I.B. number	CH_4 produced (nl./mg. protein/hr.)
Sulphate-reducing bacteria	
Desulfovibrio desulfuricans BERRE S (8388)	15–20
D. africanus BENGHAZI (8401)	0·16
D. salexigens BRITISH GUIANA (8403)	1·7
Desulfotomaculum ruminis COLEMAN 42 (10,149)	9·5–11
Others	
Clostridium pasteurianum strain W	2·65

Methane at less than 0·1 nl./mg. hr. was formed by *Desulfovibrio desulfuricans* BERRE E (8387), *Desulfovibrio vulgaris* 'HILDENBOROUGH' (8303), *Desulfovibrio gigas* (9332), *Klebsiella pneumoniae* and *Bacillus polymyxa*. No methane was formed by *Desulfovibrio desulfuricans* 'ESSEX 6' (8307) or NORWAY 4 (8310), nor by *Rhodospirillum rubrum*.

Tests of other bacteria. Batches (250 ml.) of sulphate-reducing bacteria were grown in medium C under nitrogen, harvested and disrupted in liquid nitrogen as described. The preparations were tested in the usual reaction mixture for methane formation, and pyruvic phosphoroclastic activity was tested by Warburg manometry. Table 2 lists the strains tested; activity was not restricted to any particular species or even genus and not all strains of a species were active. Some were tested twice and showed wide differences; only *Desulfotomaculum ruminis* approached the activity of the BERRE S strain of *Desulfovibrio desulfuricans*. Non-sulphate-reducing bacteria were obtained from Dr M. Kelly (this laboratory) as live cultures or frozen suspensions grown in conventional nitrogen-deficient media for 2 to 4 days at 30°; phosphoroclastic activity was obvious in the preparation of *Clostridium pasteurianum* because a 'hydrogen spike' (see Methods) was observed in the chromatographic tests. The presence of

chalk carried over from the culture medium precluded a satisfactory manometric test for pyruvic phosphoroclasm with this strain and *Bacillus polymyxa*. *Clostridium pasteurianum* was tested with sodium α-ketobutyrate, from which it formed a trace of ethane.

DISCUSSION

Methane is unequivocally a minor product of pyruvic phosphoroclasm in a majority of the strains of sulphate-reducing bacteria tested and cannot reasonably be attributed to contaminant methane bacteria. It is produced in insufficient amounts to account for the stimulation of pyruvic phosphoroclasm in *Desulfovibrio desulfuricans* strain BERRE S as recorded by Yates (1967). The strains differed in their productivities, as Sisler & ZoBell (1951) reported among the cultures they surveyed, and though amounts of methane comparable with Sisler & ZoBell's maximum yields were not encountered over live cultures, it is reasonable to suppose that differences in strains and test conditions used account for this discrepancy and for Sorokin's (1956) failure to confirm Sisler & ZoBell's report.

A soluble methane-producing system was not studied because attempts to extract a soluble pyruvic phosphoroclastic system from *Desulfovibrio desulfuricans* strain BERRE S have not so far been successful. The properties of the particulate preparations, though not always as clear-cut as might be expected from soluble enzymes, resemble in certain details those of the extracts of *Methanosarcina barkerii* studied by Blaylock & Stadtman (1963, 1964; see also Stadtman, 1967): vitamin B_{12} is involved (it stimulates and becomes bleached during the reaction), and adenine nucleotides stimulate. But the source of the methane is the 3-C group of pyruvate, which it is not in extracts of *M. barkerii*. As in true methane bacteria, the yield of methane is low in terms of pyruvate consumed. This feaure also recalls the low ammonia yield in the cell-free nitrogen-fixing phosphoroclastic system from *Clostridium pasteurianum* studied by Carnahan, Mortenson, Mower & Castle (1960), but there seems to be no direct connexion between methane formation and nitrogen fixation, since (1) ammonia-grown populations of *D. desulfuricans* strain BERRE S formed methane, (2) non-nitrogen-fixing strains such as *Desulfovibrio salexigens* formed methane, (3) several nitrogen-fixing anaerobesdid not form methane. The yield of CH_4 from pyruvate with strain BERRE S was 15 to 150 times lower than that recorded for *M. barkerii*; it might have been improved had methyl B_{12}, the functional co-factor in *M. barkerii*, been available, but this reaction is clearly a small-scale process in physiological terms. A major difference between the Desulfovibrio system and that in a true methane bacterium is its relative insensitivity to oxygen. Minor differences include activity of acetyl phosphate, not reported among the methane bacteria, which may result from a peculiarity of the phosphoroclastic system of strain BERRE S. The enhanced activity of nucleotide mixtures and the effects of GTP, UTP etc. may be related to the crudity of the test system.

Relatively little of the energy and/or reducing power of the phosphoroclastic system is channelled into methane formation and it is reasonable to assume that the extent of channelling depends on the molar ratios of ATP, acetyl phosphate, co-enzyme A and their derivatives, which will normally assume steady states during the decomposition of pyruvate. It is difficult to draw precise conclusions about the influences of these factors added as single substances, when their addition in large amounts will drastically influence the steady state concentration of the others. It seems likely that the need for

large amounts of co-enzyme A reflected a necessity to unbalance a control system which normally limits drastically the amounts of methane formed.

There are reasons for regarding methane production, pyruvic phosphoroclasm, nitrogen fixation and non-haem-iron electron transport as biologically primitive reactions. Peck (1967) and Klein & Cronquist (1967) have proposed that both methane-producing bacteria and sulphate-reducing bacteria are present-day representatives of very primitive living things. The existence of a methane-producing system, biochemically similar to that in methane bacteria, in one of the sulphate-reducing bacteria, together with the detectable activity of comparable systems in some other bacteria capable of pyruvic phosphoroclasm, adds weight to this view. Methane formation in Desulfovibrio species and the other non-methane bacteria may be a 'vestigial' biochemical process of the kind proposed by Kelly (1968) for *Thiobacillus neapolitanus* and by Gottschalk (1968) for desulfovibrios and certain clostridia.

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The Metabolism of Starch, Maltose, Glucose and Some Other Sugars by the Rumen Ciliate *Entodinium caudatum*

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SUMMARY

Washed suspensions of *Entodinium caudatum* grown *in vitro* and incubated anaerobically incorporated ^{14}C from [^{14}C]glucose into the cell pool as [^{14}C]glucose, maltose and hexose phosphate and into protozoal polysaccharide and intracellular bacteria as a glucose polymer. The uptake of sugars into the pool was by an active process (predominant at low sugar concentrations) probably into the protozoal endoplasm, and by a passive process (predominant at high concentrations) into another part of the cell. Uptake of glucose by the active process was not sufficiently rapid to increase the level of glucose in the pool by more than 17% per hour; this was probably insufficient to increase appreciably the rate of glucose utilization. These protozoa hydrolysed starch to maltose and glucose and the level of these sugars in the pool of protozoa metabolizing starch grains was controlled in part by the inhibitory effect of these sugars on the enzymes that formed them. Studies on the enzymes involved in the metabolism of starch showed that the glucose in starch was hydrolysed to free glucose before phosphorylation. In experiments on the effect of carbohydrates on the survival of protozoa, starch was the most effective in prolonging protozoal life, followed by maltose and glucose in that order. The greater effectiveness of maltose was correlated with the finding that the rate of uptake of maltose carbon was always greater than that for glucose when the two sugars were present in equimolar concentrations. However, the rate for both sugars was markedly decreased in the presence of penicillin and neomycin. Evidence is presented that the protozoal ectoplasm may be freely permeable to sugars in the medium and that there is a barrier between the ectoplasm and endoplasm. ^{14}C from [^{14}C]starch grains was incorporated by the protozoa but there was no synthesis of protozoal protein from carbohydrate.

INTRODUCTION

It has been known for many years (Sugden, 1953; Abou Akkada & Howard, 1960) that rumen Entodiniomorphid protozoa do not readily metabolize sugars as determined by sugar disappearance, by acid or gas formation or by the synthesis of polysaccharide. In contrast, starch grains as can be engulfed by the protozoa are degraded to volatile fatty acids, hydrogen and CO_2 (Abou Akkada & Howard, 1960) and have been used as principal source of carbohydrate for the maintenance of *Entodinium caudatum*, *in vitro*, for over 8 years (Coleman, 1960*a*). However, there is evidence that this protozoon could metabolize sugars since the life of starved 'bacteria free' suspensions incubated in the presence of penicillin and neomycin could be prolonged by glucose, maltose and starch in increasing order of effectiveness (Coleman,

1962). The present communication describes the metabolism of [^{14}C]labelled starch and sugars by washed suspensions of *Entodinium caudatum* with special reference to the differential effect of glucose, maltose and starch on protozoal survival. There is a permeability barrier between the medium and the protozoal endoplasm and this barrier is more permeable to maltose than glucose and is crossed by starch grains following their engulfment.

METHODS

Source of protozoa. Entodinium caudatum was grown and inoculum cultures prepared and treated as described by Coleman (1962) except that inoculum cultures were treated each day with 1·0 ml. 1·5% rice starch suspension and about 10 mg. dried grass.

Preparation of protozoa for inoculation. The protozoa were present as a loose pellet at the bottom of the tube containing the inoculum cultures. After removal of the surface scum and most of the medium, the protozoa and remaining medium were transferred to 8 × 1 in. (20 × 2·5 cm.) tubes and allowed to stand until any grass present had sunk to the bottom, leaving the protozoa in the supernatant fluid. This supernatant fluid was transferred to centrifuge tubes, the residual grass washed with salt solution B (Coleman, 1960*b*) and the washings added to the supernatant fluid. The protozoa were spun down and washed four times in salt solution B through which a gas mixture of 95% (v/v) N_2 + 5% (v/v) CO_2 had been bubbled vigorously for 3 min., on a bucket-head centrifuge for 20 sec. from starting; the maximum speed was equivalent to 200*g*. The protozoa were finally used as an inoculum to give a population density of 5 to 15 × 10^5 protozoa/ml.

Incubation conditions for incorporation experiments. The medium consisted of 0·15 to 0·4 ml. [^{14}C]sugar (0·0025 to 0·7 M and 0·25 to 6·9 μc/ml.), 1·0 ml. protozoal inoculum (suspended in salt solution B through which 95% (v/v) N_2 + 5% (v/v) CO_2 had been bubbled vigorously for 3 min.), 0·2 ml. of any other additions and 0 to 0·45 ml. salt solution B (chosen so that the final volume was 1·5 ml.). Experiments were made in 4 × 0·5 in. (10 × 1·3 cm.) thick-walled test tubes and after inoculation were gassed with 95% (v/v) N_2 + 5% (v/v) CO_2, sealed with a rubber bung and incubated at 39°, except where otherwise stated.

Incubation conditions for treatment with antibiotics. The medium consisted of 0·8 ml. penicillin G (25,000 units/ml.), 0·8 ml. 1% (w/v) neomycin sulphate and 20 ml. protozoal inoculum, all in a 100 ml. conical flask which was gassed with 95% (v/v) N_2 + 5% (v/v) CO_2, sealed with a rubber bung and incubated at 39°. At the end of the incubation (usually 18 hr) the protozoa were isolated as described previously.

Incubation conditions for experiments on protozoal survival. The medium consisted of 3·1 to 5·3 ml. (chosen so that the final volume was 10 ml.) salt solution B and 2·0 ml. water autoclaved (115° for 20 min.) in a 15 ml. centrifuge tube. Immediately after removal from the autoclave the following additions were made aseptically: 0·2 ml. 1% L-cysteine hydrochloride (neutralized and Seitz filtered), 0·2 ml. 5% (w/v) NaHCO (Seitz filtered), 0·4 ml. penicillin G (25,000 units/ml.), 0·4 ml. 1% (w/v) neomycin sulphate, 1·0 ml. autoclaved rumen fluid (PARF; Coleman, 1964*a*) and 0 to 2·2 ml. sugar solution (autoclaved at 115° for 20 min. in water) or soluble starch (autoclaved at 115° for 20 min. in salt solution B) or 0 to 0·3 ml. 7·5% (w/v) rice starch (rice starch heated dry at 120° for 24 hr and suspended in sterile salt solution B). After inoculation

to give a population density of approximately 5×10^5 protozoa/ml. the tubes were gassed with 95% (v/v) N_2 + 5% (v/v) CO_2, sealed with a rubber bung and incubated at 39°. For each point on a time course a fresh culture was sampled and the tube then discarded.

Incubation conditions for growth of protozoa. The same conditions were used as for experiments on protozoal survival except that neomycin was omitted from the incubation medium. At the end of an experiment in which protozoa had been incubated with a [^{14}C]labelled compound, the culture was centrifuged and the protozoa washed twice at 300*g* for 30 sec. in salt solution B.

Breakage of protozoa. The protozoa suspended in 2·5 ml. salt solution B (for metabolic experiments) or water (for fractionation of labelled protozoa) were broken by immersion of the tube to the depth of the liquid in the tube in a 80 kc/sec. 40 W. ultrasonic cleaning bath (KG 80/1, manufactured by Kerry's of Chester Hall Lane, Basildon, Essex) for 15 sec. Unless the crude homogenate was required, the whole was then centrifuged at 7000*g* for 20 min. The supernatant fluid from this centrifugation is hereafter referred to as the 'broken-cell supernatant fluid' and the pellet after washing once in water as the 'broken-cell pellet'. This latter fraction contained all the viable bacteria and the polysaccharide granules in the homogenate.

Estimation of ^{14}C and ^{32}P. ^{14}C or ^{32}P in any material was estimated by pipetting a sample on to an aluminium planchet of area 4·7 cm.^{2} carrying a disc of lens tissue. The sample was spread by one drop of cetyltrimethylammonium bromide solution (5 mg./ml.) and fixed by one drop of polyvinyl alcohol (2 mg./ml.). The disc was dried at 40° and the radioactivity estimated by means of an automatic flow counter (Nuclear-Chicago Corp.) with an efficiency for ^{14}C of about 20%. Over 1000 counts at a rate greater than twice background (usually greater than five times) were recorded for all fractions. Determinations were made with less than 0·5 mg. material/cm.^{2} of disc. The amount of sugar incorporated by protozoa or into the fraction sampled was calculated from the known specific activity of the sugar added initially. To determine the relative ^{14}C or ^{32}P contents of spots on a chromatogram a thin mica end-window GM tube was placed directly on the spot, the position of which had been determined by radioautography.

Fractionation of protozoa. The two fractions obtained by sonic treatment and centrifugation of a protozoal suspension were further fractionated by a method based on that of Roberts *et al.* (1955). The fraction was treated with 5% trichloroacetic acid (TCA) and allowed to stand at 4° for 30 min. The precipitate was centrifuged down and washed once in 5% TCA. The supernatant fluid and the washing formed the 'cold TCA-soluble fraction'. This fraction of the broken-cell supernatant fluid is hereafter referred to as the protozoal 'pool'. The precipitate was then extracted with 75% (v/v) ethanol in water at 40° for 30 min. The residue was centrifuged down and the supernatant fluid formed the ethanol-soluble fraction. The precipitate was then extracted with 75% ethanol + ether (1 + 1 by vol.) at 40° for 15 min. The residue was centrifuged down and the supernatant fluid formed the ethanol + ether soluble fraction. The precipitate was then extracted twice with 5% TCA at 100° for 30 min. The supernatant fluids formed 'the hot TCA-soluble fraction'. The residue after washing once with acidified ethanol formed the 'residual protein fraction'. The TCA was removed from fractions by washing three times with ether before plating out for the estimation of ^{14}C. The ethanol and ethanol + ether soluble fractions were further

fractionated by the addition of equal quantities of water and ether and the two layers so obtained separated. The aqueous layers after washing once with ether were combined and formed the 'ethanol-soluble protein' fraction. The ether layers plus the washing were combined to form the 'lipid fraction'.

Paper chromatography. The following solvents were used: (A) *sec*-butanol + formic acid + water (70 + 10 + 20, by vol.); (B) *n*-propanol + ethyl acetate + water (24 + 13 + 7, by vol.); (C) *tert*-butanol + ammonia (sp.gr. = 0·880) + water (60 + 30 − 10, by vol.) on paper impregnated with 0·01 M-$Na_2B_4O_7$ (Agarwal, Sanwal & Krishnan, 1963); (D) phenol + ammonia (sp.gr. = 0·880) + water (80 g. + 0·3 ml. + 20 ml.); (E) methanol + ammonia (sp.gr. = 0·9015) + water (60 + 10 + 30, by vol.; Bandurski & Axelrod, 1951); (F) *n*-butanol + cyclohexane + propylene glycol + water + ammonia (sp.gr. = 0·880) + morpholine (30 + 30 + 10 + 3·5 + 0·7 + 0·07, by vol.; Guillaume & Osteux, 1955); (G) *n*-butanol saturated with 1·5 N-NH_4OH; (H) isopropanol + water (80 + 20, by vol.; Smith, 1960).

Identification of [^{14}C]*glucose in protozoal fractions.* The material was first chromatographed in solvent B which separated hexoses from pentoses, disaccharides and phosphate esters, and the position of radioactive spots determined by radioautography. The spot that ran next to marker glucose was eluted and identified in the following way. It was chromatographed against markers in solvents A to H and shown to be glucose. The material was also chromatographed in solvent B before and after treatment with glucose oxidase. As a result of enzyme action the original spot disappeared and was replaced by another which ran with the same R_F as that produced from pure [^{14}C]glucose under the same conditions. When sufficient material was present the spots on the paper after chromatographic separation were shown to give the characteristic hexose reaction when treated with aniline oxalate reagent (Horrocks & Manning, 1949). When insufficient material was present the identity of the glucose was confirmed by the 'fingerprint' technique of Roberts *et al.* (1955). For this method the unknown [^{14}C]compound was mixed with a relatively large amount of the [^{12}C]-form of the compound with which it had been identified and the whole chromatographed. The ^{14}C spot was then detected by radioautography and the carrier compound by the aniline oxalate reagent. When the tracer and carrier compound are the same, then the pattern of the radioautogram must agree in every detail with the pattern produced by the chemical spray.

Identification of other [^{14}C]*sugars in the protozoal 'pool'.* The method adopted was similar to that for [^{14}C]glucose with the exception that the glucose oxidase treatment had no effect on the chromatographic behaviour of the [^{14}C]material.

Identification and estimation of ^{14}C *in volatile fatty acids.* The volatile acids produced during the metabolism of [^{14}C]carbohydrates were removed from the medium by steam distillation and then the distillate was neutralized, evaporated to dryness *in vacuo* and the residue chromatographed in solvent F, which separated formic, acetic, propionic and butyric acids. Immediately after removal from the tank and while the chromatogram was still wet, it was sprayed with 0·01 M-NaOH to fix the acids on the paper. To determine the position of the various fatty acids, marker mixtures of formic, acetic, propionic, *n*-butyric and *n*-valeric acids containing each acid in turn labelled with ^{14}C were chromatographed on the same paper. The relative [^{14}C]content of the various spots was determined by placing a thin mica end-window GM tube directly on the paper.

Protozoal counts. The number of protozoa was estimated by the method of Coleman (1958). Only those protozoa which showed no signs of disintegration were counted.

Phosphorylase assay. The incubation medium consisted of 1·5 ml. 0·1 M-$\beta\beta'$-dimethylglutarate buffer (pH 6·4), 0·5 to 1·0 ml. water (chosen so that the final volume was 3·0 ml.), 0·1 ml. ^{32}P-inorganic phosphate, 0·1 to 0·6 ml. 0·001 to 0·1 M-phosphate buffer (pH 6·4), 0·2 ml. carbohydrate solution (usually 2% soluble starch for experiments on glucan phosphorylase) and 0·1 ml. 1% L-cysteine hydrochloride (neutralized). Gas mixture 95% (v/v) N_2+5% (v/v) CO_2 was bubbled through the medium for 2 min. immediately after the addition of the cysteine and before the addition of 0·2 ml. broken protozoal preparation. The tubes were then gassed for a further 10 sec. before sealing with a rubber bung and incubation at 39° usually for 30 min. At the end of the incubation 0·2 ml. 0·5 M-NaF and 0·2 ml. 0·2 M-phosphate buffer (pH 6·4) were added and the whole mixed. Then 0·6 ml. magnesia mixture (Leloir & Cardini, 1957) was added, the contents of the tube mixed and the whole allowed to stand at 4° for 1 hr. At the end of this period the precipitate was centrifuged down and 0·2 ml. of the supernatant fluid plated out for the estimation of ^{32}P.

Glucose estimations. Glucose was estimated by the method of Huggett & Nixon (1957) by using a carbohydrase-free preparation of glucose oxidase (Type V of Sigma Chemical Co., 12 Lettice Street, London, S.W. 6).

Maltose estimation. Maltose was estimated as glucose after hydrolysis in N-H_2SO_4 for 30 min. at 100°, followed by neutralization with NaOH. The results were corrected for the amount of free glucose present before hydrolysis.

Reducing sugar estimations. Reducing sugars were estimated by the ferricyanide method of Park & Johnson (1949).

Hexokinase assay. The incubation medium contained 1·0 ml. salt solution B, 0·6 ml. 0·001 to 0·1 M [U-^{14}C]glucose or water, 0·1 ml. 0·1 M-adenosine triphosphate (ATP) and 0·2 ml. broken protozoal supernatant fluid. The reaction was usually allowed to continue for 30 min. at 39° and was then stopped by heating at 100° for 5 min. Two ml. of a suspension of Dowex 2 [acetate] was added and the whole shaken for 15 min. to remove the [^{14}C]hexose phosphates. The resin was then washed twice with water and the phosphates removed by treatment with 3 ml. N-HCl a sample of which was plated out for the estimation of ^{14}C. The ^{14}C in the eluate was used as a measure of enzyme activity.

Phosphoglucomutase assay. The reaction mixture contained 1·0 ml. 0·1 M-$\beta\beta'$-dimethylglutarate buffer (pH 6·4), 0·6 ml. 0·01 to 0·1 M-glucose-1-phosphate or water and 0·2 ml. broken protozoal supernatant fluid. Tubes were incubated at 39° for 30 min. and the reaction stopped by heating at 100° for 5 min. The precipitate was centrifuged down and 0·1 ml. of the supernatant fluid used for the estimation of reducing sugars, with glucose-6-phosphate as a standard. To correct for the reducing materials present in the reagents initially, reducing sugar estimations were done at each glucose-1-phosphate concentration before and after incubation.

Maltase assay. The reaction mixture contained 1·0 ml. salt solution B, 0·6 ml. 0·01 to 0·1 M-maltose or water and 0·2 ml. broken protozoal supernatant fluid. Tubes were incubated at 39° for 30 min. and the reaction stopped by heating at 100° for 5 min. The precipitate was centrifuged down and 0·1 ml. of the supernatant fluid used for the estimation of glucose. To correct for the glucose present in the maltose, glucose estimations were carried out at each maltose concentration before and after incubation.

Preparation of [^{14}C]*labelled starch grains.* The method was based on the observation of Oxford (1951) that rumen holotrich protozoa rapidly convert glucose into an intracellular glucosan. A sample (500 ml.) of crude rumen contents from a Clun Forest sheep fed on hay and oats was strained through muslin and allowed to stand in a separating funnel at 39° for 45 min. The sediment, which consisted principally of holotrich protozoa, was run off into an 8 × 1 in. (20 × 2·5 cm.) test tube and washed three times with salt solution B by sedimentation under gravity. The washed protozoa were inoculated into 5 ml. salt solution B (from which the sodium propionate was omitted) containing [U-^{14}C]glucose (0·5 mg. and 6 μc/ml.) and 0·2 ml. saturated $NaHCO_3$ and through which CO_2 had been bubbled for 2 min. After inoculation the tubes were gassed with CO_2 for 10 sec., sealed with a rubber bung and incubated horizontally for 2 hr at 39°. The protozoa were then harvested, washed four times in salt solution B and broken in a Potter homogenizer (Potter & Elvehjem, 1936). The homogenate was centrifuged at 1200*g* for 15 min. and the pellet of starch grains washed twice in water under the same conditions. Approximately 10% of the ^{14}C present in the glucose initially was converted into [^{14}C]starch. On fractionation of these grains (see Methods) over 96% of the ^{14}C was solubilized on treatment with 5% TCA at 100° for 20 min., and on further hydrolysis of this extract in N-HCl for 1 hr at 100° glucose was found to be the only ^{14}C-labelled monosaccharide present; only 0·4% of the ^{14}C was present in the protein fraction. These results indicated that the preparation contained [^{14}C]starch contaminated with very little protein.

Chemicals. [^{14}C]compounds were supplied by the Radiochemical Centre, Amersham, Buckinghamshire. The specific activities of the compounds, as supplied, were in μc/mg., [1-^{14}C]-D-galactose, 20·8; [U-^{14}C]-D-glucose, 16·2; [U-^{14}C]-D-maltose, 13·5; [1-^{14}C]-D-ribose, 54·9; [U-^{14}C]soluble starch, 26; [U-^{14}C]sucrose, 3·5. All sugars were usually used within 6 months of receipt and were always checked for purity by chromatography in solvents A and B.

RESULTS

Metabolism of intact starch grains

Entodinium caudatum suspensions were incubated with the ^{14}C-labelled starch grains (see Methods) for 48 hr either under standard conditions in the presence of penicillin + neomycin when there was no bacterial growth and the number of protozoa decreased by 26% or under conditions which allowed both protozoal and bacterial growth (Coleman, 1960*b*) when the number of protozoa increased by 45%. Table 1 shows that under both conditions there was no evidence for any synthesis of protein from carbohydrate. Over 80% of the protozoal ^{14}C was found in the hot TCA soluble fraction of the broken-cell pellet: ^{14}C from the original starch grains and any other granular polysaccharide would be liberated in this fraction. With non-multiplying protozoa over 50% of the ^{14}C from the [^{14}C]starch was liberated into the medium as soluble non-volatile material which was identified as glucose (65%) and maltose (35%). The volatile compounds were acetate (35%) and butyrate (65%); no propionate was detected. In an attempt to demonstrate the presence of [^{14}C]lactate, the untreated supernatant fluid was chromatographed in solvent A, which separated lactate from glucose and maltose but less than 1% of the total ^{14}C could have been present as lactate. The remainder of the ^{14}C in the presence of non-multiplying protozoa and

most of the ^{14}C in the presence of multiplying protozoa was not retained on planchetes in the presence of acid or alkali and was presumed to be CO_2.

Effect of metabolism of starch on sugar concentrations in the protozoal pool and the medium

On incubation of *Entodinium caudatum* in the absence of added carbohydrate or of antibiotics there was a steady decline in the amount of glucose and maltose in the pool accompanied by a corresponding increase in the amount in the medium such that the total amount of free + bound glucose remained almost constant over the first 90 min.

Table 1. *The metabolism of ^{14}C-starch grains by Entodinium caudatum*

Protozoa were incubated anaerobically for 48 hr in 10 ml. medium containing 10 % (v/v) autoclaved rumen fluid (PARF, Coleman, 1964*a*), 1000 units penicillin G/ml. and 0·14 μc ^{14}C-starch grains from holotrich protozoa/ml. in the presence or absence of 400 μg. neomycin sulphate/ml. With neomycin the number of protozoa decreased by 26 % and without it the number increased by 45 %. At the end of the experiment the washed protozoa were broken by ultrasonic treatment and the supernatant fluid and pellet fractions separated by centrifugation.

	Radioactivity (percentage of ^{14}C added initially)				
	No protozoa	Multiplying protozoa (−neomycin)		Non-multiplying protozoa (+neomycin)	
Non-volatile soluble material in medium	1·8	9·9		55·9	
Volatile acid	0	3·9		19·0	
	Initial starch	Supernatant fluid	Pellet	Supernatant fluid	Pellet
Cold TCA-soluble	2·8	0·45	0·14	0·33	0·11
Lipid	0·6	0·01	0·06	0·01	0·04
Hot TCA-soluble	94·4	0·06	4·4	0·05	7·4
Protein	0·4	0·10	0·30	0·03	0·15

Table 2. *The effect of the metabolism of starch grains on the concentration of maltose and glucose in the medium and pool of Entodinium caudatum*

$1·6 \times 10^6$ protozoa were incubated anaerobically in 1·2 ml. salt solution B in the presence or absence of 1·5 % suspension of rice starch. At the times shown the protozoa were centrifuged down, washed twice in salt solution B, broken by ultrasonic treatment and the supernatant fluid separated from the pellet by centrifugation. The pool was the cold TCA soluble fraction of the protozoal supernatant fluid. The concentration of maltose and glucose in the pool and the incubation medium were determined as described in Methods. All results are expressed in μmoles.

	Protozoal pool				Medium			
	−starch		+starch		−starch		+starch	
Time (min.)	Maltose	Glucose	Maltose	Glucose	Maltose	Glucose	Maltose	Glucose
0	0·368	0·412	0·378	0·425	0·040	0·100	0·176	0·100
5	0·346	0·388	0·453	0·578	0·044	0·112	0·284	0·140
15	0·382	0·418	0·408	0·480	0·056	0·100	0·306	0·160
30	0·314	0·380	0·404	0·480	0·104	0·112	0·464	0·220
90	0·312	0·328	0·422	0·348	0·080	0·132	0·746	0·408
240	0·248	0·260	0·262	0·248	0·236	0·148	1·50	0·584

In the presence of added rice starch (additional to that already present in the protozoa on harvesting) there was an initial (in first 5 min.) 20 to 25% increase in the amount of glucose and maltose in the pool but thereafter the amount of each declined as in the absence of added starch (Table 2). The amount of glucose in the pool initially was always greater, on a molar basis, than the amount of maltose. In contrast in the presence of added starch there was a large increase in the amount of maltose liberated into the medium and a smaller increase in the amount of glucose. These results indicate that the sugars were probably not liberated into the medium by an 'overflow' from the pool but may have been liberated directly without passage through the pool.

In freshly harvested protozoa the quantity of free glucose and maltose present in 10^6 protozoa was 0·26 and 0·23 μmoles, respectively. Since the volume of each protozoon was $4{\cdot}7 \times 10^4$ μ^3 (Coleman, 1967*a*) the total volume of 10^6 ciliates was $4{\cdot}7 \times 10^{10}$ μ^3 or 0·047 ml. The minimum concentration of glucose and maltose was therefore 5·5 and 4·9 μmoles/ml., respectively. Since 26% of a packed protozoal pellet was solid material (Coleman, 1967*a*) and since it is considered likely that these sugars were not present in the 37% of the cell (see below) in and out of which low molecular weight compounds could pass readily, it is probable that these sugars were present in 37% of the cell volume. If this is correct then the concentration of glucose and maltose was 14·8 and 13·2 mM, respectively.

Table 3. *Distribution of ^{14}C in Entodinium caudatum after the incorporation of [U-^{14}C]glucose*

Protozoa ($7{\cdot}6 \times 10^5$) were incubated anaerobically for 5 hr in salt solution B containing 1000 units penicillin + 400 μg. neomycin sulphate/ml. in the presence of 0·8 mM [U-^{14}C] glucose (670,000 counts/min.) or 110 mM-[U-^{14}C]glucose (670,000 counts/min.). After incubation the washed protozoa were broken by ultrasonic treatment and the supernatant fluid and pellet fractions separated by centrifugation. These were further fractionated as described in Methods.

	Radioactivity (counts/min.)			
	Broken-cell supernatant fluid		Broken-cell pellet	
Initial glucose concentration	0·8 mM	110 mM	0·8 mM	110 mM
Cold TCA-soluble fraction	1380	720	1110	540
Ethanol-soluble protein	15	5	30	0
Lipid	35	20	30	15
Hot TCA-soluble fraction	70	30	2660	1110
Residual protein	50	20	60	15

Metabolism of glucose

Washed suspensions of *Entodinium caudatum* incubated anaerobically in the presence of penicillin + neomycin for 5 hr incorporated ^{14}C from 0·8 mM and 0·11 M [U-^{14}C]glucose. Table 3 shows that the distribution of ^{14}C was similar under both conditions and that 26 to 29% was present in the cell pool and 45 to 50% in the hot TCA soluble fraction of the broken cell pellet: after further hydrolysis in N-HCl for 1 hr at 100° the material in the latter fraction was identified as [^{14}C]glucose. This suggested that in the intact protozoa the ^{14}C was present as a glucose polysaccharide. Less than 3% of the total protozoal ^{14}C was present in the protein fractions. 4% of the glucose-carbon present initially was converted into volatile fatty acids as acetate

(80%) and butyrate (20%). The different proportions of acetate and butyrate produced during the metabolism of glucose and starch could be the result of the differing action of intracellular bacteria on the hydrolysis products of starch digested in the cytoplasm and on glucose taken up from the medium.

Distribution of ^{14}C in the hot TCA soluble fraction of the broken cell pellet. In contrast to the results obtained with [^{14}C]glycine or [^{14}C]bicarbonate (Coleman, 1964*a*) when breakage of the protozoa abolished incorporation of ^{14}C into the pellet, the incorporation of ^{14}C from [^{14}C]glucose into the pellet was increased by up to 60% on protozoal breakage. This suggested that the incorporation under these conditions was into bacteria and that in the intact protozoon [^{14}C]glucose could be incorporated into intracellular bacteria and possibly protozoal polysaccharide granules, both of which would be found in the broken-cell pellet after cell breakage and centrifugation.

Incorporation of ^{14}C from [^{14}C]glucose by bacteria isolated from protozoa. White (1966) found forty viable bacteria inside each ciliate and showed that 90% of these were one or the other of two species of Gram-negative bacilli. These have been shown subsequently to be *Klebsiella aerogenes* (Bacterium 31 used by Coleman 1964*b*, 1967*b*) and *Proteus mirabilis* (R. W. White, personal communication). Cultures of these bacteria were grown anaerobically on a yeast-extract + tryptose + glucose + salts medium (Coleman, 1964*b*) and washed suspensions prepared by washing the bacteria twice on the centrifuge in salt solution B. These suspensions were incubated anaerobically for 20 min. at 39° with $0{\cdot}5 \times 10^{-3}$ to 80 mM-[U-^{14}C]glucose of known specific activity and the incorporation of ^{14}C into washed bacteria measured. Figure 1 is a reciprocal plot of ^{14}C incorporated against substrate concentration and shows that both bacterial species incorporated ^{14}C but that *K. aerogenes* was over four times as active as *P. mirabilis*. Both curves showed a very marked discontinuity at 10 to 20 mM. On incubation of *K. aerogenes* anaerobically in the presence of glucose concentrations in excess of 0·001 M, a fluffy layer of what was presumed to be a glucose polymer was found associated with the bacteria on centrifugation. On fractionation of all the sedimented material over 90% of the glucose-carbon that had been incorporated was released by treatment with hot 5% TCA and after further hydrolysis in N-HCl was found to be glucose. It was essential in these experiments to use short incubation times because after 1 hr, especially at high glucose concentrations, so much 'fluffy' material was formed that it was impossible to remove the supernatant fluid after centrifugation without removing some.

Determination of incorporation of ^{14}C into protozoal polysaccharide and intracellular bacteria. The method used to distinguish between incorporation into protozoal polysaccharide and bacteria was based on the observation that, after centrifugation of the protozoal broken-cell pellet through sucrose, much of the starch but few of the bacteria were present in the pellet as observed microscopically. To obtain a quantitative result it was necessary to assume that on incubation of broken protozoa with [^{14}C]glucose ^{14}C was incorporated only into bacteria. The washed pellet from such an incubation was resuspended in 2 ml. water and samples layered on top of 2 ml. quantities of sucrose of differing molarities and centrifuged at 1900*g* for 10 min. in a bucket-head centrifuge. The supernatant fluids were removed and the pellets, after washing once in water, plated out for the estimation of ^{14}C. They contained 73, 20, 2·5 and 1·7% of the initial ^{14}C after centrifugation through 1·0, 1·5, 2·0 and 2·5 M-sucrose, respectively. From an examination of this result it was decided to use 2 M-sucrose and by

direct microscopical examination 30% of the original number of protozoal polysaccharide granules were found in the pellet after centrifugation through sucrose. It was therefore possible to obtain an estimate of the amount of ^{14}C incorporated into the protozoal polysaccharide granules by centrifugation of the broken-cell pellet through 2 M-sucrose and multiplying the amount of ^{14}C in this second pellet by 3·3. For the purposes of these calculations the 2·5 % of the bacterial ^{14}C that centrifuged through 2 M-sucrose was ignored. The amount of ^{14}C in the intracellular bacteria was the difference between the total ^{14}C in the broken-cell pellet and the ^{14}C in the protozoal polysaccharide measured as described above.

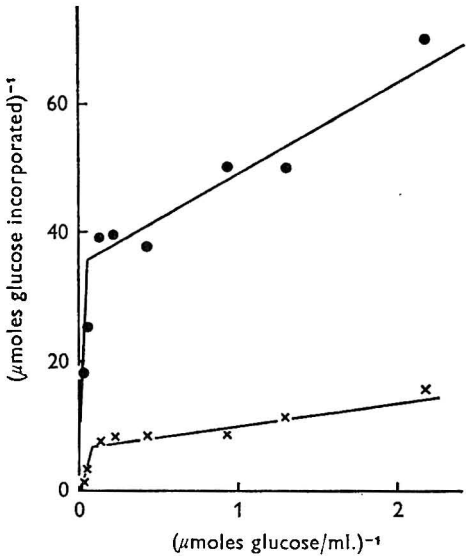

Fig. 1

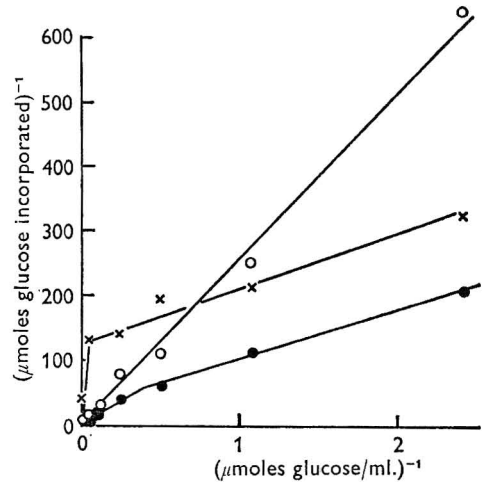

Fig. 2

Fig. 1. Effect of glucose concentration on the incorporation of ^{14}C from [U-^{14}C]glucose by washed suspension of *Proteus mirabilis* (●—●) and *Klebsiella aerogenes* (×——×) incubated anaerobically in salt solution B. Duration of experiment 20 min. Results expressed per 10^9 bacteria.

Fig. 2. Effect of glucose concentration on the incorporation of ^{14}C from [U-^{14}C]glucose by *Entodinium caudatum*. After 45 min. the washed protozoa were treated ultrasonically and separated into supernatant fluid and pellet fractions by centrifugation. The cold TCA-soluble material from the broken-cell supernatant fluid formed the pool (●——●). The pellet fraction was further fractionated by centrifugation through 2M-sucrose to give a protozoal polysaccharide (○——○) and a bacterial (×——×) fraction.

Figure 2 is a reciprocal plot of the effect of glucose concentration on the incorporation of ^{14}C from [^{14}C]glucose and shows that there was a discontinuity in the curves for incorporation into the pool and the bacteria but not for incorporation into protozoal polysaccharide. The break in the curve for uptake into the bacteria occurred at 20 mM, which was at the same concentration as was found with pure cultures of the two predominant bacterial species and may therefore not be associated with any protozoal process. The discontinuity in the curve for incorporation into the pool (at 2·5 mM) and the fact that the left-hand part passed through the origin suggested there may be an 'active' (predominant at low glucose concentrations) and a passive (predominant at

high glucose concentrations) uptake process as was found for amino acids (Coleman, 1967*a*).

In an attempt to obtain further evidence for the existence of incorporation into both subfractions of the broken-cell pellet, the effect of penicillin (1000 units/ml.)+neomycin (400 μg./ml.) on incorporation of ^{14}C from [^{14}C]glucose was investigated, since these antibiotics are known to kill the bacteria associated with the protozoa (Coleman, 1962). Unfortunately incubation of the protozoa with these antibiotics for 16 hr before incubation with [^{14}C]glucose decreased the incorporation of ^{14}C into intact protozoa, the broken-cell supernatant fluid and the broken-cell pellet by 84%, and it was not possible to measure incorporation into the bacterial and polysaccharide fractions satisfactorily. However, it was found that, whereas breakage of freshly harvested protozoa before incubation with [^{14}C]glucose stimulated incorporation of ^{14}C into the pellet, breakage of protozoa that had been pre-incubated with antibiotics for 16 hr decreased incorporation of ^{14}C into the pellet by 60%. This result suggests that in freshly harvested protozoa incorporation of ^{14}C was into two fractions, one of which was associated with living bacteria. It was also possible to show a selective effect of penicillin and neomycin when freshly harvested protozoa were incubated in the presence or absence of antibiotics for 4 hr. Under these conditions at low (0·1 mM) glucose concentrations, incorporation into broken-cell supernatant fluid, intracellular bacteria and protozoal polysaccharide was inhibited by 74, 83 and 53% respectively in the presence of antibiotics. These results show that the incorporation into the intracellular bacteria is most sensitive to penicillin and neomycin and are generally consistent with the view that incorporation in the broken-cell pellet is into a bacterial and non-bacterial component.

Effect of other sugars and of sugar analogues. For these experiments the protozoa were incubated anaerobically with 0·4 to 67 mM [U-^{14}C]glucose in the presence of the inhibitor at 67 mM, and the incorporation into the protozoal pool and polysaccharide and the intracellular bacteria measured. On the basis of results obtained in experiments on the total incorporation into protozoa over 3 to 4 hr the 'sugars' were divided into two groups. Those of group 1 which contained D-galactose and D-2-deoxyglucose inhibited incorporation at concentrations above and below the break (at 2·2 mM) in the reciprocal plot of incorporation of ^{14}C into intact protozoa/substrate concentration. Those of group 2 (i.e. D-sorbitol, D-mannose, D-maltose, D-fructose) inhibited only at concentrations below the break; L-glucose and D-ribose had no effect at any concentration. However, when the experiments were made for only 20 min. then group 2 compounds were not inhibitory and those of group 1 inhibited markedly at concentrations below the break, but only slightly at high concentrations. At low glucose concentrations 2-deoxyglucose inhibited incorporation of ^{14}C from [^{14}C]glucose into all the sub-cellular fractions equally (Table 4) and the same occurred in the presence of galactose although the over-all inhibition was always greater in the presence of 2-deoxyglucose, i.e. 71% compared with 29% (mean of six experiments). However at high glucose concentrations, where the over-all inhibition by both inhibitors was much smaller, the incorporation into the protozoal polysaccharide was always affected more than the incorporation into the other two fractions. The incorporation of ^{14}C from [^{14}C]glucose by washed broken-cell pellet after a 3-hr incubation was decreased to 54% in the presence of galactose and to 37% in the presence of 2-deoxyglucose. However, similar experiments with washed suspensions of the *Klebsiella aerogenes* and *Proteus*

mirabilis isolated from the protozoa showed that incorporation into both bacteria was not affected by 2-deoxyglucose but was decreased to 45% by galactose. As this difference may have been the result of the exposure of the intracellular bacteria, but not the washed suspensions, to ultrasonic treatment, the effect of such treatment of washed suspensions of the bacteria on their ability to incorporate [^{14}C]glucose was examined. On ultrasonic treatment of suspensions of either bacterium the rate of incorporation of ^{14}C from [^{14}C]glucose decreased progressively with time of treatment and 2-deoxyglucose became progressively more inhibitory until after 30 sec. of treatment followed by 30 min. incubation 2-deoxyglucose decreased incorporation by 30%. Under the same conditions galactose inhibited incorporation by 90%. Sorbitol had no effect on the incorporation of ^{14}C from [^{14}C]glucose by protozoal washed broken-cell pellet or by either bacterium under any conditions tested.

Table 4. *The effect of galactose and 2-deoxyglucose on the incorporation of ^{14}C from [^{14}C]glucose by Entodinium caudatum*

Protozoa (650,000/ml.) were incubated anaerobically in 1·5 ml. medium for 4 hr in the presence of 0·3 mM-[U-^{14}C]glucose (66,000 counts/min./ml.) or 67 mM-[U-^{14}C]glucose (66,000 counts/min./ml.) and in the presence or absence of 67 mM-D-galactose (Expt. B) or 67 mM-2-deoxyglucose (Expt. A). At the end of the experiment the washed protozoa were broken by ultrasonic treatment and the supernatant fluid and pellet fractions separated by centrifugation. The washed pellet fraction was further fractionated by centrifugation through 2M-sucrose, as described in the text, and the incorporation of ^{14}C into the polysaccharide and bacterial fractions determined. The 'pool' was the cold TCA-soluble fraction of the broken-cell supernatant fluid. Results are expressed relative to incorporation into fraction in absence of inhibitor at given glucose concentrations = 100.

Inhibitor	Glucose concentration	Pool	Bacteria	Polysaccharide
Expt. A				
2-Deoxyglucose	0·3 mM	7·7	7·8	5·6
2-Deoxyglucose	67 mM	73	54	28
Expt. B				
Galactose	0·3 mM	33	31	23
Galactose	67 mM	81	103	51

Nature of products in protozoal pool. The [^{14}C]labelled constituents of the protozoal pool after incubation with [^{14}C]glucose were separated into up to four compounds by chromatography in solvent B. One ^{14}C-spot remained at the origin and after elution and treatment with acid phosphatase gave rise to [^{14}C]glucose (for identification of [^{14}C]glucose see Methods). On chromatography in solvent C the untreated material was resolved into a major component (over 80%) which chromatographed next to marker glucose-6-phosphate, and a minor component which chromatographed opposite glucose-1-phosphate. On elution, this latter component was hydrolysed to [^{14}C]glucose on treatment with N-H_2SO_4 at 100° for 10 min.; the former was stable under these conditions as determined by chromatography in solvent B. Both components of the origin spot were removed from solution by treatment with Dowex 2 (acetate), showing that they were moderately strong acids. Their identity was confirmed by chromatography in solvents A to G next to marker compounds. The second spot in the original chromatographic separation ran opposite marker maltose. After elution some of this material was hydrolysed in N-HCl at 100° for 1 hr and was converted into glucose only. The remaining material was chromatographed in solvents A to H against marker

compounds and shown to be maltose. The third spot in the original was glucose. The fourth spot which never contained more than 10% of the total ^{14}C in the pool ran slightly faster than glucose in solvent B and was probably fructose, although this was not shown conclusively.

Effect of glucose concentration and inhibitors on incorporation into the pool. After 15-min. incubation in the presence of low (0·4 mM) [^{14}C]glucose 10% of the ^{14}C in the pool was present as hexose phosphate, 36% as maltose and 54% as glucose whereas in the presence of a high (40 mM) glucose concentration the corresponding proportions were 5, 20 and 75%. The proportion of the ^{14}C that was present as hexose phosphate was rather variable and in some experiments was as high as 35%. In the presence of 20 mM-2-deoxyglucose at low glucose concentrations the total incorporation into the pool was decreased by 65% and the distribution of ^{14}C was similar to that at high glucose concentrations. 2-Deoxyglucose had no effect on the pattern of incorporation at high glucose concentrations.

In an attempt to prevent the 'active' uptake of glucose by the protozoa the effect of 0·67 mM-iodoacetate was investigated. At this concentration all ciliary movement of the protozoa was abolished and they began to disintegrate after incubation for 1 hr; all experiments in the presence of iodoacetate were therefore done for 20 min. only. Under these conditions incorporation into the pool was increased up to six times by the presence of iodoacetate and there was a straight-line relationship between incorporation of glucose and glucose concentration on a reciprocal plot, and this straight line passed through the origin. These findings suggested that the 'active' uptake had been abolished by iodoacetate and the passive uptake increased. Incorporation into the hexose phosphate fraction in the pool and into the broken-cell pellet fraction was abolished. The relative incorporation into the glucose and maltose in the pool were unchanged.

Since the increased passive uptake of ^{14}C from [^{14}C]glucose may have been the result of the inhibition of some process for the 'active' removal of glucose from part of the cell, the effect of iodoacetate on the release of ^{14}C from washed protozoa which had been pre-incubated with 67 mM [^{14}C]glucose for 30 min. was investigated. It was not possible to obtain an unambiguous result because, although the addition of 0·67 mM-iodoacetate decreased the rate of loss of ^{14}C from the pool, it stimulated the release of ^{14}C from the pellet fraction of the protozoa into the medium.

Effect of salt concentration and temperature on incorporation into the pool. A decrease of 65% in the salt concentration in the incubation medium killed the protozoa after 1 hr but stimulated the incorporation of ^{14}C from [^{14}C]glucose into the pool by 260% when the incubations were continued for 20 min. only. There was a straight line relationship between incorporation of ^{14}C and glucose concentration on a reciprocal plot, and the line passed through the origin. There was no break in the curve as occurred at standard salt concentration. Almost all the increase in the incorporation of ^{14}C was accounted for by an increase in the amount of [^{14}C]glucose: this increased by 420% compared with only 55% for maltose. This result can probably be explained on the basis of a passive uptake of medium into the protozoa caused by the lowered osmotic pressure. The glucose would be carried into the protozoa with the other medium constituents and be trapped there when the protozoa were washed in salt solution of the standard concentration.

Lowering the temperature from 40° to 30° for a 4-hr incubation had no effect on the

total incorporation of ^{14}C from [^{14}C]glucose into the protozoa but increased the incorporation into the pool by 20% at low (i.e. below the break shown in Fig. 2) glucose concentrations only. Prolonged incubation at 20° was not possible because of death of the protozoa; but in experiments of 30 min. duration the total incorporation at low glucose concentrations was decreased by 35% as compared with incubation at 40° with no change in the amount of ^{14}C in the pool. At high glucose concentrations lowering the temperature to 20° had no effect on the total incorporation of ^{14}C but increased the proportion of the ^{14}C present in the pool from 78% to 88%. These results show that at all glucose concentrations the reactions by which glucose in the pool was incorporated into particulate matter were more sensitive to a decrease in temperature than the reactions by which glucose was incorporated into the pool.

Effect of particulate matter on uptake of glucose. Holter (1965) showed that in *Amoeba proteus* which was taking up protein particles from the medium by pinocytosis only 5% of the liquid in which the protein was suspended was taken up at the same time. Since *Entodinium caudatum* engulfs particulate matter and can take up 200 bacteria/min. (Coleman, 1964*b*) it was of interest to determine the effect of inert particulate matter on the rate of glucose incorporation. The material used was polystyrene latex particles of diam. 1·3 μ (Dow Chemical Co., Midland, Michigan, U.S.A.) which have been shown by light and electron microscopy to be engulfed by the protozoa. In the presence of 0·33% polystyrene latex ($5{\cdot}5 \times 10^9$ particles/ml.) there was usually a small and variable increase (0 to 25%) in the incorporation of ^{14}C from 2 to 130 mM-[^{14}C]glucose into the protozoa. However, in one experiment the stimulation increased from 28% in the presence of 130 mM-[^{14}C]glucose to 120% with 4 mM-[^{14}C]glucose. The actual stimulation probably depended on how full of starch grains the protozoa were on harvesting. Latex at 0·03% gave negligible stimulation at any glucose concentration. Since with the higher latex concentration the protozoa were completely filled with particles after incubation for 20 min. and many were still free in the medium, the stimulation obtained must have been the maximum possible associated with the uptake of particulate matter. It is therefore considered that in standard washed suspension experiments particle-stimulated incorporation was unimportant.

Effect of glucose incorporation on the level of glucose in the pool. It has been shown above that glucose in the medium was incorporated into the protozoa and that the freshly harvested protozoon had free glucose in its pool. It was therefore of interest to investigate the effect of glucose incorporation on the concentration of glucose in the pool. Figure 3 is a time course for the effect on the glucose in the pool of the incorporation of ^{14}C from 83 mM-[^{14}C]glucose and shows that after incubation for 6 hr ^{14}C equivalent to 0·18 μmole glucose was incorporated into a pool that contained 0·20 μmole glucose; 75% (i.e. 0·135 μmole) of the ^{14}C incorporated was present as glucose. This shows that over 60% of the glucose in the pool was replaceable by glucose from the medium without increase in the concentration of glucose in the pool. At external glucose concentrations over about 0·08 M the incorporation of [^{14}C] glucose was accompanied by an increase in the total glucose in the cell (Fig. 4). Inhibition of some of the metabolic activities of the protozoa by lowering the temperature to 21° or by adding 0·67 mM-iodoacetate abolished the phase at lower glucose concentrations where the concentration in the pool was not affected by glucose incorporation; for every mole of glucose incorporated there was a corresponding increase in pool glucose over the entire concentration range tested (Fig. 4). The

concentration of glucose in the pool usually decreased on incubation of freshly harvested protozoa (Table 2) but both these treatments produced an increase in pool glucose even in the absence of added glucose (Fig. 4). These results are explicable if the level of glucose in the pool represents a steady state balance between an input of glucose by hydrolysis of maltose derived from intracellular starch or from the medium, and a loss of glucose by release into the medium or conversion into protozoal or bacterial polysaccharide. Inhibition of the biosynthetic reactions by lowering the temperature or the addition of iodoacetate would tend to increase the glucose concentration in the pool because the rate of removal of glucose was decreased without alteration in the

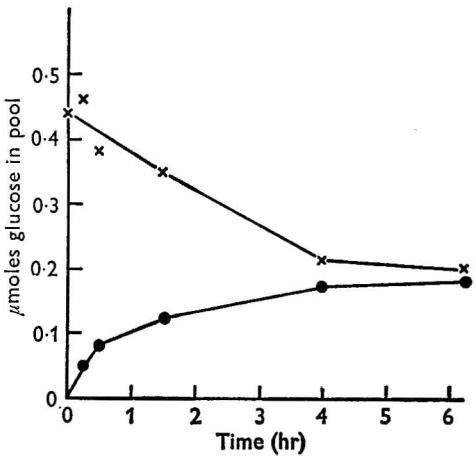

Fig. 3

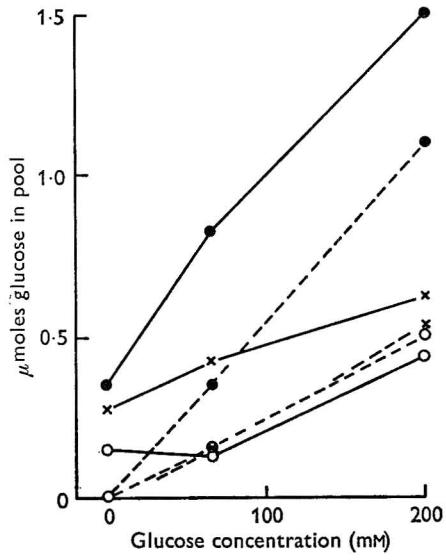

Fig. 4

Fig. 3. Effect of incubation in the presence of 80 mM-[^{14}C]glucose on the concentration of glucose in the pool of *Entodinium caudatum*. ×——×, Total glucose in pool as determined by glucose oxidase method; ●——●, [^{14}C]glucose incorporated into pool as calculated from ^{14}C found in pool and known specific activity of glucose added initially.

Fig. 4. Effect of glucose concentration in the medium on the incorporation of [^{14}C]glucose and the concentration of glucose in the protozoal pool in $1{\cdot}7 \times 10^8$ protozoa contained in 1·5 ml. during a 20 min. incubation. ——, Total pool glucose as determined by glucose oxidase method; - - - -, [^{14}C]glucose in the pool calculated from the ^{14}C found in the pool and the known specific activity of the glucose added initially; ●, in presence of 0·67 mM-iodoacetate at 40°; ○, incubated at 40° in absence of iodoacetate; ×, incubated at 21° in absence of iodoacetate. Initial pool glucose was 0·16 µmoles.

rate of input of glucose. With normal protozoa incubated at 40° in the presence of a low concentration of glucose the absence of any increase in pool glucose could be explained by (*a*) entry of [^{14}C]glucose by exchange with [^{12}C]glucose in the pool or (*b*) a feed-back mechanism in which the concentration of glucose controls the activity of the maltase which hydrolyses maltose to glucose, but not the rate of glucose uptake from the medium.

In an attempt to show an exchange reaction the protozoa were pre-incubated with 0·02 or 100 mM-[^{14}C]glucose for 50 min., then washed three times in salt solution and incubated in the presence or absence of [^{12}C]glucose for 5 or 30 min. If an exchange

reaction occurred, the release of ^{14}C from the protozoal pool into the medium would be markedly stimulated by the presence of [^{12}C]glucose in the medium. The protozoa labelled at a high glucose concentration released ^{14}C progressively into the medium and there was a decline in the ^{14}C in both the pool and the pellet fractions, but the rate of loss from the pool was not affected by the presence in the medium of 6·7 or 67 mM-[^{12}C]glucose. With protozoa labelled at low glucose concentration there was a progressive loss of ^{14}C from the pool into the protozoal pellet and the medium but neither of these reactions was affected by the presence of [^{12}C]glucose. It is therefore considered unlikely that the incorporation of [^{14}C]glucose occurred by an exchange reaction. The effect of glucose concentration on the rate of hydrolysis of maltose is considered below.

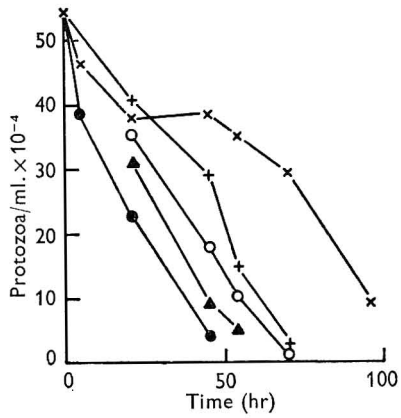

Fig. 5

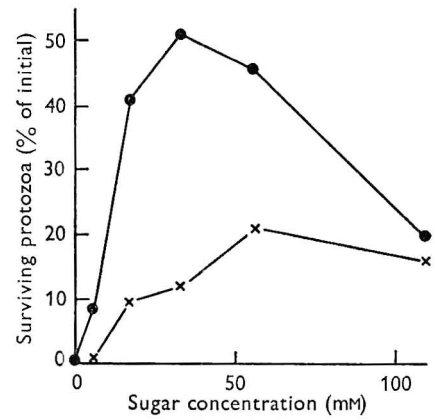

Fig. 6

Fig. 5. Effect of various carbohydrates on the survival of freshly harvested *Entodinium caudatum* incubated anaerobically in the presence of 10% (v/v) autoclaved rumen fluid (PARF, Coleman, 1964*a*)+1000 units penicillin G/ml.+400 μg. neomycin sulphate/ml. ●——●, No added carbohydrate; ▲——▲, 22 mM-galactose; ○——○, 22 mM-glucose; +——+, 11 mM-maltose; ×——×, 0·4% soluble starch.

Fig. 6. Effect of concentration of maltose (●——●) and glucose (×——×) on the survival of freshly harvested *Entodinium caudatum* incubated anaerobically in the presence of 10% (v/v) autoclaved rumen fluid (PARF)+1000 units penicillin G/ml.+400 μg. neomycin sulphate/ml. for 50 hr.

Metabolism of maltose

Effect on protozoal survival. Figure 5 shows time courses for the effect of various carbohydrates on the survival of freshly harvested and washed *Entodinium caudatum* incubated anaerobically in the presence of penicillin+neomycin+autoclaved rumen fluid (PARF). Although all the protozoa died in 5 days, soluble starch or maltose were more effective in prolonging the life of the ciliates than were either glucose or galactose. The greater effectiveness of maltose was observed at all concentrations (Fig. 6) whether these were expressed per molecule of maltose or per molecule of glucose. This suggests a difference in mode of uptake or metabolism and not that maltose acts because it contains two molecules of glucose. In experiments with [^{14}C] glucose and maltose it was possible to show that their greater effectiveness at high concentrations was not due to the complete utilization of sugars at low concentrations. In no culture in the experiment shown in Fig. 6 was more than 30% of the sugar

utilized. Of the sugar that was metabolized less than 2 % appeared in the protozoa and over 80 % was converted into material that was volatile on the addition of acid but retained on a planchet in the presence of NaOH, i.e. probably a fatty acid.

Incorporation of maltose. When incubated anaerobically in the presence of equimolar concentrations of glucose and maltose in the absence of antibiotics, *Entodinium caudatum* incorporated into the pool maltose-carbon over four times as rapidly as glucose-carbon. In one experiment $7{\cdot}4 \times 10^5$ protozoa incubated in the presence of 0·67 mM-sugar incorporated in 1 hr $0{\cdot}87 \times 10^{-2}$ μmoles glucose and $1{\cdot}96 \times 10^{-2}$ μmoles maltose. This higher incorporation of maltose was only apparent at low sugar concentrations (Fig. 7) because on a reciprocal plot of incorporation against sugar concentration both curves showed a break at 2·8 mM and above this the incorporation of both sugars was the same. The maximum 'active' uptake of maltose into the pool as calculated by extrapolation of the right-hand part of the curve to the ordinate was 0·034 μmoles/hr/10^6 protozoa, as compared with 0·046 μmoles/hr/10^6 protozoa for glucose. However the substrate concentration for half maximum uptake was 3·6 mM for glucose and 0·85 mM for maltose. There was also an increased (40 %) incorporation of carbon into the hot TCA-soluble fraction of the broken-cell pellet in the presence of maltose, but this was only into the bacterial fraction, and equimolar amounts of glucose and maltose were incorporated into protozoal polysaccharide at all concentrations. This result can be explained by the observation that although the *Proteus mirabilis* inside the protozoa does not incorporate maltose-carbon *Klebsiella aerogenes* incorporated maltose-carbon at over three times the rate for glucose-carbon when the sugars were present singly at equimolar concentration.

Effect of inhibitors and temperature. As with glucose incorporation, the above results suggested that there may be an active (predominant at low concentration) and a passive (predominant at high concentration) uptake of maltose. An attempt was therefore made to see whether the active process could be inhibited by the use of other sugars or by decreasing the temperature. Like the inhibitors of glucose incorporation, the inhibitors of maltose incorporation could be divided into two groups based on their effect when added at 25 mM in experiments of 3 hr duration. Those of group 1 which contained only 2-deoxyglucose inhibited the incorporation of ^{14}C from [^{14}C]maltose at all concentrations, whereas those of group 2 (galactose, glucose, mannose, cellobiose) inhibited only at concentrations below the break shown in Fig. 7. Similar results were obtained for incorporation into the pool alone and the relative uptakes into the pool and the broken-cell pellet were the same in the presence and absence of inhibitors. In contrast, when the protozoa were incubated at 30° there was an increased incorporation into the pool, especially at substrate concentrations of 0·5 to 1·2 mM and the position of the break in the curve of incorporation/substrate concentration on a reciprocal plot was moved to a lower concentration (Fig. 8). As this result was only obtained clearly after 4 to 5 hr incubation, the experiment was made in the presence of antibiotics in order to prevent the growth of bacteria. Incorporation into the pellet fraction of the protozoa was decreased by 20 to 25 % at all substrate concentrations, so that the total incorporation into the protozoa was unchanged or slightly stimulated. The increase in the concentration of ^{14}C in the pool shows that the mechanisms for the incorporation of [^{14}C]maltose into the pool were less sensitive to change in temperature than those for the removal of ^{14}C into the pellet fractions. The change in the position of the break in the curve for incorporation into the pool shown in Fig. 8 means that

the 'passive' uptake reaction was predominant over a longer concentration range at the lower temperature; this suggested that it was less sensitive to change of temperature than the 'active' process. The uniform effect of the temperature change on incorporation of ^{14}C into the pellet fractions at all substrate concentrations may indicate that only the [^{14}C]maltose taken up by the active process was incorporated into the pellet fractions. If this were not so, then the increased concentration of ^{14}C in the pool might be expected to nullify the effect of the lower temperature on incorporation of ^{14}C into the pellet fractions from the pool.

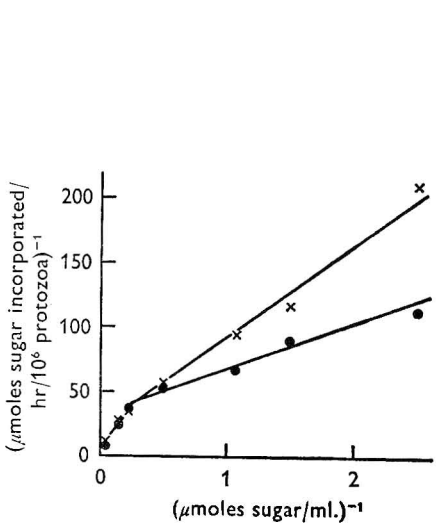

Fig. 7

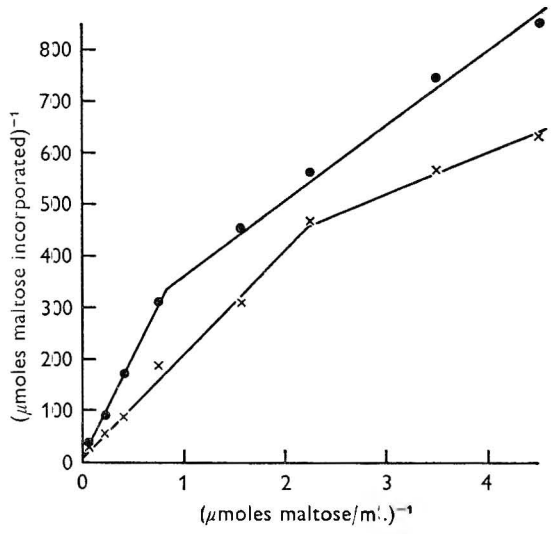

Fig. 8

Fig. 7. Effect of substrate concentration on the incorporation of [U-^{14}C]maltose (●——●) and [U-^{14}C]glucose (×——×) by *Entodinium caudatum* incubated anaerobically in the absence of antibiotics for 20 min.

Fig. 8. Effect of incubation temperature on the incorporation of ^{14}C from [U-^{14}C]maltose into the pool of *Entodinium caudatum* incubated anaerobically in the presence of 1000 units penicillin G/ml. + 400 μg. neomycin sulphate/ml. for 5 hr. ●——●, Incubated at 40°; ×——×, incubated at 30°.

Effect of antibiotics and of cell breakage. The incorporation of ^{14}C from 0·7 mM-[^{14}C]maltose into the pool of protozoa incubated in absence of antibiotics was markedly decreased by pre-incubation for 20 hr of protozoa with 1000 units penicillin/ml. + 400 μg. neomycin sulphate/ml. (Fig. 9). Similar results were obtained with glucose and with both sugars incorporation was progressive for over 4 hr without pre-incubation, whereas with antibiotic-treated protozoa incorporation reached a maximum after 20 min. and then decreased, until after 1 hr the incorporation was less than 15% of that into untreated protozoa. At high sugar concentrations (20 mM) the pre-incubation had no inhibitory effect on the incorporation of ^{14}C from [^{14}C]glucose or maltose both of which were incorporated to the same extent on a molar basis. This observation suggested that the antibiotics affected the 'active' uptake only. In contrast, pre-incubation of the protozoa with penicillin and neomycin decreased incorporation of ^{14}C from high concentrations of [^{14}C]maltose into the pellet fractions to 30% of that with normal untreated protozoa; at low maltose concentrations incorporation

was decreased to 1 to 2%. This suggested, as before, that only ^{14}C taken up by the 'active' process could be incorporated into the pellet fractions. Breakage of the protozoa before incubation with [^{14}C]maltose decreased the incorporation of ^{14}C into the pellet by between 41% (at 0·3 mM-maltose) and 72% (at 15 mM-maltose).

Nature of products in protozoal pool. Figure 10 shows a time course for the incorporation of ^{14}C from [^{14}C]maltose into various pool constituents. There was a rapid incorporation of maltose into the pool and breakdown of this maltose to glucose during the first minute of incubation. Thereafter the amounts of these [^{14}C]sugars

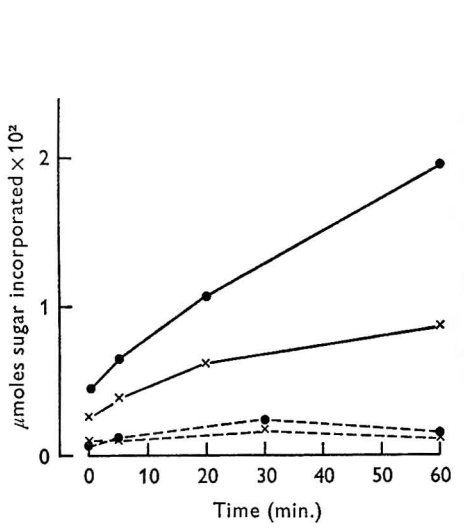

Fig. 9

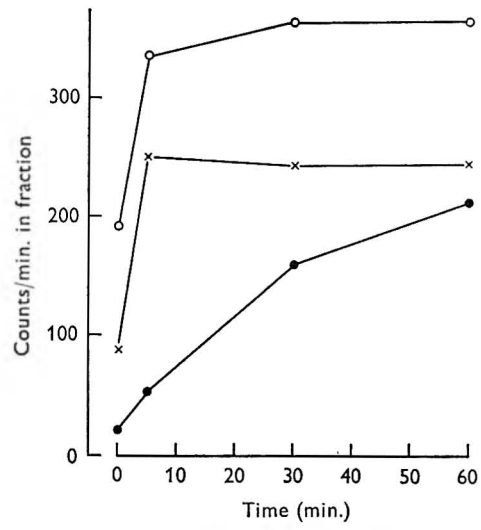

Fig. 10

Fig. 9. Effect of pre-incubation of *Entodinium caudatum* for 20 hr in the presence of 1000 units penicillin G/ml. + 400 μg. neomycin sulphate/ml. on the uptake of ^{14}C from [U-^{14}C]maltose (●) and [U-^{14}C]glucose (×) in the absence of antibiotics. ——, 4·3 × 10^6/ml. freshly harvested protozoa; – – – –, 4·0 × 10^6/ml. protozoa pre-incubated with antibiotics.

Fig. 10. Incorporation of ^{14}C from 0·15 mM-[U-^{14}C]maltose into various fractions in the pool of *Entodinium caudatum*. ○——○, Into glucose; ×——×, into maltose; ●——●, into hexose phosphate.

increased more slowly to reach a maximum after 5 min., after which time the quantities remained constant. In contrast, there was a steady increase throughout the experiment in the amount of glucose phosphate present. During incubation for 5 hr 17 mM-2-deoxyglucose inhibited incorporation into all pool constituents equally, whereas glucose specifically inhibited incorporation into pool glucose and decreased the amount of maltose in the medium that was hydrolysed to glucose from 29 to 3%. The relative incorporation into the various pool constituents was the same at 30° and 40° and at high and low maltose concentrations.

Metabolism of other sugars

Ribose. Washed suspensions of *Entodinium caudatum* incubated anaerobically in the presence of penicillin + neomycin incorporated ^{14}C from [1-^{14}C]-D-ribose progressively for at least 6 hr. After incubation for 5 hr 48% of the ^{14}C incorporated by the protozoa was present in the pool, 5% in the hot TCA-soluble fraction of the broken-cell

supernatant fluid, 25% in the hot TCA-soluble fraction of the broken-cell pellet and 6% in the combined protein fractions of the protozoa. The ^{14}C in the pool was present as ribose only. After further hydrolysis of the hot TCA-soluble fraction of the pellet in N-HCl for 1 hr at 100° all the ^{14}C was present as glucose. It was not possible to identify [^{14}C]ribose in either of the hot TCA-soluble fractions.

The pellet fraction of protozoa that had incorporated ^{14}C from [^{14}C]ribose was fractionated by centrifugation through 2·0 M-sucrose (as described above) in order to investigate the incorporation of ^{14}C into protozoal polysaccharide and intracellular bacteria. Of the ^{14}C in the pellet, 40% and 70% were present in the protozoal polysaccharide on incubation with 0·04 mM and 67 mM [^{14}C]ribose, respectively. Since glucose was the only combined [^{14}C]sugar found in the pellet fraction this suggested that both the protozoa and the bacteria converted ribose into glucose.

As with [^{14}C]glucose and maltose there was a break in the curve for incorporation of ^{14}C from [^{14}C]ribose/substrate concentration on a reciprocal plot; for ribose this was at 3 mM. The maximum rate of uptake of ribose calculated by extrapolation of the right-hand part of the curve was 0·024 μmoles/4 hr/10^6 protozoa. During a 4-hr period 25 mM-D-glucose inhibited the incorporation of ^{14}C from 0·3 mM-[^{14}C]ribose by 23% but had no effect at high ribose concentrations. 2-Deoxyglucose at 25 mM inhibited incorporation by 33% at 0·3 mM-ribose and by 22% at 0·05 M-ribose. In contrast to the results on the incorporation of [^{14}C]glucose, breakage of the protozoa before incubation or changing the incubation temperature to 30° decreased the incorporation of ^{14}C from 0·3 mM-[^{14}C]ribose to 28 and 73%, respectively.

Galactose. ^{14}C from [1-^{14}C]-D-galactose was incorporated by washed protozoal suspensions and the curve for incorporation of ^{14}C/substrate concentration on a reciprocal plot showed a break at 1·2 mM-galactose. The maximum rate of galactose uptake calculated by extrapolation of the right hand part of the curve to the ordinate was 0·023 μmole/hr/10^1 protozoa. After incubation for 4 hr 25% of the protozoal ^{14}C was present in the pool and 51% in the hot TCA-soluble fraction of the broken-cell pellet. The only ^{14}C-labelled compound in the pool and in the hot TCA-soluble fraction after hydrolysis in N-HCl for 1 hr at 100° was glucose. Of the ^{14}C in this latter fraction 30% was associated with the protozoal polysaccharide at low external galactose concentrations and 50% at high concentrations. As shown in Fig. 5, galactose has little beneficial effect on the survival of protozoa in the presence of antibiotics.

Sucrose. ^{14}C from 0·3 mM-[U-^{14}C]sucrose was incorporated into the protozoa at two-thirds of the rate for ^{14}C from [^{14}C]maltose at the same concentration. The break in the curve for incorporation of ^{14}C/substrate concentration on a reciprocal plot was 2 mM.

Permeability of protozoa to ^{14}C-labelled sugars

It has been shown (Coleman, 1967*a*) that 50 to 60% of the packed cell volume of *Entodinium caudatum* is impermeable to amino acids in experiments up to 5 min. duration. It was of interest to determine whether the same is true with sugars and whether or not this is affected by different temperatures of incubation. The experiments were done by a method similar to that described by Coleman (1967*a*). This involved preparing by the standard method a very thick protozoal suspension such that on centrifugation the packed protozoal pad was about half the total volume. Samples (0·2 ml.) of [^{14}C]sugar (at minimum concentration of 5 mM) were mixed with

1·0 ml. protozoal suspension or 1·0 ml. salts solution in a 10 ml. graduated conical centrifuge tube and incubated for 2 min. The tubes were then centrifuged for 30 sec. from starting, on a bucket-head centrifuge (final speed equivalent to 300*g*). The volume of the protozoal pellet was measured, then all the supernatant fluid removed and 0·1 ml. of it used for the estimation of ^{14}C. The sides of the tube were wiped with filter paper to remove free medium and then the protozoal pellet suspended in salts solution to a total volume of 5 ml.; 0·25 ml. of this suspension was used for the estimation of the total ^{14}C associated with unwashed protozoa. The suspension was then centrifuged, the protozoa washed once more in salt solution and treated as described under Methods for the estimation of ^{14}C in washed protozoa and in the protozoal pool. In contrast to the results obtained two years ago and reported previously (Coleman, 1967*a*) it was now possible to incubate *E. caudatum* in a thick suspension at 40° for up to 1 hr without the protozoa showing signs of disintegration. The cause of this change in protozoal behaviour is unknown but it enabled the effect of temperature on protozoal permeability to be measured with greater accuracy. The results were calculated as follows. The total amount of ^{14}C added to the system in the presence or absence of protozoa was calculated from the ^{14}C present in 0·1 ml. of medium in the absence of protozoa. From this value and the amount of ^{14}C present in 0·1 ml. supernatant fluid above the protozoa, the volume of liquid available to the sugar in the presence of protozoa was calculated. The difference between this value and the total volume of liquid gave the volume impermeable to the sugar and this was compared with the volume of protozoa present. The percentage of the protozoal volume impermeable to the sugar was given by

$$100 \times [\text{total volume} - (A/10B)]/\text{protozoal volume},$$

where in counts/min., A = total amount of ^{14}C and B = ^{14}C present in 0·1 ml. supernatant fluid in the presence of protozoa.

Under these conditions 63 ± 12% (for seven different batches of protozoa; range 46 to 81%) of the packed-cell pad volume was impermeable to glucose at 20°. In an experiment in which 27% of the protozoal volume was permeable to 0·001 M-^{14}C-glucose at 20°, 4% of this glucose was not removable by washing the protozoa twice in salt solution. If this glucose was isolated in a cellular compartment of the protozoa, then this compartment occupied only 1·7% of the volume of the protozoon. On incubation of the protozoa for 5 min. at 40° the apparent mean impermeable volume decreased to 46 ± 10%; but since the decrease in the ^{14}C in the presence of protozoa was due to a loss of ^{14}C from the complete system, presumably because of the formation of $^{14}CO_2$, this increased permeability was probably an artifact. However, the proportion of the protozoal ^{14}C that was not removable from the pool by washing increased to 7%. At high glucose concentrations (i.e. 70 mM) the permeable volume remained at the lower value even after 5 min. incubation at 40°, and 5% of the glucose associated with the protozoa was not removed from the pool by washing. Similar results were obtained at low glucose concentrations (1·0 mM) in the presence of 14 mM-2-deoxyglucose. However, in the presence of 0·8 mM-iodoacetate, although the impermeable volume remained unchanged, the proportion of the total ^{14}C in unwashed protozoa that could not be removed from the pool by washing increased to 27% after incubation for 5 min. If this glucose was isolated in a compartment of the protozoa at its original concentration it must have occupied 11% of protozoal volume.

Studies on some protozoal enzymes

A possible explanation for the comparative ineffectiveness of glucose and maltose compared with starch in maintaining protozoal life would be the absence of a glucokinase (ATP: D-glucose-6-phospho-transferase, EC 2.7.1.2) and a maltose phosphorylase (maltose: orthophosphate glucosyltransferase, EC 2.4.1.8) associated with the presence of an α glucan phosphorylase (α 1,4 glucan: orthophosphate phosphorylase, EC 2.4.1.1). Under these conditions glucose could not be phosphorylated to glucose-6-phosphate, the glucose of maltose could not be phosphorylated either directly or after liberation in the free form, but starch could undergo a phosphorolysis to glucose-1-phosphate which could be metabolized by the enzymes of the glycolytic pathway. The presence of these enzymes in the protozoal broken cell supernatant fluid was therefore investigated.

Table 5. *Phosphorylase activity of Entodinium caudatum*

The basal incubation medium contained 1·5 ml. 0·1 M-$\beta\beta'$-dimethylglutarate buffer (pH 6·4), 0·1 ml. 1 mM-phosphate, 0·1 ml. ^{32}P-inorganic phosphate (10 μC/ml.) *plus* the additions given below. 95 % (v/v) N_2 + 5 % (v/v) CO_2 was bubbled through the medium for 2 min. (except where cysteine was omitted from the medium) before the addition of 0·2 ml. of broken protozoal preparation. The tubes were then gassed for a further 10 sec. before sealing with a rubber bung and incubation at 39°; total volume was 3 ml. At the end of the experiment inorganic phosphate was precipitated with sodium fluoride and magnesia mixture (see Methods) and a sample of the supernatant fluid plated out for the estimation of ^{32}P.

Additions to the basal medium			Radioactivity (counts/min.)	
2 % soluble starch	1 % L-cysteine	Protozoal preparation	Initial	30 min.
0·1 ml.	0·2 ml.	BCS	200	1500
	0·2 ml.	BCS	180	480
0·1 ml.	—	BCS	190	900
0·1 ml.	0·2 ml.	—	200	220
	0·2 ml.	SP	250	2010
0·1 ml.	0·2 ml.	SP	300	3520
—	0·2 ml.	BCP	270	900
0·1 ml.	0·2 ml.	BCP	130	1940

SP = ultrasonically treated protozoa; BCS = broken-protozoal supernatant fluid; BCP = broken-protozoal pellet.

α-Glucan phosphorylase. Under the conditions described in Methods the values obtained before incubation and in the absence of enzyme represented 0·5 % of the total radioactivity and the amount of soluble ^{32}P usually increased linearly up to 10 times during a 2-hr incubation with enzyme (Table 5). When the sodium fluoride that was normally added at the end of the incubation was present throughout, it has been shown on a reciprocal plot of enzyme activity/phosphate concentration to act as a competitive inhibitor (Dixon & Webb, 1964). The effect of cysteine on enzyme activity was very variable and its omission decreased this by 0 to 50%. In the initial experiments the buffer used was 0·03% $NaHCO_3$ + 5% (v/v) CO_2 + 0·5% NaCl (pH 6·64). The buffering capacity of this mixture was poor: 0·11 M-malate, 0·04 M-citrate, 0·04 M-tris and 0·05 M-$\beta\beta'$-dimethylglutarate buffers were tested as possible replacements. Phosphorylase activity was detected in the presence of each of these buffers

but with the exception of dimethylglutarate the optimum pH value was at the limit of their buffering ranges. In the presence of *ββ'*-dimethylglutaric acid + NaOH buffer the optimum was pH 6·4, with 0·13 % soluble starch as substrate.

Substrate specificity. The following carbohydrates were tested as substrates in the presence of broken-cell supernatant fluid and 0·04 mM-[^{32}P]phosphate; the results are expressed relative to that with 0·13 % soluble starch = 100: 0·2 % rice starch, 10 to 30; 3·3 mM-maltose, 10 to 36; 3·3 mM-sucrose, 12 to 220; 3·3 mM-glucose, 17; 3·3 mM-lactose, 25; 3·3 mM-cellobiose, 24; 0·06 % inulin, 13; no addition, 13 to 23. The activities in the presence of sucrose were very variable. It is possible that the amount of the enzyme responsible varied in different batches of protozoa. The maximum velocities at infinite phosphate concentration and the K_m values (i.e. substrate concentration at which velocity is half the maximum) in the presence of 0·2 % carbohydrate are given in Table 6. Although these experiments were all made with broken-cell supernatant fluid as source of enzyme, 60 % of the activity present in a complete protozoal homogenate was in the broken-cell pellet (Table 5). The activity found on incubation of the pellet fraction in the absence of added carbohydrate was only stimulated three-fold by the addition of 0·2 % soluble starch, presumably because of the presence of rice starch grains and protozoal polysaccharide granules in the pellet fraction. The activity of both the supernatant fluid and pellet fractions in presence of 0·2 % soluble starch was decreased by about 20 % at all phosphate concentrations on pre-incubation of the protozoa with 1000 units penicillin/ml. + 400 μg. neomycin sulphate/ml. for 18 hr.

Table 6. *Kinetic constants of certain enzymes of Entodinium caudatum*

The enzyme activities of protozoal broken-cell supernatant fluid were measured as described in Methods. To determine the activities present in antibiotic-treated protozoa, the protozoa were harvested from the growth medium and incubated in the presence of 1000 units penicillin/ml. + 400 μg. neomycin sulphate/ml. for 18 hr before preparation of the broken-cell supernatant fluid. Maximum velocities were measured by extrapolation of a reciprocal plot of velocity/substrate concentration, to infinite substrate concentration. The K_m value was the substrate concentration at which the velocity was half the maximum.

Enzyme	Substrate	Protozoa pre-incubated with antibiotics	Maximum velocity (μmoles/hr/10^6 protozoa)	K_m (mM)
α Glucan phosphorylase	0·2 % soluble starch, phosphate	−	4·1–9·8*	5–10
	0·2 % rice starch, phosphate	−	0·53*	2·9
	0·2 % maltose, phosphate	−	0·15*	1·7
Hexokinase	Glucose, 5 mM ATP	−	1·8†	0·77
	Glucose, 5 mM ATP	+	1·4†	0·54
	Galactose, 5 mM ATP	−	1·2†	7·1
Phosphoglucomutase	Glucose-1-phosphate	−	4·1	0·63
	Glucose-1-phosphate	+	6·2	0·86
Maltase	Maltose	−	22	6·6

* Phosphate esterified at infinite phosphate concentration. † Measured at infinite sugar concentration.

Reaction products. The crude broken-cell supernatant fluid contained an active phosphoglucomutase (see below). After prolonged incubation the products of any reaction in which glucose phosphates were produced were 95 % glucose-6-phosphate

and 5% glucose-1-phosphate, which is the equilibrium mixture. To determine which phosphate was formed first during incubation of broken-cell supernatant fluid with soluble starch and [^{32}P]phosphate, samples were taken at intervals, the phosphate precipitated with fluoride+magnesia mixture and then the glucose phosphates separated by chromatography in solvent C. The position of the [^{32}P]phosphates was determined by radioautography and the radioactivity in each measured. After 5 min. incubation 61% of the ^{32}P was associated with the glucose-1-phosphate; this proportion fell progressively until after 3 hr only 10% was present, the remainder being all associated with the glucose-6-phosphate spot. This is taken as evidence that glucose-1-phosphate was the first product of the reaction and that the ^{32}P was not entering the glucose phosphates by exchange with the γ-phosphate of adenosine triphosphate (ATP) followed by phosphorylation of glucose to give glucose-6-phosphate.

Hexokinase. Glucokinase (ATP: D-glucose-6-phosphotransferase) activity was measured by the formation of [^{14}C]glucose phosphate from [^{14}C]glucose in the presence of ATP. In the presence of 1 mM-glucose and the broken-cell supernatant fluid from $2{\cdot}7 \times 10^6$ protozoa, the reaction was linear for 1 hr. Thereafter the rate of hexose phosphate formation decreased markedly and this was associated with the appearance of fructose in the medium. In the absence of ATP the amount of glucose esterified decreased to less than 5%. The optimum value was pH 6·7. The maximum velocities and K_m values for the hexokinase activity of the broken-cell supernatant fluid from normal and antibiotic treated protozoa are given in Table 6.

In view of the possibility that hexokinase might be involved in the incorporation of [^{14}C]glucose by washed protozoa it was necessary to determine whether the pattern of inhibition of the two reactions by other sugars and sugar analogues was the same. For these experiments which were of 30 min. duration [^{14}C]glucose was present at 0·5 mM, ATP at 5 mM and the inhibitors at 25 mM. The results, expressed relative to those in the absence of inhibitor = 100 were: L-glucose, 100; 3-methylglucose, 100; ribose, 100; sorbitol, 96; fructose, 90; mannose, 86; 2-deoxyglucose, 80; maltose, 40; galactose, 38; D-glucose, 6. The decrease in activity in the presence of maltose was probably due to hydrolysis of maltose to glucose as the preparation contained a maltase (see below). These results contrasted with those on the incorporation of glucose by intact protozoa, in that with intact protozoa 2-deoxyglucose was more potent than galactose at low glucose concentration. This suggests that hexokinase did not mediate the incorporation of glucose by intact protozoa. The inhibition of hexokinase activity by galactose was competitive on a reciprocal plot of activity/substrate concentration in the presence and absence of 80 mM-galactose. Galactose was also utilized as a substrate for the hexokinase reaction and the maximum velocity and K_m value are given in Table 6.

Phosphoglucomutase. Phosphoglucomutase (α-D-glucose-1,6-diphosphate:α-glucose-1-phosphate phosphotransferase, EC 2.7.5.1) activity was assayed by estimating the amount of reducing sugar, i.e. glucose-6-phosphate, formed on incubation of broken-cell supernatant fluid with glucose-1-phosphate. The rate of reaction was constant for 30 min. The kinetic constants are given in Table 6.

Maltase. Maltase (α-D-glucoside glycohydrolase, EC 3.2.1.20) activity was assayed by measuring glucose formation from maltose in presence of broken-cell supernatant fluid by means of glucose oxidase. The kinetic constants are given in Table 6. As the concentration of glucose in the protozoal pool could be controlled in part by an

inhibitory effect of glucose on the enzyme hydrolysing maltose to glucose, the effect of glucose and other monosaccharides on this reaction was investigated. This was estimated by incubation of broken-cell supernatant fluid with [^{14}C]maltose in the presence of different concentrations of [^{12}C]glucose or other sugar for 5 min. and then stopping the reaction by immersion in a boiling water bath for 5 min. Samples of the supernatant fluids after removal of the protein by centrifugation were chromatographed in solvent B, the position of the maltose and glucose spots determined by radioautography and the ^{14}C in each estimated by placing a GM tube directly on the paper. From these values the proportion of the maltose hydrolysed to glucose could be calculated. Figure 11 shows that in the presence of 12 mM-maltose the addition of 10 mM-glucose decreased the rate of maltose hydrolysis by 50%. Over the glucose concentration range of 5 to 15 mM (the probable concentration in the freshly harvested protozoon) a change of 1 mM in the glucose concentration altered the rate by 5 to 10%.

Amylase. Since the concentration of glucose in the pool would also depend on the concentration of maltose and the rate of maltose formation from starch, the effect of glucose and maltose on this latter reaction was investigated. The experiment was made in 0·4 ml. volumes containing [U-^{14}C]soluble starch (0·2 μC; 7·7 μg.), broken-cell supernatant fluid from approximately 5×10^5 protozoa and 0 to 500 mM [^{12}C]glucose or maltose. After incubation for 5 min. the reaction was stopped by immersion of the tubes in boiling water and the precipitate removed by centrifugation. The glucose and maltose were separated from the starch by chromatography in solvent B and the ^{14}C contents of the compounds measured as described above. The amount of hydrolysis was measured as the ^{14}C present in the two sugars divided by the ^{14}C present in all three compounds. [^{12}C]glucose had little effect on the rate of starch hydrolysis, but small changes in the concentration of [^{12}C]maltose had a marked effect. Over the range 5 to 20 mM a change of 1 mM in the maltose concentration altered the rate by 3 to 5% (Fig. 11).

Probable mechanism of starch metabolism

Although it has been shown above that various enzymes for the metabolism of carbohydrates exist in the cell, it is not clear whether starch was degraded initially by phosphorolysis to glucose-1-phosphate or hydrolysed *via* maltose to glucose and then phosphorylated to glucose-6-phosphate in the presence of a hexokinase. To elucidate this ^{14}C-soluble starch was incubated in salt solution B with broken-cell supernatant fluid in the presence and absence of 6 mM-ATP and the rate of [^{14}C]hexose phosphate formation measured under the two conditions. In the absence of ATP any hexose phosphate must be formed by phosphorolysis of the starch, whereas in the presence of ATP hexose phosphate could also arise by phosphorylation of glucose formed from the starch by hydrolysis.

The experiment was made in 1·7 ml. volumes containing salt solution B, 0·1% [^{14}C-] soluble starch (0·06 μC/ml.) and the broken-cell supernatant fluid from $0{\cdot}86 \times 10^6$ protozoa in the presence or absence of 6 mM-ATP. The reaction was stopped by immersing the tubes containing the reaction mixture in boiling water and the protein precipitate then removed by centrifugation. A sample of the supernatant fluid was treated with Dowex 2 (acetate) to remove hexose phosphates which were subsequently eluted by treatment with N-HCl. To obtain a measure of the amount of

starch that had been hydrolysed to maltose and glucose, a reducing sugar determination was made on the reaction mixture after removal of the hexose phosphates with the resin. Under the conditions of the reducing sugar assay the extinction obtained per mole of glucose was only 11 % less than that obtained per mole of maltose; the results have been calculated as though all the reducing sugar was glucose. Approximately equal quantities of the two sugars were found. In the experiment shown in Fig. 12 the rate of formation of hexose phosphate in the absence of added ATP was lower than in its presence and had almost ceased after 15 min.; in the presence of ATP the reaction was still proceeding after 60 min. These findings suggested that there

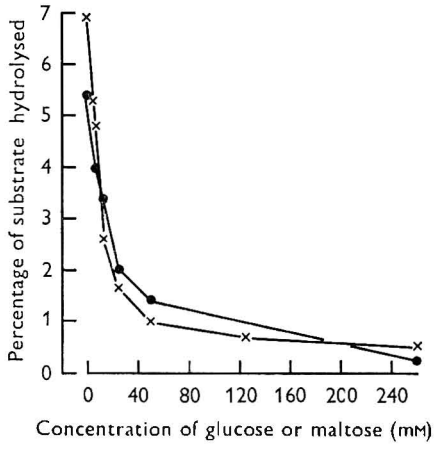

Fig. 11

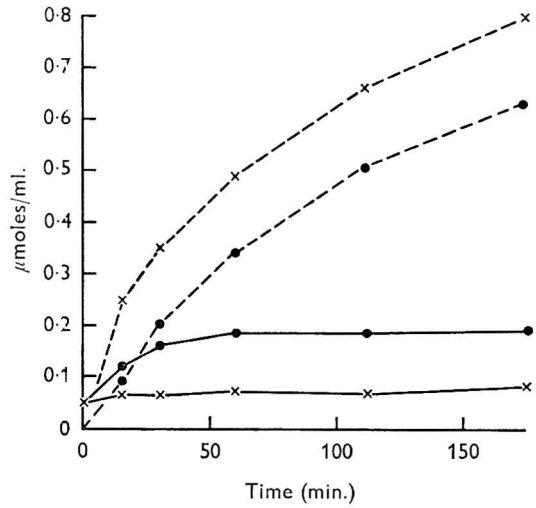

Fig. 12

Fig. 11. Effect of glucose concentration on the hydrolysis of 12 mM-[U-^{14}C]maltose (× —— ×) and of maltose concentration on the hydrolysis of 0·2 % [U-^{14}C]soluble starch (● —— ●) by broken-cell supernatant fluid during a 5 min. incubation.

Fig. 12. Effect of 10 mM-ATP on the formation of hexose phosphate (——) and reducing sugars expressed as glucose (– – –) from 0·017 % [U-^{14}C]soluble starch by broken-protozoal supernatant fluid. (●), In presence of ATP; (×), in absence of ATP.

might have been a limited amount of ATP present in the broken-cell supernatant fluid and that this was all utilized in the first 15 min. in absence of added ATP. The subsequent slow increase in the amount of hexose phosphate formed is presumed to be due to phosphorolysis. It is apparent from these results that, given ATP, the broken-cell supernatant fluid converted the glucose in starch to hexose phosphate after hydrolysis to free glucose, and that the rate of starch hydrolysis was greater than the rate of phosphorylation of glucose.

DISCUSSION

It has been shown that 63 % of the volume of a packed protozoal pad was impermeable to sugars on incubation for a few minutes at 20°. The electron microscope studies on thin sections of *Entodinium caudatum* reported previously (Coleman & Hall, 1966) showed that the organism was divided into an outer region (ectoplasm) and an inner region (endoplasm). This latter region contained all the rice starch grains which the

ciliate had engulfed and all the intracellular bacteria. From these findings it is considered likely that the membrane between the endoplasm and the ectoplasm may form the barrier between the regions which are permeable and impermeable to low molecular weight compounds such as sugars. Some information on which of the two regions was freely permeable to sugars was obtained in the investigations of the effect of inhibitors on the incorporation of ^{14}C from [^{14}C]glucose. If the endoplasm were freely permeable and the bacteria therein were exposed to the external medium then the degree of inhibition of incorporation of ^{14}C into the bacteria by any reagent should be the same in intact protozoa as in broken protozoa. If it is the ectoplasm which is freely permeable, then the effect of an inhibitor would be more complicated and would depend in part on the effect of the inhibitor on the passage of glucose through the membrane into the endoplasm, and not necessarily on the effect of the inhibitor on the bacteria. The results obtained showed that 2-deoxyglucose and galactose inhibited the incorporation of ^{14}C from [^{14}C]glucose by bacteria in intact protozoa by 71 and 29%, respectively, and into washed suspensions of the two most numerous bacteria by 30 and 90%, respectively, i.e. the pattern of inhibition was different. With intact protozoa in the presence of either inhibitor the effect on the incorporation of ^{14}C into the intracellular bacteria was the same as that on the incorporation into the protozoal pool, suggesting that the glucose utilized by the bacteria came from the pool and not from the medium. Both these results supported the view that it was the ectoplasm that was freely permeable. Unfortunately experiments on the effect of inhibitor on broken protozoa that contained intact bacteria showed that although 2-deoxyglucose and galactose gave about 20% greater inhibition than in intact protozoa, the 2-deoxyglucose was always more effective than galactose. Although this may be interpreted as supporting the view that it was the endoplasm that was permeable, two previously reported findings are not consistent with this. Firstly, the inhibitory effect of a free [^{12}C]amino acid on the incorporation of the ^{14}C-form of that amino acid from bacterial protein into protozoal protein was related to the rate at which the free amino acid was incorporated by the protozoa (Coleman, 1967*a*). Secondly, it proved difficult to kill bacteria inside intact protozoa by incubation in the presence of penicillin + neomycin, although the same bacteria were readily killed under the same conditions after protozoal breakage (Coleman, 1962). Therefore, it is considered more likely that it was the ectoplasm that was freely permeable to glucose and other low molecular weight compounds of the medium.

The incorporation of [^{14}C]glucose into the protozoal pool consisted of two processes as shown by the break in the curve on the reciprocal plot of incorporation of ^{14}C in a certain time/substrate concentration. At high concentrations, above about 2·5 mM, incorporation was proportional to glucose concentration, had an almost infinite maximum velocity and was not affected by inhibitors of group 2 such as sorbitol. It is therefore considered to be a passive uptake. At low concentrations, the reaction had a finite maximum rate, was more rapid than the passive uptake and was inhibited by inhibitors of group 2. It is therefore assumed to be a process that was dependent upon metabolic processes, i.e. 'active'. Both processes are considered to be operative at all glucose concentrations, but the 'active' process is predominant at low concentrations and the passive at high. If the [^{14}C]glucose, in protozoa that had been incubated in the presence of a high concentration of glucose and then washed, was present at its original concentration, it occupied only 5% of the protozoal volume. This means that

the passive uptake of glucose must represent penetration into a minor cell compartment and not into the complete endoplasm. However, it is considered likely that the 'active' uptake of glucose was into the endoplasm. As there was no discontinuity in the curve for incorporation of ^{14}C into protozoal polysaccharide on a reciprocal plot of incorporation/substrate concentration, it is postulated that only glucose taken up by the 'active' process can be incorporated into protozoal polysaccharide. If glucose taken up by both processes could be utilized the curve might be expected to show a break at about 2·5 mM. At low glucose concentrations more of the ^{14}C that had been incorporated was present as hexose phosphate than at high concentrations and this would be expected if only the 'active' uptake were into a compartment where further metabolism of the glucose could occur. The finding that at low glucose concentrations 2-deoxyglucose decreased the incorporation of ^{14}C into the pool but had no effect on the distribution of ^{14}C between the various pool constituents is interpreted as showing that glucose is taken up actively as such, and then phosphorylated. If the phosphorylation were obligatory for uptake then 2-deoxyglucose might have a specific effect on the concentration of hexose phosphate. This interpretation is supported by the finding that the hexokinase of the broken protozoal supernatant fluid was not very sensitive to inhibition by 2-deoxyglucose.

The action of iodoacetate in markedly increasing the amount of [^{14}C]glucose that could not be removed from the protozoa by washing, without increasing the permeability of the protozoa, was unexpected since it was used in an attempt to inhibit the 'active' uptake of glucose. Since iodoacetate is a metabolic inhibitor then its effect must result from the inhibition of some process such as a metabolic pump for the expulsion of glucose from a cell compartment. If the region of the cell into which glucose penetrated passively were the contractile vacuole then in normal protozoa much of this glucose would be lost by operation of the vacuole during washing of the protozoa. In the presence of iodoacetate the vacuole would be unable to contract and would remain full of glucose. The similar results obtained on incubation of the protozoa at very low salt concentration could be explained by the ectoplasmic/endoplasmic membrane becoming 'leaky', associated with the passage of water into the cell. Glucose would then enter the endoplasm and be trapped there on return of the protozoa to salts of standard concentration.

It has been known for many years (Abou Akkada & Howard, 1960) that the addition of starch but not glucose to washed protozoal suspensions stimulates gas production. This means that any proposed mechanism of glucose incorporation must explain why exogenous glucose and glucose formed by the hydrolysis of starch grains are metabolized differently. For the reasons given above the passive uptake of glucose is assumed to be into some compartment such as the contractile vacuole, where it is not available for further metabolism. The active incorporation of [^{14}C]glucose is probably a net incorporation of glucose into the endoplasm as no evidence was found for an exchange reaction. The maximum rate of glucose uptake at infinite glucose concentration was 0·046 μmole/hr/10^6 protozoa. As the volume of each protozoon in a packed cell pad was $4{\cdot}7 \times 10^4$ μ^3 (Coleman, 1967*a*) the volume of 10^6 protozoa was $4{\cdot}7 \times 10^{10}$ μ^3, or 0·047 ml. If, as shown above, 37 % of this volume was fluid space into which the external medium could not freely penetrate, then the volume of 10^6 protozoa that contained the incorporated glucose was $0{\cdot}047 \times 0{\cdot}37 = 0{\cdot}017$ ml. If all the glucose incorporated in 1 hr remained as free glucose, the concentration at

the end of that hour would be 0·046/0·017 = 2·7 mM compared with 14·8 mM present initially in the protozoa as the result of the hydrolysis of engulfed starch grains. It is considered that there would be no net increase in the glucose concentration in the endoplasm due to 'active' uptake of glucose on incubation with say 50 mM-glucose in the presence of antibiotics, for the following reasons. Firstly, since the incorporation quoted above was the maximum at infinite glucose concentration, the actual incorporation would be lower. Secondly, as prolonged incubations must be done in the presence of penicillin + neomycin to prevent bacterial growth, the actual rate of glucose incorporation would be decreased under these conditions. Thirdly, any increase in glucose concentration in the pool would tend to decrease the rate of glucose formation from maltose. Even if the glucose concentration were to rise as the result of uptake of free glucose, the rate of glucose phosphorylation would not rise significantly, because at 14·8 μmoles glucose/ml. the activity of the hexokinase was 96% of that at infinite glucose concentration.

The finding that little ^{14}C from [^{14}C]glucose or [^{14}C]starch was incorporated into protozoal protein is of considerable importance in considering the over-all metabolism of the rumen. Bryant & Robinson (1963) showed that many pure strains of rumen bacteria utilized ammonia in preference to amino acids for growth, and when provided with amino acids as sole source of nitrogen incorporated little amino acid-carbon into cellular materials. In contrast, *Entodinium caudatum* incorporated the amino acids of bacterial protein directly into its protein and showed very limited ability to catabolize amino acids (Coleman, 1967*a*, *b*). This means that if *E. caudatum* is typical of all the Entodiniomorphid protozoa in the rumen, then these protozoa play no role in the synthesis of those 'essential' amino acids commonly present in low concentration in plant proteins.

I wish to thank Mr G. A. Embleton for inserting permanent rumen canulas into the sheep used to provide rumen fluid for the routine maintenance of the protozoa, the members of the Sub-Department of Chemical Microbiology, Biochemistry Department, University of Cambridge, for their helpful advice and criticism, and Miss B. C. Hanzl for valuable technical assistance.

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Numerical Taxonomy of *Listeria*, Streptococci and Possibly Related Bacteria

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SUMMARY

Similarity values based on 199 features of 88 strains of bacteria, including freshly-isolated strains of streptococci and named strains of the genera *Streptococcus*, *Listeria*, *Microbacterium*, *Erysipelothrix*, *Brevibacterium* and *Arthrobacter*, were analysed by single and complete linkage sorting programmes. It is concluded that *Erysipelothrix*, *Listeria* and strains currently known as *Microbacterium thermosphactum* show closer relations to the family Lactobacillaceae than to the other representatives of the family Corynebacteriaceae here examined.

INTRODUCTION

All information is grist to the taxonomic mill. The modern mill is the computer and as long as the data are relevant and comparative and the programming adequate, digestion of data can occur and synthesis of taxa follow. This is the basis of numerical taxonomy. As currently practised, it is essentially an extension of old-fashioned taxonomy whereby phenotypic data in large volumes are sorted impartially by mechanical means.

The aim of the present work was to see whether numerical analysis of data relating to a large number of phenotypic features could contribute anything new to the question of the generic relations of *Listeria*. This genus is currently classified in the family Corynebacteriaceae with the genera *Corynebacterium*, *Erysipelothrix*, *Microbacterium*, *Cellulomonas* and *Arthrobacter*. It is monospecific and the biological characters and significance of the species *Listeria monocytogenes* were reviewed by Gray & Killinger (1966), who pointed out that although various strain to strain differences have been reported, for practical purposes the species is divisible into four serological groups and 11 serotypes on somatic and flagellar antigens. Reports of antigenic cross-reactions between Listeria serotypes and various Gram-positive and Gram-negative bacteria, notably staphylococci and streptococci, which led to suggestions of intergeneric relations and complicated the serological diagnosis of Listeria infections, are now generally considered to result from the sharing of common antigens (see for example Neter, Anzai & Gorzyniski, 1960). These and other considerations led to the studies of Hartwigk (1958) and Robin & Magard (1960) in which cultural and physiological comparisons were made between *Listeria* and possibly related (or diagnostically confusing) bacteria such as streptococci (pyogenic and faecal), corynebacteria and *Erysipelothrix*. Relationship to the family Lactobacteriaceae has also been suggested on metabolic grounds (Miller & Silverman, 1959). In a preliminary study in this

laboratory on numerical classification of coryneform bacteria (Davis & Newton, 1969) a single strain of *Listeria monocytogenes* showed highest similarity to a group of strains isolated from beef stored at low temperature (Weidemann, 1965) and which closely resembled *Microbacterium thermosphactum* (McLean & Sulzbacher, 1953). In the present work some of these points have been re-examined and the opportunity taken to apply numerical analysis methods to a group of freshly-isolated streptococci from the upper respiratory tract of man.

METHODS

(*a*) *Strains examined*

Of the 88 strains used, 54 were obtained as named cultures and their details are shown in Table 1. The remaining 34 were isolated immediately prior to the study from nose, throat and saliva cultures on Mitis Salivarius agar (Oxoid CM 157), Azide Blood agar (Oxoid CM 259), Tween-carbonate agar (Burkwall & Hartman, 1964) and blood agar (Cruickshank, 1965). Colonies were picked at random, subcultured, purified and labelled '*Streptococcus*' sp. if they exhibited the characteristic morphology in Gram-stained smears. The following strain numbers were allotted to these presumptive streptococcal cultures: S1, S9, S10/1, S10/2, S12, S13, S14, S15, S18, S19, S24, S25, S27, S28, S30, S32, S39, S42, S46, S50, S52, S53, S55, S56, S59, S61, S62, S64, S65, S67, S75, S78, S80, S83.

(*b*) *Strain growth and maintenance*

All strains grew satisfactorily on the following TY base medium, Tryptone (Difco), 1% (w/v); yeast extract (Difco), 0·5% (w/v); sodium chloride, 0·5% (w/v); pH 7·2. Many strains grew better on TY medium plus glucose 0·2% (w/v) and K_2HPO_4, 0·5% (w/v), designated TYGP medium. For solid media 1·5% (w/v) Oxoid Agar No. 3 (Oxoid L 13) was added. Media were sterilized by autoclaving at 121° for 15 min. All strains were stored at 4° on slopes of Oxoid Blood agar base No. 2 enriched with 5% (v/v) sterile horse serum. In addition all strains were lyophilized.

Preliminary tests showed that the strains marked with an asterisk in Table 1 grew better at 28°, the remainder at 37°, and were grown at these temperatures unless stated otherwise.

(*c*) *Data collection*

1. Twenty strains, selected for their ability to grow at one of the specified growth temperatures (28° or 37°), were subcultured to TYGP broth and incubated 48 hr.

2. Broth cultures from 1 were streaked one per plate on TYGP agar and incubated 48 hr. After confirming colonial purity by using a stereomicroscope each strain was subcultured to TYGP broth and incubated 24 hr (see 3 below). The agar cultures were then used to record the following (numbers in parentheses indicate the number of features recorded): diameter of discrete colonies (3); margin character (3); consistency character (4); colonies adherent (1); colony elevation (2). Smears were stained by Gram's method, from these were recorded: Gram reaction (1); cocci present (1); regular or irregular rods (coryneforms) present (2); filaments or branching present (2); chain or palisade arrangement (2). Growth from the same cultures was tested for catalase activity by smearing on a clean slide and adding one drop of 10 vol. H_2O_2, and for oxidase activity by Kovacs's (1956) method; both activities were recorded as

Table 1. *Named strains and their sources*

Study no.	Name on receipt	Donor and number
334	*Listeria monocytogenes* (serotype 1)	Foley NCTC 7973
344	*Listeria monocytogenes* (serotype 1)	Killinger F5051
335	*Listeria monocytogenes* (serotype 2)	Foley NCTC 5348
336	*Listeria monocytogenes* (serotype 3)	Foley NCTC 5103
338	*Listeria monocytogenes* (serotype 4*a*)	Foley —
337	*Listeria monocytogenes* (serotype 4*b*)	Foley —
342	*Listeria monocytogenes* (serotype 4*b*)	Killinger 660
343	*Listeria monocytogenes* (serotype 4*b*)	Killinger 671
339	*Listeria monocytogenes* (untyped)	Clark —
340	*Listeria monocytogenes* (untyped)	Clark —
341	*Listeria monocytogenes* (untyped)	Hasman —
209	*Listeria monocytogenes* (untyped)	Crowder 2681
305	*Lactobacillus* sp. (heterofermentative)	Isolated locally from saliva
34	*Erysipelothrix insidiosa*	Simmons V535
208	*Erysipelothrix insidiosa*	Crowder 2682
259	*Erysipelothrix insidiosa*	Simmons V1851
271	*Erysipelothrix insidiosa*	Simmons V2556
*286	*Microbacterium thermosphactum*	Brownlie ATCC 11509
*58	*Microbacterium thermosphactum*	Brownlie SW 26
*278	*Microbacterium lacticum*	Dick —
*277	*Microbacterium lacticum*	Dick —
*276	*Microbacterium lacticum*	Dick —
*275	*Microbacterium lacticum*	Dick —
*10	*Microbacterium lacticum*	Szabo 275
*11	*Microbacterium lacticum*	Szabo 101
*59	*Microbacterium* sp.	Brownlie 9
*60	*Microbacterium* sp.	Brownlie 10
*61	*Microbacterium* sp.	Brownlie 11
*62	*Microbacterium* sp.	Brownlie 15
*28	*Kurthia zopfii*	Szabo 92
*29	*Kurthia zopfii*	Szabo 93
*22	*Arthrobacter globiformis*	Szabo 3
*282	*Brevibacterium linens*	Dick ATCC 9174
*281	*Brevibacterium linens*	Dick ATCC 9172
*12	*Brevibacterium linens*	Szabo 25
*13	*Brevibacterium linens*	Szabo 26
*14	*Brevibacterium helvolum*	Szabo 67
*91	*B. sociovivum*	Bhat 135
*92	*B. insectiphilium*	Bhat 146
*93	*B. ammoniagenes*	Bhat —
*94	*B. imperiale*	Bhat —
*2	*Cellulomonas flavigena*	Szabo 414
*96	*C. flavigena*	— NCIB 8073
*110	*C. biazotea*	— NCIB 8077
*3	*C. biazotea*	Szabo 49
S187	*Streptococcus anginosus*	Szabo 187
S199	*S. pyogenes*	Szabo 199
*S193	*S. lactis*	Szabo 193
*S192	*S. cremoris*	Szabo 192
S397	*S. salivarius*	Szabo 397
S179	*S. agalactiae*	Szabo 179
S185	*S. faecalis*	Szabo 185
S188	*S. mitis*	Szabo 188
S418	*Streptococcus* sp. (Lancefield group C)	Szabo 418

* Denotes strains grown at 28°; the rest at 37°.

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negative, weak or strong (4). The benzidine test of Deibel & Evans (1960) was then performed (1).

3. The following characters were recorded from 24 hr TYGP broth cultures (see 2 above): uniform turbidity (1); sediment (1); sediment granular, flocculent or mucoid (3). Hanging drop preparations were observed for motility (1). Gram-stained smears were observed for shape, cocci, regular or irregular rods, filaments, branching present (5); chain formation, usually over or under 30 cells/chain (2).

Unless otherwise stated the following tests, 4 to 6 strains per plate, were made on TY agar inoculated from TYGP broth cultures, and read after 6 days of incubation. Gelatin hydrolysis (1), (Frazier, 1926); deoxyribonuclease activity (1) (Jeffries, Holtman & Guse, 1957); casein hydrolysis and solution (2) and milk hydrolysis and solution (2) (Baird-Parker, 1963) (BDH light white soluble casein and defatted fresh milk were the substrates). Tyrosine hydrolysis (1) with formation of brown diffusible pigment on tyrosine (1) after 12 days, and xanthine hydrolysis (1), at 12 days (Gordon & Smith, 1955); clearing, opacity, pearly layer and copper sulphate reaction on egg-yolk agar (4), lecithinase activity (1), and tributyrin hydrolysis (1), (Willis, 1960); hydrolysis of Tween 20, 40, 60 and 80 (Sierra, 1957), weak or strong (8); oxidation of calcium lactate to carbonate (1), (Shimwell, Carr & Rhodes, 1960); salicylate hydrolysis (1), Tsukamura, 1965) after 12 days; alginate (1), and chitin hydrolysis (1), (Skerman, 1967) using BDH granular chitin as source of colloidal chitin, observed at 12 days; starch hydrolysis (1), (Davis & Park, 1962); growth under hydrogen (1) using Baird and Tatlock cold-catalyst anaerobic jars; sensitivity to penicillin, streptomycin, erythromycin, chloramphenicol, tetracycline, oleandomycin, novobiocin (7) on TYGP agar after 2 days with Oxoid Multodisk No. 11-14C; neutral red test (1), (Cann & Willox, 1965); phosphatase and arysulphatase activity (2), on TY agar plus 1 ml./100 ml. of 1% (w/v) filter-sterilized substrate solution, tested at 6 days by flooding growth with 15% (w/v) aqueous sodium carbonate. Growth with 0·0002, 0·0001 and 0·00005% (w/v) crystal violet (3); 0·1 and 0·5% (w/v) thallous acetate (2); 0·01, 0·05, 0·1 and 0·2% (w/v) sodium azide (4); 1% (w/v) sodium tauroglycocholate (1); 0·05 and 0·1% (v/v) Teepol (2), (BDH 610); 2, 4, 6 and 8% (w/v) sodium chloride (4); 0·01, 0·05 and 0·25% (w/v) potassium tellurite (3) (BDH Analar); 0·001, 0·005, 0·01 and 0·05% (w/v) triphenyl-tetrazolium-chloride (TTC) (4). Reduction of 0·01 and/or 0·05% (w/v) TTC (1); growth in 48 hr in the dark after exposure to a standard 15 watt U.V. tube at 24 in. for 1, 2, 3, 4 and 5 min. (5); growth and characters on Mitis salivarius agar (Oxoid CM 157) (5); growth on Tween-carbonate agar (Burkwall & Hartman, 1964) (1); growth and haemolysis on azide blood agar (Oxoid CM 259) (3).

4. Approximately 10 days after these tests fresh inocula were grown in TYGP broth for all except carbohydrate fermentation tests, which were inoculated with a cell suspension in phosphate buffered saline (Cruickshank, 1965). With a few exceptions, e.g. Hugh and Leifson test, Thornley's arginine, etc., tests were made in 2 to 3 ml. of substrate media in $3 \times \frac{1}{2}$ in. tubes, and unless otherwise indicated were read after 6 days.

Hippurate hydrolysis (1), (Hare & Colebrook, 1934); Stern's glycerol fuchsin test (1), (Cruickshank, 1965); aesculin hydrolysis (1), (Davis, 1955); gluconate oxidation (1), (Shaw & Clarke, 1955); final pH and acetoin production (Barritt, 1936) in 1% (w/v) glucose (5); acetoin from citrate, (1); liquefaction of inspissated (Loeffler's) serum (1); hydrogen sulphide formation in TY broth, TY plus 0·1% (w/v) sodium

thiosulphate and TY plus 0·05% (w/v) L-cysteine HCl, detected with lead acetate papers (3); nitrate and nitrite reduction (2), (ZoBell, 1932; Cowan & Steel, 1965); acetamide deamination (1), using medium and method of Bühlmann, Vischer & Bruhin (1961); urease activity (1) as for acetamide above with 1% (w/v) urea and 0·05% (w/v) glucose; ammonia from arginine (1) in TYGP broth plus 0·3% (w/v) L-arginine HCl after 3 days tested with Nessler's reagent; indole production (1) in TY broth by Ehrlich-Bohme reagent and ether; phenylalanine deamination (1), (Shaw & Clarke, 1955) after 12 days; Thornley's (1960) arginine test, modified (1); lysine decarboxylase (Carlquist, 1956, modified) (1); litmus milk reaction (4); 0·1% (w/v) methylene blue tolerance (1), tested by reduction in skimmed milk; tolerance of 60° for 30 min. in TYGP broth as shown by growth within 48 hr. (1); growth at 4° (12 days), 28°, 37° and 45° (4 days) (4); Hugh and Leifson test using Mossel's (1962) single tube method with Tryptone, 0·2% (w/v); yeast-extract, 0·5% (w/v); NaCl, 0·5% (w/v); glucose, 1% (w/v); agar 0·5% (w/v); pH 7·1 and bromothymol blue as indicator, read at 3 days (3); fermentation (acid production) in 0·5% (w/v) xylose, arabinose, fructose, galactose, lactose, maltose, sucrose, melibiose, trehalose, melezitose, raffinose, inulin, starch, salicin, mannitol, sorbitol, inositol, glucose, glycerol, cellobiose, amygdalin, after 3 and 6 days in ammonium sulphate, 2·64 g.; potassium dihydrogen phosphate, 0·5 g.; magnesium sulphate, 0·5 g.; yeast extract, 0·2 g., distilled water, 1000 ml.; pH 7·2 and bromocresol purple as indicator (cf. Tsukamura, 1966). Media for these sugar tests were sterilized by flash autoclaving at 121° and sugar-free controls were inoculated for each strain (43). Horse blood haemolysis around submerged colonies was recorded as beta, alpha or alpha prime (3) in Oxoid Blood agar base No. 2 plus 5% horse blood (Brown, 1919).

(*d*) *Duplication of data collection*

Approximately 20% of strains were run through the test procedure twice, in some cases to test the veracity of the results; in others strains were stored frozen in glycerol for several months before retesting; in others strains were retested at different incubation temperatures. Preliminary analyses with these duplicates showed that although differences in *S* values occurred they were usually minor. The following examples have been included in this report: 91A and 91B; 187 and 187D; these were straight duplications; 341X, 338X, 335X, 342X, 337X, 336X and 334X were the *Listeria* strains (see Table 1) of those numbers tested at 28° instead of 37°.

(*e*) *Data analysis*

Every character was coded as a simple plus or minus, with no effort to reduce weighting by non-additive coding of quantitative character states. The data were analysed using two programmes written for the G.E. 225 computer (University of Queensland Centre) by Miss E. Szabo of this department. Both programmes calculated the strain similarity values using the simple matching coefficient of Sokal & Sneath (1963) but differed in that one performed cluster analysis by the single linkage method and the other by the complete linkage method. The latter was stopped after 80 min. on the computer and had not achieved a complete sort in that time.

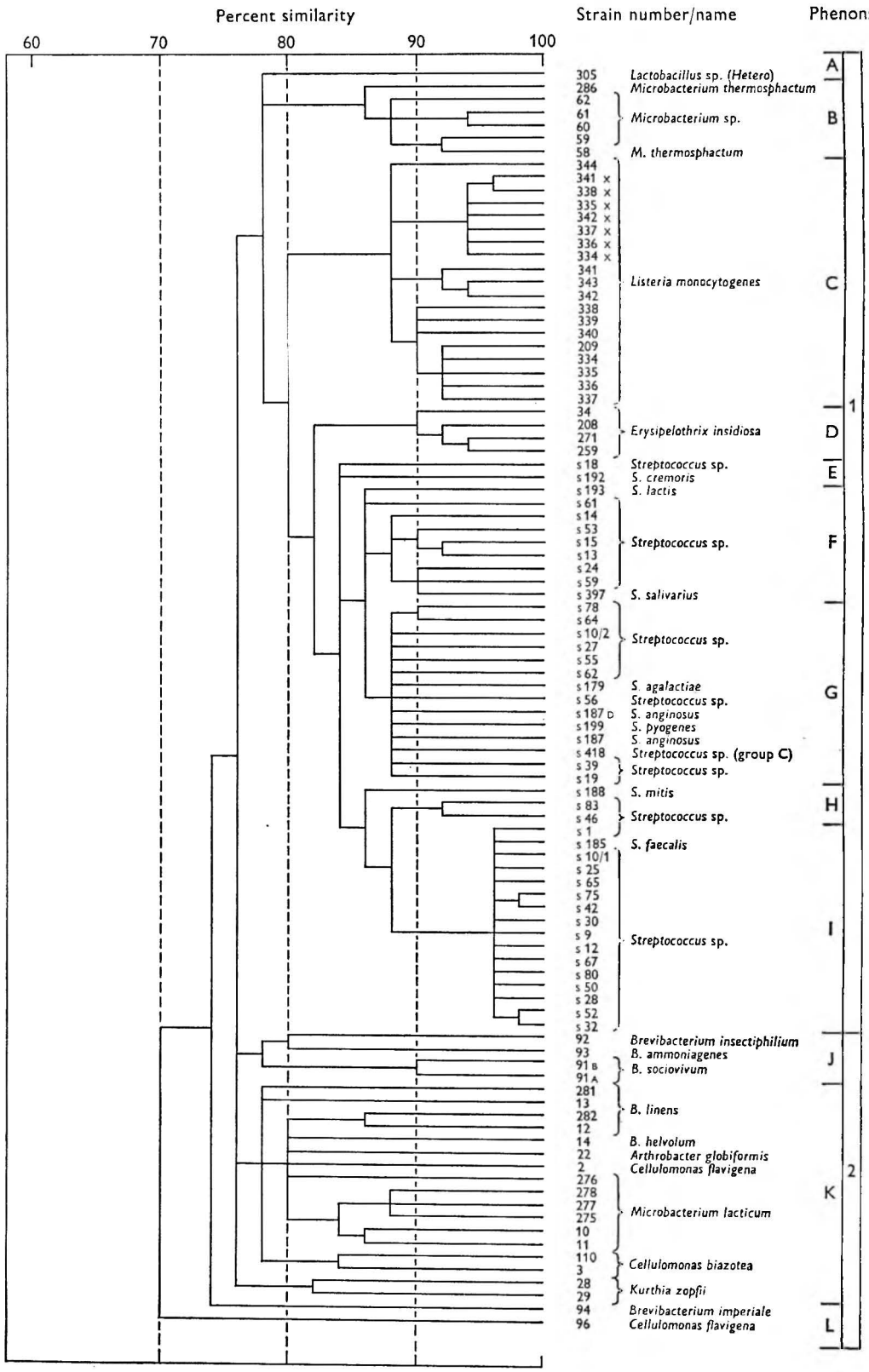

Fig. 1. Dendogram presentation of phenon formation by single linkage analysis.

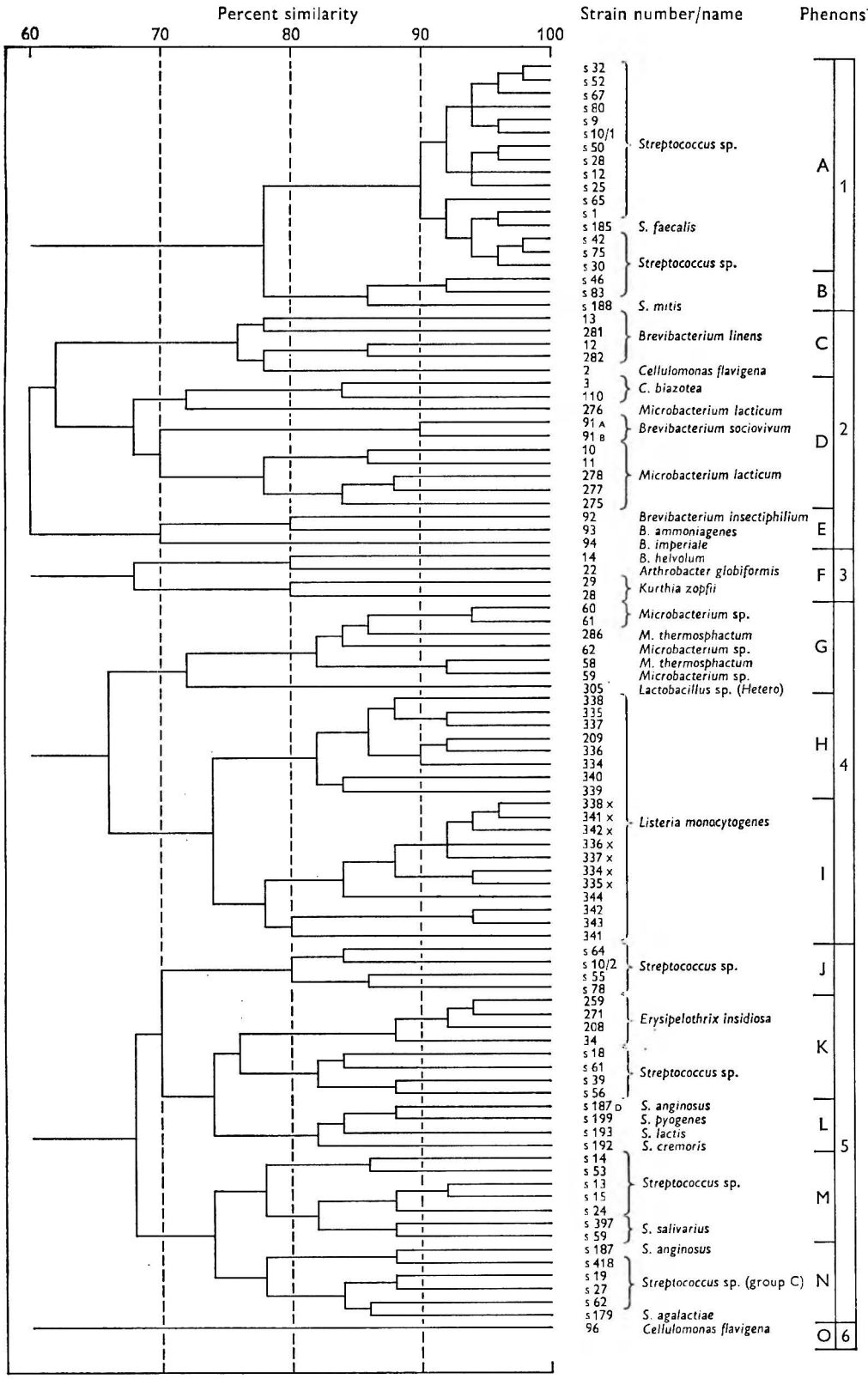

Fig. 2. Dendogram presentation of phenon formation by multiple linkage analysis.

RESULTS

Dendrograms showing cluster (phenon) formations by single and complete linkage analyses of the 97 operational taxonomic units (o.t.u.) (88 strains and 9 duplications) are shown in Fig. 1 and 2. Ignoring strain 96, which clustered poorly, single linkage analysis indicated two major phenons at the 78% *S* level. The smaller of these (phenon 2, Fig. 1) had strains labelled by five generic names, and apart from the separation of four o.t.u. in subphenon 2J little of significance can be said about them. Except for strains 2 and 96, strains bearing the same specific epithet clustered together but at relatively low % *S* levels. Duplicates of *Brevibacterium sociovivum* (91A and 91B) showed the highest similarity of any pair of o.t.u. in this phenon (90%).

Phenon 1 in Fig. 1 linked together the o.t.u. labelled *Listeria*, *Erysipelothrix*, *Streptococcus*, *Microbacterium* and the single strain of *Lactobacillus* which clustered only peripherally with this phenon. The four o.t.u. groups distinguished nomenclaturally as *Microbacterium*, *Listeria*, *Erysipelothrix* and *Streptococcus* strains formed four distinct subphenons. In the *Listeria* subphenon (1C, Fig. 1) the seven o.t.u. marked *X*, indicating that they were duplicates tested at 28°, clustered together and not with their respective duplicate o.t.u. tested at 37°. The *Streptococcus* o.t.u. were further divisible into three major and two minor clusters. The best defined of these contained 16 o.t.u. clustered at 96% *S* (phenon 1I, Fig. 1); it included the named strain of *S. faecalis* (S185) and is considered to represent that species. Although the three o.t.u. in subphenon 1H, Fig. 1 included the named strain of *S. mitis* (S188) the over-all characters of the cluster indicated that it probably represented *S. faecium*. Cluster 1G included the five o.t.u. labelled as members of the 'pyogenic' section of the genus *Streptococcus* plus nine fresh isolates and was tentatively recognized as representing the 'pyogenic streptococci'. Similarly cluster 1F was tentatively considered to include seven o.t.u. (S14, 53, 15, 13, 24, 59, 397) which could be called 'viridans streptococci' and two o.t.u. (S193 and 61) that clustered peripherally with them. These two clusters (1G and 1F) both formed at the 88% *S* level, and fused to form a single cluster at the 86% *S* level. This was the same *S* level at which clusters 1I and 1H, representing the enterococci, fused to form a single cluster. The remaining two streptococcal o.u.t. (S18 and 192) joined clusters 1F, G, H and I at the 84% *S* level to form the complete *Streptococcus* cluster at that level. The four *Erysipelothrix* o.t.u. (cluster 1D) linked with the streptococci at the 82% *S* level, followed by the *Listeria* cluster at 80% *S* and the *Microbacterium thermosphactum* and *Lactobacillus* o.t.u. at 78% *S*.

In the complete linkage analysis (Fig. 2) five distinct clusters are discernible. Phenon 1, with its two subclusters A and B, is exactly equivalent to the enterococcus clusters in the single linkage analysis (1I and H, Fig. 1). We assume that the apparent separation of this streptococcal cluster from the rest of the streptococci in this figure is an artifact caused by the incomplete nature of the complete linkage analysis (see Methods, section e). Phenon 4G in Fig. 2 represents the *Microbacterium thermosphactum*/*Lactobacillus* cluster seen in Fig. 1 as phenons 1A and B. Similarly phenons 4H and I in Fig. 2 are equivalent to phenon 1C in Fig. 1, with the separation of o.t.u. 338, 335, 337, 209, 336, 334, 340 and 339 (phenon 4H, Fig. 2) from the remaining *Listeria* o.t.u. rendered more apparent. Phenons 2 and 3 in Fig. 2 embrace 22 of the 23 o.t.u. clustered in phenon 2 in Fig. 1 but with considerable resorting of o.t.u. in the

various subclusters. The strains of *Brevibacterium helvolum* (14) and *Arthrobacter globiformis* (22) were brought together with the two strains of *Kurthia zopfii* (28 and 29) to form a distinct phenon; the four strains of *B. linens* clustered more compactly as also did the three *Brevibacterium* strains numbered 92, 93 and 94. Subphenon 2D in Fig. 2 included six strains of *Microbacterium lacticum*, two duplicate o.t.u. labelled *B. sociovivum* and two labelled *Cellulomonas biazotea*. Phenon 5 in Fig. 2 also showed some resorting of o.t.u. when compared with the equivalent phenons in Fig. 1. Thus, the four *Erysipelothrix* o.t.u. were more intimately clustered with the streptococci (5K, Fig. 2) with streptococcal strains S61 and S39 being moved from their single linkage clusters to a new association. Strains S18 and S192 (1E in Fig. 1) were redistributed. The seven o.t.u. considered as viridans streptococci in phenon 1F, Fig. 1 were more clearly demarcated (5M, Fig. 2) and the 14 o.t.u. of phenon 1G in Fig. 1 (pyogenic streptococci) were resorted rather drastically into phenons 5J, K, L and N in Fig. 2.

It was noticeable that in neither analysis were the two strains labelled *Streptococcus lactis* (S193) and *S. cremoris* (S192) clearly separated as a distinct phenon.

DISCUSSION

In general the results confirmed that the *Brevibacterium/Arthrobacter/Cellulomonas/Microbacterium lacticum* strains formed a related if heterogeneous phenon, as reported by Davis & Newton (1969). The inclusion of *Kurthia* strains in this general group differs from our earlier findings, in which closer relationship with *Listeria* was suggested. By single linkage analysis the remaining o.t.u. were all related in varying degrees. The six o.t.u. labelled *Microbacterium* sp. or *Microbacterium thermosphactum* showed greater similarity to the single *Lactobacillus* strain used than to the *Listeria* o.t.u. Apart from the tight clustering of the *Listeria* o.t.u. tested at 28°, possibly due to phenotypic similarities under the test conditions, single linkage analysis did not indicate any useful subdivision of the *Listeria* o.t.u., but in the complete linkage analysis two phenons were distinguished within this group of o.t.u. Gray & Killinger (1966) refer to a number of reports suggesting biochemical, physiological and morphological (rough/smooth) subdivision of strains of *Listeria*, but we were not able to connect the division shown in Fig. 2 into phenons 4H and 4I with these reports or with the serological data available for our o.t.u. We suspect that the division may be an artifact because the o.t.u. in phenon 4H were tested early in the study while 10 of the o.t.u. in phenon 4I were tested in two later groups. However o.t.u. 341 in phenon 4J was tested at the same time as those in 4H but did not cluster with them.

Having introduced the possibility that phenon formation in numerical analysis may be influenced by factors such as the ones just mentioned we can sympathize with those who have decried numerical taxonomy as tedious, indirect and inaccurate. As it is currently practised numerical taxonomy consists of the subjective interpretation of objective analyses of subjective data. By far the weakest links in the chain occur in the data collection area and such weaknesses are only rarely referred to in published work, e.g. Focht & Lockhart (1965); Goodfellow (1967). To take Goodfellow's example, growth at optimal conditions can only be established by basic research and is a near impossible criterion to attempt. In studies on diverse bacteria the best that can be done is to limit arbitrarily the known variations in o.t.u. comparison as, for example,

Table 2. *Distribution of characters showing 100% positive results in complete linkage phenons (cf. Fig. 2)*

Phenons	1A	1B	2C	2D	2E	3F	4G	4H	4I	5J	5K	5L	5M	5N
No. of strains	16	3	5	10	3	4	7	8	11	4	8	4	7	6
Colony under 1 mm.	−	−	−	+	−	−	−	−	−	+	−	−	−	−
Colony over 2 mm.	−	−	+	−	−	−	−	−	−	−	−	−	−	−
Margin entire	+	+	−	+	+	−	−	+	+	+	−	+	−	−
Colony butyrous	+	+	−	−	−	−	−	+	−	+	−	+	−	−
Colony viscous	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Colony corvex	+	+	+	−	+	−	−	+	+	+	−	+	−	−
Colony flat	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Gram positive	+	+	+	+	−	+	+	+	+	+	+	+	+	+
An agar:														
Cocci formed	+	+	−	−	−	−	−	−	−	+	−	+	+	+
Regular rods	−	−	+	−	−	+	+	−	+	−	−	−	−	−
Irregular rods	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Chains	−	−	−	−	−	−	−	−	−	+	−	−	−	+
Pallisades	−	−	−	−	−	−	−	+	+	−	−	−	−	−
In broth:														
Cocci formed	+	+	−	−	−	−	−	−	−	+	−	+	+	+
Regular rods	−	−	−	−	+	+	+	+	+	−	−	−	−	−
Irregular rods	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Catalase strong	−	−	+	+	+	−	−	+	+	−	−	−	−	−
Benzidine reaction	−	−	+	−	+	+	−	−	−	−	−	−	−	−
Gelatin	+	−	+	−	−	−	−	−	−	+	−	−	−	−
DNA	−	−	−	−	−	−	−	−	−	+	−	−	−	−
Casein hydrolysis	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Tyrosine cleared	−	−	+	−	+	−	−	−	−	−	−	−	−	−
Egg yolk cleared	−	−	+	−	−	−	−	−	−	−	−	−	−	−
Egg yolk opaque	−	−	−	−	−	−	−	+	−	−	−	−	−	−
Trybutyrin	−	+	+	−	−	−	−	+	+	−	−	−	−	−
Tween 20	−	−	−	−	−	+	−	−	−	−	−	−	−	−
Tween 40	−	−	−	−	−	−	−	+	+	−	−	−	−	−
Tween 60	−	−	−	−	−	−	−	+	+	−	−	−	−	−
Starch	−	−	−	+	+	−	−	−	−	−	−	−	−	−
Anaerobic growth	+	+	−	−	−	−	+	+	+	+	+	+	+	+
Phosphatase	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Arylsulphatase	−	−	−	−	−	−	−	+	−	−	−	−	−	−
Tolerate 0·0002% crystal violet	+	+	−	−	−	−	−	−	−	+	+	+	+	+
Tolerate 0·5% thallous acetate	+	+	−	−	−	−	+	+	+	+	+	+	+	+
Tolerate 0·1% sodium azide	+	+	−	−	−	−	−	−	−	+	+	−	+	+
Tolerate 0·2% sodium azide	+	+	−	−	−	−	−	−	−	−	−	−	−	−
Tolerate 0·05% Teepol	+	+	−	−	−	−	−	−	+	−	−	−	−	−
Tolerate 8% NaCl	+	+	+	−	+	−	−	+	+	−	−	−	−	−
Tolerate 0·05% potassium tellurite	+	+	−	−	+	−	−	−	+	−	+	+	+	−
Reduce triphenyl tetrazolium chloride	+	+	−	−	+	−	−	−	−	−	−	+	−	−
Tolerate u.v. 5 mins.	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Grow on mitissalivarius agar	+	+	+	−	−	+	−	+	+	+	+	+	+	+
Gum drop colony on mitissalivarius agar	−	−	+	−	−	−	−	−	−	−	−	−	+	−
Grow on Tween-carbonate agar	−	+	−	−	−	−	−	−	−	−	−	−	−	−

Table 2 (*cont.*)

Phenons	1A	1B	2C	2D	2E	2F	4G	4H	4I	5J	5K	5L	5M	5N
No. of strains	16	3	5	10	3	4	7	8	11	4	8	4	7	6
Grow on azide-blood agar	+	+	+	+	+	−	−	+	+	+	+	+	+	+
Hippurate	−	+	−	−	+	−	−	+	+	−	−	−	−	−
Aesculin	+	+	−	−	+	−	+	+	+	−	−	−	+	−
Gluconate	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Acetoin from glucose	+	+	−	−	−	−	−	−	−	−	−	−	−	−
Serum hydrolysis	+	−	−	−	−	−	−	−	−	−	−	−	−	−
H_2S from cysteine	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Acetamide	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Ammonia from arginine	+	+	−	−	−	−	−	−	−	+	−	−	−	−
Thornley's arginine test	+	+	−	−	−	−	−	−	−	+	+	−	−	−
Methylene blue tolerance	+	+	−	−	+	−	−	−	−	−	−	−	−	−
60° 30 min. tolerance	+	+	−	−	+	−	+	+	−	−	−	−	−	−
Growth at 28°	+	+	+	+	+	+	+	+	+	+	−	+	+	−
Growth at 37°	+	+	−	−	+	−	−	+	+	+	+	+	+	+
Growth at 45°	+	+	−	−	−	−	−	−	−	−	−	−	−	−
Oxidise glucose	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Ferment glucose	+	+	−	−	−	−	+	+	+	+	−	−	+	−
Arabinose acid	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Fructose acid	+	+	−	−	+	−	−	−	+	−	−	−	−	−
Galactose acid	+	+	−	−	−	−	−	−	−	−	−	−	+	−
Lactose acid	+	+	−	−	−	−	−	−	−	−	−	−	+	−
Maltose acid	+	+	−	−	−	−	+	−	+	−	−	−	+	−
Sucrose acid	+	+	−	−	−	−	+	−	−	−	−	−	+	−
Trehalose acid	+	+	−	−	−	−	+	−	+	−	−	−	-	−
Melezitose acid	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Salicin acid	+	+	−	−	−	−	+	+	+	−	−	−	−	−
Mannitol acid	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Sorbitol acid	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Inositol acid	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Glucose acid	+	+	−	−	−	−	+	+	+	−	−	−	+	−
Cellobiose acid	+	+	−	−	−	−	−	+	+	−	−	−	+	−
Amygdalin acid	+	+	−	−	−	−	+	−	+	−	−	−	−	−
Beta haemolysis	−	−	−	−	−	−	−	+	+	−	−	−	−	−
Alpha haemolysis	−	−	−	−	−	−	−	−	−	+	−	−	−	−

Key: + = all o.t.u. positive; − = one or more o.t.u. negative.

to normal growth temperature. Without this certain o.t.u. would not have grown. We recognized that the conditions were certainly not optimal (even for growth) of some strains such as *Erysipelothrix*, and may have influenced the formation of the phenon labelled 5K in Fig. 2, which is an obvious hybrid. The problem of timing tests is another example of the difficulties faced. For purely practical reasons we prefer to test a few strains by all the tests at one time rather than test all the strains in a few tests. Each alternative has its disadvantages; in the former there is danger of unconscious test variation and in the latter of undetected strain variation. To use a mixture of these alternatives has the disadvantages of both and arrangements should be designed to avoid it.

Other problems of data collection include the need to select a wide range of characters without undue weight on any one aspect and without duplication of characters. The data recorded included 34 features based on morphology, 113 on enzymatic reactions, and 52 on tolerance and growth characters. In retrospect the data

on chemical tolerance may seem to be unduly weighted in favour of the enterococci and also, in view of the inclusion of negative matches in the similarity calculation, may have led to the confusion of phenons in the viridans/pyogenic/lactic streptococci. Feature duplication can be a subtle problem; for example haemolysis may relate to phospholipase activity, tolerance and growth characters can reflect enzymic characters such as catalase activity. In dealing with diverse groups of bacteria there is the problem of over-all relevance of features; it seems likely that some form of staged analysis will be needed to allow preliminary sorting but it will require to be done carefully if the benefits of numerical taxonomy are to be retained.

Gray & Killinger (1966) comment on the relationship between *Listeria* and *Erysipelothrix*; we agree that for diagnostic purposes it is a relatively simple matter to differentiate these genera (e.g. we have confirmed the report of Fuzi (1963) that *Erysipelothrix* tolerates up to 100 μg./ml. of neomycin whereas *Listeria* is inhibited by *c.* 2 μg./ml.) but our results indicate a relationship between these genera, and their retention in the same family is considered valid. *Erysipelothrix* showed closer similarity to the streptococci than did *Listeria* and its inclusion into an otherwise streptococcal phenon (5K) in Fig. 2 is of interest (but see above). Preliminary attempts to analyse the cell walls of the *Listeria* and *Erysipelothrix* strains used were hampered by difficulties in preparing clean samples for hydrolysis; in particular, trypsin as a cleansing agent appeared to cause gross destruction of the *Listeria* cell wall fractions, and may be connected with the tryptic activation of autolysis in *Streptococcus faecalis* reported by Shockman, Pooley & Thompson (1967). In both *Listeria* and *Erysipelothrix* our results indicated the presence of lysine, DL/DD diaminopimelic acid, aspartic acid and glycine and agreed with Tinelli's (1966) results for *Listeria*. Keeler & Gray (1960) did not detect lysine or glycine in *Listeria*. Diaminopimelic acid (DAP) does not occur in streptococci. We found that two strains of *Microbacterium thermosphactum* possessed DL/DD DAP but no lysine. The occurrence of DL/DD DAP in these bacteria and their other characters suggests possible relationship with *Lactobacillus plantarum* and/or *Corynebacterium*. Against this can be set a reported % GC ratio of 38 for *Listeria*, which is essentially the same as that found in most streptococci (Hill, 1966). Clearly the problem of relationships between these genera is not yet solved.

Sherman (1937) recognized four species groups in the genus *Streptococcus*. In a numerical analysis Seyfreid (1968) successfully differentiated named strains of enterococci, lactic and pyogenic streptococci into three phenons; viridans streptococci were not included. In our analysis we consider phenons 1A and B in Fig. 2 to represent the 'enterococci' of Hartman, Reinbold & Saraswat (1966) with the larger phenon (1A) as *S. faecalis* and the smaller (1B) as *S. faecium*. Differentiation of the remaining streptococci is much less easy, except that phenon 5M, Fig. 2 represents *S. salivarius*. In general it appears that the more stringent analysis (Fig. 2) confuses rather than clarifies the groupings in phenons 1F and 1G of Fig. 1. Our failure to separate the two cultures labelled *S. cremoris* and *S. lactis* is difficult to explain, especially as these strains were tested at 28° while the other streptococci were tested at 37°; previous reports of aberrant lactic streptococci (if such they are) tend to reclassify them in the enterococcus group (see Deibel, Lake & Niven, 1963; Deibel & Silliker, 1963), not with the pyogenic streptococci as the clustering in Fig. 2 suggests.

We have constructed Table 2 for those who, like us, regret the absence of data on actual features of the bacteria studied in published numerical analyses. This was done

by the simple sorting of the IBM cards bearing o.t.u. data into the phenons shown in Fig. 2. The alligned batches of cards were then viewed against a light and results for which all o.t.u. were positive (i.e. punched out) recorded by plus signs. We emphasize that the negative symbols mean only that not all the o.t.u. of the particular phenon

Table 3. *Manual analysis of 41 streptococcal strains based on serology and eight physiological characters*

Strain (study no.)	Lancefield group	Tolerance of 0·05% Teepol	Polysaccharide from sucrose	Tolerance of 0·1% methylene blue	Aesculin hydrolysis	Beta haemolysis	Tolerance of 8% NaCl	Ammonia from arginine	Acid from arabinose	Fig. 2 phenon	Fig. 1 phenon	
s1	D	+	−	+	+	−	+	+	−	1A	1I	Enterococcus (*S. faecalis*)
S9	D	+	−	+	+	−	+	+	−	1A	1I	
s10/1	D	+	−	+	+	−	+	+	−	1A	1I	
s12	D	+	−	+	+	−	+	+	−	1A	1I	
s25	D	+	−	+	+	−	+	+	−	1A	1I	
s28	D	+	−	+	+	−	+	+	−	1A	1I	
s30	D	+	−	+	+	−	+	+	−	1A	1I	
s42	D	+	−	+	+	−	+	+	−	1A	1I	
s50	D	+	−	+	+	−	+	+	−	1A	1I	
s52	D	+	−	+	+	−	+	+	−	1A	1I	
s65	D	+	−	+	+	−	+	+	−	1A	1I	
s67	D	+	−	+	+	−	+	+	−	1A	1I	
s75	D	+	−	+	+	−	+	+	−	1A	1I	
s80	D	+	−	+	+	−	+	+	−	1A	1I	
s185	D	+	−	+	+	−	+	+	−	1A	1I	
s46	D	+	−	+	+	−	+	+	+	1B	1H	Enterococcus (*S. faecium*)
s83	D	+	−	+	+	−	+	+	+	1B	1H	
s188	N.T.	+	−	+	+	−	+	+	+	1B	1H	
s55	D	−	−	+	+	−	−	+	−	5J	1G	Enterococcus (?)
s397	−	−	−	+	±	+	−	−	−	5M	1F	Viridans (*S. salivarius*)
s15	−	−	−	+	±	+	−	−	−	5M	1F	
s53	−	−	−	+	−	+	−	−	−	5M	1F	
s59	−	−	−	+	−	+	−	−	−	5M	1F	
s24	−	−	−	+	−	+	−	−	−	5M	1F	
s13	−	−	−	+	−	+	−	−	−	5M	1F	
s61	−	−	−	+	−	+	−	−	−	5K	1F	
s10/2	−	−	−	−	−	−	−	+	−	5J	1G	Viridans (?) Pyogenic (?)
s19	−	−	−	−	−	−	−	+	−	5N	1G	
s27	−	−	−	−	±	−	−	+	−	5N	1G	
s62	−	−	−	−	−	−	−	+	−	5N	1G	
s64	−	−	−	−	+	−	−	+	−	5J	1G	
s78	−	−	−	−	−	−	−	+	−	5J	1G	
s18	−	−	−	−	−	+	+	+	−	5K	1E	Pyogenic
s418	C	−	−	−	−	+	−	+	−	5N	1G	
s39	C	−	−	−	−	+	−	+	−	5K	1G	
s187	G	−	−	−	−	+	−	+	−	5N	1G	
s56	G	−	−	−	±	N.T.	−	+	−	5K	1G	
s199	A	−	−	−	−	+	−	±	−	5L	1G	
s179	B	−	−	−	−	+	−	+	−	5N	1G	
s192	N.T.	−	−	+	+	+	−	−	−	5L	1E	Lactic
s193	N.T.	−	−	+	+	−	−	+	−	5L	1F	

Key: N.T. = not tested; − = negative; + = positive; ± = weak positive.

were positive for that feature, they do not necessarily mean that all members of a phenon were negative. This table allows the more obvious features of the phenons to be recognized and in our opinion brings out some interesting details.

Table 3 shows the results of simple manual classification of 41 of the streptococcal o.t.u. based upon eight physiological characters plus Lancefield serological grouping, using antisera A, B, C, D and G; negative signs in Table 3 do not necessarily mean that group antigen was not present in those strains. The physiological tests selected for this analysis resemble some of the characters commonly used in 'diagnostic' classifications of streptococci, e.g. Cowan & Steel (1965); Sharpe & Fryer (1966). This weighted type of classification appears to achieve its purpose quite well. The groupings produced correlate better with the phenons formed by single linkage analysis of mass data (Fig. 1) than with those produced by complete linkage analysis (Fig. 2), especially in the lower half of the table (strain S10/2 to S179). The two strains labelled *S. lactis* and *S. cremoris* (S192 and S193) are differentiated better in Table 3 than in either of the computer analyses. Strain S55 may represent an atypical enterococcus similar to those described by Kenner, Clark & Kabler (1960) although by computer analysis it appears to be more similar to the pyogenic streptococci.

In conclusion, we consider our results indicate that *Erysipelothrix*, *Listeria* and *Microbacterium thermosphactum* show closer phenotypical relations to the family Lactobacilleceae than they do to certain members of the family Corynebacteriaceae, e.g. *Arthrobacter*. Subdivision of the *Listeria* strains used was not achieved. Separation of the enterococci from the remaining streptococococci appeared to be relatively simple when compared with the more difficult task of separating the viridans streptococci (other than *S. salivarius*) from the pyogenic group.

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Numerical Taxonomy of Genera *Micrococcus* Cohn and *Sarcina* Goodsir

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SUMMARY

A group of 62 strains, mostly members of the genera *Micrococcus* and *Sarcina*, has been subjected to a numerical–taxonomic analysis. All 57 characters have been scored according to the scale 0 to 5 and an appropriate formula has been used for the calculation of similarity. The method of clustering of organisms applied here is a modification of the original 'single-linkage method'. Eleven 85-phenons were found and the rank of species has been attributed to them. On the basis of existing taxonomic studies it appears probable that aerobic sarcinas must be considered as members of the genus *Micrococcus* and that they are R-dissociated forms of proteolytic micrococci.

INTRODUCTION

The extensive literature existing today on the taxonomy of micrococci includes such comprehensive papers as those by Abd-el-Malek & Gibson (1948), Shaw, Stitt & Cowan (1951), Kocur & Martinec (1962), Baird-Parker (1963, 1965). The numerical taxonomy introduced in the classification of bacteria by Sneath (1957*a*, *b*) and broadly used in the present taxonomy of micro-organisms, was for the first time applied to micrococci by Hill (1959) and later by Pohja (1960), Pohja & Gyllenberg (1962), Hubálek (1964), Hill *et al.* (1965). The results obtained in the application of numerical taxonomy were compared with the ratio of DNA bases in this group of bacteria by Silvestri & Hill (1965) and Rosypal, Rosypalová & Hořejš (1966). This communication is based on an earlier study (Hubálek, 1964) and applies the modified Sneath's method to non-pathogenic micrococci and sarcinas.

METHODS

A total of 62 strains of the genera *Micrococcus* Cohn, *Sarcina* Goodsir, and *Staphylococcus* Rosenbach from the Czechoslovak Collection of Micro-organisms (CCM) have been studied and a list of them is presented in Table 1. All the tests were performed two or three times and in the same way as by Kocur & Martinec (1962) unless otherwise indicated. The cultivation temperature was 30° and both a 'positive' culture and a negative control in each test were used for comparison. A complete description of the methods used has been given by Hubálek (1964).

Characters

The final set of 57 characters does not comprise 'unsuitable' ones, i.e. those which in all strains were negative (e.g. acetoin and indole production, benzoate utilization, phenylalanine decomposition and presence of lecithinase), inconstant or difficult to classify (milk decomposition, colony profile and margin).

All characters have been evaluated according to the scoring scale of 0 to 5 with the degrees 0, 1, 2, 3, 4, 5 (using intergrades of 0·5) corresponding to the increasing intensity of character manifestation. The degree 0 represents the absence of a character, the degree 5 its highest intensity. The same scoring system has been used by Poncet (1967).

Table 1. *Test organisms*

Original names	CCM numbers
Micrococcus citreus	105, 642, 644, 683 (ATCC 9510), 848
M. cyaneus	852
M. epidermidis	253, 257
M. flavus	210 (ATCC 400), 291, 531, 546, 555 (ATCC 10240 a), 617, 732 (ATCC 10240), 819, 1424 (NCTC 7743), 1681
M. luteus	294, 810 (ATCC 398), 824
M. lysodeikticus	169 (NCTC 2665), 276, 530, 550, 656, 1335, 1423 (ATCC 12698), 1680
M. sodonensis	144 (ATCC 11880)
Micrococcus sp.	46, 52, 80, 134, 135, 183, 661,
Sarcina citrea	248, 631
S. flava	163, 166, 296, 308, 335, 351 (ATCC 540), 410 (ATCC 272)
S. lutea	149, 249, 310, 336, 337 (ATCC 382), 357, 362, 407, 443, 523, 536
S. marginata	265
S. subflava	209
S. variabilis	266
Staphylococcus citreus	688
S. flavocyaneus	247 (NCTC 7011, ATCC 8676)

Survey of characters

1. *Gram reaction.* The preparations were made from meat-peptone agar (MPA) and meat-peptone broth (MPB) from 20-, 45- and 115-hr-old cultures. From each strain six preparations were made, stained, and the Gram reaction has been calculated as the arithmetic mean of all these values and rounded to the nearest degree of the scoring scale.

Scoring: 0, typically Gram-negative; 1, Gram-negative; 2, Gram-variable, tending to Gram-negative; 3, Gram-variable, tending to Gram-positive; 4, Gram-positive; 5, strongly Gram-positive.

2. *Occurrence of packets in preparations from MPA.* Average value of three wet-mount preparations (1-, 2- and 5-day-old cultures).

Scoring: 0, typical grape-like clusters; 1, less typical grape-like clusters; 2, small clusters, less abundant tetrads and also short chains; 3, prevailing tetrads; 4, typical tetrads, less abundant octads; 5, packets of minimal number of 16/32 cells.

3. *Occurrence of packets in preparations from MPB.* Scoring as character 2. The score was different in some strains from that in character 2, therefore the character has been included.

4. '*Membranes*'. In preparations of some strains (CCM 294, 824, 848) membranes

surrounding several cocci have been found. Scoring 0 to 5 according to occurrence frequency of this structure, by mutual comparison of the strains.

5. '*Compressed tetrads*'. The cells of some strains (CCM 253, 257, 247, 852) appeared constantly to be just dividing into tetrads; the whole grouping looked like a very 'compressed' tetrad. Scoring 0 to 5, analogically as character 4.

6. *Turbidity in MPB.* Estimated at the 1st, 2nd and 5th day, from these values the arithmetic mean has been calculated and rounded to the nearest degree of the scoring scale.

Scoring: 0, none; 1, weak; 2, rather weak; 3, moderate; 4, dense; 5, very dense.

7. *Conglomerates in MPB.* In liquid medium typical sarcinas produce small conglomerates, often visible by naked eye. Evaluation was made on the 5th day by means of a $\times 6$ hand lens.

Scoring: 0, homogenous turbidity; 1, very fine conglomerates hardly discernible by lens; 2, rather fine conglomerates visible only by lens; 3, moderately large conglomerates scarcely visible by naked eye; 4, large conglomerates visible by naked eye; 5, very conspicuous conglomerates easily visible by naked eye.

8. *Ring pellicle in MPB.* The highest value attained within 5 days.

Scoring: 0, none; 1, a trace on the wall of the test-tube; 2, slight, disconnected; 3, medium, uninterrupted or disconnected; 4, strong, consistent; 5, very strong.

9. *Intensity of sediment in MPB.* Average value from data on the 1st, 2nd and 5th day.

Scoring: 0, slight or none; 1, weak; 2, rather weak; 3, medium; 4, strong; 5, very strong.

10. *Character of sediment in MPB.* Evaluated on the 5th day according to the following scheme:

Presence of viticula	Dispersion of sediment on shaking	Appearance and character of sediment	Scoring
±	−	Mucous	0
+	−	Viscid	1
+	±	Viscid	2
±	+	Slightly granular	3
−	+	Granular	4
−	++	Strongly granular	5

Graded response: ++, +, ±, −.

The term 'viticula' designates a corkscrew-like formation of sediment created on the bottom of the test-tube after careful rotatory shaking.

11. *Transparency of colony.* From 3-day cultures on MPA.

Scoring: 0, very opaque; 1, opaque; 2, slightly translucent; 3, translucent; 4, slightly transparent; 5, clearly transparent.

12. *Surface roughness of colony.* The data are average values obtained after 5-day cultivation on MPA and glucose yeast-extract agar.

Scoring: 0, surface mucous, very glossy; 1, glossy; 2, less glossy; 3, matt; 4, strikingly matt to granular; 5, strongly granular.

13. *Yellow pigmentation of colony.* Inoculated MPA slants were incubated for 2 days in thermostat at 30° and then kept at room temperature in diffuse light for 5 days. The evaluation is the average value obtained from several repeated observations.

Scoring: 0, colourless; 1, white, whitish or greyish; 2, slightly yellowish; 3, pale lemon; 4, lemon to yolk yellow; 5, pure yolk yellow.

14. *The highest grade of yellow pigment.* Seen on a number of solid media. In several strains the pigmentation on media such as glucose yeast-extract agar or starch agar was deeper than on the MPA. Scoring as character 13.

15. *Orange pigment on MPA.* Scoring 0 to 5 according to intensity. Estimated by mutual comparison of all the cultures.

16. *Brown exopigment on MPA.* Scoring as for character 15.

17. *Violet exopigment on glucose yeast-extract agar.* Scoring as for character 15.

18. *Colony diameter on MPA.* An inoculum from a streak culture was inoculated onto three spots on one 15 ml. MPA plate in a 10 cm. Petri dish; after 48-hr cultivation the diameter of the three colonies was measured and the arithmetic mean of these values calculated for each strain.

Scoring: 0, no growth, never seen; 1, diam. 1 mm.; 2, diam. 2 mm.; 3, diam. 3 mm.; 4, diam. 4 mm.; 5, diam. 5 and more mm.

19. *Optimal temperature.* Determined on MPA.

Scoring: 0, about 18 to 20°; 1, about 21 to 23°; 2, about 24 to 27°; 3, about 28 to 31°; 4, about 32 to 35°; 5, about 36° and more.

20. *NaCl tolerance.* Determined on MPA with various concentrations of salt.

Scoring	5% NaCl	7·5% NaCl	10% NaCl	12·5% NaCl
0	−	−	−	−
1	±	−	−	−
2	+	±	−	−
3	+	+	±	−
4	+	+	+	±
5	+	+	+	+

Legend: +, growth; ±, weak growth; −, no growth.

21. *Aesculin hydrolysis.*

Scoring: 0, medium without colour change; 1, a trace of brown coloration within 25 days; 2, brown coloration within 14 days; 3, black coloration in 6 to 14 days; 4, black coloration in 2 to 5 days; 5, black coloration within 1 day.

22. *Starch hydrolysis.* After 5-day cultivation the breadth of zone with degraded starch was measured.

Scoring: 0, no zone; 1, zone 0·5–1·0 mm.; 2, zone 1·5–2·5 mm.; 3, zone 3·0–4·5 mm.; 4, zone 5·0–10·5 mm.; 5, zone 11·0 mm. and more.

23. *Glucose 'oxidation'* (formation of acids in Hugh–Leifson medium with glucose and bromthymol-blue).

Scoring: 0, without colour change of indicator in 30 days; 1, green-yellow coloration of indicator in 30 days; 2, yellow coloration of indicator not before 15 days; 3, yellow coloration of indicator in 10 to 14 days; 4, yellow coloration of indicator in 5 to 9 days; 5, yellow coloration of indicator in 1 to 4 days.

24. *Glucose 'fermentation'* (formation of acids in Hugh–Leifson medium, over which a 2 cm. column of liquid paraffin was poured). Scoring as character 23.

25. *Methyl red test.* 1-, 3-, 5- and 7-day-old cultures were tested in each strain.

Scoring: Colour of the indicator added—0, yellow in all the cultures; 1, very slight

orange at most; 2, orange at most; 3, red not before the 5th day; 4, red in a 3-day culture; 5, red in a 1-day culture.

26. *Gluconate oxidation and growth in the medium.* None of the strains used oxidized gluconate; growth was scored after 10 days.

Scoring: 0, no growth; 1, weak growth; 2, good growth. Degrees 3 to 5 would correspond to positive biochemical reactions which were not found in the set of the strains under investigation.

27 to 34. *Utilization of salts of organic acids.* Concentrations (%, w/v): sodium acetate 0·2, sodium lactate 1·0, sodium potassium tartrate 1·0, ammonium tartrate 0·5, sodium formate 0·1, ammonium succinate 1·0, sodium citrate 0·5, sodium citrate 0·5 in the presence of Difco-yeast extract 0·01.

Scoring: 0, without colour change of indicator within 30 days and not growing; 1, without colour change of indicator or trace—growing weakly; 2, without colour change of indicator or trace—growing well; 3, colour change of indicator in 15 to 20 days; 4, colour change of indicator in 7 to 14 days; 5, colour change of indicator in 2 to 6 days.

35. *Hucker's test.* (Utilization of $NH_4H_2PO_4$ as a sole source of nitrogen in the presence of glucose.) Scoring as characters 27 to 34.

36. *Hucker's test, with Difco-yeast extract* (0·01 %) *in medium.* Scoring as characters 27 to 34.

37. *Tyrosine oxidation.* Scoring as character 26.

38. *Gelatin hydrolysis.* After 3-day incubation the diameters of the hydrolytic zones round the colonies on plates of MPA plus 10% gelatin after pouring on $HgCl_2$ reagent were measured. Scoring as character 22.

39. *Halo-zones on MPA.* After inoculating a thin MPA layer in a Petri dish in a straight streak, some strains show turbid zones probably due to the activity of proteolytic enzymes. Scoring as character 22.

40. *Casein hydrolysis.* Read after 3 days; scoring as character 22.

41. *Proteolytic-lipolytic activity in egg agar.* Scoring 0 to 5 by comparison of cultures.

42. *H_2S production.* This was determined by a rather sensitive microtest (MPB plus 0·1 % sodium thiosulphate, with a suspended lead acetate paper). Scoring according to the amount of PbS produced, estimated by visual comparison of the indicator papers.

43, 44. *Lysine and arginine decarboxylation.*

Scoring: 0, without colour change of indicator in 50 days; 1, colour change of indicator not before 40 days; 2, colour change of indicator in 30 to 39 days; 3, colour change of indicator in 15 to 29 days; 4, colour change of indicator in 5 to 14 days; 5, colour change of indicator in 1 to 4 days.

45. *Haemolysis of sheep red blood corpuscles.*

Scoring: 0, no zone in 5 days; 1, a slight trace of zone in 5 days; 2, a well discernible trace in 5 days; 3, a distinct transparent zone in 4 to 5 days; 4, a distinct transparent zone in 3 days; 5, a zone in 1 to 2 days, never seen.

46. *Urea decomposition.* Studied by two methods: the resulting value is the average of both.

(*a*) Microtest after Cowan.

Scoring: 0, without colour change of indicator in 40 hr; 1, orange coloration of indicator in 30 to 40 hr; 2, orange coloration of indicator within 29 hr; 3, red

coloration of indicator not before 16 hr; 4, red coloration of indicator in 9 to 15 hr; 5, red coloration of indicator within 8 hr.

(*b*) The test with liquid medium. Concentration (%, w/v): urea 2·0, KH_2PO_4 0·0091, $(NH_4)_2HPO_4$ 0·0095, Difco-yeast extract 0·01, phenol red 0·001.

Scoring: 0, without colour change of indicator and not growing; 1, without colour change of indicator, growing; 2, colour change of indicator in 5 to 14 days; 3, colour change of indicator in 2 to 4 days; 4, colour change of indicator in 13 to 24 hr; 5, colour change of indicator in 12 hr.

47. *Nitrate reduction* (0·05% KNO_3). Read after 5 days incubation, after the addition of Griess–Illosway reagent.

Scoring; 0, no reduction; 1, medium slightly reddish; 2, medium weakly reddish; 3, medium red; 4, red-brown precipitate in medium; 5, very intensive tufts of red-brown precipitate or simultaneous absence of NO_2^- and NO_3^- as demonstrated by means of zinc powder.

48. *Nitrite decomposition.* Semisolid medium with 0·0005% $NaNO_2$. 2-, 5- and 7-day cultures were tested in each strain.

Scoring: Colour of medium after addition of Griess-Ilosvay reagent— 0, deeply red in each culture (2, 5, 7 day); 1, red; 2, faintly red at most; 3, without colour change not before the 7th day; 4, without colour change in a 5-day-old culture; 5, without colour change in a 2-day-old culture.

49. *Methylene-blue reduction.* Scoring 0 to 5 by comparison of cultures.

50. *Catalase production.* Scoring 0 to 5 by comparison of cultures.

51 to 55. *Sensitivity to antibiotics.* The test was performed on MPA plates with 'Spofa' test tablets: penicillin 10 i.u., chloramphenicol 20 μg., neomycin 40 μg., nystatin 20 i.u., bacitracin 5 i.u. per tablet.

Scoring: 0, inhibition zone 0 to 1 mm.; 1, inhibition zone 1·5 to 2 mm. ;2, inhibition zone 2·5 to 6 mm.; 3, inhibition zone 6·5 to 11 mm.; 4, inhibition zone 11·5 to 16 mm.; 5, inhibition zone 16·5 mm and more.

56. *Sensitivity to lysozyme.* Lysozyme solution was placed in wells in inoculated agar plates (about 20 μg. of the substance per well). Scoring as for characters 51 to 55.

57. *Sensitivity to N-6 phage* (host strain is *Micrococcus lysodeikticus* CCM 1335). One drop of phage preparation was placed on a lawn on MPA plates.

Scoring: 0, growth unaffected; 1, a trace of weaker growth; 2, distinctly weaker growth; 3, translucent plaque in the spot covered by drop, but with more than 50 microcolonies in it; 4, 6 to 50 microcolonies in plaque; 5, 0 to 5 microcolonies in plaque. This evaluation is a rough expression of the frequency of sensitive cells in the population.

Numerical methods

In contrast to the existing most usual formula for calculating similarity (Sneath, 1957*b*):

$$S = \frac{n_s}{n_s + n_d},$$

where n_s = number of characters possessed by both individuals, n_d = number of different characters, another formula has been applied (Hubálek, 1964), in order to use the scale of 0 to 5, in scoring characters:

$$S = \frac{r.\Sigma p - \Sigma|d|}{r.\Sigma p},$$

where r = scoring scale range (in this study $r = 5$), Σp = number of comparable characters in the two individuals, $\Sigma|d|$ = sum of absolute values of differences in the characters of the two individuals. It is important to keep the range of the classification scale (r) the same for all characters.

The similarity values between all pairs of strains have been analysed not by the method of 'single linkage' (Sneath, 1957*b*), which partially distorts the complicated spatial relations expressed by the mutual similarity of strains (Lysenko & Sneath, 1959), but using another clustering method. In order to add a strain to a cluster (one strain or a group of strains), it must have a minimum number of links to the members of the cluster that are above the similarity value that is being examined. This minimum number is defined by the following scheme:

No. in cluster	Minimum links
1	1
2	1
3	2
4	2
5 and more	3

In this way the deformation of the actual spatial relations caused by their arrangement in a linear scale is decreased. Apart from this, another rule was made. In each step of clustering (1 % S) clusters were checked and when a strain in any cluster had a greater number of links to another cluster than to its own, it was transferred. In general, strains from smaller clusters were transferred to bigger ones. By means of these two rules about the addition and the transfer of strains the whole process has been done without using a computer.

RESULTS

The similarity values and the clustering are given in Tables 2 and 3. After some consideration the value $S = 85\%$ has been chosen as the 'species' level. Consequently, there are 11 groups (85-phenons according to the nomenclature of Sneath & Sokal, 1962) in the set.

While designating the groups I have primarily relied upon *Bergey's Manual* (1948, 1957) and tried to find for each 85-phenon the name of a species whose original description best corresponds with that phenon. The view of Evans, Bradford & Niven (1955) that *Micrococcus* and *Staphylococcus* constitute two different genera which can be with certainty differentiated on the basis of the 'fermentative' technique of glucose utilization, has been accepted. Validity of this point of view was clearly demonstrated by the quite different ratio of DNA bases in these genera (Silvestri & Hill, 1965).

A survey of characters of the phenons is shown in Table 4.

DISCUSSION

Classification techniques. The first clustering technique used in numerical taxonomy was 'single linkage' (Sneath, 1957*b*), later modified to the 'average linkage' method (Sokal & Michener, 1958) or that given by Hubálek (1964). A more objective determination of groups connected with a 'reasonable measure of inhomogeneity' has been suggested by Rogers & Tanimoto (1960). The use of factorial analysis has made possible a fully automatic and objective analysis of data (Hill *et al.* 1965; Gyllenberg, 1965; Poncet, 1967). Other methods introduced include 'probabilistic similarity

Table 2 (part 1). *Similarity values.*

$S \cdot 10^3$	80	105	144	149	209	248	265	291	296	335	337	351	357	407	410	443	523	531	536	546	555
80																					
105	867																				
144	833	861																			
149	861	886	827																		
209	828	870	830	875																	
248	832	895	882	836	863																
265	821	832	845	846	875	879															
291	833	791	821	823	788	798	809														
296	837	840	884	834	812	847	884	821													
335	814	856	861	850	846	877	857	770	882												
337	768	829	820	800	848	879	843	741	816	871											
351	826	865	870	846	847	851	868	775	877	846	836										
357	836	814	798	839	850	832	850	798	816	807	800	818									
407	788	795	764	798	830	802	829	768	825	798	777	798	873								
410	821	854	859	843	848	836	875	795	866	864	836	861	829	830							
443	788	812	807	818	858	823	843	730	856	861	825	874	829	848	886						
523	807	829	834	807	836	861	886	759	863	889	886	877	807	809	909	864					
531	786	818	813	814	850	843	864	750	830	871	879	968	832	841	868	871	896				
536	846	836	820	846	868	861	871	818	845	839	825	854	954	895	850	854	839	857			
546	872	835	840	814	814	825	839	833	875	811	754	840	800	805	843	837	811	796	818		
555	856	821	847	804	793	818	818	868	854	804	746	830	789	770	811	784	786	793	811	909	
617	868	854	863	841	875	833	852	797	835	809	770	835	813	786	834	814	809	795	834	854	851
631	800	849	840	807	853	842	868	735	847	867	864	872	796	798	882	884	893	871	825	839	804
683	813	848	818	813	873	841	849	805	850	870	860	850	838	851	875	854	882	871	871	829	811
732	840	791	846	805	777	788	795	870	853	781	777	814	784	793	788	765	784	770	813	854	865
819	852	855	893	848	813	834	863	864	893	838	788	863	784	782	848	795	838	825	809	884	891
1424	849	791	839	784	746	791	788	821	863	819	738	804	759	757	813	793	795	759	770	879	840
1681	818	793	840	768	754	793	811	830	840	807	750	809	743	752	829	767	814	786	768	884	874
169	847	787	811	809	787	798	824	898	849	769	769	776	824	778	813	761	794	783	839	833	830
276	866	845	849	822	845	818	855	889	853	815	778	807	845	784	844	786	829	822	826	887	895
530	829	786	805	778	767	793	815	844	816	775	753	796	789	751	818	764	800	767	807	858	864
550	813	780	782	769	762	776	813	833	807	758	755	791	787	749	798	758	776	762	802	851	840
656	805	777	782	773	745	780	773	864	771	738	752	759	791	739	766	734	755	745	802	813	823
1335	845	795	796	780	769	795	816	833	811	773	762	798	795	746	816	765	809	780	805	858	858
1423	820	791	793	780	784	788	820	818	821	777	780	788	777	754	834	784	816	780	791	852	830
1680	833	791	786	765	762	787	798	831	796	751	769	791	791	753	805	758	787	769	802	840	847
134	863	827	832	848	795	827	813	829	839	813	773	791	845	796	802	795	780	770	841	823	827
163	856	832	837	861	853	839	836	840	816	804	796	823	825	791	814	798	786	796	843	842	825
166	849	809	814	789	798	805	820	853	793	749	763	758	821	779	791	758	759	763	841	812	823
183	865	847	853	855	840	830	827	832	863	840	784	818	820	811	827	818	802	788	839	833	840
210	835	832	823	821	800	825	821	847	812	807	793	812	793	752	804	774	779	771	814	828	821
266	842	779	795	782	779	797	796	854	802	768	761	777	804	770	764	739	761	750	829	809	846
247	877	816	804	813	820	830	813	843	800	770	763	766	827	800	795	769	777	773	848	838	823
852	816	798	809	759	805	788	802	807	783	763	763	765	766	764	798	765	763	748	795	816	812
249	814	789	816	807	782	811	782	873	813	761	761	782	825	791	796	766	757	757	839	829	839
308	833	819	779	841	823	805	802	744	842	875	780	846	795	807	820	849	827	830	816	840	823
294	740	698	725	693	674	705	693	826	735	688	721	714	693	684	689	682	704	682	729	765	786
688	788	713	761	738	691	730	718	839	757	713	736	736	729	709	718	686	729	704	753	788	827
810	756	697	733	702	689	721	705	835	730	693	702	702	720	704	695	677	695	677	748	774	802
824	748	698	711	691	677	705	688	814	725	680	727	702	695	700	691	680	705	680	727	759	777
642	758	744	767	773	696	740	707	813	753	722	715	740	707	691	693	671	693	678	733	760	796
848	722	711	724	700	645	678	649	773	709	660	685	704	649	647	649	627	653	627	678	720	756
310	756	725	735	739	695	715	702	795	720	689	687	727	698	674	683	669	670	659	709	747	771
644	780	757	763	769	739	732	725	804	725	718	698	739	716	700	731	704	691	709	738	770	796
46	765	712	714	693	684	698	686	774	728	688	654	714	686	677	693	668	671	671	721	761	768
52	789	707	723	706	696	689	695	805	738	686	655	709	695	685	695	663	662	662	724	763	791
135	730	677	689	675	628	656	643	784	672	639	625	665	661	655	639	630	629	596	675	740	751
253	767	746	737	738	696	718	720	751	733	693	666	716	691	671	709	688	680	688	705	739	760
257	779	761	739	757	712	734	729	760	739	719	696	735	704	680	739	700	700	700	714	765	772
336	725	728	737	752	739	721	734	691	726	746	720	765	720	689	730	747	734	738	727	739	702
362	730	779	795	764	765	754	764	753	756	747	729	760	743	709	761	735	743	754	750	772	786
661	740	695	714	736	702	663	696	756	682	663	654	675	664	641	686	672	668	675	675	726	754

Approximative similarity expression in the upper right half of the table:
■ $S \geq 0{\cdot}860$: the two strains are very similar; □ $0{\cdot}740 < S < 0{\cdot}860$; ◪ $S \leq 0{\cdot}740$: the two strains are remarkably different.

Table 2 *cont.* (part 2)

$S \cdot 10^3$	617	631	683	732	819	1424	1681	169	276	530	550	656	1335	1423	1680	134	163	166	183	210	266
80	■								■							■			■		
105																					
144	■				■																
149																	■				
209	■		■																		
248																					
265		■			■																
291		◢		■	■			■	■			■									
296					■	■													■		
335		■	■									◢									
337		■				◢															
351		■			■																
357																					
407												◢									
440		■	■																		
443		■										◢									◢
523		■																			
531		■																			
536			■																		
546					■	■	■		■												
555				■			■		■	■											
617	■				■												■				
631	816	■																			
663	811	884	■																		
732	793	788	814	■	■				■												
819	861	830	811	893	■		■		■												
1424	811	795	804	828	857	■	■														
1681	842	800	820	844	877	882	■														
169	785	769	819	837	837	765	809	■	■	■	■	■	■	■	■			■			■
276	842	812	830	864	896	849	855	867	■	■	■	■	■	■	■						
530	843	778	795	838	858	824	851	871	902	■	■	■	■	■	■						
550	775	776	786	836	844	793	816	896	882	940	■	■	■	□	■						
656	754	752	800	819	807	782	843	904	860	882	902	■	■	□	■						
1335	822	795	807	816	847	829	845	874	900	936	943	876	■	■	■						
1423	825	816	829	819	839	848	834	874	864	893	858	836	902	■	■						
1680	778	780	793	821	829	789	843	869	870	922	931	901	935	876	■						
134	814	788	782	825	839	804	784	822	827	791	789	800	800	804	793	■	■	■	■	■	■
163	879	804	805	837	841	777	779	843	829	845	798	798	805	820	791	855	■	■	■	■	
166	844	770	786	842	825	781	788	863	849	824	845	836	841	807	824	879	872	■	□	■	
183	853	802	821	814	843	807	802	841	809	780	764	786	782	791	798	889	865	839	■	■	
210	802	779	802	846	841	798	807	850	848	815	813	827	843	823	827	877	867	879	868	■	
266	805	744	795	830	823	763	765	861	826	800	804	823	805	809	795	870	858	847	834	846	■
267	829	788	845	850	829	796	788	856	845	845	836	836	836	829	840	836	895	850	829	852	823
852	835	777	848	807	807	790	842	804	841	791	764	768	778	841	771	805	854	856	832	833	809
244	805	743	796	852	834	788	841	872	840	829	831	852	813	791	835	834	835	863	845	829	821
308	793	830	820	790	841	800	791	743	795	756	747	714	751	743	729	800	809	737	814	790	784
294	689	688	716	798	763	749	733	813	789	775	784	813	755	741	791	720	709	746	728	765	797
688	714	705	753	854	841	771	802	827	820	820	804	849	796	782	807	755	754	764	746	763	830
810	802	696	732	807	775	754	749	841	805	802	807	850	789	761	807	736	753	779	737	774	811
828	696	680	717	793	750	732	723	849	776	795	793	821	778	761	807	736	759	764	739	784	809
642	722	689	720	833	841	753	775	794	791	787	786	833	767	760	793	749	769	771	731	773	784
848	698	656	684	796	767	727	744	735	734	736	727	774	746	743	738	691	745	720	695	748	747
310	704	678	700	802	756	711	720	800	757	764	785	804	732	749	774	765	780	789	753	769	844
644	771	714	731	780	776	738	761	764	776	756	737	776	754	755	741	742	843	755	739	770	848
46	721	670	705	795	738	707	733	769	742	753	755	792	725	741	744	748	740	741	739	775	786
52	748	682	705	813	751	743	739	778	763	760	756	755	733	751	744	773	800	846	755	775	825
135	668	646	666	770	709	728	730	769	738	727	748	759	741	700	704	709	705	735	696	716	783
253	744	693	682	751	754	749	748	787	748	772	691	704	720	736	743	757	774	755	761	746	790
257	742	746	746	770	763	742	730	778	767	753	748	770	744	766	729	766	793	742	760	775	841
336	765	721	741	691	748	733	741	684	741	700	669	671	695	736	655	721	788	702	746	728	725
362	795	730	752	746	773	767	765	735	764	738	741	746	729	766	707	734	775	735	756	723	747
661	763	649	677	724	741	725	742	726	769	746	704	743	736	730	704	680	754	744	734	742	744

Table 2 *cont.* (part 3)

S·10⁷	247	852	249	308	294	688	810	824	642	848	310	644	46	52	135	253	257	336	362	661
80																				
105																				
144																				
149																				
209																				
248																				
265																				
291																				
296																				
335																				
337																				
351																				
357																				
407																				
410																				
443																				
523																				
531																				
536																				
546																				
555																				
617																				
631																				
683																				
732																				
819																				
1424																				
1681																				
169																				
276																				
530																				
550																				
656																				
1335																				
1423																				
1680																				
134																				
163																				
166																				
183																				
210																				
266																				
247																				
852	864																			
249	845	802																		
308	764	740	741																	
294	759	704	800	686																
688	793	739	813	729	873															
810	796	730	800	688	896	879														
824	764	707	788	686	948	869	889													
642	767	727	809	707	787	867	844	782												
848	716	702	744	664	835	859	826	822	880											
310	759	733	774	702	805	844	838	793	816	800										
644	780	759	747	741	754	831	802	762	802	780	817									
46	759	763	746	714	698	766	742	713	787	740	784	754								
52	780	777	771	718	732	802	770	747	773	755	831	780	943							
135	702	711	754	693	715	791	809	755	747	736	805	759	761	800						
253	739	747	702	709	707	736	719	696	706	664	778	791	732	773	739					
257	759	760	714	739	719	766	746	716	718	689	816	811	751	786	768	935				
336	682	712	666	747	623	655	632	636	684	622	669	732	672	663	672	723	742			
362	720	753	725	704	670	693	675	652	696	675	718	768	684	686	677	760	761	840		
661	709	718	700	707	721	720	719	713	741	700	736	793	663	696	737	773	783	738	807	

Table 3. *Clustering of micro-organisms*

Number of clusters in the set is given in parentheses.

$S \geqq$ 0·990: — (62)
0·980: — (62)
0·970: — (62)
0·960: — (62)
0·950: A. 357, 536 (61)
0·940: A. ditto; B. 46, 52; C. 294, 824; D. 530, 550 (58)
0·930: A., B., C. ditto; D. ditto+1335, 1680; E. 253, 257 (55)
0·920: ditto (55)
0·920: ditto (55)
0·900: A., B., C. ditto; D. ditto+276; E. ditto; F. 169, 656; G. 410, 523; H. 546, 555 (51)
0·890: A. ditto+407; B. ditto; C. ditto+810; D., E. ditto; F. ditto+291; G. ditto+531; H. ditto; I. 105, 248; J. 144, 296, 819; K. 335, 351 (43)
0·880: A., B., C. ditto; D. ditto+169, 656; E. ditto; F. 291; G. ditto+443, 631; H. ditto+1681; I. ditto+149; J., K. ditto; L. 134, 183; M. 642, 848 (37)
0·870: A., B. ditto; C. ditto+688; D. ditto+1423; E. ditto; F. 291, 249, 732; G. ditto+683, 335, 337, 351; H. ditto+819, 1424; I. ditto+209, 265; J. 144, 296; K. —; L. ditto+166, 210; M. ditto; N. 163, 617; ·80, 247 (23)
0·860: A. 357, 407; B., C. ditto; D. ditto+291, 555, 732; E. ditto; F. 249; G. ditto+105, 144, 209, 248, 265, 296, 536, 819; H. 546, 1424, 1681; I. 149; J., K. —; L. ditto+80, 163; M. ditto; N. 617; ·247, 852 (21)
0·850: A. —; B., C. ditto; D. —; E. ditto; F. —; G. ditto+276, 555, 546, 732, 1424, 1681; 291, 530, 169, 550, 1335, 1680, 656, 1423, 357, 407, 617, 80, 183, 163, 134, 166, 210, 149 249, 247, 852, 266; H., I., J., K., L. —; M. ditto; N., O. —; P. 336, 362 (11)
0·840: B. ditto; C. ditto+642; E. ditto; G. ditto+308; M. 848; P. ditto. A., D., F., H., I., J., K., L., N., O. — (10)
0·830: B., E. ditto; G. ditto+294, 310, 642, 688, 810, 824, 848; P. ditto. The remaining — (7)
0·820: ditto (7)
0·810: B. 46; E. 253; G. ditto+52, 257, 644; P. ditto (6)
0·800: B. 46; E. 253; G. ditto+135; P. 336, 362, 661 (4)
0·790: B. —; E. 253; G. ditto+46; P. ditto (3)
0·780: E. —; G. ditto+253, 336, 362, 661; P. — (1)

'ditto': The same strains as the previous step.

index' (Goodall, 1966) for direct significance testing of classification relationships; *t*-test for a statistical comparison of more or less similar groups of strains (Hubálek, 1964; Tsukamura, 1967); calculation of taxonomic relations derived from the theory of entropy (Hall, 1967); 'furthest neighbour sorting' and 'flexible sorting' (Lance & Williams, 1967). At present it is difficult to judge which of these methods is most advantageous. A comparison of some of them has been made for example by Hill *et al.* (1965) and 'tMannetje (1967), but without any definite conclusion. However, the results obtained by various grouping techniques may be different to a certain extent. Up to now, the 'principal component analysis' and 'gradient method' (Hill *et al.* 1965) seem to be very objective methods in taxometrics.

Taxonomic problems. The view that 85-phenons in this study would be about at the level of species seems to be supported by the results of Rosypal *et al.* (1966). They have studied, among others 11 strains also common to the set in this investigation; the strains fell on DNA base composition into three different groups:

Strain CCM no.	85-phenon	Rosypal *et al.* (1966) *Micrococcus* group
144, 210, 247, 248, 265, 266, 523, 852, 1335	I	1: 70·8 to 73·3 moles % GC
810	III	3: 65·8 to 67·0 moles % GC
310	V	2: 67·5 to 69·5 moles % GC

Table 4. *Scoring of characters in 85-phenons*

Characters no.	Mean	85-phenons I: 45 strains (see Table 1)	II: 308	III: 294, 688 810, 824	IV: 642, 848	V: 310	VI: 644	VII: 46, 52	VIII: 135	IX: 253, 257	X: 336, 362	XI: 661
			Strains CCM no.									
			Scores and range									
1	3·9	3–5	4	2·5–4	4	3	4	4	3	5	4–5	3·5
2	2·5	(0) 1–4·5	5	0–1	0·5–1	3	2	1	0	3–4	3–4	0·5
3	2·7	(0) 1–5	4	0·5–1·5	1–2	2·5	3	1	1	3 5–4	3–4·5	2·5
4	0·1	0 (1·5)	0	0–3	0–4	0	0	0	0	0	0	0
5	1·0	0–4	0	0	0	3·5	1·5	3·5	0·5	4 5–5	0	1
6	1·8	0–3·5 (4)	1	2–3	3–4	1·5	2	3	2·5	2–3	3	3
7	1·7	0–4 (5)	4	0	0	0	1	0	0	0 5–1	1–4	0
8	1·4	0–3 (5)	2·5	0 1	0	0	2	2–3	0	2 5–3·5	3	5
9	2·8	(1) 2–4	3	2–3	2	2	2	3	2	2–3	3	2
10	2·3	0–5	4·5	0–1·5	0·5–1	1	2·5	2–3	1	1·5–3	3–4·5	1
11	0·2	0–1 (2)	0	0·5–3	3–5	2	2·5	3–4	1·5	0	0–1	0
12	2·0	(0·5) 1–3 (4)	3	1	1	1	1	1	1	2	2	1
13	3·8	(1) 3–5	4	2–4	3–4	—	3	1	4	1	3–5	3
14	4·4	(2) 3–5	5	4·5–5	4	—	3·5	1–2	5	3	3–5	4
15	0·3	0–1 (2)	0	0	0–1·5	4	0	0–2	1	0	0–1	0
16	0·2	0–1 (2)	0	0	0	0	0	0	0	0	0	0
17	0·2	0 (5)	0	0	0	0	0	0	0	0	0	0
18	4·1	(2) 3–5	3	2–2·5	2·5–3	2·5	1	4–5	2	3·5–5	2–4	3·5
19	3·9	2·5–5	5	2–3	2–3	3	1	3–3·5	4	3–4	2–4	3
20	3·0	2–4	3	2–3	2	3·5	—	2–2·5	2·5	3	3·5–4	3
21	0·6	0–2 (4)	2	0	0–2·5	0	0	0	5	1	0	0
22	0·6	0–3	3·5	0	0–1·5	0	0	0	0	0	0	0
23	0·4	0–2 (2·5)	0	0	0	0	0	0–1	0·5	3	4–4·5	4·5
24	0·1	0–0·5 (1)	0	0	0	0	0	0	3	3·5	4–4·5	4·5
25	0·0	0	0	0	0	0	0	0	1	0	3	0·5
26	1·1	0 2	1	0–2	0–2	0	2	0	0	1–2	2	2·5
27	3·4	2–4 (4·5)	4	2–3	2·5	2	2·5	1–2	1·5	2–2·5	3	4
28	3·2	(1·5) 2–4 (4·5)	4	2–3	2	2	2	1·5–2	1	2·5–3	1–2·5	3
29	1·6	0·5–2	0·5	1·5–4	1–2	2	3	0·5–1	0	3·5–4	0·5–2	2
30	1·2	(0) 0·5–2	1	1–4·5	4·5	1	3	0–0·5	1·5	2·5–4·5	1–1·5	2
31	1·7	(0) 0·5–2 (4)	1	2–4	2–4	3	0·5	1·5	4	2·5	0–3	4
32	1·7	0–2 (3)	3	0–2	2	0·5	2	1·5–2	0	0·5–1	2–2·5	4
33	0·4	0–1 (2)	1	0–0·5	2·5	2	0	0	0	1	5	5
34	1·8	0–5	1	0–2	5	3	5	0	0	2	4–5	5
35	2·2	0–4 (4·5)	1·5	0	0	0	4	0	0	3	2·5–4	5
36	3·3	1·5–4 (5)	5	0–2·5	0–1	0	4	0	4	4	3·5–4	5
37	1·3	0–2·5	2	0	0	0	0	0	0	2	2	2
38	3·4	(0) 2–5	1	0	2·5–3·5	0	0	1·5–4	0	0	4	0·5
39	3·0	(0) 1·5–4 (4·5)	3	0	0	0	0	0	0	0	0	0
40	3·0	(0) 2–4	2·5	0	—	0	0	5	0	0	3–4	0
41	2·9	(0) 2–4	3	0	—	0	2	2–3	0	0	1–2	0
42	2·6	(0) 1–4 (5)	4	0–4	0	0	0	3–3·5	2	0–0·5	2–4	4
43	0·8	0 2·5	2	0–0·5	0–0·5	0	0·5	0	0	0	0–2	1·5
44	0·7	0–2 (2·5)	0	0–0·5	0–0·5	0	0	0	0	1	0–0·5	1
45	0·9	0–3	0	0	0–1	0	0	0	0	0	0	0
46	2·4	0–5	0	0–2	0	0·5	2	0	0	0	2·5–3·5	3
47	0·3	0–2 (2·5)	5	0	3–4	3	5	5	5	3	0·5–2·5	5
48	0·0	0 (1)	0	0	0	0	0	5	4·5	3–4	0	0
49	2·7	(1·5) 2–3 (5)	2	1·5–3	2·5	3	2·5	3	5	2·5–3	2·5–5	3
50	4·0	(2·5) 3–5	4	3·5–5	2·5	4	4	3	4	4	4–5	5
51	3·1	(1) 2–4 (5)	3	2	2	4	3	3	4	3	5	4
52	3·7	3–5	4	4–5	4	3	4	3	5	3	3–4	4
53	1·6	0–3	2	3	3	3	3	2	3	2–3	2–4	3
54	3·4	(2) 3–4 (5)	3	0–3	0–3	3	4	3	4	3–4	5	4
55	2·6	1–4	2	0	1–2	2	3	3	3	3	4	3
56	3·4	0 5	1·5	3–4·5	4–5	0	0	0	0	0	0	0
57	0·8	0–5	0	0–1	0	0	0	0	0	0	0	0

Legend: number in parentheses: the score is achieved by 1 or 2 strains only; —, could not be scored.
Determination of phenons in accordance with *Bergey's Manual* (1952; 1948):
85-phenon I: Approximately as *Micrococcus flavus*; 85-phenon II: *M. citreus*; 85-phenon III: *M. luteus*; 85-phenon V: *M. aurantiacus*; 85-phenons IV, VI, VII: *Micrococcus* spp.; 85-phenon IX: *Staphylococcus epidermidis*; 85-phenon X: *S. conglomeratus*; 85-phenon XI: *S. varians*; 85-phenon VIII: *Staphylococcus* sp.

85-phenon I (*Micrococcus flavus*) includes a number of strains with cells occurring in the form of packets and which are usually placed into the genus *Sarcina*. The whole phenon I may be divided in three morphological types:

1. 'Sarcinas'—strains with cells arranged in typical packets of cocci: 19 strains (CCM 134, 149, 163, 209, 248, 265, 296, 335, 337, 351, 357, 410, 443, 523, 531, 536 631, 683, 819).

2. 'Micrococci'—strains with cells arranged in more or less typical clusters of cocci: 17 strains (CCM 80, 169, 247, 276, 291, 530, 546, 550, 555, 656, 732, 852, 1335, 1423, 1424, 1680, 1681).

3. Strains with 'intermediary' arrangement of cocci: 9 strains (CCM 105, 144, 166, 183, 210, 249, 266, 407, 617).

The formation of packets is not a quite constant feature in aerobic sarcinas (Kocur & Martinec, 1962; Hubálek, 1964) and correlates with a number of other morphological properties (turbidity in broth is weak or none, there are conglomerates in liquid medium, sediment is grainy, colonies matt to grainy) but not with physiological or biochemical properties (only the activity of proteolytic enzymes in sarcinas, as well as the production of hydrogen sulphide, appears to be somewhat higher; it is interesting to note that all the sarcinas studied, without exception, were proteolytic). In order to compare both basic morphological types in the 85-phenon I, six strains of the morphological type 1 and six strains of the type 2 were randomly selected and the similarity was calculated between all these strains, after the characters nos. 2, 3, 6, 7, 10 and 12 (which are directly influenced by the morphological type) had been eliminated. Then the mean intragroup similarity values were found for both types and also the mean value of intergroup similarity ($\bar{x}_{1\cdot2}$), and their standard deviations:

$$\bar{x}_1 = 86{\cdot}487\,\%\ S, \qquad s_1 = \pm 3{\cdot}895\,\%\ S,$$
$$\bar{x}_2 = 84{\cdot}527\,\%\ S, \qquad s_2 = \pm 3{\cdot}406\,\%\ S,$$
$$\bar{x}_{1\cdot2} = 82{\cdot}939\,\%\ S, \qquad s_{1\cdot2} = \pm 1{\cdot}897\,\%\ S.$$

While comparing these S-mean values by t-test, the Cochran modification of the Behrens–Fisher method (see Snedecor, 1957) has been used because

$n_1 \neq n_2$ ($n_1 = 15$, $n_2 = 36$) and $\sigma_1 \neq \sigma_2$ ($F_{1/1\cdot2} = 4{\cdot}22^{**}$, $F_{2/1\cdot2} = 3{\cdot}22^{**}$).

The formula is written as follows:

$$t' = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\left(s^2_{\bar{x}_1} + s^2_{\bar{x}_2}\right)}},$$

and the corresponding values are

$$t'_{1/1\cdot2} = 3{\cdot}366, \quad t'_{2/1\cdot2} = 1{\cdot}699.$$

Distribution of t'-values is different from the Student's t; the t'-tabular values have been estimated by the formula:

$$t'_{0\cdot05} = \frac{s^2_{\bar{x}_1} \cdot t_{0\cdot05_1} + s^2_{\bar{x}_2} \cdot t_{0\cdot05_2}}{s^2_{\bar{x}_1} + s^2_{\bar{x}_2}} \doteq 2{\cdot}13$$

for $t'_{0\cdot05_{1'1\cdot2}}$ and $t'_{0\cdot05_{2/1\cdot2}}$, too.

According to Tsukamura (1967), two samples of more or less taxonomically similar strains may be assumed to be two distinct subspecies if *both* intragroup similarity

mean values are different from the intergroup one; the difference is estimated by t-test for $P = 0{\cdot}05$. In our case, the value $t'_{2/1\cdot2}$ indicates that a classification of both samples (randomly drawn from the morphological types 1 and 2) as two different subspecies is not justified and obviously they cannot be considered as separate species or genera.

Discussions on the unsuitability of placing aerobic sarcinas into a separate genus may be found in the literature (Winslow & Winslow, 1908; Shaw *et al.* 1951; Evans *et al.* 1955) but only Kocur & Martinec (1962) transferred them into the genus *Micrococcus*, and the present communication has confirmed this view. It is probable (Hubálek, 1964) that aerobic 'sarcinas' are R-dissociated forms of proteolytic micrococci.

The genus *Sarcina*, however, should be kept for the anaerobic sarcinas: *S. ventriculi*, *S. maxima*, *S. methanica*, *S. barkeri*.

I wish to thank Dr M. Kocur for making available strains from the Czechoslovak Collection of Micro-organisms. I am also indebted to Dr O. Lysenko of the Entomological Institute, Czechoslovak Academy of Sciences, for valuable advice and for checking the text of manuscript, and to Mrs E. Bělyjová of the Institute of Parasitology for translation into English.

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With 1 plate
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Formation of Fragile Cysts by a Strain of *Azotobacter chroococcum*

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SUMMARY

A strain of *Azotobacter chroococcum* (designated *A. chroococcum* NTS) which produces fragile cysts was isolated from soil. Fragility of the cysts was measured by ultrasonic treatment and compared to the fragility of cysts of a typical strain of *A. chroococcum*. Differences in fragility and resistance to ultraviolet radiation were observed but differences in desiccation resistance were not perceptible between the two strains. Electron micrographs of cysts of the new isolate revealed a structural aberration in the exine area of the cyst coat which may be associated with the physiological differences described. The morphological aberration and the fragility of the cysts are inheritable traits of this strain of *A. chroococcum*.

INTRODUCTION

Spores and cysts are dormant forms of some bacteria which are highly resistant to adverse environmental conditions. The cyst has been described as the dormant stage in the life-cycle of some members of the Azotobacteraceae, and Socolofsky & Wyss (1962) have shown it to be resistant to both chemical and physical agents. The basis for this resistance is not known but Parker & Socolofsky (1966) attribute it to the cyst coat: the encystment process, implying the formation of a cyst coat, is correlated with the acquisition of resistance to ultrasonic treatment, ultraviolet radiation, and desiccation; removal of the cyst coat by certain chemical agents makes the surviving central body as vulnerable as the vegetative form to deleterious agents.

This paper describes a strain of *Azotobacter chroococcum* which produces cysts which are as resistant to desiccation and ultraviolet radiation as the typical cysts of *A. chroococcum* but which are mechanically very fragile. These data are presented because they indicate that the structure of the cyst coat may not be the entire basis of the resistant properties of the azotobacter cyst.

METHODS

Organisms and culture conditions. Two organisms were used in this study. *Azotobacter chroococcum* 75–1 was obtained from the stock culture collection, Department of Microbiology, University of Texas, Austin, U.S.A., and maintained on Burk basal medium (Wilson & Knight, 1952) with 1 % glucose as the carbon source and 2 % agar as the solidifying agent. This organism produced cysts readily when grown on the same medium with 0·2 % *n*-butanol instead of 1 % glucose as the carbon source. The second organism, *A. chroococcum* strain NTS, was isolated from a soil sample obtained

from the state of Louisana, U.S.A. It was isolated on Burk medium with glucose and induced to encyst by culturing it on plates of Burk medium with *n*-butanol. All cultures were incubated at 30°. Purity of all cultures was established by colonial morphology, microscopic examination, and subculture on Tryptic Soy Agar (Difco).

Determination of fragility. Cyst fragility was first noted microscopically when cysts were seen to rupture during preparation of thin wet mounts between cover glass and microscope slide. Subsequently, cysts were ruptured by ultrasonic treatment 20,000 cyc./sec. delivered into an aqueous suspension of cysts at approximately 0°, with a Branson Model S-75 Sonifier (Branson Instruments, Inc., Stamford, Connecticut, U.S.A.). Disruption of cysts was monitored by changes in extinction at a wavelength of 540 mμ and confirmed by microscopic examination.

Ultraviolet irradiation resistance. The resistance of cysts to ultraviolet radiation was determined by measuring survival after exposure to radiation of approximately 2537 Å wavelength. Cysts were washed by centrifugation and resuspension in sterile water, placed in Petri dishes to a layer depth of approximately 1 mm., irradiated at room temperature, and kept constantly in motion to minimize exposure differences. All radiation studies were done in a darkened room to avoid photoreactivation. (Recent work in this laboratory shows that cysts of Azotobacter can be photoreactivated: unpublished data.) Radiation survival was determined by spread plate counts using Burk basal agar with 1 % glucose and the death curves obtained were not unlike those reported previously (Socolofsky & Wyss, 1962; Vela & Wyss, 1965).

Desiccation resistance. Resistance of cysts to desiccation was determined by the method of Socolofsky & Wyss (1962).

Electron micrography. Cysts were collected from the surface of agar plates and fixed in 3 % glutaraldehyde buffered with 0·1 M-sodium cacodylate at pH 7·2 for 1 hr at 3°. They were washed several times in 0·2 M-cacodylate buffer at 3° and suspended in Bouin fluid, pH 3·7, for 15 min. at 3°. They were washed several times with cacodylate buffer at 3°, fixed with 1 % OsO_4 buffered with 0·1 M-cacodylate at pH 7·2 for 2 hr at 3°, then dehydrated by passage through a series of graded ethanol solutions terminating in absolute ethanol. Embedding was accomplished by infiltration with a 1 + 1 mixture of propylene oxide and Epon 812 followed by serial increases of Epon 812. Polymerization was done in a drying oven at 60° overnight. Thin sections were prepared with the Porter–Blum ultramicrotome equipped with a diamond knife (Ivan Sorvall, Inc., Norwalk, Connecticut, U.S.A.). The sections were treated with a saturated ethanolic solution of uranyl acetate for 30 min. and then with an aqueous saturated solution of lead tartrate for 7 minutes. All preparations were examined with an RCA-EMU-3G electron microscope.

RESULTS AND CONCLUSIONS

Gross observations. The preliminary observations of mechanically fragile cysts were made during routine examinations of soil isolates. Encystment of newly isolated cultures of Azotobacter was determined by microscopic examination of wet mounts using a cyst stain previously described (Vela & Wyss, 1964). Large numbers of cysts from isolate NTS were ruptured but preparations from other isolated cultures of Azotobacter yielded whole normal cysts which, like those from known encysting

Azotobacter species, were not ruptured by the staining and mounting procedure. The extent of cyst rupture was roughly correlated with the amount of pressure used in attempting to remove excess stain from the coverslip preparations. The newly isolated organism and *Azotobacter chroococcum* 75–1 produced cysts which were indistinguishable when examined with the light microscope, with the cyst stain, and with the phase-contrast microscope. In cultural characteristics, cell morphology, and colonial morphology strain NTS was *A. chroococcum* according to the criteria given in *Bergey's Manual* (1957). The isolate was subcultured every day or two for a total of 47 transfers using Burk medium with 1 % glucose without detectable changes in the relative fragility of its cysts as compared to those of *A. chroococcum* 75–1.

Cyst fragility and desiccation resistance. Figure 1 records quantitative comparisons of the fragility of *Azotobacter chroococcum* strains 75-1 and NTS to ultrasonic treatment in an aqueous medium; the correlation between changes in extinction and cyst breakage was established by Socolofsky & Wyss (1962). Cysts of *A. chrococcum* 75-1 were much more resistant to sonication than those of *A. chroococcum* NTS. No difference was detected in the desiccation resistance of cysts of *A. chroococcum* strains 75-1 and NTS.

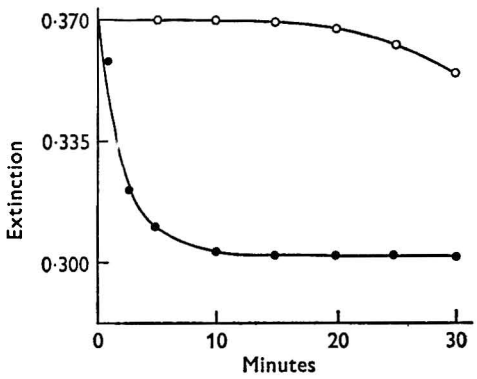

Fig. 1

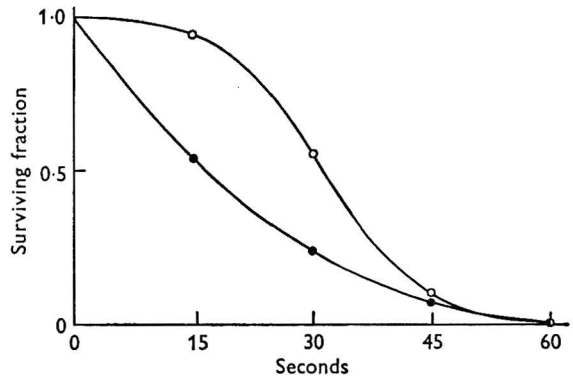

Fig. 2

Fig. 1. Effect of ultrasonic treatment on the cysts of *A. chroococcum* 75–1 (○) and those of *A. chroococcum* NTS (●). Changes in extinction were indicative of cyst rupture as verified by microscopic examination.

Fig. 2. Relative resistance of cysts of *A. chroococcum* 75–1 (○) and those of *A. chroococcum* NTS (●) to ultraviolet radiation. The abscissa indicates the number of seconds of exposure to the flux of ultraviolet light established by a Model R-51 Mineralight at a distance of approximately 15 in. from the cyst suspensions while the ordinate indicates the surviving fraction of bacteria after exposure.

Ultraviolet radiation resistance. The data from an experiment typical of several (Fig. 2) indicate that, while there was a difference between the two strains in their response to the bactericidal effect of ultraviolet radiation, the difference was qualitative and not quantitative, since small doses of radiation produced a sigmoidal survival curve in *Azotobacter chroococcum* 75-1 but a parabolic survival curve in NTS. With higher doses of radiation the survival curves of the two strains were essentially identical. Thus the two organisms reacted differently to ultraviolet irradiation, but their resistance was approximately the same.

Ultrastructure of the cysts. Under the light microscope the two strains of *Azotobacter chroococcum* appeared identical. The morphologies of the cyst coats were different and easily distinguishable when examined by electron microscopy: the cyst coats possessed structurally different exines. The exine of *A. chroococcum* NTS (Pl. 1, fig. 1) appeared to be a loose non-laminar structure. *Azotobacter chroococcum* 75-1 (Pl. 1 fig. 2) possessed a well-defined compact exine in agreement with previous descriptions of Azotobacter cysts (Wyss, Newmann & Socolofsky, 1961; Socolofsky & Wyss, 1962; Tchan, Birch-Andersen & Jensen, 1962; Parker & Socolofsky, 1966).

Conclusions. Resistance of *Azotobacter chroococcum* NTS to various deleterious agents may be attributed to diverse morphological characteristics. Parker & Socolofsky (1966) stated that the resistance of the Azotobacter cyst to deleterious agents resides in the cyst coat. The data presented here indicate that resistances to desiccation, ultraviolet radiation, and ultrasonic treatment are not entirely due to the same structural entity, the cyst wall. There is an ultrastructural difference between the two Azotobacter strains and these two strains show marked differences in their resistance to ultrasonic treatment. It seems reasonable therefore to state that the resistance to desiccation is apparently not entirely a function of the cyst wall structure, that the resistance to ultraviolet radiation is apparently affected by the ultrastructure of the cyst wall, and that the resistance to mechanical stress is apparently due to the configuration of the exine.

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EXPLANATION OF PLATE

(Magnification, × 25,000)

Fig. 1. Thin section of the cyst of *Azotobacter chroococcum* NTS.

Fig. 2. Thin section of the cyst of *Azotobacter chroococcum* 75–1.

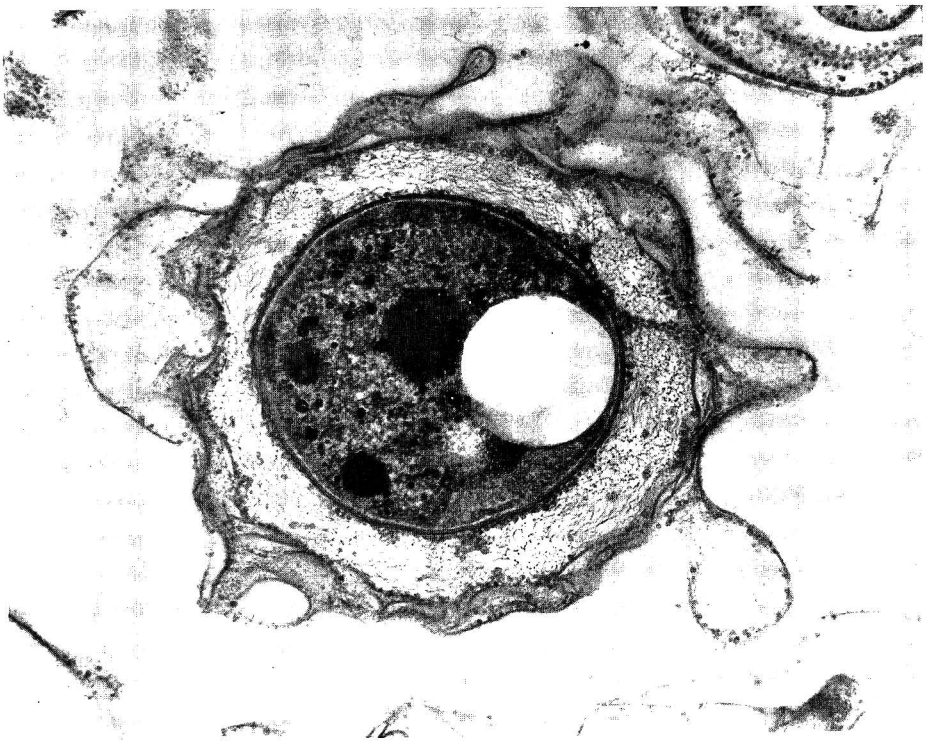

Fig. 1

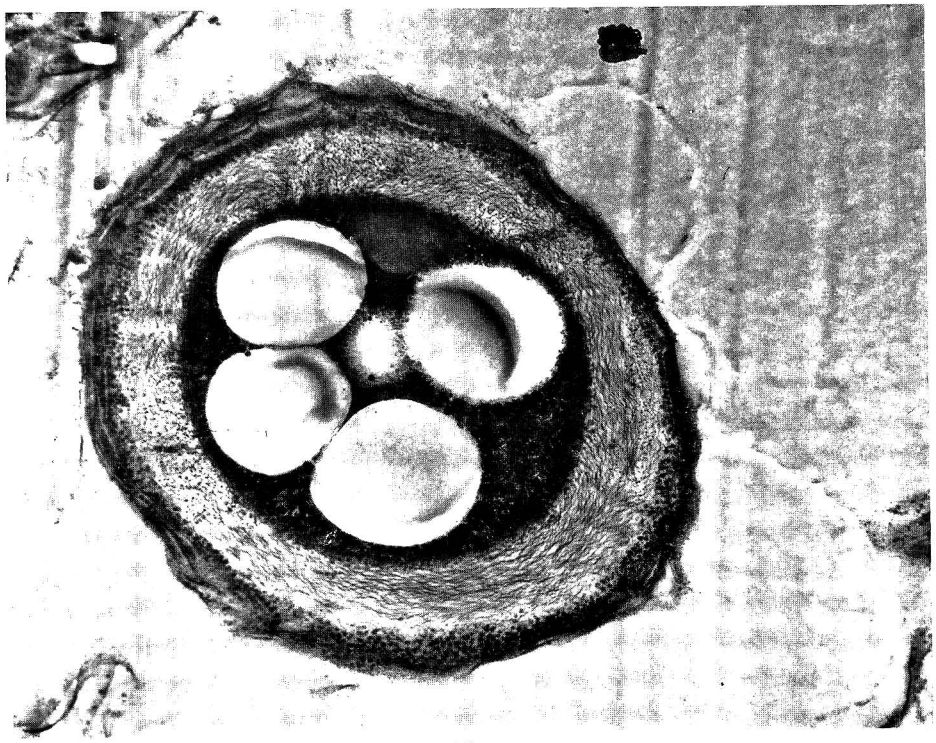

Fig. 2

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A Group of Klebsiella Mutants Showing Temperature-dependent Polysaccharide Synthesis

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SUMMARY

Several mutants isolated from three *Klebsiella aerogenes* strains showed characteristics differentiating them from the wild type and also from the normal type of non-mucoid mutants. These mutants synthesize much less polysaccharide slime or capsule than the parent bacteria at low incubation temperature, but similar amounts at 37°. The colonies are of rough appearance at 20° and liquid cultures at this temperature autoagglutinate. At 37°, mutant and parental types are indistinguishable. The mutants show altered phage sensitivity patterns at 20° and the yield of lipopolysaccharide appears to be decreased at lower temperatures. Double mutants retaining certain of these characteristics but devoid of exopolysaccharide-synthesizing activity have also been isolated. The properties of the parent and mutant types are compared.

INTRODUCTION

In studies on polysaccharide biosynthesis, frequent use has been made of mutants unable to synthesize the exopolysaccharide slime or capsule characteristic of the parent organism, for example, *Diplococcus pneumoniae* capsular polysaccharide (Smith, Mills & Harper, 1957), the slime polysaccharide synthesized by *Escherichia* strains and by other species of the Enterobacteriaceae (Beiser & Davis, 1957). During studies on exopolysaccharide biosynthesis by strains of *Klebsiella aerogenes* a number of mutants differing from normal non-mucoid variants were isolated. The unusual properties of these mutants and their relationship to the parent strains are reported.

METHODS

Bacteria and methods of culture. Three strains of *Klebsiella aerogenes* have been used. Strains A3(SL) and A4 are laboratory strains used in several earlier studies (Wilkinson, Dudman & Aspinall, 1955; Sutherland, 1967). Strain W70 was kindly provided by Dr D. G. McPhee, School of Biological Sciences, University of East Anglia. Strains KP1 and A4-O are non-mucoid mutants of A3(SL) and A4, respectively, and are included in certain experiments for comparison. All strains were cultured routinely on nutrient agar at 35°. For production of extracellular polysaccharide, strains were grown on a solid nitrogen-deficient medium (Sutherland & Wilkinson, 1965). A liquid version of this medium was also used for some experiments. The minimal medium used was that described by Davis & Mingioli (1950). Liquid cultures were grown in Erlenmeyer flasks containing half the nominal volume and shaken at 200–300 rev./min. in an orbital incubator. Initial isolation of mutants was made on

eosin methylene blue (EMB) agar with glucose as carbon source or on nitrogen-deficient medium.

Bacteriophages. The phages having *Klebsiella aerogenes* A3 (SL) as host have been described earlier (Sutherland, 1967). Bacteriophage active against other *K. aerogenes* strains were isolated from raw sewage by the same techniques except that membrane filtration and not chloroform was used to remove bacteria from the preparations. The phage-induced fucosidase preparation was that used by Sutherland (1967).

Mutagenesis. 'Old cultures' were nutrient-broth tube cultures held at 35° for 10 to 20 days. Dilutions were made into sterile 0·9 % (w/v) saline and 0·1 ml. samples were spread over the surface of suitable solid media. 2-Aminopurine mutagenesis was performed on overnight nutrient broth cultures which were diluted to approximately 10^4 bacteria/ml. in broth containing 2-aminopurine (200 μg./ml.), and incubated 48 hr at 35°. Samples were then spread over nitrogen-deficient solid medium.

Preparation of polysaccharides and lipopolysaccharides. The exopolysaccharides produced by the three strains were of two types: slime or capsule. These were isolated and purified by the techniques originally described by Wilkinson *et al.* (1955). Lipopolysaccharides were extracted from lyophilized bacterial cells by the phenol + water method using a 5 min. extraction time (Lüderitz *et al.* 1965). After removal of the phenol by dialysis, the extracts were concentrated under reduced pressure and the lipopolysaccharide was freed from other material by ultracentrifugation at 100,000 *g* for 4 hr, then lyophilised. The supernatant fluid was also freeze-dried and assayed for glucose as a measure of glycogen.

Microanalysis. The constituent sugars of the polysacharides and lipopolysaccharides were identified by paper chromatography in the solvent of Fischer & Dörfel (1955), after hydrolysis in N-H_2SO_4. The individual sugars were determined as described previously (Sutherland, 1967). In addition, mannose was determined by the cysteine + sulphuric acid assay (Dische, Shettles & Osnos, 1949) and total polysaccharide by the anthrone method. The *O*-acetyl groups found in two of the three exopolysaccharides were estimated by the hydroxylamine-ferric chloride assay (Hestrin, 1949). Total protein was assayed by the Folin technique. All colorimetric results were read in glass cells in a Zeiss PMQII spectrophotometer or a Unicam SP500 instrument. These were also used for determining the turbidity of cell suspensions and for enzyme assays.

Paper chromatography and paper electrophoresis. Paper chromatograms on Whatman no. 1 paper were run in ethyl acetate + acetic acid + pyridine + water (5 + 1 + 5 + 3, by vol.) (Fischer & Dörfel, 1955) or ethyl acetate + acetic acid + formic acid + water (18 + 3 + 1 + 4, by vol.) (Feather & Whistler, 1962) for 24 or 40 hr respectively. Partial acid hydrolysates (N-H_2SO_4 for 15 min. at 100°.) were neutralized with $Ba(OH)_2$ solution and compared by paper electrophoresis in pyridinium acetate at pH 5·3 using the conditions previously described (Sutherland & Wilkinson, 1968), and by paper chromatography in the acid solvent.

RESULTS

Isolation of colonies with crenated colony type (CR mutants).

The parent strains were subjected to mutagenesis and spread, after expression of mutations, on nitrogen-deficient or EMB medium and the colonies examined for the presence of non-mucoid types (O mutants). These occurred at low frequency and were

slightly smaller than the wild-type colonies, lacking the mucoidness and consequent glossy appearance. The non-mucoid cells grew in liquid culture with uniform turbidity and had a growth rate similar to the parental type. Several hundred O mutants have been isolated using various mutagens. During examination of colonies following mutagenesis, another type of mutant was observed. These colonies (CR mutants) were detected on plates incubated at 30° or 20° and had a very characteristic appearance. They were of the same size as those of non-mucoid mutants (10 to 12 mm. diam. after 48 hr growth on nutrient agar at 20°). The outline of the colonies was irregular and the consistency more friable than either the parental or O type, and ridges radiated from a central apex. The CR mutants were only isolated following treatment with aminopurine and not with other mutagens. One CR mutant was obtained as a spontaneous mutant from an 'old culture'. From two of the CR mutants further mutagenesis with aminopurine led to the isolation of another form which we have designated CR-O mutants. These cultures possessed properties common to CR mutants and O mutants. The derivation of mutants and their type is shown in Table 1. The frequency of occurrence of either CR or CR-O mutants was very much lower than that of the O mutants when aminopurine was used as mutagen. Attempts to derive a CR-O mutant from *Klebsiella aerogenes* W70 have been unsuccessful.

Table 1. *The type and mode of isolation of the mutants used in the present study*

Parent	Mutant	Type	Mutagen
A3(SL)	A3-CR	CR	2-Aminopurine
A3-CR	A3-CRO	CR-O	2-Aminopurine
W70	W70-CR1	CR	2-Aminopurine
W70	W70-CR2	CR	2-Spontaneous
A4	A4-CR	CR	2-Aminopurine
A4-CR	A4-CRO	CR-O	2-Aminopurine

Cultural characteristics of CR mutants

The characteristic colonial appearance of CR mutants on solid media was only observed following incubation at 20 to 30° for 24 to 48 hr. After longer incubation at these temperatures some mucoidness was noticed. The colonies also become less friable and very adhesive to the medium. Incubation at 37° led to cultures almost indistinguishable from the wild-type strains. The CR-O mutants were recognizable as their characteristic colonial appearance was retained on incubation at 37°. They thus appeared to lack the ability to synthesise extracellular polysaccharide, so resembling O mutants. Colonies on solid media were very friable.

Liquid cultures of CR mutants at 20° in all the media tested underwent auto-agglutination and sedimentation as a granular aggregate of bacteria. At 37° liquid cultures were indistinguishable from the wild type, being turbid and viscous due to polysaccharide excretion. The CR-O mutants showed the same autoagglutination and lack of polysaccharide production at all incubation temperatures. The autoagglutination of CR or CR-O mutants in liquid defined media was unaffected by altering the concentration of Ca^{2+} or Mg^{2+} ions between 0 and 0·5 mM and by the addition of EDTA up to 3 mg/ml. (w/v). Addition of Tween 80 in concentrations up to 1 % (w/v) also failed to prevent autoagglutination. All strains grew in the same manner using glucose, galactose, mannose or glycerol as carbon and energy source in defined media.

Reversion

A marked difference between CR and O mutants was in the stability of the mutation. In the *Klebsiella aerogenes* A4-O and other non-mucoid variants spontaneous revertants were never detected despite examination of more than 10,000 colonies. Application of various mutagens did not induce reversion. The same was true of the CR-O mutants. However the CR mutants reverted to the parental type with varying frequencies. Thus A3-CR reverted to A3(SL) spontaneously with 0·01 % frequency, whereas no spontaneous revertants from A4-CR were observed. The use of acriflavin, 2-aminopurine or ethanemethanesulphonate resulted in a reversion frequency of 0·3 % to 0·4 % in the mutant A4-CR. Lower frequencies were observed using other mutagens. The mutants from W70 behaved similarly.

Table 2. *The composition of slime and capsule exopolysaccharides*

Strain	Type	Growth temperature	Constituents	Molar ratio (approx.)
A4	Capsule, slime	All	Glc:Gal:GlcUA	1:2:1
A4-CR	Capsule, slime	30 to 35°	Glc:Gal:GlcUA	1:2:1
A3(S1)	Slime	All	Glc:Fuc:GlcUA:acetyl	4:2:2:1
A3-CR	Slime	30 to 35°	Glc:Fuc:GlcUA:acetyl	4:2:2:1
W70	Capsule, slime	All	Gal:Mann:GlcUA:acetyl	1:1:1:1
W70-CR2	Capsule, slime	30 to 35°	Gal:Mann:GlcUA:acetyl	1:1:1:1

Exopolysaccharide synthesis

It was obvious during the initial examination of cultures that differences in exopolysaccharide synthesis occurred between parent and CR strains. It was thus possible that the mutants might synthesize polysaccharides of altered composition and structure. The repeating unit of *Klebsiella aerogenes* A3(SL) slime polysaccharide has already been identified (Sutherland, 1967; Sutherland & Wilkinson, 1968). Hydrolysates of A4 polysaccharide contained glucose, galactose and glucuronic acid, while acetyl and pyruvoyl groups were absent. The capsular polysaccharide of strain W70 contained galactose, mannose, glucuronic acid and *O*-acetyl groups. The composition and approximate molar ratios of the polysaccharides are shown in Table 2 together with the composition of the material secreted by the three corresponding CR mutants when grown at 35°. The parent and mutant strains secrete polysaccharides of the same chemotype with sugars present in the same molar ratio. Further proof of the similarity of the pairs of polysaccharides was obtained by comparison of partial acid hydrolysates by paper electrophoresis and paper chromatography. The fragments obtained were identical for each parent and mutant strain. In addition, phage-induced fucosidases active against A3(SL) polysaccharide yielded the same hydrolysis products from A3(SL) and A3-CR polysaccharides.

On nitrogen-limited defined media with glucose as carbon and energy source, the production of polysaccharide was determined after separation from cells by the anthrone technique. The slime or capsular material was removed by boiling and the bacterial deposits by centrifugation. For all the strains examined, the greatest differences between parent and mutant strains were observed at 20° at which temperature the CR mutants produced only about 30 % that excreted by the parent in liquid medium. Similar results were obtained for each set of mutants and values for A4, A4-CR and

A4-CRO are given in Table 3. The CR-O mutants produced no polysaccharide detectable by the techniques used, and so resemble O mutants.

Lipopolysaccharides and glycogen

The occurrence of autoagglutination and 'roughness' in other species of Enterobacteriaceae in usually associated with the production of incomplete lipopolysaccharides (Lüderitz, Staub & Westphal, 1966). Thus examination of the mutant lipopolysaccharides might reveal loss of part of the macromolecule. An earlier study of the A3 and A4 strains showed that they contained 17 to 23 % galactose but only 2 % glucose (Sutherland & Wilkinson, 1966). They thus differed from the types of polymer common to members of the genera *Salmonella*, *Shigella* and *Escherichia*, which all

Table 3. *Exopolysaccharide production on nitrogen-limited media*

		Incubation temperature							
		35°			30°			20°	
Strain	Medium	24 hr	48 hr	72 hr	48 hr	72 hr	96 hr	72 hr	96 hr
A4	Solid	2·8	3·5	3·0	1·6	2·0	2·8	3·7	3·7
	Liquid	2·1	2·3	2·4	2·1	1·9	2·0	2·2	2·2
A4-CR	Solid	3·3	2·1	2·6	1·4	2·0	2·5	1·0	0·9
	Liquid	2·9	2·0	2·1	1·8	2·0	2·6	1·2	1·1
A4-CRO	Solid or liquid	0·1	0·1	0·1	0·1	0·05	0·05	0·05	0·05

All values are given as mg. polysaccharide (glucose anthrone value)/mg. cell protein.

Table 4. *The yield of lipopolysaccharides by CR and CR-O strains*

			Lipopolysaccharide		
Strain	Growth temperature	Lipopoly-saccharide (% dry cells)	Glucose	Composition galactose	Glycogen (% dry cells)*
A3-CRO	20°	1·10	2·4	19·0	2·2
	35°	3·10	1·2	15·0	5·5
W70-CR2	20°	1·45	1·1	14·0	2·8
	35°	5·09	1·0	16·3	1·0
A4-CRO	20°	2·57	1·6	17·9	5·4
	35°	3·33	1·2	18·6	1·9
A4-CR	20°	1·2	2·5	22·5	6·6
	35°	2·1	3·7	20·7	6·0

* The values for glycogen were derived by multiplying the glucose content of the ultracentrifuge supernatants by the weight of dry material.

contain approximately equal quantities of glucose and galactose in their basal structures (Lüderitz *et al.* 1965). The galactan nature of the lipopolysaccharide of Klebsiella A3(SL) and another strain has been confirmed by structural studies (Koeltzow, Epley & Conrad, 1968). Strain W70 also proved to have a similar lipopolysaccharide.

Several of the CR and CR-O strains were grown in nutrient broth containing 1% (w/v) glucose in shaken culture at 20° or 35°. The bacteria were harvested by centrifugation, washed and lyophilized. The yields of lipopolysaccharide extracted by phenol/water and purified by ultracentrifugation are shown in Table 4. It can be seen that the polymer yields were generally lower following incubation at 20° than those from 35°. The composition of the macromolecules as measured by their glucose and galactose contents appeared to be constant. The yields of glycogen varied considerably and do not show any distinct pattern.

Phage sensitivity

One of the methods used to check the identity of mutants with the wild type was to test the sensitivity of both strains to one or more bacteriophages. The bacterial culture was incubated in nutrient broth then spread over the surface of plates of nutrient agar. These were allowed to dry at room temperature for 30 min. Drops (0·02 ml.) of bacteriophage suspensions containing approximately 10^6 plaque-forming units were applied and incubation continued at varying temperatures. Initial tests showed no difference in phage sensitivity after incubation at 20° or 37°. When, however, the parent cells were incubated through two subcultures in nutrient broth at 20° then used to seed the plate, there were several differences from the sensitivity pattern observed at 37°. These results are shown in Table 5. It seems probable that in

Table 5. *Phage sensitivity patterns of mutant and wild-type strains*

(+, Confluent lysis; −, no lysis; ±, very small numbers of plaques.)

	Incubation 37°, phage:								Temperature 20°, phage							
Host strain	31	33	36	33	38	40	41	42	31	33	36	37	38	40	41	42
A3(S1)	+	+	+	+	+	+	−	−	+	+	−	+	+	+	−	−
A3-CR	+	+	+	+	+	+	−	−	+	−	+	±	−	+	−	−
W70-CR1	−	−	·	−	−	+	+	+	−	−	−	−	−	+	−	+
W70-CR2	−	−	−	−	−	+	+	−	−	−	−	−	−	+	+	−
A3-CRO	−	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−
W70	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+
A4	−	−	−	−	−	−	+	−	−	−	−	·	−	−	+	−
A4-O	−	−	−	−	−	+	+	−	−	−	−	−	−	+	±	−
A4-CR	−	−	−	−		·	+	−	−	−		−	−	−	−	−
A4-CRO	−	−	−	−	−	+	+	−	−	−	−	−	−	+	+	−
Original Host	←		A3St.		→	A1	A4	W70								

certain of the mutants the phage receptor is no longer synthesized at the lower temperature or alternatively the mutants are unable to liberate mature phage. If the defect is one of surface properties it is presumably due to a loss of carbohydrate metabolism at a step which is also essential for exopolysaccharide synthesis. Thus strain A3-CR was no longer susceptible to phages F33 and 38, which both acted on the parent and on A3-CR at 37°. It is interesting that in some cases the mutants are susceptible to more phages than the mucoid parent. This is presumably due to occlusion of the bacterial surface by the capsule preventing phage uptake as it is not seen in the A3 mutants where the parent strain produces slime only.

DISCUSSION

Mutations involving exopolysaccharide biosynthesis in *Klebsiella aerogenes* appear to occur in two ways. The wild-type organisms producing capsules and slime may undergo a mutation causing loss of the ability to form a discrete capsule. Such a slime-forming (SL type) mutants are exemplified by *K. aerogenes* A 3 (SL) which secretes an exopolysaccharide identical in all respects tested with the capsular polysaccharide synthesized by the parent cells (Dudman & Wilkinson, 1956). Either the wild type or the SL type can undergo a mutation causing complete loss of polysaccharide-synthesizing capacity. Such non-mucoid (O) mutants show no tendency to revert. Attempts to render them mucoid by transduction were unsuccessful, although other markers were transferred (McPhee, Sutherland & Wilkinson, 1969). Such O mutants deficient in precursor-synthesizing or transferase activity are analagous to many of the mutants isolated during studies on lipopolysaccharide biosynthesis (Lüderitz *et al.* 1966). Due to the lack of suitable selection procedures no mutants synthesizing in complete exopolysaccharide have yet been identified. Indeed by virtue of their repeating unit structure it is probable that the mutation is an 'all or none' phenomenon causing complete loss of polysaccharide-synthesizing capability. Nothing analagous to the 'rough' mutants isolated in studies on lipopolysaccharide biosynthesis has yet been found in exopolysaccharide studies.

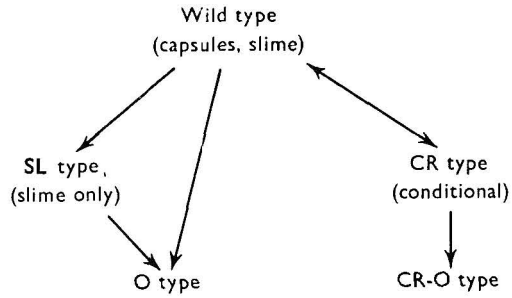

Fig. 1. Scheme for mutations involving polysaccharide synthesis in the genus *Klebsiella*.

The second and apparently unusual form of mutation is that involved in CR mutants. These apparently only arise spontaneously or under the influence of aminopurine. CR clones appear to be much less stable than O mutants. Further mutagenesis can, albeit rarely, lead to isolation of a double mutant retaining the characteristic colonial morphology of the CR mutant but having completely lost exopolysaccharide-synthesizing capacity even at 37°. The scheme involving such mutations is shown in Fig. 1. The CR mutants are obviously conditional mutants and are unusual in that the mutation appears to have two distinct effects, assuming that no double mutation has occurred. The first effect is on exopolysaccharide synthesis, which is greatly reduced by lower incubation temperature but returns to the level of the wild type on temperature up-shift. The second effect is on one of the surface components of the bacterial cells, apparently the lipopolysaccharide. This effect appears to be a quantitative one rather than an alteration to the lipopolysaccharide structure. A consequence of the lower lipopolysaccharide content is the agglutinability and 'roughness' of the cultures. These

observed results from the conditional mutation are consistent with an alteration to an enzyme involved in the formation of nucleotide precursors for the lipopolysaccharide and the exopolysaccharide or alternatively an altered regulator. The lipopolysaccharides of the Klebsiella strains used in this study contain galactose, heptose, 2-ketodeoxyoctonic acid and *N*-acetylglucosamine (Sutherland & Wilkinson, 1965). The sugars found in the slime or capsular polysaccharides are fucose, glucose, galactose glucuronic acid and mannose. Thus galactose is the only sugar common to both lipopolysaccharide and exopolysaccharide, but only in strains A4 and W70, *not* in A3(SL). We therefore conclude that the mutation occurs in the sequence of reactions.

glucose → glucose-6-phosphate → glucose-1-phosphate → UDP-glucose

Alteration in any of the three enzymes involved, hexokinase, phosphoglucomutase or UDP-Glc pyrophosphorylase would affect either polymer if UDP-Glc is the glucosyl donor involved in exopolysaccharide biosynthesis. The results for glycogen yields were not consistent enough to determine whether a significant reduction had occurred at lower temperatures. The donor for glycogen synthesis being ADP-glucose (Gahan & Conrad, 1968), a reduction in glycogen would favour the first two enyzmes in the sequence as the possible sites of mutation. One similar conditional alteration in surface property involving what is also probably a polysaccharide, has been reported (Knolle & Ørskov, 1967). The f^+ antigen of *Escherichia coli* is lost at lowered incubation temperature but is again formed on return of the cells to 37°.

Further experiments to identify the enzyme involved in the 'CR' conditional mutation of *Klebsiella aerogenes* strains are proceeding.

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The Influence of Various Moulds on the Multiplication of Some Mycophagous Mites

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SUMMARY

The aflatoxins of *Aspergillus flavus* are very toxic for mycophagous mites Glyciphagus and less toxic for Histiostoma. Some Penicillium species producing mycotoxins were not favourable for the multiplication of Glyciphagus, but other moulds such as *Penicillium roqueforti* were suitable.

INTRODUCTION

It is known that some species of moulds produce a series of substances known as mycotoxins, harmful to different animals (Wogan, 1964) and to some plants. Among these, the aflatoxins are considered to be amongst the strongest of biological poisons; they are produced by *Aspergillus flavus* (Hesseltine, Shotwell, Ellis & Stubblefield, 1966) and by some Penicillium strains (Hodges *et al.* 1964).

In our previous research on the mould flora of raw and smoked salami we found that the normal mould layer was consumed by some species of mycophagous mites (Racovitza & Racovitza, 1968). This raised the question whether mycophagous mites are sensitive to mycotoxins and especialy to aflatoxins.

METHODS

An aqueous suspension of mould spores (obtained in pure culture) was inoculated on Czapek-medium in test-tubes, so that the whole surface was evenly covered. The excess liquid was drained off to prevent the mites getting drowned. The tubes were kept in the laboratory for 3 to 4 days till the beginning of the sporulation. At that time a known number of adult mites, male and female, were introduced into each tube on the pointed end of the thin glass rod and shaken on the upper side of the culture. The tubes were then kept in a cool place in the laboratory for 23 to 70 days.

The tubes were divided by 4 to 5 rings with a diamond and each area was examined separately under a lens to observe the multiplication of the mites. The living larvae, nymphs, adults and hypopi (resistant stage) were counted separately. Then the medium was removed from each ring and examined under the binocular microscope to count the mites on the media as well as those on the glass. The end of the cotton-wool plug was carded with a needle over black paper and all the mites shaken out counted with a lens.

The influence on mite multiplication of *Aspergillus flavus* Link, *Penicillium commune* Thom, *P. cyclopium* Westling, *P. expansum* Lk., *P. roqueforti* and *P. viridicatum* Westling was examined on *Glyciphagus domesticus* de Geer and a *Histiostoma* sp. (fam. Tyroglyphidae).

The *Aspergillus flavus* strain was isolated from soil and was found positive for aflatoxin by tests on day-old ducklings (Racovitza, Racovitza & Constantinescu, 1969). The other species were isolated from salami surface.

RESULTS

The results showing the influence of the moulds on the multiplication of the Glyciphagus mite are seen in Table 1. In four experiments totalling 128 mites, the cultures of *Aspergillus flavus* were extremely toxic for this mite, which died shortly after feeding. After 23 days, only 2/10 were left and after 47 to 50 days all were dead. It was noticed that, although the females laid their eggs, the neonate larvae died.

Table 1. *Influence of various moulds on the multiplication of the mite Glyciphagus domesticus*

Experiment	Temperature	Mould	No. of tubes	Initial no. of mites in each tube	Time (days)	Average no. of living mites: Larvae	Average no. of living mites: Adult hypopi
1	18–28°	*A. flavus*	1	10	23	0	2
		P. roqueforti	1	10	23	0	16
		P. expansum	1	10	23	0	8
2	18–21°	*A. flavus*	4	12	50	0	0
		P. roqueforti	1	12	50	283	2
3	18–21°	*A. flavus*	1	10	47	0	0
		P. expansum	1	10	47	148	2
4	18–21°	*A. flavus*	6	10	42	0	0·3
5	14–22°	*P. commune*	3	10	50	91	35
		P. cyclopium	3	10	50	91	9
		P. expansum	3	10	50	108	14
		P. viridicatum	3	10	50	32	5

Table 2. *Influence of Aspergillus flavus and Penicillium roqueforti on the multiplication of the mite Histiostoma sp.*

Experiment	Temperature	Mould	No. of tubes	Initial no. of mites in each tube	Times (days)	Average no. of living mites
1	18–21°	*A. flavus*	4	5	70	71·5
		P. roqueforti	3	5	70	745·3
2	18–21°	*A. flavus*	10	10	42	86

In the experiment with *Penicillium roqueforti*, 10 mites increased to 16 after 23 days; no hypopus stage was found. In a second 50-day experiment, 12 mites grew to two nymphs and 283 hypopi.

Experiment 1 with *Penicillium expansum* showed no multiplication in 23 days, but in Expts. 3 and 5 the initial number of 10 increased, after 50 days, to 136 individuals, an increase of 13·6-fold.

The initial number of 10 increased to 126 (12·6-fold) on *Penicillium commune;* 10 to 100 (10-fold) on *Penicillium cyclopium*, and 10 to 37 (3·7-fold) on *Penicillium viridicatum.*

Table 2 shows the influence of *Aspergillus flavus* and *Penicillium roqueforti* on the multiplication of a *Histiostoma* sp. mite.

CONCLUSIONS

From these preliminary results, the following conclusions may be drawn:

The *Aspergillus flavus* strain which produces aflatoxin is extremely toxic for *Glyciphagus domesticus*, though less toxic for *Histiostoma* sp. Therefore within this group of Arthropoda there are differences in resistance to aflatoxins.

Penicillium viridicatum, which produces a toxin, viridicatine (Wogan, 1964), and *P. commune*, which produces a toxin causing gastrointestinal disturbancies to animals (Wogan, 1964), were both toxic to *Glyciphagus domesticus*, as was *P. cyclopium*, which probably produced a toxin.

Penicillium expansum and *P. roqueforti* did not appear to produce toxins and were suitable moulds for the growth and multiplication of the mites.

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Acceleration of Morphogenesis in *Dictyostelium discoideum* by Exogenous Mononucleotides

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SUMMARY

Ribose mononucleotides accelerated morphogenesis in *Dictyostelium discoideum* at 10^{-4} M and above; equimolar purines, pyrimidines, ribose, phosphate, ribose-5-phosphate and nucleosides either slowed morphogenesis or had no effect; 5′-GMP, 5′-UMP and 5′-CMP were about as effective as AMP. The position of attachment of the phosphate to the ribose moiety was not critical; 3′,5′-cyclic mononucleotides influenced chemotaxis and accelerated morphogenesis.

INTRODUCTION

Numerous low molecular weight organic or inorganic compounds accelerate morphogenesis in *Dictyostelium discoideum* (Bradely, Sussman & Ennis, 1956; Krichevsky & Wright, 1963; Krichevsky & Love, 1964*a*). For example, imidazole-containing compounds (imidazole, D- or L-histidine, urocanic acid, histamine), glucose, Mg^{2+}, K^{+}, PO_4^{3-}, are representative of those increasing the rate of sorocarp formation without affecting the quality of morphogenesis. They also prevent the efflux of RNA and protein from washed amoebae (Krichevsky & Love, 1965) with a concomitant internal accumulation of 2′,3′-mononucleotides (Pannbacker, 1966; Krichevsky & Love, 1968). The present communication describes the ability of mononucleotides to stimulate the rate of morphogenesis at much lower concentrations than any other single stimulant found.

METHODS

The procedures for growing and harvesting the myxamoebae, as well as for determining the effect of materials on the rate of morphogenesis, were described previously (Krichevsky & Wright, 1963). An outline of the rate assay follows. Amoebae from one 9 cm. diam. Petri dish were harvested, washed and resuspended in 10 ml. cold distilled water. Six 0·01 ml. samples were placed on the surface of each 2·5 % Noble agar plate containing the concentrations of compounds indicated. The unspread plates were incubated at 19° for about 26 hr until plates without added compound exhibited the first complete sorocarp formation. The total number of sorocarps (fruits) on each plate were determined. Compounds were tested for ability to prevent efflux of either RNA or protein from washed amoebae by assaying cell-free filtrates of amoebal suspensions containing the test compound (Krichevsky & Love, 1965); pentose-containing materials were assayed by the orcinol reaction of Mejbaum as modified by Horecker, Smyrniotis & Klenow (1953), protein was determined by the method of Sutherland, Cori, Haynes & Olsen (1949).

RESULTS

Krichevsky & Love (1968) postulated that one or more naturally occurring mononucleotides would stimulate the rate of morphogenesis if supplied exogenously. The rate of morphogenesis was indeed stimulated by 0·1 to 6·0 mM-5′-AMP, 5′-GMP, 5′-UMP, or 5′-CMP in agar test plates at pH 7·0, pH 6·0 or pH 5·0 (Table 1). The stimulations observed were solely upon the rate of morphogenesis and had little effect upon the ultimate pattern of sorocarp formation. The pH of the test plate had no marked effect on stimulation, though small quantitative differences were observed. The acceleration of the rate of morphogenesis by 5′-nucleotides was reproducible but subject to variation in quantitative detail.

Structural components of the 5′-nucleotides did not enhance the rate of sorocarp formation (Table 1): D-ribose and D-ribose-5-phosphate, at concentrations up to 6 mM, were inert; deoxyribose stimulated at low concentrations but gave more

Table 1. *Stimulation of the rate of morphogenesis by ribose-5′-mononucleotides and related compounds*

Samples were placed on the surface of agar plates containing the concentrations noted in the table. The pH was adjusted as indicated for the ionic compounds. For non-ionic compounds the pH was not adjusted. The plates (unspread) were incubated until the control plate exhibited the first complete sorocarp formation. The number of fruits on each plate were determined.

Addition	Concentration (mM)	No. of fruits pH 5	pH 6	pH 7
None	—	27	3	0
5′-AMP	0·1	11	—	36
	0·25	—	39	—
	0·5	95	59	91
	1·5	87	—	102
	3·0	—	85	—
	6·0	—	91	—
5′-GMP	0·1	12	—	41
	0·5	79	—	63
	1·5	167	—	135
5′-CMP	0·1	5	—	15
	0·5	85	—	39
	1·5	118	—	69
5′-UMP	0·1	88	—	5
	0·5	116	—	47
	1·5	126	—	42
Deoxyribose	0·25	-	25	—
	0·5	—	38	—
	3·0	—	10	—
	6·0	—	8	—
Ribose	0·25	—	14	—
	0·5	—	2	—
	3·0	—	12	—
	6·0	—	9	—
Ribose-5-PO_4	0·25	—	5	—
	0·5	—	3	—
	3·0	—	0	—
	6·0	—	7	—

variable results at high concentrations. Adenosine inhibited morphogenesis (data not shown) in a manner similar to that previously reported for adenine (Krichevsky & Love, 1964*b*); equimolar phosphate ion had no effect (Krichevsky & Wright, 1963).

Adenosine 2′- and 3′-phosphates accelerated differentiation both as single nucleotides and mixtures (Table 2). While the enhancements were similar to those observed with 5′-AMP, they were more variable. The position of the phosphate group on the pentose moiety of the adenine nucleotide is thus not critical since all adenosine mononucleotides accelerated morphogenesis.

Replacing a portion of the 5′-AMP with equimolar concentrations of other 5′-mononucleotides either singly or in various mixtures in most cases did not diminish the stimulatory effect and often enhanced it. The data given in Table 2 show the effects just discussed; many other combinations tested yielded similar results.

Table 2. *Stimulation of the rate of morphogenesis by adenosine-2′,3′ or 5′-monophosphate or mixtures of 5′-mononucleotides*

The same conditions described for Table 1 were used. The pH was adjusted to 6·0.

Additions	Concentration (mM as total phosphate)	No. of fruits
None	—	11
5′-AMP	0·5	38
	3·0	59
2′-AMP	0·5	76
	3·0	52
3′-AMP	0·5	15
	3·0	68
2′-AMP + 3′-AMP	0·5	110
	3·0	31
None	—	—
5′-AMP	0·25	93
	0·5	76
5′-AMP + 5′-GMP	0·25	115
	0·5	132
5′-UMP + 5′-GMP + 5′-CMP	0·25	36
	0·5	140
5′-AMP + 5′-GMP + 5′-UMP	0·25	146
	0·5	179
5′-AMP + 5′-GMP + 5′-CMP	0·25	160
	0·5	194
5′-AMP + 5′-GMP + 5′-CMP + 5′-UMP	0·25	39
	0·5	248

Previous work (Krichevsky & Love, 1965) demonstrated that stimulants of the rate of morphogenesis also prevented the leakage of RNA and protein from washed cells. Mg^{2+} is quite effective in blocking efflux and Fig. 1 compares 5′-AMP with Mg^{2+}. The combination of 5′-AMP plus Mg^{2+} was better able to prevent the RNA leakage than the Mg^{2+} alone. The efflux of protein followed a qualitatively similar pattern (Fig. 2). Efflux of RNA was not diminished by 5′-AMP in the range of 5×10^{-4} M through 1×10^{-2} M when compared to the efflux from cells suspended in distilled water.

Adenine is a potent inhibitor of the rate of morphogenesis in *Dictyostelium discoideum* (Bradley, Sussman & Ennis, 1956; Krichevsky & Love, 1964*b*). Figure 3 illustrates the effect of combining adenine with 5′-AMP: at the higher concentrations of 5′-AMP the ability of adenine to slow sorocarp formation was lessened, thus representing an apparent competition between the two compounds.

In all the experiments described so far, the added materials only affected the rate of morphogenesis. The sorocarp formation was qualitatively identical to that in the control agar gel containing only water. The differentiating multicellular structures were

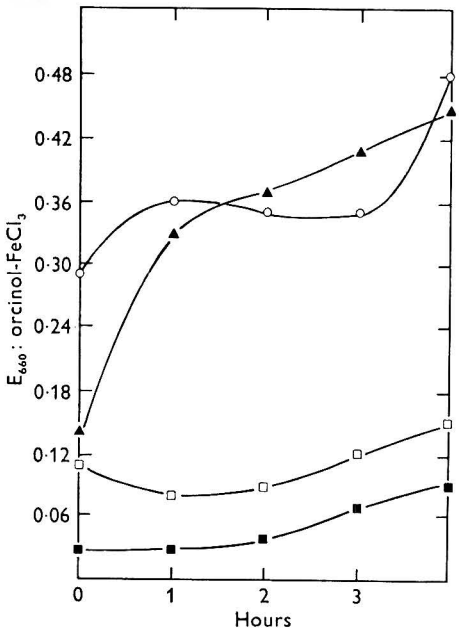

Fig. 1

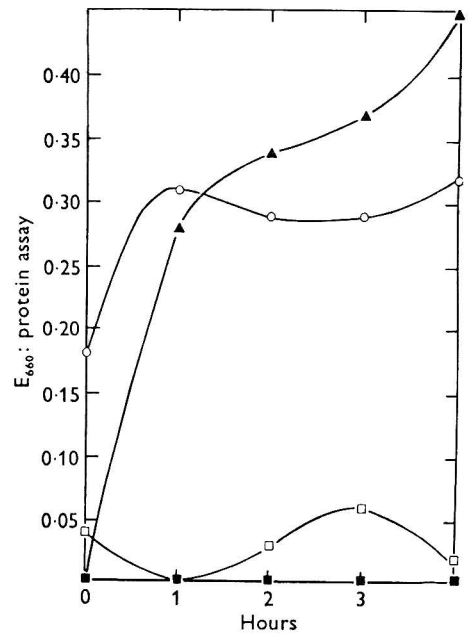

Fig. 2

Fig. 1. Efflux of RNA in the presence of various stimulants of the rate of morphogenesis. Amoebal suspensions were incubated for the times shown. Cell-free filtrates from suspensions of washed amoebae were obtained at the times indicated by the method described in Krichevsky & Love (1965). One-half ml. of filtrate was combined with 0·5 ml. of cold 3% perchloric acid. The precipitated RNA was recovered by a second filtration on polyvinyl chloride discs (1 in. diam., type VM-6, Gelman Instrument Co.). The pentose content of the precipitate was assayed directly from the filter. Previous work had established that no detectable low-molecular-weight, pentose-containing compounds are released by cell suspensions under these conditions. The additions were: none (▲); 5′-AMP, pH 6·0, 2×10^{-4} M (○); $MgCl_2$, 0·1 M (□); 5′-AMP, pH 6·0, 2×10^{-4} M + $MgCl_2$, 0·1 M (■) (final concentrations).

Fig. 2. Efflux of protein in the presence of various stimulants of the rate of morphogenesis. The incubations were the same ones described for Fig. 1. The same procedure was used to obtain the samples for protein assay, except that the perchlorate-treated samples were placed in a boiling water bath for 15 min., cooled, filtered through a 25 mm. type HA Millipore membrane filter and the precipitate redissolved in 0·3 ml. 0·5 M-NaOH.

randomly distributed within the original spots formed by placing the 0·01 ml. drop of suspension on the agar surface. A few pseudoplasmodia migrated outside the confines of the periphery. The individual amoebae did not.

An exception to the preceding description was observed with 3′,5′-cyclic AMP. Figure 4 shows that increasing concentrations of 3′,5′-cyclic AMP were about as

effective as 5′-AMP in accelerating morphogenesis. The common pattern of morphogenesis, as observed at the periphery of a spot, is shown in Pl. 1. In contrast, the inclusion of 3′,5′-cyclic AMP in the agar produced the result shown in Pl. 2, evenly spaced groups of cells migrated out from the edge of the original spot. Even though differentiation appeared to proceed normally within the confines of the original spot, the groups of cells which migrated outside did not differentiate further.

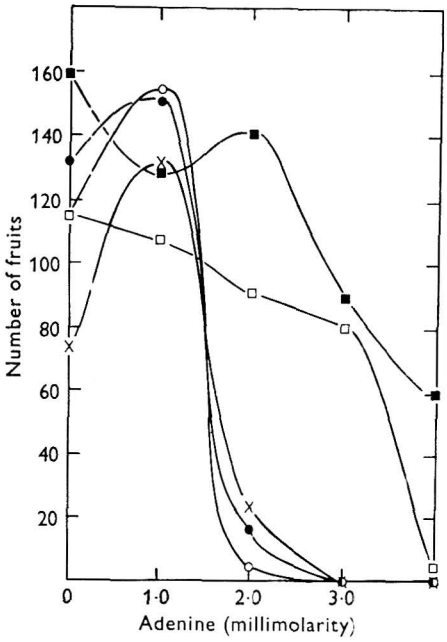

Fig. 3

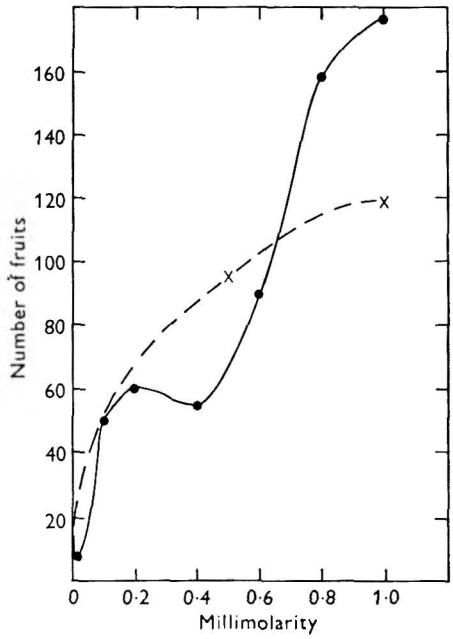

Fig. 4

Fig. 3. Reversal of adenine inhibition by AMP. The conditions were those as described for Table 2. The adenine concentrations were those indicated on the figure. The concentrations of 5′-AMP (pH 6·0) were 0 (×), 0·25 mM (○), 0·5 mM (●), 3·0 mM (□), 6·0 mM (■).

Fig. 4. Stimulation of the rate of morphogenesis by 3′,5′-cyclic AMP. The conditions were those described for Table 2. The additions were 5′-AMP (× – – ×) or 3′,5′-AMP (●—●).

DISCUSSION

A mononucleotide must be complete to stimulate the rate of morphogenesis in *Dictyostelium discoideum*, because purines, pyrimidines, ribose nucleosides, D-ribose, D-ribose-5-phosphate and phosphate either do not stimulate or actually inhibit at comparable concentrations. Deoxyribose is the sole exception.

The structural requirements for mononucleotide activity do not appear to be specific: a variety of purine and pyrimidine moieties are approximately equivalent and the position of the phosphate attachment to the pentose is not critical. Whether the non-specificity observed reflects actual non-specificity or is a consequence of the organism's ability to interconvert mononucleotides is not known.

Those substances which prevent the loss of protein and RNA from suspensions of amoebae enhance the accumulation of mononucleotides at the onset of morphogenesis and accelerate morphogenesis (Krichevsky & Love, 1968). These observations

suggest a causal relationship between the conservation of RNA within the cells and the stimulation of the rate of morphogenesis. The stimulation is presumably mediated by the resultant constituents of the RNA. Two observations from the present study are consistent with this postulated role of mononucleotides.

First, mononucleotides are the most efficient exogenously supplied stimulants of the rate of morphogenesis yet found. The effective concentrations are at least 10 times lower than those necessary for other stimulants (excepting deoxyribose) to show any stimulation and 100 times less when optimum concentrations are compared.

The second point is that 5′-AMP is ineffective in preventing leakage of RNA and protein from washed cells. That 5′-AMP does not prevent efflux indicates that the RNA conserved within the cells acts as a source of mononucleotides. When the mononucleotides are exogenously supplied it should be immaterial whether the RNA leaks out or is conserved.

A variety of stimulants of the rate of morphogenesis could still stimulate, albeit to a lesser extent, in the presence of normally inhibitory concentrations of adenine (Krichevsky & Love, 1964*b*). The ability of these stimulants partially to reverse the adenine inhibition is probably a reflection of the increase in the pool level of mononucleotides. It is the nucleotides themselves which actually annul the adenine inhibition. Thus any material which stimulates the rate of morphogenesis by preventing the efflux of RNA can be expected to annul adenine inhibition.

The work of Bonner and his associates (Konijn, van de Meene, Bonner & Barkley, 1967; Chang, 1968; Konijn, Barkley, Chang & Bonner, 1968) provides a partial explanation for the observations with cyclic-3′,5′-AMP. In these papers the ability of cyclic-3′,5′-AMP to act as the long-sought acrasin and the appropriate cyclic monophosphate phosphodiesterase to act as the acrasin-destroying enzyme are described. In the last paper referred to (Konijn *et al.* 1968) the authors describe ring formation similar to that illustrated in Pl. 2 and postulate that 'the cells deplete the concentration of cyclic AMP at the site of the drop by means of their phosphodiesterase and that this produces an ever widening circular gradient which orients the ring of amoebae in their outward expansion'. This postulate can be expanded to include the stimulation of the rate of differentiation inside the spot of amoebae. Since the product of the phosphodiesterase reaction is 5′-AMP and this compound is a stimulant of the rate of differentiation, it is not surprising that morphogenesis accelerates within the spot.

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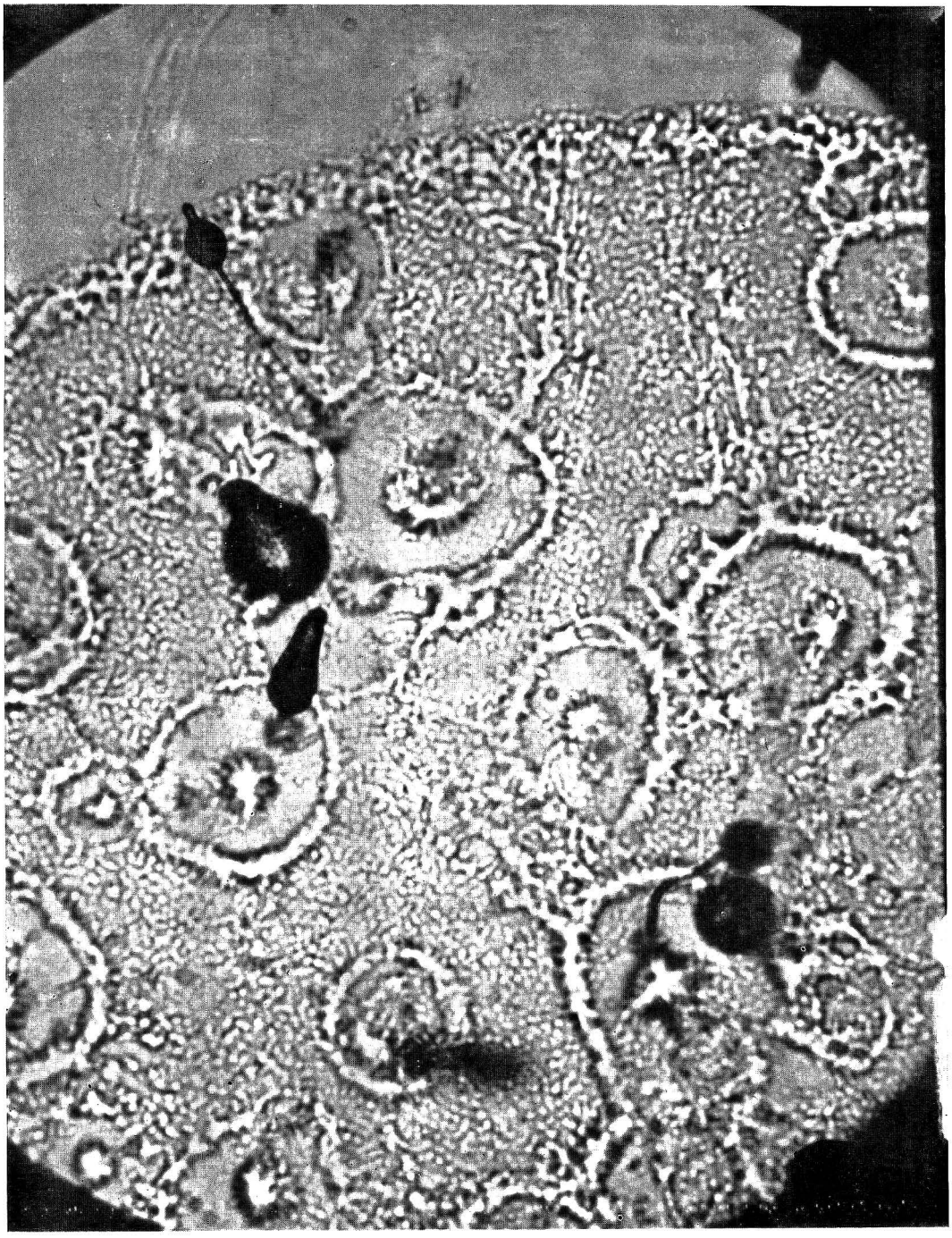

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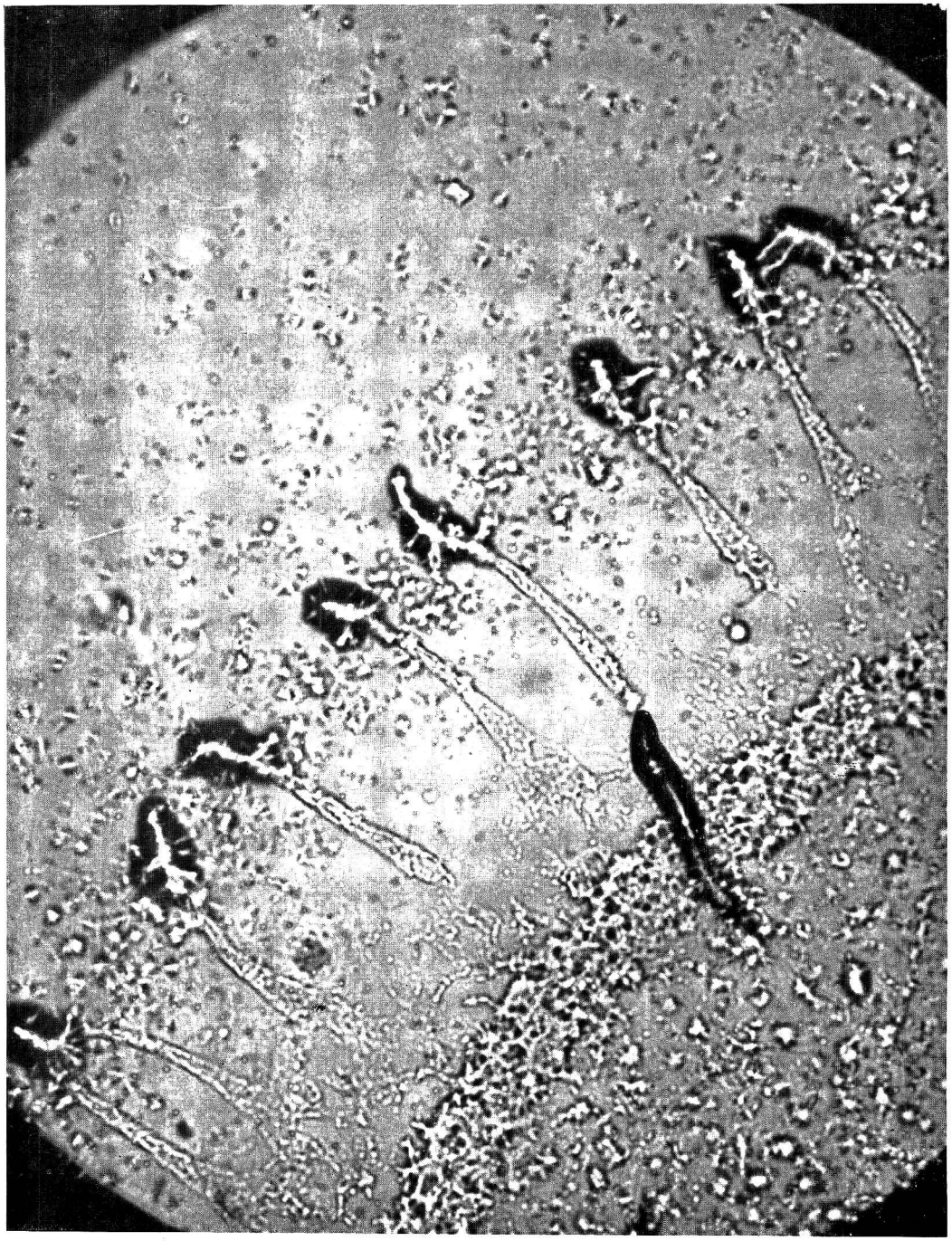

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EXPLANATION OF PLATES

PLATE 1

The periphery of a spot of amoebal suspension placed on washed agar. The conditions were those described for the morphogenetic rate assay. The agar gel contained only water.

PLATE 2

The periphery of a spot of amoebal suspension placed on washed agar containing 5×10^{-4}M-3′,5′-cyclic AMP. This photograph was taken at the same time as Pl. 1. The concentration of amoebae was identical. The magnification was 40× using bright field optics, polaroid 3000 film in Zeiss WL stand fitted with 1·3 NA condenser.

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Proposal for Classifying Organisms Related to *Mycoplasma laidlawii* in a Family Sapromycetaceae, Genus *Sapromyces*, within the Mycoplasmatales

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SUMMARY

It is proposed that a second family be re-established within the Mycoplasmatales for those strains not requiring sterol. Following the nomenclature of Sabin, who first made this proposal, the sewage strains of Laidlaw and Elford are renamed *Sapromyces laidlawii* and assigned to a family Sapromycetaceae. The status of strains not requiring sterol is discussed, including that of sewage A and B. In the light of recent evidence the pig isolates, originally named *Mycoplasma granularum*, seem to belong to the genus *Sapromyces*. Recently described tissue-culture isolates, as yet unnamed, represent another species within the Sapromycetaceae. It is questionable whether the organisms classified as *M. laidlawii* var. *inocuum* deserve the status of a named variety of *S. laidlawii*.

In the first three decades of this century two organisms with unique properties were recognized, namely those causing contagious bovine pleuropneumonia and contagious agalactia. Ledingham (1933) suggested assigning them to the family Actinomycetaceae, but Turner (1935) proposed a new order, since he did not consider their unique properties permitted their inclusion in any existing order of the Schizomycetes. Subsequently other similar organisms were isolated from a variety of sources, leading to the recognition of a group of organisms which came to be known as pleuropneumonia-like organisms (PPLO) because of their similarity to the organism causing contagious bovine pleuropneumonia. Isolations included those made by Laidlaw & Elford (1936) from sewage and by Seiffert (1937*a*, *b*) from soil, compost and manure, and which were regarded as saprophytes.

Sabin (1941*a*) proposed a classification and nomenclature for those organisms of the pleuropneumonia group known at that time. Regarding them as distinct from the Schizomycetes, he suggested placing them in a new class with two families, one containing those species parasitic for animals and the other with one species represented by the sewage isolates of Laidlaw and Elford. Believing the sewage organisms to be saprophytic, the species was named *Sapromyces laidlawi* and the family Saprophytaceae, later amended to Sapromycetaceae to conform to the Rules of Bacteriological Nomenclature (Sabin, 1941*b*).

Edward & Freundt (1956) in their classification recognized only a number of species

of one genus *Mycoplasma*, belonging to an order Mycoplasmatales. They discussed whether the saprophytic strains should be placed in a separate genus, but did not consider that at that time there was sufficient evidence to justify it. At its first meeting in 1966 the Subcommittee on Taxonomy of Mycoplasmatales, while approving the system of classification and nomenclature proposed by Edward and Freundt and recommending its extension and also proposing that the Mycoplasmatales be assigned to a new class, took the view that it was still premature to establish a more elaborate taxonomic scheme for the mycoplasmas, such as placing *M. laidlawii* in a higher taxon (*Minutes*, 1967; Edward *et al.* 1967).

Proposal for the establishment of a second family

A classification in which only a number of species of one genus in a whole biological class is recognized is obviously unsatisfactory and can only be regarded as a temporary measure (see Edward & Freundt, 1969). Moreover, there have recently been numerous isolations of strains similar to *Mycoplasma laidlawii* from mammals, birds, man and tissue cultures and their investigation has revealed certain differences from the classic sewage strains. We therefore consider that the time has now arrived to propose that *M. laidlawii* should be placed in a higher taxon to permit further differentiation and classification of the strains related to it.

It has long been recognized that the 'saprophytic' sewage strains do not require media enriched with serum for growth, whereas other mycoplasmas are serum-dependent. It is now known that the latter need sterol for growth, but sterol is not required by *Mycoplasma laidlawii* and related strains. The requirement of most *Mycoplasma* species for sterol, all of which is incorporated in the membrane lipids, indicates that they differ in membrane composition from *M. laidlawii*. Differences in membrane structure are also suggested by the finding of different locations of nicotinamide adenine dinucleotide phosphate oxidase activity between *M. laidlawii* and six other species examined (Pollack, Razin & Cleverdon, 1965). We have pointed out elsewhere that sterol dependence is a character of fundamental importance. It is one shared by most mycoplasmas and thus helps to distinguish them from other micro-organisms and to justify the Mycoplasmatales being placed in a separate class from the Schizomycetes (Edward & Freundt, 1969). Since *M. laidlawii* and strains like it do not share this property it would seem justifiable to assign them to a taxon of at least as high a rank as a separate family within the Mycoplasmatales.

It has already been pointed out that Sabin (1941 *a*, *b*) had proposed assigning the sewage strains of Laidlaw and Elford to a separate family and genus, named Sapromycetaceae and *Sapromyces* respectively. Since many of the strains that now require to be placed in this genus have been isolated from animals and it is even possible that some are pathogenic, a name suggesting a saprophytic role is unfortunate. However, as the name of the genus was validly published, it cannot be rejected as inappropriate under Rule 23 of the International Code of Nomenclature of Bacteria (1966).

It is therefore proposed that a family Sapromycetaceae be re-established in the order Mycoplasmatales with a genus *Sapromyces*, the type species being *Sapromyces laidlawii*.

Classification within the second family

Placing the non-sterol requiring strains in a separate family permits their further classification into other genera and species; investigation of such strains is therefore to be encouraged. A beginning has already been made by Tully and Razin. Some 24 strains, mostly isolated from animal and human materials and from tissue cultures, but including the classic strains from sewage and soil, had with few exceptions the same biological properties and behaved similarly when tested serologically by the indirect fluorescent antibody technique (Tully & Razin, 1968). These strains can therefore be regarded as representing the type species *Sapromyces laidlawii*.

The sewage isolates of Laidlaw & Elford (1936) fell into three serological groups. Sewage C appeared distinct by the agglutination reaction, but is no longer available for study. Sewage B was agglutinated only to low titre by an antiserum to Sewage A. In more recent tests of Sewage A and B strains by growth inhibition contradictory results have been obtained. Whereas Morton (1966) and Tully & Razin (1969) obtained equally sized zones of inhibition in reciprocal tests, Clyde (1964) and Argaman & Razin (1969) found only partial inhibition with the heterologous antisera. J. Fabricant (personal communication) was unable to distinguish between Sewage A and B by the metabolic inhibition test. The patterns of cell proteins of Sewage A and B produced by polyacrylamide-gel electrophoresis, although showing a basic similarity, differed in detail, and the many strains of *S. laidlawii* studied by Tully & Razin (1969) exhibited identical or very similar patterns to either Sewage A and B. In view of these findings, more particularly the contradictory serological results, it is not clear whether Sewage A and B strains should be established as distinct varieties or types of *S. laidlawii*.

Tully & Razin (1968) also examined four strains previously classified as *Mycoplasma granularum*. They found them indistinguishable from Laidlaw's sewage strains in their ability to grow on serum-free media and in their fermentation patterns. Like the sewage strains they produced carotenoids, whereas those serum-requiring *Mycoplasma* species tested did not. The electrophoretic patterns of the sewage strains and the *M. granularum* strains showed a basic similarity. When colonies of the two groups of organisms were tested with specific antisera by the direct fluorescent antibody technique there were no cross-reactions. However, there was a partial one-way cross between the *M. granularum* strains and a *S. laidlawii* antiserum in the indirect fluorescent antibody test. There was a similar one-way cross in the growth-inhibition test using one antiserum to a sewage strain, although no inhibition was found later using three other antisera (Tully & Razin, 1969). Similar crossing between the *M. granularum* strains and a *S. laidlawii* antiserum was noted in the complement fixation test without there being any fixation with *S. laidlawii* antigen and a high titred *M. granularum* antiserum (J. G. Tully, personal communication). These findings provide confirmation that the *M. granalarum* strains represent a distinct species. This species must be reclassified as belonging to the genus *Sapromyces* and will be renamed *Sapromyces granularum*.

Tully & Razin (1969) investigated two tissue-culture isolates, serologically identical with each other but distinct from the 43 other species and serotypes of Mycoplasmatales tested. Both were independent of sterol for growth, but unlike the sewage and *S. granularum* strains did not form carotenoids. It is clear that these two strains can be regarded as belonging to a distinct new species within the Sapromycetaceae, al-

though no name has yet been proposed. Only in the light of further work on the non-sterol requiring Mycoplasmatales will it be possible to evaluate the taxonomic importance of ability to synthesize carotenoids and to determine whether it is justifiable to place this species in a separate genus.

Consideration must also be given to the status of *Mycoplasma laidlawii* var. *inocuum* (Adler & Shifrine, 1964), isolated from chickens and capable of growth in serum-free media. Although originally designated as a separate species, *Mycoplasma inocuum* (Adler, Shifrine & Ortmayer, 1961) it was later recognized to be serologically similar to *M. laidlawii*. On the basis that it differed in regard to a biochemical property, namely an ability to metabolize glucose through a hexose monophosphate shunt, this shunt being absent from the representative strain of *M. laidlawii* (Castrejon-Diez, Fisher & Fisher, 1963), it was regarded as a variety of *M. laidlawii* and renamed *M. laidlawii* var. *inocuum* (Adler & Shifrine, 1964). This organism was included in the investigations of Tully & Razin (1968, 1969), where it behaved similarly to other strains of *S. laidlawii* in regard to carbohydrate fermentation, serology and electrophoretic pattern of cell proteins. At this stage, in the absence of detailed biochemical studies of the metabolism of other Sapromyces strains, it is questionable whether the chicken organism deserves the status of a named variety of *S. laidlawii*.

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Taxonomy of the Genus *Thiobacillus*: the Outcome of Numerical Taxonomy Applied to the Group as a Whole

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SUMMARY

Data from previous numerical analyses have been combined to produce an over-all classification of the genus *Thiobacillus*; 93 strains were examined using 38 tests and 106 character states. The results indicated the possible existence of a new species and demonstrated an unexpectedly close similarity between *T. neapolitanus* and *T. thio-oxidans*.

A number of diagnostic tests have been selected from those used for the numerical analysis. A brief description of the other important features of each species recognized is also given.

INTRODUCTION

The reasons for this investigation of the taxonomy of the thiobacilli and a description of the numerical methods used were given in an earlier paper (Hutchinson, Johnstone & White, 1965). Originally it had been intended to examine all the species using the same series of tests but this was not possible because of the differing physiological requirements of the various species. For this reason *Thiobacillus novellus*, *T. thioparus*, *T. neapolitanus* and '*T. trautweinii*' were tested with thiosulphate as the substrate at pH 6·6 (Hutchinson *et al.* 1965), whereas *T. thio-oxidans* and *T. ferro-oxidans* had to be tested in a similar medium at pH 5 (Hutchinson, Johnstone & White, 1966). The anaerobic species *T. denitrificans* and certain other strains were tested both aerobically and anaerobically at pH 6·6 and 7·0, respectively (Hutchinson, Johnstone & White, 1967). The data from these tests were analysed numerically and the results were highly significant for the groups of species under test. It was apparent, however, that an over-all indication of the interrelationships of the various species could only be obtained from a single numerical analysis which included all the species.

In an attempt to devise a test series which would be suitable for all the species, serious consideration was given to pH values in the range between 5·0 and 6·6, but no one value was satisfactory. The choice, therefore, was between a test series in which some of the species would not grow because of the initial pH value, and a numerical analysis in which the various groups of species were tested at, or near, their optimum pH value. The latter course was regarded as most appropriate although this involved the abandonment of strict Adansonian principles. To achieve further

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uniformity, thiosulphate was chosen as the basal substrate for these tests in that this substrate is utilized by all of the strains.

Numerical methods in taxonomy are designed to remove personal bias from the final result and the authors believe that the present study has been made as objectively as possible. In one instance, however, a separation between two species has been maintained despite the evidence from the numerical analysis.

METHODS

Organisms

From the 128 strains available 87 were selected for inclusion in the analysis together with six strains not described previously. The latter are listed in Table 1. The strains omitted were mainly from groups 3 and 4 and were in most cases duplicates of strains included in the analysis.

The results for all the tests based on thiosulphate were pooled, irrespective of the pH value at which the tests were made. Where necessary, new limits were drawn up for the quantitative tests and the data re-scored. Details of the tests are given in Table 2. In this final analysis 106 characters were scored for the 93 strains.

Table 1. *Strains not previously described and their sources of origin*

Source	Species	Code no.
R. L. Starkey, Rutgers University, New Jersey	*T. novellus**	S 1
	T. novellus†	S 2
From lagoon system treating carbonization liquor	*T. neapolitanus*	6 G
	T. neapolitanus	7 G
Natural spring of sulphuretted water	*T. neapolitanus*	1 R
Laboratory scale plant treating gas liquor	*T. neapolitanus*	1 V

* Maintained on thiosulphate agar. † Maintained on nutrient agar.

Analysis of data

The results were scored according to the method of Beers & Lockhart (1962) and the S values computed according to the method given by Sneath (1957). The order of the rearranged matrices was obtained by inspection.

RESULTS

From the rearranged matrix of S values given in Fig. 1 it is apparent that the groups 0 to 7 which were found corresponded with those reported previously for the individual series of numerical analyses (Hutchinson *et al.* 1965, 1966, 1967). A new group was found which had been overlooked in the previous analyses and this has been designated group 1*a*. This new group does not correspond to any named species and is represented by only two isolates, 10G and 11G, from a lagoon system treating carbonization effluent.

The essential data from the rearranged matrix of S values are listed in Table 3. This method, which has been used previously, permits the comparison of two adjacent groups with reference to the central strain of one of the groups. Thus, in the comparison of groups 3 and 4, strain P2 was selected as the central strain, and the S values

Table 2. *Key to tests used in the final analysis of the thiobacilli*

Test	Concentration (%)	Comments	Characters
Sulphur	Excess	Sterilized by steaming on 3 successive days (Hutchinson *et al.* 1965)	2
Ammonium thiocyanate	0·02	Previously described (Hutchinson *et al.* 1965)	2
Hydrogen sulphide	.	Previously described (Hutchinson *et al.* 1965)	2
Ferrous iron	2	Growth determined visually	2
Thioacetamide	0·02	Titrated with iodine	4
Nutrient agar	.	Visible colonies	3
Citrate utilization	.	Growth with citrate as sole energy source	2
Thiosulphate liquid medium	1	Final pH attained	6
	1	Amount of thiosulphate used at 19°	3
	1	Amount of thiosulphate used at 29°	3
	1	Amount of thiosulphate used at 35°	3
	1	Rate of thiosulphate oxidation at 19°	3
	1	Rate of thiosulphate oxidation at 29°	3
	1	Rate of thiosulphate oxidation at 35°	3
	0·5	Amount of thiosulphate used	3
	2	Amount of thiosulphate used	3
	6	Amount of thiosulphate used	2
Anaerobic growth	.	Amount of thiosulphate used in S 8 liquid (Hutchinson *et al.* 1967)	3
	.	Gas production under anaerobic conditions	2
Thiosulphate agar	.	Sulphur deposition	2
	.	Irregular margin to the colony	2
Inhibitors			
Streptomycin	.	These tests were done on plates using 'Sentest' tablets (Evans Medical Ltd.)	2
Chloramphenicol	.		2
Bacitracin	.		2
Novobiocin	.		2
Ampicillin	.		2
Sulphonamide	.		2
Elemental sulphur	Excess	The results were scored as a percentage of the amount of thiosulphate oxidized relative to a parallel control series grown under the standard conditions with no inhibitor present	4
Nutrient broth	1		4
Mixed phosphate	4		4
Sodium chloride	5		2
Ammonium thiocyanate	0·02		3
Potassium nitrate	1		3
Calcium chloride	1		2
Ammonium chloride	2·5		3
Sodium glutamate	1		4
Phenol	0·0025		3
Phenol	0·01		

of group 3 to this strain ranged from 74 to 89, with a mean value of 83. The highest S values of group 4 to strain P2 was 57, indicating a gap between the two groups of 17 units. If one assumes a normal distribution of S values about the central organism of each group of plus or minus three standard deviations, the separation of the groups is not as precise as that for the previous individual studies (Hutchinson *et al.* 1965, 1966, 1967). A certain degree of overlap has arisen largely as a result of the adaptation

of the scoring system to encompass the wide variety of strains. However, on the basis of the lowest S value of the group with the highest range of S values and the highest S value of the lower group, there is reasonable agreement between all the results of this and the previous analyses.

The overlap between groups 4 and 5 was rather unexpected because, as explained previously, these two groups cannot be tested at the same pH value. However, once this difference has been removed the two groups are of sufficient similarity to overlap each other on several important points; notably their tolerance of high salt concentrations and growth in 6 % (w/v) $Na_2S_2O_3.5H_2O$.

Table 3. *Comparison of S values to the central strains for adjacent groups*

		S values of group A to central strain of group A				S values of group B to central strain of group A			
A	B	1	2	3	4	1	2	3	4
Group 0 (1A is central strain)	Group 1*a*	89	78	6·1	68	55	52	*	49
Group 1 (a1 is central strain)	Group 1*a*	97	87	7·6	78	55	54	*	52
Group 0 (1A is central strain)	Group 1	89	78	6·1	68	69	61	5·4	52
Group 1 (a1 is central strain)	Group 2	97	87	7·6	78	64	60	2·8	58
Group 2 (8G is central strain)	Group 3	94	93	1·0	92	73	58	6·8	47
Group 3 (p2 is central strain)	Group 4	89	83	5·0	74	57	50	3·0	43
Group 4 (1I is central strain)	Group 5	97	85	5·7	74	81	74	4·6	66
Group 5 (2P is central strain)	Group 6	89	84	4·9	75	61	50	6·8	38
Group 6 (b7F is central strain)	Group 7	91	86	4·3	77	53	46	*	40

* Not applicable.
1 = Highest S value in the set.
2 = Mean S value in the set.
3 = One standard deviation of the S values for the set.
4 = Lowest S value in the set.

If the groups are drawn out spatially they form a network, not the succession of different types as the linear order of the rearranged S value matrix might suggest. An attempt to represent this in a two-dimensional plan is given in Fig. 2. This construction is based on the central strain of each group, i.e. the organism possessing the least standard deviation in its S values to the remainder of the strains of the group. These have been arranged relative to each other by way of 'best fits' using $\log_2 1/S$ (Rogers & Tanimoto, 1960) as the measure of distance. As the production of these diagrams involves the condensation of large amounts of data into two dimensions, it is considered that the method used here is sufficient to achieve a simple over-all picture of interrelationships. A more precise method for calculating the relative positions of the groups has been suggested by Rayner (1965).

The heterogeneity of the groups themselves is represented by the circle of radius

$2\sqrt{(\Sigma d^2/n)}$ (Gyllenberg, 1965), where d is the distance of the strains from the central strain of the group and n the number of strains within the group. Any overlap of these circles does not necessarily indicate that the two groups concerned merge into each other, the circles only indicate the diversity of the individual groups: in most cases the groups concerned are possibly in different planes. When drawn out in the two dimensional state, planes may be drawn which serve to segregate the species according to certain characteristics which were highly correlated with other tests in the analyses, e.g. autotrophy/heterotrophy and final pH attained in culture; tests which would be termed 'key features' of conventional systematics.

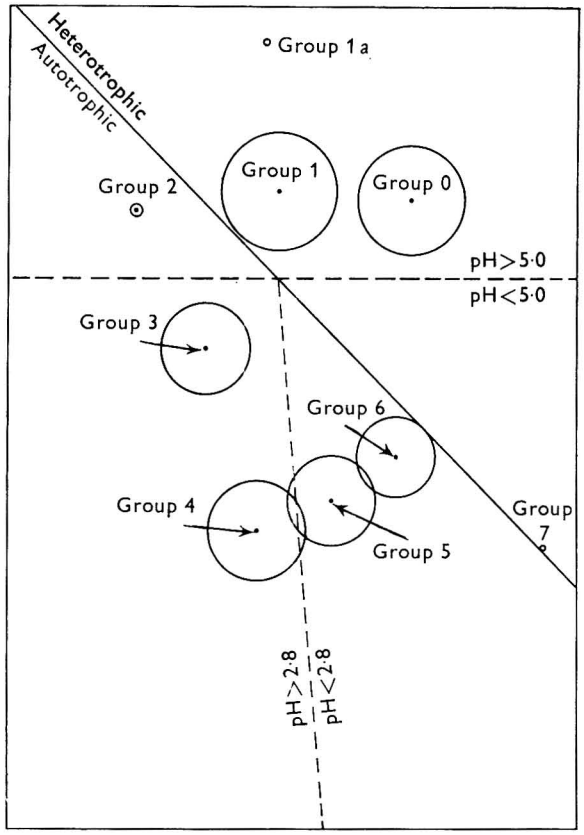

Fig. 2. Plan of the relationships of the groups of thiobacilli showing planes which separate the groups according to specific characters. Relative distance of the groups is based on the relationship $\log_2 1/S$ for the centrotype of each group. The area of the circles is proportional to the range of S values within the group according to the expression: radius $= 2\sqrt{(\Sigma d^2 n)}$, where d is the distance in terms of difference in S value between each strain and the centrotype of the group and n is the number of strains within the group.

For the reasons given, it would be unwise to regard this total analysis as representing a true picture of the relationships of the various groups of thiobacilli. Therefore greater attention should be paid to the individual analyses where the strains have been compared directly with others under the same condition of growth. This is not so much a failing of Adansonian principles but is specific to the groups of organisms

involved, for there can be few genera of bacteria in which the species cannot be tested under the same physiological conditions so that direct comparisons may be made between them.

The results of this work indicate that the classification of Parker & Temple (1957) is basically correct, though certain of the diagnostic criteria have been found to be invalid. Our assessment of the status of the various species is given in Table 4.

Table 4. *List of species and redundant names of the thiobacilli*

Species	Original description	Comments
Thiobacillus novellus	Starkey (1934, 1935*a*, *b*)	Good species
T. denitrificans	Beijerinck (1904*a*, *b*)	Good species
T. thioparus	Starkey (1935*a*, *b*)	Type species
T. neapolitanus	Parker & Prisk (1953)	Good species
T. thio-oxidans	Waksman & Joffe (1921, 1922)	Good species
T. ferro-oxidans	Temple & Colmer (1951)	Good species
T. intermedius	London (1963)	Good species
Group 1 a	Hutchinson *et al.* (this work)	Doubtful species
Ferrobacillus ferro-oxidans	Leathen & Braley (1954)	Redundant, new name *T. ferro-oxidans*
F. sulfo-oxidans	Kinsel (1960)	Redundant, new name *T. ferro-oxidans*
T. thiocyanoxidans	Happold *et al.* (1954)	Redundant, new name *T. thioparus*
T. concretivorus	Parker (1945)	Not examined in the present study
T. coproliticus	Lipman & McLees (1940)	Not examined in the present study
T. thermophilica	Egorova & Deryugina (1963)	Probably different genus
Strain 1D	Hutchinson & White (1964)	Probably different genus
T. trautweinii	Trautwein (1921)	Many heterotrophic strains are of this type and should not be regarded as thiobacilli

Procedure for the identification of an unknown strain of Thiobacillus

To achieve an unambiguous identification, the following procedure should be strictly adhered to, paying particular attention to the time factor.

The culture should first be inoculated into both S5 and S6 media to determine the appropriate pH value for the tests. Experience has shown that it is necessary to passage iron oxidizing strains suspected of being thiobacilli, through a sulphur medium before testing them in thiosulphate.

Test media should be inoculated with approximately 0·02 ml. of a 3-day-old culture under the following standard conditions which should be adhered to throughout the test series. These conditions consist of growing the strains at 28° in 10 ml. quantities of the appropriate medium contained in 50 ml. conical flasks in static culture to ensure an adequate but uniform, degree of aeration. Growth is determined by the amount of thiosulphate oxidized and the pH value after 28 days.

The deposition of sulphur intercolonially is determined by microscopical examination of the culture on thiosulphate agar over a period of up to 28 days. Sulphur appears, by transmitted light, as a black opaque material which may be of a crystalline or globular form. Both of these forms have proved to be sulphur by X-ray analysis. Certain strains of group 0 produce a white intercolonial deposit which appears as a 'brown' amorphous deposit on microscopical examination by transmitted light. This material has been identified as sodium sulphate by X-ray analysis.

Table 5. *Useful diagnostic tests for the thiobacilli*

Species ...		A	B	C	D	E	F	G	H	I
Group ...		0	1*a*	1	2	3	4	5	6	7
Medium	Criterion									
S6/S5	Final pH	> 6·6	6·5–5·0	6·6–5·0	6·6–5·0	6·6–3·5	3·5–2·8	< 2·0	< 2·0	> 2·0–< 2·8
S6/S5	% thiosulphate oxidation	< 90%	< 50%	< 30%	< 90%	> 90%	> 90%	> 90%	> 90%	> 90%
So+6% thiosulphate	% thiosulphate	< 10%	< 10%	< 10%	< 10%	< 10%	> 10%	> 10%	< 10%	< 10%
S6/S5+4% phosphate	Inhibition	+/−	+	+	+	+	−	−	+	+
S6/S5+5% NaCl	Inhibition	+	+	+	+	+	−	+/−	+	+
Sulphur	Final pH	ng	6·1–6·25	ng	5·15	4·6–4·1	3·2–2·8	< 2·0	< 2·0	> 2·0–< 2·8
S8	Growth	+	+	−	+	+/−	−	−	−	−
Anaerobic conditions	Gas formation	−	−	−	+	−	−	−	−	−
S7	Thiocyanate oxidation	−	−	−	+	+	−	−	−	−
So+O5% dithionate	pH change	−	−	−	−	+/−	−	+	−	−
Iron	Iron oxidation	−	−	−	−	−	−	−	+	−
Nutrient agar plate	Growth	+	+	+	−	−	−	−	−	+
Kosers citrate	Growth	+	−	−	−	−	−	−	−	+
Thiosulphate agar	Sulphur deposition	−	−	+	+	+	+	+	−	+

KEY TO SPECIES. A = 'trautweinii' types: B = group 1*a*: C = *Thiobacillus novellus*: D = *T. denitrificans*: E = *T. thioparus*: F = *T. neapolitanus*: G = *T. thio oxidans*: H = *T. ferro-oxidans*: I = *T. intermedius*: ng = no growth.

The identification and description of the species of the genus Thiobacillus

In view of the extended period for which the diagnostic tests must be run, the normal dichotomous key has been discarded in favour of the diagnostic table suggested by Cowan & Steel (1961). The relevant test media and diagnostic criteria are listed in Table 5.

The most important of these criteria is the final pH attained in the S6 or S5 medium and the majority of the other tests should be regarded as confirmatory. Data on the individual species which are not easily tabulated are included in the following brief descriptions.

Table 6. *The effect of the initial pH of the medium and its effect on the utilization of thiosulphate by certain facultative autotrophic strains*

Group...	0				1	
Strain...	d1		P4		52	
Initial pH of thiosulphate medium	Thio-sulphate used (%)	Final pH	Thio-sulphate used (%)	Final pH	Thio-sulphate used (%)	Final pH
5·05	0	5·05	0	5·05	0	5·05
6·5	42	7·65	44	7·7	10	5·5
7·6	17	8·2	20	8·55	19	5·15

Results were read after 28 days incubation at 28°.

Group 0 (*'T. trautweinii'*). The members of this group are facultatively autotrophic and oxidize thiosulphate to tetrathionate with a corresponding increase of the pH value. As reported previously (Hutchinson *et al.* 1965), this group is very heterogenous and subsequent electron micrographs have shown that one of the two authentic strains of *'T. trautweinii'* is peritrichously flagellated whereas the genus *Thiobacillus* is defined as consisting of Gram-negative, rod-shaped cells; non-motile or motile, by means of a single polar flagellum (Parker & Temple, 1957). For these reasons it is proposed to exclude these organisms from the genus *Thiobacillus* even though certain of them may oxidize greater amounts of thiosulphate than other undoubted thiobacilli, as shown in Table 6. They have been included in this study for completeness because representatives of this group are commonly encountered in nature (Trudinger, 1967).

Group 1 (*T. novellus*). These organisms are facultative autotrophs which oxidize thiosulphate with the production of a small amount of acid; the reaction of S6 medium may fall below that of the control but never below pH 5·0. Thiosulphate utilization is small (usually less than 20 %) though this is probably a reflexion of the limited acid tolerance of these strains (Table 6). The addition of greater amounts of buffer does not significantly increase the amount of thiosulphate used, because these strains are very sensitive to increased salt concentrations. They do not tolerate the inhibitory compounds used in the test scheme.

Heterotrophically, these organisms are very limited in their reactions; they are oxidase-positive, catalase-negative, unable to utilize citrate as sole source of carbon, do not reduce nitrate, do not hydrolyse arginine, tributyrin, tallow or starch, acid is not formed in glucose or lactose, acetylmethylcarbinol and indole are not produced,

gelatin is not liquefied and there is no change in litmus milk. Contrary to the findings of Starkey (1935*a*), growth on a potato plug has not been observed and no pigments were formed on any media. The only readily discernable heterotrophic property for these strains is their growth in proteinaceous media, which is characterized by the formation of streamer like growths extending from the surface of the medium to the bottom of the vessel. This form of growth was described by Starkey (1935*b*).

The original strain isolated by Starkey is widely available. An organism (1 L) was isolated in the course of the present study which resembled the authentic strains.

Group 1a. Although only two isolates of this group have been examined, the characters studied would suggest that they represent a valid group in that they are facultatively autotrophic and produce a slight acidity (pH 6·0) in S6 medium. Moreover, the amount of thiosulphate oxidized is greater than that of *T. novellus* under standard conditions. These strains differ from *T. novellus* in their ability to grow anaerobically in thiosulphate media, but fail to produce visible evidence of denitrification. In nitrate peptone broth, however, nitrogen gas is formed. Citrate is utilized as the sole source of carbon. The numerical analysis has shown that these strains may represent a new species, but it is thought that more strains should be examined before a name is given. Similar strains were described by Tjulpanova-Mossevitch (1930) and Sijderius (1946).

Group 2 (*T. denitrificans*). These are autotrophic strains which are able to oxidize sulphur, thiosulphate and thiocyanate under anaerobic conditions in the presence of nitrate. This latter anion is reduced to nitrogen gas which is clearly visible when these organisms are grown in completely filled and stoppered bottles. Good growth is obtained on solid media containing thiosulphate and nitrate when incubated in a McIntosh & Fildes jar and large amounts of elementary sulphur are deposited intercolonially.

Organisms of this group are also capable of aerobic growth but under these conditions the amount of thiosulphate used is considerably reduced. This is a reflexion on the unfavourable pH attained (pH 5·0). This does not occur under anaerobic conditions as the fall in pH is moderated by the products of the nitrate reduction. (Baalsrud & Baalsrud, 1954). This species resembles *T. thioparus* in its sensitivity to inhibitory compounds.

Strains of this species appear to have been isolated at intervals since the original description by Beijerinck (1904*a*, *b*), but no authentic strains of *T. denitrificans* could be located. The strains of this group isolated during the course of this work have been deposited with the National Collection of Industrial Bacteria, accession numbers 9546, 9547 and 9548.

Group 3 (*T. thioparus*). As with group 2, these organisms oxidize thiocyanate, thiosulphate and sulphur under aerobic conditions. The final pH value with the two latter substrates is always below 5·0 but never less than pH 3·5. Although essentially aerobic, they possess a limited ability to grow anaerobically in that colonies are produced on thiosulphate-nitrate agar in a McIntosh & Fildes jar; unlike *T. denitrificans*, they die out with prolonged anaerobic cultivation. In liquid anaerobic culture, gas production has never been observed, nor appreciable amounts of thiosulphate or thiocyanate used. These strains are inhibited by many of the compounds listed in Table 2, and in some cases the final pH is greater than 7·0, indicating that the oxidation of thiosulphate has not proceeded beyond the tetrathionate stage.

Thiobacillus thioparus is regarded as the type species of this genus. Many authentic strains of this species are available and it is readily isolated from a wide variety of sources. All the named strains of *T. thiocyanoxidans* examined were grouped with *T. thioparus*.

Group 4 (*T. neapolitanus*). This species differs from *T. thioparus* in that it is unable to oxidize thiocyanate, or grow anaerobically. Thiosulphate and sulphur are oxidized with the production of acid which becomes inhibitory when the pH falls to 2·8. In 1 % thiosulphate liquid medium the amount of sulphur deposited varies from strain to strain. Strains of *T. neapolitanus* are very resistant to inhibitors and in general are not affected by 6 % thiosulphate, 4 % phosphate, 5 % sodium chloride, 1 % sodium glutamate or 100 p.p.m. phenol.

They have been isolated from many sources, and some strains of this species have been incorrectly described as *T. thioparus*.

Group 5 (*T. thio-oxidans*). This species oxidizes sulphur, hydrogen sulphide and thiosulphate with the production of pH values below 2·0. As Kempner (1966) has shown, when grown in a medium containing sulphur pH values below 1·0 may be recorded and viable bacteria recovered. Initiation of growth in thiosulphate media only occurs in the pH range 4·0 to 5·7 but in sulphur-containing media this pH range extends from 3·0 to 6·4.

Thiobacillus thio-oxidans shares with group 4 a resistance to high salt concentrations which are inhibitory to most other species of thiobacilli. Tolerance to intense acidities is the only reliable character which serves to differentiate these two groups. On solid media this intense acid formation by *T. thio-oxidans* produces a characteristic fogging of the agar around the colonies.

Strains of this species are easily isolated from acidic environments, particularly those associated with mining operations. The only named culture of *T. concretivorus* which could be included in the analysis was classified in group 5 and no new isolate corresponding to this species was found.

Group 6 (*T. ferro-oxidans*). In sulphur- and thiosulphate-containing media this species resembles *T. thio-oxidans* with respect to final pH value but, unlike the latter species, ferrous iron may also be used as the sole energy source. It also differs in that hydrogen sulphide is not metabolized by *T. ferro-oxidans* and growth is inhibited by the high concentrations of salts and organic compounds used in this study.

A further diagnostic criterion is their colony form on thiosulphate agar on which characteristic colonies are produced with a flat profile, irregular shape and a crystalline intercolonial deposit. This material gives them the frosty appearance described by Colmer, Temple & Hinkle (1950); Colmer (1962).

Strains of this species have been isolated from acid mine waters, a natural ferruginous water and domestic sewage. All the named strains of the iron-oxidizing species *T. ferro-oxidans*, *Ferrobacillus ferro-oxidans* and *F. sulfo-oxidans*, were placed in one group by the numerical analysis. Because all of these species oxidize sulphur, thiosulphate and ferrous iron, it is considered that certain of these names are redundant. A possible explanation of the reported differences in the substrate utilization by these species was reported previously (Hutchinson *et al.* 1966).

Group 7 (*T. intermedius*). Only two strains of this group have been examined; the original isolate of London (1963) and a similar strain isolated during this study. They both possess the unusual combination of characters of facultative heterotrophy and

acid tolerance. In sulphur or thiosulphate media they produce final pH values in the range 2·0 to 2·8.

In thiosulphate media these strains are inhibited by the various inorganic compounds used in the test scheme but, in general, the organic inhibitors have no effect.

Heterotrophically, these organisms grow to a limited extent in nutrient broth or nutrient agar and this is marginally improved by the addition of yeast extract. Citrate is utilized as the sole source of carbon.

Thiobacillus thermophilica (Egorova & Deryugina, 1963) was also examined and it was confirmed that this organism is strictly a thermophilic autotroph, though its exceptional morphology in being a spore former probably singles it out as belonging to a different genus. Similarly, the evidence for strain ID, a heterotrophic isolate which possesses the ability to grow autotrophically on thiocyanate but not on thiosulphate, is against its inclusion in the genus *Thiobacillus* (Hutchinson & White, 1964).

DISCUSSION

The apparent degree of success achieved by the application of numerical analysis in this genus may be attributed to the occurrence of species which form discrete groups with no intermediate forms. In certain other analyses the species, and even genera, were not discontinuous and in these cases numerical analysis was of limited applicability (Graham, 1964; Kreig & Lockhart, 1966). Although numerical taxonomy gives rise to groupings it does not in any way identify them. This may be done by the inclusion of authentic cultures in the analysis or by the comparison of the original test results with descriptions from the literature. Both methods have been used to name the groups found in this analysis and in general, most of the groups correspond to the species recognized by Parker & Temple (1957).

A recent adjunct to systematics utilizes DNA base composition in that related bacterial species should have a similar over-all base composition. This concept was applied by Jackson, Moriarty & Nicholas (1968) to test the homogeneity of certain autotrophs, notably authentic cultures of thiobacilli. Their findings are almost in complete agreement with the present multivariate analysis. However, although it is possible for two species to have the same over-all base composition, the base sequences can be entirely different resulting in completely different phenotypic expression. This is well illustrated in the work of Jackson *et al.* (1968), who found G+C base compositions of 56 % and 57 % (± 1 %) for *Thiobacillus neapolitanus* and *T. ferro-oxidans* which are very different physiologically and biochemically. It is considered that DNA base ratios may not in themselves be diagnostic at the species level.

For the purpose of numerical analysis, several new differential tests have been investigated and certain of these have proved to be diagnostic. The diagnostic tests are listed in Table 5. Of all the tests, the most conclusive are those which depend upon the utilization of an unusual substrate such as thiocyanate or ferrous iron. The differentiation based on final pH attained is also important, provided a strict experimental procedure is adopted which allows growth to proceed to completion without any physical or chemical constraints being applied. Inhibitory conditions tend to produce an incomplete metabolism of thiosulphate to tetrathionate with a corresponding rise in pH, $4Na_2S_2O_3 + 2H_2O + O_2 = 2Na_2S_4O_6 + 4NaOH$. Vishniac & Trudinger (1962) have described a similar phenomenon with respect to the availability of oxygen.

Several of the diagnostic tests which have been suggested previously for this genus were found to be unsatisfactory. All the strains examined produced polythionates under our test conditions with the possible exception of *Thiobacillus novellus*. With this organism the amount of thiosulphate metabolized was so small that the detection of polythionates was outside the experimental error of the analytical method. Although these results differ from those of Parker & Temple (1957) similar findings have been reported by Pankhurst (1964).

It has been suggested (Parker & Prisk, 1953) that the form of the nitrogen source might be used as a diagnostic test, but our experiments suggest that it is difficult to obtain an absence of growth in nitrogen free media used for control purposes despite all reasonable precautions to exclude possible sources of nitrogen contamination.

Baalsrud (1954) suggested that the thiobacilli may represent a spectrum of types in that he could arrange all of his isolates in a series according to the final pH attained in culture. In this study considerable attention has been given both to the pH value at which growth may be initiated and also the final value after 28 days. The latter closely paralleled the findings of Baalsrud (1954). Our work would indicate, however, that the spectrum is not continuous as there has been no evidence of interchange of strains of one group with another and the lower limits of the final pH values are extremely stable. This property has proved the most fundamental and reliable diagnostic feature of these organisms and is in every case correlated with other characteristics.

Bunker (1936) was of the opinion that a spectrum of types merging from the autotrophic thiobacilli to common heterotrophs may be possible. The number of thiobacilli which possess heterotrophic abilities is increasing with the discovery of new species such as *T. intermedius*, and possibly the group 1*a* and strain 1D in the present study. This is further supported by reports in the literature of an iron-oxidizing strain which has been reported to grow on glucose (Remsen & Lundgren, 1963) and similarly for a strain of *T. thio-oxidans* (Borichewski & Umbreit, 1964, 1966). This suggests that there may be a series of these organisms, each of which could conceivably represent a heterotrophic analogue of the unknown obligate autotrophs.

No difficulty was experienced in isolating a sufficient number of strains of most species with the exception of *T. novellus*, *T. intermedius* and the new group 1*a*. This may have occurred because they may be comparatively rare in nature, or alternatively special techniques are required for their isolation. Such species, however, do give rise to difficulties in numerical analysis because the possible extent of the group cannot be judged from only one or two strains. These considerations suggest that the absence of intermediate forms between the groups in this study may simply be a function of the number of isolates examined, and increasing this by a suitable factor may have led to the recognition of intermediate forms.

As interspecies change had been encountered previously in this genus (Johnstone, Townshend & White, 1961) all the stock cultures were carefully observed at frequent intervals for a repetition of this phenomenon. However, there were only four instances in which unusual strains were found associated with the stock cultures which, in view of the maintenance of over 100 cultures for a period of three years, could possibly represent contamination. No unusual strains were encountered when a number of representative isolates were regularly subcultured either on solid media, liquid then solid media, or solely in liquid over a period of six months.

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A Medium for the Study of the Ecology of Human Cutaneous Diphtheroids

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SUMMARY

A new medium (FTO), selective for Gram-positive bacilli from human skin, was developed, consisting of trypticase-soy, yeast-extract agar supplemented with (i) Tween 80 (0·5 %) to support growth of lipophilic corynebacteria, (ii) Oil Red O (0·0005 %) to distinguish lipophilic from non-lipophilic bacteria, and (iii) Furoxone (50 to 100 μg./ml.) to inhibit growth of Gram-negative bacilli and Gram-positive cocci. Furoxone may be used to render other media selective for more fastidious diphtheroids.

INTRODUCTION

With the exception of media for lactobacilli, there are few media that selectively isolate Gram-positive bacilli from both Gram-positive cocci and Gram-negative bacilli. Several media select for cocci or Gram-negative bacilli by inhibiting Gram-positive bacilli. Selective media continue to play a vital role in studying the ecology of bacteria indigenous to man. Ritz (1966) developed a polymyxin + gluconate medium deficient in organic nitrogen to isolate oral Nocardia, Corynebacterium and Bacterionema species. Staphylococci and micrococci are regularly found in the oral cavity (Pike, 1962) but their numbers are low and they can be excluded from oral bacterial enumerations by a few tenfold dilutions (Handelman & Mills, 1965).

On human skin, the predominant autochthonous bacteria are the Gram-positive cocci and corynebacteria (Sarkany & Gaylarde, 1968). Despite much research dealing with the sterilizing properties of skin and its vulnerability to infection, surprisingly little attention has been given to studying the ecology of the resident cutaneous bacteria (Marples, 1965). There appear to be many more lipophilic corynebacteria on human skin than was once thought (Smith, 1969). Information on the distribution and types of these bacilli relative to cocci is lacking as is knowledge of ecological factors that may regulate these two major groups of skin bacteria.

As a preliminary step in these studies, a medium was sought to select cutaneous Gram-positive bacilli but inhibit the often abundant Gram-positive cocci; its development and evaluation is reported here.

METHODS

Organisms and cultures. The sources of the reference strains used in this study were previously reported (Smith, 1969). This group included the following: American Type Culture Collection (ATCC)—*Listeria monocytogenes* ATCC 15313, *Kurthia zopfii*

ATCC 6900, *Microbacterium lacticum* ATCC 8180, *Brevibacterium linens* ATCC 9172, *Mima polymorpha* ATCC 14291, *Propionibacterium acnes* ATCC 11828, *Corynebacterium parvum* ATCC 11829, *C. striatum* ATCC 6940, *C. minutissimum* ATCC 23347, *C. xerosis* ATCC 373 and 7064, *C. bovis* ATCC 7715; *C. xerosis*-29 (Midwest Culture Service), *C. bovis*-65 (National Institute for Research in Dairying, England, and *C. diphtheriae* COBE. Non-designated strains were part of the departmental collection. In addition, the following anaerobic Corynebacterium species were kindly supplied by C. H. Zierdt, Clinical Pathology Dept., Clinical Center, National Institutes of Health, Bethesda, Md., U.S.A.: *C. granulosum* 3024B, *C. diphtheroides* 2764, *C. liquefaciens* 3044B, *C. pyogenes* 637B and *C. lymphophilium* PRÉVOT. Gram-positive cocci isolated from human skin were identified by the methods of Baird-Parker (1962). Diphtheroids were identified by the methods of Smith (1969). Most aerobic bacteria were maintained on Todd-Hewitt slopes (Difco). Lipophilic corynebacteria (LC) were stored on the same medium supplemented with 0·1 % Tween 80. Anaerobes were maintained in thioglycollate medium (Difco) and experiments with the latter group were conducted in Brewer jars. All tests were made at 35°.

Antibiotic sensitivity tests. Sensi-discs (BBL, Baltimore Biological Laboratory, Baltimore, Md., U.S.A.) were used for screening facultative cultures. Strains were grown 24 or 48 hr in trypticase-soy broth (TS) without glucose supplemented with 0·1 % Tween 80. Cultures were diluted 1/10 in sterile broth and 0·1 ml. of the dilution was spread on Mueller-Hinton agar (Difco) containing 0·1 % Tween 80. After the inoculum dried, antibiotic discs were placed on the plates and incubated 1 to 3 days. Strains were considered inhibited when sharp well-defined zones at least 2 mm. in width were observed. Anaerobes were tested on 5 % (v/v) whole unwashed human blood agar. In other experiments, certain antibiotic compounds were tested in TS-Tween 80 broth using conventional twofold dilution methods. Growth was measured turbidimetrically.

Skin enumeration technique. Skin swabbings were made by the method of Smith & Willett (1968) and samples were diluted in tenfold amounts. One-tenth ml. amounts of certain dilutions were spread on various media and incubated aerobically 1 to 5 days. Colony counts were expressed as colonies per sample derived from elution of the swabs. Total counts were made using TS agar containing 0·25 % glucose, 0·2 % Tween 80 and 0·1 % yeast extract. Gram-positive cocci were isolated on mannitol salt agar (BBL), coliform bacilli and Mima species were isolated on Herellea agar (Difco) and diphtheroids on the media described below.

Special test media. For reasons presented in the results, the following media were prepared and studied. The TS agar without glucose was supplemented with 0·1 % yeast extract, 50 to 100 μg./ml. Furoxone (*N*-[5-nitro-2-furfurylidene]-3-amino-2-oxazolidone, courtesy of H. R. Bennett, Norwich Pharmacal Co., Norwich, N.Y., U.S.A.), 0·5 % Tween 80 and 0·0005 % Oil Red O (Fisher Scientific Co., King of Prussia, Pa., U.S.A.). Oil Red O was used in the concentration recommended by Del Giudice & Carski (1968). Furoxone and Oil Red O were each dissolved in acetone. No other form of sterilization was required. These two compounds were added to the basal medium after sterilization. The medium containing Furoxone, Tween 80 and Oil Red O is referred to as FTO agar, medium without Furoxone as TO and without Oil Red O as FT. Each medium had a final pH of 7·0. The medium of Ritz (1966) was evaluated and compared to FTO agar in certain experiments. The composition of

Ritz agar was (g./l.): potassium gluconate, 10·0; KNO_3, 10·0; K_2HPO_4, 1·0; NaCl, 0·5; $MgSO_4.7\ H_2O$, 0·8; $MnCl_2.4\ H_2O$, 0·14; $FeSO_4.7\ H_2O$, 0·04; yeast extract, 0·5; Noble agar (Difco), 20. After sterilization, polymyxin B sulphate, 1·75 μg. (13 units)/ml. was added; final pH 7·2. In testing this medium, 0·1 % Tween 80 was sometimes added or the yeast extract or polymyxin were sometimes omitted.

Table 1. *Sensitivity of Gram-positive cocci and bacilli to nitrofuran compounds*

Organisms	No. of strains tested	Compound-100 (μg. per disc)			
		Furacin	Furadantin	Furoxone	Furamazone
Staphylococci					
Subgroup					
I	4	0	0	0	0
II	5	0	0	0	0
IV	3	0	0	0	0
VI	4	0	0	0	0
Micrococci					
Subgroup					
1	2	0	0	0	0
3	2	0	0	0	0
Lipophilic corynebacteria					
Group					
I	1	0	0	1	1
II	8	0	0	0	0
III	9	0	0	0	0
IV	12	0	1	0	1
IV*a*	12	6*	8	11	1
V	9	0	0	0	0
VI	8	1	3	4	1
VII	7	1	3	5	1
Reference bacilli					
K. zopfii	1	—	—	+	—
M. lacticum	1	—	+	+	—
B. linens	1	+	+	+	+
C. striatum	1	—	—	+	—
C. minutissimum	1	—	—	+	—
C. bovis	2	—	+	+	—
C. diphtheriae	1	—	—	+	—
C. xerosis	3	—	2	+	—

* Number indicates strains resistant out of total tested.
+, Resistant; —, inhibited.

RESULTS

Twenty Gram-positive cocci and 66 LC strains previously isolated from human skin together with 11 reference bacilli were compared for their sensitivity to 16 antibacterial drugs. Most of the cocci were resistant to triple sulphonamide, sulphathiazole and gantrisin but the bacilli were inhibited. Neomycin, penicillin, aureomycin, chloramphenicol, streptomycin, erythromycin, tetracycline and bacitracin were inhibitory to 96 % of the tested strains. Some exceptions were three penicillin-resistant staphylococci and *Brevibacterium linens*, which was resistant to penicillin, triple sulphonamide and bacitracin. Approximately one-half of the cocci and bacilli showed a partial resistance to polymyxin. The cocci were uniformly inhibited by four nitrofurans but many bacilli were resistant to one or more of these compounds (Table 1). The strains initially

tested and others were examined for their sensitivity to furoxone in broth culture. Cocci were inhibited by 1 to 10 μg. furoxone/ml. but all of the bacilli including those inhibited by furoxone sensi-discs were resistant to 50 μg. furoxone/ml. in broth.

Anaerobic corynebacteria grew poorly in the test broth but when tested by the disc method on blood plates were also resistant to 50 μg. furoxone./ml. *Mima polymorpha* and one strain each of *Klebsiella* (*Aerobacter*) *aerogenes* and *Escherichia coli* were inhibited by 25 μg./ml. furoxone in broth culture.

Table 2. *Selectivity of FT agar in the isolation of cutaneous bacteria*

Cutaneous site sampled	No. of subjects sampled	Growth on media			
		Blood*	Mannitol salt*	Herellea	FT
Scalp	10	+	+	0	1
Face	6	+	+	1	0
Nasal	6	+	+	0	5
Ear canal	6	+	+	0	3
Forearm	6	+	5*	1	0
Interdigital	6	+	+	0	1
Groin	1	+	+	0	1
Axilla	1	+	+	0	0

Swabbings from each area were washed in 5 ml. buffer and 0.1 ml. of the washing was spread on each medium. The FT agar contained 100 μg./ml. furoxone. All plates were incubated aerobically for 5 days. Plates containing 30 to 300 colonies were scored as positive.

* + = all samples positive.

† Number of plates positive out of total tested.

Furoxone was used in FT medium for further studies as a selective medium for Gram-positive bacilli. Skin swabbings from eight sites of 37 males and 5 females were inoculated on FT agar and other media (Table 2). All 42 samples produced heavy abundant growth on blood agar and mannitol salt agar. Compared with these media, FT agar was selective permitting growth from only 11 samples. A total of 144 colonies were randomly taken from FT agar and 131 were Gram-positive bacilli. The other 13 colonies, considered to be contaminants, originated from five subjects and consisted of either yeasts or yellow pigmented tetrad-forming cocci. Both types were recognized by their colonial morphology.

Since many cutaneous bacilli or diphtheroids are lipophilic, Oil Red O, a fat dye was added to the furoxone and Tween 80 agar (FTO). On FTO agar, strains of *Corynebacterium striatum* and *C. xerosis* produced slight pink hues partly because of their production of tan-beige pigments, partly because of some dye absorption. One strain of *Bacillus subtilis* was inhibited on FTO agar for 36 hr but eventually grew, producing a colourless colony. None of the anaerobic corynebacteria produced a positive dye reaction on FTO medium during 12 days incubation. When furoxone was omitted from the agar (TO type), 20 staphylococci and micrococci grew but did not absorb the dye to the extent that the LC strains did. Cutaneous LC strains and strains of *C. bovis* produced uniformly pink or red colonies, indicating uptake or concentration of lipid materials in the colonies. Colonies of 6 enteric Gram-negative bacilli of the coliform type also produced colourless colonies on TO agar.

Skin swabbings were taken from six sites (ear canal, face, nares, scalp, toeweb and groin) of six subjects and inoculated on TO and FTO agars. Only pink or dye-positive

colonies were taken from the TO media and compared to pink or colourless colonies isolated on FTO agar (Table 3). Sixty-one isolates were identified and represented seven of eight provisional groups of LC strains. There were no contaminants found on either medium.

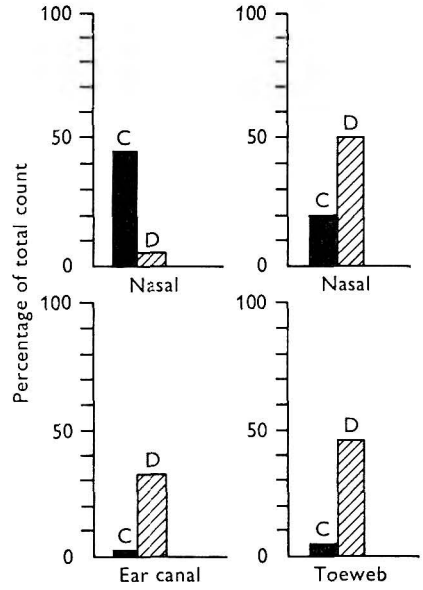

Fig. 1. Frequency and distribution of Gram-positive bacilli relative to Gram-positive cocci on human skin. Samples were taken from four individuals. Dark areas, C = cocci; cross-hatched areas, D = bacilli or diphtheroids. The latter were counted on FTO agar with 50 μg./ml. furoxone.

Table 3. *Isolation and identification of cutaneous lipophilic corynebacteria on TO and FTO media*

LC group identified	No. of isolates	
	TO agar	FTO agar
I	0	0
II	1	0
III	1	1
IV	7	2
IV*a*	4	21
V	5	1
VI	1	0
VII	4	13

From TO agar only those colonies absorbing Oil Red O were isolated. Isolates from FTO plates were randomly selected regardless of extent of dye absorption.

Skin bacteria were counted using FTO agar for diphtheroids (Fig. 1). Total counts from four samples ranged from 320,000 to 3,000,000 bacteria per specimen; FTO agar demonstrated the relative numbers of diphtheroids present at the various sites compared to the Gram-positive cocci. Nasal samples from two individuals revealed inverse relationships in the numbers of diphtheroids and cocci present.

In experiments with Ritz agar, Tween 80 was a required supplement to support

growth of the LC strains. *Kurthia zopfii*, *Brevibacterium linens*, *Klebsiella aerogenes* and *Mima polymorpha* grew on the medium without yeast extract but were inhibited by polymyxin. Yeast extract was essential for the growth of the remaining organisms (Table 4). Some reference bacilli and LC strains grew poorly on Ritz complete medium containing Tween 80 and all of the strains recorded as growing adequately developed more rapidly on control plates. Skin swabbings of nasal, groin and anal areas were streaked on Ritz's complete medium and incubated aerobically for 5 days. Gram-positive cocci and bacilli were present equally in randomly selected colonies.

Table 4. *Growth of various bacteria on Ritz agar and its modifications*

Organisms tested	Complete medium	With yeast extract minus polymyxin	Without yeast extract	
			polymyxin +	polymyxin −
Cocci				
I S	+	+	−	−
II S	+	+	−	−
IV S	+	+	−	−
VI S	W	W	−	−
1 M	+	+	−	−
3 M	+	+	−	−
LC strains				
II–143	+	+	−	−
III–65	+	+	−	−
IV–33	W	W	−	−
IV*a*–171	W	W	−	−
IV*a*–112	+	+	−	−
V–27	+	+	−	−
VI–31	W	W	−	−
VII–140	+	+	−	−
Reference strains				
C. minutissimum	W	W	−	−
L. monocytogenes	W	W	−	−
K. zopfii	−	+	−	+
B. linens	−	+	−	+
C. xerosis	+	+	−	−
C. bovis	+	+	−	−
C. striatum	+	+	−	−
M. polymorpha	−	+	−	+
K. aerogenes	−	+	−	+

All media contained 0·1% Tween 80 to support growth of the LC strains. Cultures were incubated for 5 days and compared to growth on TS-Tween agar. +, Growth; −, no growth; W, weak or poor growth.

DISCUSSION

There are probably no infallibly selective media. Ritz's agar has some use in isolating cutaneous diphtheroids but certain Gram-positive bacilli did not grow on the complete medium containing polymyxin. Among strains that did grow, none developed as rapidly on Ritz agar as they did on peptone media. In addition, stock cultures of Gram-positive cocci grew on Ritz agar and were identified on the medium from skin swabbings. This would make the medium unsuitable for presumptive separation and counting of cutaneous diphtheroids. The polymyxin concentration can not be increased because bacilli rather than cocci would then be inhibited (Finegold & Sweeney, 1961; Hobbs, Kendall & Gilbert, 1968).

Considerable variation in bacterial counts from skin occur because of different sampling techniques and the exact layer or area of skin that is examined (Updegraff, 1964). The FTO medium appears to be selective for Gram-positive bacilli of human skin and appears suitable for counting them. This can be accomplished without time-consuming subcultures and Gram staining from colonies for morphological separation from cocci. In this study it was repeatedly observed that some diphtheroids, because of their small size and rounded ends, are often difficult to distinguish microscopically from cocci. No Gram-negative bacteria were detected on FTO agar during its evaluation, which included samples from more than 50 individuals.

The inverse relationship in the numbers of cocci and diphtheroids observed from two different nasal samples does not appear to be a random event. Studies of the nasal flora of 12 subjects has indicated that the inverse relationships in most subjects remain constant over a period of several months (unpublished data) and a possible ecological basis for these data is now being studied.

Many selective media are effective only within a limited incubation period. After 5 days incubation, the contaminants found on FTO agar were the yellow sarcina-like cocci and yeasts. These groups were infrequently found, but when present, could be distinguished by colonial morphology.

FTO agar is relatively simple to prepare and can be modified in several ways. If Oil Red O is omitted, lipophilic bacteria cannot be directly counted. If furoxone is omitted, all types of common skin bacteria will grow but the lipophilic types can be differentiated by their dye absorption. Furoxone may also be incorporated into other media that will support the growth of more fastidious diphtheroids. Preliminary studies indicate that FTO agar (50 μg. furoxone/ml.) is effective in isolating Gram-positive bacilli and filamentous forms from the oral cavity.

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Catabolite Repression in Antibiotic-limited Streptomycin-dependent *Escherichia coli* B

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SUMMARY

The half-maximal growth rate of a streptomycin-dependent mutant of *Escherichia coli* B on limiting concentrations of dihydrostreptomycin varied with the nature (but not with the initial concentration) of the carbon source. It was highest with gluconate, lower with glucose and glycerol and lowest with lactate. Glucose-sensitive enzymes (acetohydroxy acid synthetase, fumarase, aconitase, citrate synthase and isocitric dehydrogenase) were specifically repressed by antibiotic limitation. Parallelism was observed between decreasing dihydrostreptomycin concentration, decreasing growth rate and increasing catabolite repression of certain glucose-sensitive enzymes. The results are not incompatible with the hypothesis that the primary site of action of dihydrostreptomycin in the dependent organism is an anabolic process (e.g. protein synthesis). However, the growth-limiting effect of antibiotic deprival appears to be augmented by catabolite repression.

INTRODUCTION

A recent publication from this laboratory (Coukell & Polglase, 1969*b*) reported that a number of catabolite repression-sensitive enzymes were specifically derepressed in a streptomycin-dependent mutant of *Escherichia coli* B growing exponentially in a medium containing a non-limiting concentration of antibiotic. Relaxation of catabolite repression in this mutant was attributed to a deficiency in glucose metabolism observed through a study of cell yields (Coukell & Polglase, 1969*b*). One of the enzymes derepressed in this streptomycin-dependent mutant was the glucose-sensitive acetohydroxy acid (AHA) synthetase (Coukell & Polglase, 1969*a*), a biosynthetic enzyme involved in the formation of the aliphatic amino acids (valine, leucine and isoleucine) and pantothenate (Umbarger & Davis, 1962). Earlier studies on AHA synthetase in a streptomycin-dependent strain of *E. coli* grown with limiting antibiotic indicated that the formation of this enzyme was strongly repressed and that the degree of repression was inversely related to the concentration of antibiotic on which the culture was grown (Polglase, 1966). The present study reports an investigation of the effect of antibiotic-limitation on the growth of a streptomycin-dependent mutant of *E. coli* and on catabolite repression in these cells.

METHODS

Organisms. Wild-type *Escherichia coli* B (ATCC 11303) and a spontaneous streptomycin-dependent mutant derived from this organism (Coukell & Polglase, 1965), were used throughout this work.

Media, cultures and extraction. Cultures were grown on a minimal salts medium described by Davis & Mingioli (1950) but with the omission of citrate. The carbon source (glucose, sodium gluconate, glycerol or sodium lactate) and dihydrostreptomycin sulphate were autoclaved separately in concentrated solution and added to the minimal medium. Growth was determined turbidimetrically at 420 mμ in a Beckman B spectrophotometer (light path, 1·0 cm.). The procedures for growing streptomycin-sensitive cultures, and for growing streptomycin-dependent cultures on non-limiting concentrations (> 100 μg./ml.) of antibiotic, and the procedures for harvesting cultures and for preparation of crude sonic extracts have been described previously (Coukell & Polglase, 1969*a*). All cultures were grown at 37° with vigorous aeration.

Growth of antibiotic-limited and depleted cells. The streptomycin-dependent strain was grown in approximately 50 ml. of minimal salts medium containing 30 μg. of dihydrostreptomycin/ml. and the appropriate carbon source at 37° without agitation for 24 to 48 hr. A portion (2 to 4 ml.) of this culture was transferred to another 50 ml. of similar medium and the procedure was repeated. After three or four such transfers, the entire culture (50 ml.) was added to 500 ml. of similar medium and grown for 18 hr at 37°. The resulting bacteria were centrifuged at 16,000 *g* for 20 min. and resuspended in 6 l. of fresh, warm (37°) medium to give an E_{420} of approximately 0·1. The culture was divided into six 1 l. volumes, and supplemented with the required amount of dihydrostreptomycin (usually in the range of 3 to 25 μg./ml.). A carbon source was added and the cultures were grown with forced aeration to an E_{420} of 0·9 to 1·0. The bacteria were harvested and stored as described by Coukell & Polglase (1969*a*). Bacteria grown in this way are referred to as 'antibiotic-*limited*, streptomycin-dependent'. Bacteria referred to as 'antibiotic-*depleted* streptomycin-dependent' were obtained by growth of the mutant on antibiotic-free medium as described by Bragg & Polglase (1963).

Enzyme assays. All enzyme activities were determined on crude ultrasonic extracts. AHA-synthetase activity was measured in fresh extracts as described elsewhere (Coukell & Polglase, 1969*a*). One unit of activity is equivalent to the formation of 1 μmole acetoin/hr at 37°. Fumarase and aconitase activities were determined spectrophotometrically on fresh extracts at 240 mμ as described by Hanson & Cox (1967). A unit of activity for each enzyme is defined as an extinction change of 0·001/min. Isocitric dehydrogenase, glucose-6-phosphate dehydrogenase (Coukell & Polglase, 1969*b*) and glucokinase (Coukell & Polglase, 1965) activities were estimated on fresh extracts or on extracts which had been stored at −20° for not more than 48 hr. A unit of activity for each enzyme is equivalent to the formation of 1 mμmole NADPH/min. The condensing enzyme, citrate synthase, was determined spectrophotometrically in extracts by measuring the change in extinction at 233 mμ (Coukell & Polglase, 1969*a*). A unit of enzyme activity is equivalent to the amount of enzyme which catalyses the formation of 1 mμmole citrate/min. All spectrophotometric determinations were made with a Cary 15 recording spectrophotometer at 25°. Specific activities were expressed as units enzyme/mg. of protein. Protein was estimated by the procedure of Lowry, Rosebrough, Farr & Randall (1951), with bovine γ-globulin as a standard.

Chemicals. Dihydrostreptomycin sulphate was a gift from Merck, Sharp and Dohme, Montreal, Canada. All other chemicals were obtained from Calbiochem., Los Angeles.

RESULTS

Antibiotic-requirement for growth on various carbon sources. Preliminary experiments indicated that the streptomycin-dependent mutant, which was isolated initially on medium containing 1 mg. dihydrostreptomycin/ml., had comparable growth rates at dihydrostreptomycin concentrations of 1 mg./ml. or 100 μg./ml. Below 50 μg./ml., however, the growth rate of this mutant was diminished (Fig. 1). When antibiotic was not added to the culture, only slight growth occurred during the period

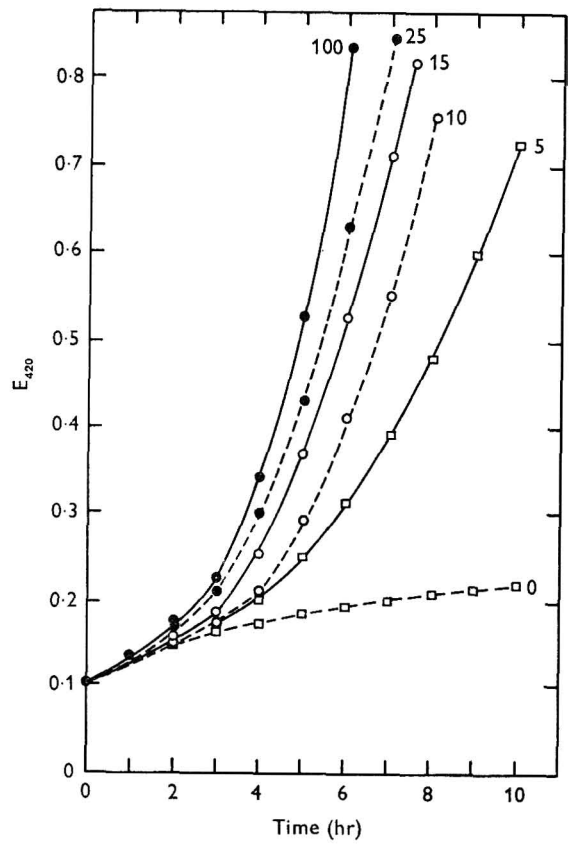

Fig. 1. Growth curves for streptomycin-dependent *Escherichia coli* B growing on glucose (0·4%, w/v) salts medium supplemented with rate-limiting concentrations of dihydrostreptomycin. The numbers on each curve indicate the concentration (μg./ml.) of antibiotic added at zero time. Further experimental details are given in Methods.

of incubation. Cultures with 5 to 100 μg. dihydrostreptomycin/ml. exhibited exponential growth. When the growth rates of these cultures were plotted against the concentration of antibiotic on which they were grown, a hyperbolic, saturation curve was obtained (Fig. 2A). The corresponding double reciprocal plot of these data is shown in Fig. 2B from which two constants may be calculated: K_{DHSm}, equivalent to the concentration of dihydrostreptomycin which permitted half the maximum growth rate, and V_{max}, the growth rate at a non-limiting concentration of dihydrostrepto-

mycin. With glucose as the carbon source, K_{DHSm} and V_{max} were 5·3 μg./ml. and 0·645 hr^{-1} respectively.

A tenfold increase in initial glucose concentration had no significant effect on K_{DHSm} but increased V_{max}. K_{DHSm} and V_{max} values obtained employing different carbon sources are listed in Table 1. Average K_{DHSm} values ranged from 12·6 when gluconate was the carbon source to 1·5 with lactate. Thus, in order to attain one-half maximal growth rates, 8·4 times as much dihydrostreptomycin was needed when gluconate was the carbon source as when lactate was used. The order of increasing growth rate was lactate < glycerol < glucose < gluconate, the same order as the K_{DHSm} values. The values obtained for K_{DHSm} and V_{max} were highly reproducible.

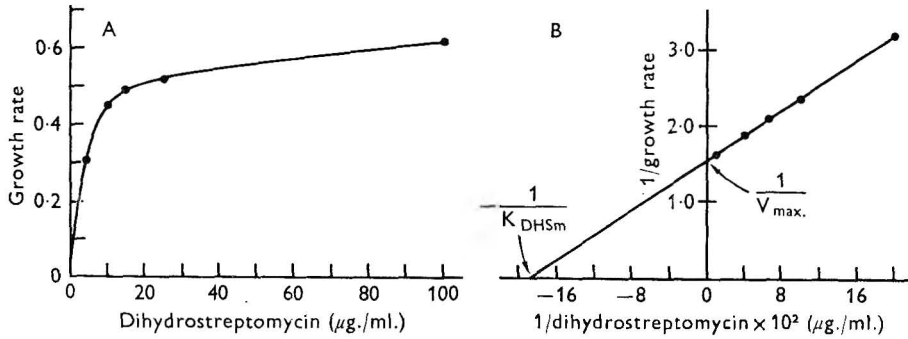

Fig. 2. Relationship between the growth rate of streptomycin-dependent *Escherichia coli* and the concentration of dihydrostreptomycin in the medium. The data were obtained from the experiment shown in Fig. 1. A, Plot of growth rate versus antibiotic concentration; B, Reciprocal of growth rate plotted against reciprocal of dihydrostreptomycin concentration.

Table 1. *Effect of carbon source on the antibiotic requirement for growth of the streptomycin-dependent mutant of Escherichia coli* B

Growth experiments were performed as described in Methods. Each carbon source was initially 0·4% (w/v). V_{max} is the growth rate on non-limiting antibiotic, K_{DHSm} is the antibiotic concentration required to give one-half the maximum growth rate, calculated as shown in Fig. 2B.

Carbon source	V_{max} (hr^{-1})	K_{DHSm} (μg./ml.)
Gluconate	0·914	12·6
Glucose	0·661	5·3
Glycerol	0·558	2·0
Lactate	0·485	1·5

Effect of growth on limiting-dihydrostreptomycin on the formation of enzymes of carbohydrate metabolism. Table 2 records the specific activities of several catabolite repression-sensitive enzymes of carbohydrate metabolism (Coukell & Polglase, 1969*b*) which were determined after growth on various carbon sources in the presence of growth-rate-limiting or non-limiting concentrations of dihydrostreptomycin. The specific activities of two glucose-insensitive enzymes—glucokinase and glucose-6-phosphate dehydrogenase (Coukell & Polglase, 1969*b*)—were determined in the same extracts. In general, the specific activities of the catabolite repression-sensitive enzymes (AHA synthetase, isocitrate dehydrogenase, aconitase, fumarase and citrate synthase)

from cells grown in the presence of 100 μg. dihydrostreptomycin/ml. were lowest when K_{DHSm} values for the various carbon sources were highest. For any particular carbon source, except gluconate, the percentage repression of the glucose-sensitive enzymes (isocitrate dehydrogenase excluded) from cells grown with 3 μg. dihydrostreptomycin/ml. compared with cells grown with 100 μg. antibiotic/ml. was greater the higher the value of K_{DHSm}. Gluconate exhibited a relatively high degree of repression even when the dihydrostreptomycin concentration was non-limiting (100 μg./ml.) (Table 2). Aconitase was repressed when streptomycin-dependent cells were grown on glucose or gluconate at a concentration of 3μg. dihydrostreptomycin/ml. but was derepressed when they were grown on glycerol or lactate at the same antibiotic concentration.

Table 2. *The effect of dihydrostreptomycin concentration on enzyme formation on different carbon sources in streptomycin-dependent Escherichia coli* B

Carbon sources were initially 0·4% (w/v). The cultures were grown on either a limiting (3 μg./ml.) or a non-limiting (100 μg./ml.) concentration of dihydrostreptomycin (DHSm). The procedures for growing and harvesting cultures, preparing sonic extracts, and assaying enzymes are described in Methods. Specific activities are expressed as units of enzyme/mg. bacterial protein.

	DHSm (μg./ml.)	Specific activity and repression (percentage in parenthesis) with carbon source			
		Gluconate	Glucose	Glycerol	Lactate
AHA synthetase	100	6·0	11·4	11·0	12·9
	3	1·5 (75)	2·5 (78)	7·5 (32)	11·4 (12)
Isocitric dehydrogenase	100	564	547	800	817
	3	607 (−8)	462 (16)	883 (−11)	815 (0)
Aconitase	100	270	216	478	434
	3	61 (77)	128 (41)	530 (−11)	638 (−47)
Fumarase	100	552	1942	2260	2170
	3	134 (76)	670 (65)	1590 (30)	1935 (11)
Citrate synthase	100	197	320	523	365
	3	84 (57)	89 (72)	426 (19)	287 (21)
Glucose-6-phosphate dehydrogenase	100	125	109	120	79
	3	118 (6)	109 (0)	122 (−2)	70 (11)
Glucokinase	100	79	115	81	75
	3	77 (3)	92 (20)	79 (2)	76 (−1)

Glucokinase and glucose-6-phosphate dehydrogenase formation were relatively unaffected by dihydrostreptomycin-limitation. Although the specific activities of both enzymes varied slightly on the different carbon sources, these differences showed no correlation with either the antibiotic requirements or the growth rates of the streptomycin-dependent cultures (Table 2).

To determine whether the enhanced catabolite repression resulting from growth on gluconate was a general characteristic of *Escherichia coli* strain B, or a specific property of the streptomycin-dependent mutant, the specific activity of AHA synthetase was determined in extracts of streptomycin-sensitive and streptomycin-dependent cultures grown on glucose and on gluconate (Table 3). As shown previously (Coukell & Polglase, 1969*b*), the AHA synthetase of the streptomycin-dependent mutant was derepressed in extracts of glucose-grown cells, when compared with the wild-type

organism. In extracts of the streptomycin-dependent mutant grown on gluconate, however, AHA synthetase was repressed almost as strongly as was this enzyme in the wild-type organism grown on either glucose or gluconate. Furthermore, the growth rate of the streptomycin-dependent culture was considerably higher on gluconate than on glucose, while the streptomycin-sensitive culture grew at the same rate on either carbon source (Table 3). Cell-yield experiments, however, indicated that gluconate metabolism, like glucose metabolism, was 38 % less efficient in the mutant than in the wild-type organism (Coukell & Polglase, unpublished observation). Therefore, the enhanced growth rate and the increased catabolite repression observed in the streptomycin-dependent mutant growing on gluconate did not result from an increased yield of energy from this carbon source.

Table 3. *Growth rates of Escherichia coli strains on glucose and gluconate and the formation of AHA synthetase*

Cultures were grown aerobically at 37° on salts medium supplemented as indicated. The media for growth of the streptomycin-dependent cultures were supplemented with dihydrostreptomycin (1 mg./ml.). AHA synthetase was assayed in crude sonic extracts (see Methods).

Strain	Carbon source (0·4 %, w/v)	Growth rate (hr^{-1})	AHA synthetase (μmoles acetoin/mg. protein/hr)
Streptomycin-sensitive	Glucose	1·11	5·00
	Gluconate	1·11	5·90
Streptomycin-dependent	Glucose	0·66	14·1
	Gluconate	0·81	7·85

Relationship between growth rate and enzyme synthesis in streptomycin-dependent cells grown with limiting-antibiotic. A direct relationship was observed between the diminished growth rate in the presence of limiting-antibiotic and the degree of repression of certain glucose-sensitive enzymes. This relationship is illustrated in Fig. 3 for AHA synthetase and fumarase activities in extracts of glucose and gluconate-grown cells. In general, both growth rate and enzyme formation were lower on gluconate than on glucose at the same antibiotic concentration. Not all glucose-sensitive enzymes exhibited this relationship, however. While the degree of repression of citrate synthase under these conditions often correlated with the growth rate of the culture, aconitase and isocitric dehydrogenase rarely displayed this parallelism. Except for fumarase, this relationship was seldom observed in cultures grown on the poor carbon sources glycerol and lactate.

Response of catabolite repression-sensitive enzymes of antibiotic-depleted cells to dihydrostreptomycin supplementation. After 10 to 12 hr of growth in medium containing glucose but no antibiotic, the streptomycin-dependent cells exhibited the filamentous growth which is characteristic of antibiotic depletion (Delaporte, 1949). The addition of dihydrostreptomycin (1 mg./ml.) to antibiotic-starved cultures resulted in a progressive breakdown of the elongated cells which was usually complete within 3 hr. The effect of dihydrostreptomycin-supplementation of antibiotic-depleted cells on the formation of certain glucose-sensitive enzymes in strain DB is shown in Table 4. The difference in specific activities of these enzymes in extracts of antibiotic-depleted and antibiotic-supplemented cells after 3 hr indicated that the synthesis of these enzymes undergoes a rapid and dramatic derepression during this period. The glucose-

insensitive enzymes, glucokinase and glucose-6-phosphate dehydrogenase, were relatively unaffected by dihydrostreptomycin-depletion or by the subsequent antibiotic-supplementation.

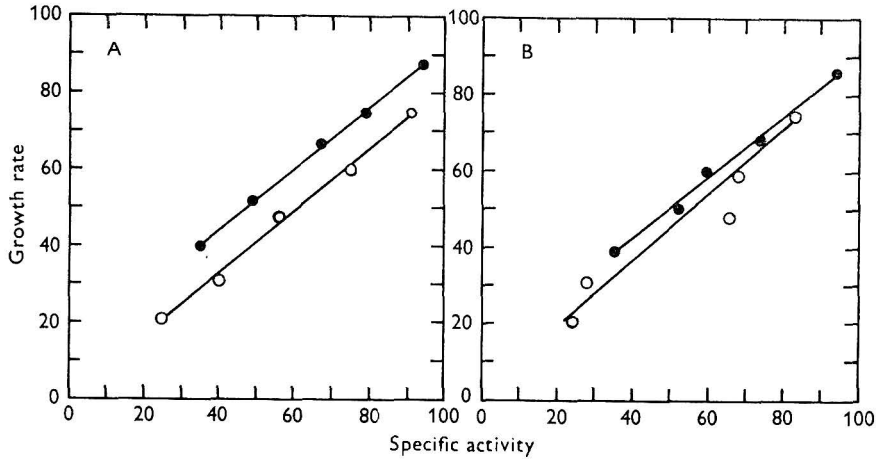

Fig. 3. Relationship between the decrease in growth rate and enzyme repression in antibiotic-limited cultures of streptomycin-dependent *Escherichia coli* B. Growth experiments were carried out as described in Methods. Glucose (●) or gluconate (○) were added to give an initial concentration of 0·4% (w/v). Growth rates and specific activities are expressed as percentages of the values obtained with cultures grown on 100 μg. dihydrostreptomycin/ml. The antibiotic concentrations employed were 25, 15, 10, 5 and 3 μg./ml., respectively. A, AHA synthetase; B, Fumarase.

Table 4. *Effect of antibiotic-supplementation on enzyme formation in streptomycin-depleted cells grown on glucose*

Preparation of antibiotic-depleted streptomycin-dependent *Escherichia coli* and methods of enzyme assay are described in Methods. Supplemented cells received 1 mg. dihydrostreptomycin/ml. 3 hr before harvesting.

Enzyme	Specific activity (units/mg. of protein)		Ratio, supplemented: depleted
	Depleted	Supplemented	
AHA synthetase	1·68	11·7	7·0
Fumarase	200	1622	8·1
Aconitase	34·4	182	5·3
Isocitric dehydrogenase	183	490	2·7
Citrate synthase	118	426	3·6
Glucose-6-phosphate dehydrogenase	75·2	81·4	1·1
Glucokinase	64·3	91·6	1·4

DISCUSSION

The value obtained for K_{DHSm} depended on the nature of the carbon source, being highest for energy-rich, rapidly metabolizable compounds such as gluconate and glucose. This finding contrasts with a related study by Spotts (1962) on streptomycin-dependent mutants of *Escherichia coli* K 12, in which the growth rates of these mutants on glycerol were a direct function of the streptomycin concentration up to

approximately 100 μg./ml., at which value ('critical concentration') streptomycin was no longer the rate-limiting factor. When glucose was the carbon source, streptomycin remained rate-limiting up to about 250 μg./ml. At rate-limiting concentrations of antibiotic, however, growth rates were *independent* of the nature of the carbon source (Spotts, 1962).

Goodman & Spotts (1967) reported that the synthesis of both inducible and constitutive β-galactosidase and of constitutive alkaline phosphatase were inhibited during the growth of streptomycin-dependent strains of *Escherichia coli* transferred to medium containing no added antibiotic. They attributed some, but not all, of this inhibition to an increase in catabolite repression in the antibiotic-deprived cells. They suggested that, in addition to inhibition of enzyme synthesis by catabolite repression, enzyme inhibition could occur randomly due to the misreading of certain codons by the abnormal streptomycin-deficient, streptomycin-dependent ribosomes. However, they were unable to detect cross-reacting material by serological methods or to find any other evidence for infidelity of translation *in vivo*, either in the presence or absence of streptomycin. The results of the present investigation suggest that catabolite repression without misreading can explain the enzyme imbalance (Tables 2, 4). All of the glucose-sensitive enzymes examined were repressed whereas glucose-insensitive enzymes were not affected by growth with limiting antibiotic. Furthermore, dihydrostreptomycin supplementation of antibiotic-starved cells resulted in a rapid and specific derepression of the catabolite repression-sensitive enzymes (Table 4).

Glucose generally is considered to be the most effective source of catabolite co-repressor (Magasanik, 1961). Therefore, it was surprising that growth of the streptomycin-dependent mutant (but not of the wild-type parent strain) on gluconate resulted in a higher rate of growth and stronger catabolite repression than when the same organism was grown on glucose (Table 3). This response was not due to a higher yield of energy from gluconate since the yield of streptomycin-dependent cells was the same with both carbon sources (Coukell & Polglase, unpublished observation). Gluconate therefore must be metabolized more rapidly than glucose by this mutant. Eisenberg & Dobrogosz (1967) reported that, in *Escherichia coli*, gluconate is metabolized via the inducible Entner-Doudoroff pathway rather than by the hexose monophosphate pathway, the major aerobic route of glucose dissimilation. Since pyruvate is an early product of the Entner-Doudoroff pathway, the rate of aerobic pyruvate-coupled energy formation may be higher in the antibiotic-dependent mutant growing on gluconate than in the same mutant growing on glucose. An increase such as this in rate of energy formation could explain both the higher rate of growth and the stronger catabolite repression observed in gluconate-grown cultures of the streptomycin-dependent mutant.

The current hypothesis concerning the mechanism of action of streptomycin (or dihydrostreptomycin) has been deduced mainly from studies of ribosomal preparations of streptomycin-sensitive and resistant (indifferent) cells (Pestka, 1967). Streptomycin has been shown to affect reactions of protein synthesis in ribosomal preparations from streptomycin-sensitive cells whereas these reactions were not affected in similar preparations from streptomycin-resistant cells. The results presented here indicate that catabolite repression may play an important role in establishing the quantitative requirement for antibiotic for growth of streptomycin-dependent *Escherichia coli*. In order to incorporate these results into the hypothesis which indicates protein

synthesis to be the primary process affected by streptomycin, one may postulate that, *in vivo*, the primary effect of the antibiotic on protein synthesis is augmented by the secondary effect of catabolite repression. In the streptomycin-dependent cells the limitation of antibiotic initially may impair ribosomal function without immediately affecting catabolic processes. The result of limitation of anabolism while catabolism continues would be severe repression of the glucose-sensitive enzymes ultimately resulting in a decreased rate of both catabolic and anabolic reactions and leading to a decrease in the growth rate of the organism.

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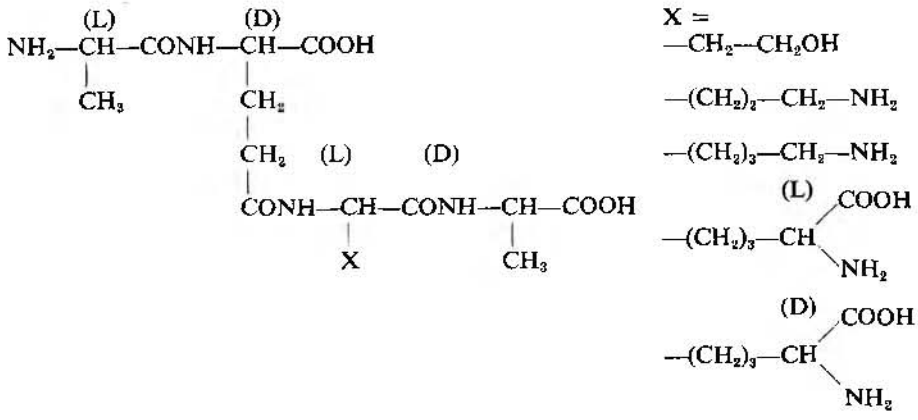
SYMPOSIUM: THE CELL WALL AND THE CYTOPLASMIC MEMBRANE OF BACTERIA

Introduction. By M. R. J. SALTON (*Department of Microbiology, New York University School of Medicine, New York, U.S.A.*)

The Primary Structure of Bacterial Wall Peptidoglycans. By JEAN-MARIE GHUYSEN and MELINA LEYH-BOUILLE. (*Service de Bactériologie, 32 Bvd de la Constitution, Université de Liège, Belgium*)

The bacterial wall peptidoglycan is an insoluble network composed of: (i) glycan chains of alternating β-1,4-linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues, i.e. a chitin-like structure except that every other sugar is substituted by a 3-*O*-D-lactyl group and that the average chain length is small (20 to 140 Hexosamine residues, depending upon the bacterial species). Variations so far encountered include the possible presence of *O*-acteyl substituents on C-6 of some of the *N*-acetylmuramic acid residues (*Staphylococcus aureus*; some strains of *Lactobacillus acidophilus* (unpublished) and of *Micrococcus lysodeikticus*), and the replacement of the *N*-acetylmuramic acid residues by another derivative of muramic acid, possibly *N*-glycolylmuramic acid (*Mycobacterium smegmatis*) (ii) tetrapeptide subunits which substitute through their *N*-termini the D-lactic acid groups of the glycan chains. (iii) peptide bridges which cross-link tetrapeptide subunits of adjacent glycan chains (average size of the peptide moieties: 1·5 to 10 cross-linked peptide subunits).

The tetrapeptide subunits have the general sequence R_1-γ-D-glutamyl-R_3-D-alanine. The R_3 residue is sometimes a neutral amino acid such as homoserine (Perkins, H. R. (1965), *Biochem. J.* 95, 876), most often a diamino acid such as L-ornithine, L-lysine LL-diaminopimelic acid, *meso*-diaminopimelic acid. Irrespective of the nature of the R_3 residue, however, both the amino group and the carboxyl group engaged in linkages to the γ-COOH of D-glutamic acid and to the NH_2 of D-alanine, respectively, are located on the same carbon which has always an L configuration. It follows that, with the possible exceptions that the R_1 residue is not always L-alanine, but sometimes L-serine or glycine, and that glutamic acid may be replaced by 3-hydroxy-glutamic acid (Schleifer, K. H., Plapp, R. & Kandler, O. (1967), *Biochem. biophys. Res. Commun.* 28, 566), the tetrapeptide units have the following general structure:



The main variations thus so far encountered lie in the nature of the side chain of the third amino acid in the sequence. Depending upon the bacterial species, the general tetrapeptide unit may have additional substituents. Amide ammonia, glycine or D-serine are possible substituents of the α-COOH group of D-glutamic acid. Similarly, the carboxyl group of LL or *meso* diaminopimelic acid which is not engaged in peptide linkage, may also be substituted by an amide group.

The following peptide subunits have been fully characterized: N^{α}-[L-Ala-γ-(α-D-Glu-amide)]-L-Lys-D-Ala (*Micrococcus*, *Staphylococcus*, *Gaffkya*, *Streptococcus*, *Lactobacillus* sp.); N^{α}-[L-Ala-γ(α-D-Glu-Gly)]-L-Lys-D-Ala (*Micrococcus lysodeikticus* and related *Micrococci*); L-Ala-γ-D-Glu-(L)-*meso*-DAP-(L)-D-Ala (*Escherichia coli*; probably all the Gram-negative bacteria; *Bacillus* sp.); L-Ala-γ-(α-D-Glu-amide)-(L)-LL'DAP-(L)-D-Ala (*Clostridium welchii*; *Streptomyces* sp.) (unpublished experiments); N^{α}-(L-Ser-γ-D-Glu)-L-Orn-D-Ala (*Butyribacterium rettgeri*); Gly-γ-D-Glu-homo-Ser-D-Ala (plant pathogenic *Corynebacteria*).

The peptide bridges always involve the carboxyl group of the terminal D-alanine residue of one peptide subunit and either the free amino group of the diamino acid (peptidoglycans of type I, II and III) or the α carboxyl group of the D-glutamic acid (peptidoglycan of type IV) of another peptide subunit. The cross-linking between two peptide subunits may consist: (i) in a direct bond such as a D-alanyl-(D)-*meso*-diaminopimelic acid linkage, that is to say a peptide bond which extends from the amino group located on the D carbon of *meso*-diaminopimelic acid of one peptide subunit, to the C terminal D-alanine of another peptide subunit (van Heijenoort, J., Elberz, L., Dezélée, Ph., Petit, J. F., Bricas, E. & Ghuysen, J. M. (1969), *Biochemistry*, 8, 200. (Gram negative bacteria; *Bacillus* sp.) (Peptidoglycan of type I). (ii) in a single additional amino acid (a glycine, a neutral L-amino acid, or a D-isoasparagine residue) or an intervening peptide chain composed of glycine and/or neutral amino acid residues and containing up to 5 residues. This bridging frequently occurs between N^{α}-[L-Ala-γ-(α-D-Glu-amide)]-L-Orn or L-Lys or LL-DAP)-D-Ala peptide subunits. It extends from the free amino group of the diamino acid of one peptide subunit to the C terminal D-alanine of another peptide subunit (Peptidoglycan of type II). (iii) in one or several peptides, each having the same amino acid sequence as the peptide subunit (Peptidoglycan of type III). The location of the bridging is identical to that of type II peptidoglycan. When the bridge is composed of several peptide subunits, these subunits are linked to each other through 'head to tail' D-alanyl-L-alanine linkages. One peculiarity of type III peptidoglycans (*Micrococcus lysodeikticus* and related *Micrococci*) is that the number of peptide subunits may be greater than the number of disaccharide units of the glycan moiety and that not all the *N*-acetylmuramic acid residues are peptide substituted (Campbell, J. M., Leyh-Bouille, M. & Ghuysen, J. M. (1969), *Biochemistry*, 8, 193). (iiii) in a diamino acid residue, D-ornithine or D-lysine, extending between the α-COOH group of D-glutamic acid and C terminal D-alanine (Peptidoglycan of type IV) (some plant pathogenic *Corynebacteria* (2) and *Butyribacterium rettgeri* (Guinand, M., Ghuysen, J. M., Schleifer, K. H. & Kandler, O. (1969), *Biochemistry*, 8, 207)). The bond to the C terminal D-alanine always involves the α-amino group of the intervening diamino acid.

The peptidoglycans of the Gram-positive bacteria are at least 100 Å thick and, thus, probably occur as three-dimensional multilayered structures. It may be that some of the bridges which characterize the peptidoglycans of type II, III and IV, are used to interconnect several superimposed monolayers. The peptidoglycans of type I have no additional amino acids to insure the bridging between the peptide subunits. This might be related to the fact that the peptidoglycan in the Gram-negative bacteria probably occurs as a two-dimensional monolayer (20 Å thick). The 100 Å thick peptidoglycans in the Gram positive *Bacillaceae* might then be visualized as made up of several interlinked *E. coli*-like peptidoglycan monolayers. DD-diaminopimelic acid which has been characterized in walls of *Bacillus megaterium* KM could be involved in these cross-linkages. Similarly, the peptide cross-linking within the *Micrococcus varians meso*-diaminopimelic acid containing peptidoglycan might be mediated through a polyglutamic acid sequence (with the amino terminus linked to COOH group of D-alanine; unpublished).

Structure, Function and Biosynthesis of the Rigid Layer of the *Escherichia coli* Cell Wall.

By V. BRAUN and U. SCHWARZ (*Max-Planck-Institut für Biologie Tübingen, Germany.*)

The rigid layer of the cell wall of *Escherichia coli* consists of a macromolecule the size and the shape of the cell, which is held together exclusively by covalent bonds. The repeating unit (*N*-Acetylglucosamine-*N*-Acetylmuramic acid)$_n$ forms polysaccharide strands which are cross-linked by the peptide L-Ala-D-Glu-meso-DAP-(D-Ala)-D-Ala-meso-DAP-D-Glu-L-Ala. The resulting network is called murein (peptido-glycan, mucopeptide or glycopeptide). Data will be presented which show that in addition to the murein network (sacculus) the rigid layer contains a lipoprotein which is covalently bound to the murein. Enzymic degradation of the rigid layer with trypsin or pronase revealed that on the average there is one lipoprotein molecule bound to every tenth repeating unit of the murein. The lipoprotein is linked by its *N*-terminal lysine to the carboxyl group of 2·6 diaminopimelic acid of the murein and the following sequence has been established for the murein-lipoprotein link: DAP-Lys-Arg. The size of the protein is about 7000 and the absolute amino acid composition has been determined. There are strong indications that also a part of the lipid is covalently bound to the protein. A model of the rigid layer will be given in molecular dimensions in which about 10^5 lipoprotein molecules are evenly distributed over the entire surface of the rigid layer and where the lipoprotein molecules are spaced 96 Å apart along the polysaccharide chains of the murein.

The rigid layer essentially determines the shape of the cell. This function is not only due to its chemical structure but is also based on its interaction with the other components of the cell wall. Our results suggest that this interaction is primarily mediated by the lipoprotein. When cell walls are incubated with trypsin we found a rapid decrease in the optical density. The rate of the reaction is very high and can only be obtained with trypsin. The target of the rapid trypsin reaction is the above mentioned Lys-Arg bond. After a short trypsin incubation of cell walls (20 min., room temp., ratio of enzyme to total cell wall protein = 1:50) all the lipoprotein is removed except lysine for which one residue remains for every tenth murein building block. In ultra-thin sections of such trypsin treated cell walls, all cell walls are split into two well separated layers which otherwise are closely adjacent to one another. The fast decrease of the optical density and the parallel appearance of two layers in thin-sections are consistent with the view that the lipoprotein has an important function in stabilizing the cell wall.

The enlargement of the macromolecule during cell growth and division should depend on the action of at least two enzymic systems. The one should provide both space and acceptor sites for incoming material; this can be done only by hydrolysis of covalent bonds in the pre-existing murein. The second system would then insert the new precursors. The composition of these precursors is known. They are put together in the cytoplasm and transported by carrier molecules to the place where they are used. It is only partially known how these precursors are polymerized into the sacculus.

The essential participation of murein hydrolases in the process of murein synthesis has been proved. *In vivo* studies with a DAP-requiring mutant of *E. coli* have demonstrated the activity of at least five murein-degrading enzymes in growing cells. They seem to be pacemakers of murein biosynthesis. In a mutant with temperature dependent murein hydrolase activity, murein neosynthesis comes to a stop by preventing murein hydrolases from acting.

Furthermore, murein hydrolases play a key role in bacterial morphogenesis. Experiments dealing with morphogenetic aspects of murein synthesis have shown that the growth of the sacculus is accomplished by several functionally different systems which can be distinguished from each other by their differential sensitivity to penicillin. One is involved in cell elongation, the others in cell division. Penicillin was also used to localize the site of action of murein hydrolases. It was demonstrated that an exact splitting of the sacculus by such enzymes is a step preceding cell division, and the topology and timing of this splitting are correlated with DNA replication.

The Organisation of the Polymers in Gram-positive and Gram-negative bacteria. By H. J. ROGERS (*National Institute for Medical Research, Mill Hill, London, Great Britain*)

The structures of the mucopeptides, teichoic acids and polysaccharides in the walls of a number of Gram-positive bacteria are now known, and likewise in a number of Gram-negative species the structures of the mocupeptides and of much of the lipopolysaccharide molecules are now known. Among the Gram-negative species divalent cations appear to play a considerable role in linking together some of the components. A large proportion of the lipopolysaccharide can be removed from the walls of pseudomonads and by chelating agents such as EDTA (Gray, G. W. & Wilkinson, S. G. (1965), *J. gen. Microbiol.* 39, 385; Asbell, M. A. & Eagon, R. G. (1966), *Biochem. Biophys. Res. Commun.* 22, 664), whilst treatments involving EDTA, osmotic and cold shock, can release enzymes from Gram-negative cells that often appear as truly exo-cellular enzymes in Gram-positive species, e.g. ribonuclease, alkaline phosphatase, penicillinase (Neu, H. C. & Heppel, L. A. (1964*a*), *Biochem. Biophys. Res. Commun.* 17, 215; (1964*b*), *Proc. natn. Acad. Sci. U.S.A.* 51, 1267). Likewise penetration into the cytoplasmic membrane by substances which inhibit mucopeptide synthesis such as the penicillins meet diffusion barriers in Gram-negative bacteria which are not present in Gram-positive species and which can be damaged by chelating agents (Hamilton-Miller, J. M. T. (1966), *Biochem. J.* 100, 675). Thus the outermost layers of Gram-negative cells possess some of the properties usually associated with membranes rather than with walls. The functional wall behaves as if it were a region between two membranes rather than a region existing outside a membrane and which is freely permeable to smaller molecules as with Gram-positive species.

In a number of examples the polymers making up the walls of Gram-positive species have been shown to be linked together by firm covalent linkages which need relatively drastic treatment such as long exposure to low pH or treatment with hot formamide to break them. Recent work with *Bacillus licheniformis* and *B. subtilis*, for example, shows that both the teichoic acid and in the former, the teichuronic acid are linked by covalent linkages to the mucopeptide. When the walls are incubated with an autolytic enzyme hydrolysing the linkage between the terminal L-alanine of the peptide and the polysaccharide backbone (amidase), relatively small fragments can be isolated which contain teichoic acid, and teichuronic acid attached to the polysaccharide backbones of mucopeptide (Hughes, R. C., Pavlik, J. G., Rogers, J. H. & Tanner, P. J. (1968), *Nature. Lond.* 219, 642). The teichoic acid can be removed by treatment under very mild alkaline conditions leaving a teichuronic acid molecule joined by its terminal reducing *N*-acetylgalactosamine molecule probably through a phosphate group to the mucopeptide backbone (Hughes, R. C. & Tanner, P. J. (1968), *Biochem. Biophys. Res. Commun.* 33, 22).

The teichoic acid linked to a mucopeptide fraction containing an excess of glucosamine can be isolated and separated from the teichuronic acid-mucopeptide compound after the combined action of lysozyme, amidase and a ribonuclease preparation. Thus it appears that the teichuronic acid-mucopeptide, and the teichoic acid-*N*-acetyl glucosamine-mucopeptide may be linked together by a bond sensitive either to ribonuclease or to an enzyme present in preparations of crystalline ribonuclease.

The question arises as to how far the structure and organization of the various polymers in the walls defines the morphology and cell division of the micro-organisms. We have recently been examining morphological mutants of *Bacillus subtilis* 168 try$^-$ which grow in high NaCl concentration as rods, but in minimal medium of low tonicity as round cocci, these have been called rod$^-$. (Rogers, H. J., McConnell, M. & Burdett, I. D. J. (1968), *Nature, Lond.* 219, 285). The shape of the mutants can also be corrected from spherical to rod form by including a sufficient concentration of casein hydrolysate in the growth medium. The casein hydrolysate can be replaced by 0·2 % glutamate, or by 0·2 % of the amino acids, L-proline, L-arginine or DL-ornithine. The mutants in both morphological forms contain a higher proportion of wall per unit weight of micro-organism than the wild type. The mucopeptide in the round forms, in one class of rod$^-$ mutant, is much less cross-linked than that in the rod-shaped cells grown in the presence of either high salt concentration or with casein hydrolysate. Grown in the presence of salt the walls contain much less ester-alanine in the phosphate containing polymers and more

N-acetylgalactosamine. Another class of rod⁻ mutants has also been isolated for which salt or casein hydrolysate are ineffective in correcting the shape back to a rod. In these the walls have the same degree of cross-linking as is present in the parent. This second class of mutant has nutritional requirements not shared by the parent.

Sructural Studies on some Salmonella lipopolysaccharides. By BENGT LINDBERG (*Institutionen för organisk kemi, Stockholms Universitet, Stockholm, Sweden*)

Lipopolysaccharides (LPS) from Gram-negative bacteria consist of a lipid part, an O-specific side chain and a 'basal core', linking the O-specific side chain to the lipid part. The O-specific side chains, which carry the O-antigens of the bacteria, are composed of oligosaccharide repeating units, containing three or more sugar residues. Structural studies on the O-specific side chains of LPS from different Salmonella bacteria, belonging to groups A, B, C, D and E are presented.

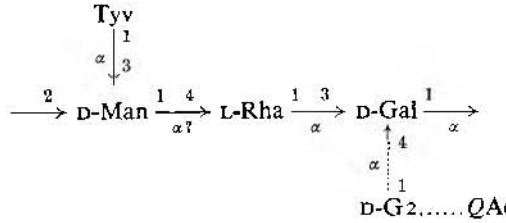
Methylation analysis has provided the essential informaion in these studies and has been performed by a new technique, whereby the mixture of methylated sugars, obtained on hydrolysis of the fully methylated polysaccharide, is qualitatively and quantitatively analysed GLC-mass spectrometry of their alditol acetates (Björndal, H., Lindberg, B. & Svensson, S. (1968), *Acta chem. scand.* **21**, 1801; (1968), *Carbohyd. Res.* 5, 433).

By methylation analysis of the original LPS and of the partially hydrolysed LPS, it has been possible to determine the structures of the 'biological repeating unit' and the average number of repeating units in the O-specific side chains of several LPS.

Methylation analysis does not provide any information on the anomeric nature of the sugar residues. For some sugar residues, these have been determined by following the change in optical rotation on acid hydrolysis. For other sugar residues, this has been done, mostly by other investigators, by partial hydrolysis and structural determination of the resulting oligosaccharides.

Several LPS contain *O*-acetyl groups, which may be immunologically significant. The positions of such groups have been determined by protecting all hydroxyl groups as acetals, removing the *O*-acetyl groups by alkaline hydrolysis and subjecting the derived polysaccharide to methylation analysis (de Belder, A. N. & Norrman, B. (1968), *Carbohyd. Res.* 8, 1).

As a result of these studies, complete or partial structures of O-specific side chains in several LPS have been determined. (Hellerqvist, C. G., Lindberg, B., Svensson, S., Holme, T. & Lindberg, A. A. (1968), *Carbohyd. Res.* 8, 43; (1969) 9, 237). Some results from previous studies have been confirmed, others have been revised and new structural features have been demonstrated. As an example, the structure of the repeating unit in a strain of *S. typhi* (9, 12_2) is given below. A dotted line



connecting a residue to the repeating unit indicates that only some of the repeating units contain this residue. The terminal α-tyvelose residue, linked to the 3-position in D-mannose, and the terminal α-D-glucose residue, linked to the 4-position in D-galactose, are associated with the presence of O-factors 9 and 12_2, respectively. The immunological significance of the 2-*O*-acetyl groups, present in some of the D-glucose residues, is not known.

How Genes Determine the Structure of the Salmonella Lipopolysaccharide By P. HELENA MÄKELÄ and B. A. D. STOCKER (*State Serum Institute, Helsinki, Finland, and Department of Medical Microbiology, Stanford University, California*)

The genetic determination of the polysaccharide part of the Salmonella lipopolysaccharide (LPS) has been studied over a period of 6–7 years. Mutants affected in various steps of LPS synthesis as well as representatives of several Salmonella species belonging to different O antigen groups have been used to identify the genes involved.

The polysaccharide consists of a *core*, common to all Salmonella serogroups and containing ketodeoxyoctonate, heptose, phosphate, glucose, galactose and *N*-acetylglucosamine, and of O *side-chains* attached to the second glucose of the core and differing in structure between different O groups. Corresponding to these two parts, two gene clusters, *rfa* and *rfb*, were identified.

The core is synthesized by sequential addition of monosaccharides from their nucleotide donors to the unfinished core, each addition requiring the action of a specific transferase. The *rfa* cluster, located on the bacterial chromosome between the loci *cysE* and *pyrE* and cotransducible with these, contains genes determining the synthesis of most of these transferases, including the transferase of the phosphate to heptose. The gene *rfaH* required for the transfer of one of the sugars (the first galactose) in the main chain of the core does, however, not appear to be in the same cluster.

The O side-chains are synthesized separately, on an intermediate lipid carrier called ACL. An oligosaccharide, the *repeating unit*, is first assembled by sequential addition of its component monosaccharides, and then polymerized still on the ACL. The polymer is then 'translocated' onto the core; if the core is not complete, this step cannot be performed and the polymer-ACL accumulates as O-specific 'hapten'. The *rfb* cluster, co-transducible with the histidine operon, contains several (at least ten in *Salmonella typhimurium*) genes for enzymes required in the synthesis of the monosaccharide-nucleotides, and the genes specifying the transferases needed for the assembly of the repeating unit. Hybrids of two Salmonella species belonging to different O groups have established that the *rfb* cluster contains all the information necessary to specify the structure of the repeating unit.

The polymerization of the repeating units requires a polymerase, which in groups B and D is specified by *rfc* gene(s) between *gal* and *trp*. The translocation of the polymer onto the core appears to require the cooperation of two genes, one in the *rfa* cluster, the other in the *rfb* cluster—each one perhaps specifying a subunit of the ligase enzyme. Finally, a *rfe* gene or genes close to *ilv* are required for the synthesis of O-specific material at least in O groups C_1 and L. It seems possible that their products might participate in the synthesis or preparation of ACL for LPS synthesis. T 1-specific side-chains are found attached to the core in the same manner as are O chains. Their synthesis is dependent on gene(s) in a separate cluster, called *rft*[1].

Mutations in any one of the genes mentioned result in the synthesis of a very deficient LPS without long O-specific side-chains, and leading to a rough (*R*) phenotype of the bacteria. A basic structure of the repeating units (and of the core) is therefore necessary for their utilization in the synthesis. Modifications of the basic structure are, however, permissible, and are recognized as O antigen factors such as 5 or 10 (O-acetylation of certain sugars in the O chain), 1 or 12_2 (glucose side-branches). Variants with or without these factors are found in nature or as laboratory mutants. Many of them are variable even within one clone, and many are determined by temperate bacteriophages. The *oaf* genes responsible for these factors are found outside the *rf.* clusters, and several of them are located in the upper right-hand quadrant of the chromosome.

Structure of the Cytoplasmic Membrane and Mesosomes. By D. J. ELLAR (*University of Cambridge, Great Britain*)

In the search for an understanding of the principles governing the morphogenesis and function of cellular structures, the cytoplasmic membrane of bacteria continues to provide valuable insights. Early electron microscope studies revealed an apparent absence in bacteria

of those organelles typically found in animal and plant cells and supported the classification of bacteria as prokaryotic cells. This classification elaborated by Stanier and van Niel in their classic paper ((1960), *Archiv. Mikrobiol.* **42**, 17) included as one of the distinguishing features of the prokaryotic cell a lack of internal membranes separating the cytoplasm from both the nucleus and the machinery of photosynthesis and respiration. While subsequent refinements in cytological techniques have confirmed the absence of a nuclear membrane in bacteria, the situation is not so unequivocal with regard to photosynthetic and other functional organelles. There is in fact considerable evidence that bacteria may contain units of cellular function enclosed within a membrane which forms a barrier of sorts between these structures and other regions of the cell. A careful study of the photosynthetic bacterium *Ectothiorhodospira mobilis* (Remsen, C. C. *et al.* (1968), *J. Bact.* **95**, 2374) revealed a photosynthetic lamellar system very similar in appearance to the aukaryotic chloroplast. Nevertheless, no simple analogy can be drawn between these two photosynthetic structures, since it is clear that in *E. mobilis* the lamellar stack originates from one or more plasma membrane invaginations. In the fully developed stack these invaginations persist to constitute an opening into the special environment which lies between the plasma membrane and the external layers of the cell envelope. This persistent connexion with the extracellular space also appears to occur in the vesicular membrane network found in other photosynthetic bacteria, but it is not a feature of eukaryotic chloroplasts. Such connexions have been repeatedly demonstrated in the case of bacterial mesosomes (Ellar, D. J. *et al.* (1967), *J. Bact.* **94**, 1189), although the function of these structures is still not clear. It appears therefore that the prokaryotic bacteria may indeed contain organelles isolated from the surrounding cytoplasm in the rather special sense that their interior, together with the outer surface of the plasma membrane is in direct communication with the extracytoplasmic space. The elimination of this communication by a fusion of apposed membranes at the base of the original invagination, could create a second type of specialized environment isolated now from both the cytoplasm and the extracellular space. In prokaryotic cells these phenomena may fulfil a particular evolutionary requirement for spatial organization, or for transport of certain molecules to and from the external environment.

Unfortunately these connexions between the plasma membrane and intracellular bacterial membrane structures have frequently hampered attempts to separate and purify them. Recently, however, by exploiting this connexion it has been possible to isolate the membranes of the mesosome in pure form using the techniques of plasmolysis and protoplast formation. (Ellar, D. J. & Freer, J. H. (1969), *J. gen. Microbiol.* (in press); Ferrandes, B. *et al.* (1966), *C. r. Acad. Sci., Paris* **263**, 1632); Reavely, D. A. (1968), *Biochem. biophys. Res. Commun.* **30**, 649). In this procedure the membrane bounding the mesosome evaginates, becoming part of the protoplast membrane and expelling its contents into the suspending medium. The influence of cation concentration on this expulsion will be discussed. Mesosomal membranes isolated in this way can now be compared chemically and functionally with the plasma membrane freed from mesosomal material. Furthermore, suggestions as to a mesosomal role in membrane synthesis, the uptake of transforming DNA, transverse septum synthesis and the partitioning of the bacterial genome are now more amenable to experimental study.

The results of comparisons of mesosomal and plasma membranes from *Micrococcus lysodeikticus* and *Bacillus megaterium* will be presented. These studies reveal significant differences in protein and lipid content which are in turn reflected in density differences upon sucrose gradient centrifugation. Mesosomes and plasma membranes have also been compared with respect to their content of enzymes and respiratory pigments, their ability to synthesise cell wall peptidoglycan *in vitro* and their ability to incorporate radioactivity into membrane lipids. The results of an electron microscope examination of these fractions will be reported. Experiments will be discussed which indicate considerable structural similarity between the plasma membrane of *M. lysodeikticus* and the inner mitochondrial membrane of eukaryotic cells. A number of enzymes including ATP'ase, $NADH_2$'ase, succinic dehydrogenase and cytochromes have been shown to be associated with the *M. lysodeikticus* membrane. Of these, the ATP'ase and $NADH_2$'ase can be selectively dissociated by washing with buffers and appear to represent 'detachable' components existing as granular substructure on the membrane (Muñoz, E. *et al.* (1968), *Biochem. biophys. Res. Commun.* **32**, 539; Ellar, D. J. *et al.* (in preparation)). Extensive washing to remove these components results in the production of

a smooth-surfaced residue resistant to further washing which contains the components of the electron transport system including the total succinic dehydrogenase activity. A markedly similar residue can be obtained by controlled washing of the freshly isolated plasma membrane with deoxycholate, (Salton, M. R. J. *et al.* (1968), *Biochem. biophys. Res. Commun.* **36**, 909). Evidence to be discussed suggests that certain components of the electron transport chain in *M. lysodeikticus* exist in a relatively rigid membrane fraction which resists various procedures designed to remove 'detachable' proteins. The relatively mild washing techniques required to produce this fraction favours the view that it constitutes an important functional and structural component of the native membrane. The use of low glutaraldehyde concentrations to modify the strength of attachment of membrane associated enzymes will be discussed.

Organization of Protein and Lipid in the Mycoplasma Membrane. By SHMUEL RAZIN (*Department of Clinical Microbiology, The Hebrew Universtiy-Hadassah Medical School, Jerusalem, Israel*)

Devoid of a cell wall and intracytoplasmic membranes, the mycoplasmas have only one type of membrane—the plasma membrane. This makes them excellent models for membrane study (Razin, S. (1969), *The Mycoplasmatales and L-Phase of Bacteria*, ed. L. Hayflick, Appelton-Century-Crofts: New York). Highly purified membranes have been isolated from several mycoplasmas by osmotic lysis, and were shown to consist of 50 to 60 % protein and 30 to 40 % lipid (Razin, S. (1967), *Ann. N.Y.Acad. Sci.* **143**, 115). Some of the biophysical and catalytic properties of the membrane proteins have recently been characterized (Rodwell, A. W. *et al.* (1967), *Archs Biochem. Biophys.* **122**, 621; Rottem, S. & Razin, S. (1967), *J. Bact.* **94**, 359; Engelman, D. M. ; Morowitz, H. J. (1968), *Biochim. biophys. Acta.* **150**, 385), and considerable information on the chemical nature of the membrane lipids is available (Smith, P. F. (1967), *Ann. N.Y. Acad. Sci.* **143**, 139). However, the organization of the protein and lipid in the membrane is not yet clear. Its investigation is likely to be of special interest as it might contribute to the solution of the highly disputed problem whether the biological membrane is built of a bimolecular leaflet of lipid coated with protein on both sides, of repeating lipoprotein subunits, or of some combination of both.

Morphological evidence for a subunit architecture is scanty and inconclusive. The subunits observed in negatively-stained membranes of *Mycoplasma pulmonis* (Hummeler, K. *et al.* (1965), *J.. Bact.* **90**, 517) and *M. gallisepticum* (Chu, H. P. & Horne, R. W. (1967), *Ann. N.Y. Acad. Sci.* **143**, 190) probably are surface projections, while the structure of the membrane *per se* may still conform to the leaflet model. On the other hand, the fact that the triple-layered structure is preserved in sectioned *M. laidlawii* membranes after about 95 % of the membrane lipids have been removed with aqueous acetone (Terry, T. M. (1966), Ph.D. Thesis, Yale Univ.) does not favour the leaflet theory.

Solubilization of mycoplasma membranes by sodium dodecyl sulphate (SDS) has yielded a clear solution exhibiting a single symmetrical schlieren peak of about 3 *S*. The initial interpretation that this represented lipoprotein subunits (Razin, S. *et al.* (1965), *Proc. natn. Acad. Sci. U.S.A.* **54**, 219) was disproven by showing that by ultracentrifugation or electrophoresis the protein could be separated from the lipid in the SDS-solubilized material (Engelman, D. M. *et al.* (1967), *Biochim. biophys. Acta* **135**, 381; Rottem, S. *et al.* (1968), *Arch. Biochem. biophys.* **125**, 46). Nevertheless, on removal of the detergent by dialysis, in the presence of Mg^{2+} or some other divalent or polyvalent cation, the lipid-SDS and protein-SDS micelles reaggregated spontaneously to structures morphologically and chemically resembling the original membranes (Razin, S. *et al.* (1965), *Proc. natn. Acad. Sci. U.S.A.* **54**, 219; Terry, T. M. *et al.* (1967), *Biochim. Biophys. Acta* **135**, 391). At a low Mg^{2+} concentration (5×10^{-3} M) the membraneous structures formed had a higher lipid to protein ratio than at the optimal Mg^{2+} concentration (2×10^{-2} M; Rottem, S. *et al.* (1968), *Arch. Biochem. Biophys.* **125**, 46). Electrophoretic and enzymic analyses tend to support the conclusion that the incorporation of different proteins into the reformed membranes at various Mg^{2+} concentrations is selective. Study of the reaggregation kinetics during dialysis against 2×10^{-2} M–Mg^{2+} has shown that initially the lipid-rich membranes are formed, which upon further dialysis are apparently transformed into membranes whose lipid to protein ratio resembles that of the original

membranes (Razin, S. *et al.* to be published). The reformation phenomenon may perhaps be visualized as a multi-step process which involves the formation of a primary lipid-rich membraneous structure containing only part of the membrane protein species. More protein can be bound to this structure when enough Mg^{2+} ions become available to neutralize electrostatic repulsive forces interfering with this binding. Though it is still doubtful whether such a mechanism also operates in membrane formation *in vivo*, it may be safely concluded that membrane protein and lipid molecules contain sufficient structure-determining information to interact spontaneously to form membrane-like structures in the absence of a pre-existing membrane template.

Recent studies (Kahane, I. & Razin, S. (1969), *Biochim, biophys. Acta.* in the Press) have shown that membrane protein and lipid biosynthesis in *Mycoplasma laidlawii* are not necessarily synchronized, so that plasma membranes having a different lipid to protein ratio can be formed. This finding speaks against the hypothesis that the membrane is built of lipoprotein subunits having a constant lipid to protein ratio.

SYMPOSIUM: ANIMAL DNA VIRUSES

A memorial review of the work of the late Dr Robin Valentine.

Internal Components of Adenovirus By W. C. RUSSELL (*National Institute for Medical Research, Mill Hill, London, Great Britain*)

At least 25 % of the total protein of adenovirus resides within the viral capsid in close association with the nucleic acid. By acrylamide gel electrophoresis it can be shown that this 'core' protein consists of five components two of which are rich in arginine. These components are soluble in mineral acids and possess some of the characteristics of the arginine-rich histones; in some other respects they bear similarities to the protamines. Amino acid analyses have been carried out on the acid-soluble components and these have been compared with the amino acid patterns characteristic of histones and protamines. A number of methods of preparing viral 'cores' have been investigated, the primary purpose being to obtain the structures in their original configuration and free of contaminating capsid components. Attempts have also been made to obtain viral 'cores' from infected cells but these have not been successful. Some physical and biological properties of the 'cores' obtained by disruption of the capsid will be described.

The relationship of the core components to events in the infected cells have been investigated by 'pulse' labelling and serological techniques. Thus, immunofluorescence can detect an antigen whose production depends on the presence of exogenous arginine and which is probably related to the 'core' components. Early in infection cellular DNA synthesis is inhibited but this event does not appear to be related to the production of the antigen requiring exogenous arginine. The possibility that the arginine-rich core components are concerned essentially with the packaging of the viral nucleic acid has also been investigated and the results of some preliminary experiments will be described.

Capsid Mosaics of Human Adenoviruses. BY ERLING NORRBY (*Department of Virology, Karolinska institutet, Faculty of Medicine, Stockholm University, Stockholm, Sweden*)

Two distinct antigen specifications of type-specific nature are available at the surface of adenovirus virions. One is carried by fibres and can be demonstrated in hemagglutination-inhibition (HI) tests by use of soluble components as antigen and the other is carried by hexons. Antibodies against the latter can not inhibit the hemagglutinin (HA) activity of soluble components, but still in most cases (some members of Rosen's subgroup III form an exception) effectively inhibit the HA activity carried by homotypic virions. Antibodies against hexons play the major role as concerns neutralization of adenovirus infectivity.

Prototype strains can be specified by reference to the two type-specific antigens mentioned, viz. by neutralization tests (or virion-HI tests in most cases) and by HI tests with soluble components. A number of adenovirus strains have been identified in which

(*a*) only one of the two type-specific antigens are related to that of a prototype strain, or (*b*) the two antigen specificities are related to two *different* prototype strains. The latter type of strains have been called intermediate strains. By use of different immunological techniques including immune electron microscopy, attempts have been made to characterize, on the level of structural components, the relationship between prototype strains and strains displaying immunological characteristics of the kind mentioned above. As an example of strains sharing only one of the two type-specific antigens, the prototype strains 4 and 16 were chosen. These two strains which display a relationship (more pronounced in one direction) in neutralization tests were found to share a unique antigen specificity carried by hexons. Similarly intermediate strains (types 3–16 and 15–9 were studied) were found to carry hexons similar to, but not identical with hexons of the prototype strains to which they were related in neutralization tests. They furthermore carried fibres (alternatively pentons) immunologically related to those of prototype strains, with which they cross-reacted in HI tests.

Phenotype mixing experiments have revealed that mixing between structural components can be readily established both within the capsid of virions and within polymers of soluble components. Phenotypic mixing occurred even between distantly related serotypes, e.g. representative members of different subgroups. The occurrence of capsids including pentons of different prototype origin was readily demonstrated by identification of fibres of different lengths in isolated particles and of the occurrence of different HA activities carried by them. Phenotypic mixing between hexons was analysed by immune electron microscopy. Examples of mixing between structural components of different serotype origin into soluble polymers were found. For example, components of type 16, which normally form a soluble HA represented by dimers of pentons or dimers of fibres, were found to mix with type 4 pentons into dodecons.

Fate of Adenovirus During the Early Phase of Infection. By L. PHILIPSON and K. LONBERG-HOLM (*Department of Microbiology, The Wallenberg Laboratory, University of Uppsala, Uppsala, Sweden*)

Adenovirion type 2 are uncoated by KB or HeLa cells to essentially free DNA. About half of this DNA is located within the nucleus. At least 3 intermediates can be resolved by appropriate gradient centrifugation techniques. The schematic overall sequence is:

A free virus
↓
B virus-receptor complex
↓
C partly uncoated intracellular virus
↓
D lipid complex with virus core
↓
E viral DNA

The virion (A) attaches to specific receptors on the plasma membrane via the fibre antigen to form component B. There are about 10^4 receptor sites per cell and these are mainly on the cell surface where they can be digested by subtilisin. B can be formed in cell free systems and this is blocked by pure fibre antigen.

The breakdown of B to C appears to be first order with a half life of the order of 10 min. Component C is partly DNase sensitive, has a buoyant density about 0·01 g./c.c. greater than A and probably lacks some or all penton units. There is no A within the cell. About half of component C is rapidly distributed into the cell nucleus.

The third virion product behaves in sucrose and CsCl gradients as to suggest that it is the virion core bound to a membrane component.

The fourth step involves the weakening of binding, or removal, of virion core proteins from the DNA. This goes to completion in about two hours in the nucleus but is slower in the cytoplasm. Even during the last step there is no major degradation of the DNA as is shown by alkaline-rate-zonal centrifugation. There is also no evidence for the formation of covalent circles or integrated forms with cellular DNA.

The proposed steps during the first 2 hr of infection are thought to have biological significance. It cannot yet, however, be proven that they are obligatory in the infective process because the ratio of infective to physical particles is only about 0·03 in this system.

Synthesis and Morphogenesis of Adenovirus Capsid Proteins. By H. S. GINSBERG and L. F. VELICER (*Department of Microbiology, University of Pennsylvania, Philadelphia, Pennsylvania, U.S.A.*)

The icosahedral adenovirus capsid is composed of 240 face capsomers, the hexons, and 12 corner units, the pentons. Each penton consists of a base unit and a fibre of varying length. Although viral DNA is synthesized in the nucleus, the capsid proteins are only detected in the nucleus by immunofluorescent techniques, and the virions are assembled in the nucleus, autoradiography demonstrates that the viral proteins are synthesized in the cytoplasm and rapidly transported into the nucleus (Velicer, L. F. & Ginsberg, H. S. (1968), *Proc. natn. Acad. Sci. U.S.A.* **61**, 1264). The viral proteins are synthesized on polyribosomes with an average sedimentation coefficient of 200S, and the polypeptide chains synthesized have an average sedimentation coefficient of about 3·4S.

The viral polypeptide chains are synthesized within 1 min. after which they are rapidly released from the polyribosomes. After release from the site of their biosynthesis the nascent polypeptide chains acquire immunological reactivity of a rate similar to that at which they are transported from the cytoplasm into the nucleus. Utilizing isotope labeling pulse-chase techniques, the newly made polypeptide chains, which are of relatively uniform size, can be followed into 4 species of multimeric proteins having sedimentation coefficients of 6S, 9S, 10·5S, and 12S. When purified I^{125}-labelled fibres and hexons are used as markers the 6S and 12S proteins are seen to sediment in sucrose gradients as fibre and hexon proteins, respectively. The 6S and 12S proteins can also be identified immunologically as fibre and hexon respectively. The 9S protein is trypsin sensitive and in pulse-chase experiments it appears to be a precursor of the 10·5S macromolecule which on reaction with trypsin yields a 6S fibre protein and acid-soluble material. Hence, the 10·5S protein may be identified as the penton capsid structure.

It is striking that the hexon, which has a molecular weight of 300,000 daltons, and the fibre, which is about 65,000 daltons, can be disrupted into polypeptide chains with sedimentation coefficients of 3–3·5S. The hexon consists of 12 identical subunits of 25,400 daltons, and preliminary data indicate that the fibre is made from 3 polypeptide chains each with a molecular weight of about 22,000 daltons. Thus, the adenovirus capsid proteins are polymers composed of similar sized polypeptide chains which are synthesized on cytoplasmic polyribosomes after which they rapidly move into the nucleus and undergo immunological and morphological maturation before assembly into the virion.

Interactions Between γ-Globulins and Adenovirus Virions. By L. KJELLÉN (*University of Lund, Sweden*)

Reactions between adenovirus type 5 and separated fractions of rabbit IgC will be described.

Heavy chain fractions of rabbit IgC, rendered soluble in glycine-HCl buffer at pH 3·0, retain antiviral activity. No activity is found in the light chain fractions. The power to inactivate adenovirus is not limited to H chains, separated from anti adenovirus IgC molecules. H-chains separated from IgG's of rabbits, immunized with unrelated antigens or rabbits not immunized at all, show activity as well. The 'specificity' of the reactions will be discussed. A detailed report will be published elsewhere.

***In vitro* Transformation by Human Adenoviruses.** By J. VAN DER NOORDAA (*University of Amsterdam, Holland*)

Oncogenic human adenoviruses can be classified in three subgroups: A, B, and C. The viruses of subgroup A (types 12, 18,31) are highly oncogenic in hamsters, those of subgroup B (types 3, 7, 14, 16, 21) are weakly oncogenic and the 'non-oncogenic' subgroup C viruses (types 1, 2, 5) have the ability to transform rat cells *in vitro*. A number of adenoviruses of these

three subgroups have been shown to transform several types of cells derived from various species of animals.

Of the subgroup A, adenovirus type 12 transforms hamster, rat, and rabbit cells. The *in vitro* transformation studies with adenovirus type 12 and other types of adenovirus have been facilitated by the use of media containing low concentrations of calcium (0·1 mM). The adeno-12 transformed cells contain virus specific nuclear antigens (T, or tumour antigen) and virus specific messenger RNA. The cells transformed by adenovirus type 12 are oncogenic when introduced into the appropriate host system. Recently it has been demonstrated that adenovirus type 12 is able to induce foci of altered cells in certain susceptible human cell cultures. As yet it has not been possible to establish lines of transformed cells from these altered foci.

Of subgroup B, adenovirus type 3 transforms rat embryo and rat kidney cells. The adeno-3 transformed rat cells are oncogenic; they contain a subgroup B specific tumour antigen. Transformation of rat embryo cells by adenovirus type 7 has been mentioned.

The so-called non-oncogenic subgroup C adenoviruses types 1, 2 and 5 share the ability to induce morphological transformation of both rat embryo and rat kidney cells. By employing sera from hamsters carrying tumours induced by the adeno-1-SV40 and adeno-2-SV40 hybrid viruses a common T antigen has been detected in rat cells transformed by adenoviruses types 1, 2, and 5. The rat cells, morphologically transformed by subgroup C viruses, have not yet been proved to be oncogenic.

Recent studies in our laboratory have revealed the ability of the non-oncogenic adenovirus type 4 to transform rat kidney cells. Of interest was the enhancing effect of u.v.-irradiation of the virus. A decline of infectivity, caused by u.v.-irradiation of the virus, was accompanied by an enhanced transforming ability of the virus. The antigenic and oncogenic properties of the adeno-4 transformed cells are currently studied. Preliminary CF tests seem to indicate the presence of a T antigen in the adeno-4 transformed cells which is related to the T antigen of adeno-3 transformed cells.

Infectious adenovirus has not yet been recovered from any of the transformed or tumour cells.

The Molecular Structure of SV_{40} Virions. By M. A. Koch and F. A. Anderer (*Justus Liebig-Universität, Giessen, Germany*)

The simian virus 40 (SV_{40}) is a naked DNA containing virus with a diameter of 42 mμ. The molecular weight of the particle is $17{\cdot}3 \times 10^6$. Purified virus consists only of DNA and protein.

The DNA of SV_{40} is doublestranded, forming circular, super-coiled molecules with a S_{20} of 21 S. The molecular weight of the DNA was found to be $2{\cdot}3 \times 10^6 \pm 15\,\%$.

Under conditions known to dissociate an assembly of polypeptide chains into monomers the protein moeity of SV_{40} consists of three different species of polypeptide chains, which can be separated by electrophoresis and by chromatography. These polypeptide chains were designated A, B and C. The polypeptides A and B are present in equimolar amounts and constitute together 90 % of the protein moiety. The polypeptide chain C is a basic protein containing more than 20 % basic amino acid residues. The molecular weights of the polypeptide chains were determined: M = 16, 800 ± 10 % for C, M = 16,900 ± 10 % for B and M = 16,400 for type A polypeptide chains.

Exposure of purified SV_{40} to pH 10·5 at low ionic strength results in degradation of the virion. The resulting degradation products can be separated in two fractions by sucrose density gradient centrifugation. One fraction contains material sedimenting with 30–40 S, while the second fraction sediments with 2·5–5·0 S. This slower sedimenting fraction contains no DNA and consists only of the A- and B-polypeptide chains. The faster sedimenting fraction contains the viral DNA together with the C-polypeptide chains. The molecular weight of the alkali-stable DNA-protein complex was determined to be $3{\cdot}7 \times 10^6$. Assuming that all C-polypeptide chains of the virion are bound to the viral DNA in this complex, a value of $3{\cdot}65 \times 10^6$ can be calculated for the molecular weight. The conclusion that the polypeptide chains type C are fixed to the viral DNA is supported by the finding that on tryptic digestion of virions the A and B chains are more rapidly degraded than the C-chains.

Electron microscopic investigation of the SV_{40} virion revealed that 72 morphological units compose the icosahedral viral particle. The symmetry of the icosahedral surface lattice is $T = 7d$.

To form the $T = 7$ icosahedral surface lattice 420 structural units are required. If one assumes that one A and one B polypeptide chain form together one structure unit, then the weight of the particle shell can be calculated to be $420 \times (16{,}400 + 16{,}900) = 13{\cdot}98 \times 10^6$. Together with the DNA C-polypeptide complex the calculated molecular weight is $17{\cdot}6 \times 10^6$.

Purified preparations of SV_{40} particles lacking DNA (= empty particles) were also investigated. Such particles were composed, as the SV_{40} virions, only of the three known polypeptides. As in full particles the polypeptide C constitutes 10 % of the viral protein. From this observation it is concluded that the C polypeptide chains interact specifically with the particle shell. These interactions are broken during alkaline degradation.

On the basis of these findings it is proposed that the structure units consist each of one A- and one B-polypeptide chain. 420 structure units are arranged to form a particle with a $T = 7d$ icosahedral surface lattice. The C-polypeptide chains, being affixed to shell and DNA, orient the DNA within the particle.

Pox Viruses. K. DUMBELL (*Wright Fleming Institute of Microbiology, London, Great Britain*)

No abstract.

ORIGINAL PAPERS

SESSION A

Three-dimensional Molecular Models of the Mucopeptide from *Staphylococcus aureus*. By MARIANNE V. KELEMEN and H. J. ROGERS (*Department of Pharmaceutics, The School of Pharmacy, and The National Institute for Medical Research, Mill Hill, London, Great Britain*).

The composition and the chemical fine structure of some mucopeptides have been well established. However, no detailed study has yet been made of the secondary structures involved. As a preliminary step before undertaking physical measurements on mucopeptides, and in the light of the difficulties likely to be met in the interpretation of results of examining undergraded material, models of the mucopeptides from *Staphylococcus aureus* and Bacillus species have been constructed to see whether it is possible to distinguish the most likely secondary structures.

For *Staphylococcus aureus* the model consists of three polysaccharide chains, each containing eight monosaccharides, *N*-acetylglucosamine being placed at the non-reducing end of the chain. The peptide attached to the *N*-acetylmuramic acid residue contains one molecule of L-alanine, D-isoglutamate, L-lysine and D-alanine. The α-carboxyl group of D-isoglutamate is amidated, cross-linking between peptide chains is obtained by linking the carboxyl end of the pentaglycine to the ϵ-amino group of L-lysine in one chain and the amino group of the pentaglycine to the carboxyl group of D-alanine in another chain.

The polysaccharide chains run head to tail and can be hydrogen bonded as in cellulose or chitin. Two spatial configurations of the peptide chain have been constructed, a helical and a pleated (β-configuration) sheet structure. The helical structure forms few hydrogen bonds, and the required repeating unit for an α-helix is absent as within the chain peptide bonds are formed from the γ-carboxyl of D-glutamate, and the ϵ-amino group of L-lysine. The pleated sheet or β-configuration containing trans carboxyl groups provides a regular net-like structure in which 60 % of the amino groups are hydrogen bonded.

In mucopeptide from various species of bacilli, the L-lysine in the peptide chains is replaced by DL2,6 diaminopimelic acid (DAP) and no D-alanine exists with a free carboxyl group. (Hughes, R. C., Pavlick, J. G., Rogers, H. J. & Tanner, P. J. (1968), *Nature, Lond*, **219**, 642). The cross-linking is directly between the DAP 6-amino group and the carboxyl group

of the D-alanine in the next peptide chain. Again, when extended in the β configuration extensive hydrogen bonding is possible, but only between pairs of heptapeptide chains.

It is hoped that speculations such as these will lead to further experiments which could test how mocopeptides provide both shape and rigidity to the bacterial cell wall.

Erythrocyte-sensitizing Activity of Staphylococcal Mucopeptide. By ARNE GROV (*The University of Bergen, School of Medicine, The Gade Institute, Department of Microbiology, Bergen, Norway*)

Teichoic acid-mucopeptide (T–M) complexes of staphylococci sensitize tanned sheep erythrocytes (TSE) for agglutination by antisera to staphylococci. A ^{32}P-labelled T–M complex from *Staphylococcus aureus* strain WOOD 46 sensitized TSE, but no radioactivity was detectable in the sensitized and washed TSE. Radioactivity was detected in dilutions of the ^{32}P-labelled complex corresponding to a content of 0·05 μg. The minimal sensitizing dose of the ^{32}P-labelled complex was 10 μg., and the minimal inhibitory dose was 0·5 μg. These results indicate that the teichoic acid is not attached to the TSE and that the mucopeptide moiety is responsible for the present serological activity. This conclusion is supported by the lack of precipitins against teichoic acid in antiserum to TSE sensitized with T–M complex, the inability of sensitized TSE to absorb teichoic acid precipitins, and by the ability of TSE to remove haemagglutinogens but not precipitinogens from a solution of T-M complex.

A TSE-sensitizing peptide, shown to be a mucopeptide subunit, has been isolated from *S. aureus*. This substance cross-reacted serologically with the T-M complex in indirect haemagglutination. Absorption and inhibition experiments revealed that the mucopeptide moiety of the T-M complex exhibits two antigenic specificities: one is due to the peptide subunit and cross-reacts with the isolated TSE-sensitizing substance, the other is due to the amino sugars.

Substrate Dependent Modifications of the Amino Acid Sequence of the Murein of Staphylococci. By K. H. SCHLEIFER (*Botanisches Institut der Universität München, Germany*)

The interpeptide chains in the murein (peptidoglycan) of staphylococci depend qualitatively and quantitatively on the amino acid content of the medium.

When *Staphylococcus aureus*, strain COPENHAGEN, is grown in the presence of an unfavourable ratio of glycine to alanine only one-third of the ϵ-amino groups of lysine is substituted by a pentaglycine, while one-third remains free and one-third is substituted by L-alanine. These L-alanine residues remain *N*-terminal and do not contribute to cross-linkage. Even on a defined medium which contains 50 mg. glycine/l. (enough for optimal growth) but no alanine, some ϵ-amino groups of lysine carry an L-alanine. When the glycine content is increased to 0·1 % almost all ϵ-amino groups of lysine are substituted by pentaglycine. The same is true when D,L-serine is added, however, about 0·5 mole of serine per mole of peptide subunit are incorporated into the interpeptide chains in this case. The morphology of the cell remains unchanged under the various conditions, although the percentage of cross-linkages differs greatly. A similar influence of the amino acid content of the medium on the cross-linkage of the murein was found in *S. epidermidis* strain 24. The murein of this strain contains about 0·5 mole of serine per mole peptide subunit even when no serine is added. In addition less L-alanine is bound to the ϵ-amino groups of lysine as compared to *S. aureus* when grown under identical conditions.

S. epidermidis strain 66 represents a different type. Here the interpeptide chain is Gly-Gly-Gly-Gly-L-Ala and, therefore, the ϵ-amino group of lysine is obligatory substituted by L-alanine. Even when a large excess of glycine is added the amount of L-alanine remains constant and the interpeptide chain is not changed to a pentaglycine. When serine is added to the medium, it can replace some glycine (about 0·3 mole per mole of peptide subunit) similar to the other strains mentioned above.

Biosynthesis of Mucopeptide by *Pediococcus cerevisiae* and by a Substrain requiring Methicillin for Growth. By B. J. WILKINSON and P. J. WHITE (*Department of Microbiology, The University, Sheffield, Great Britain*)

From the parent strain *P. cerevisiae* ATCC 8081 a substrain (8081 CRD) was developed that required methicillin (or some other penicillins) for growth in a partly defined medium, and comparisons were made of cell walls from the two strains (White, P. J. (1968), *J. gen. Microbiol.* 50, 85 and 107).

Organisms of each strain were harvested during exponential growth, washed and incubated at 37° in a solution containing: potassium phosphate buffer, pH 6·5; glucose; $(NH_4)_2SO_4$; $MgSO_4$; L-alanine; L-glutamic acid; L-lysine and L-aspartic acid (derived from work of Shockman, G. D., Conover, M. J., Kolb, J. J., Riley, L. S. & Toennies, G. (1961), *J. Bact.* 81, 44). Little protein synthesis occurred in this solution, but mucopeptide was synthesized as shown by increase of turbidity of the suspended organisms or increase of bound hexosamine and ninhydrin-positive material in mucopeptide isolated by the method of Park J. T. & Hancock, R. (1960), *J. gen. Microbiol.* 22, 249.

Similar rates of mucopeptide synthesis (about 10 % increase of turbidity/hr) occurred with both strains, but parent organisms formed mucopeptide for only 2–3 hr whereas organisms of strain 8081 CRD continued for about 2 hr longer. With both strains addition of other amino acids or acetate was not stimulatory, and chloramphenicol, streptomycin and aminopterin were not inhibitory, but synthesis was considerably reduced if any one component (except aspartate) were omitted from the suspending solution. Methicillin was not needed for mucopeptide formation by strain 8081 CRD whether organisms had been grown in presence of drug or without it (in medium without acetate supplemented with yeast extract). Benzyl penicillin, cloxacillin and cycloserine inhibited mucopeptide synthesis; strain 8081 CRD was not resistant to these compounds. Methicillin (above 250 μg./ml.) also inhibited synthesis by parent organisms but strain 8081 CRD was relatively resistant (4 mg. of methicillin/ml. caused only partial inhibition).

Influence of Growth Environment on the Cell Wall Anionic Polymers in some Gram-positive Bacteria. By D. C. ELLWOOD and D. W. TEMPEST (*Microbiological Research Establishment Porton, nr Salisbury, Great Britain*)

When cultured in a simple salts medium, in a chemostat, the cell wall composition of Bacillus species varied with the chemical nature of the environment. In particular, cell wall teichoic acid, which was present in substantial amounts when the organisms were limited in their growth by the availability of magnesium, was totally replaced by a teichuronic acid-type compound when growth was limited by the supply of phosphate. Other interesting Gram-positive organisms (Staphylococcus, Micrococcus) would not grow in a simple salts medium; nevertheless, it was possible to arrange chemostat conditions in which growth in a complex medium was limited by the availability of either magnesium or phosphate.

When *Staphylococcus aureus* H organisms were cultured in a Mg-limited environment in a chemostat (D = 0·1 hr^{-1}, 35°, pH 7·0) their walls (3·0 % phosphorus) contained a ribitol teichoic acid which accounted for over 30 % of the wall weight. But as with cultures of Bacillus species, when *S. aureus* H was cultured in a phosphate-limited medium, the wall phosphorus content fell to a low level (0·4 %) and the teichoic acid was totally replaced by a teichuronic acid.

The walls of *Micrococcus lysodeikticus* are known to contain small amounts of a teichuronic acid but not teichoic acid. As expected *M. lysodeikticus* walls contained little phosphorus (0·1 %) when the organisms were grown in a phosphate-limited medium in a chemostat (D = 0·1 hr^{-1}, 35°, pH 7·0); these walls contained an anionic polymer (30 % of wall weight) which was composed of glucose and a amino hexuronic acid. Most surprisingly, however, when grown in a Mg-limited medium, *M. lysodeikticus* synthesised a wall which contained 3·2 % phosphorus; extraction of these walls with 10 % trichloroacetic acid (4°, 24 hr.) yielded a polymer which was characterised as a glycerol teichoic acid.

Action of Uranyl Acetate on the Murein of *Spirillum serpens*. By H. J. PREUSSER and H. H. MARTIN (*Institut für Mikrobiologie, Technische Hochschule, Darmstadt, Germany*)

In cell walls of Gram-negative bacteria murein (peptidoglycan) is present as a thin, sheet-like polymer forming the cell-shaped 'murein sacculus'. Sectioned murein sheets of isolated sacculi from enteric bacteria and *Spirillum serpens* have a thickness of only 20–25 Å, suggesting that they may be monolayer complexes of polysaccharide and peptide moieties covalently cross-linked in two dimensions (Frank, H. & Dekegel, D. (1967), *Fol. Microbiol. (Prague)*, **12**, 227; Hofschneider, P. H. & Martin, H. H. (1968), *J. gen. Microbiol.* **51**, 23; Frank, H. & Martin, H. H., unpublished). Precise geometrical order and orientation patterns with respect to the overall shape of the cell can be expected from such a polymer and invite electron microscopical investigation. Untreated isolated sacculi of *Spirillum serpens* were highly transparent and virtually invisible in the electron microscope. 'Staining' with a variety of heavy metal salts revealed that uranyl acetate has a special affinity for murein. Treatment with 0·5 % aqueous uranyl acetate at pH 4·5 caused the following effects: (1) strong increase in electron density of the sacculi, (2) specific shrinkage in longitudinal direction, (3) cleavage into hoops of varying breadth, (4) complete disintegration. The significance of these observations for the interpretation of murein ultrastructure and the use of uranyl acetate as contrasting agent in the electron microscopy of bacterial cell walls will be discussed.

The Action of Penicillin on the Mucopeptide of *Escherichia coli*. By W. ROBERTS, A. DAVIES and M. K. R. BURNHAM (*Imperial Chemical Industries Ltd., Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire, Great Britain*)

Gram negative organisms are, in general, less susceptible than Gram-positive organisms to penicillin although low concentrations of penicillin reduce the cross-linking of mucopeptide synthesised by a particulate enzyme system from *Escherichia coli* (Izaki, K., Matsuhashi, M. & Strominger, J. L. (1968), *J. biol. Chem.* **243**, 3180). In *Staphylococcus aureus* penicillin reduces both the cross-linking and the rate of synthesis of mucopeptide (Tipper, D. J. & Strominger, J. L. (1968), *J. biol. Chem.* **243**, 3169). The low penicillin sensitivity of *E. coli* could be due to enzymes being less affected by penicillin, to permeability barriers or a decreased requirement for cross-linking. *E. coli* ATCC 11229 was grown in glucose (uniformly labelled with ^{14}C) ammonium salts medium without and with sodium penicillin G 10 (30) μg./ml. for 90 min. at 37°: viabilities were then 20 (1·0) % of the control. Relative radioactivities with respect to the control were: whole bacteria 1·23 (0·86); whole walls 1·44 (0·80) mucopeptide 0·64 (0·24). The ratio alanine: glutamic acid (1·84 in control) was 2·28 (2·63) and the percentage of diaminopimelic acid found as the dinitrophenyl derivative following dinitrophenylation of the mucopeptide was 26·5 (26·0) against 15·7 in the control. The percentage of mucopeptide hydrolysed to monomer by lysozyme increased from 10 % to about 25 % following penicillin treatment.

These results indicate that penicillin although at less than the minimal inhibitory concentration (at 10 μg./ml.) causes some killing during the experiment, reduces both the cross-linking and the rate of synthesis of mucopeptide while at above the minimal inhibitory concentration (which was rapidly lethal) has only little more effect on cross-linking but greatly reduces the rate of synthesis of mucopeptide.

Bacterial N-Acetyl-β-Glucosaminidases. By R. C. W. BERKELEY, J. W. DROZD and J. M. BROUGHALL (*Department of Bacteriology, The Medical School, University Walk, Bristol Great Britain*)

N-acetyl-β-glucosaminidases have been detected by Woollen, J. W., Walker, P. G. & Heyworth, R. (1961), *Biochem. J.* **79**, 294) in cells of several bacteria, but with preparations of several others no activity was found under the test conditions which involved incubation of the enzyme preparation with *p*-nitrophenyl-*N*-acetylglucosaminide at pH 4·5. The pH optimum of some bacterial *N*-acetyl-β-glucosaminidases is, however, more alkaline than this ranging from pH 6·3 to pH 7·5. The higher value is the optimum of the *E. coli* B enzyme which

exhibited no activity at pH 4·5 (Maass, D., Pelzer, H. & Weidel, W. (1964), *Z. Natuff.* **19***b*, 413). Some strains of certain of the organisms in which Woollen *et al.* (1961) failed to find *N*-acetyl-*β*-glucosaminidase are known to hydrolyse chitin. Almost invariably two enzymes are involved in this hydrolysis—a chitinase and a chitobiase which hydrolyses other *β*-*N*-acetylglucosaminides in addition to *N*,*N*'-diacetylchitobiose.

We have grown several bacteria in a chitin-salts medium supplemented where necessary by 0·5 % casein hydrolysate and tested for *N*-acetyl-*β*-glucosaminidase under conditions allowing detection of enzymes with pH optima of both about pH 4·3 and about pH 7·3. Many, including some of those previously regarded as lacking this enzyme, were found to exhibit a high level of activity towards *p*-nitrophenyl-*N*-acetyl-*β*-glucosaminide. The pH optima of crude enzyme preparations from 5-day-old cultures of several Bacillus species and one *Escherichea coli* strain were found to be between pH 7·0 and pH 7·9. *N*-acetyl-*β*-glucosaminidase from *B. subtilis* has been examined in more detail and there is evidence that more than one enzyme is produced. The role of these enzymes and the possible involvement of *N-acetyl-β*-glucosaminidases in cell wall metabolism will be discussed.

Behaviour of some Enzymes of the Cell Envelope of Baker's Yeast During the Digestion of the Cell Wall. By T. NURMINEN and H. SUOMALAINEN (*Research Laboratories of the State Alcohol Monopoly, Alko, Helsinki, Finland*)

The carefully isolated cell wall of baker's yeast contains fragments of plasma membrane (Suomalainen, H., Nurminen, T. & Oura, E. (1967), Federation of European Biochemical Societies, Fourth Meeting, Oslo, *Abstr. Commun.*, p. 111, and *Suomen Kemistilehti* B **40**, 323; Nurminen, T., Oura, E. & Suomalainen, H. (1968), Federation of European Biochemical Societies, Prague, *Abstr. Commun.*, p. 115). The isolated cell walls showed no activities of intracellular enzymes investigated. The isolated cell walls were found to contain considerable amounts of saccharase and phosphatases hydrolysing *p*-nitrophenyl phosphate, ATP, ADP, thiamine pyrophosphate and inorganic pyrophosphate with optimum activity at pH 3–4, and an activity of Mg^{2+}-dependent ATPase at neutral pH. The activities appearing in the cell walls were mostly released into the medium during enzymic digestion, as has become apparent in the preparation of yeast protoplasts. The bulk of Mg^{2+}-dependent ATPase remained in the plasma membrane preparation. Accordingly, it may be assumed that the enzymes released into the medium are located in the cell wall outside the plasma membrane, whereas the Mg^{2+}-dependent ATPase is an enzyme of the plasma membrane.

The Chemical Characteristics of Endotoxin from *Bacteroides fragilis* By T. HOFSTAD (*Laboratory of Oral Microbiology, The Gade Institute, The University of Bergen, Schools of Medicine and Dentistry, Bergen, Norway*)

Endotoxin has been prepared from *Bacteroides fragilis*, strain NCTC 9343, by phenol–water extraction of acetone-dried cells, followed by purification by ultracentrifugation and subsequent treatment with ribonuclease and deoxyribonuclease.

The lyophilized endotoxin contains 6 % nitrogen, 40 % protein (Folin), 33 % neutral sugar (orcinol), 15 % lipid as fatty acid esters, and 7 % hexosamine. Paper chromatography of acid hydrolysates revealed glucose, galactose, rhamnose, fucose, trace amounts of mannose, glucosamine and galactosamine, of which glucose was the major sugar component. In addition, a fast-moving compound has been detected on paper chromatography of acid hydrolysates. The compound, which possibly is a novel amino sugar, reduces silver nitrate and gives a positive ninhydrin reaction. In the Elson–Morgan reaction it produces an absorption spectrum similar to that of glucosamine. On Dowex 50 (H^+ form) the compound has a chromatographic mobility of 1·37 with respect to glucosamine when eluted with 0·33 N-HCl.

Heptose and 2-keto-3-deoxy-octonate (KDO) has not been demonstrated. The absence of these sugars, together with the presence of the novel sugar compound, suggest that *B. fragilis* endotoxin has a structure quite different from that of endotoxins of aerobic Gram-negative bacilli. In this connection it is of great interest that heptose and KDO are lacking also in

endotoxin from *B. melaninogenicus* (Hofstad, T.) 1968, *Arch. oral Biol.* **13**, 1149), In contrast, endotoxin from oral strains of *Fusobacterium* contains both KDO and heptose (Kristoffersen, T. 1969. *Acta path. microbiol. scand.* in Press).

The Structure of Colanic Acid. By I. W. SUTHERLAND (*Department of General Microbiology, University of Edinburgh, Great Britain*)

The formation of a polysaccharide slime common to many species of Enterobacteriaceae has been noted by several workers (Kauffmann, F. (1954), *Enterobacteriaceae.* Copenhagen: Munksgaard; Anderson, E. S. & Rogers, A. H. (1963), *Nature, Lond.* **198**, 714). Although the control of synthesis of this polysaccharide has been studied, there has been little reported on its structure other than identification of the component sugars and the aldobiuronic acid 3-*O*-*β*-D-glucuronosyl-galactose (Roden, L. H. & Markovitz, A. (1966) *Biochim. biophys. Acta*, **127**, 252). We have confirmed the presence of D-glucose, D-galactose, D-glucuronic acid and L-fucose. In addition an *O*-acyl group has been identified as acetyl and Rees and his colleagues (unpublished) have isolated a complex of pyruvate and galactose.

The colanic acid from representative strains of *Escherichia coli*, *Aerobacter cloacae* and *Salmonella typhimurium* has been isolated, purified and found to be identical in all respects tested. By partial acid hydrolysis a number of neutral and charged oligosaccharides have been obtained from all three preparations. The structure of these fragments has been determined and several were found to contain pyruvate. No acetylated fractions were obtained. Oligosaccharides were also isolated from partial acid hydrolysates of carboxyl reduced colonic acid. The glucose emanating from glucuronic acid being identified by tritiation. On the basis of these results, together with periodate oxidation and other tests, a hexasaccharide repeating unit containing 2 moles fucose, 2 moles galactose and 1 mole each of glucose, glucuronic acid, acetyl and pyruvate is proposed.

The significance of this structure in a biosynthetic system for colanic acid will be discussed.

The Relation of K Antigens to the Complement Sensitivity of Strains of *Escherichia coli* By A. A. GLYNN and C. J. HOWARD (*Wright-Fleming Institute, St Mary's Hospital Medical School, London, Great Britain*)

The structure of the bacterial cell wall is of great importance in the analysis of the bactericidal and bacteriolytic action of fresh serum. Previous work (Glynn (1969), *Immunology*, **16**, in Press) suggests that the probable sequence of events in *Escherichia coli* is damage to the lipopolysaccharide lipoprotein layer by complement followed by lysozyme action on the mucopeptide. However, some smooth strains of *E. coli* are resistant to complement and antibody and Sjostedt (1946, *Acta. path. Scand.* Suppl. 63) suggested that this was due to K antigens. Later work by Muschel (1960, *Proc. Soc. exp. Biol. Med.* **103**, 632) confirmed this.

Complement resistance in *E. coli* is not related to any particular antigenic type of K, but resistant strains in general contain more K than do sensitive strains. This has been shown most clearly using 4 strains of O6 K 13 differing widely in complement resistance. However, qualitative differences are also important. A significant property of K antigens is their ability to suppress agglutination in heterologous systems. Pure K antigen from a complement resistant strain of *E. coli* possessed more agglutination inhibiting activity weight for weight than purified K from a sensitive strain. Using ^{125}I labelled antibody it has been shown that K antigens decrease the amount of antibody bound by red cells and also affect complement action directly. Chromatographic analysis and chemical modifications suggest that both molecular size and charge may be involved.

SESSION B

Egg Production and Disease: Adenovirus. By D. M. BERRY (*Virus Vaccines Department, Glaxo Laboratories, Ltd., Greenford, Middlesex, Great Britain*)

The finding that adenoviruses were frequently isolated, together with infectious bronchitis (I.B.), virus in laying flocks apparently showing typical I.B. infection (Berry, D. M. & Stokes, K. J. (1968), *Vet. Rec.* 82, 157) prompted this study to investigate the effect of adenovirus on egg production in chicken. Attempts were made to isolate virus from laying flocks with severe depression of egg production. Adenovirus isolates were compared with G.A.L. virus supplied by I. A. Macpherson, Institute of Virology, Glasgow, and C.E.L.O. virus (E.V. 89) supplied by D. A. McMartin, Veterinary Laboratory, Lasswade, in cross-neutralization tests, but no antigenic differences were demonstrable.

Pullets from a flock free from I.B. virus, adenovirus and *Mycoplasma gallisepticum* were raised in isolation and transferred to individual laying cages at point of lay. Individual egg production records were kept from the point of lay until the end of each experiment. Birds laying less than 5 eggs/week before challenge were excluded from the experiments. Groups of twenty pullets were exposed either to intranasal infection and intramuscular inoculation of 10^9 EID_{50} adenovirus/bird alone, or together with the inoculation of 10^9 ID_{50} *M. gallisepticum* strain s6 into the thoracic air sacs, or together with intranasal and intramuscular inoculation of 10^6 EID_{50} I.B. virus Massachusetts strain/bird and 10^9 ID_{50} *M. gallisepticum* into the thoracic air sac of each bird.

In susceptible laying hens, adenovirus infection by itself showed a 10 % depression of egg production which lasted 3 weeks.

The egg production of birds infected with both adenovirus and *M. gallisepticum* was reduced for a longer period, and this trend was accentuated further in the group infected with adenovirus, I.B. virus and *M. gallisepticum*. The egg production of birds infected with the three agents was more severely depressed than the egg production of birds infected with I.B. virus and *M. gallisepticum*, but not with adenovirus. This is important in view of our finding adenovirus in three out of twelve infections of laying hens characterised by depressed egg production, in which I.B. virus had also been isolated.

It is clear that adenovirus infection affects egg production in a way indistinguishable from I.B. infection, particularly when such infection is enhanced by infection with *M. gallisepticum*. It is seen that the depth and duration of any depression of egg production due to infection is dependent on the number of agents present.

Comparison of Two Canine Adenoviruses. By R. MARUSYK and E. NORRBY (*Department of Virology, Karolinska Institutet, Stockholm, Sweden*)

Morphological and biophysical studies have revealed a unique relationship between infectious canine hepatitis (ICH) virus and the lesser known canine adenovirus, designated infectious canine laryngotracheitis (ICL) virus. Serological testing has shown the two virus types to be very closely related with regards to neutralization and haemagglutination-inhibition tests. However, anion-exchange chromatography has revealed a distinct difference in the elution pattern of the soluble components of each virus type. Electron micoscopic examination of the virions and components has also shown a substantial difference in the length of the fibre components. Preliminary data from further studies with these types will also be presented.

Structural Aspects of the Adenovirus type 2 Penton Antigen. By U. PETTERSSON and S. HÖGLUND (*The Wallenberg Laboratory, University of Uppsala, Sweden*)

The penton antigen from adenovirus type 2 has been purified by DEAE-chromatography, agarose chromatography and preparative polyacrylamide electrophoresis. The final product is homogeneous by electronmicroscopy, immunoelectrophoresis, polyacrylamide electrophoresis and analytical ultracentrifugation.

Penton antigen so prepared has a sedimentation coefficient of 10·5 and a molecular weight around 400,000. A minimum of three antigenic specificities can be demonstrated with immunodiffusion.

Electron microscopy shows a complex structure with a prismatic head, sometimes showing five-fold symmetry, and a threadlike tail. Low concentrations of trypsin selectively inactivates the cytopathic effect of the type 2 penton. At high trypsin concentrations the main part of the vertex capsomer antigenicity and morphology is also lost. Rabbit antisera to pure pentons contain low titres of neutralizing antibodies when assayed by inhibition of plaque formation. The use of the fluorescent focus assay reveals, in contrast, high neutralizing activity in these sera. Treatment of penton antigen with pyridine releases free vertex capsomers which are immunologically and morphologically intact and have the ability to induce cytopathic changes in KB-cell cultures.

Aspects on the Neutralization of Adenoviruses. By GÖRAN WADELL (*Department of Virology Karolinska Institutet, Stockholm, Sweden*)

Non-vertex capsomeres, hexons, have been considered to contain the antigenic specificity, which can induce the production of antibodies capable of neutralizing the infectivity of adenoviruses (Wilcox & Ginsberg (1963), *Proc. Soc. expt. Biol. Med.* **114**, 37–42; Kjellen & Pereira (1968), *J. gen. Virol.* **2**, 117–185; Norrby, (1969), *Virology*, in Press). Hexons of adenovirus type 2 have been separated into two different populations by anion exchange chromatography and by iso-electric focusing techniques. The immunological properties of these two populations of hexons have been characterized in complement-fixation and neutralization tests.

In further studies the enhancing capacity of sheep anti-rabbit sera on the neutralizing capacity of adenovirus specific rabbit-antisera was examined (Ashe & Notkins (1966), *Proc. nat. Acad. Sci. U.S.A.* **56**, 447). The relative effect on antisera against different structural components of various representative members of Rosen's three subgroups was evaluated.

Adsorption and Fate of Adenovirus type 5 Hexon antigen in HeLa cells. By P. J. SANDERSON (*National Institute for Medical Research, Mill Hill, London, Great Britain*)

Crystalline Hexon antigen (kindly supplied by Dr H. G. Pereira) was labelled with ^{131}I by the method of Greenwood, Hunter & Glover (1963, *Biochem. J.* **89**, 114) and mixed with suspensions of HeLa cells under different conditions. Adsorption of the labelled antigen was determined by the loss of radioactivity in the supernatants of test and control mixtures. Break down and elution of adsorbed antigen by cells was determined by changes in the amount of antigen precipitated by 10 % Trichloracetic acid in supernatants and cells.

It was found that up to 3 % of the labelled antigen added to a HeLa cell suspension, equilibriated to a temperature of 4°C, was adsorbed after 1 h by $1{\cdot}0 \times 10^8$ cells and that adsorption was dose dependant over the range of antigen concentrations tested. Adsorption was increased by mixing the antigen with specific antibody and by pre-exposing cells to partially purified preparation of adenovirus type 5 Penton antigen.

In experiments where the antigen was adsorbed to cells at a temperature of 4° and the cells washed and transferred to a temperature of 37°, it was found that a significant proportion of the adsorbed antigen was converted to acid soluble products, some of which appeared in the suspending medium. Thus adsorption studies carried out at a temperature of 37° may underestimate or fail to show adsorption unless the extent of cellular break down of the adsorbed antigen is determined also.

Cellular break down of the antigen was temperature dependant and inhibited by sodium fluoride. Break down was found in HeLa cell monolayers, in suspensions of chick fibroblasts, monkey kidney cells and mouse peritoneal exudate cells. It did not occur in medium from which cells shown to be capable of breaking down antigen had been removed. When different quantities of antigen were adsorbed to cells a similar proportion of adsorbed antigen was broken down, indicating that the amount of break down was dependant upon the quantity of antigen adsorbed over the range of antigen concentrations tested.

In experiments where adsorption and break down were measured concurrently in cell suspensions equilibriated to a temperature of 37° it was found that antigen adsorption and break down both occurred rapidly and it was concluded that these events are closely associated.

These results and those obtained with partially purified preparations of the other adenovirus type 5 capsid antigens will be discussed.

Cross-reaction Between TSTAs of Tumors Induced by Adenoviruses of Groups A and B. By J. ANKERST and H. O. SJÖGREN (*Department of Medical Microbiology, University of Lund, Sweden*)

Viral neoplasms are characterized by tumor specific transplantation antigens (TSTA) which are identical, or have at least some identical components in all the neoplasms induced by the same virus but are usually different in tumors induced by different viruses. Adeno 12 tumors possess this type of cross-reacting TSTA. The adeno viruses might be divided in three groups with regard to oncogenicity: group A (including type 12), group B (including weakly oncogenic virus types), and finally the non-oncogenic virus types. It has been studied whether the TSTAs of tumors induced by adenovirus types belonging to groups A and B might cross-react or whether the TSTA are 'subgroup' specific. It was demonstrated by isograft rejection tests that hamster adenovirus group B tumor cells are immunogenic with regard to the TSTA adenovirus group A tumors. Furthermore, by use of ^{51}Cr-cytotoxic tests *in vitro* it was shown that sera of animals immunized against adenovirus group A tumors were cytotoxic to adenovirus group B tumor cells.

Factors Affecting Replication of the Lactate Dehydrogenase-elevating Virus (LDH virus) in Peritoneal Macrophage. By R. EVANS (*Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Sutton, Surrey*)

Replication of the LDH-virus in peritoneal macrophage cultures to high levels comparable to those seen in the infected mouse has been previously reported (Evans, R. (1967), *J. gen. Virology* **1**, 363). Further experiments have shown that a consistent response to a standard of virus, $10^{6\cdot0}$ ID_{50} ml. was obtained when cultures were inoculated 24 h after preparation. Peak titres up to 10^{8} ID_{50}/ml. were reached by 3 days, followed by a decline to below the ID_{50} within 21 days. This decline could not be explained by the production of interferon or DNA-directed protein inhibitors since actinomycin-D was without effect on LDH-virus replication in these cultures. Lymphocytes, frequently found in macrophage cultures during the first few days, were shown not to be involved in the replicative process. Peak virus titres were reached more quickly when doses of virus greater than 10^{6} ID_{50}/ml. were inoculated into cultures, though the yield of virus was no greater. Doses less than 10^{3} ID_{50}/ml. failed to infect cultures. The possibility that susceptibility is related to the length of the time the cells are maintained *in vitro*, and the presence of an apparently small percentage of susceptible cells will be discussed.

Inhibition by Frog Virus 3 of Vaccinia Virus and Host Cell DNA Replication in KB Cells. By ANDRÉ KIRN and ANNE-MARIE AUBERTIN (*Groupe de Recherches de l'I.N.S.E.R.M. sur la Pathogénie des Infections Virales-3, rue Koeberlé 67-Strasbourg, France*)

Frog virus 3 (FV_3) a desoxyribovirus which replicates in the cytoplasm of amphibian and mammalian cells at low temperature (Granoff, A. *et al.* (1966), *Virology*, **29**, 133) produces an inhibition of host cell DNA synthesis (Macauslan, B. & Smith, W. R. (1968), *J. Virol.* **2**, 1006).

It was of interest to know if FV_3 had also an effect on viral DNA replication. We have undertaken a study on the action of FV_3 on host cell and vaccinia virus DNA replication in a non-permissive system (KB cells) at non permissive temperature (37°) for FV_3 development.

When monolayers of KB cells were preinfected during 4 hours with FV_3 (10 p.f.u./cell) and then infected with vaccinia virus and labelled, ^{3}H thymidine uptake in the nuclei as well as in the cytoplasmic fraction of the cells was completely inhibited.

However, vaccinia virus was uncoated as it could be demonstrated by electron microscope examinations. A similar inhibition of cellular and viral DNA synthesis was noted when both viruses were mixed and put on KB cells. Moreover, in suspended KB cells infected with vaccinia virus, host cell and vaccinia virus DNA replication were completely blocked by superinfecting the cells 1 h later with FV_3. We have shown that the fraction of the virus responsible for inhibition is thermolabile.

Compositional Heterogeneity of the DNA of Phage P2. By R. B. INMAN (*Department of Biophysics, University of Wisconsin, Madison, U.S.A.*) and G. BERTANI (*Department of Microbiology, Karolinska Institutet, Stockholm, Sweden*

The DNA of bacteriophage P2 (which is double stranded, linear, approximately 13 μ long, and able to form rings reversibly *in vitro* through terminal cohesive sites) showed at least three well-defined steps in optical density with progressive heat denaturation (melting curves). This suggested heterogeneity of composition in respect to nucleobase ratio.

Electron microscope studies of partially denatured P2 DNA showed localized zones, clearly identifiable by the temperature at which they start denaturing. These observations indicate that the heterogeneity of composition is intramolecular, and yield highly specific denaturation maps for the P2 chromosome.

P2 *Hy dis*, a phage believed to be genetically identical to P2 except for a small segment of its chromosome near one end of the genetic map, gives multistep denaturation curves, by optical density, and denaturation maps, by electron microscopy, which are almost superimposable to those of P2. A small, but probably significant difference exists, however, and this leads to a tentative orientation of the physical (electron microscopy) map in respect to the genetic map of these phages.

Further Studies of P2 Associated Eduction in *Escherichia coli* K12. By M. G. SUNSHINE (*Department of Microbiology, Karolinska Institutet*) *and* B. L. KELLY (*Department of Microbiology, San Diego State College, California, U.S.A.*)

Escherichia coli K-12 strains lysogenic for phage P2 in chromosite H, closely linked to histidine, can spontaneously lose several adjacent genes when being cured of their prophage. This phenomenon, termed eduction, also occurs upon infection of sensitive K-12 strains with P2. Eduction has not been observed to occur with K-12 strains lysogenic for P2 in other chromosites or with *E. coli* C (Kelly, B. L. T. & Sunshine, M. G. (1967), *Biochem. biophys. Res. Comm.* **28**, 237). The deletions have now been shown to include the closely linked genes for histidine biosynthesis, 6-phospho-gluconic dehydrogenase (*gnd*), and part or all of the *rfb* locus governing the biosynthesis of nucleotide sugars. Eduction occurs with several different mutants of P2 which are capable of lysogenization, but not with P2 mutants unable to lysogenize. Specifically, it does not occur upon infection with P2 *int* mutants, i.e. mutants which have lost the ability to integrate as prophages in the host chromosome. All *E. coli* K-12 strains so far tested, including one recombination deficient strain, give rise to eductants. The mechanism favored for eduction is a recombination event, promoted by the *int* function of the phage, involving not only the normal chromosite but also another homology region on the host chromosome which defines the end of the deletion.

Control of Prophage Integration in Bacteriophage P2. By L. ELIZABETH BERTANI (*Institutionen för Mikrobiologi, Karolinska Institutet, Stockholm, Sweden*)

Following superinfection of an immune host, bacteriophage P2 is able to attach with great efficiency to an unoccupied attachment site. The attachment depends on the activity of a gene that presumably determines synthesis of P2 integrating enzyme (integrase). This result suggests that, unlike the case for phage λ, the synthesis of P2 integrase is not under the control of the ordinary phage repressor. However, no integrase activity can be found in P2 lysogens. Models to explain this paradoxical situation will be discussed.

SESSION C

The Location of Chemical Components on Ultra-thin Sections of *Bacillus cereus* Embedded in Glycol Methacrylate By P. D. WALKER (*Wellcome Research Laboratories, Beckenham, Kent, Great Britain*)

The details of sporulation and germination in bacteria have been extensively investigated using the technique of ultra-thin sectioning. These investigations have invariably used osmic acid as a fixative and epoxy resins for embedding. The structure of *Bacillus cereus* has been investigated during various stages of sporulation and germination after aldehyde fixation and embedding in a water soluble medium, Granboulan & Leduc (1967). In addition the susceptibility of ribosomes to digestion by ribonuclease has been investigated by floating ultra-thin sections of the organism on to the enzyme. Ribosomes were readily removed from ultra-thin sections of young vegetative cells by digestion of ribonuclease. Increased resistance to digestion was observed during development of the spore coat which was associated with the beginning of refractability under phase contrast. Similar differences in susceptibility were observed during germination of spores.

Polysaccharides were located on the ultra-thin sections of the organisms using the silver-methenamine staining technique, Walker & Short (1968). Their location corresponds to that previously described using osmic acid fixed material in epoxy resins.

Structural Changes in *Escherichia coli* Cells Infected with ØX-174 type Bacteriophages. By D. E. BRADLEY (*Department of Zoology, University of Edinburgh, Great Britain*)

The ØX-174 type *Escherichia coli* bacteriophages are morphologically identical, being 250 Å icosahedra with large apical capsomeres. They contain single-stranded DNA. Three serologically unrelated groups, represented by phages $\alpha 3$, ØX-174 and St/1, have been identified; each has a different host range. St/1 alone growing on male strains. The electron microscopy of infected bacteria shows that phage $\alpha 3$ produces somewhat different morphological changes compared to ØX-174 and St/1.

The cytological effects associated with infection by ØX-174 and St/1 are similar. First, intracellular phage is assembled there being no obvious changes in the structure of the cell envelope. After assembly, however, the plasma membrane begins to break up and small holes appear in the cell wall. The cell contents and phage progeny pass through the holes. Alternatively, a single hole may form in the cell wall and plasma membrane at the midpoint of the cell. In this case, the plasma membrane remains intact, but retracts from the poles.

With phage $\alpha 3$ the first signs of infection are a retraction of the plasma membrane from the poles of the cell and the appearance of a bulge at the midpoint. This swells up until a typical spheroplast is formed. Lysis occurs in two stages. First, the cell wall breaks and curls back from the spheroplast bulge, then the plasma membrane disintegrates releasing the contents. Spheroplast formation is in complete contrast to the production of excess cell wall and plasma membrane associated with infection by the single-stranded DNA filamentous phages.

The significance of these observations is that morphologically identical phages have a noticeably different effect on the host cell.

The Ultrastructure of the Protein-lipopolysaccharide Surface of *Spirillum serpens*. By R. G. E. MURRAY and F. L. A. BUCKMIRE. (*Statens Seruminstitut, Copenhagen, Denmark and Department of Bacteriology, University of Western Ontario, London, Canada*)

The outer surface of Spirillum species presents a hexagonal array of macromolecules (Houwink, A. L. (1953), *Biochim. biophys. Acta*, **10**, 360); details of this regular structure (RS) have been described for *Spirillum serpens* VH (Murray, R. G. E. (1963), *Can. J. Microbiol.* **9**, 381). Ring-like units (*c.* 85 Å diameter) are arrayed with a repeat interval of *c.* 145 Å and have Y-form linkers. This type of surface has been identified on 6 of 12 strains of *S. serpens* and it has also been found that the integrity of the RS layer requires Ca^{2+} (Steed-Glaister, P. D., Maier, S. M. & Murray, R. G. E., unpublished). The structural description is

now amplified by micrographs of freeze etching preparations and of isolated and reaggregated components of the RS layer.

The RS layer, removed cleanly from growing cells by heat treatment (65° for 1 hr) in 0·01 M-$CaCl_2$, consisted of sheets of units or tubes of units arrayed on an amorphous backing material; the layers and components can be identified by freeze etching of intact or treated cells. RS material definitely lies outside the 'double track' profile of the cell wall. The units were removed and dissolved by M-guanidine-HCl without solution of LPS backing layer (apparently lipopolysaccharide). The solution of RS units (protein in nature) obtained by differential centrifugation and after dialysis was free of identifiable ring-like units and was monodisperse in the ultracentrifuge (4·1 S); yet units complete with Y-linkers of expected size and spacing were formed from this solution when interacting with the surface of the RS-free VHL mutant (which appears to have the LPS layer).

The concept of Y-linkers as a fabric on which the units sit (Murray, R. G. E. *op. cit.*) is untenable; they may be a consequence of assembly of the open V or short stemmed Y forms visible in preparations of the protein. Both hydrogen and salt (Ca^{2+}) bonds may unite RS and LPS components and Ca^{2+} is required for RS production.

Some Electron Microscopic Features of *Cardiobacterium hominis*. By Alice Reyn, R. G. E. Murray and A. Birch-Andersen (*Neisseria and Biophysics Departments, Statens Seruminstitut, 2300 Copenhagen S, Denmark*)

Cardiobacterium hominis (Slotnick, I. J. and Dougherty, M. (1964), *Antonie van Leeuwenhoek* **30**, 261) was chosen for study because it was reported to be Gram-negative but 'with many cells retaining discrete Gram-positive areas'. In our hands, three strains (NCTC 10426 and 10427, SSI 1742) of these organisms were small pleomorphic but definitely Gram-negative organisms without any Gram-positive patches when grown on 10 % horse blood broth agar and on two kinds of 'chocolate agar'.

Twenty-hour cultures were harvested from these media with 0·1 % YAP medium (yeast extract-sodium acetate-peptone medium), the resulting suspensions fixed with osmium tetroxide (Ryter, A. & Kellenberger, E. J. (1957), *Z. Natur.* **13***b*, 597) or prefixed with 1 % glutaraldehyde for one hour preparatory to fixation with 1 % osmium tetroxide. Profiles from sections showed similar structures in each of the three strains. The cell wall was of a coherent Gram-negative type, each of the elements remaining closely apposed in sections. The distinctive features were as follows: The 'doublet' layer of the cell wall (unit membrane ~ 75 Å) was sandwiched between dense outer and inland layers, of which the former (~40–50 Å showed a hint of repeating structure, and the latter was very dense, of variable thickness (50–100 Å) and usually adherent to the plasma membrane. Intrusions of the plasma membrane formed irregular masses near the periphery or partially filled membrane-lined vesicles; one strain (NCTC 10426) showed numerous membranous lamellae at the periphery. Each strain showed caps of 2–300 Å thickness and irregular, tufted density restricted to the poles. Cytoplasm and nucleoplasm was not remarkable.

Cell wall fragments were obtained from ultrasonically disrupted cell suspensions by differential centrifugation and negatively stained with 1 % ammonium molybdate pH 6·5, 1 % lithium tungstate pH 7·5 and 1 % uranyl formate pH 4·5–5 with excellent and equivalent differentiation of surface structure. The outer layer of the wall consisted of a rectangular close packing array of 30–40 Å particles with a repeating distance of 50–60 Å. This represents a new type of surface structure on a Gram-negative bacterium. The distinctive polar caps were not found in negatively stained preparation of fragmented or whole cells.

A Bacteriolytic Hexosaminidase from *Staphylococcus aureus*. By T. Wadström and K. Hisatsune (*Department of Bacteriology, Karolinska Institutet, Stockholm, Sweden*)

The production of extracellular lysozyme-like enzyme(s) is a frequent property of staphylococcal strains and has been reported to be strongly correlated to pathogenicity. *Staphylococcus aureus* strain M 18 has been cultivated in CCY-medium in a batch and a continuous process.

The lytic activity was assayed on whole cells of *Micrococcus lysodeikticus*. Ethanol precipitation (35 %, v/v; 0–to -10°; pH 7·0), CM-Sephadex chromatography, isoelectric focusing, and Sephadex G-150 gel filtration were used in this order to purify an extracellular hexosaminidase (pI 9·5 ± 0·05). The purity of this enzyme was demonstrated by analytical acrylamide electrophoresis in several buffer systems and gel concentrations. The release of reducing sugar and *N*-acetylglucosamine from *M. lysodeikticus* cell walls by a partly purified enzyme has earlier been reported (Wadström and Hisatsune, FEBS congress, Prague, 1968, abstract 422). The purified enzyme has been used for investigation of the bacteriolytic spectrum. Degradation products of *M. lysodeikticus* cell walls and *S. aureus* strain 3528 mucopeptide are analysed after chromatography on Sephadex G-10 and Dowex 50 W-X8.

Different strains (v 8, Wood, 46, Foggie, R 1, 524 and Duncan) of *S. aureus* earlier investigated for the production of enzymes and toxins, were all found to produce an activity which was lytic for *M. lysodeikticus* and was isoelectric at pH 9·5. This was shown by the method of isoelectric focusing.

Some data will be presented on the autolytic system in some of the strains investigated.

The Production of Bacteriolytic Enzymes by *Staphylococcus aureus* in Batch and Continuous Culture. By S. Arvidsson, T. Holme and T. Wadström (*Department of Bacteriology, Karolinska Institutet, Stockholm, Sweden*)

The production of extracellular bacteriolytic enzymes by *Staphylococcus aureus* strain M 18 has been studied. *Micrococcus lysodeikticus* was used as the test organism. One of the enzymes has been shown to be a hexosaminidase. Preliminary experiments showed that CCY medium gave a better enzyme yield than Brain Heart Infusion Broth (Difco) and Trypticase Soy Broth (BBL). Batch cultivations performed in a stirred fermenter increased the yield five-fold compared to that obtained in shaken flasks. A rapid increase in lytic activity was obtained during the last three hours of the logarithmic phase, followed by a rapid decrease. Less than 50 % of the activity remained two hours after the onset of the stationary phase. An increased aeration with formation of an excess of foam was found to speed up this process. Maximal lytic activity was produced at pH 6·5–7·5 and was also influenced by the carbon source. These and further recent results obtained in continuous culture experiments will be discussed. In continuous culture, grown under similar conditions as in batch cultivation, the yield of lytic activity was significantly increased. The dilution rate applied was 0·5 hp^{-1}. When the dilution rate was changed to 0·3 hp^{-1}, a rapid increase in bacterial density and lytic activity was obtained.

Effects of Antibiotics on Bacterial Lysis and Intracellular Ribosomes. By Margot Kogut (*Department of Biochemistry, King's College, Strand, London, Great Britain*)

Rapid release of ribosomes from bacteria by lytic methods has facilitated examination of ribosome patterns under different growth conditions and antibiotic treatment (Luzzatto *et al.* (1968), *Proc. natn. Acad. Sci.*, **60**, 873). However, interpretation of ribosome profiles obtained by these methods has assumed equal extent of lysis and release of ribosomes in treated and control cultures. By prelabelling treated cultures with ^{14}C-uracil and harvesting together with a ten-fold excess of unlabelled control cultures in exponential growth, and differences due to harvesting, lysis and extraction of ribosomes can be minimized. After lysis of the combined cultures (Godson & Sinsheimer (1967), *Biochim. biophys. Acta* **149**, 476, 489) centrifugation through sucrose gradients yields simultaneous ribosome profiles of controls and treated culture. Since treatment of sensitive cultures of *Escherichia coli* during growth on defined media with 20–30 μg./ml. of dihydrostreptomycin produces gradual decline of growth rates (Kogut, Lightbown & Isaacson, (1965), *J. gen. Microbiol.* **39**, 165; Kogut & Harris, (1969), *Europ. J. Biochem.* in Press) ribosome profiles were obtained as above at various times during streptomycin treatment. They showed, compared with controls, a gradual diminution of the larger polysomes concomitant with increasing reduction in growth rate. Complete disappearance of polysomes was only observed some time after growth had ceased. These patterns resembled those obtained during growth inhibition by chloramphenicol, but differed from those

produced by progressive exhaustion of nitrogen source. Quantitative estimation of the extent of lysis showed a progressive decrease during growth inhibition by dihydrostreptomycin; this was more extensive during chloramphenicol treatment, but amino acid exhaustion had a lesser effect. Sucrose gradient centrifugation of material re-extracted from the cell-wall-membrane fraction showed similar patterns to those obtained immediately after lysis. Thus no preferential retention of certain ribosome classes had occurred in antibiotic-treated cultures.

Effect of Light on Malate Oxidation by Membranes of a Carotenoidless Mutant of *Sarcina lutea*. By J. PREBBLE and S. HUDA (*Department of Biochemistry, Bedford College, University of London, Great Britain*)

Protection of bacteria by carotenoids against photodynamic action of light has been demonstrated in several organisms including *Sarcina lutea*. Illumination of carotenoidless cultures resulted in death at very high light intensities (sunlight) or at low light intensities in the presence of a sensitizer, toluidine blue. From experiments with whole cells, the site of light action was believed to be the cell membrane where carotenoids are located. (Mathews, M. M. & Sistrom, W. R. (1960), *Arch. Mikrobiol.* **35**, 139).

The light sensitivity of malate oxidase activity (demonstrated by a depressed oxidation rate) in membranes prepared by a lysozyme method from a strain of *S. lutea* and a u.v.-induced carotenoidless mutant have been compared manometrically at 28° and 660 lumens/sq. ft. The malate oxidase of whole cells and membranes of the pigmented strain is slightly sensitive to illumination in the presence of toluidine blue at concentrations used by Mathews and Sistrom but mutant cells and membranes both show a greater sensitivity. At toluidine blue concentrations between 10^{-6} and 10^{-5} M, the sensitivities of mutant and pigmented membranes increased with sensitizer concentration, but mutant membranes showed much greater sensitivity particularly at lower concentrations.

With 10^{-5} M toluidine blue, malate oxidation by mutant membranes, as compared with a dark control, declines after 30–40 min. irradiation. Short exposures to light show that up to 10 min. illumination has no effect while 20–30 min. shows a depressed oxidation rate which is reversible in the dark. Illumination for longer than 40 min. renders the effect irreversible.

It is suggested that an initial reversible effect on electron transport, normally inhibited by carotenoids in the wild type, could lead to secondary irreversible effects including membrane damage, increased permeability, inactivation of membrane enzymes and death.

The Preparation and Use of a New Type of Bacterial Immunoabsorbent Column. By R. O. THOMSON (*Wellcome Research Laboratories, Beckenham, Kent, Great Britain*).

A number of methods have been described for the preparation of columns containing bacteria for the absorption of specific agglutinins (Morrison, R. B. (1961), *Lancet*, 1288; Weetall, H. H. (1967), *J. Bact.* **93**, 1876). In general these columns have low absorptive capacities because of the difficulty of incorporating sufficiently high concentrations of organisms into them.

The type of column used in the present work was prepared from an immunoabsorbent which consisted of organisms dispersed in agar subsequently converted to a bead form by a method similar to that of Hjerten, S. (1964, *Biochem. biophys. Acta* 79, 393). The concentration of organisms could be made extremely high and the columns prepared had sufficient capacity to enable them to be used (*a*) for the removal of cross-reacting agglutinins from production batches of type specific antisera, (*b*) for the large-scale isolation of specific agglutinins from crude sera by absorption-elution and (*c*) for the fractionation of such agglutinins. Columns were regenerated after absorption by removing the agglutinins by elution with acid buffers.

Although the organism used in this work was *Escherichia coli* it is felt that the technique could be applied with equal success in studies of other bacterial, viral and tissue antigens and antibodies.

Effect of azide on anaerobic respiratory enzymes in *Proteus mirabilis*. By G. N. DE GROOT and A. H. STOUTHAMER (*Microbiology Department, Botanical Laboratory, Free University, Amsterdam, The Netherlands*)

Proteus mirabilis can form four reductases after anaerobic growth, which are linked to the electron transparent chain of the cytoplasmic membrane. Chlorate-resistant mutants are blocked in the synthesis of nitrate reductase A and chlorate reductase C. Formate dehydrogenase, hydrogenase, thiosulphate reductase and tetrathionate reductase are formed by only some of the chlorate-resistant mutants. Azide (1 mM) fully inhibits nitrate reductase A, but it has no effect on the other reductases.

Anaerobic growth of the wild type in the presence of azide gives a strong derepression of cytochrome b_1 and nitrate reductase A. Simultaneously the formation of chlorate-reductase C is fully repressed. Azide has no influence on the formation of thiosulfate reductase and tetrathionate reductase. In chlorate-resistant mutants azide does not stimulate the formation of cytochrome b_1 (and nitrate reductase). Both in the wild type and some chlorate-resistant mutants the formation of cytochrome a_2 (and a_1) is repressed by azide. The oxidation of several metabolites is not influenced by azide after growth with or without azide. This suggests that the electron flow from these metabolites to oxygen is not mediated by those cytochromes which are influenced by azide.

Cells grown with azide also show a much higher formate-nitrate reductase activity, but it appears that this is not a consequence of increased amounts of formic dehydrogenase. On the other hand the formation of formic hydrogenlyase is diminished in cells grown with azide. Azide does not inhibit formic dehydrogenase, the reduction of cytochrome b_1 by formate, nor hydrogenase, but it inhibits completely formic hydrogenlyase. Possibly the formation of the cytochrome b_1-nitrate reductase complex is regulated by one of the components of the formic hydrogenlyase complex.

A Bacteriocinogenic Factor of *Enterobacter cloacae*. By G. A. TIEZE and A. H. STOUTHAMER (*Biological Laboratory, Microbiology Department, Free University, Amsterdam*), H. S. JANSZ (*Laboratory for Physiological Chemistry, State University, Utrecht*), *and* E. F. J. VAN BRUGGEN (*Laboratory of Structural Chemistry, The University, Groningen, The Netherlands*)

Enterobacter cloacae strain DE 13 produces a bacteriocin which is able to kill some strains of Enterobacter, Klebsiella, and Escherichia (Stouthamer, A. H. & Tieze, G. A. (1966). *Antonie van Leeuwenhoek; J. Microbiol. Serol.* **32**, 171). Using the method of Mulczyk & Duguid (1966), this property can be transferred to another strain of *Ent. cloacae* (up to 90 % of the acceptor-population became bacteriocinogenic and to *Escherichia coli* K 12 (transfer frequency about 3×10^{-3}). Transfer of chromosomal material was never observed, suggesting that the production of the bacteriocin is determined by a plasmid, which is mostly in a repressed state (Graaf, F. K. De *et al.* (1968), *J. Bact.* **95**, 631). However, all attempts to eliminate this plasmid failed. The plasmid F'trp cys Col B Col V could be transferred from *E. coli* into *Ent. cloacae* strain DF 13, and subsequently it could be eliminated by acridine orange treatment. By CsCl gradient centrifugation in the presence of ethidium bromide (Jansz, H. S. *et al.* (1969), *Europ. J. Biochem.* in Press) closed circular DNA molecules were selected from native DNA isolated from these strains. Electron microscopic investigaton was done by a modified Kleinschmidt method. *Ent. cloacae* strain DF 13 harbours small closed circular DNA molecules of different length classes (0·65 μ, 1·35 μ, 2·65 μ, and 3·0 μ). One of these rings with a molecular weight of about $5{\cdot}2 \times 10^6$ daltons (length 2·65 μ, S_{20w} *c.* 26S) was transferred concomitantly with the ability to produce the bacteriocin DF 13 to *E. coli*. It is concluded that this DNA represents the bacteriocinogenic factor Bac DF 13, of which there are at least six copies per cell. Similar results were obtained with *Ent. cloacae* strain 02, which also contains small closed circular DNA molecules (0·9 μ, 1·3 μ, 1·8 μ, and 2·5–3·0 μ). After this strain had become bacteriocinogenic, a significant increase was found in the amount of the DNA rings in the 2·5–3·0 range.

Germination of Bacterial Spores by High Hydrostatic Pressures. By G. W. GOULD and A. J. SALE (*Unilever Research Laboratory Colworth/Welwyn, Sharnbrook, Bedford*) and W. A. HAMILTON (*Department of Biological Chemistry, Marischal College, University of Aberdeen, Great Britain*)

Bacterial spores have generally been found to be more resistant hydrostatic pressure than vegetative forms. However, under certain conditions, low pressures (100 to 2000 atmos.) may heat-sensitize or inactivate spores even more than high pressures (e.g. 8000 atmos.). Inactivation at low pressures resulted from the effect of pressure in causing germination of spores (Clouston, J. G. & Wills, P. A. (1969). *J. Bact.* in Press), and subsequent loss of viability of the germinated forms. At temperatures below about 50° high pressures were generally less germinative, and therefore less lethal, than low pressures. In contrast, at temperatures above about 50° germination and inactivation of spores generally increased with pressure at least up to 8000 atmos.

Spores germinated by pressure became heat-sensitive, excreted dipicolinic acid, calcium and peptidoglycan components, and showed phase and electronmicroscopic changes similar to those typical of spores germinated by nutrient germinants at 1 atmos. pressure. Pressure germination showed well-defined highly temperature-dependent pressure optima, temperature optima and pH optima; was inhibited by metabolic poisons, high concentrations of ionic but not of non-ionic solutes, extreme pH values and low temperatures; but was strongly potentiated by certain nutrient germinants (in particular by L-alanine and some other amino acids) at concentrations well below those effective at 1 atmos. Pressure increased the rate of racemization of alanine by spores and thereby allowed D-alanine, which inhibits L-alanine-initiated germination at 1 atmos., to potentiate germination under pressure.

It was concluded that low pressures caused germination by (1) increasing the rate of a germination reaction which was negligibly slow at 1 atmos. and (2) increasing the permeability of a barrier within the spore to exogenous and endogenous L-alanine or other germinative L-amino acids.

Influence of O- and T1 Side-Chains on the attachment of Bacteriophages to the Somatic Antigen of Salmonella. By A. A. LINDBERG (*Department of Bacteriology, Statens Bakteriologiska Laboratorium, Stockholm, Sweden*), M. SARVAS (*Department of Bacteriology and Serology, University of Helsinki*) and P. HELENA MÄKELÄ, (*State Serum Institute, Helsinki, Finland*)

The phage adsorption ability and serological specificity of different Salmonella strains having incomplete, leaky blocks in their lipopolysaccharide (LPS) synthesis were compared with their genotype and sugar composition to provide a set of standards relating these parameters to LPS structure. Strains that had T1-specific side-chains in their LPS, both with or without O side-chains, were examined in the same way to learn more about the organization of these two side-chains in the LPS.

Phage P22 adsorption was found to be dependent on the presence of a nearly complete O side-chain complement. The adsorption of the FO (Felix o-1), 6SR and Br2 bacteriophages, attaching to structures in the LPS core, was a sensitive indication of any defect in O side-chain synthesis. Strains with T1 side-chains adsorbed the FO and 6SR phages efficiently whereas the adsorption of the Br2 phage was blocked to a large extent. Strains with both O and T1 side-chains showed that as the amount of O side-chains increased there was a reduction of the 6SR, Br2 and FO attachment with a concomitant increase in Pss attachment. The results suggest that the apparent competition between O- and T1 side-chains may not be a competition for available sites in the LPS.

Virulence of Salmonella strains with a reduced amount of O-antigen By VILLE VALTONEN (*Department of Serology and Bacteriology, University of Helsinki, Finland*)

It is well known that when the lipopolysaccharide (LPS) of Salmonella loses its O-antigenic side-chains the bacteria lose their virulence. The present report concerns the effect of less

complete alterations of the O side-chains on virulence. Ten-fold dilutions of an overnight culture of bacteria were injected intraperitoneally into groups of ten adult SAW mice, and the LD_{50} values were calculated from the 10-day survival. All the strains were derivatives of SL 1027 of *S. typhimurium* line LT 2; this strain and its *rfb* and *rfc* mutants were obtained from Dr B. A. D. Stocker, Stanford University. The LD_{50} of the smooth virulent strain was 5×10^4, and the LD_{50} of its avirulent *rfb* mutant, which has no O side-chains, 5×10^7.

A *rfc* mutant, with only one repeating unit in each O side-chain (in contrast to the average ten units in the S form) had an intermediate virulence, $LD_{50} = 10^6$.

If smooth bacteria acquire a *rfi*− locus by genetic recombination they will produce both T1-specific and O side chains. (Sarvas, M. (1967), *Ann. Med. exp. Fenn.* **45**, 447–471). These T1, S forms produce about 10 times fewer O side-chains than the smooth parent. (Lindberg, A. A., Sarvas, M., Mäkelä, P. H. (1969), *J. Bact.* in Press). An F+, T1 form of *S. paratyphi* B was used as a genetic donor to a *gal*- derivative of the smooth *S. typhimurium*, gal+ recombinants were selected. Some recombinants were T1, S, having inherited the *rfi*+ of the donor, others were S like the recipient. The S recombinants and the recipient parent were virulent ($LD_{50} = 10^5$), while the T1, S, recombinants were almost avirulent ($LD_{50} = 10^7$). Thus the reduction in the O side-chain material had a deleterious effect on virulence, which could not be compensated for by the T1 side-chains. Preliminary results indicate, however, that T1 side-chains alone confer a slight degree of virulence on a *rfb* mutant.

Phage Receptor Development after the addition of Galactose to a Galactose-Epimeraseless Mutant of *Salmonella typhimurium*. By T. HOLME (*Department of Bacteriology, Karolinska Institutet*) and A. A. LINDBERG (*Department of Bacteriology, Statens Bakteriologiska Laboratorium, Stockholm, Sweden*)

A rapid incorporation of galactose and S-specific sugars in the cell wall polysaccharide was observed after addition of galactose to cultures of the UDP-Gal-4-epimeraseless mutant *Salmonella typhimurium* LT 2–M 1. The incorporation velocity was higher when galactose was added to bacteria in the log. phase of growth than when it was added to stationary phase bacteria. The adsorption rate constants for S-specific phages increased from zero to a maximum value in 20–40 min. after addition of galactose. Adsorption of phage C 21, which attacks only Rd mutants and Rc mutants grown without galactose, decreased to zero in 10 min. after the addition. The FO-1 phage, which adsorbs rapidly only to Ra mutants by slowly to the parent S-form, displayed low adsorption rate constant at all times after the addition of galactose. Passive hemagglutination inhibition tests revealed the rapid synthesis of the O-antigenic side-chains but also that the immunological specificity characteristic of the LT 2–M 1 cells was still present 60 min. after the addition of galactose. The LPS were further analysed by g.l.c.-mass spectrometry.

A Genetic Analysis of the O-antigenic Conversion by Phage 27 In Group B Salmonellas. By G. BAGDIAN and P. HELENA MÄKELÄ (*State Serum Institute, Helsinki, Finland*)

When the temperate bacteriophage P 27 lysogenises Salmonellas of group B, a new O-antigenic factor, 27_B, appears on the surface of the converted bacteria. Previous work had shown that the neo-antigen probably corresponds to an altered linkage between the repeating units of the lipopolysaccharide O side-chain. This linkage is in nonconverted bacteria formed by a polymerase coded by gene(s) at a *rfc* locus between *gal* and *trp*.

We could show that the site of integration of the prophage 27 (att*P*27) in the bacterial chromosome is far removed from the *rfc* locus, *attP*27 being contransducible with *purE* between *pro* and *gal*. This finding excludes a direct modification of the polymerase gene(s) through the integration of the prophage.

By means of F-mediated crosses we introduced the prophage 27 into group B Salmonella strains lacking the polymerase gene(s) because of a previous recombination event with a group C_1 Salmonellas and therefore having a semirough phenotype. We could show that recombinants which had the prophage 27 but no bacterial polymerase gene(s) were still semirough. The same result was obtained with two other strains that were lacking polymerase activity because

of a mutation in the *rfc* gene(s): they too remained semirough even after acquiring the prophage 27.

Transduction and Dominance Studies of the envA Gene Present in a Chain-Forming Mutant of *Escherichia coli* K 12. By STAFFAN NORMARK, (*Department of Microbiology, University of Umeå, Sweden*)

We have recently reported a genetical and physiological characterisation of a chain-forming mutant of *Escherichia coli* K 12. The gene responsible for chain formation *envA*, was located at 2–4 min. and besides the chain formation it also mediated a drastically decreased resistance to ampicillin and several other antibiotics (Normark, S., Boman, H. G. & Matsson, E. (1969), *J. Bact.* 97, 1334.)

The *envA* gene has now been found to be contransducible with leucine requirement (*leu*) and with azide resistance (*azi*). A low number of cotransductants was also obtained with threonine requirement (*thr*) but these recombinants did not show the normal phenotypic properties of the *envA* strain D 22. The available frequencies of cotransduction indicate the following gene order: *the-leu-envA-azi*.

Using the method of Low with strains carrying the *recA*⁻ gene episomes were isolated with different alleles of the *envA* gene. Studies of the morphology and antibiotic resistance of these partial diploids indicate that *envA* is recessive to its wild type allele. When the *envA* gene was integrated into a highly ampicillin resistant strain its ampicillin resistance was decreased by a 100-fold.

In addition to the antibiotics previously studied it has now been found that the *envA* gene also mediates sensitivity to actinomycin D and rifamycin. *EnvA* strains also show a drastically increased uptake of crystal violet. The results now reported are consistent with the previous hypothesis that *envA* strains carry a defect in their envelope formation which in term is responsible both for the chain formation and for a changed penetration of actinomycin D, rifamycin and crystal violet.

Cell Division and DNA Synthesis in a Chain-forming Derivative of *Escherichia coli* K 12. By HANS G. BOMAN, GUNNAR BLOOM, STAFFAN NORMARK, and EVA MATSSON. (*Departments of Microbiology and Histology, University of Umeå, Sweden*)

We have recently described a mutant of *Escherichia coli* K 12 carrying the *envA* gene which mediates chain-formation and decreased antibiotic resistance (Normark, S., Boman, H. G. & Matsson, E. (1969), *J. Bact.* 97, 1334). Strain D 223 is a thymine requiring (*thy*) *envA*-strain obtained in a cross between KL 16 (*thy*) and D 22 (*ampA*, *envA*, *proB*, *trp*, *his*) followed by scoring for a *his*⁺ and *thy* recombinant. Strain D 212, containing the wild type allele of *ampA* was obtained from strain D 21 by an analogous cross and similar scoring.

Strain D 223 can form chains consisting of more than ten cell units. Experimental conditions have been obtained during which D 223 can give less than one doubling of cell mass and DNA synthesis, but more than a 10-fold increase in the number of viable cells. This process of cell division was also followed by electron microscopy. Strain D 213 was used as a control both in the physiological and morphological work. The present results indicate that the *envA* gene, in some way, affects the association of the inner and outer layers of the cell envelope and that cell division follows after a constant period of DNA synthesis.

AmpB a Mutation which Affects the Cell Envelope and Ampicillin Resistance in *Escherichia col*
By KURT NORDSTRÖM, LARS G. BURMAN and KERSTIN G. GRENNBERG (*Department of Microbiology, University of Umeå, Sweden*)

Development of ampicillin resistance in *Escherichia coli* is a stepwise process involving at least two types of mutations. The first type was called *ampA* (Eriksson-Grennberg, K. G., (1968), *Genet. Res. Camb.* 12, 147) and raises ampicillin resistance by a factor of 10 due to an increased synthesis of penicillinase.

Without affecting penicillinase production the second mutant type, *ampB*, doubles the resistance of *ampA*$^+$ and *ampA* cells as well as that of cells with R-factor mediated penicillinase in tests where resistance is defined as ability to form colonies on ampicillin plates. However, in liquid medium *ampB* reduces ampicillin resistance in all cases (Nordström, K. Eriksson-Grennberg, K. G. & Boman, H. G. (1968) *Genet. Res., Camb.* **12**, 158). Normally, both chromosome- and episome-mediated penicillinases are strictly cell-bound in *E. coli*, but *ampB* causes a considerable liberation of these enzymes. This leakage seems to be an advantage for cells on ampicillin plates, while in liquid medium resistance of the individual cell correlated only to cell-bound penicillinase activity.

AmpB cells are osmotically fragile, especially after EDTA-treatment or wash with NA^+ ions. Osmotic stability is restored by 10^{-3} M Mg^{2+}. Moreover, these cells produce mucoid colonies and show low plating efficiency of phage T4 due to impaired adsorption. They are also 'tolerant' to colicin E2 and E3 although these are adsorbed normally. Finally *ampB* cells are cholate sensitive and show changed resistance to different cell wall antibiotics as well as chloramphenicol and streptomycin.

These data indicate that *ampB* affects the cell envelope. It cannot be judged at present whether *ampB* is a structural or a regulatory mutation or which part of the cell envelope is primarily affected.

Preliminary mapping experiments indicate that mutations in several loci can give the *ampB* phenotype. One locus is located in the *gal* region and two loci in the *trp* region.

Use of Sex Specific Phages for Demonstration of Cell Surface Changes Mediated by Genes for Ampicillin Resistance in *Escherichia coli*. By DAVID MONNER, HANS G. BOMAN and STAFFAN JONSSON (*Department of Microbiology, University of Umeå, Sweden*)

The phages used were MS2, which is male specific, and ØII, which is female specific. The main experimental approaches were to study efficiency of plating (EOP) and adsorption of phage to strains of bacteria with known genetic constitution.

It was found that in Hfr strains the *ampA* gene improved adsorption of MS2 from 85 to 90 %. In strains, carrying both *ampA* and *ampB*, adsorption of MS2 was decreased to 75 %. The presence of the *ampAB* genotype reduced adsorption to 40 %. The corresponding variations in EOP range from 1 for *ampA* strains (0·8 for wild type) to 0·03 for *ampAB* mutants (for gene symbols see *Genet. Res.* (1968), **12**, 169).

Only the restricted form of phage ØII (see next abstract) was used in this study. This phage was not adsorbed to male or female cells with *ampA* or wild type genes. Female strains with both *ampA* and *ampB*, or *ampAB*, and male strains with *ampAB*, gave all three 80–85 % adsorption. A corresponding all-or-none pattern was found for EOP values, which varied from 1 to 10^{-7}. The insertion of F-factor in female strains did not alter the EOP values.

Taken together the male and the female specific phages show a minor-pattern behaviour with respect to *ampAB* strains. In addition the adsorption data show that all presently known genes for ampicillin resistance also mediate changes on the cell surface of the bacteria.

A Mutated R-factor Mediating Increased Resistance to Several Antibiotics. By KURT NORDSTRÖM (*Department of Microbiology, University of Umeå, Sweden*)

An F$^-$-strain of *Escherichia coli* (D 1) carrying the R-factor R 1 was treated with ethylmethane sulphonate. A number of clones wtih increased ampicillin resistance were isolated. Most of them were mutated in the R-factor since the increased resistance was infective. One mutated R-factor, R1 B1, was selected for further studies and was transferred to an Hfr strain (G 11). All four resistancies mediated by R 1 (ampicillin, chloramphenicol, streptomycin, sulphonamides) (Meynell, E. & Datta, N. (1966), *Genet. Res.* 7, 134) were increased 2–3 times. R-factor mediated ampicillin resistance is due to the production of penicillinase (Datta, N. & Kontomichalou, P. (1965), *Nature, Lond.* **208**, 239). The activity of this enzyme was two times higher in G 11–R 1 B 1 than in G 11–R 1. There was no indication of any difference in specific activity or in other properties of the pure penicillinases mediated by R 1 B 1 and R 1. Chloramphenicol resistance is also due to metabolizing enzymes (Okamoto, S. & Suzuki, Y. (1964)

Nature, Lond. 208, 1301) and indirect data showed that this activity was about two times higher in R 1 B 1 than in R 1 strains. R-factor transfer and transfer of an early chromosomal gene (*proB*) was increased about three times. These results indicate that more gene product is formed from RTF genes as well as from resistance genes of the R-factor. Several explanations for this result are possible. An increased transcription of the genes in R 1 B 1 is rather unlikely. It is more reasonable that the number of R 1 copies per cell is increased in R 1 B 1 strains either by changes in the regulation of the multiplication of the episome or by the formation of a dimer of R 1. The increased resistance was transduced as one block by phage P 1, favouring the former possibility but a definite conclusion about the character of the mutation in R 1 B 1 cannot be drawn at present. Mutants of this type may perhaps be valuable in studies of the regulation of episome multiplication.

Regulation of RNA Formation in T-even Phage Infected Bacteria: Shut-off of Early mRNA Synthesis. By OLA SKÖLD (*Department of Microbiology, Faculty of Pharmacy, University of Uppsala, Sweden*)

The formation of T-even phage specific, DNA-synthesizing enzymes in *Escherichia coli* B at 37° abruptly ceases 12–15 min. after phage infection in a rich medium. This regulation could act by a restriction either of the transcription of early genes or of the translation of early mRNA. The present investigation was undertaken with the aim of measuring the time course of early mRNA synthesis. Formation of phage RNA was followed either by the cumulative incorporation of ^{14}C-uracil into acid-insoluble RNA or by short time incorporation of pulses of ^{14}C-5-fluorouracil or 5-^{3}H-uracil. Wild type and several mutants of phage T4 was used to infect *E. coli* B. It could be seen with early gene, phage mutants, both of the amber and of the temperature sensitive type, none of which will produce late mRNA under non-permissive conditions, that early mRNA formation diminished rapidly with time after infection. This restriction in early mRNA synthesis was almost abolished if gene 55 was also damaged by mutation. This could be observed both by the use of a single mutant and of a double mutant, combining the gene 55 lesion with another early gene mutation. It was concluded that the T4 phage seems to have a transcriptional regulation mechanism for early mRNA synthesis, and that this mechanism is related to the gene 55 function.

Regulation of early mRNA formation in T-even Phage Infected Bacteria: Dependence on Protein Synthesis. By CHRISTINA BOLUND and OLA SKÖLD (*Department of Microbiology, Faculty of Pharmacy, University of Uppsala, Sweden*)

In the previous communication evidence was obtained for a transcriptional regulation mechanism controlling early mRNA synthesis in T4 phage infected bacteria. This regulatory phenomenon was studied by a different approach in the present investigation. The phenotypic reversion of amber mutations in early T4 phage genes by 5-fluorouracil, was studied as a function of time after infection. Several early gene mutants were used, which could not produce DNA under nonpermissive conditions. Reversion by 5-fluorouracil was measured as the rescue of DNA synthesis, determined as the incorporation of ^{3}H-thymidine into acid-insoluble product. The 5-fluorouracil was added at different times after the infection of *Escherichia coli* B cells with the early gene mutant. The rescuability of DNA synthesis was found to decrease rapidly with time after infection. This is interpreted as a reflexion of an mRNA synthesis regulation, because 5-fluorouracil rescue of early genes could take place only as long as the analog is actively incorporated into early mRNA under synthesis. This decrease in 5-fluorouracil rescuability was almost abolished by chloramphenicol. It was, furthermore, demonstrated that the restriction in early mRNA synthesis determined by pulse-labelling of RNA in early amber mutant infected *E. coli* B, was abolished by the presence of chloramphenicol.

In conclusion, the observed regulation mechanism for early mRNA synthesis in T4 phage infected bacteria seems to require an undisturbed protein synthesis.

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